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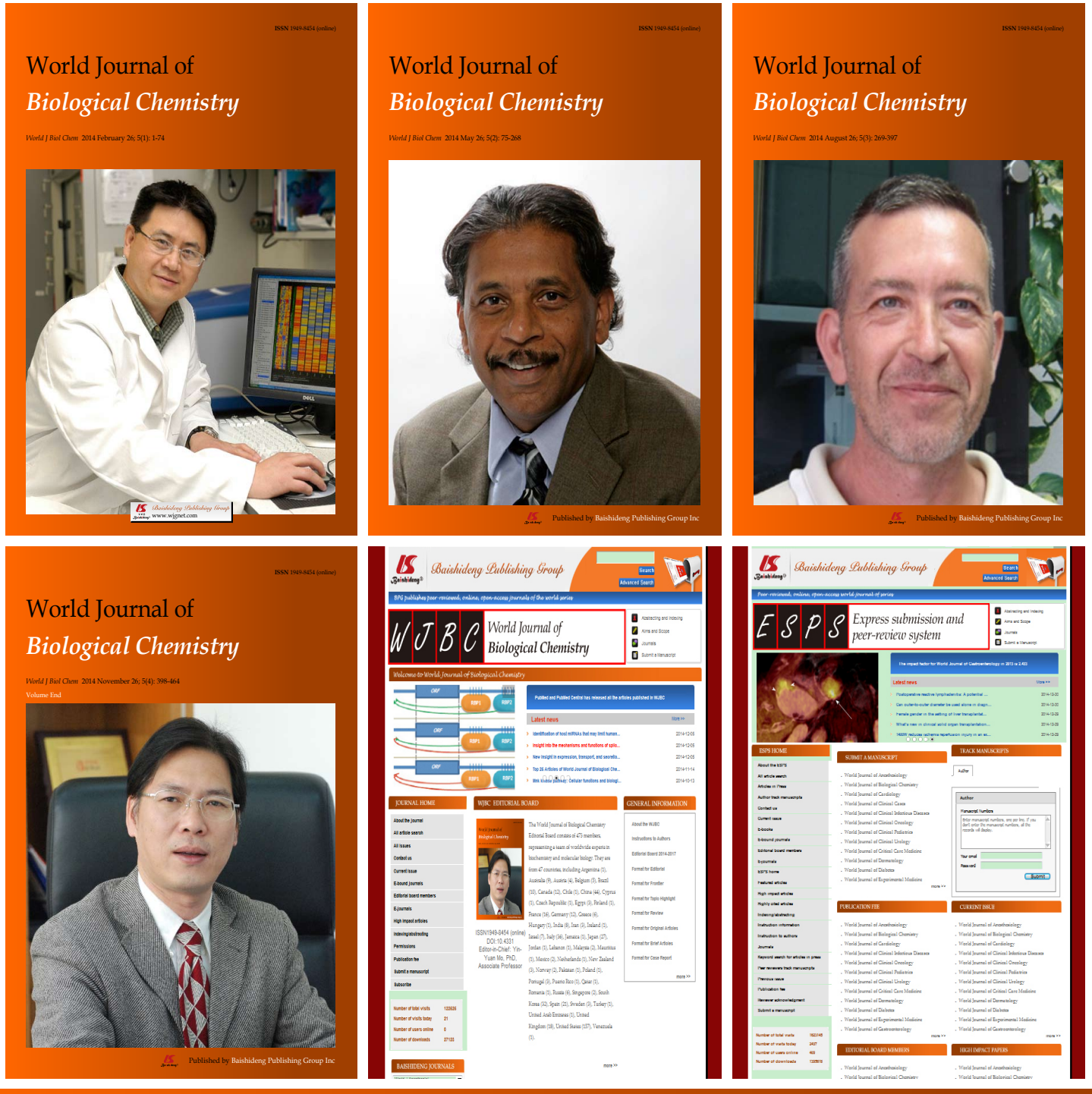

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Contents

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REVIEW

MicroRNA signature and function in retinal neovascularization

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Abstract

Ischemic retinopathies are clinically well-defined chronic microvascular complications characterized by gradually progressive alterations in the retinal microvasculature and a compensatory aberrant neovascularization of the eye. The subsequent metabolic deficiencies result in structural and functional alterations in the retina which is highly susceptible to injurious stimuli such as diabetes, trauma, hyperoxia, inflammation, aging and dysplipidemia. Emerging evidence indicates that an effective therapy may require targeting multiple components of the angiogenic pathway. Conceptually, mircoRNA (miRNA)-based therapy provides the rationale basis for an effective antiangiogenic treatment. miRNAs are an evolutionarily conserved family of short RNAs, each regulating the expression of multiple protein-coding genes. The activity of specific miRNAs is important for vascular cell signaling and blood vessel formation and function. Recently, important progress has been made in mapping the miRNA-gene target network and miRNA-mediated gene expression control. Here we highlight the latest findings on angiogenic and antiangiogenic miRNAs and their targets as well as potential implications in ocular neovascular diseases. Emphasis is placed on how specific vascular-enriched miRNAs regulate cell responses to various cues by targeting several factors, receptors and/or signaling molecules in order to maintain either vascular function or dysfunction. Further improvement of our knowledge in not only miRNA specificity, turnover, and transport but also how miRNA sequences and functions can be altered will enhance the therapeutic utility of such molecules.

Key words: MircoRNA; Angiogenesis; Retinal neovascularization; Vascular endothelial growth factor; Ischemia; Endothelial cell

Core tip: This review examines the critical regulatory role of microRNAs (miRNAs) in the process of normal and pathological angiogenesis and the prospects that they provide for the development of new treatments. miRNAs are both upstream and downstream of multiple growth factors in regulating endothelial proliferation, migration, and vascular patterning, processes critical for normal and abnormal formation of blood vessels. Emphasis in this review is placed on how specific vascular-enriched miRNAs regulate cell responses to various cues by targeting several factors, receptors and/or signaling molecules in order to maintain either vascular function or dysfunction.

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INTRODUCTION

Angiogenesis is the generation of new blood vessels from pre-existing ones, a process initiated by branching "decisions" of endothelial cells (ECs) to undergo proliferation, guided migration, tubulogenesis, vessel fusion and pruning. Physiological angiogenesis is crucial in maintaining normal vascular growth and homeostasis from embryogenesis to postnatal life, especially in instances of fetal development, wound healing, transplantation, post-ischemic tissue repair and the menstrual cycle[1-4]. However, excessive angiogenesis is a commonly occurring pathogenic condition in more than 30 diseases, including eye diseases, cancer, rheumatoid arthritis, atherosclerosis, diabetic nephropathy, pathologic obesity, asthma, cystic fibrosis, inflammatory bowel disease, psoriasis, endometriosis, vasculitis and vascular malformations. In particular, the vascular beds supplying the retina often sustains injury as a result of underlying diseases such as diabetes, trauma, hyperoxia, aging, dyslipidemia, or the interaction of genetic predisposition, environmental insults and age. The high metabolic and oxygen demands make the retina highly susceptible to these injurious stimuli which lead to an arrest of vascular development, vaso-obliteration and/or vascular occlusion. The subsequent vascular pathological response observed, especially in intraocular vascular diseases, generates disorganized, leaky, and tortuous vessels that leak into the interface between the vitreous and the retinal tissue, attracting fibroglial elements causing severe hemorrhage, retinal detachment, and vision loss. These are the characteristic features of neovascular and fibrovascular diseases of the eye such as retinopathy of prematurity and proliferative diabetic retinopathy. The exudative or "wet" form of age-related macular degeneration (AMD) which largely affects choroidal vessels and cause blindness in elderly populations is characterized by the overgrowth of the choriocapillaris that invade the Bruch's membrane and grow into subretinal spaces[5,6].

GROWTH FACTOR EXPRESSION AS A DETERMINANT FACTOR OF NORMAL AND PATHOLOGICAL ANGIOGENESIS IN THE RETINA

The formation of an aberrant and dysfunctional vasculature is commonly initiated by the uncontrolled expression or, lack thereof of growth factors including vascular endothelial growth factor (VEGF), Notch and Wnt signaling components, bone morphogenic protein, thrombospondins and insulin-like growth factors (IGFs)[7-11]. In particular, VEGF, a highly specific mitogen for ECs, is a major determinant of normal and pathological formation of the retinal vasculature[12]. Loss of VEGF attenuates blood vessel formation in mice embryos leading to early embryonic lethality and causes defective vascularization in adults[13-16]. Conversely, high expression of VEGF is common in avascular peripheral hypoxic regions of the retina compared to already vascularized areas[17]. Under conditions of oxygen deprivation, hypoxia-inducible factor 1α (HIF-1α) is activated and binds to its responsive elements in the promoter region of VEGF and other hypoxia-responsive genes, causing their upregulation and subsequent abnormal vessel growth[18]. Anti-VEGF treatments have been useful in reducing neovascularization of the eye. However, not all patients have achieved an optimal response. Safety data from several studies identified ocular and systemic adverse events including subretinal fibrosis, endophthalmatis, traumatic cataract, non-ocular hemorrhage, *etc*. Additionally, the use of anti-VEGF treatments, in the case of AMD in diabetic patients, interfered with myocardial revascularization and, in some cases, worsened the pathology in the diabetic eyes as a result of VEGF-dependent loss of neurotrophic and vasculotropic factors[19].

There are numerous other factors that contribute to neovascular growth. The erythropoietin (Epo) and *VEGF* genes, for instance, exhibit a similar expression pattern during both physiological and pathological vessel growth and inhibition of Epo suppressed retinal neovascularization both *in vivo* and *in vitro*[20,21]. Other factors such as basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), transforming growth factor alpha, interleukin 8 (IL-8), connective tissue growth factor (CTGF), pigment epithelium-derived factor, IGF- I , and matrix metalloproteinase (MMP)-2 were similarly implicated in the neovascular response and are considered as potential therapeutic targets. In addition, inflammation-mediated cyclooxygenase-2 (COX-2) can modulate angiogenesis *via* its interaction with VEGF[22] and important pro-angiogenic and neovascular functions have been associated with the activation of the renin-angiotensin system, ephrins, tyrosine kinase receptors and ligands (*e.g.*, tie/angiopoietin receptors). Together, all these factors form a well-coordinated and functional network of molecules affecting the process of normal and pathological angiogenesis. Emerging evidence indicates that antiangiogenic therapy may require therapeutic approaches that target multiple components of the angiogenic pathway[23-26]. Conceptually, microRNA-based approaches may potentially provide the rationale basis for such approaches.

MICRORNA BIOGENESIS AND FUNCTION IN THE MODULATION OF GENE EXPRESSION

Key events in gene regulation depend on specific small non-coding RNA-guided posttranscriptional regulators, commonly referred to as miRNAs that target a "mixture" of diverse growth and differentiation factor mRNAs encoding networks[27]. MicroRNAs are a relatively abundant class of gene expression regulators that function as "micromanagers" of gene expression[28]. These are short non-coding RNAs (18-25 nucleotides) which work

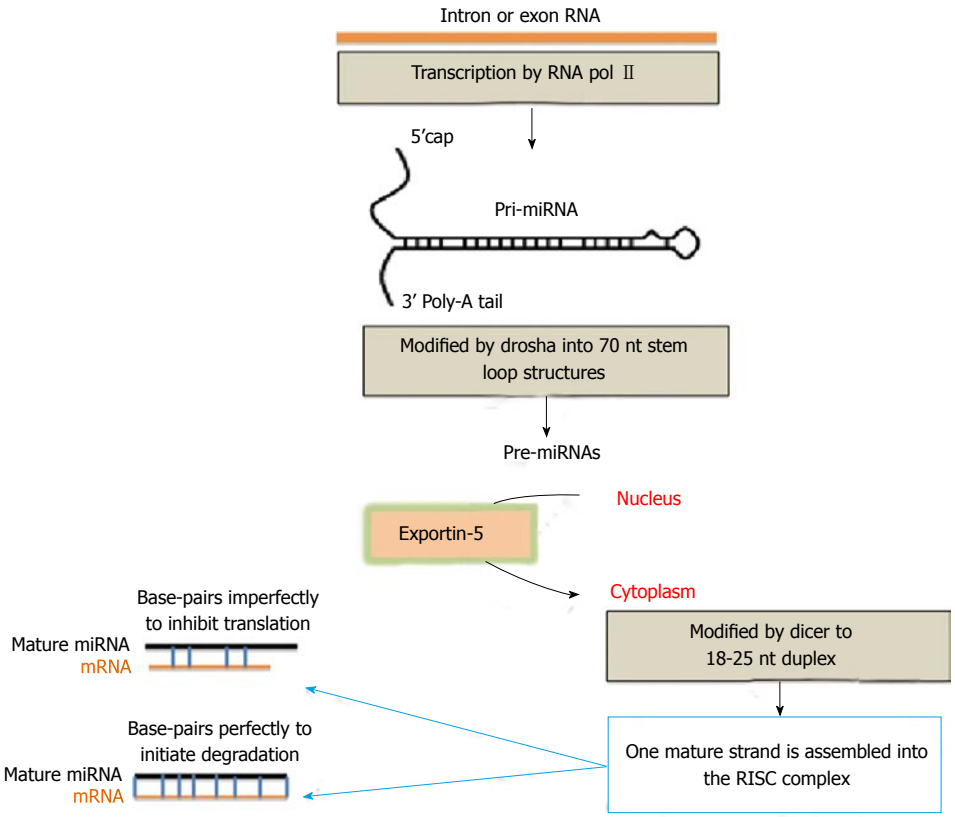

Figure 1 Schematic representation of microRNA (miRNA) biogenesis. *miRNA* genes are transcribed into large pre-miRNA (capital R) that are cleaved by a protein complex containing the endonuclease Drosha into shorter pre-miRNAs. The latter are then transported to the cytoplasm by exportin-5. A complex containing the endonuclease, Dicer, then cleaves the loop portion of the pre-miRNA (capital R) to form a short duplex molecule that is unwound, and the single-stranded mature mirNA is then passed to Argonaute to from a functional mature, approximately 22 nucleotide, miRNA that inhibit translation after base-pairing with the 3' UTR of the miRNA (capital R) target.

post-transcriptionally to negatively regulate gene expression through translational inhibition or degeneration of mRNAs. They might act as on-off switches to eliminate mRNAs that should not be expressed in a particular cell type or at a particular moment. MicroRNAs can also act to fine tune mRNA abundance and adjust the levels of their mRNA targets within a physiological range in response to environmental cues. A single miRNA has the capacity to target multiple target mRNAs, which can themselves be targeted by numerous other miRNAs. To date, 1186 mouse miRNA and 1872 human miRNA sequences have been noted on the miRBase database and may control at least 30% of all the protein-coding genes[29].

Since the discovery of miRNAs, their biogenesis has been thoroughly examined and it is now known that both miRNAs and small interfering (si) RNAs share the same cellular machinery[4,30]. Most *miRNA* genes are transcribed by RNA polymerase Ⅱ, which is usually responsible for the transcription of protein coding genes, to yield several kilobase-long primary miRNA (pri-miRNA) transcripts (Figure 1). Pri-miRNAs have characteristic loop stem (or hairpin) morphology and contain the mature miRNA sequence in the stem portion near the loop. The microprocessor, containing the endonuclease Drosha, cleaves the pri-miRNA into shorter pre-miRNAs that are transported to the cytoplasm by exportin-5. Once in the cytoplasmic compartment, pre-miRNAs undergo the final steps towards maturation. The first step involves "dicing" of the loop portion of the molecule by another endonuclease, Dicer and the transactivation response RNA binding protein (TRBP). A miRNA-miRNA duplex that is unwound is released together with the single-stranded mature miRNA. The latter is then passed to Argonaute to from a functionally mature, approximately 22 nucleotide miRNA. The 2-8-bp "seed" region in the 5' end of miRNAs binds to target 3'UTR of mRNA sequences and inhibits translation if base-pairing is imperfect or initiates mRNA cleavage if base-pairing is perfect.

REGULATION OF ANGIOGENESIS BY MICRORNAS

The first studies of the functional significance of the miRNA pathway in angiogenesis were performed using conditional deletion of Dicer alleles, as complete loss of Dicer resulted in a significant reduction of the mature miRNA profile and early embryonic lethality[31,32]. Yang *et al*[32] have shown that mice with Dicer gene deletion lack adequate blood vessel formation in embryos and yolk sacs and die between 12.5 and 14.5 d post-gestation, thus implicating Dicer-dependent miRNA genesis in the regulation of blood vessel formation. Defects in these mice were due to dysregulation of VEGF

and its receptors, KDR and FLT-1, along with Tie-1, an angiopoietin-2 receptor[32]. Similarly, silencing of Dicer or Drosha in (ECs) using siRNA significantly inhibited capillary sprouting and altered expression patterns of Tie-2, VEGF receptor 2 (VEGFR2/KDR), Tie-1, endothelial nitric oxide synthase (eNOS) and IL-8 *in vitro*[33,34]. Another study by Otsuka *et al*[35] showed that in female Dicer hypomorphic mice, infertility ensued from lack of angiogenesis in the ovaries. Further analysis revealed that impaired angiogenesis resulted from the absence of two pro-angiogenic miRNAs, miR17-5p and let-7b, which target anti-angiogenic factors[35]. Additionally, nude mice subcutaneously injected with siRNA-transfected ECs showed reduced angiogenic sprouting of transplanted cells[33]. In two EC-specific Dicer knock-out mouse models generated by Suarez *et al*[34], postnatal angiogenesis significantly decreased in response to multiple stimuli. In this study, transfection of cells with miR-18a, miR-17-5p, and miR-20a (collectively forming the miR-17-92 cluster) restored normal angiogenesis in Dicer knockout mice[34]. Taken together, these studies established a role of Dicer-dependent miRNA biogenesis in the control of angiogenesis *in vitro* and *in vivo*.

MICRORNA SIGNATURE IN NORMAL AND PATHOLOGICAL ANGIOGENESIS

Recent studies have examined miRNA expression profiles and patterns during retinal angiogenesis[8,36-38]. More than 250 miRNAs have been enumerated in the retina and new information on the regulation and mode of action of those miRNAs is progressively emerging[38]. Specific functions have been attributed to individual angiogenic miRNAs, although the challenge still remains in validating their protein targets[23,36,39-46]. Similarly, differential expression of miRNAs during retinal neovascularization has been studied in the mouse model of oxygen-induced retinopathy (OIR). In this model, seven miRNAs were upregulated, including miR-451, -424, -146, -214, -199a, -181 and -106a, when compared to control retinas, while miR-31, -150 and -184 were downregulated. However, this study provided only an exhaustive list of potentially key angiogenic miRNAs whose expression patterns, localization, and actual targets remain unclear.

Greater insights on angiogenic and antiangiogenic miRNA expression and function have been obtained from *in vitro* studies and other *in vivo* models of pathological angiogenesis. Poliseno *et al*[47] have performed the first large-scale analysis of miRNA expression in human umbilical vein endothelial cells using miRNA arrays. Twenty seven highly expressed miRNAs were identified, 15 of which were predicted to regulate the expression of receptors for angiogenic factors (*e.g.*, Flt-1, Nrp-2, FGF-R, c-Met, c-Kit). Additional studies from other groups have identified a total of 200 miRNAs that are expressed in ECs[4,24]. Overall 28 endothelial-specific miRNAs were highly expressed in 5 out of 8 of the profiling studies including miR-221/222, miR-21, the let-7 family, miR-126, miR-17-92 cluster, and the miR 23-27-24 cluster[4,24,25]. Angiogenic factors and receptors are putative targets of those miRNAs[1,7,48,49]. However, it should be noted that both abundantly expressed miRNAs as well as the rarely expressed ones play important regulatory roles and the exact *in vivo* relevance of all miRNAs expressed in ECs remains to be determined. Since angiogenesis involves complex and intertwined pathways, we have classified the currently known endothelial-specific miRNAs based on the context/conditions of their expression (Figure 2).

Hypoxia-sensitive miRNAs

Microarray-based expression profiling revealed that specific miRNAs are induced under hypoxic conditions and target angiogenic factors produced by ECs[50]. In particular, miR-15b, -16, -20a and -20b were shown to be upregulated under hypoxic conditions and target VEGF[50]. Additionally, miR-15b and miR-16 are predicted to be putative regulatory miRNAs of *uPAR*, *COX2*, and *c-MET*, which themselves are induced in response to hypoxic conditions[50]. Upregulation of these miRNAs is p53- and HIF-1α-dependent. Other microarray-based expression profiles have also revealed a set of hypoxia-induced miRNAs which are also over-expressed in tumors[51]. In particular, miR-210 is hypoxia-induced in all cell types tested[41,52]. In ECs subjected to hypoxia, miR-210 regulates the tyrosine kinase receptor ephrin-A3 that contributes to vascular remodeling. miR-210 promotes the formation of capillary-like structures in cultured ECs but, under hypoxic conditions, it decreases ECs tube formation and migration[52,53]. miR-100 is another hypoxia-sensitive miRNA that was shown to be significantly down-regulated after hind-limb ischemia[54]. Under these conditions, miR-100 repressed the expression of an angiogenic serine/threonine protein kinase targeted by rapamycin[55]. Furthermore, Shen *et al*[39] reported a dramatic increase in the expression of miR-106a, -146, -181, -199a, -214, -424 and -451 in a model of retinal ischemia suggesting their potential roles in the pathogenesis of neovascular diseases of the eye. Similarly, the hypoxia-induced miR-424 and miR-200 target the protein complex that stabilizes HIF-α and promote angiogenesis[56,57].

Growth factor-sensitive miRNAs

The effects of several angiogenic factors are mediated by miRNAs such as miR-155, -191, -21, -18a, -130a, -17-5p, -20a, -296, -101, -125b and -132[58]. In particular, serum, VEGF, and bFGF increased the expression of miR-130a, which enhances angiogenesis by downregulating the expression of anti-angiogenic homeobox proteins such as growth arrest-specific homeobox and Homeobox protein Hox-A5[52,59]. In the presence of VEGF or epidermal growth factor (EGF), the levels of miR-296 were significantly up-regulated in primary human brain microvascular ECs[60]. miR-296 was also found to be up-regulated in tumors and targets the hepatocyte growth factor-regulated tyrosine kinase substrate that inhibits degradation of key angiogenic growth factor recep-

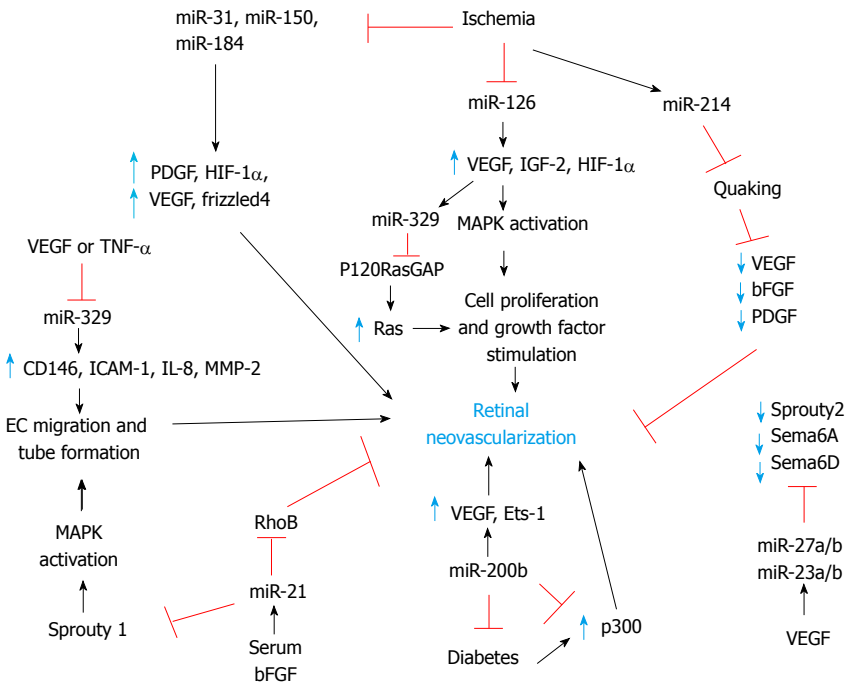

Figure 2 Overview of major angiogenic and antiangiogenic miRNAs and their targets in promoting or suppressing retinal neovascularization. VEGF: Vascular endothelial growth factor; IGFs: Insulin-like growth factors; HIF-1α: Hypoxia-inducible factor 1α; bFGF: Basic fibroblast growth factor; PDGF: Platelet derived growth factor; IL-8: Interleukin 8; MMP: Matrix metalloproteinase; ICAM-1: Intercellular adhesion molecule-1; TNF-α: Tumour necrosis factor-alpha; MAPK: Mitogen-activated protein kinase.

tors such as VEGF receptor 2 and PDGF receptor β[60]. Conversely, miR-101 was found to be down-regulated by VEGF which then allows the expression of histone-methyltransferase enhancer of zest homolog 2, increasing methylation of histone H3 and enhancing a pro-angiogenic response[61]. miR-125b was induced transiently by VEGF and negatively regulates vascular endothelial (VE)-cadherin to suppress tube formation *in vitro* and tumor growth *in vivo*[62]. A prolonged over-expression of miR-125b *in vivo* resulted in blood vessel regression. Transforming growth factor β (TGF-β) which is best known for its profibrotic activities, is a potent inducer of *VEGF* gene expression in retinal pigment epithelial cells[63]. However, numerous miRNAs have also been found to regulate and participate in TGF-β-induced VEGF expression[64]. Such effect is mediated by miR-29a which targets the phosphatase and tensin homolog (*PTEN*) gene, leading to the activation of the protein kinase B pathway, increased VEGF expression and angiogenesis[64]. Similarly, miR-132, which is undetectable in normal endothelium, was shown to be induced by VEGF and FGF in ECs and trigger neovascularization in the retina[65,66].

Inflammation and cytokines

Inflammation typically has beneficial effects on an acute basis, but it produces undesirable effects if persisting chronically. Angiogenesis sustains inflammation by providing oxygen and nutrients for inflammatory cells which, in turn, stimulates pathological angiogenesis[67]. The increased expression of many inflammatory proteins such as IL-1, IL-3 and tumour necrosis factor-alpha (TNF-α), is regulated at the level of gene transcription through the activation of proinflammatory transcription factors, including nuclear factor-kappa-B (NF-κB). In retinal ECs of diabetic rats, the expression of miR-146, -155, -132 and -21 up-regulates *NF-κB* gene expression and activity[67]. In contrast, miR-146 negatively regulates IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6 which are themselves induced following NF-κB activation[52,67]. Thus, targeting miR-146 may have an anti-inflammatory potential.

Meanwhile, T cell derived cytokine IL-3, a pro-inflammatory and a pro-angiogenic cytokine, was reported to down-regulate the expression of miR-296, miR-126, and -miR-221/222 in ECs[68]. The miR-222 exhibited anti-angiogenic effects by negatively regulating STAT5A in a mouse model of retinal neovascularization[68]. miR-126 has been portrayed as an anti-inflammatory molecule because it suppresses TNF-α mediated vascular cell adhesion molecule 1 (VCAM-1) expression and leukocyte interactions with ECs[26,31,52].

Reactive oxygen species as inducers of EC senescence

There is considerable evidence that increased production of reactive oxygen species (ROS) in the retina affects retinal vessel formation, although the mechanisms by which this occurs are not fully understood[69]. ROS such as superoxide anions such as H_2O_2 inhibit EC growth and increased cell death which are commonly associated with vaso-obliteration preceding ischemia[70]. Over-expression of miR-23a from the miR-23-27-24 cluster inhibits H_2O_2-induced apoptosis in retinal pigment epithelial cells from AMD patients *via* the repression of Fas, an activator of the apoptotic pathway[71]. Similarly, the

miR-200c is up-regulated in ECs by oxidative stress and affects EC proliferation and death by inhibiting ZEB1[72].

The miRNA profiling of aging human primacy ECs revealed that miR-17,-21,-216,-217,-31b, and-181a/b are highly expressed[73]. In particular, miR-217 is progressively expressed in response to EC stimulation by ROS and targets Sirt1 (silent information regulator 1) that regulates angiogenic gene expression *via* deacetylation of histones[31,73]. Inhibition of miR-217 in ECs reduced senescence and enhanced angiogenesis[73]. Likewise, miR-34a targets Sirt1 and impairs angiogenesis which leads to the onset of senescence[31,74].

OTHER MIRNAS WITH POTENTIALLY IMPORTANT ANGIOGENIC FUNCTIONS IN THE RETINA

Other miRNAs with potentially important angiogenic functions in the retina were shown in Figure 2.

miR-221/222

miR-221 and miR-222 are two paralogue miRNAs located in close proximity to one another on Xp11.3 chromosome[26,47]. Over-expression of miR-221/222 reduced EC growth *in vitro* by targeting the c-Kit receptor, a tyrosine kinase receptor for stem cell factor which regulates EC migration, and survival as well as tube formation[47,52]. EC transfection with miR-221/222 inhibits tube formation, migration, and wound healing[47,52]. Conversely, miR-221/222 positively regulates proliferation and migration of cultured vascular smooth muscle cells, suggesting a cell type-specific function[68,75]. The proangiogenic effects of miR-221/222 in smooth muscle cells are p27 and p57-dependent. A recent study in zebra fish showed that miR-221 deficiency resulted in drastic developmental vascular defects which underscore an important function of miR-221/222 in angiogenesis[11]. In the latter study, miR-221 acts autonomously on the VEGF-C/Flt4 signaling pathway, altering endothelial tip and stalk cell phenotypes[11]. miR-221 promotes tip cell migration and proliferation by negatively regulating cyclin dependent kinase inhibitor 1b and phosphoinositide-3-kinase regulatory subunit 1[11]. The discrepancy between the *in vitro* and *in vivo* activities of miR-221/222 may be due to a differential effect on the mature and non-mature circulatory system. Further studies are needed to ascertain the regulation and function of miR-221/222 in developmental and pathological angiogenesis in the retina.

miR-17-92 cluster

The miR-17-92 cluster is a polycistronic *miRNA* gene located in intron 3 of chromosome 13 in humans, and contains six mature miRNAs, miR-17, -18a, -19a, -19b-1, -20a and -92a[3,4,26]. This cluster is highly expressed in ECs and tumor cells and is strongly up-regulated by ischemia[28,31,52,76]. Ectopic expression of the miR-17-92 cluster partially rescued the angiogenic phenotype of Dicer-deficient ECs[58]. Similarly, restoration of miR-17 in combination with let-7b in Dicer knockout mice also partially normalized corpus luteum angiogenesis by targeting the tissue inhibitor metalloproteinase-1, an anti-angiogenic factor[35]. The pro-angiogenic function of this cluster is due to the inhibition of the anti-angiogenic molecules thrombospondin-1 and CTGF by miR-18 and miR-19, respectively[58]. However, the function of this miRNA cluster in retinal angiogenesis remains to be elucidated.

miR-126

miR-126 is the best characterized EC-specific miRNA and is known to be highly conserved among species[1,26]. It is encoded by intron 7 of the EGF-like domain 7. miR-126 enhanced VEGF signaling by directly targeting the 3'UTR of Sprouty-related EVH1 domain containing protein-1 and phosphoinositol-3-kinase regulatory subunit 2[1,7,26,31,49]. Thus, miR-126 promotes angiogenesis by targeting negative regulators of the angiogenic pathway. miR-126 affects cell migration, reorganization of the cytoskeleton, capillary network stability, and cell survival *in vitro*[7]. It also altered developmental angiogenesis and vascular integrity. Fifty percent of miR-126 null mice died as a result of severe systemic edema, ruptured blood vessels and multifocal hemorrhages[49]. Vascularization of the retina was shown to be severely impaired in mice that survived the miR-126 deletion[49]. An intravitreal injection of miR-126 in the retina reduced the levels of VEGF, IGF-2, and HIF-1α[77]. Additionally, miR-126 exhibited tumor suppressor functions in lung cancer cells by negatively regulating VEGF both *in vivo* and *in vitro*[26,78]. Hence, strategies to modulate miR-126 levels may hold a great therapeutic value against retinal neovascular diseases.

miR-200b

The miR-200 family is up-regulated by stimuli such as TGF-β1 and PDGF and suppresses growth of human microvascular ECs[57]. Hypoxia inhibits miR-200b expression, prompting an elevated *Ets-1* gene expression and its downstream target genes such as *MMP1* and *VEGFR2*[57]. Intravitreal injection of miR-200b mimicked reduced elevated levels of VEGF and prevented angiogenesis in a model of diabetic retinopathy[79]. Thus, the regulation of miR-200b in retinal neovascular diseases may prevent the aberrant expression of critical factors associated with pathological angiogenesis.

miR-214

miR-214 is located on a non-coding intronic Dynamin-3 gene sequence and its expression is controlled by the transcription factor Twist-1. HIF-1α mediates Twist-1 transcription, which then allows miR-214 expression[80]. Concordantly, miR-214 was shown to be up-regulated in ischemic conditions when HIF-1α was stabilized[80]. A recent study has shown that miR-214 directly targets Quaking (QKI) and regulates the expression and secretion of angiogenic growth factors such as VEGF, bFGF

and PDGF[81]. Quaking plays an essential role in vascular development[82]. *In vivo* silencing of miR-214 enhanced the formation of blood vessels on Matrigel plugs and increased the secretion of pro-angiogenic growth factors[81]. Additionally, miR-214 is substantially increased in the mouse model of OIR[39]. Inhibition of miR-214 enhanced normalization of the vascularization of the retina through the expression of QKI, suggesting that miR-214 may function directly to either block pathological neovascularization or prevent hyperoxia-induced vasoobliteration[81].

miR-329

miR-329 targets the important pro-angiogenic gene, CD146, and inhibits angiogenesis *in vitro* and *in vivo*[83]. CD146 is an adhesion molecule and an endothelial biomarker which actively participates in the angiogenic process[83,84]. CD146 functions as a co-receptor for VEGFR2 and activates the p38/IκB kinase/NF-κB signaling pathway leading to increased EC migration and tube formation. A study by Wang *et al*[83] has shown that exposure of ECs to VEGF represses endogenous miR-329 expression, resulting in the simultaneous up-regulation of CD146 and treatment with miR-329 significantly reduced retinal neovascularization. miR-329 is thought to inhibit the expression of many downstream pro-angiogenic genes including intercellular adhesion molecule-1 (ICAM-1), IL-8, and MMP-2, among others. Thus, miR-329 serves as a potential therapeutic target in pathological retinal angiogenesis.

miR-21

miR-21 is located on chromosome 17q23.2 within the protein-coding region of the transmembrane protein 49[85]. miR-21 promotes angiogenesis by inhibiting phosphate and tensin homolog deleted on chromosome 10 (PTEN), a potent negative regulator of the phosphatidyl inositol-3 kinase/AKT signaling pathway. By blocking Akt signaling, PTEN decreases both eNOS activity and VCAM-1 expression[31,86,87]. In tumor cells, overexpression of miR-21 significantly increased the levels of HIF-α and VEGF. In primary bovine retinal microvascular ECs, inhibition of miR-21 drastically reduced proliferation, migration, and tube-forming capacity reinforcing the important pro-angiogenic role of miR-21 in the retinal microvasculature[88].

miR-23-27-24 cluster

The miR-23-27-24 cluster is highly enriched in ECs and is well conserved between rodent and humans[40]. There are two paralogs of the clusters: an intergenic miR-23a-27a-24-2 cluster and an intronic miR-23b-27b-24-1 cluster on vertebrate chromosomes 8 and 13 respectively[40]. miR-27a/b and miR-23a/b mediate proper capillary formation in response to VEGF *in vitro*[40]. miR-27a/b and miR-23a/b repress anti-angiogenic gene expression such as SPROUTY2, SEMA6A and SEMA6D[40]. These anti-angiogenic genes inhibit the mitogen-activated protein kinase pathway and VEGF pathway[40]. Additionally, miR-23a/b and miR-27a/b also promote choroidal neovascularization (CNV)[40]. Silencing of miR-23a/b and miR-27a/b suppressed CNV in mice[40]. Thus, targeting the miR-23-27-24 cluster may have beneficial therapeutic applications in the treatment of AMD.

miR-132

miR-132 is highly up-regulated in human embryonic stem cells and tumors whereas it was undetectable in a normal endothelium[26,65]. However, stimulation of ECs by growth factors increased the levels of miR-132 which then activates quiescent endothelium by suppressing p120RasGAP[26,65,66]. Suppression of p120RasGAP led to the activation of Ras which then increases VEGF-mediated phosphorylation of mitogen-activated protein kinase extracellular related protein kinase kinase-1[65]. Ectopic expression of miR-132 was sufficient to induce EC proliferation *in vitro* and its inhibition significantly reduced growth factor-mediated angiogenesis *in vivo* and *in vitro*[65]. Additionally, inhibition of miR-132 also greatly decreased retinal neovascularization in mice[65]. Thus, early detection and modulation of this miRNA may inhibit the onset of neovascularization.

CONCLUSION

Treatment and management of neovascular diseases rely mainly on pharmacotherapy and/or surgical procedures. However, these treatments are seldom efficacious and they often are plagued by unwanted side effects and/or insurmountable complications. The use of miRNAs that specifically target a set of angiogenic genes appears to be a viable alternative approach. Currently, there are numerous ongoing clinical trials designed to test the efficacy and effectiveness of such approach in the treatment of various disorders (*e.g.*, atherosclerosis, cancer, inflammatory diseases) and the preliminary results are promising[89-91]. Neovascular diseases including those of the eye will likely test/use such approach in a near future as our understanding of miRNA regulation and the molecular mechanisms underpinning their functions increases every day.

MicroRNAs are also increasingly considered as potential diagnostic markers of disease stages. Indeed, miRNAs have been discovered in a wide variety of extracellular body fluids such as saliva, serum, plasma, milk, and urine as nuclease resistant entities[24,31,92]. These extracellular circulating miRNAs enable cell-to-cell communication and also provide insight into the physiological states or progression of pathological diseases within the secreting cells[92-94]. miRNAs are thought to be secreted from cells in three possible ways: (1) *via* passive leakage from cells resulting from injury, inflammation, apoptosis or necrosis; (2) *via* an active secretion method in membrane-bound vesicles such as exosomes, shedding vesicles and apoptotic bodies; and (3) *via* an active secretion method of protein-miRNA complexes[92]. Exosomes are 30 nm-100 nm ves-

icles, arising from multivesicular bodies and their release is mediated by the enzyme sphingomyelinase-2[31,92-95]. Shedding vesicles, arising from the plasma membrane, is facilitated *via* a ligand-receptor method. Further insight into the exosomal miRNA formation and circulation may not only validate their prognostic potential in the slowly developing neovascular diseases of the eye but, will also help design optimal delivery systems of miRNAs *in vivo*.

REFERENCES

1 **Suárez Y**, Sessa WC. MicroRNAs as novel regulators of angiogenesis. *Circ Res* 2009; **104**: 442-454 [PMID: 19246688 DOI: 10.1161/CIRCRESAHA.108.191270]
2 **Saint-Geniez M**, D'Amore PA. Development and pathology of the hyaloid, choroidal and retinal vasculature. *Int J Dev Biol* 2004; **48**: 1045-1058 [PMID: 15558494 DOI: 10.1387/ijdb.041895ms]
3 **Fish JE**, Srivastava D. MicroRNAs: opening a new vein in angiogenesis research. *Sci Signal* 2009; **2**: 52; pe1 [PMID: 19126861 DOI: 10.1126/scisignal.252pe1]
4 **Heusschen R**, van Gink M, Griffioen AW, Thijssen VL. MicroRNAs in the tumor endothelium: novel controls on the angioregulatory switchboard. *Biochim Biophys Acta* 2010; **1805**: 87-96 [PMID: 19782719 DOI: 10.1016/j.bbcan.2009.09.005]
5 **Campochiaro PA**. Retinal and choroidal neovascularization. *J Cell Physiol* 2000; **184**: 301-310 [PMID: 10911360]
6 **Grossniklaus HE**, Kang SJ, Berglin L. Animal models of choroidal and retinal neovascularization. *Prog Retin Eye Res* 2010; **29**: 500-519 [PMID: 20488255 DOI: 10.1016/j.preteyeres.2010.05.003]
7 **Fish JE**, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, Ivey KN, Bruneau BG, Stainier DY, Srivastava D. miR-126 regulates angiogenic signaling and vascular integrity. *Dev Cell* 2008; **15**: 272-284 [PMID: 18694566 DOI: 10.1016/j.devcel.2008.07.008]
8 **Karali M**, Peluso I, Gennarino VA, Bilio M, Verde R, Lago G, Dollé P, Banfi S. miRNeye: a microRNA expression atlas of the mouse eye. *BMC Genomics* 2010; **11**: 715 [PMID: 21171988 DOI: 10.1186/1471-2164-11-715]
9 **Dang LT**, Lawson ND, Fish JE. MicroRNA control of vascular endothelial growth factor signaling output during vascular development. *Arterioscler Thromb Vasc Biol* 2013; **33**: 193-200 [PMID: 23325476 DOI: 10.1161/ATVBAHA.112.300142]
10 **Bouïs D**, Kusumanto Y, Meijer C, Mulder NH, Hospers GA. A review on pro- and anti-angiogenic factors as targets of clinical intervention. *Pharmacol Res* 2006; **53**: 89-103 [PMID: 16321545 DOI: 10.1016/j.phrs.2005.10.006]
11 **Nicoli S**, Knyphausen CP, Zhu LJ, Lakshmanan A, Lawson ND. miR-221 is required for endothelial tip cell behaviors during vascular development. *Dev Cell* 2012; **22**: 418-429 [PMID: 22340502 DOI: 10.1016/j.devcel.2012.01.008]
12 **Hoeben A**, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev* 2004; **56**: 549-580 [PMID: 15602010 DOI: 10.1124/pr.56.4.3]
13 **Uemura A**, Kusuhara S, Katsuta H, Nishikawa S. Angiogenesis in the mouse retina: a model system for experimental manipulation. *Exp Cell Res* 2006; **312**: 676-683 [PMID: 16337189 DOI: 10.1016/j.yexcr.2005.10.030]
14 **Carmeliet P**, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 1996; **380**: 435-439 [PMID: 8602241 DOI: 10.1038/380435a0]
15 **Ferrara N**, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996; **380**: 439-442 [PMID: 8602242 DOI: 10.1038/380439a0]
16 **Lee S**, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, Ferrara N, Nagy A, Roos KP, Iruela-Arispe ML. Autocrine VEGF signaling is required for vascular homeostasis. *Cell* 2007; **130**: 691-703 [PMID: 17719546 DOI: 10.1016/j.cell.2007.06.054]
17 **Fruttiger M**. Development of the retinal vasculature. *Angiogenesis* 2007; **10**: 77-88 [PMID: 17322966 DOI: 10.1007/s10456-007-9065-1]
18 **Yamakawa M**, Liu LX, Date T, Belanger AJ, Vincent KA, Akita GY, Kuriyama T, Cheng SH, Gregory RJ, Jiang C. Hypoxia-inducible factor-1 mediates activation of cultured vascular endothelial cells by inducing multiple angiogenic factors. *Circ Res* 2003; **93**: 664-673 [PMID: 12958144 DOI: 10.1161/01.RES.0000093984.48643.D7]
19 **Gariano RF**, Gardner TW. Retinal angiogenesis in development and disease. *Nature* 2005; **438**: 960-966 [PMID: 16355161 DOI: 10.1038/nature04482]
20 **Watanabe D**. Erythropoietin as a retinal angiogenic factor in proliferative diabetic retinopathy. *Nihon Ganka Gakkai Zasshi* 2007; **111**: 892-898 [PMID: 18051819]
21 **Watanabe D**, Suzuma K, Matsui S, Kurimoto M, Kiryu J, Kita M, Suzuma I, Ohashi H, Ojima T, Murakami T, Kobayashi T, Masuda S, Nagao M, Yoshimura N, Takagi H. Erythropoietin as a retinal angiogenic factor in proliferative diabetic retinopathy. *N Engl J Med* 2005; **353**: 782-792 [PMID: 16120858 DOI: 10.1056/NEJMoa041773]
22 **Qazi Y**, Maddula S, Ambati BK. Mediators of ocular angiogenesis. *J Genet* 2009; **88**: 495-515 [PMID: 20090210]
23 **Wang S**, Koster KM, He Y, Zhou Q. miRNAs as potential therapeutic targets for age-related macular degeneration. *Future Med Chem* 2012; **4**: 277-287 [PMID: 22393936 DOI: 10.4155/fmc.11.176]
24 **Landskroner-Eiger S**, Moneke I, Sessa WC. miRNAs as modulators of angiogenesis. *Cold Spring Harb Perspect Med* 2013; **3**: a006643 [PMID: 23169571 DOI: 10.1101/cshperspect.a006643]
25 **Caporali A**, Emanueli C. MicroRNA regulation in angiogenesis. *Vascul Pharmacol* 2011; **55**: 79-86 [PMID: 21777698 DOI: 10.1016/j.vph.2011.06.006]
26 **Hartmann D**, Thum T. MicroRNAs and vascular (dys)function. *Vascul Pharmacol* 2011; **55**: 92-105 [PMID: 21802526 DOI: 10.1016/j.vph.2011.07.005]
27 **Roy S**, Sen CK. miRNA in wound inflammation and angiogenesis. *Microcirculation* 2012; **19**: 224-232 [PMID: 22211762 DOI: 10.1111/j.1549-8719.2011.00156.x]
28 **Kuehbacher A**, Urbich C, Dimmeler S. Targeting microRNA expression to regulate angiogenesis. *Trends Pharmacol Sci* 2008; **29**: 12-15 [PMID: 18068232 DOI: 10.1016/j.tips.2007.10.014]
29 **Ullah S**, John P, Bhatti A. MicroRNAs with a role in gene regulation and in human diseases. *Mol Biol Rep* 2014; **41**: 225-232 [PMID: 24197698 DOI: 10.1007/s11033-013-2855-1]
30 **Bernstein E**, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. Dicer is essential for mouse development. *Nat Genet* 2003; **35**: 215-217 [PMID: 14528307 DOI: 10.1038/ng1253]
31 **Yamakuchi M**. MicroRNAs in Vascular Biology. *Int J Vasc Med* 2012; **2012**: 794898 [PMID: 23056947 DOI: 10.1155/2012/794898]
32 **Yang WJ**, Yang DD, Na S, Sandusky GE, Zhang Q, Zhao G. Dicer is required for embryonic angiogenesis during mouse development. *J Biol Chem* 2005; **280**: 9330-9335 [PMID: 15613470 DOI: 10.1074/jbc.M413394200]
33 **Kuehbacher A**, Urbich C, Zeiher AM, Dimmeler S. Role of Dicer and Drosha for endothelial microRNA expression

and angiogenesis. *Circ Res* 2007; **101**: 59-68 [PMID: 17540974 DOI: 10.1161/CIRCRESAHA.107.153916]
34 **Suárez Y**, Fernández-Hernando C, Pober JS, Sessa WC. Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. *Circ Res* 2007; **100**: 1164-1173 [PMID: 17379831 DOI: 10.1161/01.RES.0000265065.26744.17]
35 **Otsuka M**, Zheng M, Hayashi M, Lee JD, Yoshino O, Lin S, Han J. Impaired microRNA processing causes corpus luteum insufficiency and infertility in mice. *J Clin Invest* 2008; **118**: 1944-1954 [PMID: 18398510 DOI: 10.1172/JCI33680]
36 **Maiorano NA**, Hindges R. Non-Coding RNAs in Retinal Development. *Int J Mol Sci* 2012; **13**: 558-578 [PMID: 22312272 DOI: 10.3390/ijms13010558]
37 **Xu S**. microRNA expression in the eyes and their significance in relation to functions. *Prog Retin Eye Res* 2009; **28**: 87-116 [PMID: 19071227 DOI: 10.1016/j.preteyeres.2008.11.003]
38 **Sundermeier TR**, Palczewski K. The physiological impact of microRNA gene regulation in the retina. *Cell Mol Life Sci* 2012; **69**: 2739-2750 [PMID: 22460583 DOI: 10.1007/s00018-012-0976-7]
39 **Shen J**, Yang X, Xie B, Chen Y, Swaim M, Hackett SF, Campochiaro PA. MicroRNAs regulate ocular neovascularization. *Mol Ther* 2008; **16**: 1208-1216 [PMID: 18500251 DOI: 10.1038/mt.2008.104]
40 **Zhou Q**, Gallagher R, Ufret-Vincenty R, Li X, Olson EN, Wang S. Regulation of angiogenesis and choroidal neovascularization by members of microRNA-23~27~24 clusters. *Proc Natl Acad Sci USA* 2011; **108**: 8287-8292 [PMID: 21536891 DOI: 10.1073/pnas.1105254108]
41 **Decembrini S**, Bressan D, Vignali R, Pitto L, Mariotti S, Rainaldi G, Wang X, Evangelista M, Barsacchi G, Cremisi F. MicroRNAs couple cell fate and developmental timing in retina. *Proc Natl Acad Sci USA* 2009; **106**: 21179-21184 [PMID: 19965369 DOI: 10.1073/pnas.0909167106]
42 **Conte I**, Carrella S, Avellino R, Karali M, Marco-Ferreres R, Bovolenta P, Banfi S. miR-204 is required for lens and retinal development via Meis2 targeting. *Proc Natl Acad Sci USA* 2010; **107**: 15491-15496 [PMID: 20713703 DOI: 10.1073/pnas.0914785107]
43 **Lumayag S**, Haldin CE, Corbett NJ, Wahlin KJ, Cowan C, Turturro S, Larsen PE, Kovacs B, Witmer PD, Valle D, Zack DJ, Nicholson DA, Xu S. Inactivation of the microRNA-183/96/182 cluster results in syndromic retinal degeneration. *Proc Natl Acad Sci USA* 2013; **110**: E507-E516 [PMID: 23341629 DOI: 10.1073/pnas.1212655110]
44 **La Torre A**, Georgi S, Reh TA. Conserved microRNA pathway regulates developmental timing of retinal neurogenesis. *Proc Natl Acad Sci USA* 2013; **110**: E2362-E2370 [PMID: 23754433 DOI: 10.1073/pnas.13018371]
45 **Arora A**, Guduric-Fuchs J, Harwood L, Dellett M, Cogliati T, Simpson DA. Prediction of microRNAs affecting mRNA expression during retinal development. *BMC Dev Biol* 2010; **10**: 1 [PMID: 20053268 DOI: 10.1186/1471-213X-10-1]
46 **Liu D**, Krueger J, Le Noble F. The role of blood flow and microRNAs in blood vessel development. *Int J Dev Biol* 2011; **55**: 419-429 [PMID: 21858767 DOI: 10.1387/ijdb.103220dl]
47 **Poliseno L**, Tuccoli A, Mariani L, Evangelista M, Citti L, Woods K, Mercatanti A, Hammond S, Rainaldi G. MicroRNAs modulate the angiogenic properties of HUVECs. *Blood* 2006; **108**: 3068-3071 [PMID: 16849646 DOI: 10.1182/blood-2006-01-012369]
48 **Heinke J**, Patterson C, Moser M. Life is a pattern: vascular assembly within the embryo. *Front Biosci* (Elite Ed) 2012; **4**: 2269-2288 [PMID: 22202036]
49 **Wang S**, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R, Olson EN. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev Cell* 2008; **15**: 261-271 [PMID: 18694565 DOI: 10.1016/j.devcel.2008.07.002]
50 **Hua Z**, Lv Q, Ye W, Wong CK, Cai G, Gu D, Ji Y, Zhao C, Wang J, Yang BB, Zhang Y. MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. *PLoS One* 2006; **1**: e116 [PMID: 17205120 DOI: 10.1371/journal.pone.0000116]
51 **Kulshreshtha R**, Ferracin M, Wojcik SE, Garzon R, Alder H, Agosto-Perez FJ, Davuluri R, Liu CG, Croce CM, Negrini M, Calin GA, Ivan M. A microRNA signature of hypoxia. *Mol Cell Biol* 2007; **27**: 1859-1867 [PMID: 17194750 DOI: 10.1128/MCB.01395-06]
52 **Urbich C**, Kuehbacher A, Dimmeler S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovasc Res* 2008; **79**: 581-588 [PMID: 18550634 DOI: 10.1093/cvr/cvn156]
53 **Fasanaro P**, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, Capogrossi MC, Martelli F. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. *J Biol Chem* 2008; **283**: 15878-15883 [PMID: 18417479 DOI: 10.1074/jbc.M800731200]
54 **Blick C**, Ramachandran A, Wigfield S, McCormick R, Jubb A, Buffa FM, Turley H, Knowles MA, Cranston D, Catto J, Harris AL. Hypoxia regulates FGFR3 expression via HIF-1α and miR-100 and contributes to cell survival in non-muscle invasive bladder cancer. *Br J Cancer* 2013; **109**: 50-59 [PMID: 23778527 DOI: 10.1038/bjc.2013.240.]
55 **Grundmann S**, Hans FP, Kinniry S, Heinke J, Helbing T, Bluhm F, Sluijter JP, Hoefer I, Pasterkamp G, Bode C, Moser M. MicroRNA-100 regulates neovascularization by suppression of mammalian target of rapamycin in endothelial and vascular smooth muscle cells. *Circulation* 2011; **123**: 999-1009 [PMID: 21339483 DOI: 10.1161/CIRCULATIONAHA.110.000323]
56 **Ghosh G**, Subramanian IV, Adhikari N, Zhang X, Joshi HP, Basi D, Chandrashekhar YS, Hall JL, Roy S, Zeng Y, Ramakrishnan S. Hypoxia-induced microRNA-424 expression in human endothelial cells regulates HIF-α isoforms and promotes angiogenesis. *J Clin Invest* 2010; **120**: 4141-4154 [PMID: 20972335 DOI: 10.1172/JCI42980]
57 **Chan YC**, Khanna S, Roy S, Sen CK. miR-200b targets Ets-1 and is down-regulated by hypoxia to induce angiogenic response of endothelial cells. *J Biol Chem* 2011; **286**: 2047-2056 [PMID: 21081489 DOI: 10.1074/jbc.M110.158790]
58 **Suárez Y**, Fernández-Hernando C, Yu J, Gerber SA, Harrison KD, Pober JS, Iruela-Arispe ML, Merkenschlager M, Sessa WC. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. *Proc Natl Acad Sci USA* 2008; **105**: 14082-14087 [PMID: 18779589 DOI: 10.1073/pnas.0804597105]
59 **Chen Y**, Gorski DH. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood* 2008; **111**: 1217-1226 [PMID: 17957028 DOI: 10.1182/blood-2007-07-104133]
60 **Würdinger T**, Tannous BA, Saydam O, Skog J, Grau S, Soutschek J, Weissleder R, Breakefield XO, Krichevsky AM. miR-296 regulates growth factor receptor overexpression in angiogenic endothelial cells. *Cancer Cell* 2008; **14**: 382-393 [PMID: 18977327 DOI: 10.1016/j.ccr.2008.10.005]
61 **Smits M**, Mir SE, Nilsson RJ, van der Stoop PM, Niers JM, Marquez VE, Cloos J, Breakefield XO, Krichevsky AM, Noske DP, Tannous BA, Würdinger T. Down-regulation of miR-101 in endothelial cells promotes blood vessel formation through reduced repression of EZH2. *PLoS One* 2011; **6**: e16282 [PMID: 21297974 DOI: 10.1371/journal.pone.0016282]
62 **Muramatsu F**, Kidoya H, Naito H, Sakimoto S, Takakura N. microRNA-125b inhibits tube formation of blood vessels through translational suppression of VE-cadherin. *Oncogene* 2013; **32**: 414-421 [PMID: 22391569 DOI: 10.1038/

onc.2012.68]
63 **Nagineni CN**, Samuel W, Nagineni S, Pardhasaradhi K, Wiggert B, Detrick B, Hooks JJ. Transforming growth factor-beta induces expression of vascular endothelial growth factor in human retinal pigment epithelial cells: involvement of mitogen-activated protein kinases. *J Cell Physiol* 2003; **197**: 453-462 [PMID: 14566975 DOI: 10.1002/jcp.10378]
64 **Wang J**, Wang Y, Wang Y, Ma Y, Lan Y, Yang X. Transforming growth factor β-regulated microRNA-29a promotes angiogenesis through targeting the phosphatase and tensin homolog in endothelium. *J Biol Chem* 2013; **288**: 10418-10426 [PMID: 23426367 DOI: 10.1074/jbc.M112.444463]
65 **Anand S**, Majeti BK, Acevedo LM, Murphy EA, Mukthavaram R, Scheppke L, Huang M, Shields DJ, Lindquist JN, Lapinski PE, King PD, Weis SM, Cheresh DA. MicroRNA-132-mediated loss of p120RasGAP activates the endothelium to facilitate pathological angiogenesis. *Nat Med* 2010; **16**: 909-914 [PMID: 20676106 DOI: 10.1038/nm.2186]
66 **Anand S**, Cheresh DA. MicroRNA-mediated regulation of the angiogenic switch. *Curr Opin Hematol* 2011; **18**: 171-176 [PMID: 21423013 DOI: 10.1097/MOH.0b013e328345a180]
67 **Kovacs B**, Lumayag S, Cowan C, Xu S. MicroRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. *Invest Ophthalmol Vis Sci* 2011; **52**: 4402-4409 [PMID: 21498619 DOI: 10.1167/iovs.10-6879]
68 **Dentelli P**, Rosso A, Orso F, Olgasi C, Taverna D, Brizzi MF. microRNA-222 controls neovascularization by regulating signal transducer and activator of transcription 5A expression. *Arterioscler Thromb Vasc Biol* 2010; **30**: 1562-1568 [PMID: 20489169 DOI: 10.1161/ATVBAHA.110.206201]
69 **Wilkinson-Berka JL**, Deliyanti D, Rana I, Miller AG, Agrotis A, Armani R, Szyndralewiez C, Wingler K, Touyz RM, Cooper ME, Jandeleit-Dahm KA, Schmidt HH. NADPH Oxidase, NOX1, Mediates Vascular Injury in Ischemic Retinopathy. *Antioxid Redox Signal* 2013 Oct 30; Epub ahead of print [PMID: 24053718 DOI: 10.1089/ars.2013.5357]
70 **Maulik N**, Das DK. Redox signaling in vascular angiogenesis. *Free Radic Biol Med* 2002; **33**: 1047-1060 [PMID: 12374616]
71 **Lin H**, Qian J, Castillo AC, Long B, Keyes KT, Chen G, Ye Y. Effect of miR-23 on oxidant-induced injury in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 2011; **52**: 6308-6314 [PMID: 21693609 DOI: 10.1167/iovs.10-6632]
72 **Magenta A**, Cencioni C, Fasanaro P, Zaccagnini G, Greco S, Sarra-Ferraris G, Antonini A, Martelli F, Capogrossi MC. miR-200c is upregulated by oxidative stress and induces endothelial cell apoptosis and senescence via ZEB1 inhibition. *Cell Death Differ* 2011; **18**: 1628-1639 [PMID: 21527937 DOI: 10.1038/cdd.2011.42]
73 **Menghini R**, Casagrande V, Cardellini M, Martelli E, Terrinoni A, Amati F, Vasa-Nicotera M, Ippoliti A, Novelli G, Melino G, Lauro R, Federici M. MicroRNA 217 modulates endothelial cell senescence via silent information regulator 1. *Circulation* 2009; **120**: 1524-1532 [PMID: 19786632 DOI: 10.1161/CIRCULATIONAHA.109.864629]
74 **Zhao T**, Li J, Chen AF. MicroRNA-34a induces endothelial progenitor cell senescence and impedes its angiogenesis via suppressing silent information regulator 1. *Am J Physiol Endocrinol Metab* 2010; **299**: E110-E116 [PMID: 20424141 DOI: 10.1152/ajpendo.00192.2010]
75 **Liu X**, Cheng Y, Yang J, Xu L, Zhang C. Cell-specific effects of miR-221/222 in vessels: molecular mechanism and therapeutic application. *J Mol Cell Cardiol* 2012; **52**: 245-255 [PMID: 22138289 DOI: 10.1016/j.yjmcc.2011.11.008]
76 **Doebele C**, Bonauer A, Fischer A, Scholz A, Reiss Y, Urbich C, Hofmann WK, Zeiher AM, Dimmeler S. Members of the microRNA-17-92 cluster exhibit a cell-intrinsic antiangiogenic function in endothelial cells. *Blood* 2010; **115**: 4944-4950 [PMID: 20299512 DOI: 10.1182/blood-2010-01-264812]
77 **Bai Y**, Bai X, Wang Z, Zhang X, Ruan C, Miao J. MicroRNA-126 inhibits ischemia-induced retinal neovascularization via regulating angiogenic growth factors. *Exp Mol Pathol* 2011; **91**: 471-477 [PMID: 21586283 DOI: 10.1016/j.yexmp.2011.04.016]
78 **Liu B**, Peng XC, Zheng XL, Wang J, Qin YW. MiR-126 restoration down-regulate VEGF and inhibit the growth of lung cancer cell lines in vitro and in vivo. *Lung Cancer* 2009; **66**: 169-175 [PMID: 19223090 DOI: 10.1016/j.lungcan.2009.01.010]
79 **McArthur K**, Feng B, Wu Y, Chen S, Chakrabarti S. MicroRNA-200b regulates vascular endothelial growth factor-mediated alterations in diabetic retinopathy. *Diabetes* 2011; **60**: 1314-1323 [PMID: 21357793 DOI: 10.2337/db10-1557]
80 **Lee YB**, Bantounas I, Lee DY, Phylactou L, Caldwell MA, Uney JB. Twist-1 regulates the miR-199a/214 cluster during development. *Nucleic Acids Res* 2009; **37**: 123-128 [PMID: 19029138 DOI: 10.1093/nar/gkn920]
81 **van Mil A**, Grundmann S, Goumans MJ, Lei Z, Oerlemans MI, Jaksani S, Doevendans PA, Sluijter JP. MicroRNA-214 inhibits angiogenesis by targeting Quaking and reducing angiogenic growth factor release. *Cardiovasc Res* 2012; **93**: 655-665 [PMID: 22227154 DOI: 10.1093/cvr/cvs003]
82 **Noveroske JK**, Lai L, Gaussin V, Northrop JL, Nakamura H, Hirschi KK, Justice MJ. Quaking is essential for blood vessel development. *Genesis* 2002; **32**: 218-230 [PMID: 11892011]
83 **Wang P**, Luo Y, Duan H, Xing S, Zhang J, Lu D, Feng J, Yang D, Song L, Yan X. MicroRNA 329 suppresses angiogenesis by targeting CD146. *Mol Cell Biol* 2013; **33**: 3689-3699 [PMID: 23878390 DOI: 10.1128/MCB.00343-13]
84 **Zheng C**, Qiu Y, Zeng Q, Zhang Y, Lu D, Yang D, Feng J, Yan X. Endothelial CD146 is required for in vitro tumor-induced angiogenesis: the role of a disulfide bond in signaling and dimerization. *Int J Biochem Cell Biol* 2009; **41**: 2163-2172 [PMID: 19782948 DOI: 10.1016/j.biocel.2009.03.014]
85 **Fujita S**, Ito T, Mizutani T, Minoguchi S, Yamamichi N, Sakurai K, Iba H. miR-21 Gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. *J Mol Biol* 2008; **378**: 492-504 [PMID: 18384814 DOI: 10.1016/j.jmb.2008.03.015]
86 **Liu LZ**, Li C, Chen Q, Jing Y, Carpenter R, Jiang Y, Kung HF, Lai L, Jiang BH. MiR-21 induced angiogenesis through AKT and ERK activation and HIF-1α expression. *PLoS One* 2011; **6**: e19139 [PMID: 21544242 DOI: 10.1371/journal.pone.0019139]
87 **Weber M**, Baker MB, Moore JP, Searles CD. MiR-21 is induced in endothelial cells by shear stress and modulates apoptosis and eNOS activity. *Biochem Biophys Res Commun* 2010; **393**: 643-648 [PMID: 20153722 DOI: 10.1016/j.bbrc.2010.02.045]
88 **Guduric-Fuchs J**, O'Connor A, Cullen A, Harwood L, Medina RJ, O'Neill CL, Stitt AW, Curtis TM, Simpson DA. Deep sequencing reveals predominant expression of miR-21 amongst the small non-coding RNAs in retinal microvascular endothelial cells. *J Cell Biochem* 2012; **113**: 2098-2111 [PMID: 22298343 DOI: 10.1002/jcb.24084]
89 **Ling H**, Fabbri M, Calin GA. MicroRNAs and other non-coding RNAs as targets for anticancer drug development. *Nat Rev Drug Discov* 2013; **12**: 847-865 [PMID: 24172333]
90 **Séguin RM**, Ferrari N. Emerging oligonucleotide therapies for asthma and chronic obstructive pulmonary disease. *Expert Opin Investig Drugs* 2009; **18**: 1505-1517 [PMID: 19715448 DOI: 10.1517/13543780903179294]
91 **Thum T**. MicroRNA therapeutics in cardiovascular medicine. *EMBO Mol Med* 2012; **4**: 3-14 [PMID: 22162462 DOI: 10.1002/emmm.201100191]
92 **Redis RS**, Calin S, Yang Y, You MJ, Calin GA. Cell-to-cell miRNA transfer: from body homeostasis to therapy. *Pharmacol Ther* 2012; **136**: 169-174 [PMID: 22903157 DOI: 10.1016/j.pharmthera.2012.08.003]
93 **Creemers EE**, Tijsen AJ, Pinto YM. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? *Circ Res* 2012; **110**: 483-495 [PMID:

22302755 DOI: 10.1161/CIRCRESAHA.111.247452]

94 **Zhu H, Fan GC.** Extracellular/circulating microRNAs and their potential role in cardiovascular disease. *Am J Cardiovasc Dis* 2011; **1**: 138-149 [PMID: 22059153]

95 **Russo F**, Di Bella S, Nigita G, Macca V, Laganà A, Giugno R, Pulvirenti A, Ferro A. miRandola: extracellular circulating microRNAs database. *PLoS One* 2012; **7**: e47786 [PMID: 23094086 DOI: 10.1371/journal.pone.0047786]

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REVIEW

Systems biology unravels interferon responses to respiratory virus infections

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Abstract

Interferon production is an important defence against viral replication and its activation is an attractive therapeutic target. However, it has long been known that viruses perpetually evolve a multitude of strategies to evade these host immune responses. In recent years there has been an explosion of information on virus-induced alterations of the host immune response that have resulted from data-rich omics technologies. Unravelling how these systems interact and determining the overall outcome of the host response to viral infection will play an important role in future treatment and vaccine development. In this review we focus primarily on the interferon pathway and its regulation as well as mechanisms by which respiratory RNA viruses interfere with its signalling capacity.

Key words: Respiratory virus; Interferon; Systems biology; Proteomics; Genomics; Innate immunity

Core tip: Many novel regulators of innate immune signalling pathways, such as the interferon signalling pathway, have been discovered recently. These advances may be in part attributed to high-throughput systems biology techniques including genomic, proteomic, miRNA and siRNA screens, as well as through various confirmatory methods using quantitative polymerase chain reaction, microscopy, and animal models. Collectively, these studies have provided insights into novel drug targets that could boost host innate immunity or could potentially serve as broad-spectrum anti-virals against RNA respiratory viruses.

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INTRODUCTION TO SYSTEMS BIOLOGY AND INTERFERONS

Virus-host studies of a wide range of viruses have identified many host changes that occur upon infection, including the induction of a variety of anti-viral pathways. For example, these include autophagy, apoptosis, endoplasmic-reticular stress, nuclear-factor kappa B (NF-κB) and proteasomal degradation pathways as well as the topic of this review, interferon signalling. Some of these studies have utilized global genomic, transcriptomics and proteomic technologies and have led to the characterizations of "infectomes", "interactomes" and "interferomes". One of the great advantages to systems biology tools is that they can provide a relatively unbiased "bottom-up" discovery approach such as with global transcriptome and siRNA screens. These have proven useful in the characterization of innate immune responses. Biological tools for detection of specific subsets of the cell are also continually being developed, including probes for specific

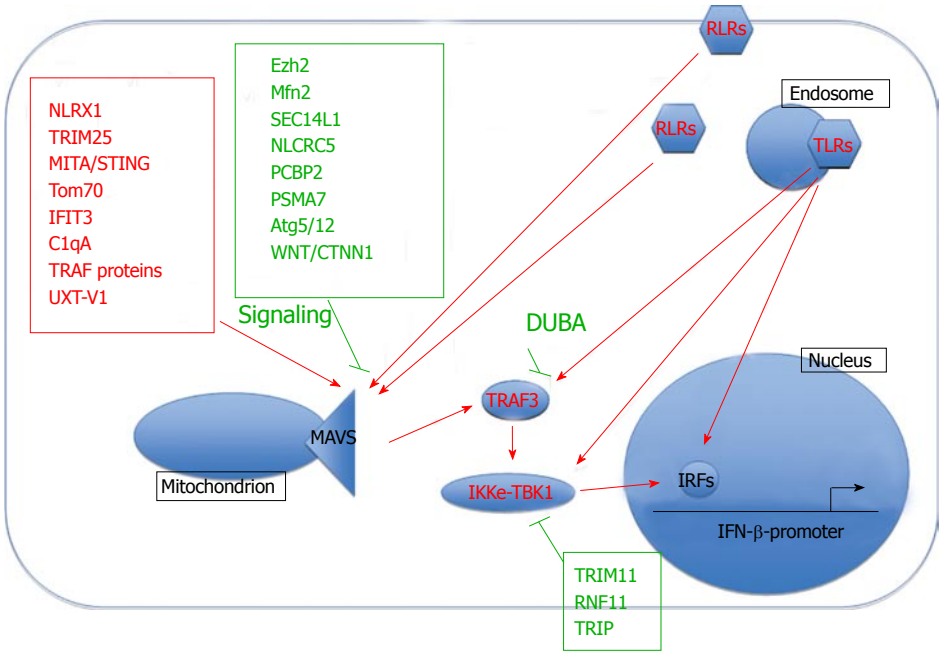

Figure 1 Interferon activation. NLRX1 : Nucleotide-binding oligomerization domain, leucine rich repeat containing X1; TRIM25: Tripartite motif-containing 25; MITA/STING: Mediator of IRF3 activation/stimulator of interferon genes protein; TRIM11: Tripartite motif containing 11; RNF11: Ring finger protein 11; TRIP: Thyroid receptor-interacting protein; DUBA: Deubiquitinating enzyme A; RLRs: RIG-like receptors; TLRs: Toll-like receptors; IFIT3: Interferon-inducible transmembrane protein 3; MAVS: Mitochondrial antiviral signaling protein; NLRC5: Nod-like receptor C5; PCBP2: Poly(rC)-binding protein 2; PSMA7: Proteasome subunit alpha type-7; TBK1: Tank binding kinase; IKKe: Inhibitor of nuclear factor kappa-B kinase; IRFs: Interferon regulatory factors; WNT/CTNNB1: Wnt/beta-catenin.

classes of enzymes, methods to detect different protein post-translational and epigenetic modifications, and subcellular fractionation techniques. As will be discussed below, many studies have begun to characterize gene transcription programs in response to viruses, have identified novel anti-viral proteins and regulators of interferon production and have experimented with novel approaches to treatment of viral infection.

The study of interferons (IFN) is one of the oldest known family of proteins with anti-viral properties. They are produced and released in response to pathogens, such as viruses and bacteria, and function in establishing an anti-viral state in host cells and activating immune cells (for review see[1]). Type I interferons in humans include IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω and are classified as such by their ability to bind the IFNAR1-IFNAR2 interferon receptor complex[2]. IFN-γ is a type Ⅱ interferon and signals through the IFNGR1-IFNGR2 receptor complex. A third class of interferons, type Ⅲ, has been proposed and would likely contain IFN-λ1, -λ2 and -λ3, which are also known as interleukin-29 (IL-29), IL-28A and IL-28B, respectively, and bind IFNLR1 (also known as IL-28 receptor-α, IL-28Rα) and IL-10Rβ[3]. Effects of interferons are numerous and depend on downstream signaling pathways. The canonical activation of Janus-Kinase-Signal Transduction Activator (JAK-STAT) signalling[4], for example, induces a variety of interferon-stimulated genes (ISGs) of which some have known anti-viral activities. Activation of mitogen-activated protein kinases[5] has also been shown to have anti-viral as well as anti-proliferative effects. In contrast, phosphatidylinositol 3-kinase activation[6] induces cell proliferation and increased protein synthesis (for review see[7]). Autophagy has also been described as an inducer of interferon[8,9] as well as being induced by interferons[10,11]. The interactions and cross-regulation of these pathways are complex and are not well defined but overall, the ability of the host to mount an effective interferon response typically plays a significant protective role against viral pathogenicity.

Regulation of the interferon signalling pathway is influenced by multiple cellular regulatory systems including phosphorylation, ubiquitination and miRNA silencing. In addition, viral components such as viral proteins and viral RNA can also significantly impact interferon production by the infected host cell. Systems biology approaches have substantially contributed to understanding the interactions of these various regulatory networks, the overall outcome of their actions, and their impact on respiratory virus replication. For example, it is becoming increasingly popular to combine various omics technologies such as transcriptome and proteomic screens with functional validation using techniques such as siRNA screens, pPCR and microscopy imaging.

REGULATION OF INTERFERON INDUCTION

Activation of viral pattern recognition receptors

Innate immune responses are initially triggered in response to viral infection through the recognition of highly conserved pathogen association molecular patterns (PAMPs). In terms of RNA viruses this typically involves activation of RIG-like (RLR), Toll-like (TLR) and Nod-like receptors (NLR) in the cytoplasm and at membranous surfaces such as the plasma membrane, endosomes and endoplasmic reticulum. A major outcome of RLR and TLR activation is the production of interferons. This induction, and its regulation, will be the focus of this review (summarized in Figure 1).

Coordination of antiviral responses at the mitochondrial outer membrane

An important event following RLR activation consists

of the formation of mitochondrion-centric anti-viral signalling complexes that regulate interferon and NF-κB signalling cascades and subsequent immune responses. The mitochondrial anti-viral signaling protein (MAVS)/virus-induced signaling adaptor/interferon-beta promoter stimulator protein 1/Cardif protein is central to this process. Located at the outer mitochondrial membrane, it acts as a scaffolding protein that interacts with a variety of different host proteins that regulate downstream signalling pathways. There are many activators and facilitators of MAVS-mediated signalling and some of the most recently discovered ones include retinoic-acid inducible gene I (RIG-I), nucleotide-binding oligomerization domain, leucine rich repeat containing X1 (NLRX1), MITA/Stimulator of interferon genes protein[12], Tom70[13], interferon-induced protein with tetratricopeptide repeats 3 (IFIT3)[14], C1qA[15], tumor necrosis factor receptor associated factor (TRAF) proteins[16] and UXT-V1[17]. The formation of MAVS-mediated complexes can subsequently lead to the recruitment of tank binding kinase (TBK1) and inhibitor of nuclear factor kappa-B kinase (IKKe). However, this process is also carefully controlled through recruitment of negative regulators such as Ezh2[18], Mfn2[19], SEC14L1[20] and Wnt/beta-catenin (WNT/CTNNB1) signalling[21]. MAVS has also been described to associate with the endoplasmic reticulum[12, 22-24], peroxisomes[22], and autophagosomes[25], although the outcome of these events are beyond the scope of this review. For further details we direct readers to a review by Belgnaoui[26]. Overall, MAVS-interacting partners influence the extent of activation or inhibition of downstream interferon and NF-κB anti-viral pathways.

Activation of interferon regulatory factors

RLR and TLR activation culminate in the phosphorylation, activation and nuclear translocation of various IRF transcription factors. Two well-known factors are IRF3 and IRF7, which can be activated by kinases TBK1, IKKi, TAK1, and interleukin-1 receptor-associated kinase. This activation is carefully controlled through ubiquitin-mediated degradation of TBK1, which can be negatively regulated by tripartite motif containing 11 (TRIM11)[27], ring finger protein 11 (RNF11)[28] and thyroid receptor-interacting protein[29]. Interaction with other molecules such as TRAF3, DDX3 [(DEAD (Asp-Glu-Ala-Asp) box polypeptide 3][30] and nef-associated protein 1[31] can also modulate downstream signalling. Interestingly, a recent study using triple IRF3/IRF5/IRF7 knockout mice[32] demonstrated a formerly unappreciated role of IRF5 in interferon induction in myeloid dendritic cells. Genome-wide IRF1 binding sites have also been characterized in primary monocytes[33]. Overall, the IRF family members are essential mediators of interferon signalling in response to RNA viral infection.

Other regulators of interferon production

Numerous other proteins have been described in regulating interferon production including activators Gab1[34] and suppressors protein tyrosine phosphatase 1[35], forkhead box protein O3[36], and toll/interleukin-1 receptor domain containing adaptor molecule 2 (TRIF) degradation[37]. Several E3 ligases promote interferon signalling such as Pellino1[38], TRIM25[39], TRIM32[40] and Riplet[41]. Other E3 ligases have been characterized with a negative regulatory role in interferon production, such as Smurf1[42], RNF125[43], disintegrin and metalloproteinase domain-containing protein 15[44], TRIM38[37], TRIM11[27] and TRIM21[45]. Finally, several deubiquitinases appear to negatively regulate interferon responses, for example OTUB1[46] and UCHL1[47]. In addition, miRNAs are emerging as important regulators of interferon-mediated anti-viral responses such as miR-155[48], miR-21[49,50], miR-146[51] and miR-466l[52].

JAK-STAT signalling

Secreted type I interferons bind to interferon receptors at the cell-membrane and induce the janus activated kinase-signal transducer and activator (JAK-STAT) pathway. The bound receptor activates self-catalyzed kinase activity and causes phosphorylation, dimerization and nuclear translocation of STAT proteins. Ubiquitination has also been demonstrated to negatively regulate this pathway, for example, by ubiquitinating JAK1[53] and STAT1[54,55] as well as through binding of suppressor of cytokine signaling and protein inhibitor of activated STAT proteins, which recruit E3 ligases[56]. In addition, mir-19a has been identified as a JAK-STAT regulator[57].

ISG-induced gene transcription

There are many different interferon transcriptional programs that depend on factors such as the receptor and JAK isoforms, as well as the type of STAT dimer[58] that are induced. These in turn are dependent on the stimulus, species, cell type, and co-stimuli. Because of this complexity, the study of interferon-stimulated gene (ISG) transcription patterns has benefited greatly from omics studies and has begun to provide powerful insights into the effects of interferons on host transcription. The response to interferon-gamma, for example, has been a source of recent interest and has been demonstrated to regulate ISGs at both the mRNA[59] and miRNA level[59,60]. A few specific miRNAs that have been identified as interferon regulators include miR-203[61] and miR-9[62]. Genome-wide DNA-binding sites for STAT1 have also been characterized using ChIP-Seq[63]. Many quantitative proteomic studies have also identified altered expression patterns of interferon-induced proteins upon various stimuli, especially after viral infection; some of these genes have also been found to be dependent upon NF-κB signalling[64].

Microarrays and quantitative proteomics: Identifying global viral-induced alterations to the host response

A variety of models have been used to study the induction of innate immune pathways following virus infection, including epithelial cells, productive and abortive

Table 1 For example references

	Cell type	Proteomics	Genomics
Respiratory syncytial virus	Epithelial cells	[95-98]	[99]
	Macrophages	-	[65,100]
	Cord blood	-	[101]
Coronavirus	Epithelial cells	[79,80,102-104]	[76-78]
	Pro-monocytes		-
Influenza	Macrophages	[70,73,105,106]	[110]
	Epithelial cells	[71, 72, 107]	[111]
	Mice	[108]	[112,113]
	Ferrets	-	[114,115]
	Macaques	[109]	[112,116]
Reovirus	Epithelial cells	[117-120]	[64]
	Myocytes	[119]	-
	Mice	-	[121]
Rhinovirus	Epithelial cells	-	[74,122,123]
	Dendritic cells	[66]	-
	Human nasal cells	-	[75]

infections in macrophages[65], dendritic cells[66] and animal models (see Table 1). Microarrays have been particularly popular for these studies due to its ability to provide a comprehensive analysis of the entire cellular genome with relatively sensitive quantification of gene expression (see[67] for review of microarray technologies). Quantitative proteomic studies have also been important in validating these findings at the protein level and have been useful, for example, in the search for biomarkers. Many respiratory viruses, such as influenza[68-73], reovirus, and rhinovirus[74,75], demonstrate a robust activation of antiviral pathways and pro-inflammatory cytokines. Both genomic and proteomic analyses have demonstrated hubs of gene and protein induction that are induced by key transcriptional factors such as IRFs, STAT proteins, NF-κB and JNK. On the other hand, genomic profiling of respiratory syncytial virus[65] and pathogenic coronaviruses such as severe acute respiratory syndrome (SARS) and EMC strains have been reported to elicit weaker innate immune responses[76-78]. The absence of interferon signalling has also been recapitulated in several proteomic viral-host studies[79-81].

Analyses of microRNA expression during influenza have recently begun to emerge in a variety of models including respiratory epithelial cells[82-85], human blood[86], immune cells[87-89] and lung tissue in animal models[90,91]. Collectively these have identified roles for miR-18a[86,92] and miR-223[86,93] in negative regulation of STAT3, mir-29 in IFN-γ1 production[89], and miR-449b as a positive regulator of IFN-β production[85]. miR-23b has also been identified as a novel anti-viral molecule that is induced through RLR signaling during rhinovirus infection[94].

Strain differences: One of the fundamental questions of virology revolves around deciphering factors of pathogenesis. Hence, some studies have attempted to identify pathways that are differentially altered by pathogenic viral strains compared to less pathogenic strains. Influenza has been particularly well studied in this respect and several host factors have been identified that are unique to the replication of strains such as the pathogenic avian H5N1, the p2009 swine flu and the 1918 strain[69,70,108,124]. However, rather than inducing radically different cell responses, many different influenza strains have been found to activate surprisingly similar immune signatures (reviewed in[125]). It was, instead, the degree and timing of activation and resolution[125] of these pathways that was found to significantly impact the severity of disease[126]. Dysregulation of the host inflammatory response in particular is a major determinant of influenza pathogenicity and is influenced by both viral and host factors[127]. Different rhinovirus strains, for example type 14 and 1B[128,129], have also been demonstrated to have different abilities to attenuate interferon production and secretion from epithelial cells. This effect has been attributed to the inhibition of IRF3 dimerization[74,129] but the viral mechanism leading to this is unknown.

Cell type differences: Cell types have also been demonstrated to express different basal levels of interferon and hence, have different innate susceptibilities to viral infection[130,131]. For example, a direct comparison of interferon signaling between primary bronchial lung epithelial cells and the A549 continuous alveolar epithelial cell line suggested differences between either primary and cancer cell lines and/or epithelial cells of different origins in the lung[72]. Additionally, different cell types have been shown to influence the degree of interferon activation after reovirus infection[132].

Correlation of interferon signaling with pathogenesis: Generally interferon production is considered protective against viral infections. It has been shown numerous times that cells that produce less interferon, such as Vero cells, are more susceptible to viral infection and produce high titers of the virus[133]. The extent of interferon inhibition by the influenza non-structural (NS)-1 protein[134] and RSV NS1 and NS2 proteins[135,136] has also been extensively studied and correlates negatively with pathogenicity[137,138]. Similarly, models in which interferon signaling has been disrupted, such as by deleting IFNR, can produce high viral titers[139] and display increased lung tissue pathology[140]. Conversely, type I interferon signaling has also been shown to contribute to secondary bacterial infections[141,142]. In some studies the degree of interferon induction correlated positively with the degree of pathogenicity. For example, the reovirus T3D strain is considered more pathogenic the T1L strain, but the T3D strain was found to induce higher levels of innate immunity proteins[64,117,118]. The role of interferons in these situations is not currently understood.

Altered innate immune responses in chronic lung diseases: Many studies with rhinovirus have investigated differences in the immune response between healthy and non-healthy donor cells. In one study, infection of chronic obstructive pulmonary disorder (COPD) epithelial cells induced higher transcription levels of cytokines, chemokines, RNA helicases, interferons and increased apoptosis compared to infection of healthy control cells. In addi-

tion, basal levels of several antiviral signalling pathways were altered in COPD patients[128]. Similarly, asthma-derived epithelial cells also showed altered expression of several immunity genes both at basal levels and during rhinovirus infection[122,143]. Modulation of rhinovirus-induced host responses has also been investigated in the presence of Echinacea extracts and cigarette smoke[123].

Core innate immune responses shared by multiple respiratory viruses: While many studies that have been discussed in this review have focused on identifying global host responses towards a single virus, a few studies have directly compared viruses from multiple families. For example, Smith *el al*[144]. identified common gene networks that were activated in response to seven respiratory viruses: influenza A virus, respiratory syncytial virus, rhinovirus, SARS-coronavirus, metapneumonia virus, coxsackievirus and cytomegalovirus[144]. Among those responses were pathways associated with a general immune response including interferon signalling[144]. A second study also identified core immune responses to four respiratory viruses including apoptosis induction, endoplasmic reticulum stress and interferon signalling[98]. In addition several host interferon-induced proteins have been tested against multiple families and strains of viruses. For example, IFIT1[145], Interferon-inducible transmembrane (IFITM) proteins[146], ISG15[147,148] and Viperin[149-152] protect against multiple virus families.

Overall, microarrays and quantitative proteomics have allowed sensitive and comprehensive analyses of the host genome, and have contributed substantially to understanding the types and kinetics of signaling pathways that are activated upon viral infections.

Identification of host-virus interactions and novel restriction factors

Interactomes, viral-mediated antagonism of interferon signaling: As many viruses encode interferon-antagonizing proteins, there has been significant interest in defining their interacting partners in the host cell. Several studies have also been undertaken to identify host proteins that recognize dsRNA and 5'pppRNA. This has, for example, led to the discovery and characterization of the IFIT family[145] and their role anti-viral innate immunity.

Influenza: The influenza NS1 protein is a well-known antagonist of interferon signalling and is able to interfere with multiple anti-viral pathways. Viral-host studies have identified additional host proteins that interact with the influenza NS1 protein, using either plasmid-based expression of NS1[153-155] or during whole virus infection[153,156]. Collectively, the integration of multiple interactome studies has allowed networks such as Flu-Pol to be established which provide the basis for comparing differences and commonalities between influenza strains and cell types and are useful for targeted drug design.

RSV: RSV proteins NS1 and NS2 strongly inhibit IFN α/β by preventing the phosphorylation of the IFN regulatory factor-3[157,158] as well as activation of NLRX1 and RIG-I[35]. Additionally, the RSV NS1 protein interferes with interferon signaling through interaction with an elonginC-cullin2 E3 ligase complex that ubiquitinates and degrades STAT2[97,159]. RSV NS1 and NS2 have also been shown to alter miRNA expression, which can contribute to antagonism of interferon and NF-κB responses[160].

Coronavirus: In studies with coronaviruses, it has been previously proposed that the viral deubiquitinase, PLpro, plays a major role in suppressing interferon-alpha induction. In support of this idea, Li *et al*[161] recently demonstrated that PLpro overexpression mediated the down-regulation of mitogen-activated protein kinase and up-regulation of the ubiquitinase Ubiquitin ligase (UBC E2-25k). The open reading frame 6 protein has also been shown to attenuate antiviral responses by sequestering host nuclear impact factors including STAT1[162], vitamin D receptor, cyclic AMP-responsive element-binding protein 1, mothers against decapentaplegic homolog 4, p53, Epas I and Oct3/4[163].

Rhinovirus: Despite induction of interferon gene transcription, rhinovirus (type 14) infection can strongly attenuate interferon secretion from epithelial cells. This effect has been attributed to the inhibition of IRF3 dimerization[74,129] but the viral mechanism leading to this is unknown. In contrast, rhinovirus 1B readily stimulated interferon production in bronchial smooth muscle cells[164], suggesting different interferon regulation between strains and/or cell types.

Reovirus: The degree of IFN-α/β induction after reovirus infection has been attributed to both host and viral factors but is not well understood. However, repression of interferon signaling has been mapped to the *M1*, *L2* and *S2*[132,165] genes.

Knockdown/Knockout studies: siRNA technology has been important in testing functional effects of interferon-induced proteins. Both whole genome siRNA screens, and individual knockdown experiments have discovered and validated anti-viral effects of many including interferon-induced proteins such as the IFITM1-3 proteins[166], IRF3 and IRF2 (Shapira), ISG15[147] and Viperin[167]. In contrast, several interferon pathway members have been assigned pro-viral functions such as MxB[168] and IFIT2[156,168].

Knock-out animals have also underscored the protective effects of interferon signaling during respiratory virus infections, for example, ISG15$^{-/-}$[147,169], IFNAR$^{-/-}$[170], and MxA$^{-/-}$[171]. In addition to its role in innate immunity, interferons have also been demonstrated to have profound effects on the adaptive immune system, for example, by priming CD^{+} T-cells during influenza infection[172] and inhibiting neurotropism of reovirus infection[173,174]. Although discussion of the effects of interferon on whole host immunity is beyond the scope of this review, further discussion can be found in several comprehensive reviews[175,176].

Collectively, these studies have provided fundamental insights into how cells respond to RNA virus infection and have highlighted the importance of interferon induction in restricting virus replication and activating an appropriate host immune response. Many new and unex-

pected regulators of interferon signalling have been discovered and have demonstrated how multiple anti-viral networks interact such as ubiquitin-mediated regulation of interferon signalling molecules. As large omics studies move forward, it will become possible to compare and draw connections between anti-viral networks that are induced by different viruses.

FUTURE DIRECTIONS: INTERFERON SIGNALING AS A BROAD-SPECTRUM ANTI-VIRAL PATHWAY?

Using interferons therapeutically has been most extensively studied in models of hepatitis. However, it has also shown some promise in protecting against a variety of other virus families, including the respiratory viruses discussed in this review. For example, exogenous IFN-alpha treatment has proven effective against influenza[177-179], rhinovirus[128,180] and coronavirus[181-183]. Interferons are also important in protecting against reovirus infections[184]. The role of type Ⅲ interferons is generally not as well understood as type I but may also afford protection against respiratory viruses[185].

Interferons can also be endogenously elicited through a variety of RLR and TLR agonists. 5'pppRNA, for example, is a well-known and potent RIG-I agonist and has been demonstrated to protect against both RNA and DNA viruses, including Dengue virus, influenza, hepatitis C and human immunodeficiency virus-1[186]. Similarly, TLR agonists such as dsRNA[187,188] or inosine-containing ssRNA[189] have been shown to protect against coronavirus, influenza, and respiratory syncytial virus infections in mice. A commercial compound, Arbidol, has also had some success in neutralizing various respiratory viruses such as influenza, rhinovirus, adenovirus, coxsackie virus and RSV[190]. Additional small molecules that induce type I interferons have recently been identified using high-throughput screens[191,192]. Alternatively, inhibiting antagonists of interferon signaling can also boost the production of interferon. As discussed above, these antagonists can either be host molecules or viral proteins, and inhibitors to each have been described[193]. Interestingly, ribavirin treatment of RSV-infected epithelial cells was shown to enhance interferon-stimulated gene expression[194] and treating RSV-infected macrophages with lovastatin was shown to blunt pro-inflammatory cytokine gene expression[100]. These therapies may have potential for broad-spectrum anti-viral properties.

Despite successfully treating some viral infection with interferon, it has also been noted that interferon stimulation can increase lung inflammation. Many gene array studies have also positively correlated pathogenicity or cytopathology with the induction of interferon and/or inflammatory genes. For example, the severe pathology of the 1918 influenza pandemic and of H5N1 (bird flu) viruses has been attributed to a "cytokine storm" (reviewed by[125]). It is therefore important to identify the mechanisms behind interferon-dependent protection against viruses. Numerous studies, for example, have suggested that MxA is a major effector of INF-α pre-treatment against influenza[195-197]; other newly identified interferon-induced anti-viral proteins include IFITM proteins[146,198], ISG15[147] and Viperin[149-152]. It may also be useful to combine interferon treatment with anti-inflammatory compounds such as curcumin [199-201], resveratrol[202], S1P agonists[203,204], COX-2 inhibitors[205,206] and statins[100,207].

CONCLUSION

The study of immune responses to viral infection has benefited greatly from viral proteomic studies. However, knowledge of proteomic subsets is still limited and future studies could provide more detailed insight into the dynamics of protein localization, activity and regulation through post-translational modifications during virus infection. Based on current technologies and identified networks, it may be beneficial to also investigate alterations of the phosphoproteome, ubiquitome, and the activity of proteasomes after viral infection. The development of broad-spectrum anti-virals has also shown some potential and could benefit from comparative analyses of multiple viruses.

REFERENCES

1 **Durbin RK**, Kotenko SV, Durbin JE. Interferon induction and function at the mucosal surface. *Immunol Rev* 2013; **255**: 25-39 [PMID: 23947345 DOI: 10.1111/imr.12101]

2 **de Weerd NA**, Samarajiwa SA, Hertzog PJ. Type I interferon receptors: biochemistry and biological functions. *J Biol Chem* 2007; **282**: 20053-20057 [PMID: 17502368 DOI: 10.1074/jbc.R700006200]

3 **Kotenko SV**, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 2003; **4**: 69-77 [PMID: 12483210 DOI: 10.1038/ni875]

4 **Au-Yeung N**, Mandhana R, Horvath CM. Transcriptional regulation by STAT1 and STAT2 in the interferon JAK-STAT pathway. *JAKSTAT* 2013; **2**: e23931 [PMID: 24069549 DOI: 10.4161/jkst.23931]

5 **Arthur JS**, Ley SC. Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol* 2013; **13**: 679-692 [PMID: 23954936 DOI: 10.1038/nri3495]

6 **Uddin S**, Yenush L, Sun XJ, Sweet ME, White MF, Platanias LC. Interferon-alpha engages the insulin receptor substrate-1 to associate with the phosphatidylinositol 3'-kinase. *J Biol Chem* 1995; **270**: 15938-15941 [PMID: 7608146 DOI: 10.1074/jbc.270.27.15938]

7 **Platanias LC**. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* 2005; **5**: 375-386 [PMID: 15864272 DOI: 10.1038/nri1604]

8 **Henault J**, Martinez J, Riggs JM, Tian J, Mehta P, Clarke L, Sasai M, Latz E, Brinkmann MM, Iwasaki A, Coyle AJ, Kolbeck R, Green DR, Sanjuan MA. Noncanonical autophagy is required for type I interferon secretion in response to DNA-immune complexes. *Immunity* 2012; **37**: 986-997 [PMID: 23219390 DOI: 10.1016/j.immuni.2012.09.014]

9 **Law AH**, Lee DC, Yuen KY, Peiris M, Lau AS. Cellular response to influenza virus infection: a potential role for autophagy in CXCL10 and interferon-alpha induction. *Cell Mol Immunol* 2010; **7**: 263-270 [PMID: 20473322 DOI: 10.1038/

cmi.2010.25]
10 **Schmeisser H**, Fey SB, Horowitz J, Fischer ER, Balinsky CA, Miyake K, Bekisz J, Snow AL, Zoon KC. Type I interferons induce autophagy in certain human cancer cell lines. *Autophagy* 2013; **9**: 683-696 [PMID: 23419269 DOI: 10.4161/auto.23921]
11 **Kalvakolanu DV**, Gade P. IFNG and autophagy: a critical role for the ER-stress mediator ATF6 in controlling bacterial infections. *Autophagy* 2012; **8**: 1673-1674 [PMID: 22874566 DOI: 10.4161/auto.21403]
12 **Zhong B**, Yang Y, Li S, Wang YY, Li Y, Diao F, Lei C, He X, Zhang L, Tien P, Shu HB. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* 2008; **29**: 538-550 [PMID: 18818105 DOI: 10.1016/j.immuni.2008.09.003]
13 **Liu XY**, Wei B, Shi HX, Shan YF, Wang C. Tom70 mediates activation of interferon regulatory factor 3 on mitochondria. *Cell Res* 2010; **20**: 994-1011 [PMID: 20628368 DOI: 10.1038/cr.2010.103]
14 **Liu XY**, Chen W, Wei B, Shan YF, Wang C. IFN-induced TPR protein IFIT3 potentiates antiviral signaling by bridging MAVS and TBK1. *J Immunol* 2011; **187**: 2559-2568 [PMID: 21813773 DOI: 10.4049/jimmunol.1100963]
15 **Wang Y**, Tong X, Zhang J, Ye X. The complement C1qA enhances retinoic acid-inducible gene-I-mediated immune signalling. *Immunology* 2012; **136**: 78-85 [PMID: 22260551 DOI: 10.1111/j.1365-2567.2012.03561.x]
16 **Liu S**, Chen J, Cai X, Wu J, Chen X, Wu YT, Sun L, Chen ZJ. MAVS recruits multiple ubiquitin E3 ligases to activate antiviral signaling cascades. *Elife* 2013; **2**: e00785 [PMID: 23951545 DOI: 10.7554/eLife.00785]
17 **Huang Y**, Liu H, Ge R, Zhou Y, Lou X, Wang C. UXT-V1 facilitates the formation of MAVS antiviral signalosome on mitochondria. *J Immunol* 2012; **188**: 358-366 [PMID: 22131337 DOI: 10.4049/jimmunol.1102079]
18 **Chen CH**, Zhang XQ, Lo CW, Liu PF, Liu YT, Gallo RL, Hsieh MF, Schooley RT, Huang CM. The essentiality of alpha-2-macroglobulin in human salivary innate immunity against new H1N1 swine origin influenza A virus. *Proteomics* 2010; **10**: 2396-2401 [PMID: 20391540 DOI: 10.1002/pmic.200900775]
19 **Yasukawa K**, Oshiumi H, Takeda M, Ishihara N, Yanagi Y, Seya T, Kawabata S, Koshiba T. Mitofusin 2 inhibits mitochondrial antiviral signaling. *Sci Signal* 2009; **2**: ra47 [PMID: 19690333 DOI: 10.1126/scisignal.2000287]
20 **Li MT**, Di W, Xu H, Yang YK, Chen HW, Zhang FX, Zhai ZH, Chen DY. Negative regulation of RIG-I-mediated innate antiviral signaling by SEC14L1. *J Virol* 2013; **87**: 10037-10046 [PMID: 23843640 DOI: 10.1128/JVI.01073-13]
21 **Baril M**, Es-Saad S, Chatel-Chaix L, Fink K, Pham T, Raymond VA, Audette K, Guenier AS, Duchaine J, Servant M, Bilodeau M, Cohen E, Grandvaux N, Lamarre D. Genome-wide RNAi screen reveals a new role of a WNT/CTNNB1 signaling pathway as negative regulator of virus-induced innate immune responses. *PLoS Pathog* 2013; **9**: e1003416 [PMID: 23785285 DOI: 10.1371/journal.ppat.1003416]
22 **Horner SM**, Liu HM, Park HS, Briley J, Gale M. Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus. *Proc Natl Acad Sci USA* 2011; **108**: 14590-14595 [PMID: 21844353 DOI: 10.1073/pnas.1110133108]
23 **Sun W**, Li Y, Chen L, Chen H, You F, Zhou X, Zhou Y, Zhai Z, Chen D, Jiang Z. ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. *Proc Natl Acad Sci USA* 2009; **106**: 8653-8658 [PMID: 19433799 DOI: 10.1073/pnas.0900850106]
24 **Ishikawa H**, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* 2008; **455**: 674-678 [PMID: 18724357 DOI: 10.1038/nature07317]
25 **Jounai N**, Takeshita F, Kobiyama K, Sawano A, Miyawaki A, Xin KQ, Ishii KJ, Kawai T, Akira S, Suzuki K, Okuda K. The Atg5 Atg12 conjugate associates with innate antiviral immune responses. *Proc Natl Acad Sci USA* 2007; **104**: 14050-14055 [PMID: 17709747 DOI: 10.1073/pnas.0704014104]
26 **Belgnaoui SM**, Paz S, Hiscott J. Orchestrating the interferon antiviral response through the mitochondrial antiviral signaling (MAVS) adapter. *Curr Opin Immunol* 2011; **23**: 564-572 [PMID: 21865020 DOI: 10.1016/j.coi.2011.08.001]
27 **Lee Y**, Song B, Park C, Kwon KS. TRIM11 negatively regulates IFNβ production and antiviral activity by targeting TBK1. *PLoS One* 2013; **8**: e63255 [PMID: 23675467 DOI: 10.1371/journal.pone.0063255]
28 **Charoenthongtrakul S**, Gao L, Parvatiyar K, Lee D, Harhaj EW. RING finger protein 11 targets TBK1/IKKi kinases to inhibit antiviral signaling. *PLoS One* 2013; **8**: e53717 [PMID: 23308279 DOI: 10.1371/journal.pone.0053717]
29 **Zhang M**, Wang L, Zhao X, Zhao K, Meng H, Zhao W, Gao C. TRAF-interacting protein (TRIP) negatively regulates IFN-β production and antiviral response by promoting proteasomal degradation of TANK-binding kinase 1. *J Exp Med* 2012; **209**: 1703-1711 [PMID: 22945920 DOI: 10.1084/jem.20120024]
30 **Gu L**, Fullam A, Brennan R, Schröder M. Human DEAD box helicase 3 couples IκB kinase ε to interferon regulatory factor 3 activation. *Mol Cell Biol* 2013; **33**: 2004-2015 [PMID: 23478265 DOI: 10.1128/MCB.01603-12]
31 **Zhao W**, Wang L, Zhang M, Wang P, Yuan C, Qi J, Meng H, Gao C. Tripartite motif-containing protein 38 negatively regulates TLR3/4- and RIG-I-mediated IFN-β production and antiviral response by targeting NAP1. *J Immunol* 2012; **188**: 5311-5318 [PMID: 22539786 DOI: 10.4049/jimmunol.1103506]
32 **Lazear HM**, Lancaster A, Wilkins C, Suthar MS, Huang A, Vick SC, Clepper L, Thackray L, Brassil MM, Virgin HW, Nikolich-Zugich J, Moses AV, Gale M, Früh K, Diamond MS. IRF-3, IRF-5, and IRF-7 coordinately regulate the type I IFN response in myeloid dendritic cells downstream of MAVS signaling. *PLoS Pathog* 2013; **9**: e1003118 [PMID: 23300459 DOI: 10.1371/journal.ppat.1003118]
33 **Shi L**, Perin JC, Leipzig J, Zhang Z, Sullivan KE. Genome-wide analysis of interferon regulatory factor I binding in primary human monocytes. *Gene* 2011; **487**: 21-28 [PMID: 21803131 DOI: 10.1016/j.gene.2011.07.004]
34 **Zheng Y**, An H, Yao M, Hou J, Yu Y, Feng G, Cao X. Scaffolding adaptor protein Gab1 is required for TLR3/4- and RIG-I-mediated production of proinflammatory cytokines and type I IFN in macrophages. *J Immunol* 2010; **184**: 6447-6456 [PMID: 20435932 DOI: 10.4049/jimmunol.0901750]
35 **Xu H**, An H, Hou J, Han C, Wang P, Yu Y, Cao X. Phosphatase PTP1B negatively regulates MyD88- and TRIF-dependent proinflammatory cytokine and type I interferon production in TLR-triggered macrophages. *Mol Immunol* 2008; **45**: 3545-3552 [PMID: 18571728 DOI: 10.1016/j.molimm.2008.05.006]
36 **Luron L**, Saliba D, Blazek K, Lanfrancotti A, Udalova IA. FOXO3 as a new IKK-ε-controlled check-point of regulation of IFN-β expression. *Eur J Immunol* 2012; **42**: 1030-1037 [PMID: 22531926 DOI: 10.1002/eji.201141969]
37 **Xue Q**, Zhou Z, Lei X, Liu X, He B, Wang J, Hung T. TRIM38 negatively regulates TLR3-mediated IFN-β signaling by targeting TRIF for degradation. *PLoS One* 2012; **7**: e46825 [PMID: 23056470 DOI: 10.1371/journal.pone.0046825]
38 **Enesa K**, Ordureau A, Smith H, Barford D, Cheung PC, Patterson-Kane J, Arthur JS, Cohen P. Pellino1 is required for interferon production by viral double-stranded RNA. *J Biol Chem* 2012; **287**: 34825-34835 [PMID: 22902624 DOI: 10.1074/jbc.M112.367557]
39 **Castanier C**, Zemirli N, Portier A, Garcin D, Bidère N, Vazquez A, Arnoult D. MAVS ubiquitination by the E3 ligase TRIM25 and degradation by the proteasome is involved in type I interferon production after activation of the

antiviral RIG-I-like receptors. *BMC Biol* 2012; **10**: 44 [PMID: 22626058 DOI: 10.1186/1741-7007-10-44]
40 **Zhang J**, Hu MM, Wang YY, Shu HB. TRIM32 protein modulates type I interferon induction and cellular antiviral response by targeting MITA/STING protein for K63-linked ubiquitination. *J Biol Chem* 2012; **287**: 28646-28655 [PMID: 22745133 DOI: 10.1074/jbc.M112.362608]
41 **Oshiumi H**, Matsumoto M, Hatakeyama S, Seya T. Riplet/RNF135, a RING finger protein, ubiquitinates RIG-I to promote interferon-beta induction during the early phase of viral infection. *J Biol Chem* 2009; **284**: 807-817 [PMID: 19017631 DOI: 10.1074/jbc.M804259200]
42 **Wang Y**, Tong X, Ye X. Ndfip1 negatively regulates RIG-I-dependent immune signaling by enhancing E3 ligase Smurf1-mediated MAVS degradation. *J Immunol* 2012; **189**: 5304-5313 [PMID: 23087404 DOI: 10.4049/jimmunol.1201445]
43 **Luo H**, Zhang Z, Zheng Z, Ke X, Zhang X, Li Q, Liu Y, Bai B, Mao P, Hu Q, Wang H. Human bocavirus VP2 upregulates IFN-β pathway by inhibiting ring finger protein 125-mediated ubiquitination of retinoic acid-inducible gene-I. *J Immunol* 2013; **191**: 660-669 [PMID: 23772026 DOI: 10.4049/jimmunol.1202933]
44 **Ahmed S**, Maratha A, Butt AQ, Shevlin E, Miggin SM. TRIF-mediated TLR3 and TLR4 signaling is negatively regulated by ADAM15. *J Immunol* 2013; **190**: 2217-2228 [PMID: 23365087 DOI: 10.4049/jimmunol.1201630]
45 **Young JA**, Sermwittayawong D, Kim HJ, Nandu S, An N, Erdjument-Bromage H, Tempst P, Coscoy L, Winoto A. Fas-associated death domain (FADD) and the E3 ubiquitin-protein ligase TRIM21 interact to negatively regulate virus-induced interferon production. *J Biol Chem* 2011; **286**: 6521-6531 [PMID: 21183682 DOI: 10.1074/jbc.M110.172288]
46 **Li S**, Zheng H, Mao AP, Zhong B, Li Y, Liu Y, Gao Y, Ran Y, Tien P, Shu HB. Regulation of virus-triggered signaling by OTUB1- and OTUB2-mediated deubiquitination of TRAF3 and TRAF6. *J Biol Chem* 2010; **285**: 4291-4297 [PMID: 19996094 DOI: 10.1074/jbc.M109.074971]
47 **Karim R**, Tummers B, Meyers C, Biryukov JL, Alam S, Backendorf C, Jha V, Offringa R, van Ommen GJ, Melief CJ, Guardavaccaro D, Boer JM, van der Burg SH. Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1 to suppress the keratinocyte's innate immune response. *PLoS Pathog* 2013; **9**: e1003384 [PMID: 23717208 DOI: 10.1371/journal.ppat.1003384]
48 **Wang P**, Hou J, Lin L, Wang C, Liu X, Li D, Ma F, Wang Z, Cao X. Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. *J Immunol* 2010; **185**: 6226-6233 [PMID: 20937844 DOI: 10.4049/jimmunol.1000491]
49 **Yang CH**, Yue J, Fan M, Pfeffer LM. IFN induces miR-21 through a signal transducer and activator of transcription 3-dependent pathway as a suppressive negative feedback on IFN-induced apoptosis. *Cancer Res* 2010; **70**: 8108-8116 [PMID: 20813833 DOI: 10.1158/0008-5472.CAN-10-2579]
50 **Lu TX**, Hartner J, Lim EJ, Fabry V, Mingler MK, Cole ET, Orkin SH, Aronow BJ, Rothenberg ME. MicroRNA-21 limits in vivo immune response-mediated activation of the IL-12/IFN-gamma pathway, Th1 polarization, and the severity of delayed-type hypersensitivity. *J Immunol* 2011; **187**: 3362-3373 [PMID: 21849676 DOI: 10.4049/jimmunol.1101235]
51 **Hou J**, Wang P, Lin L, Liu X, Ma F, An H, Wang Z, Cao X. MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. *J Immunol* 2009; **183**: 2150-2158 [PMID: 19596990 DOI: 10.4049/jimmunol.0900707]
52 **Li Y**, Fan X, He X, Sun H, Zou Z, Yuan H, Xu H, Wang C, Shi X. MicroRNA-466l inhibits antiviral innate immune response by targeting interferon-alpha. *Cell Mol Immunol* 2012; **9**: 497-502 [PMID: 23042536 DOI: 10.1038/cmi.2012.35]
53 **Lee Y**, Hyung SW, Jung HJ, Kim HJ, Staerk J, Constantinescu SN, Chang EJ, Lee ZH, Lee SW, Kim HH. The ubiquitin-mediated degradation of Jak1 modulates osteoclastogenesis by limiting interferon-beta-induced inhibitory signaling. *Blood* 2008; **111**: 885-893 [PMID: 17928529 DOI: 10.1182/blood-2007-03-082941]
54 **Kim TK**, Maniatis T. Regulation of interferon-gamma-activated STAT1 by the ubiquitin-proteasome pathway. *Science* 1996; **273**: 1717-1719 [PMID: 8781235 DOI: 10.1126/science.273.5282.1717]
55 **Yeh HM**, Yu CY, Yang HC, Ko SH, Liao CL, Lin YL. Ubiquitin-specific protease 13 regulates IFN signaling by stabilizing STAT1. *J Immunol* 2013; **191**: 3328-3336 [PMID: 23940278 DOI: 10.4049/jimmunol.1300225]
56 **Kile BT**, Schulman BA, Alexander WS, Nicola NA, Martin HM, Hilton DJ. The SOCS box: a tale of destruction and degradation. *Trends Biochem Sci* 2002; **27**: 235-241 [PMID: 12076535 DOI: 10.1016/S0968-0004(02)02085-6]
57 **Collins AS**, McCoy CE, Lloyd AT, O'Farrelly C, Stevenson NJ. miR-19a: an effective regulator of SOCS3 and enhancer of JAK-STAT signalling. *PLoS One* 2013; **8**: e69090 [PMID: 23894411 DOI: 10.1371/journal.pone.0069090]
58 **Murray PJ**. The JAK-STAT signaling pathway: input and output integration. *J Immunol* 2007; **178**: 2623-2629 [PMID: 17312100]
59 **Nazarov PV**, Reinsbach SE, Muller A, Nicot N, Philippidou D, Vallar L, Kreis S. Interplay of microRNAs, transcription factors and target genes: linking dynamic expression changes to function. *Nucleic Acids Res* 2013; **41**: 2817-2831 [PMID: 23335783 DOI: 10.1093/nar/gks1471]
60 **Reinsbach S**, Nazarov PV, Philippidou D, Schmitt M, Wienecke-Baldacchino A, Muller A, Vallar L, Behrmann I, Kreis S. Dynamic regulation of microRNA expression following interferon-γ-induced gene transcription. *RNA Biol* 2012; **9**: 978-989 [PMID: 22767256 DOI: 10.4161/rna.20494]
61 **Buggele WA**, Horvath CM. MicroRNA profiling of Sendai virus-infected A549 cells identifies miR-203 as an interferon-inducible regulator of IFIT1/ISG56. *J Virol* 2013; **87**: 9260-9270 [PMID: 23785202 DOI: 10.1128/JVI.01064-13]
62 **Gao F**, Zhao ZL, Zhao WT, Fan QR, Wang SC, Li J, Zhang YQ, Shi JW, Lin XL, Yang S, Xie RY, Liu W, Zhang TT, Sun YL, Xu K, Yao KT, Xiao D. miR-9 modulates the expression of interferon-regulated genes and MHC class I molecules in human nasopharyngeal carcinoma cells. *Biochem Biophys Res Commun* 2013; **431**: 610-616 [PMID: 23291181 DOI: 10.1016/j.bbrc.2012.12.097]
63 **Satoh J**, Tabunoki H. A Comprehensive Profile of ChIP-Seq-Based STAT1 Target Genes Suggests the Complexity of STAT1-Mediated Gene Regulatory Mechanisms. *Gene Regul Syst Bio* 2013; **7**: 41-56 [PMID: 23645984 DOI: 10.4137/GRSB.S11433]
64 **O'Donnell SM**, Holm GH, Pierce JM, Tian B, Watson MJ, Chari RS, Ballard DW, Brasier AR, Dermody TS. Identification of an NF-kappaB-dependent gene network in cells infected by mammalian reovirus. *J Virol* 2006; **80**: 1077-1086 [PMID: 16414985 DOI: 10.1128/JVI.80.3.1077-1086.2006]
65 **Ravi LI**, Li L, Sutejo R, Chen H, Wong PS, Tan BH, Sugrue RJ. A systems-based approach to analyse the host response in murine lung macrophages challenged with respiratory syncytial virus. *BMC Genomics* 2013; **14**: 190 [PMID: 23506210 DOI: 10.1186/1471-2164-14-190]
66 **Gundacker NC**, Haudek VJ, Wimmer H, Slany A, Griss J, Bochkov V, Zielinski C, Wagner O, Stöckl J, Gerner C. Cytoplasmic proteome and secretome profiles of differently stimulated human dendritic cells. *J Proteome Res* 2009; **8**: 2799-2811 [PMID: 19351150 DOI: 10.1021/pr8011039]
67 **Coombs A**. Transcriptomics and quantitative proteomics in virology. *Future Virol* 2012; **7**: 1193-1204 [DOI: 10.2217/fvl.12.112]
68 **Baskin C**. The role and contributions of systems biology to the non-human primate model of influenza pathogenesis

and vaccinology. *Curr Top Microbiol Immunol* 2013; **363**: 69-85 [PMID: 22890718 DOI: 10.1007/82_2012_248]
69 **Bortz E**, Westera L, Maamary J, Steel J, Albrecht RA, Manicassamy B, Chase G, Martínez-Sobrido L, Schwemmle M, García-Sastre A. Host- and strain-specific regulation of influenza virus polymerase activity by interacting cellular proteins. *MBio* 2011; **2**: pii: e00151-11 [PMID: 21846828]
70 **Cheung CY**, Chan EY, Krasnoselsky A, Purdy D, Navare AT, Bryan JT, Leung CK, Hui KP, Peiris JS, Katze MG. H5N1 virus causes significant perturbations in host proteome very early in influenza virus-infected primary human monocyte-derived macrophages. *J Infect Dis* 2012; **206**: 640-645 [PMID: 22822004 DOI: 10.1093/infdis/jis423]
71 **Coombs KM**, Berard A, Xu W, Krokhin O, Meng X, Cortens JP, Kobasa D, Wilkins J, Brown EG. Quantitative proteomic analyses of influenza virus-infected cultured human lung cells. *J Virol* 2010; **84**: 10888-10906 [PMID: 20702633 DOI: 10.1128/JVI.00431-10]
72 **Kroeker AL**, Ezzati P, Halayko AJ, Coombs KM. Response of primary human airway epithelial cells to influenza infection: a quantitative proteomic study. *J Proteome Res* 2012; **11**: 4132-4146 [PMID: 22694362 DOI: 10.1021/pr300239r]
73 **Lietzén N**, Ohman T, Rintahaka J, Julkunen I, Aittokallio T, Matikainen S, Nyman TA. Quantitative subcellular proteome and secretome profiling of influenza A virus-infected human primary macrophages. *PLoS Pathog* 2011; **7**: e1001340 [PMID: 21589892 DOI: 10.1371/journal.ppat.1001340]
74 **Peng T**, Kotla S, Bumgarner RE, Gustin KE. Human rhinovirus attenuates the type I interferon response by disrupting activation of interferon regulatory factor 3. *J Virol* 2006; **80**: 5021-5031 [PMID: 16641293 DOI: 10.1128/JVI.80.10.5021-5031.2006]
75 **Proud D**, Turner RB, Winther B, Wiehler S, Tiesman JP, Reichling TD, Juhlin KD, Fulmer AW, Ho BY, Walanski AA, Poore CL, Mizoguchi H, Jump L, Moore ML, Zukowski CK, Clymer JW. Gene expression profiles during in vivo human rhinovirus infection: insights into the host response. *Am J Respir Crit Care Med* 2008; **178**: 962-968 [PMID: 18658112 DOI: 10.1164/rccm.200805-670OC]
76 **Josset L**, Menachery VD, Gralinski LE, Agnihothram S, Sova P, Carter VS, Yount BL, Graham RL, Baric RS, Katze MG. Cell host response to infection with novel human coronavirus EMC predicts potential antivirals and important differences with SARS coronavirus. *MBio* 2013; **4**: e00165-e00113 [PMID: 23631916 DOI: 10.1128/mBio.00165-13]
77 **Lau SK**, Lau CC, Chan KH, Li CP, Chen H, Jin DY, Chan JF, Woo PC, Yuen KY. Delayed induction of proinflammatory cytokines and suppression of innate antiviral response by the novel Middle East respiratory syndrome coronavirus: implications for pathogenesis and treatment. *J Gen Virol* 2013; **94**: 2679-2690 [PMID: 24077366 DOI: 10.1099/vir.0.055533-0]
78 **Tang BS**, Chan KH, Cheng VC, Woo PC, Lau SK, Lam CC, Chan TL, Wu AK, Hung IF, Leung SY, Yuen KY. Comparative host gene transcription by microarray analysis early after infection of the Huh7 cell line by severe acute respiratory syndrome coronavirus and human coronavirus 229E. *J Virol* 2005; **79**: 6180-6193 [PMID: 15858003]
79 **Jiang XS**, Tang LY, Dai J, Zhou H, Li SJ, Xia QC, Wu JR, Zeng R. Quantitative analysis of severe acute respiratory syndrome (SARS)-associated coronavirus-infected cells using proteomic approaches: implications for cellular responses to virus infection. *Mol Cell Proteomics* 2005; **4**: 902-913 [PMID: 15784933]
80 **Lai CC**, Jou MJ, Huang SY, Li SW, Wan L, Tsai FJ, Lin CW. Proteomic analysis of up-regulated proteins in human promonocyte cells expressing severe acute respiratory syndrome coronavirus 3C-like protease. *Proteomics* 2007; **7**: 1446-1460 [PMID: 17407183 DOI: 10.1002/pmic.200600459]
81 **Zhang L**, Zhang X, Ma Q, Ma F, Zhou H. Transcriptomics and proteomics in the study of H1N1 2009. *Genomics Proteomics Bioinformatics* 2010; **8**: 139-144 [PMID: 20970742 DOI: 10.1016/S1672-0229(10)60016-2]
82 **Lam WY**, Yeung AC, Ngai KL, Li MS, To KF, Tsui SK, Chan PK. Effect of avian influenza A H5N1 infection on the expression of microRNA-141 in human respiratory epithelial cells. *BMC Microbiol* 2013; **13**: 104 [PMID: 23663545 DOI: 10.1186/1471-2180-13-104]
83 **Terrier O**, Textoris J, Carron C, Marcel V, Bourdon JC, Rosa-Calatrava M. Host microRNA molecular signatures associated with human H1N1 and H3N2 influenza A viruses reveal an unanticipated antiviral activity for miR-146a. *J Gen Virol* 2013; **94**: 985-995 [PMID: 23343627 DOI: 10.1099/vir.0.049528-0]
84 **Buggele WA**, Johnson KE, Horvath CM. Influenza A virus infection of human respiratory cells induces primary microRNA expression. *J Biol Chem* 2012; **287**: 31027-31040 [PMID: 22822053 DOI: 10.1074/jbc.M112.387670]
85 **Buggele WA**, Krause KE, Horvath CM. Small RNA profiling of influenza A virus-infected cells identifies miR-449b as a regulator of histone deacetylase 1 and interferon beta. *PLoS One* 2013; **8**: e76560 [PMID: 24086750 DOI: 10.1371/journal.pone.0076560]
86 **Tambyah PA**, Sepramaniam S, Mohamed Ali J, Chai SC, Swaminathan P, Armugam A, Jeyaseelan K. microRNAs in circulation are altered in response to influenza A virus infection in humans. *PLoS One* 2013; **8**: e76811 [PMID: 24116168 DOI: 10.1371/journal.pone.0076811]
87 **Rosenberger CM**, Podyminogin RL, Navarro G, Zhao GW, Askovich PS, Weiss MJ, Aderem A. miR-451 regulates dendritic cell cytokine responses to influenza infection. *J Immunol* 2012; **189**: 5965-5975 [PMID: 23169590 DOI: 10.4049/jimmunol.1201437]
88 **Song H**, Wang Q, Guo Y, Liu S, Song R, Gao X, Dai L, Li B, Zhang D, Cheng J. Microarray analysis of microRNA expression in peripheral blood mononuclear cells of critically ill patients with influenza A (H1N1). *BMC Infect Dis* 2013; **13**: 257 [PMID: 23731466 DOI: 10.1186/1471-2334-13-257]
89 **Fang J**, Hao Q, Liu L, Li Y, Wu J, Huo X, Zhu Y. Epigenetic changes mediated by microRNA miR29 activate cyclooxygenase 2 and lambda-1 interferon production during viral infection. *J Virol* 2012; **86**: 1010-1020 [PMID: 22072783 DOI: 10.1128/JVI.06169-11]
90 **Langlois RA**, Varble A, Chua MA, García-Sastre A, tenOever BR. Hematopoietic-specific targeting of influenza A virus reveals replication requirements for induction of antiviral immune responses. *Proc Natl Acad Sci USA* 2012; **109**: 12117-12122 [PMID: 22778433 DOI: 10.1073/pnas.1206039109]
91 **Wu Z**, Hao R, Li P, Zhang X, Liu N, Qiu S, Wang L, Wang Y, Xue W, Liu K, Yang G, Cui J, Zhang C, Song H. MicroRNA expression profile of mouse lung infected with 2009 pandemic H1N1 influenza virus. *PLoS One* 2013; **8**: e74190 [PMID: 24066118 DOI: 10.1371/journal.pone.0074190]
92 **Brock M**, Trenkmann M, Gay RE, Gay S, Speich R, Huber LC. MicroRNA-18a enhances the interleukin-6-mediated production of the acute-phase proteins fibrinogen and haptoglobin in human hepatocytes. *J Biol Chem* 2011; **286**: 40142-40150 [PMID: 21953462 DOI: 10.1074/jbc.M111.251793]
93 **Bhaumik D**, Scott GK, Schokrpur S, Patil CK, Orjalo AV, Rodier F, Lithgow GJ, Campisi J. MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8. *Aging* (Albany NY) 2009; **1**: 402-411 [PMID: 20148189]
94 **Ouda R**, Onomoto K, Takahasi K, Edwards MR, Kato H, Yoneyama M, Fujita T. Retinoic acid-inducible gene I-inducible miR-23b inhibits infections by minor group rhinoviruses through down-regulation of the very low density lipoprotein receptor. *J Biol Chem* 2011; **286**: 26210-26219 [PMID: 21642441 DOI: 10.1074/jbc.M111.229856]
95 **Munday DC**, Emmott E, Surtees R, Lardeau CH, Wu W, Duprex WP, Dove BK, Barr JN, Hiscox JA. Quantitative

proteomic analysis of A549 cells infected with human respiratory syncytial virus. *Mol Cell Proteomics* 2010; **9**: 2438-2459 [PMID: 20647383]

96 **Munday DC**, Hiscox JA, Barr JN. Quantitative proteomic analysis of A549 cells infected with human respiratory syncytial virus subgroup B using SILAC coupled to LC-MS/MS. *Proteomics* 2010; **10**: 4320-4334 [PMID: 21110324 DOI: 10.1002/pmic.201000228]

97 **Ternette N**, Wright C, Kramer HB, Altun M, Kessler BM. Label-free quantitative proteomics reveals regulation of interferon-induced protein with tetratricopeptide repeats 3 (IFIT3) and 5′-3′-exoribonuclease 2 (XRN2) during respiratory syncytial virus infection. *Virol J* 2011; **8**: 442 [PMID: 21933386]

98 **van Diepen A**, Brand HK, Sama I, Lambooy LH, van den Heuvel LP, van der Well L, Huynen M, Osterhaus AD, Andeweg AC, Hermans PW. Quantitative proteome profiling of respiratory virus-infected lung epithelial cells. *J Proteomics* 2010; **73**: 1680-1693 [PMID: 20470912]

99 **Huang YC**, Li Z, Hyseni X, Schmitt M, Devlin RB, Karoly ED, Soukup JM. Identification of gene biomarkers for respiratory syncytial virus infection in a bronchial epithelial cell line. *Genomic Med* 2008; **2**: 113-125 [PMID: 19459069 DOI: 10.1007/s11568-009-9080-y]

100 **Ravi LI**, Li L, Wong PS, Sutejo R, Tan BH, Sugrue RJ. Lovastatin treatment mitigates the pro-inflammatory cytokine response in respiratory syncytial virus infected macrophage cells. *Antiviral Res* 2013; **98**: 332-343 [PMID: 23523944]

101 **Fjaerli HO**, Bukholm G, Skjaeret C, Holden M, Nakstad B. Cord blood gene expression in infants hospitalized with respiratory syncytial virus bronchiolitis. *J Infect Dis* 2007; **196**: 394-404 [PMID: 17597454 DOI: 10.1086/519168]

102 **Emmott E**, Smith C, Emmett SR, Dove BK, Hiscox JA. Elucidation of the avian nucleolar proteome by quantitative proteomics using SILAC and changes in cells infected with the coronavirus infectious bronchitis virus. *Proteomics* 2010; **10**: 3558-3562 [PMID: 20827733 DOI: 10.1002/pmic.201000139]

103 **Vogels MW**, van Balkom BW, Kaloyanova DV, Batenburg JJ, Heck AJ, Helms JB, Rottier PJ, de Haan CA. Identification of host factors involved in coronavirus replication by quantitative proteomics analysis. *Proteomics* 2011; **11**: 64-80 [PMID: 21182195 DOI: 10.1002/pmic.201000309]

104 **Zhang L**, Zhang ZP, Zhang XE, Lin FS, Ge F. Quantitative proteomics analysis reveals BAG3 as a potential target to suppress severe acute respiratory syndrome coronavirus replication. *J Virol* 2010; **84**: 6050-6059 [PMID: 20392858]

105 **Liu L**, Zhou J, Wang Y, Mason RJ, Funk CJ, Du Y. Proteome alterations in primary human alveolar macrophages in response to influenza A virus infection. *J Proteome Res* 2012; **11**: 4091-4101 [PMID: 22709384 DOI: 10.1021/pr3001332]

106 **Ohman T**, Rintahaka J, Kalkkinen N, Matikainen S, Nyman TA. Actin and RIG-I/MAVS signaling components translocate to mitochondria upon influenza A virus infection of human primary macrophages. *J Immunol* 2009; **182**: 5682-5692 [PMID: 19380815]

107 **Kroeker AL**, Ezzati P, Coombs KM, Halayko AJ. Influenza A infection of primary human airway epithelial cells up-regulates proteins related to purine metabolism and ubiquitin-related signaling. *J Proteome Res* 2013; **12**: 3139-3151 [PMID: 23750822 DOI: 10.1021/pr400464p]

108 **Zhao D**, Liang L, Li Y, Liu L, Guan Y, Jiang Y, Chen H. Proteomic analysis of the lungs of mice infected with different pathotypes of H5N1 avian influenza viruses. *Proteomics* 2012; **12**: 1970-1982 [PMID: 22623221 DOI: 10.1002/pmic.201100619]

109 **Baas T**, Baskin CR, Diamond DL, García-Sastre A, Bielefeldt-Ohmann H, Tumpey TM, Thomas MJ, Carter VS, Teal TH, Van Hoeven N, Proll S, Jacobs JM, Caldwell ZR, Gritsenko MA, Hukkanen RR, Camp DG, Smith RD, Katze MG. Integrated molecular signature of disease: analysis of influenza virus-infected macaques through functional genomics and proteomics. *J Virol* 2006; **80**: 10813-10828 [PMID: 16928763]

110 **Wang J**, Nikrad MP, Travanty EA, Zhou B, Phang T, Gao B, Alford T, Ito Y, Nahreini P, Hartshorn K, Wentworth D, Dinarello CA, Mason RJ. Innate immune response of human alveolar macrophages during influenza A infection. *PLoS One* 2012; **7**: e29879 [PMID: 22396727 DOI: 10.1371/journal.pone.0029879]

111 **Yang XX**, Du N, Zhou JF, Li Z, Wang M, Guo JF, Wang DY, Shu YL. Gene expression profiles comparison between 2009 pandemic and seasonal H1N1 influenza viruses in A549 cells. *Biomed Environ Sci* 2010; **23**: 259-266 [PMID: 20934112]

112 **Kash JC**, Basler CF, García-Sastre A, Carter V, Billharz R, Swayne DE, Przygodzki RM, Taubenberger JK, Katze MG, Tumpey TM. Global host immune response: pathogenesis and transcriptional profiling of type A influenza viruses expressing the hemagglutinin and neuraminidase genes from the 1918 pandemic virus. *J Virol* 2004; **78**: 9499-9511 [PMID: 15308742 DOI: 10.1128/JVI.78.17.9499-9511.2004]

113 **Belisle SE**, Tisoncik JR, Korth MJ, Carter VS, Proll SC, Swayne DE, Pantin-Jackwood M, Tumpey TM, Katze MG. Genomic profiling of tumor necrosis factor alpha (TNF-alpha) receptor and interleukin-1 receptor knockout mice reveals a link between TNF-alpha signaling and increased severity of 1918 pandemic influenza virus infection. *J Virol* 2010; **84**: 12576-12588 [PMID: 20926563 DOI: 10.1128/JVI.01310-10]

114 **Rowe T**, León AJ, Crevar CJ, Carter DM, Xu L, Ran L, Fang Y, Cameron CM, Cameron MJ, Banner D, Ng DC, Ran R, Weirback HK, Wiley CA, Kelvin DJ, Ross TM. Modeling host responses in ferrets during A/California/07/2009 influenza infection. *Virology* 2010; **401**: 257-265 [PMID: 20334888 DOI: 10.1016/j.virol.2010.02.020]

115 **Cameron CM**, Cameron MJ, Bermejo-Martin JF, Ran L, Xu L, Turner PV, Ran R, Danesh A, Fang Y, Chan PK, Mytle N, Sullivan TJ, Collins TL, Johnson MG, Medina JC, Rowe T, Kelvin DJ. Gene expression analysis of host innate immune responses during Lethal H5N1 infection in ferrets. *J Virol* 2008; **82**: 11308-11317 [PMID: 18684821 DOI: 10.1128/JVI.00691-08]

116 **Baskin CR**, García-Sastre A, Tumpey TM, Bielefeldt-Ohmann H, Carter VS, Nistal-Villán E, Katze MG. Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (Macaca nemestrina). *J Virol* 2004; **78**: 10420-10432 [PMID: 15367608 DOI: 10.1128/JVI.78.19.10420-10432.2004]

117 **Berard AR**, Cortens JP, Krokhin O, Wilkins JA, Severini A, Coombs KM. Quantification of the host response proteome after mammalian reovirus T1L infection. *PLoS One* 2012; **7**: e51939 [PMID: 23240068 DOI: 10.1371/journal.pone.0051939]

118 **Coombs KM**. HeLa cell response proteome alterations induced by mammalian reovirus T3D infection. *Virol J* 2013; **10**: 202 [PMID: 23799967]

119 **Li L**, Sevinsky JR, Rowland MD, Bundy JL, Stephenson JL, Sherry B. Proteomic analysis reveals virus-specific Hsp25 modulation in cardiac myocytes. *J Proteome Res* 2010; **9**: 2460-2471 [PMID: 20196617 DOI: 10.1021/pr901151k]

120 **Jiang J**, Opanubi KJ, Coombs KM. Non-Biased Enrichment Does Not Improve Quantitative Proteomic Delineation of Reovirus T3D-Infected HeLa Cell Protein Alterations. *Front Microbiol* 2012; **3**: 310 [PMID: 23024642 DOI: 10.3389/fmicb.2012.00310]

121 **Tyler KL**, Leser JS, Phang TL, Clarke P. Gene expression in the brain during reovirus encephalitis. *J Neurovirol* 2010; **16**: 56-71 [PMID: 20158406 DOI: 10.3109/13550280903586394]

122 **Bochkov YA**, Hanson KM, Keles S, Brockman-Schneider RA, Jarjour NN, Gern JE. Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma. *Mucosal Immunol* 2010; **3**: 69-80 [PMID: 19710636 DOI: 10.1038/mi.2009.109]

123 **Proud D**, Hudy MH, Wiehler S, Zaheer RS, Amin MA,

Pelikan JB, Tacon CE, Tonsaker TO, Walker BL, Kooi C, Traves SL, Leigh R. Cigarette smoke modulates expression of human rhinovirus-induced airway epithelial host defense genes. *PLoS One* 2012; **7**: e40762 [PMID: 22808255 DOI: 10.1371/journal.pone.0040762]

124 **Go JT**, Belisle SE, Tchitchek N, Tumpey TM, Ma W, Richt JA, Safronetz D, Feldmann H, Katze MG. 2009 pandemic H1N1 influenza virus elicits similar clinical course but differential host transcriptional response in mouse, macaque, and swine infection models. *BMC Genomics* 2012; **13**: 627 [PMID: 23153050 DOI: 10.1186/1471-2164-13-627]

125 **Korth MJ**, Tchitchek N, Benecke AG, Katze MG. Systems approaches to influenza-virus host interactions and the pathogenesis of highly virulent and pandemic viruses. *Semin Immunol* 2013; **25**: 228-239 [PMID: 23218769 DOI: 10.1016/j.smim.2012.11.001]

126 **Huang Y**, Zaas AK, Rao A, Dobigeon N, Woolf PJ, Veldman T, Øien NC, McClain MT, Varkey JB, Nicholson B, Carin L, Kingsmore S, Woods CW, Ginsburg GS, Hero AO. Temporal dynamics of host molecular responses differentiate symptomatic and asymptomatic influenza a infection. *PLoS Genet* 2011; **7**: e1002234 [PMID: 21901105 DOI: 10.1371/journal.pgen.1002234]

127 **Fukuyama S**, Kawaoka Y. The pathogenesis of influenza virus infections: the contributions of virus and host factors. *Curr Opin Immunol* 2011; **23**: 481-486 [PMID: 21840185 DOI: 10.1016/j.coi.2011.07.016]

128 **Baines KJ**, Hsu AC, Tooze M, Gunawardhana LP, Gibson PG, Wark PA. Novel immune genes associated with excessive inflammatory and antiviral responses to rhinovirus in COPD. *Respir Res* 2013; **14**: 15 [PMID: 23384071 DOI: 10.1186/1465-9921-14-15]

129 **Kotla S**, Peng T, Bumgarner RE, Gustin KE. Attenuation of the type I interferon response in cells infected with human rhinovirus. *Virology* 2008; **374**: 399-410 [PMID: 18272195 DOI: 10.1016/j.virol.2008.01.022]

130 **Stewart MJ**, Smoak K, Blum MA, Sherry B. Basal and reovirus-induced beta interferon (IFN-beta) and IFN-beta-stimulated gene expression are cell type specific in the cardiac protective response. *J Virol* 2005; **79**: 2979-2987 [PMID: 15709018]

131 **van Boxel-Dezaire AH**, Rani MR, Stark GR. Complex modulation of cell type-specific signaling in response to type I interferons. *Immunity* 2006; **25**: 361-372 [PMID: 16979568]

132 **Zurney J**, Kobayashi T, Holm GH, Dermody TS, Sherry B. Reovirus mu2 protein inhibits interferon signaling through a novel mechanism involving nuclear accumulation of interferon regulatory factor 9. *J Virol* 2009; **83**: 2178-2187 [PMID: 19109390]

133 **Montomoli E**, Khadang B, Piccirella S, Trombetta C, Mennitto E, Manini I, Stanzani V, Lapini G. Cell culture-derived influenza vaccines from Vero cells: a new horizon for vaccine production. *Expert Rev Vaccines* 2012; **11**: 587-594 [PMID: 22827244 DOI: 10.1586/erv.12.24]

134 **Rajsbaum R**, Albrecht RA, Wang MK, Maharaj NP, Versteeg GA, Nistal-Villán E, García-Sastre A, Gack MU. Species-specific inhibition of RIG-I ubiquitination and IFN induction by the influenza A virus NS1 protein. *PLoS Pathog* 2012; **8**: e1003059 [PMID: 23209422 DOI: 10.1371/journal.ppat.1003059]

135 **Boyapalle S**, Wong T, Garay J, Teng M, San Juan-Vergara H, Mohapatra S, Mohapatra S. Respiratory syncytial virus NS1 protein colocalizes with mitochondrial antiviral signaling protein MAVS following infection. *PLoS One* 2012; **7**: e29386 [PMID: 22383950 DOI: 10.1371/journal.pone.0029386]

136 **Ling Z**, Tran KC, Teng MN. Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I. *J Virol* 2009; **83**: 3734-3742 [PMID: 19193793 DOI: 10.1128/JVI.02434-08]

137 **Durbin JE**, Johnson TR, Durbin RK, Mertz SE, Morotti RA, Peebles RS, Graham BS. The role of IFN in respiratory syncytial virus pathogenesis. *J Immunol* 2002; **168**: 2944-2952 [PMID: 11884466]

138 **Wolff T**, Ludwig S. Influenza viruses control the vertebrate type I interferon system: factors, mechanisms, and consequences. *J Interferon Cytokine Res* 2009; **29**: 549-557 [PMID: 19708812 DOI: 10.1089/jir.2009.0066]

139 **Koerner I**, Kochs G, Kalinke U, Weiss S, Staeheli P. Protective role of beta interferon in host defense against influenza A virus. *J Virol* 2007; **81**: 2025-2030 [PMID: 17151098 DOI: 10.1128/JVI.01718-06]

140 **Arimori Y**, Nakamura R, Yamada H, Shibata K, Maeda N, Kase T, Yoshikai Y. Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice. *Antiviral Res* 2013; **99**: 230-237 [PMID: 23721943 DOI: 10.1016/j.antiviral.2013.05.007]

141 **Li W**, Moltedo B, Moran TM. Type I interferon induction during influenza virus infection increases susceptibility to secondary Streptococcus pneumoniae infection by negative regulation of γδ T cells. *J Virol* 2012; **86**: 12304-12312 [PMID: 22951826 DOI: 10.1128/JVI.01269-12]

142 **Shahangian A**, Chow EK, Tian X, Kang JR, Ghaffari A, Liu SY, Belperio JA, Cheng G, Deng JC. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. *J Clin Invest* 2009; **119**: 1910-1920 [PMID: 19487810]

143 **Sykes A**, Macintyre J, Edwards MR, Del Rosario A, Haas J, Gielen V, Kon OM, McHale M, Johnston SL. Rhinovirus-induced interferon production is not deficient in well controlled asthma. *Thorax* 2014; **69**: 240-246 [PMID: 24127021 DOI: 10.1136/thoraxjnl-2012-202909]

144 **Smith SB**, Dampier W, Tozeren A, Brown JR, Magid-Slav M. Identification of common biological pathways and drug targets across multiple respiratory viruses based on human host gene expression analysis. *PLoS One* 2012; **7**: e33174 [PMID: 22432004 DOI: 10.1371/journal.pone.0033174]

145 **Pichlmair A**, Lassnig C, Eberle CA, Górna MW, Baumann CL, Burkard TR, Bürckstümmer T, Stefanovic A, Krieger S, Bennett KL, Rülicke T, Weber F, Colinge J, Müller M, Superti-Furga G. IFIT1 is an antiviral protein that recognizes 5′-triphosphate RNA. *Nat Immunol* 2011; **12**: 624-630 [PMID: 21642987]

146 **Huang IC**, Bailey CC, Weyer JL, Radoshitzky SR, Becker MM, Chiang JJ, Brass AL, Ahmed AA, Chi X, Dong L, Longobardi LE, Boltz D, Kuhn JH, Elledge SJ, Bavari S, Denison MR, Choe H, Farzan M. Distinct patterns of IFITM-mediated restriction of filoviruses, SARS coronavirus, and influenza A virus. *PLoS Pathog* 2011; **7**: e1001258 [PMID: 21253575 DOI: 10.1371/journal.ppat.1001258]

147 **Lenschow DJ**, Lai C, Frias-Staheli N, Giannakopoulos NV, Lutz A, Wolff T, Osiak A, Levine B, Schmidt RE, García-Sastre A, Leib DA, Pekosz A, Knobeloch KP, Horak I, Virgin HW. IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc Natl Acad Sci USA* 2007; **104**: 1371-1376 [PMID: 17227866]

148 **Yángüez E**, García-Culebras A, Frau A, Llompart C, Knobeloch KP, Gutierrez-Erlandsson S, García-Sastre A, Esteban M, Nieto A, Guerra S. ISG15 regulates peritoneal macrophages functionality against viral infection. *PLoS Pathog* 2013; **9**: e1003632 [PMID: 24137104 DOI: 10.1371/journal.ppat.1003632]

149 **Wang X**, Hinson ER, Cresswell P. The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. *Cell Host Microbe* 2007; **2**: 96-105 [PMID: 18005724 DOI: 10.1016/j.chom.2007.06.009]

150 **Teng TS**, Foo SS, Simamarta D, Lum FM, Teo TH, Lulla A, Yeo NK, Koh EG, Chow A, Leo YS, Merits A, Chin KC, Ng LF. Viperin restricts chikungunya virus replication and pathology. *J Clin Invest* 2012; **122**: 4447-4460 [PMID: 23160199 DOI: 10.1172/JCI63120]

151 **Nasr N**, Maddocks S, Turville SG, Harman AN, Woolger N, Helbig KJ, Wilkinson J, Bye CR, Wright TK, Rambukwelle D, Donaghy H, Beard MR, Cunningham AL. HIV-1 infection of human macrophages directly induces viperin which inhibits viral production. *Blood* 2012; **120**: 778-788 [PMID: 22677126 DOI: 10.1182/blood-2012-01-407395]

152 **Helbig KJ**, Eyre NS, Yip E, Narayana S, Li K, Fiches G, McCartney EM, Jangra RK, Lemon SM, Beard MR. The antiviral protein viperin inhibits hepatitis C virus replication via interaction with nonstructural protein 5A. *Hepatology* 2011; **54**: 1506-1517 [PMID: 22045669 DOI: 10.1002/hep.24542]

153 **Tafforeau L**, Chantier T, Pradezynski F, Pellet J, Mangeot PE, Vidalain PO, Andre P, Rabourdin-Combe C, Lotteau V. Generation and comprehensive analysis of an influenza virus polymerase cellular interaction network. *J Virol* 2011; **85**: 13010-13018 [PMID: 21994455 DOI: 10.1128/JVI.02651-10]

154 **de Chassey B**, Aublin-Gex A, Ruggieri A, Meyniel-Schicklin L, Pradezynski F, Davoust N, Chantier T, Tafforeau L, Mangeot PE, Ciancia C, Perrin-Cocon L, Bartenschlager R, André P, Lotteau V. The interactomes of influenza virus NS1 and NS2 proteins identify new host factors and provide insights for ADAR1 playing a supportive role in virus replication. *PLoS Pathog* 2013; **9**: e1003440 [PMID: 23853584 DOI: 10.1371/journal.ppat.1003440]

155 **Mok BW**, Song W, Wang P, Tai H, Chen Y, Zheng M, Wen X, Lau SY, Wu WL, Matsumoto K, Yuen KY, Chen H. The NS1 protein of influenza A virus interacts with cellular processing bodies and stress granules through RNA-associated protein 55 (RAP55) during virus infection. *J Virol* 2012; **86**: 12695-12707 [PMID: 22973032 DOI: 10.1128/JVI.00647-12]

156 **Shapira SD**, Gat-Viks I, Shum BO, Dricot A, de Grace MM, Wu L, Gupta PB, Hao T, Silver SJ, Root DE, Hill DE, Regev A, Hacohen N. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. *Cell* 2009; **139**: 1255-1267 [PMID: 20064372 DOI: 10.1016/j.cell.2009.12.018]

157 **Liu P**, Jamaluddin M, Li K, Garofalo RP, Casola A, Brasier AR. Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. *J Virol* 2007; **81**: 1401-1411 [PMID: 17108032 DOI: 10.1128/JVI.01740-06]

158 **Rudd BD**, Burstein E, Duckett CS, Li X, Lukacs NW. Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. *J Virol* 2005; **79**: 3350-3357 [PMID: 15731229 DOI: 10.1128/JVI.79.6.3350-3357.2005]

159 **Elliott J**, Lynch OT, Suessmuth Y, Qian P, Boyd CR, Burrows JF, Buick R, Stevenson NJ, Touzelet O, Gadina M, Power UF, Johnston JA. Respiratory syncytial virus NS1 protein degrades STAT2 by using the Elongin-Cullin E3 ligase. *J Virol* 2007; **81**: 3428-3436 [PMID: 17251292 DOI: 10.1128/JVI.02303-06]

160 **Thornburg NJ**, Hayward SL, Crowe JE. Respiratory syncytial virus regulates human microRNAs by using mechanisms involving beta interferon and NF-κB. *MBio* 2012; **3**: [PMID: 23249809]

161 **Li SW**, Lai CC, Ping JF, Tsai FJ, Wan L, Lin YJ, Kung SH, Lin CW. Severe acute respiratory syndrome coronavirus papain-like protease suppressed alpha interferon-induced responses through downregulation of extracellular signal-regulated kinase 1-mediated signalling pathways. *J Gen Virol* 2011; **92**: 1127-1140 [PMID: 21270289 DOI: 10.1099/vir.0.028936-0]

162 **Frieman M**, Yount B, Heise M, Kopecky-Bromberg SA, Palese P, Baric RS. Severe acute respiratory syndrome coronavirus ORF6 antagonizes STAT1 function by sequestering nuclear import factors on the rough endoplasmic reticulum/Golgi membrane. *J Virol* 2007; **81**: 9812-9824 [PMID: 17596301 DOI: 10.1128/JVI.01012-07]

163 **Sims AC**, Tilton SC, Menachery VD, Gralinski LE, Schäfer A, Matzke MM, Webb-Robertson BJ, Chang J, Luna ML, Long CE, Shukla AK, Bankhead AR, Burkett SE, Zornetzer G, Tseng CT, Metz TO, Pickles R, McWeeney S, Smith RD, Katze MG, Waters KM, Baric RS. Release of severe acute respiratory syndrome coronavirus nuclear import block enhances host transcription in human lung cells. *J Virol* 2013; **87**: 3885-3902 [PMID: 23365422 DOI: 10.1128/JVI.02520-12]

164 **Calvén J**, Yudina Y, Uller L. Rhinovirus and dsRNA induce RIG-I-like receptors and expression of interferon β and λ1 in human bronchial smooth muscle cells. *PLoS One* 2013; **8**: e62718 [PMID: 23658644 DOI: 10.1371/journal.pone.0062718]

165 **Irvin SC**, Zurney J, Ooms LS, Chappell JD, Dermody TS, Sherry B. A single-amino-acid polymorphism in reovirus protein μ2 determines repression of interferon signaling and modulates myocarditis. *J Virol* 2012; **86**: 2302-2311 [PMID: 22156521 DOI: 10.1128/JVI.06236-11]

166 **Brass AL**, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* 2009; **139**: 1243-1254 [PMID: 20064371 DOI: 10.1016/j.cell.2009.12.017]

167 **Tan KS**, Olfat F, Phoon MC, Hsu JP, Howe JL, Seet JE, Chin KC, Chow VT. In vivo and in vitro studies on the antiviral activities of viperin against influenza H1N1 virus infection. *J Gen Virol* 2012; **93**: 1269-1277 [PMID: 22377585 DOI: 10.1099/vir.0.040824-0]

168 **Tran AT**, Rahim MN, Ranadheera C, Kroeker A, Cortens JP, Opanubi KJ, Wilkins JA, Coombs KM. Knockdown of specific host factors protects against influenza virus-induced cell death. *Cell Death Dis* 2013; **4**: e769 [PMID: 23949218 DOI: 10.1038/cddis.2013.296]

169 **Lai C**, Struckhoff JJ, Schneider J, Martinez-Sobrido L, Wolff T, García-Sastre A, Zhang DE, Lenschow DJ. Mice lacking the ISG15 E1 enzyme UbE1L demonstrate increased susceptibility to both mouse-adapted and non-mouse-adapted influenza B virus infection. *J Virol* 2009; **83**: 1147-1151 [PMID: 19004958 DOI: 10.1128/JVI.00105-08]

170 **Bartlett NW**, Slater L, Glanville N, Haas JJ, Caramori G, Casolari P, Clarke DL, Message SD, Aniscenko J, Kebadze T, Zhu J, Mallia P, Mizgerd JP, Belvisi M, Papi A, Kotenko SV, Johnston SL, Edwards MR. Defining critical roles for NF-κB p65 and type I interferon in innate immunity to rhinovirus. *EMBO Mol Med* 2012; **4**: 1244-1260 [PMID: 23165884 DOI: 10.1002/emmm.201201650]

171 **Staeheli P**, Haller O, Boll W, Lindenmann J, Weissmann C. Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. *Cell* 1986; **44**: 147-158 [PMID: 3000619 DOI: 10.1016/0092-8674(86)90493-9]

172 **Wei J**, Waithman J, Lata R, Mifsud NA, Cebon J, Kay T, Smyth MJ, Sadler AJ, Chen W. Influenza A infection enhances cross-priming of CD8+ T cells to cell-associated antigens in a TLR7- and type I IFN-dependent fashion. *J Immunol* 2010; **185**: 6013-6022 [PMID: 20956347 DOI: 10.4049/jimmunol.1002129]

173 **Shmulevitz M**, Pan LZ, Garant K, Pan D, Lee PW. Oncogenic Ras promotes reovirus spread by suppressing IFN-beta production through negative regulation of RIG-I signaling. *Cancer Res* 2010; **70**: 4912-4921 [PMID: 20501842 DOI: 10.1158/0008-5472.CAN-09-4676]

174 **Li S**, Sun F, Zhang YB, Gui JF, Zhang QY. Identification of DreI as an antiviral factor regulated by RLR signaling pathway. *PLoS One* 2012; **7**: e32427 [PMID: 22412872 DOI: 10.1371/journal.pone.0032427]

175 **Yoo JK**, Kim TS, Hufford MM, Braciale TJ. Viral infection of the lung: host response and sequelae. *J Allergy Clin Immunol* 2013; **132**: 1263-176; quiz 1277 [PMID: 23915713 DOI: 10.1016/j.jaci.2013.06.006]

176 **See H**, Wark P. Innate immune response to viral infection of the lungs. *Paediatr Respir Rev* 2008; **9**: 243-250 [PMID: 19026365]

177 **Haasbach E**, Droebner K, Vogel AB, Planz O. Low-dose interferon Type I treatment is effective against H5N1 and swine-origin H1N1 influenza A viruses in vitro and in vivo. *J Interferon Cytokine Res* 2011; **31**: 515-525 [PMID: 21323570 DOI: 10.1089/jir.2010.0071]

178 **Matzinger SR**, Carroll TD, Fritts L, McChesney MB, Miller CJ. Exogenous IFN-alpha administration reduces influenza A virus replication in the lower respiratory tract of rhesus macaques. *PLoS One* 2011; **6**: e29255 [PMID: 22220209 DOI: 10.1371/journal.pone.0029255]

179 **Jiang H**, Yang H, Kapczynski DR. Chicken interferon alpha pretreatment reduces virus replication of pandemic H1N1 and H5N9 avian influenza viruses in lung cell cultures from different avian species. *Virol J* 2011; **8**: 447 [PMID: 21939525]

180 **Gaajetaan GR**, Geelen TH, Vernooy JH, Dentener MA, Reynaert NL, Rohde GG, Beuken EV, Grauls GE, Bruggeman CA, Stassen FR. Interferon-β induces a long-lasting antiviral state in human respiratory epithelial cells. *J Infect* 2013; **66**: 163-169 [PMID: 23201152]

181 **Falzarano D**, de Wit E, Rasmussen AL, Feldmann F, Okumura A, Scott DP, Brining D, Bushmaker T, Martellaro C, Baseler L, Benecke AG, Katze MG, Munster VJ, Feldmann H. Treatment with interferon-α2b and ribavirin improves outcome in MERS-CoV-infected rhesus macaques. *Nat Med* 2013; **19**: 1313-1317 [PMID: 24013700 DOI: 10.1038/nm.3362]

182 **de Wilde AH**, Raj VS, Oudshoorn D, Bestebroer TM, van Nieuwkoop S, Limpens RW, Posthuma CC, van der Meer Y, Bárcena M, Haagmans BL, Snijder EJ, van den Hoogen BG. MERS-coronavirus replication induces severe in vitro cytopathology and is strongly inhibited by cyclosporin A or interferon-α treatment. *J Gen Virol* 2013; **94**: 1749-1760 [PMID: 23620378 DOI: 10.1099/vir.0.052910-0]

183 **Kuri T**, Weber F. Interferon interplay helps tissue cells to cope with SARS-coronavirus infection. *Virulence* 2010; **1**: 273-275 [PMID: 21178452 DOI: 10.4161/viru.1.4.11465]

184 **Johansson C**, Wetzel JD, He J, Mikacenic C, Dermody TS, Kelsall BL. Type I interferons produced by hematopoietic cells protect mice against lethal infection by mammalian reovirus. *J Exp Med* 2007; **204**: 1349-1358 [PMID: 17502662 DOI: 10.1084/jem.20061587]

185 **Empey KM**, Orend JG, Peebles RS, Egaña L, Norris KA, Oury TD, Kolls JK. Stimulation of immature lung macrophages with intranasal interferon gamma in a novel neonatal mouse model of respiratory syncytial virus infection. *PLoS One* 2012; **7**: e40499 [PMID: 22792355 DOI: 10.1371/journal.pone.0040499]

186 **Goulet ML**, Olagnier D, Xu Z, Paz S, Belgnaoui SM, Lafferty EI, Janelle V, Arguello M, Paquet M, Ghneim K, Richards S, Smith A, Wilkinson P, Cameron M, Kalinke U, Qureshi S, Lamarre A, Haddad EK, Sekaly RP, Peri S, Balachandran S, Lin R, Hiscott J. Systems analysis of a RIG-I agonist inducing broad spectrum inhibition of virus infectivity. *PLoS Pathog* 2013; **9**: e1003298 [PMID: 23633948 DOI: 10.1371/journal.ppat.1003298]

187 **Zhao J**, Wohlford-Lenane C, Zhao J, Fleming E, Lane TE, McCray PB, Perlman S. Intranasal treatment with poly(I•C) protects aged mice from lethal respiratory virus infections. *J Virol* 2012; **86**: 11416-11424 [PMID: 22915814 DOI: 10.1128/JVI.01410-12]

188 **Boukhvalova MS**, Sotomayor TB, Point RC, Pletneva LM, Prince GA, Blanco JC. Activation of interferon response through toll-like receptor 3 impacts viral pathogenesis and pulmonary toll-like receptor expression during respiratory syncytial virus and influenza infections in the cotton rat Sigmodon hispidus model. *J Interferon Cytokine Res* 2010; **30**: 229-242 [PMID: 20038196 DOI: 10.1089/jir.2009.0025]

189 **Liao JY**, Thakur SA, Zalinger ZB, Gerrish KE, Imani F. Inosine-containing RNA is a novel innate immune recognition element and reduces RSV infection. *PLoS One* 2011; **6**: e26463 [PMID: 22028885 DOI: 10.1371/journal.pone.0026463]

190 **Brooks MJ**, Burtseva EI, Ellery PJ, Marsh GA, Lew AM, Slepushkin AN, Crowe SM, Tannock GA. Antiviral activity of arbidol, a broad-spectrum drug for use against respiratory viruses, varies according to test conditions. *J Med Virol* 2012; **84**: 170-181 [PMID: 22028179 DOI: 10.1002/jmv.22234]

191 **Shi L**, Xiong H, He J, Deng H, Li Q, Zhong Q, Hou W, Cheng L, Xiao H, Yang Z. Antiviral activity of arbidol against influenza A virus, respiratory syncytial virus, rhinovirus, coxsackie virus and adenovirus in vitro and in vivo. *Arch Virol* 2007; **152**: 1447-1455 [PMID: 17497238 DOI: 10.1007/s00705-007-0974-5]

192 **Martínez-Gil L**, Ayllon J, Ortigoza MB, García-Sastre A, Shaw ML, Palese P. Identification of small molecules with type I interferon inducing properties by high-throughput screening. *PLoS One* 2012; **7**: e49049 [PMID: 23145065 DOI: 10.1371/journal.pone.0049049]

193 **Teng MN**. The non-structural proteins of RSV: targeting interferon antagonists for vaccine development. *Infect Disord Drug Targets* 2012; **12**: 129-137 [PMID: 22335499 DOI: 10.2174/187152612800100170]

194 **Zhang Y**, Jamaluddin M, Wang S, Tian B, Garofalo RP, Casola A, Brasier AR. Ribavirin treatment up-regulates antiviral gene expression via the interferon-stimulated response element in respiratory syncytial virus-infected epithelial cells. *J Virol* 2003; **77**: 5933-5947 [PMID: 12719586 DOI: 10.1128/JVI.77.10.5933-5947.2003]

195 **Kugel D**, Kochs G, Obojes K, Roth J, Kobinger GP, Kobasa D, Haller O, Staeheli P, von Messling V. Intranasal administration of alpha interferon reduces seasonal influenza A virus morbidity in ferrets. *J Virol* 2009; **83**: 3843-3851 [PMID: 19193792 DOI: 10.1128/JVI.02453-08]

196 **Matzinger SR**, Carroll TD, Dutra JC, Ma ZM, Miller CJ. Myxovirus resistance gene A (MxA) expression suppresses influenza A virus replication in alpha interferon-treated primate cells. *J Virol* 2013; **87**: 1150-1158 [PMID: 23152507 DOI: 10.1128/JVI.02271-12]

197 **Xiao H**, Killip MJ, Staeheli P, Randall RE, Jackson D. The human interferon-induced MxA protein inhibits early stages of influenza A virus infection by retaining the incoming viral genome in the cytoplasm. *J Virol* 2013; **87**: 13053-13058 [PMID: 24049170 DOI: 10.1128/JVI.02220-13]

198 **Anafu AA**, Bowen CH, Chin CR, Brass AL, Holm GH. Interferon-inducible transmembrane protein 3 (IFITM3) restricts reovirus cell entry. *J Biol Chem* 2013; **288**: 17261-17271 [PMID: 23649619 DOI: 10.1074/jbc.M112.438515]

199 **Narayanan A**, Kehn-Hall K, Senina S, Lundberg L, Van Duyne R, Guendel I, Das R, Baer A, Bethel L, Turell M, Hartman AL, Das B, Bailey C, Kashanchi F. Curcumin inhibits Rift Valley fever virus replication in human cells. *J Biol Chem* 2012; **287**: 33198-33214 [PMID: 22847000 DOI: 10.1074/jbc.M112.356535]

200 **Si X**, Wang Y, Wong J, Zhang J, McManus BM, Luo H. Dysregulation of the ubiquitin-proteasome system by curcumin suppresses coxsackievirus B3 replication. *J Virol* 2007; **81**: 3142-3150 [PMID: 17229707]

201 **Obata K**, Kojima T, Masaki T, Okabayashi T, Yokota S, Hirakawa S, Nomura K, Takasawa A, Murata M, Tanaka S, Fuchimoto J, Fujii N, Tsutsumi H, Himi T, Sawada N. Curcumin prevents replication of respiratory syncytial virus and the epithelial responses to it in human nasal epithelial cells. *PLoS One* 2013; **8**: e70225 [PMID: 24058438 DOI: 10.1371/journal.pone.0070225]

202 **Xie XH**, Zang N, Li SM, Wang LJ, Deng Y, He Y, Yang XQ, Liu EM. Resveratrol Inhibits respiratory syncytial virus-induced IL-6 production, decreases viral replication, and downregulates TRIF expression in airway epithelial cells. *Inflammation* 2012; **35**: 1392-1401 [PMID: 22391746 DOI: 10.1007/s10753-012-9452-7]

203 **Carr JM**, Mahalingam S, Bonder CS, Pitson SM. Sphingosine kinase 1 in viral infections. *Rev Med Virol* 2013; **23**: 73-84

[PMID: 22639116 DOI: 10.1002/rmv.1718]
204 **Maceyka M**, Harikumar KB, Milstien S, Spiegel S. Sphingosine-1-phosphate signaling and its role in disease. *Trends Cell Biol* 2012; **22**: 50-60 [PMID: 22001186]
205 **Shirey KA**, Pletneva LM, Puche AC, Keegan AD, Prince GA, Blanco JC, Vogel SN. Control of RSV-induced lung injury by alternatively activated macrophages is IL-4R alpha-, TLR4-, and IFN-beta-dependent. *Mucosal Immunol* 2010; **3**: 291-300 [PMID: 20404812]
206 **Verma S**, Kumar M, Nerurkar VR. Cyclooxygenase-2 inhibitor blocks the production of West Nile virus-induced neuroinflammatory markers in astrocytes. *J Gen Virol* 2011; **92**: 507-515 [PMID: 21106803]
207 **Nabatov AA**, Pollakis G, Linnemann T, Paxton WA, de Baar MP. Statins disrupt CCR5 and RANTES expression levels in CD4(+) T lymphocytes in vitro and preferentially decrease infection of R5 versus X4 HIV-1. *PLoS One* 2007; **2**: e470 [PMID: 17520029 DOI: 10.1371/journal.pone.0000470]

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REVIEW

Cystic fibrosis transmembrane conductance regulator chloride channel blockers: Pharmacological, biophysical and physiological relevance

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Abstract

Dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel causes cystic fibrosis, while inappropriate activity of this channel occurs in secretory diarrhea and polycystic kidney disease. Drugs that interact directly with CFTR are therefore of interest in the treatment of a number of disease states. This review focuses on one class of small molecules that interacts directly with CFTR, namely inhibitors that act by directly blocking chloride movement through the open channel pore. In theory such compounds could be of use in the treatment of diarrhea and polycystic kidney disease, however in practice all known substances acting by this mechanism to inhibit CFTR function lack either the potency or specificity for *in vivo* use. Nevertheless, this theoretical pharmacological usefulness set the scene for the development of more potent, specific CFTR inhibitors. Biophysically, open channel blockers have proven most useful as experimental probes of the structure and function of the CFTR chloride channel pore. Most importantly, the use of these blockers has been fundamental in developing a functional model of the pore that includes a wide inner vestibule that uses positively charged amino acid side chains to attract both permeant and blocking anions from the cell cytoplasm. CFTR channels are also subject to this kind of blocking action by endogenous anions present in the cell cytoplasm, and recently this blocking effect has been suggested to play a role in the physiological control of CFTR channel function, in particular as a novel mechanism linking CFTR function dynamically to the composition of epithelial cell secretions. It has also been suggested that future drugs could target this same pathway as a way of pharmacologically increasing CFTR activity in cystic fibrosis. Studying open channel blockers and their mechanisms of action has resulted in significant advances in our understanding of CFTR as a pharmacological target in disease states, of CFTR channel structure and function, and of how CFTR activity is controlled by its local environment.

Key words: Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator; Chloride channel; Open channel block; Channel pore; Permeation; Anion secretion; Potentiators

Core Tip: This review summarizes our understanding of small molecules that inhibit the cystic fibrosis transmembrane conductance regulator (CFTR) by blocking the channel pore. It describes how such inhibitors could be used in the treatment of diarrhea and hereditary kidney disease; how studying these inhibitors' mechanisms of action has led to advances in our understanding of CFTR channel structure and function; and how substances acting *via* this mechanism could contribute to the physiological control of CFTR function in epithelial cells. Ironically, studying channel inhibitors has recently led to the discovery of a new class of CFTR potentiators that could be used to treat cystic fibrosis.

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INTRODUCTION

Cystic fibrosis (CF) is the most common fatal autosomal recessive disease affecting Caucasians, with around 80000 CF sufferers in the world today. CF is caused by mutations that cause loss of function of the CF transmembrane conductance regulator (CFTR) protein[1]. Over 1900 different mutations that affect the transcription, synthesis, trafficking, turnover, or function of CFTR have been shown to cause CF. CFTR is expressed in the apical membrane of many different epithelial tissues, where it plays a central role in epithelial Cl^-, HCO_3^-, and fluid transport[2]. As a consequence, CF is associated with respiratory, pancreatic, gastrointestinal, and reproductive disease that results from deficient salt and fluid secretion in these epithelia[1,3]. Conversely, inappropriately elevated CFTR function results in excessive intestinal fluid secretion in secretory diarrhoeas such as that associated with cholera[4]. CFTR-mediated Cl^- transport by renal epithelial cells also underlies fluid accumulation and growth of renal cysts in autosomal dominant polycystic kidney disease (ADPKD), the most common hereditary kidney disease[5]. The involvement of CFTR in such common and serious disease states makes it an attractive target for therapeutic intervention. Many different small molecules interact directly with the CFTR protein, and these have proven useful experimental tools. The therapeutic potential of drugs that act directly with CFTR is also receiving increasing interest. This review focuses on one class of small molecules interacting with CFTR-those that directly block Cl^- movement through the open channel pore.

OVERVIEW OF CFTR ARCHITECTURE

CFTR is a member of a large family of membrane proteins, the adenosine triphosphate (ATP)-binding cassette (ABC) proteins, most members of which function as active transport ATPases[6,7]. CFTR appears to be unique within the ABC family in functioning instead as an ATP-dependent Cl^- channel[8]. The structure and function of CFTR has been reviewed in detail recently[8-12] and will be described only briefly here. In common with other ABC proteins, CFTR has a modular architecture, consisting of two membrane-spanning domains (MSDs) each comprising six transmembrane α-helices (TMs) (Figure 1). Each MSD is followed by a cytoplasmic nucleotide binding domain (NBD), and the two MSD-NBD modules are joined by a cytoplasmic regulatory domain (R domain) that is unique to CFTR. The modular architecture of CFTR also corresponds with its defining functional features. The R domain contains multiple consensus phosphorylation sites for protein kinase A and protein kinase C, allowing the channel to be regulated physiologically by hormones that act through these protein kinases. Phosphorylation of the R domain is a prerequisite for channel activity. Following R domain phosphorylation, CFTR channel gating (opening and closing) is controlled by ATP binding and hydrolysis at a dimer of the two NBDs. The NBDs also make physical contact with the long intracellular loops (ICLs) that join individual TMs (Figure 1). The channel pore that forms the transmembrane pathway for the movement of Cl^- ions is formed by a pseudo-symmetrical arrangement of the two MSDs. Recent evidence suggests that the ICLs form a functional link that allows a conformational rearrangement initiated by ATP interaction with the NBDs to be transmitted to the TMs, controlling the opening and closing the channel pore.

The channel pore itself has been studied using a combination of structural[10,13], functional[8,14], substituted cysteine accessibility[8,15,16] and molecular modeling[17-21] approaches. A simple model of the proposed overall functional architecture of the pore is shown in Figure 1C. The pore is thought to have a relatively narrow region over which discrimination between different anions is predominantly determined. This region is flanked by outer and inner vestibules, with functional evidence suggesting that the inner vestibule is both deeper and wider. Of the 12 TMs (Figure 1), TM6 appears to play a dominant role in determining functional interactions between the narrow pore region and permeating anions[15,22]. TM1, TM6, TM11 and TM12 all contribute to the inner vestibule[15,23-29], while TM1, TM6, TM11, TM12, and the extracellular loops (ECLs) adjacent to these TMs contribute to the outer vestibule[16,30-33]. As described in detail below (see "*Biophysical Relevance*"), residues from TM1 (K95), TM5 (R303), TM6 (S341) and TM12 (S1141) have all been proposed to interact with CFTR open channel blockers (Figure 1D and E).

CFTR CHANNEL BLOCKERS

The first kinds of CFTR inhibitors to be identified were those that act as open channel blockers[34,35] (Figure 2). These are substances that enter into the open channel pore and physically occlude it, temporarily preventing the flow of Cl^- ions until the blocker molecule dissociates from the pore. Many diverse substances share this mechanism of CFTR channel block, the best known (and best studied) of which are sulfonylureas such as glibenclamide[36-42] and related substances[36,42-44], arylaminobenzoates such as 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and diphenylamine-2-carboxylate[23,45-48], and disulfonic stilbenes such as 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid and 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS)[49]. Detailed biophysical analysis of the blocking effects of these groups of negatively charged substances reveal a number of common features that may reflect a common mechanism of action. In each case the blocker enters the pore only from its cytoplasmic end to reach its binding site inside the channel pore (Figure 2); block is voltage-dependent, being stronger at more hyperpolarized voltages that favour entry of negatively charged substances into the pore from its cytoplasmic

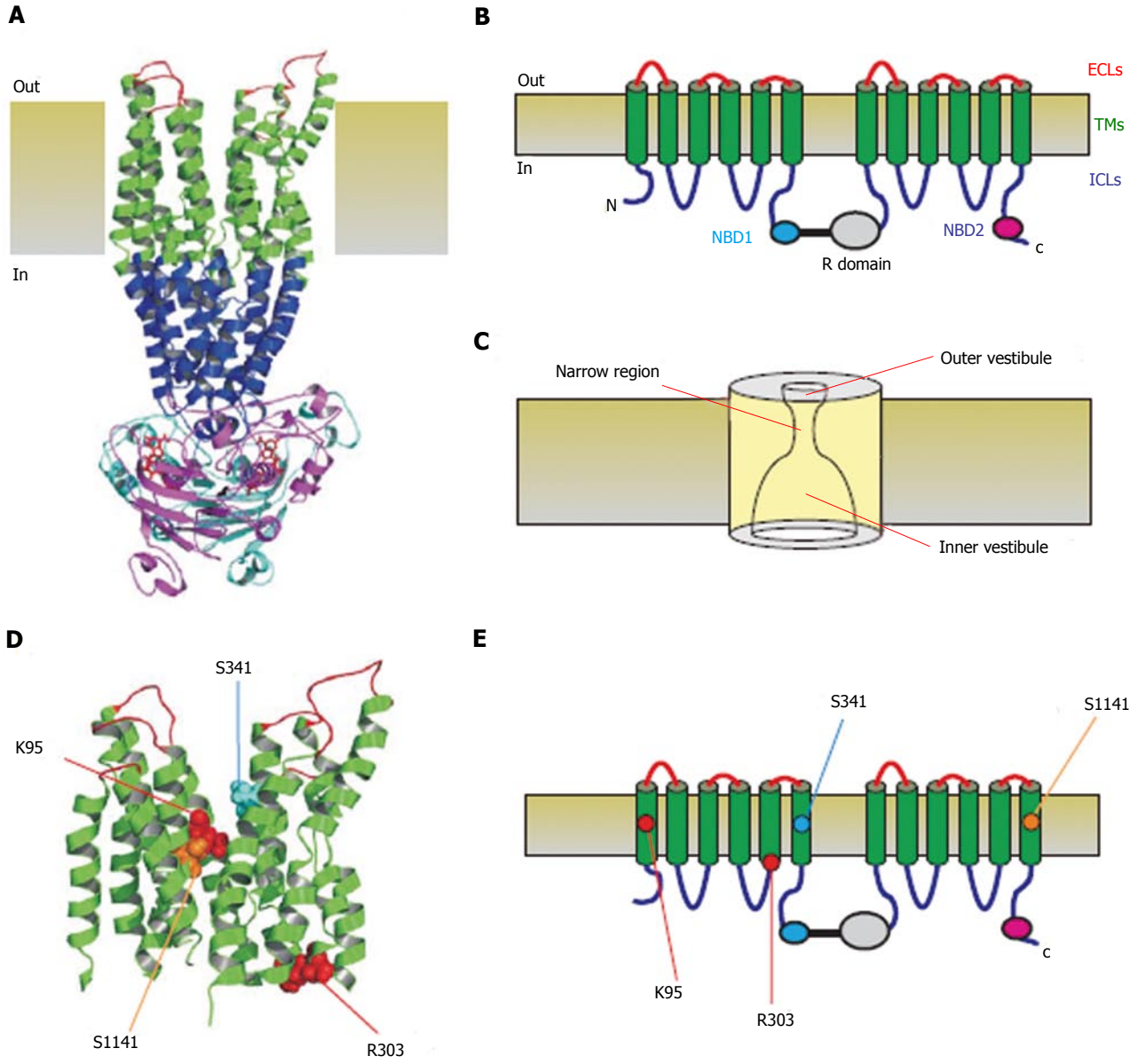

Figure 1 Three-dimensional and two-dimensional representations of cystic fibrosis transmembrane conductance regulator structure. A: Atomic homology model of cystic fibrosis transmembrane conductance regulator in a so-called "channel like" conformation[20]. Different colours are used to illustrate the approximate extent of the extracellular loops (ECLs, red), transmembrane domains (TMs, green), intracellular loops (ICLs, blue), and two nucleotide binding domains (NBD1, cyan; NBD2, magenta). The cytoplasmic R domain is not included in this homology model; B: Schematic representation of these different domains (and the R domain), using the same colour scheme; C: Functional model of pore architecture. As described in the text, experimental evidence suggests that the pore has a narrow region that is connected to the cytoplasmic and extracellular solutions by a wide inner vestibule and a narrower outer vestibule, respectively; D: Location of putative blocker-interacting residues in the TMs (K95-TM1; R303-TM5; S341-TM6; S1141-TM12) within the same homology model shown in A. E: Location of these same residues in the same schematic model shown in B.

end; and block is sensitive to the extracellular Cl^- concentration, being stronger at low Cl^- and weaker at higher Cl^-. Each of these defining features tells us something about the mechanism of inhibition and the location of the blocker binding site. Inhibition from the cytoplasmic side of the membrane was originally used to suggest that the open CFTR channel pore is structurally asymmetric, with a wide inner vestibule that is easily accessible from the cytoplasm[35,49], and a narrower extracellular entrance that prevents the entry of large substances from the extracellular solution (Figure 2). Voltage-dependent block suggests that the blocker binding site is located within the transmembrane electric field, such that the blocker apparently experiences at least part (generally about 20%-50%) of this electric field as it moves between the cytoplasm and its binding site inside the pore. While the relationship between distance across the transmembrane electric field and physical distance across the membrane itself is neither direct nor straightforward, this voltage-dependence is consistent with the blocker moving into the membrane-spanning parts of CFTR to access the blocker binding site. Finally, sensitivity of block to the extracellular Cl^- concentration is usually ascribed to repulsive electrostatic interactions between Cl^- and the negatively

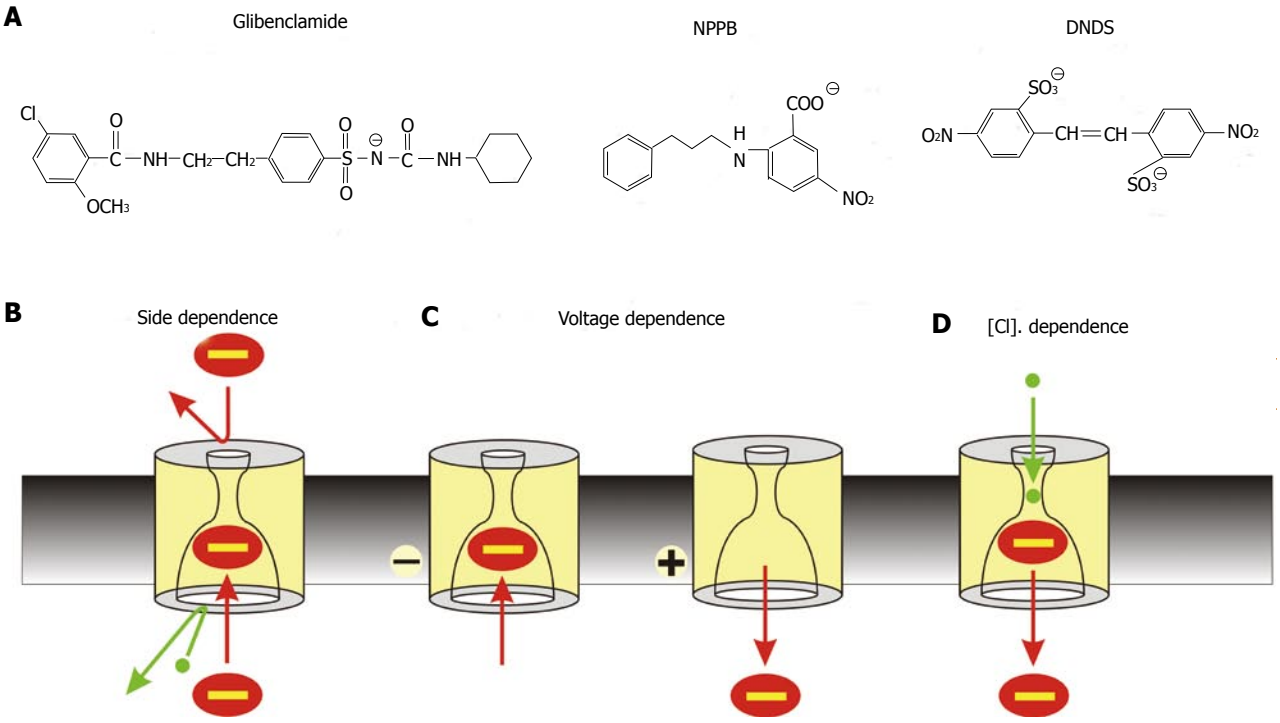

Figure 2 Mechanism of open channel blocker action. A: Chemical structures of three well-known voltage-dependent cystic fibrosis transmembrane conductance regulator (CFTR) channel blockers: the sulfonylurea glibenclamide, the aryl amino benzoate 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), and the disulfonic stilbene 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS). B-D: Characteristic functional properties of block shared by these and other CFTR open channel blockers: block is side-dependent, voltage-dependent, and sensitive to extracellular Cl^- concentration; B: Blockers enter the pore only from its cytoplasmic end, likely because the extracellular entrance to the pore is too small and/or the narrow pore region prevents them from accessing their binding site in the wide inner vestibule; C: Block is relatively strengthened at hyperpolarized membrane potentials that favour entry of negative substances into the pore from the cytoplasm, and weakened at depolarized membrane potentials that favour anion retention in the cytoplasm; D: Block is weakened at higher extracellular Cl^- concentrations; this is usually ascribed to a "knock-off" mechanism whereby Cl^- entering the pore from its extracellular end electrostatically repels negatively charged blockers back into the cytoplasm, destabilizing blocker binding inside the pore.

charged blocker molecule that take place when both are bound simultaneously within the open channel pore.

Many or all of these features of the blocking reaction have been observed with other, unrelated blocking anions, including substrates of related ABC proteins[50,51] such as the conjugated bile salt taurolithocholate-3-sulfate (TLCS) and the conjugated steroid β-estradiol 17-(β-D-glucuronide), indazole compounds such as lonidamine[52,53], short-chain fatty acids butyrate and 4-phenylbutyrate[54], the fluorescein derivative phloxine B[55], and even commonly used experimental compounds such as the pH buffer 3-(4-morpholino) propane sulfonic acid[56] and the negatively charged cysteine-reactive reagent (2-sulfatoethyl) methanesulfonate (MTSES)[57]. Together these open channel blocking substances represent a large and structurally diverse group of organic anions, suggesting that entry of anions into the CFTR Cl^- channel pore from its cytoplasmic end is a process that shows little specificity or size discrimination. Furthermore, most of these blockers show relatively low potency (dissociation constants usually in the micromolar to millimolar range, depending on voltage). At the single channel level, these blockers may cause discrete "flickers" in the open channel current (due to resolved individual blocking events)[3,7,38,44,45,47,49,51,52], or an apparent reduction in single channel current amplitude where blocking and unblocking are too fast to be resolved at the bandwidth used for patch clamp recording[44,48,50,54,56-58]. These effects reflect kinetically "intermediate" and "fast" blocking and unblocking reactions, respectively, according to the scheme proposed by Hille[59]. The low apparent affinity of CFTR open channel blockers limits their potential for use *in vivo*. Furthermore, many of these substances also block other classes of Cl^- channels[34,60], perhaps reflecting some structural similarity amongst Cl^- channel pores that results in similar sensitivity to block by organic anions[14].

In more recent years, high-throughput screening technologies have been used to identify more potent CFTR inhibitors[61]. The thiazolidinone $CFTR_{inh}$-172 inhibits CFTR from the cytoplasmic side of the membrane at sub-micromolar concentrations[62], due to a voltage-independent effect on channel gating[63,64]. Glycine hydrazides such as GlyH-101 cause voltage-dependent block from the extracellular side of the membrane at low micromolar concentrations[65]. These substances appear to inhibit CFTR by different mechanisms than that described above and in Figure 2 for intracellular open channel blockers. $CFTR_{inh}$-172 has been shown to bind preferentially to open channels, perhaps triggering a conformational change to an "inactivated" nonconducting state[64]. GlyH-101 does appear to act as an open channel blocker, however acting from the extracellular side of the mem-

brane[65], perhaps becoming lodged close to the narrow pore region to occlude Cl^- permeation[66]. These two potent and relatively selective inhibitors have become drugs of choice for experimental inhibition of CFTR activity; because of their different sidedness of action, $CFTR_{inh}$-172 is preferred when applied to the intracellular side of the membrane, and GlyH-101 for extracellular application.

Finally, a 3.7 kDa peptide toxin isolated from scorpion venom and named GaTx1 inhibits CFTR channels from the cytoplasmic side of the membrane at sub-micromolar concentrations[67]. Although the molecular mechanism of GaTx1 inhibition is not well defined, this substance has been described as acting as a non-competitive inhibitor of channel gating[67,68], with no demonstrated open channel blocking action. Currently GaTx1 is the only known peptide inhibitor of CFTR.

PHARMACOLOGICAL RELEVANCE

Because of the inviolable relationship between loss of CFTR function and CF, there is tremendous current interest in the identification and development of small molecules that directly interact with the CFTR protein to increase its function (known as CFTR "potentiators")[1,69-72]. On the other hand, it has long been suggested that CFTR channel blockers could (at least in theory) be used in the treatment of secretory diarrhoea and ADPKD[35,73]. CFTR inhibitors have also been suggested as potential male contraceptives[53,74]. As described above, known intracellular-active open channel blockers lack either the potency or the specificity for *in vivo* use. However, the higher affintiy CFTR inhibitors $CFTR_{inh}$-172 and GlyH-101 have been shown to be effective in *in vitro* and *in vivo* models of secretory diarrhea[62,65,75]. Moreover, non-absorbable lectin conjugated forms of GlyH-101 were active against cholera-induced fluid secretion and mortality in mice when administered orally[76]. Similarly, $CFTR_{inh}$-172 and GlyH-101 (or closely related substances) have been shown to retard cyst growth in *in vitro*[77,78] and *in vivo*[78] models of ADPKD. The therapeutic potential of potent and specific CFTR inhibitors has been discussed in several recent reviews[61,79,80].

BIOPHYSICAL RELEVANCE

Since open channel blockers bind to specific sites within the channel pore with relatively high affinity (compared to Cl^- and other permeant anions), they have proven invaluable probes of the structure and function of the Cl^- permeation pathway. Mutations in TM6 and TM12 have been shown to alter the affinity of block by arylaminobenzoates[23,46], sulfonylureas[42,81] and lonidamine[52], consistent with functional evidence[25-28,82] and molecular models[17-21] that suggest that these two TMs make substantial contributions to the inner vestibule of the pore where the blocker binding site is thought to reside (Figures 1 and 2). Because open channel blockers are anions, and because positively charged amino acid side chains in the CFTR channel pore are known to play important roles in electrostatic attraction of Cl^- ions[24,30,31,82,83], much attention has also been placed on the role of such fixed positive charges in interactions with blockers. In particular, mutations that remove the positive charge at lysine residue K95 in TM1 (Figure 1D and E) dramatically reduce the channel blocking affinity of glibenclamide, DNDS, lonidamine, NPPB and TLCS[24,82]. This finding suggests that these structurally diverse open channel blockers share a common molecular mechanism of block - they are attracted into the wide inner vestibule by electrostatic attraction between the negative charge on the blocker molecule and the positive charge on the lysine side chain at K95, and once in the inner vestibule they bind tightly enough to occlude the pore and temporarily prevent Cl^- permeation (Figure 3). This model of blocker binding in the pore inner vestibule is also supported by a recent *in silico* investigation of blocker docking inside the pore of an atomic homology model of CFTR[20]. Neutralization of fixed positive charge in the inner vestibule by mutagenesis of K95 also decreases single channel Cl^- conductance by about 85%[22,29,82], suggesting that this positive charge also plays an important role in the normal Cl^- permeation mechanism, most likely due to electrostatic attraction of Cl^- ions. The functional importance of the positive charge on the side chain of K95 may explain the sensitivity of CFTR to broad range of intracellular anionic blockers: a positive charge in the inner vestibule is necessary to attract Cl^- ions and so maximize the rate of Cl^- permeation, however, this fixed positive charge also attracts all anions in the cytoplasm, many of which reside within the wide inner vestibule for long enough to temporarily block the passage of Cl^- ions beyond into the narrow pore region. Mutagenesis of all positively charged lysine and arginine residues within the TMs suggests that K95 plays a unique role within the pore inner vestibule in attracting permeant and blocking ions[31,83], although other positive charges may also play somewhat analogous roles in attracting cytoplasmic ions to more superficial parts of the pore close to its intracellular mouth.

If K95 does play a unique role in attracting anions into the pore inner vestibule - suggesting that it might be the only fixed positive charge located close to the blocker binding site within this vestibule[82] then what would be the effect of adding a second positive charge to the walls of this vestibule? This question has been addressed by using mutagenesis to introduce additional positively charged lysine residues at positions that have been shown to donate pore-lining side chains to the pore inner vestibule. Initially it was demonstrated that the unique, important role played by the positive charge at K95 could be "moved" from TM1 to TM12. Thus, while the charge-neutralizing K95S mutation dramatically decreased both Cl^- conductance and sensitivity to open channel blockers, the double mutant K95S/S1141K showed similar single channel conductance and open channel blocker binding properties as wild type CFTR[82]. This "rescue" of channel function suggests that these two amino acids play

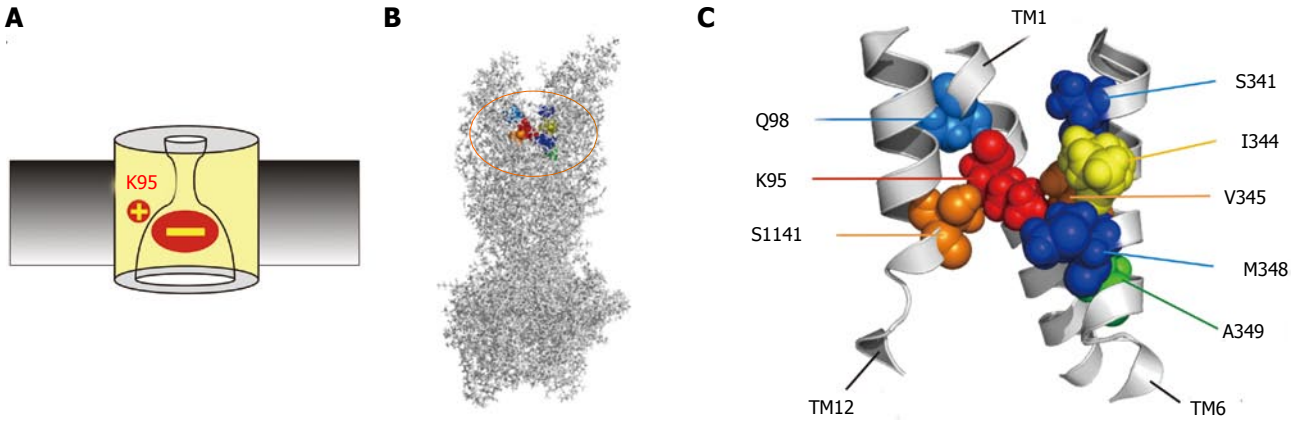

Figure 3 Location of amino acid residues key for blocker interactions in the pore inner vestibule. A: The positively charged side chain of lysine residue K95 is essential for block, due to electrostatic attraction between this positive charge and the negatively charged blocker. However, this important charge can also be supported by other amino acid side chains that line the pore inner vestibule. B, C: Sites that have been shown to host positive charge that can support block are shown in an atomic homology model of the whole cystic fibrosis transmembrane conductance regulator protein (B) and in a detailed view of the central portions of TMs 1, 6 and 12 (C) the area highlighted in (B). The endogenous positively charged side chain of K95 is shown in red; those residues that were deemed best able to support this functionally important positive charge in orange (V345, S1141) or yellow (I344); and those that were able to host this positive charge to a lesser extent in blue (Q98, S341, M348) or green (A349). The homology model used here is the "channel like" conformation presented by ref[20] and shown in Figure 1A; other models give similar relative positions of these pore-lining side chains.

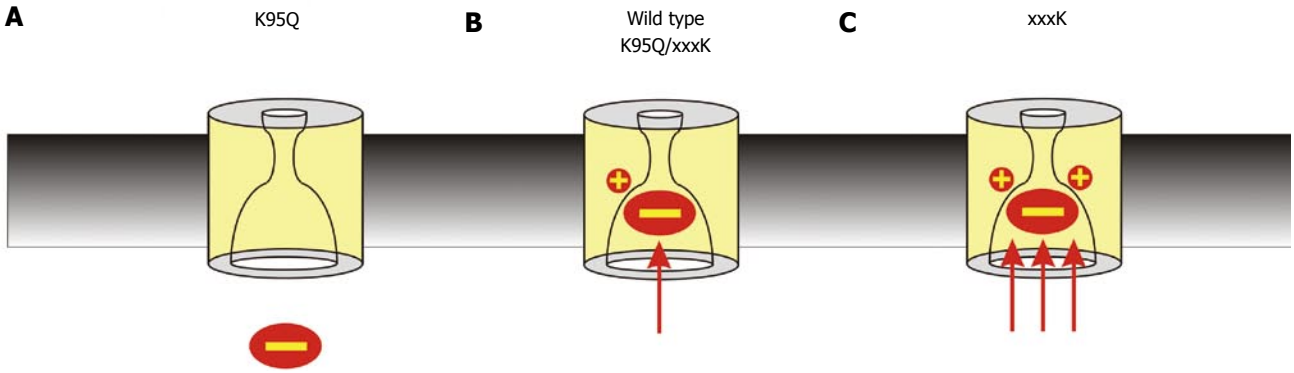

Figure 4 Importance of the number of fixed positive charges in the pore inner vestibule. The importance of electrostatic interactions with the pore inner vestibule is demonstrated by the finding that the strength of block can be decreased or increased by mutations that decrease or increase, respectively, the number of positively charged amino acid side chains in the pore inner vestibule area shown in Figure 3C. A: Block is relatively weak in when the endogenous positive charge is removed, for example as in the K95Q mutation; B: Block is of similar strength to that observed in wild type cystic fibrosis transmembrane conductance regulator when the positive charge is "transplanted" to other, nearby sites, for example as in the double mutants K95Q/I344K, K95Q/V345K, and K95S/S1141K; C: Block is relatively strong when a second positive charge is introduced, for example as in I344K, V345K and S1141K.

interchangeable roles within the pore inner vestibule, in that either could effectively host the positive charge that supports interactions with Cl^- and blocking anions[82] (Figure 4). Substituted cysteine accessibility mutagenesis and disulfide cross-linking experiments indicated that the amino acid side chains at these two positions line the inner vestibule in open channels and that these side chains are in close physical proximity[82]. Subsequent experiments showed that the positive charge from K95 could similarly be transplanted to different pore-accessible positions in TM6 (I344, V345, M348, A349), as well as a site closer to the extracellular end of TM1 (Q98)[29]. Thus it appears that the exact location of the positive charge in the pore inner vestibule is not critical to support channel function. The ability of other sites in TMs 1, 6 and 12 to accommodate the positive charge that normally resides at K95 then allowed investigation of the effects of introducing a second positive charge at these sites (by mutagenesis to lysine) while retaining the positive charge at K95 - in effect, increasing the number of positive charges located deep in the pore inner vestibule from one to two (Figure 4). Interestingly, at no site tested (Q98K, I344K, V345K, M348, A349K, S1141K) did the addition of a second positive charge increase single channel conductance[29,82]. This suggests that, while the presence of one positive charge is essential for normal Cl^- conductance, a second positive charge provides no additional benefit. However, a second fixed positive charge (in S1141K) increased the strength of block by cytoplasmic NPPB, and also induced apparent voltage-dependent channel block by polyvalent anions present in the experimental solutions (ATP, pyrophosphate)[82], suggesting that the number of positive charges was correlated with open channel blocker potency (Figure 4). This suggestion was most strongly supported using the small divalent anion $Pt(NO_2)_4^{2-}$, which also causes voltage-dependent open

channel block in a K95-dependent manner[84,85]. Addition of a second positive charge to nearby pore-lining sites in TM6 (I344K, V345K) or TM12 (S1141K) led to a dramatic (40-100 fold) increase in the apparent affinity of intracellular $Pt(NO_2)_4^{2-}$ block[29,82], suggesting that increasing the number of positive charges in the pore has a greater impact on interactions with multivalent anions (such as $Pt(NO_2)_4^{2-}$ and ATP) than monovalent anions such as Cl^-. Positive charges introduced at other sites within the pore inner vestibule (Q98K, S341K, M348K, A349K) also supported strengthened $Pt(NO_2)_4^{2-}$ block, albeit to a lesser extent. These findings, summarized in Figure 4, led to the hypothesis that one positive charge in the inner vestibule (as in wild type CFTR) was optimum for CFTR channel function[82]. Thus, removal of the one endogenous positive charge (as in K95Q or K95S) decreases channel function due to reduced electrostatic attraction of Cl^- ions and a resulting dramatic decrease in channel conductance. This effect can be "rescued" by introducing a positive charge at other, nearby positions (as in K95S/S1141K and K95Q/V345K). Conversely, addition of a second positive charge (as in I344K, V345K, S1141K and other lysine substitutions) results in no further increase in Cl^- conductance but increases the electrostatic attraction of multivalent anions that block the pore, resulting in an overall decrease in channel function. Thus the greatest importance of a single fixed positive charge in the inner vestibule may be in conferring preference for monovalent anions, including the physiological channel transport substrates Cl^- and HCO_3^-.

If one is the optimum number of positive charges in the inner vestibule to maximize channel function, where is the optimal location for this charge? Since normal channel function can be rescued by moving the positive charge to other, nearby sites in TM1, TM6 or TM12, the exact location of this charge does not appear to be critical[29]. Of all sites tested as hosts of the positive charge (K95, Q98 in TM1; S341, I344, V345, M348, A349 in TM6; S1141 in TM12), K95 appears optimal in terms of maximizing single channel conductance[29]. In terms of both single channel conductance and blocker binding properties, I344, V345 and S1141 appeared to be the best locations for a positive charge to reproduce wild type properties, with Q98, S341, M348 and A349 also being able to host this positive charge to some extent[29]. Similarly, a second positive charge at I344, V345 or S1141 had the greatest impact on divalent $Pt(NO_2)_4^{2-}$ block[29]. These ideas are presented graphically, within the framework of a recent atomic homology model of CFTR, in Figure 3C. Within this model, the side chains of I344, V345 and S1141 appear to be at approximately the same "depth" into the channel pore as K95; with Q98 and S341 being located more deeply into the pore from its cytoplasmic end, and M348 and A349 closer to the cytoplasmic mouth of the pore. This relative location of amino acids is also supported by experimental evidence that disulfide bonds can be formed between cysteine side chains substituted for K95 and S1141[82], as well as between K95C and I344C and between Q98C and I344C[86]. This model suggests that it is location along the axis of the channel pore that is most important in defining the functional effects of a positive charge in the pore inner vestibule: residues close to the endogenous site at K95 are best able to substitute and rescue pore function, while residues either further from, or closer to, the cytoplasmic entrance of the pore are less able to host this important positive charge. This is consistent with molecular modeling studies that show open channel blockers docked within the pore inner vestibule and with their negative charges close to the positive charge of K95[20].

As described above, interaction with the positive charge at K95 and occlusion of the pore inner vestibule appears to be the molecular mechanism of many different kinds of CFTR open channel blockers, including those shown in Figure 2. However, a second blocker binding site was identified using the large, polyvalent organic anion suramin[87]. Suramin causes potent, voltage-independent block of CFTR channels exclusively from the intracellular side of the membrane[87,88]. Furthermore, block by intracellular suramin is independent of extracellular Cl^- concentration, and completely unaffected by removal of the key positive charge in the inner vestibule in the K95Q mutant[87]. This suggests that suramin does not enter deeply enough into the pore inner vestibule to experience electrostatic interaction with K95, perhaps because the suramin molecule is simply too big to pass into this restricted pore region. In contrast, suramin block was weakened in an electrostatic fashion by mutagenesis of another positively charged amino acid, R303 at the cytoplasmic end of TM5[87] (Figure 1D and E). This result was consistent with the previous finding that the positive charge of R303 attracts intracellular Cl^- ions to the cytoplasmic entrance of the pore[83] and suggests that the large suramin molecule may be able to occlude the cytoplasmic mouth of the pore to prevent Cl^- movement into or out of the pore. As shown in Figure 5, this proposed molecular mechanism of suramin action is consistent with observed biophysical differences between suramin block and block by other (smaller) open channel blockers that interact with K95 (see above). Because of its size, suramin does not penetrate deeply into the inner vestibule; as a result, it does not traverse enough of the transmembrane electric field to generate measurable voltage-dependence of block, it does not reside in close proximity to Cl^- ions bound within the channel pore (perhaps in the narrow pore region or close to the outer extent of the inner vestibule) and so does not experience the kind of repulsive electrostatic interactions that are thought to underlie extracellular Cl^- dependence, and it does not approach close enough to K95 to experience attractive electrostatic interactions with this positively charged residue (Figure 5). Electrostatic interaction with R303 near the cytoplasmic mouth of the pore may also contribute to the inhibitory effects of other substances on CFTR, for example arachidonic acid[89].

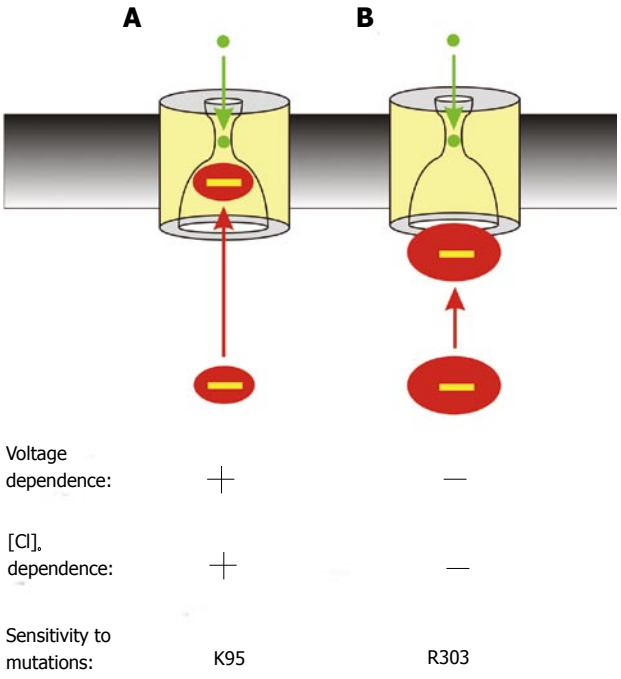

Figure 5 Two distinct proposed mechanisms of block by cytoplasmic anions. A: The effects of most blockers are voltage- and Cl^- dependent (as described in Figure 2) and are sensitive to mutations that remove the positive charge at K95; B: The large multivalent anion suramin blocks the channel in a voltage- and Cl^- independent fashion, and its effects are dependent on a positive charge at R303 but independent of K95. This is interpreted as the large suramin molecule blocking the cytoplasmic entrance to the pore; at a site that does not involve entering significantly into the transmembrane electric field or approaching close enough to Cl^- ions inside the pore to experience repulsive electrostatic interactions.

PHYSIOLOGICAL RELEVANCE

CFTR channel currents are routinely studied in excised, inside-out membrane patches, where the current-voltage relationship is uniformly linear[90,91] (Figure 6). Conversely, CFTR channel currents in intact cells, including native epithelial cells[92-94], cardiac myocytes[95,96], and many different heterologous expression systems[82,97-99], exhibit outward rectification of the current-voltage relationship, such that outward currents (carried by Cl^- influx) show greater conductance than inward currents (carried by Cl^- efflux) (Figure 6). This rectification - and its disappearance in cell-free membrane patches - led to the longstanding suggestion that CFTR channels in intact cells are subject to voltage-dependent block by unknown cytosolic anions[58,97,100]. This appears to reflect predominantly a voltage-dependent flickery block by cytosolic anions that is lost when the membrane patch is excised from the cell[94,97,98,100], although differences in single channel conductance in cell-attached and excised patches have also been reported[92,95,97]. Detailed single channel recording experiments from cell-attached membrane patches suggested that the flickery blocking mechanism was functionally analogous to that generated by exogenous voltage-dependent blocking anions with intermediate blocking and unblocking kinetics[100]. Open channel block as the mechanism of outward rectification was further

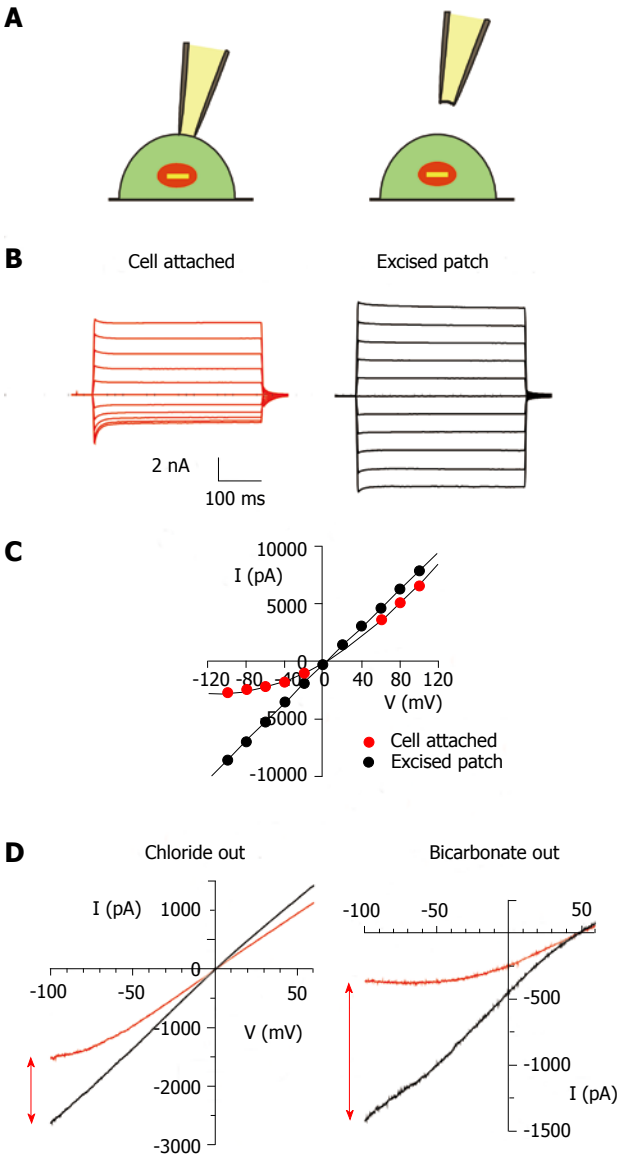

Figure 6 Channel block by cytoplasmic anions in intact cells and its dependence on extracellular anions. A: During patch clamp recording from intact cells, cystic fibrosis transmembrane conductance regulator (CFTR) channels in the cell membrane are subject to block anions present in the cytoplasm of the cell (left). This blocking effect is lost when the patch of membrane is excised into the inside-out patch configuration (right); B: Example of this effect during macroscopic CFTR current recording from a baby hamster kidney cell expressing human CFTR, as described in detail[82]. Currents were recorded before (red) and after (black) excision of the patch from the cell, during voltage steps to between -100 mV and +100 mV in 20 mV increments from a holding potential of 0 mV. Dotted line represents the zero current level; C: Current-voltage relationships for the currents shown in (B). Note the outward rectification of the relationship in cell-attached recording (red) due to voltage-dependent channel block, and loss of this blocking effect following patch excision (black); D: Similar example current-voltage relationships from baby hamster kidney cell membrane patches when the extracellular solution contained 150 mmol/L NaCl (left) or 150 mmol/L $NaHCO_3^-$ (right), as described in detail[101]. Note that the apparent degree of block in cell attached patches (red) is stronger when the extracellular solution contains HCO_3^- compared to Cl^-, an effect quantified in detail in ref. [101].

supported by the more recent finding that inhibition of currents in intact cells was reduced in K95Q-CFTR and (to a lesser extent) R303Q-CFTR[101]. This indicates that the unknown cytosolic blocking anions interact with

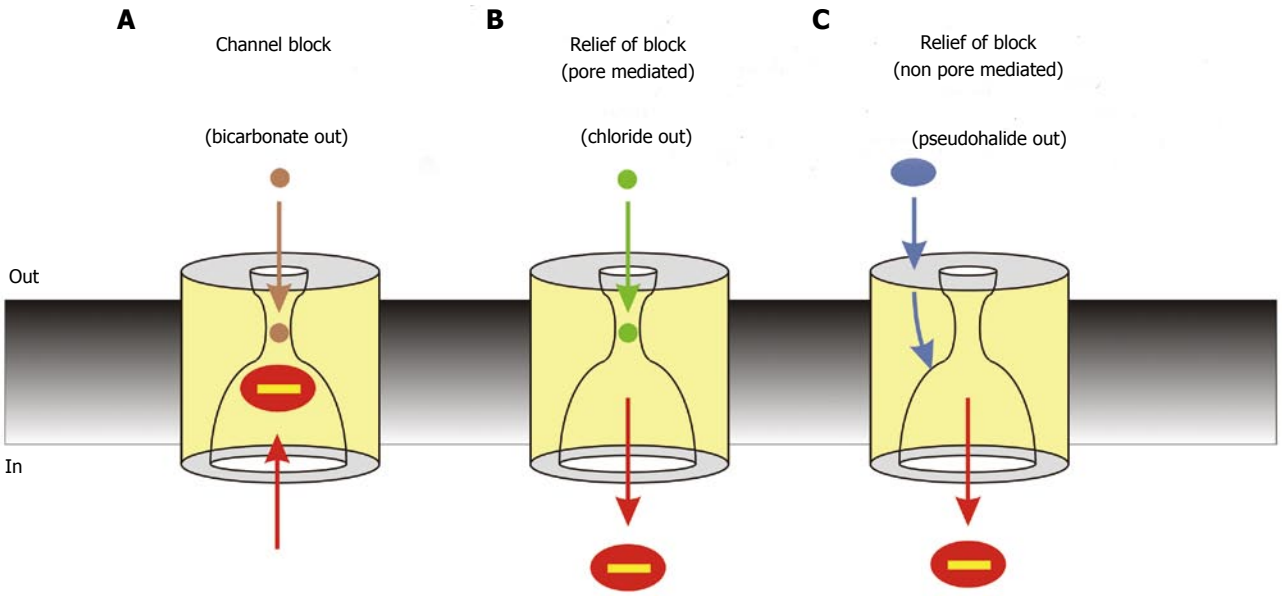

Figure 7 Interactions between cytoplasmic blocking anions and extracellular anions. Cytoplasmic block is modified by extracellular anions by different mechanisms, leading to different degrees of block under different conditions. A: Block is strong in the absence of modulation of block by extracellular anions; physiologically, such a condition may occur during periods of epithelial HCO_3^- secretion, leading to strong block of cystic fibrosis transmembrane conductance regulator (CFTR) channel currents under these conditions[101]; B: Block is weakened by extracellular anions that can enter the channel pore, such as Cl^-, due to an electrostatic "knock-off" mechanism. This may lead to increased CFTR channel currents during periods of epithelial Cl^- secretion[101]; C: Block is weakened by extracellular anions that interact with an extracellular part of the protein involving extracellular loop 4. This is presumed to result in a long-range conformational change in CFTR that decreases the affinity of the cytoplasmic blocker binding site. This mechanism may allow pharmacological manipulation of CFTR activity by compounds that interact with the extracellular anion binding site[114]. Note that Cl^- ions may also interact with intracellular blocking anions by the non-pore mediated effect shown in (C)[114].

these positively charged residues in the CFTR pore in intact cells, much as had previously been shown for exogenous channel blockers.

While outward rectification of CFTR currents in intact cells-and the weak form of voltage-dependence it confers on CFTR channel currents (Figure 6) has long been recognized, only recently has it been suggested that the voltage-dependent channel block that underlies this voltage-dependence might fulfil some kind of channel regulatory role. Just as block by exogenously applied open channel blockers is sensitive to extracellular Cl^- (Figure 2D), so too is block by endogenous cytosolic anions in intact cells[82,100,101] (Figures 6D and Figure 7). This is not surprising if, as described above, these two intracellular voltage-dependent blocking effects share a common molecular mechanism. Recently it was proposed that this Cl^- dependence might be one mechanism that allows CFTR conductance to be regulated by the composition of secreted fluid bathing the extracellular face of epithelial cells[101]. Most CFTR-expressing epithelia secrete substantial amounts of Cl^- and HCO_3^- (up to 140 mmol/L HCO_3^- in the case of the pancreas[102]) in a CFTR-dependent fashion[2,103,104]. Furthermore, in many epithelia the concentrations of Cl^- and HCO_3^- in secreted fluid vary greatly under physiological conditions[102,104-110]. Interestingly, it was shown that voltage-dependent block of CFTR in intact cells was significantly stronger under high extracellular (HCO_3^-) conditions than under high extracellular Cl^- conditions[101]. This suggests that extracellular HCO_3^- is unable to substitute for Cl^- in relieving the blocking effects of endogenous cytoplasmic blocking anions. As a result, overall CFTR activity will be increased under high extracellular Cl^- conditions (*i.e.*, during periods of epithelial Cl^- secretion) and decreased under high HCO_3^- conditions (*i.e.*, during periods of secretion of relatively HCO_3^--rich fluid)[101] (Figure 7). These findings led to the suggestion that endogenous cytoplasmic blocking anions are physiologically relevant regulators of CFTR channel function, in that they confer upon the channel sensitivity to physiologically relevant changes in extracellular fluid composition[101]. In epithelial cells, this may be one mechanism by which CFTR channel function is fine-tuned by the concentration of its transport substrates Cl^- and HCO_3^- at the apical face of these cells[111-113].

While extracellular Cl^- may be an endogenous substance regulating CFTR channel function *via* modulation of the blocking effects of cytoplasmic anions, this mechanism of channel regulation may also be subject to pharmacological manipulation. Thus, millimolar concentrations of extracellular multivalent psuedohalide anions ($Co(CN)_6^{3-}$, $Co(NO_2)_6^{3-}$, $Fe(CN)_6^{3-}$, $IrCl_6^{3-}$, $Fe(CN)_6^{4-}$) were shown to mimic the effects of high extracellular Cl^- on channel block in intact cells, leading to an overall stimulation of CFTR channel function[114] (Figure 7). It was suggested that these anions represent the founder members of a new class of CFTR potentiators, and that their effects identify a novel mechanism by which CFTR function could potentially be increased therapeutically in the treatment of CF. Interestingly, these pseudohalide anions do not enter into the CFTR channel pore[114] and as such presumably do not interact electrostatically with blocking anions inside the channel pore; such an electro-

static "knock off" mechanism is commonly proposed to underlie the effect of extracellular Cl^- ions on intracellular open channel blockers[115,116] (Figure 7), as well as permeant ion effects on blocker binding in many other ion channel types[59]. Instead, pseudohalide anions were shown to exert their effects *via* interation with extracellular parts of CFTR, in particular ECL4[114,117]. The molecular mechanism of action of these substances, acting on extracellular parts of the protein, is therefore distinct from those of known CFTR potentiators, perhaps allowing additive or synergistic effects with other types of potentiators. Furthermore, the suggestion that a novel potentiator "binding site" might exist on ECL4 raises the possibility that this externally-accessible part of the CFTR protein could in future be targetted by drugs that can manipulate CFTR function therapeutically.

CONCLUSION

The architecture and Cl^- permeation mechanism of CFTR likely results in a susceptibility to relatively low affinity, voltage dependent open channel block by a very broad range of structurally diverse organic anions, including unidentified anions that the channel normally encounters in the cytoplasm of the cell. Because the channel is normally involved in the secretion of Cl^- and HCO_3^- ions at hyperpolarized cell membrane potentials, the channel has a relatively large intracellular vestibule that contains fixed positive charges to allow it to capture these anions from the cytoplasm by the process of electrostatic attraction. As the vestibule narrows toward the centre of the pore (Figure 1C and D), a single, functionally unique positive charge (K95 in TM1) ensures efficient attraction of monovalent anions (Figure 3). Beyond this point, permeating anions pass into a narrow, uncharged pore region that may allow some level of discrimination between different anions, and also acts as a size selectivity filter to prevent larger organic anions from escaping the cell. While this architecture appears efficient at maximizing channel Cl^- conductance (Figure 4), it also probably results in some degree of channel inhibition by cytoplasmic anions that are attracted by the positive charge at K95, but which are too large to pass into the narrow pore region (Figures 2, 3, 4 and 5). CFTR experimentalists have long taken advantage of these voltage dependent blocking anions to investigate CFTR-dependent processes, to think about the possible advantages of inhibiting CFTR function in disease states associated with inappropriately elevated CFTR function, and as relatively high-affinity probes to investigate the structure and function of the wide inner vestibule of the channel pore. This has allowed the development of functional (Figures 3, 4 and 5) and structural[20] models of the pore. More recently, it has been suggested that endogenous substances that act in this fashion may in fact play a role in tying CFTR function to the content of epithelial cell secretions (Figures 6 and 7), perhaps allowing CFTR activity to be fine-tuned directly by the amount of its substrate(s) being secreted from epithelial cells. In the future, this mechanism of CFTR regulation could be targetted by new drugs that act at an extracellular site on the CFTR protein to reduce the voltage-dependent blocking effects of endogenous cytoplasmic anions and so increase overall CFTR function in CF patients.

REFERENCES

1 **Lubamba B**, Dhooghe B, Noel S, Leal T. Cystic fibrosis: insight into CFTR pathophysiology and pharmacotherapy. *Clin Biochem* 2012; **45**: 1132-1144 [PMID: 22698459 DOI: 10.1016/j.clinbiochem.2012.05.034]

2 **Frizzell RA**, Hanrahan JW. Physiology of epithelial chloride and fluid secretion. *Cold Spring Harb Perspect Med* 2012; **2**: a009563 [PMID: 22675668 DOI: 10.1101/cshperspect.a009563]

3 **O'Sullivan BP**, Freedman SD. Cystic fibrosis. *Lancet* 2009; **373**: 1891-1904 [PMID: 19403164 DOI: 10.1016/S0140-6736(09)60327-5]

4 **Field M**. Intestinal ion transport and the pathophysiology of diarrhea. *J Clin Invest* 2003; **111**: 931-943 [PMID: 12671039 DOI: 10.1172/JCI18326]

5 **Terryn S**, Ho A, Beauwens R, Devuyst O. Fluid transport and cystogenesis in autosomal dominant polycystic kidney disease. *Biochim Biophys Acta* 2011; **1812**: 1314-1321 [PMID: 21255645 DOI: 10.1016/j.bbadis.2011.01.011]

6 **Dean M**, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res* 2001; **11**: 1156-1166 [PMID: 11435397 DOI: 10.1101/gr.184901]

7 **Rees DC**, Johnson E, Lewinson O. ABC transporters: the power to change. *Nat Rev Mol Cell Biol* 2009; **10**: 218-227 [PMID: 19234479 DOI: 10.1038/nrm2646]

8 **Hwang TC**, Kirk KL. The CFTR ion channel: gating, regulation, and anion permeation. *Cold Spring Harb Perspect Med* 2013; **3**: a009498 [PMID: 23284076 DOI: 10.1101/cshperspect.a009498]

9 **Chong PA**, Kota P, Dokholyan NV, Forman-Kay JD. Dynamics intrinsic to cystic fibrosis transmembrane conductance regulator function and stability. *Cold Spring Harb Perspect Med* 2013; **3**: a009522 [PMID: 23457292 DOI: 10.1101/cshperspect.a009522]

10 **Hunt JF**, Wang C, Ford RC. Cystic fibrosis transmembrane conductance regulator (ABCC7) structure. *Cold Spring Harb Perspect Med* 2013; **3**: a009514 [PMID: 23378596 DOI: 10.1101/cshperspect.a009514]

11 **Patrick AE**, Thomas PJ. Development of CFTR Structure. *Front Pharmacol* 2012; **3**: 162 [PMID: 22973227 DOI: 10.3389/fphar.2012.00162]

12 **Jih KY**, Hwang TC. Nonequilibrium gating of CFTR on an equilibrium theme. *Physiology* (Bethesda) 2012; **27**: 351-361 [PMID: 23223629 DOI: 10.1152/physiol.00026.2012]

13 **Rosenberg MF**, O'Ryan LP, Hughes G, Zhao Z, Aleksandrov LA, Riordan JR, Ford RC. The cystic fibrosis transmembrane conductance regulator (CFTR): three-dimensional structure and localization of a channel gate. *J Biol Chem* 2011; **286**: 42647-42654 [PMID: 21931164 DOI: 10.1074/jbc.M111.292268]

14 **Linsdell P**. Mechanism of chloride permeation in the cystic fibrosis transmembrane conductance regulator chloride channel. *Exp Physiol* 2006; **91**: 123-129 [PMID: 16157656 DOI: 10.1113/expphysiol.2005.031757]

15 **Wang W**, El Hiani Y, Rubaiy HN, Linsdell P. Relative contribution of different transmembrane segments to the CFTR chloride channel pore. *Pflugers Arch* 2014; **466**: 477-490 [PMID: 23955087 DOI: 10.1007/s00424-013-1317-x]

16 **Gao X**, Bai Y, Hwang TC. Cysteine scanning of CFTR's first transmembrane segment reveals its plausible roles in gat-

ing and permeation. *Biophys J* 2013; **104**: 786-797 [PMID: 23442957 DOI: 10.1016/j.bpj.2012.12.048]
17 **Mornon JP**, Lehn P, Callebaut I. Atomic model of human cystic fibrosis transmembrane conductance regulator: membrane-spanning domains and coupling interfaces. *Cell Mol Life Sci* 2008; **65**: 2594-2612 [PMID: 18597042 DOI: 10.1007/s00018-008-8249-1]
18 **Serohijos AW**, Hegedus T, Aleksandrov AA, He L, Cui L, Dokholyan NV, Riordan JR. Phenylalanine-508 mediates a cytoplasmic-membrane domain contact in the CFTR 3D structure crucial to assembly and channel function. *Proc Natl Acad Sci USA* 2008; **105**: 3256-3261 [PMID: 18305154 DOI: 10.1073/pnas.0800254105]
19 **Mornon JP**, Lehn P, Callebaut I. Molecular models of the open and closed states of the whole human CFTR protein. *Cell Mol Life Sci* 2009; **66**: 3469-3486 [PMID: 19707853 DOI: 10.1007/s00018-009-0133-0]
20 **Dalton J**, Kalid O, Schushan M, Ben-Tal N, Villà-Freixa J. New model of cystic fibrosis transmembrane conductance regulator proposes active channel-like conformation. *J Chem Inf Model* 2012; **52**: 1842-1853 [PMID: 22747419 DOI: 10.1021/ci2005884]
21 **Norimatsu Y**, Ivetac A, Alexander C, Kirkham J, O'Donnell N, Dawson DC, Sansom MS. Cystic fibrosis transmembrane conductance regulator: a molecular model defines the architecture of the anion conduction path and locates a "bottleneck" in the pore. *Biochemistry* 2012; **51**: 2199-2212 [PMID: 22352759 DOI: 10.1021/bi201888a]
22 **Ge N**, Muise CN, Gong X, Linsdell P. Direct comparison of the functional roles played by different transmembrane regions in the cystic fibrosis transmembrane conductance regulator chloride channel pore. *J Biol Chem* 2004; **279**: 55283-55289 [PMID: 15504721 DOI: 10.1074/jbc.M411935200]
23 **McDonough S**, Davidson N, Lester HA, McCarty NA. Novel pore-lining residues in CFTR that govern permeation and open-channel block. *Neuron* 1994; **13**: 623-634 [PMID: 7522483 DOI: 10.1016/0896-6273(94)90030-2]
24 **Linsdell P**. Location of a common inhibitor binding site in the cytoplasmic vestibule of the cystic fibrosis transmembrane conductance regulator chloride channel pore. *J Biol Chem* 2005; **280**: 8945-8950 [PMID: 15634668 DOI: 10.1074/jbc.M414354200]
25 **El Hiani Y**, Linsdell P. Changes in accessibility of cytoplasmic substances to the pore associated with activation of the cystic fibrosis transmembrane conductance regulator chloride channel. *J Biol Chem* 2010; **285**: 32126-32140 [PMID: 20675380 DOI: 10.1074/jbc.M110.113332]
26 **Bai Y**, Li M, Hwang TC. Dual roles of the sixth transmembrane segment of the CFTR chloride channel in gating and permeation. *J Gen Physiol* 2010; **136**: 293-309 [PMID: 20805575 DOI: 10.1085/jgp.201010480]
27 **Qian F**, El Hiani Y, Linsdell P. Functional arrangement of the 12th transmembrane region in the CFTR chloride channel pore based on functional investigation of a cysteine-less CFTR variant. *Pflugers Arch* 2011; **462**: 559-571 [PMID: 21796338 DOI: 10.1007/s00424-011-0998-2]
28 **Bai Y**, Li M, Hwang TC. Structural basis for the channel function of a degraded ABC transporter, CFTR (ABCC7). *J Gen Physiol* 2011; **138**: 495-507 [PMID: 22042986 DOI: 10.1085/jgp.201110705]
29 **El Hiani Y**, Linsdell P. Tuning of CFTR chloride channel function by location of positive charges within the pore. *Biophys J* 2012; **103**: 1719-1726 [PMID: 23083715 DOI: 10.1016/j.bpj.2012.09.020]
30 **Smith SS**, Liu X, Zhang ZR, Sun F, Kriewall TE, McCarty NA, Dawson DC. CFTR: covalent and noncovalent modification suggests a role for fixed charges in anion conduction. *J Gen Physiol* 2001; **118**: 407-431 [PMID: 11585852 DOI: 10.1085/jgp.118.4.407]
31 **Zhou JJ**, Fatehi M, Linsdell P. Identification of positive charges situated at the outer mouth of the CFTR chloride channel pore. *Pflugers Arch* 2008; **457**: 351-360 [PMID: 18449561 DOI: 10.1007/s00424-008-0521-6]
32 **Fatehi M**, Linsdell P. Novel residues lining the CFTR chloride channel pore identified by functional modification of introduced cysteines. *J Membr Biol* 2009; **228**: 151-164 [PMID: 19381710 DOI: 10.1007/s00232-009-9167-3]
33 **Wang W**, Linsdell P. Relative movements of transmembrane regions at the outer mouth of the cystic fibrosis transmembrane conductance regulator channel pore during channel gating. *J Biol Chem* 2012; **287**: 32136-32146 [PMID: 22843683 DOI: 10.1074/jbc.M112.385096]
34 **Schultz BD**, Singh AK, Devor DC, Bridges RJ. Pharmacology of CFTR chloride channel activity. *Physiol Rev* 1999; **79**: S109-S144 [PMID: 9922378]
35 **Hwang TC**, Sheppard DN. Molecular pharmacology of the CFTR Cl- channel. *Trends Pharmacol Sci* 1999; **20**: 448-453 [PMID: 10542444 DOI: 10.1016/S0165-6147(99)01386-3]
36 **Sheppard DN**, Welsh MJ. Effect of ATP-sensitive K+ channel regulators on cystic fibrosis transmembrane conductance regulator chloride currents. *J Gen Physiol* 1992; **100**: 573-591 [PMID: 1281220 DOI: 10.1085/jgp.100.4.573]
37 **Schultz BD**, DeRoos AD, Venglarik CJ, Singh AK, Frizzell RA, Bridges RJ. Glibenclamide blockade of CFTR chloride channels. *Am J Physiol* 1996; **271**: L192-L200 [PMID: 8770056]
38 **Sheppard DN**, Robinson KA. Mechanism of glibenclamide inhibition of cystic fibrosis transmembrane conductance regulator Cl- channels expressed in a murine cell line. *J Physiol* 1997; **503** (Pt 2): 333-346 [PMID: 9306276 DOI: 10.1111/j.1469-7793.1997.333bh.x]
39 **Zhou Z**, Hu S, Hwang TC. Probing an open CFTR pore with organic anion blockers. *J Gen Physiol* 2002; **120**: 647-662 [PMID: 12407077 DOI: 10.1085/jgp.20028685]
40 **Zhang ZR**, Zeltwanger S, McCarty NA. Steady-state interactions of glibenclamide with CFTR: evidence for multiple sites in the pore. *J Membr Biol* 2004; **199**: 15-28 [PMID: 15366420 DOI: 10.1007/s00232-004-0672-0]
41 **Zhang ZR**, Cui G, Zeltwanger S, McCarty NA. Time-dependent interactions of glibenclamide with CFTR: kinetically complex block of macroscopic currents. *J Membr Biol* 2004; **201**: 139-155 [PMID: 15711774 DOI: 10.1007/s00232-004-0712-9]
42 **Cui G**, Song B, Turki HW, McCarty NA. Differential contribution of TM6 and TM12 to the pore of CFTR identified by three sulfonylurea-based blockers. *Pflugers Arch* 2012; **463**: 405-418 [PMID: 22160394 DOI: 10.1007/s00424-011-1035-1]
43 **Venglarik CJ**, Schultz BD, DeRoos AD, Singh AK, Bridges RJ. Tolbutamide causes open channel blockade of cystic fibrosis transmembrane conductance regulator Cl- channels. *Biophys J* 1996; **70**: 2696-2703 [PMID: 8744307 DOI: 10.1016/S0006-3495(96)79839-9]
44 **Cai Z**, Lansdell KA, Sheppard DN. Inhibition of heterologously expressed cystic fibrosis transmembrane conductance regulator Cl- channels by non-sulphonylurea hypoglycaemic agents. *Br J Pharmacol* 1999; **128**: 108-118 [PMID: 10498841]
45 **McCarty NA**, McDonough S, Cohen BN, Riordan JR, Davidson N, Lester HA. Voltage-dependent block of the cystic fibrosis transmembrane conductance regulator Cl- channel by two closely related arylaminobenzoates. *J Gen Physiol* 1993; **102**: 1-23 [PMID: 8397274 DOI: 10.1085/jgp.102.1.1]
46 **Walsh KB**, Long KJ, Shen X. Structural and ionic determinants of 5-nitro-2-(3-phenylprophyl-amino)-benzoic acid block of the CFTR chloride channel. *Br J Pharmacol* 1999; **127**: 369-376 [PMID: 10385235 DOI: 10.1038/sj.bjp.0702562]
47 **Zhang ZR**, Zeltwanger S, McCarty NA. Direct comparison of NPPB and DPC as probes of CFTR expressed in Xenopus oocytes. *J Membr Biol* 2000; **175**: 35-52 [PMID: 10811966 DOI: 10.1007/s002320001053]

48 **Scott-Ward TS**, Li H, Schmidt A, Cai Z, Sheppard DN. Direct block of the cystic fibrosis transmembrane conductance regulator Cl(-) channel by niflumic acid. *Mol Membr Biol* 2004; **21**: 27-38 [PMID: 14668136 DOI: 10.1080/09687680310001597758]

49 **Linsdell P**, Hanrahan JW. Disulphonic stilbene block of cystic fibrosis transmembrane conductance regulator Cl- channels expressed in a mammalian cell line and its regulation by a critical pore residue. *J Physiol* 1996; **496** (Pt 3): 687-693 [PMID: 8930836]

50 **Linsdell P**, Hanrahan JW. Substrates of multidrug resistance-associated proteins block the cystic fibrosis transmembrane conductance regulator chloride channel. *Br J Pharmacol* 1999; **126**: 1471-1477 [PMID: 10217542 DOI: 10.1038/sj.bjp.0702458]

51 **Diena T**, Melani R, Caci E, Pedemonte N, Sondo E, Zegarra-Moran O, Galietta LJ. Block of CFTR-dependent chloride currents by inhibitors of multidrug resistance-associated proteins. *Eur J Pharmacol* 2007; **560**: 127-131 [PMID: 17320853 DOI: 10.1016/j.ejphar.2007.01.051]

52 **Gong X**, Burbridge SM, Lewis AC, Wong PY, Linsdell P. Mechanism of lonidamine inhibition of the CFTR chloride channel. *Br J Pharmacol* 2002; **137**: 928-936 [PMID: 12411425 DOI: 10.1038/sj.bjp.0704932]

53 **Gong XD**, Linsdell P, Cheung KH, Leung GP, Wong PY. Indazole inhibition of cystic fibrosis transmembrane conductance regulator Cl(-) channels in rat epididymal epithelial cells. *Biol Reprod* 2002; **67**: 1888-1896 [PMID: 12444067 DOI: 10.1095/biolreprod.102.007450]

54 **Linsdell P**. Direct block of the cystic fibrosis transmembrane conductance regulator Cl(-) channel by butyrate and phenylbutyrate. *Eur J Pharmacol* 2001; **411**: 255-260 [PMID: 11164382 DOI: 10.1016/S0014-2999(00)00928-6]

55 **Cai Z**, Sheppard DN. Phloxine B interacts with the cystic fibrosis transmembrane conductance regulator at multiple sites to modulate channel activity. *J Biol Chem* 2002; **277**: 19546-19553 [PMID: 11904291 DOI: 10.1074/jbc.M108023200]

56 **Ishihara H**, Welsh MJ. Block by MOPS reveals a conformation change in the CFTR pore produced by ATP hydrolysis. *Am J Physiol* 1997; **273**: C1278-C1289 [PMID: 9357772]

57 **Li MS**, Demsey AF, Qi J, Linsdell P. Cysteine-independent inhibition of the CFTR chloride channel by the cysteine-reactive reagent sodium (2-sulphonatoethyl) methanethiosulphonate. *Br J Pharmacol* 2009; **157**: 1065-1071 [PMID: 19466983 DOI: 10.1111/j.1476-5381.2009.00258.x]

58 **Linsdell P**, Hanrahan JW. Flickery block of single CFTR chloride channels by intracellular anions and osmolytes. *Am J Physiol* 1996; **271**: C628-C634 [PMID: 8770004]

59 **Hille B**. Ion channels of excitable membranes. 3rd ed. Sunderland (MA): Sinauer Assoc, 2001

60 **Alexander SP**, Mathie A, Peters JA. Guide to Receptors and Channels (GRAC), 5th edition. *Br J Pharmacol* 2011; **164** Suppl 1: S1-324 [PMID: 22040146 DOI: 10.1111/j.1476-5381.2011.01649_5.x]

61 **Verkman AS**, Galietta LJ. Chloride channels as drug targets. *Nat Rev Drug Discov* 2009; **8**: 153-171 [PMID: 19153558 DOI: 10.1038/nrd2780]

62 **Ma T**, Thiagarajah JR, Yang H, Sonawane ND, Folli C, Galietta LJ, Verkman AS. Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J Clin Invest* 2002; **110**: 1651-1658 [PMID: 12464670 DOI: 10.1172/JCI16112]

63 **Taddei A**, Folli C, Zegarra-Moran O, Fanen P, Verkman AS, Galietta LJ. Altered channel gating mechanism for CFTR inhibition by a high-affinity thiazolidinone blocker. *FEBS Lett* 2004; **558**: 52-56 [PMID: 14759515 DOI: 10.1016/S0014-5793(04)00011-0]

64 **Kopeikin Z**, Sohma Y, Li M, Hwang TC. On the mechanism of CFTR inhibition by a thiazolidinone derivative. *J Gen Physiol* 2010; **136**: 659-671 [PMID: 21078867 DOI: 10.1085/jgp.201010518]

65 **Muanprasat C**, Sonawane ND, Salinas D, Taddei A, Galietta LJ, Verkman AS. Discovery of glycine hydrazide pore-occluding CFTR inhibitors: mechanism, structure-activity analysis, and in vivo efficacy. *J Gen Physiol* 2004; **124**: 125-137 [PMID: 15277574 DOI: 10.1085/jgp.200409059]

66 **Norimatsu Y**, Ivetac A, Alexander C, O'Donnell N, Frye L, Sansom MS, Dawson DC. Locating a plausible binding site for an open-channel blocker, GlyH-101, in the pore of the cystic fibrosis transmembrane conductance regulator. *Mol Pharmacol* 2012; **82**: 1042-1055 [PMID: 22923500 DOI: 10.1124/mol.112.080267]

67 **Fuller MD**, Thompson CH, Zhang ZR, Freeman CS, Schay E, Szakács G, Bakos E, Sarkadi B, McMaster D, French RJ, Pohl J, Kubanek J, McCarty NA. State-dependent inhibition of cystic fibrosis transmembrane conductance regulator chloride channels by a novel peptide toxin. *J Biol Chem* 2007; **282**: 37545-37555 [PMID: 17951250 DOI: 10.1074/jbc.M708079200]

68 **Fuller MD**, Zhang ZR, Cui G, McCarty NA. The block of CFTR by scorpion venom is state-dependent. *Biophys J* 2005; **89**: 3960-3975 [PMID: 16183882 DOI: 10.1529/biophysj.105.060731]

69 **Derichs N**. Targeting a genetic defect: cystic fibrosis transmembrane conductance regulator modulators in cystic fibrosis. *Eur Respir Rev* 2013; **22**: 58-65 [PMID: 23457166 DOI: 10.1183/09059180.00008412]

70 **Hanrahan JW**, Sampson HM, Thomas DY. Novel pharmacological strategies to treat cystic fibrosis. *Trends Pharmacol Sci* 2013; **34**: 119-125 [PMID: 23380248 DOI: 10.1016/j.tips.2012.11.006]

71 **Rowe SM**, Verkman AS. Cystic fibrosis transmembrane regulator correctors and potentiators. *Cold Spring Harb Perspect Med* 2013; **3**: [PMID: 23818513 DOI: 10.1101/cshperspect.a009761]

72 **Sermet-Gaudelus I**. Ivacaftor treatment in patients with cystic fibrosis and the G551D-CFTR mutation. *Eur Respir Rev* 2013; **22**: 66-71 [PMID: 23457167 DOI: 10.1183/09059180.00008512]

73 **Super M**. CFTR and disease: implications for drug development. *Lancet* 2000; **355**: 1840-1842 [PMID: 10866434 DOI: 10.1016/S0140-6736(00)02282-0]

74 **Chen H**, Ruan YC, Xu WM, Chen J, Chan HC. Regulation of male fertility by CFTR and implications in male infertility. *Hum Reprod Update* 2012; **18**: 703-713 [PMID: 22709980 DOI: 10.1093/humupd/dms027]

75 **Thiagarajah JR**, Broadbent T, Hsieh E, Verkman AS. Prevention of toxin-induced intestinal ion and fluid secretion by a small-molecule CFTR inhibitor. *Gastroenterology* 2004; **126**: 511-519 [PMID: 14762788 DOI: 10.1053/j.gastro.2003.11.005]

76 **Sonawane ND**, Zhao D, Zegarra-Moran O, Galietta LJ, Verkman AS. Lectin conjugates as potent, nonabsorbable CFTR inhibitors for reducing intestinal fluid secretion in cholera. *Gastroenterology* 2007; **132**: 1234-1244 [PMID: 17408659 DOI: 10.1053/j.gastro.2007.02.018]

77 **Li H**, Findlay IA, Sheppard DN. The relationship between cell proliferation, Cl- secretion, and renal cyst growth: a study using CFTR inhibitors. *Kidney Int* 2004; **66**: 1926-1938 [PMID: 15496164 DOI: 10.1111/j.1523-1755.2004.00967.x]

78 **Yang B**, Sonawane ND, Zhao D, Somlo S, Verkman AS. Small-molecule CFTR inhibitors slow cyst growth in polycystic kidney disease. *J Am Soc Nephrol* 2008; **19**: 1300-1310 [PMID: 18385427 DOI: 10.1681/ASN.2007070828]

79 **Li H**, Sheppard DN. Therapeutic potential of cystic fibrosis transmembrane conductance regulator (CFTR) inhibitors in polycystic kidney disease. *BioDrugs* 2009; **23**: 203-216 [PMID: 19697963 DOI: 10.2165/11313570-000000000-00000]

80 **Thiagarajah JR**, Verkman AS. CFTR inhibitors for treating diarrheal disease. *Clin Pharmacol Ther* 2012; **92**: 287-290

[PMID: 22850599 DOI: 10.1038/clpt.2012.114]
81 **Gupta J**, Evagelidis A, Hanrahan JW, Linsdell P. Asymmetric structure of the cystic fibrosis transmembrane conductance regulator chloride channel pore suggested by mutagenesis of the twelfth transmembrane region. *Biochemistry* 2001; **40**: 6620-6627 [PMID: 11380256 DOI: 10.1021/bi002819v]
82 **Zhou JJ**, Li MS, Qi J, Linsdell P. Regulation of conductance by the number of fixed positive charges in the intracellular vestibule of the CFTR chloride channel pore. *J Gen Physiol* 2010; **135**: 229-245 [PMID: 20142516 DOI: 10.1085/jgp.200910327]
83 **Aubin CN**, Linsdell P. Positive charges at the intracellular mouth of the pore regulate anion conduction in the CFTR chloride channel. *J Gen Physiol* 2006; **128**: 535-545 [PMID: 17043152 DOI: 10.1085/jgp.200609516]
84 **Gong X**, Linsdell P. Mutation-induced blocker permeability and multiion block of the CFTR chloride channel pore. *J Gen Physiol* 2003; **122**: 673-687 [PMID: 14610019 DOI: 10.1085/jgp.200308889]
85 **Zhou JJ**, Fatehi M, Linsdell P. Direct and indirect effects of mutations at the outer mouth of the cystic fibrosis transmembrane conductance regulator chloride channel pore. *J Membr Biol* 2007; **216**: 129-142 [PMID: 17673962 DOI: 10.1007/s00232-007-9056-6]
86 **Wang W**, El Hiani Y, Linsdell P. Alignment of transmembrane regions in the cystic fibrosis transmembrane conductance regulator chloride channel pore. *J Gen Physiol* 2011; **138**: 165-178 [PMID: 21746847 DOI: 10.1085/jgp.201110605]
87 **St Aubin CN**, Zhou JJ, Linsdell P. Identification of a second blocker binding site at the cytoplasmic mouth of the cystic fibrosis transmembrane conductance regulator chloride channel pore. *Mol Pharmacol* 2007; **71**: 1360-1368 [PMID: 17293558 DOI: 10.1124/mol.106.031732]
88 **Bachmann A**, Russ U, Quast U. Potent inhibition of the CFTR chloride channel by suramin. *Naunyn Schmiedebergs Arch Pharmacol* 1999; **360**: 473-476 [PMID: 10551285 DOI: 10.1007/s002109900096]
89 **Zhou JJ**, Linsdell P. Molecular mechanism of arachidonic acid inhibition of the CFTR chloride channel. *Eur J Pharmacol* 2007; **563**: 88-91 [PMID: 17397825 DOI: 10.1016/j.ejphar.2007.02.048]
90 **McCarty NA**. Permeation through the CFTR chloride channel. *J Exp Biol* 2000; **203**: 1947-1962 [PMID: 10851114]
91 **Gadsby DC**, Vergani P, Csanády L. The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature* 2006; **440**: 477-483 [PMID: 16554808 DOI: 10.1038/nature04712]
92 **Gray MA**, Harris A, Coleman L, Greenwell JR, Argent BE. Two types of chloride channel on duct cells cultured from human fetal pancreas. *Am J Physiol* 1989; **257**: C240-C251 [PMID: 2475028]
93 **Tabcharani JA**, Low W, Elie D, Hanrahan JW. Low-conductance chloride channel activated by cAMP in the epithelial cell line T84. *FEBS Lett* 1990; **270**: 157-164 [PMID: 1699790 DOI: 10.1016/0014-5793(90)81257-O]
94 **Haws C**, Krouse ME, Xia Y, Gruenert DC, Wine JJ. CFTR channels in immortalized human airway cells. *Am J Physiol* 1992; **263**: L692-L707 [PMID: 1282304]
95 **Ehara T**, Matsuura H. Single-channel study of the cyclic AMP-regulated chloride current in guinea-pig ventricular myocytes. *J Physiol* 1993; **464**: 307-320 [PMID: 8229803]
96 **Overholt JL**, Hobert ME, Harvey RD. On the mechanism of rectification of the isoproterenol-activated chloride current in guinea-pig ventricular myocytes. *J Gen Physiol* 1993; **102**: 871-895 [PMID: 8301261 DOI: 10.1085/jgp.102.5.871]
97 **Tabcharani JA**, Chang XB, Riordan JR, Hanrahan JW. Phosphorylation-regulated Cl- channel in CHO cells stably expressing the cystic fibrosis gene. *Nature* 1991; **352**: 628-631 [PMID: 1714039 DOI: 10.1038/352628a0]
98 **Fischer H**, Machen TE. CFTR displays voltage dependence and two gating modes during stimulation. *J Gen Physiol* 1994; **104**: 541-566 [PMID: 7528783 DOI: 10.1085/jgp.104.3.541]
99 **Overholt JL**, Saulino A, Drumm ML, Harvey RD. Rectification of whole cell cystic fibrosis transmembrane conductance regulator chloride current. *Am J Physiol* 1995; **268**: C636-C646 [PMID: 7534982]
100 **Zhou Z**, Hu S, Hwang TC. Voltage-dependent flickery block of an open cystic fibrosis transmembrane conductance regulator (CFTR) channel pore. *J Physiol* 2001; **532**: 435-448 [PMID: 11306662 DOI: 10.1111/j.1469-7793.2001.0435f.x]
101 **Li MS**, Holstead RG, Wang W, Linsdell P. Regulation of CFTR chloride channel macroscopic conductance by extracellular bicarbonate. *Am J Physiol Cell Physiol* 2011; **300**: C65-C74 [PMID: 20926782 DOI: 10.1152/ajpcell.00290.2010]
102 **Argent BE**, Gray MA, Steward MC, Case RM. Cell physiology of pancreatic ducts. In: Physiology of the gastrointestinal tract. 5th edition. Johnson L, editor. San Diego: Elsevier, 2012: 1399-1423
103 **Tang L**, Fatehi M, Linsdell P. Mechanism of direct bicarbonate transport by the CFTR anion channel. *J Cyst Fibros* 2009; **8**: 115-121 [PMID: 19019741 DOI: 10.1016/j.jcf.2008.10.004]
104 **Bridges RJ**. Mechanisms of bicarbonate secretion: lessons from the airways. *Cold Spring Harb Perspect Med* 2012; **2**: [PMID: 22908201 DOI: 10.1101/cshperspect.a015016]
105 **Cuthbert AW**. Bicarbonate secretion in the murine gallbladder--lessons for the treatment of cystic fibrosis. *JOP* 2001; **2**: 257-262 [PMID: 11875268]
106 **Wang XF**, Zhou CX, Shi QX, Yuan YY, Yu MK, Ajonuma LC, Ho LS, Lo PS, Tsang LL, Liu Y, Lam SY, Chan LN, Zhao WC, Chung YW, Chan HC. Involvement of CFTR in uterine bicarbonate secretion and the fertilizing capacity of sperm. *Nat Cell Biol* 2003; **5**: 902-906 [PMID: 14515130 DOI: 10.1038/ncb1047]
107 **Allen A**, Flemström G. Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. *Am J Physiol Cell Physiol* 2005; **288**: C1-19 [PMID: 15591243 DOI: 10.1152/ajpcell.00102.2004]
108 **Banales JM**, Prieto J, Medina JF. Cholangiocyte anion exchange and biliary bicarbonate excretion. *World J Gastroenterol* 2006; **12**: 3496-3511 [PMID: 16773707]
109 **Fischer H**, Widdicombe JH. Mechanisms of acid and base secretion by the airway epithelium. *J Membr Biol* 2006; **211**: 139-150 [PMID: 17091214 DOI: 10.1007/s00232-006-0861-0]
110 **Chan HC**, Ruan YC, He Q, Chen MH, Chen H, Xu WM, Chen WY, Xie C, Zhang XH, Zhou Z. The cystic fibrosis transmembrane conductance regulator in reproductive health and disease. *J Physiol* 2009; **587**: 2187-2195 [PMID: 19015188 DOI: 10.1113/jphysiol.2008.164970]
111 **O'Reilly CM**, Winpenny JP, Argent BE, Gray MA. Cystic fibrosis transmembrane conductance regulator currents in guinea pig pancreatic duct cells: inhibition by bicarbonate ions. *Gastroenterology* 2000; **118**: 1187-1196 [PMID: 10833494 DOI: 10.1016/S0016-5085(00)70372-6]
112 **Shcheynikov N**, Kim KH, Kim KM, Dorwart MR, Ko SB, Goto H, Naruse S, Thomas PJ, Muallem S. Dynamic control of cystic fibrosis transmembrane conductance regulator Cl(-)/HCO3(-) selectivity by external Cl(-). *J Biol Chem* 2004; **279**: 21857-21865 [PMID: 15010471 DOI: 10.1074/jbc.M313323200]
113 **Wright AM**, Gong X, Verdon B, Linsdell P, Mehta A, Riordan JR, Argent BE, Gray MA. Novel regulation of cystic fibrosis transmembrane conductance regulator (CFTR) channel gating by external chloride. *J Biol Chem* 2004; **279**: 41658-41663 [PMID: 15286085 DOI: 10.1074/jbc.M405517200]
114 **Li MS**, Cowley EA, Linsdell P. Pseudohalide anions reveal a novel extracellular site for potentiators to increase CFTR function. *Br J Pharmacol* 2012; **167**: 1062-1075 [PMID:

22612315 DOI: 10.1111/j.1476-5381.2012.02041.x]
115 **Linsdell P**, Tabcharani JA, Hanrahan JW. Multi-Ion mechanism for ion permeation and block in the cystic fibrosis transmembrane conductance regulator chloride channel. *J Gen Physiol* 1997; **110**: 365-377 [PMID: 9379169 DOI: 10.1085/jgp.110.4.365]
116 **Gong X**, Linsdell P. Coupled movement of permeant and blocking ions in the CFTR chloride channel pore. *J Physiol* 2003; **549**: 375-385 [PMID: 12679371 DOI: 10.1113/jphysiol.2002.038216]
117 **Zhou JJ**, Linsdell P. Evidence that extracellular anions interact with a site outside the CFTR chloride channel pore to modify channel properties. *Can J Physiol Pharmacol* 2009; **87**: 387-395 [PMID: 19448737 DOI: 10.1139/y09-023]

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REVIEW

Role of 3'-untranslated region translational control in cancer development, diagnostics and treatment

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Abstract

The messenger RNA 3'-untranslated region (3'UTR) plays an important role in regulation of gene expression on the posttranscriptional level. The 3'UTR controls gene expression *via* orchestrated interaction between the structural components of mRNAs (cis-element) and the specific trans-acting factors (RNA binding proteins and non-coding RNAs). The crosstalk of these factors is based on the binding sequences and/or direct protein-protein interaction, or just functional interaction. Much new evidence that has accumulated supports the idea that several RNA binding factors can bind to common mRNA targets: to the non-overlapping binding sites or to common sites in a competitive fashion. Various factors capable of binding to the same RNA can cooperate or be antagonistic in their actions. The outcome of the collective function of all factors bound to the same mRNA 3'UTR depends on many circumstances, such as their expression levels, affinity to the binding sites, and localization in the cell, which can be controlled by various physiological conditions. Moreover, the functional and/or physical interactions of the factors binding to 3'UTR can change the character of their actions. These interactions vary during the cell cycle and in response to changing physiological conditions. Abnormal functioning of the factors can lead to disease. In this review we will discuss how alterations of these factors or their interaction can affect cancer development and promote or enhance the malignant phenotype of cancer cells. Understanding these alterations and their impact on 3'UTR-directed posttranscriptional gene regulation will uncover promising new targets for therapeutic intervention and diagnostics. We will also discuss emerging new tools in cancer diagnostics and therapy based on 3'UTR binding factors and approaches to improve them.

Key words: Translational control; 3'-untranslated region; MicroRNAs; RNA binding proteins; Cancer

Core tip: The messenger RNA 3'-untranslated region (3'UTR) plays an important role in regulation of gene expression on the posttranscriptional level. 3'UTR controls gene expression *via* orchestrated interaction between structural components mRNAs (cis-element) and specific trans-acting factors (RNA binding proteins and non-coding RNAs). Alteration of any of these components can lead to various pathologies. In this review we will discuss how alteration of these factors or a change in the crosstalk between them can affect cancer development and promote or enhance the malignant phenotype of cancer cells. Understanding these regulatory mechanisms and their impact on 3'UTR-directed posttranscriptional gene regulation may uncover promising new targets for therapeutic intervention and diagnostics.

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INTRODUCTION

During tumor growth, characteristic alterations in gene expression result in modification of the quantity of the corresponding proteins. The alterations have been extensively documented at the mRNA transcription and protein degradation levels; both have a strong impact on the accumulation of critical proteins involved in tumorigenesis. While translational control is a key mechanism involved in the regulation of the gene expression[1], the impact of the misregulation of gene expression during carcinogenesis at the translational level has long been widely underestimated.

Translation of mRNA into proteins can be specifically regulated by a combination of RNA-binding factors (proteins and antisense RNA) that act positively or negatively on translation initiation and elongation, mRNA stability and mRNA localization. This regulation is mostly controlled by sequence elements in 3'-untranslated region (3'UTR) of the transcripts, located downstream from the open reading frame. The importance of the 3'UTR was not fully appreciated until the discovery of small non-coding regulatory RNAs (microRNAs or miRNAs). MiRNAs interact with a protein complex called RNA-induced silencing complex (RISC), which controls gene expression by binding to miRNA target sites in mRNA 3'UTRs. MiRNAs have proven to be not only important markers but also key players in the control of gene expression during cancer development. Multiple 3'UTR regulatory elements are usually involved in the regulation of translation. One of the best characterized of them is the cytoplasmic polyadenylation element (CPE) which, upon binding by the CPE-binding protein (CPEB), regulates specific target mRNAs. CPEB1 directly controls the mammalian cell cycle, particularly during senescence, suggesting a role in cancer and aging. According to the literature and to our unpublished data, members of CPEB family are misregulated in many cancers and can play important role in carcinogenesis[2,3].

The insulin-like growth factor (IGF)-2 mRNA-binding proteins 1, 2 and 3 [IGF2BP1-3/Insulin-like growth factor 2 mRNA-binding protein 1 (IMP1-3)] belong to another well-known family of proteins that bind to 3' UTR and control the expression of proteins important in the normal cell cycle and in cancerous transformation[4,5]. IGF2BP1-3 and IMPs are highly over-expressed in a number of cancers[6].

The aim of this review is to show that regulatory factors controlling gene expression *via* binding to 3'UTR do not act separately but in cooperation. Crosstalk of these factors is based on the binding sequences and direct protein-protein interaction. The functional and physical interactions of factors binding to 3'UTR can change the character of their action, according to physiological conditions[7]. Disruption of the coordinated action of these factors can have a big impact on the expression of proteins involved in cancer induction and development. A detailed understanding of these mechanisms can help in development of new tools for cancer diagnostics and treatment.

MIRNA AND CANCER

One of the main breakthroughs in cellular and molecular biology in the last decade was the discovery of gene expression regulation by non-coding RNAs. The number of classes of non-coding RNAs continues to grow rapidly. Major among them are miRNAs, piRNAs, endo-siRNAs, exo-siRNAs, rasiRNAs, scnRNAs, tasiRNAs, natsiRNAs, 21U-RNA, lncRNAs and tRNA-derived RNA fragments[8]. We will focus this review on miRNAs, which is the most widely studied group of non-coding regulatory RNAs. MiRNAs are small (21-23 nt) RNAs. MiRNAs originate from Pol Ⅱ-transcribed precursors (pri-miRNAs). Then the Drosha enzyme recognizes a 70 nt stem-loop structure and produces pre-miRNA, which is transported from the nucleus by Exportin 5. In the cytoplasm, Dicer enzyme forms a double-stranded 22 nt RNA from pre-miRNA. One of the RNA strands is degraded, whereas the other one inserts into the RISC complex, binds to the target sequence in 3'UTR, and carries out its regulatory function[9]. These tiny molecules are involved in the regulation of almost all cellular processes[10-12]. Since single miRNA can potentially have hundreds of targets, alteration of its expression can easily influence cellular homeostasis, which in the most extreme case may result in cell death or in malignant transformation of the cell. Indeed, the first evidence of involvement of miRNAs in tumorigenesis was shown in 2002 by Calin *et al*[13]. These authors found that in 68% of chronic lymphocytic leukemia (CLL) cases, deletions and down-regulation of miRNA genes *miR-15* and *miR-16* at 13q14 locus were observed. Since then, thousands of publications have been devoted to miRNAs involvement in various types of cancer.

Misregulation of miRNA expression in various cancers

Involvement of miRNA in cancer has been proven by genome-wide expression studies using microarray technology and techniques based on quantitative polymerase chain reaction (qPCR), which have helped to establish the miRNA profiles of normal and neoplastic tissues[14,15]. These studies revealed a global decrease in miRNA expression in many tumors. Various tumors also correlate with changes in specific miRNA expression. The above studies were supported by a number of investigations of individual types of neoplasms[16-29] (and many others). About 200 miRNAs have at least once been reported as being up- or down-regulated in tumors.

Overall, these studies prove that each neoplasm could exhibit a distinct miRNA expression profile that differs from one of the other neoplasms and its normal tissue counterpart. However, a group of miRNAs was shown to have a similar expression profile in multiple cancers, suggesting that their involvement in tumorigenesis is common for many cancer types. At the same time, there are many miRNAs that are differentially misregulated in different cancers[30]. The reason for this is not yet clear, but it is likely that the function of a miRNA may vary because of tissue-specific expression of their targets. On the other hand, specific miRNAs can have different cofactors and build different networking in different cancers. Thus, it becomes possible for a given miRNA to act either as an oncogene or as a tumor suppressor, according to the context.

One of the best examples for tissue-specific target regulation is the let-7 family of miRNAs, which according to many reports acts as tumor suppressors[31-34]. It has been shown that let-7 is frequently down-regulated in many cancers, leading to up-regulation of the proto-oncogenes RAS[35], High Mobility Group A2 (HMGA2)[36-38], Myc[39], integrin beta 3[40], the oncofetal gene *IMP-1*[41] and the miRNAs maturation enzyme Dicer[42]. Let-7b was shown to down-regulate the expression of cyclin D1, D3, A and cyclin-dependent kinase (Cdk 4) in melanoma cells[43].

A similar effect was observed for the miR-34 family, another potential tumor-suppressor in a variety of cancers. Localized to chromosomes 1 (34a) and 11 (34c and b), this family is frequently deregulated in various cancers, including lung, ovarian, CLL and colorectal[44-47]. In addition, miR-34b/c polymorphism has been linked to risk of developing hepatocellular carcinoma[48]. The miR-34 family appears to be the direct transcriptional target of p53[49,50] and has few validated targets, SNAIL (zing finger protein SNAL1, epithelial-mesenchymal transition), Wnt, SIRT1 (silent mating type information regulation homolog), cyclin-dependent kinase 6 (CDK6) and others[51-54].

The miR-29 family (a, b and c) also has often been found to be decreased in tumors, such as CLL, hepatocellular carcinoma and breast cancer[55-57], and has been validated to target key components of cellular survival as MCL-1 (induced myeloid leukemia cell differentiation protein), cell cycle CDK6 and dedifferentiation Krüppel-like factor 4[26,55,58]. The most interesting observation concerning miR-29 is that it can globally alter methylation status through targeting of DNA methyltransferases 3A and B (DNA methyltransferases 3A and B) and lead to the derepression of critical tumor suppressors[59].

The miR-17-92 cluster acts as a group of oncogenes when over-expressed. This group includes 14 homologous miRNAs that are encoded by three gene clusters on chromosomes 7, 13 and X[25,60]. The cluster on chromosome 13 is amplified in human B cell lymphomas[61], which leads to increased expression of various miRNA members. Forced expression of the miR17-92 cluster along with myc proto-oncogene (MYC) accelerates tumor development in mouse B cell lymphoma[62], thus acts as an oncogene. Up-regulation of members of this large miRNA group protects cells from apoptosis by inhibiting the expression of E2F, p21 and Bim[63,64].

Among oncogenic miRNAs families, the most therapeutic and diagnostic potential is the miR-21 family, located on chromosome 17. It is over-expressed in several cancers, including breast, colorectal and lung[65-67], and has few validated targets: TPMI (tropomyosin), PDCD4 (program cell death protein 4) and PTEN (phosphatase and tensin homolog)[68-70].

These and other observations found in the literature prove that miRNAs play very important roles in cancer, although their mode of action can differ according to the composition of the targets and a combination of other factors. Knowledge of the mechanisms of miRNA action in particular cancers, especially understanding of their collaborators or inhibitors, will help to develop proper tools for miRNA-based therapy and diagnostics.

Cancer processes associated with misbalance of miRNA expression

Epithelial-mesenchymal transition: To date, it is believed that one of the causes of failure in the treatment of cancer is the existence of cancer stem cells[71]. In cancer, epithelial-mesenchymal transition (EMT) is a process by which epithelial cells are reprogrammed to lose their differentiation and become undifferentiated stem cells with mesenchymal properties. Despite the fact that genes responsible for EMT are well known[72], data devoted to the involvement of miRNAs in this process are still accumulating. Thus, Nairismägi *et al*[73] showed that miR-580 and CPEB1/2 down-regulate TWIST1 expression, one of the main inductors of EMT in a cooperative way. Another miRNA that suppresses EMT belongs to the miR-200 family. These miRNAs increase E-cadherin expression by targeting the mRNA of the E-cadherin transcriptional repressors zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2[74,75]. It was later shown that the miR-200 family is downregulated in the initial stages of stromal invasion, but restored at metastatic sites[76]. In cases of hepatocellular carcinoma, miR-612 was found to have an inhibitory effect on EMT targeting of the AKT (also known as protein kinase B) signal cascade[77]. On the other hand, a set of miRNAs is correlated with EMT progression. MiR-21 is thus over-expressed during EMT, whereas blockage of miR-21 inhibits metastasis development[78,79]. During EMT, the Twist transcription factor induces expression of miR-10b. In turn, over-expression of miR-10b in non-metastatic breast tumors initiates intense invasion and metastasis[80]. Furthermore, in hepatocellular carcinoma, miR-106b promotes cell migration and metastasis by activating the EMT process[81].

Angiogenesis and proliferation: The tumor growth rate is one of the most critical characteristics that define the level of cell malignancy. However, while growing, a tumor must supply itself with nutrients, which are provided by active angiogenesis. Deregulation of miRNA

expression is also involved in these processes. For instance, upregulation of the miR-17-92 cluster in adenocarcinoma leads to downregulation of its predicted targets: anti-angiogenic thrombospondin-1 and connective tissue growth factor, resulting in enhanced neovascularization[82]. Lee *et al*[83] showed that miR-378 increases cell survival, tumor growth and angiogenesis. Detailed analyses revealed that the main targets of the miR-378 were SuFu (inhibitor of Hedgehog signal cascade) and tumor suppressor Fus-1. Regulation of proliferation is mainly carried out by forced entry and progression of the cell cycle. Cyclins and their CDKs regulators are key players in the above-mentioned process. Thus, miRNAs can potentially inhibit key components, resulting in the inhibition of proliferation or in decreased expression of cyclin inhibitors. Indeed, Linsley *et al*[84] showed that the miR-16 family regulates cell cycle progression. Furthermore, the miR-497-195 cluster has been shown to target multiple cell cycle regulators, including CDKs, but it is transcriptionally silenced in hepatocellular carcinoma[85]. In the case of breast cancer, the same miRNA was able to decrease the cyclin E1 level[86]. Wee-1, a well-known cell cycle regulator, is also a target of miR-497[87]. There are other cases in which miRNAs inhibit cell cycle inhibitors. Thus, analyses of miRnome from a broad set of different cancer samples demonstrated that a number of miRNAs were over-expressed in all cases. Interestingly, one of the main targets of these miRNAs was RB1, a well-known negative regulator of the cell cycle[15].

Mechanisms of alterations of miRNA-mediated control of gene expression in cancer

Genetic mechanisms: It is well known that genome instability characterizes malignant cells. The discovery of DNA alteration involvement in trespassing of miRNA gene expression came from the observation that 30%-50% of *miRNA* genes are located in fragile sites[88,89]. Fragile DNA sites are regions that possess high levels of instability and are susceptible to such processes as genomic rearrangement, which include multiplication and deletion of loci, translocation, high rates of mutation *etc*[90]. Such a process is deletion of oncosuppressive *miR-15a*/*miR-16a* miRNAs that target the anti-apoptotic B-cell lymphoma 2 (BCL-2) protein[91], which was found in the majority of CLL cases[13]. Another rearrangement, translocation, was shown to alter the 17-92 cluster that contains a set of miRNAs among which is leukemogenic miR-19[92]. Translocation can also alter miRNA targets, which results in the disruption of miRNA-mediated proto-oncogenes repression. For instance, Mayr showed that translocation of High Mobility Group A2 (HMGA2) 3'UTR disrupts its repression by let-7 miRNA[37]. During amplification, the number of pro-oncogenic miRNA genes is often increased. Thus, miR-26a, a direct regulator of PTEN, is frequently amplified at the DNA level in human glioma[93]. Amplification of growth-promoting miR-23a is widely observed in gastric cancer[94]. While there is little data concerning the role of mutations in miRNA-mediated control in cancer, the number of publications dedicated to the role of single-nucleotide polymorphism (SNP) on miRNA action is growing fast. SNPs are single-nucleotide variations that naturally occur in the genome. They can potentially alter miRNA seed sequence, which results in alterations in miRNA target sites and deprivation of proto-oncogene expression control. It may also influence miRNA secondary structure and cause disruption of pri-miRNA recognition by miRNA processing enzymes. So far, numerous genomic studies have shown that SNPs in the miRNA seed sequence or target site may be associated with the risk for different types of cancer and in the prognosis of cancer treatment[95-99].

Transcriptional mechanisms: MiRNAs can be processed from RNA intron (mirtron) or transcribed as independent transcripts. In the latter case, an *miRNA* gene has its own promoter and is transcribed by Pol Ⅱ[9]. Since tissue transcription factors in cancer are often misregulated, it is logical to assume that this also influences miRNA expression. Indeed, regulation of miRNA expression by such well-known cancer-related transcription factors such as E2F, RAS, MYC and P53 has been shown[100,101]. Moreover, miRNAs and their transcription factors often work in feedback loops. Thus, E2F is responsible for up-regulation of the above-mentioned 17-92 cluster of miRNA in gliomas. E2F1 acts as a transcriptional activator of the *miR-17-92* cluster and binds directly to the *miR-17-92* promoter[102]. However, the set of miRNAs produced from this cluster directly inhibits E2F1. This is an example of a negative feedback loop[102,103]. Since E2F1 activates its own transcription by a positive feedback loop, miRNAs in this case act as a fuse for E2F1 over-saturation. MiRNAs miR-449a and miR-449b are other targets of E2F1. In this case, both miRNAs form a negative feedback loop indirectly by targeting the pRb-E2F1 pathway through cell cycle arrest[104]. High expression of miR-375 and estrogen receptor α (ERα) in breast cancer cells is an example of a positive feedback circuit. MiR-375 targets dexamethasone-induced ras-related protein 1 mRNA, an ERα inhibitor, whereas ERα increases *miR-375* expression[105].

Epigenetic mechanisms: Methylation of DNA, especially gene promoter regions of the genes, causes alteration in gene expression[106]. During cancer progression, two cases could potentially be realized: hypermethylation of oncosuppressors and hypomethylation of oncogenes. The fact that most miRNAs are associated with CpG islands[107] allows us to assume that miRNA genes are potential targets of DNA methylation machinery. Indeed, treatment of cells with inhibitors of DNA methylation (5-aza-2'deoxycytidine) led to upregulation of the subset of oncosuppressor miRNAs in human cancer cells[108]. Another example is the oncosuppressor *miR-663* gene, which targets well-known proto-oncogenes such as *EEF1A2, TGFβ, JunB* and *JunD*[109-111]. It was found to be downregulated *via* methylation in samples of human acute myeloid leukemia, hepatocellular carcinoma

and breast cancer, as well as in the K-562 leukemia cell line[112-115]. Similar processes occur with miR-129-2, a tumor-suppressive miRNA that is frequently methylated in lymphoid but not myeloid malignancies[116]. The process of hypomethylation can also be influenced in cancer-related alterations of miRNA expression. Thus, Li *et al*[117] observed hypomethylation of miR-200a/200b promoters with subsequent overexpression of these miRNAs. MiR-200a and miR-200b target *SIP1*, a protein product that suppresses E-cadherin expression and contributes to epithelial mesenchymal transition[74,117]. In renal cell carcinoma, the promoter of the well-known oncogene miR-21 was found to be hypomethylated, which correlates with upregulated miRNA expression level[118].

The stoichiometry of miRNAs and their targets: Each miRNA potentially targets hundreds of transcripts. Depending on the strength of the miRNA binding site, the target can be more or less inhibited. Thus, constant levels of miRNAs and mRNAs expression are in equilibrium, which provides cell homeostasis. However, several mechanisms that might decrease the miRNA level by using "miRNA sponges" have been discovered. The most well-known example is regulation of PTEN oncosuppressor expression by its pseudogene *PTENP1,* which harbors the same conserved miRNA binding site as *PTEN* mRNA[119]. In samples of colon cancer, a decrease in the *PTENP1* pseudogene copy number was found, which potentially increases the miRNA pool that targets PTEN. A pseudogene sequestering the miRNA pool was also shown in the case of *KRAS1P* pseudogene that possess binding sites for miR-143 and let-7 family[120]. Another example of a "miRNA sponge" is circular RNAs (circRNAs). These non-coding RNAs are processed from introns during splicing and carry multiple miRNA binding sites. Hansen *et al*[121] has shown that ciRS-7 (circular RNA sponge for miR-7) contains more than 70 selectively conserved miRNA target sites and strongly inhibits miR-7 oncosuppressor activity[122,123].

3'UTR BINDING PROTEINS AND CANCER

Modulation of the protein expression on the posttranscriptional level during oncogenic transformation often depends on 3'UTR and takes place by changing cis-elements or trans-binding factors that dictate stability and translation efficiency of cancer-related protein mRNAs.

There are few well-characterized cis-elements present in the 3'UTR region. One of them is the CPE, which has a consensus sequence of U4-8A1-2U and is located in relatively close proximity to the ubiquitous nuclear polyadenylation hexanucleotide AAUAAA[124-126].

CPE binds CPEB, one member of a family of four conserved sequence-specific RNA-binding proteins that contain a zinc finger and two RNA recognition motifs[127]. During Xenopus oocyte maturation, CPEB controls meiosis progression from prophase I to metaphase Ⅱ[127]. Translational control by CPEBs was later also shown to be involved in learning and memory[128,129] and in the regulation of the mammalian cell cycle[130]. CPEB is also implicated in senescence in mammals[131,132] and in controlling the translation of proteins involved in cell cycle checkpoints[133]. Xenopus studies have shown that CPEB can both promote and inhibit RNA translation by respectively elongating and shortening the poly(A) tail. The balance between the two CPEB-associated activities is altered during progression of the cell cycle, depending on post-transcriptional modifications as well as on the number and location of CPEs to which CPEB binds and recruits associated adenylating and de-adenylating protein complexes. The CPEB-containing complex in Xenopus include: symplekin, which may be a platform protein upon which multi-component complexes are assembled, poly(A) ribonuclease, a de-adenylating enzyme and germline-development factor 2 (Gld2), an atypical poly(A) polymerase[134,135]. The induction of cytoplasmic polyadenylation is mediated by activation of the serine/threonine kinase Aurora A/Eg2, possibly through repression of glycogen synthase kinase 3[136,137]. When phosphorylated on S174 or T171 (species-dependent), CPEB promotes polyadenylation by stimulating the activity of Gld-2[138]. The newly elongated tail bound by the poly(A)-binding protein promotes translation by augmenting the assembly of the eIF4F initiation complex[139].

CPEB family members were found to be misregulated in various cancers[3]. One of them, CPEB4, was recently shown to be not only over-expressed in pancreatic cancer and glioblastoma in comparison with healthy pancreatic and brain tissues, but also plays a role as a key regulator of cancer transformation and controls translation of hundreds of mRNAs. SiRNA down-regulation of CPEB4 expression in RWP-1 (human pancreatic cancer lines) and Capan pancreatic cancer cells reduce their ability to form tumors after injection into nude mice[2]. This group found that one of the most enriched CPEB4-associated mRNAs, tissue type plasminogen activators (tPA), which is known to be over-expressed in pancreatic tumors, has a short poly(A) tail in normal tissue, whereas in ductal tumors and pancreatic ductal adenocarcinoma cell lines, the tPA poly(A) tail is elongated. This observation supports the idea that misregulation of protein expression during cancer transformation can be controlled by the length of the poly(A) tail, which depends on the presence of CPEB proteins[2].

Insulin-like growth factor-2 mRNA-binding proteins (IGF2BPs or IMPs) are oncofetal proteins that were first discovered in human embryonic Rhabdomyosarcoma and are highly expressed in a number of human cancers[6]. IMPs belong to a conserved family of RNA-binding proteins implicated in the post-transcriptional regulation of multiple mRNAs, IGF2, MYC, CD44, PTEN, G_1/S-specific cyclin-D1 (CCND1), CCND3, G_1/S-specific cyclin-G1 (CCNG1) and others[4,5,140,141]. All these IMP targets are implicated in the proliferation and invasion of human cancer cells. Moreover, several studies have shown that IMPs participate in essential cell functions alienated during cancer transformation, such as cell polarization, migration, morphology, metabolism, proliferation and

differentiation[142].

IMPs are mainly expressed in the embryo and are important during development. However, because of their abnormal re-expression in several types of cancer, IMPs are considered as oncofetal proteins. Typically, IMP1 and IMP3 have been implicated in colon, liver, kidney, pancreas and female reproductive tissue cancers. IMP3 is reported in over 50 publications as being over-expressed in multiple cancer types. IMP3 expression actually correlates with tumor aggressiveness. Concerning IMP2, a few studies have linked its expression to liposarcoma, liver cancer and endometrial adenocarcinomas[142].

IMPs are generally observed in the cytoplasm, where they associate with target mRNAs in cytoplasmic ribonucleoprotein complexes (mRNPs). Actually, in complex with a wide range of other RNA binding proteins (RBPs), IMPs are able to control mRNA turnover, transport, localization and translation.

Other studies provide evidence suggesting an important role for IMPs in cell migration. For instance, IMP2 binds and controls the expression of PINCH-2 (particularly interesting new cysteine-histidine-rich protein) and MURF-3 (muscle specific RING finger protein2) mRNAs to modulate cell motility[143].

Despite controversial observations regarding a potential nuclear role of IMPs, increasing evidence suggests that IMPs can recruit their target mRNAs in the nucleus during their transcription[144-146]. Moreover, a recent study actually shows that in contrast with IMP1 and IMP2, IMP3 has nuclear localization in a large number of human cancer cell lines. For example, IMP3 is almost 100% nuclear in hepatocellular carcinoma, breast and ovarian cancer cells[4].

Among other well known proteins that bind mostly to the AU-rich sequences in 3'UTR and are involved in cancer transformations are Hu/elav proteins, known to bind AU-rich sequences in the 3'UTR and enhance mRNA translation or increase its stability[147,148]. HuR is ubiquitously expressed and HuB, -C and -D are primarily neuronal. HuR is also known as embryonic lethal, abnormal vision, Drosophila-like 1. A link between HuR and malignant transformation has been suggested in cancers such as breast, colon, lung and ovary[149]. Their targets are involved in several processes, such as cell growth and survival, proliferation, stress response, senescence and cancer[150,151].

AU-binding factor 1 (AUF1), also known as heterogeneous nuclear ribonucleoprotein D, belongs to a big family of hnRNPs that includes hnRNNP A, B, C, D, E, F, H, I, K, L, M, Q and R. AUF1 binds to the AU-rich sequence in the 3'UTR of target mRNAs and promotes degradation of the target transcript, most probably by recruiting them to exosomes for degradation[152,153]. However, AUF1 was found to enhance stability and translation of some mRNAs[154,155]. AUF1 was also shown to be involved in several processes: cell cycle, stress response, apoptosis and carcinogenesis.

T-cell intracellular antigen 1 (TIA-1) TIA-1-related (TIAR) binds to AU/U-rich sequences in the 3'UTR of the target transcript and suppresses mRNA translation[156]. Under stress conditions, these proteins are thought to halt mRNA-to-protein aggregations known as stress granules[157].

Nuclear factors 90 interacts with AU rich sequences and suppresses translation of mRNAs involved in the cell cycle[158].

Tristetraprolin (TTP), zinc finger protein, binds AU-rich sequences in mRNAs to promote their decay. It is involved in the cell cycle, inflammation and carcinogenesis[159,160].

KH-type splices regulatory protein (KSRP). RBP binds to AU-rich sequences of target transcripts, promoting mRNA decay. Its targets encoded cytokines, transcription factors, proto-oncogenes and cell cycle regulators[161].

Nucleolin interacts with mRNAs bearing AU-rich or G-rich sequences and regulates mRNA stability and translation. Its targets are involved in the cell cycle, cell morphology, development, cell proliferation and cancer genesis[148].

Obviously, two or more RBP may functionally interplay among themselves and with microRNAs through binding to the same mRNA 3'UTR.

INTERACTION BETWEEN 3'UTR BINDING FACTORS AND THEIR FUNCTION IN NEOPLASTIC TRANSFORMATION

RNA binding proteins interaction

Significant evidence has accumulated to support the idea that several RNA binding proteins can bind the same mRNA target on either the non-overlapping binding sites or on common sites in a competitive fashion. Different RBPs that are capable of binding to the same RNA can cooperate or compete in their actions (Figure 1). The outcome of the combined action of all factors bound to the same mRNA 3'UTR depends on many circumstances, such as expression of different RBPs, their affinity for the binding sites, and their localization in the cells. This can be controlled by different physiological conditions.

For instance, interleukin (IL)-8 plays an integral role in promoting a malignant phenotype in breast cancer and its production is directly influenced by inflammatory cytokines in the tumor microenvironment. Subsequently, activation of the IL-1 receptor on malignant breast cancer cells strongly induces IL-8 mRNA expression. HuR, KSRP and TIAR were found to bind one or more locations within the IL-8 3'UTR, although affinity of the stabilizing factor HuR was 20-fold stronger than that of the KSRP destabilizing factor[162]. HuR, AUF1 and nucleolin bind to BCL-2 mRNAs. HuR and nucleolin both stabilized the BCL-2 transcript, while AUF1 enhanced degradation[163-166]. Thus HuR and nucleolin can have a cooperative effect that is antagonized by AUF1. Another example is related to regulation of GADD45A mRNA stability and translation efficiency. Nucleolin stabilized

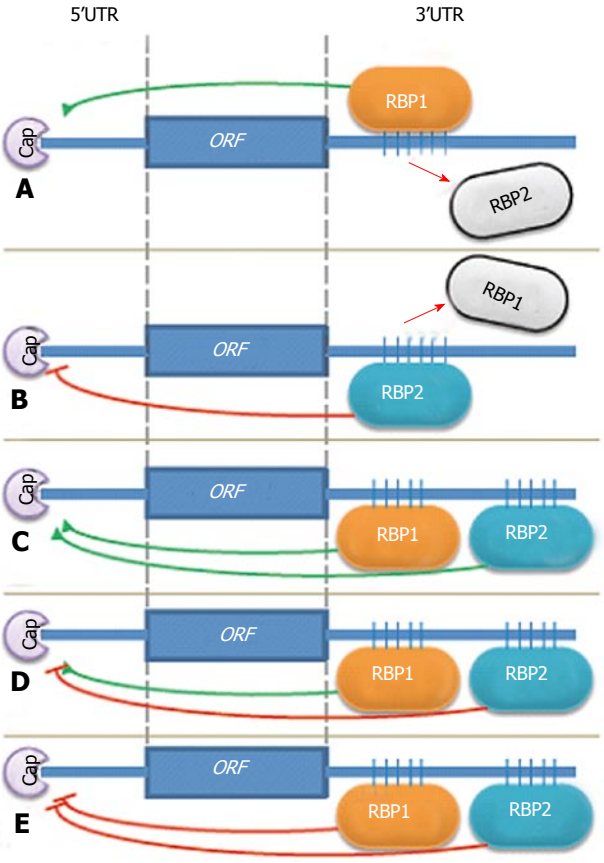

Figure 1 Models of RNA binding proteins interplay in regulation of the same target. A, B: Two RBPs compete for the same binding site in 3'UTR; A: RBP1 binds to the site, repels RBP2, and stimulates translation; B: RBP2 binds to the site, repels RBP1, and inhibits translation; C, D, E: Two RBPs bind to the different sites and cooperate (C, E) or compete (D) in their actions; C: Cooperative action of two RBPs stimulating expression; D: Two RBPs antagonized their effects; E: Cooperative action of two RBPs inhibiting expression. RBPs: RNA binding proteins; 3'UTR: 3'-untranslated region; ORF: Open reading frame.

GADD45A mRNA and was antagonized by AUF1, which promotes decay of this mRNA, and by TIAR, which suppresses translation[167].

In addition, HuR and AUF1 formed a stable ribonucleoprotein complex in the nucleus, whereas in the cytoplasm, HuR and AUF1 bound to target mRNAs individually. HuR colocalizes with the translational apparatus and AUF1 with exosomes[168].

The nuclear localization of IMP3 depends on its protein partner, HNRNPM. Nuclear IMP3 is important for the efficient synthesis of CCND1, D3 and G1 proteins and for the proliferation of human cancer cells. Curiously, IMP3 can be differentially localized in normal versus cancerous adult cells, which in turn will determine the efficiency of protein synthesis of CCND1, D3 and G1 in these cells and have an impact on their proliferation[4]. These studies suggested that IMPs are controlling the transcript destiny of targeted mRNAs in the nucleus and subsequently influence their stability and translation in the cytoplasm. IMP is also found in complex with other RNA-binding proteins, such as HNRNP A2/B1, HNRNP A1, HNRNP A3, Polypyrimidine tract-binding protein 1, interleukin enhancer-binding factor 3, an RNA helicase DHX9 and a mRNA-stabilizing protein HuR[4]. Some of these IMP3 partners, such as HuR, are already known to positively regulate CCND1 mRNA stability and translation[168].

Members of the CPEB and PUF (drosophila pumilio (Pum) protein is a founder member of a novel family of RNA-binding proteins, known as the PUF family.) (Pomelia/Fem-3 mRNA-binding factor 1) families collaborate to regulate mRNA expression throughout eukaryotes. PUF was shown to directly interact with CPEB in C. elegans and humans (CPEB3) and to inhibit translation of common targets[169].

3'UTR binding factors can control translation efficiency *via* interaction with translation, initiation and elongation factors. An example of the interaction with initiation factors has been described for CPEB1 in a previous chapter. Recently, the eukaryotic translation elongation factor 1A1 (eEF1A1) was shown to be involved in EMT regulation. The main function of eEF1A1 is delivery of aminoacyl tRNA to the A-site of the ribosome[170-172]. However, Hussey *et al*[173] discovered a new mechanism of EMT control when eEF1A1 in complex with hnRNP E1 binds to the BAT element in the 3'UTR of the EMT, inducing Dab2 and ILEI transcripts. This results in the inhibition of eEF1A1 release from the ribosomal A site, which causes a stall in translational elongation of the above-mentioned transcripts[173].

Moreover, PUF and Ago can interact with eEF1A proteins to repress translation elongation in both C. elegans and mammalians. This repression occurred after translation initiation and led to ribosome accumulation within the open reading frame, roughly at the site where the nascent polypeptide emerged from the ribosomal exit tunnel. Together, these data suggest that a conserved PUF-Ago-eEF1A complex attenuates translation elongation[174].

RNA binding protein and microRNA interaction

Proteins that bind to the same mRNAs 3'UTR can modulate the function of miRNAs. They either enhance the inhibitory function of miRNA or prevent it. On the other hand, miRNA also can assist the function of RNA binding protein or inhibit it. This can happen simply through binding site competition or collaboration (*via* RNA remodeling), direct protein-protein interaction of 3'UTR-binding complexes, or just functional interplay when a few factors act separately but their actions augment or negate each other (Figure 2).

Interestingly there are few cases described in the literature in which miRNA in collaboration with RNA binding proteins can change their mode of action during the cell cycle or under physiological conditions such as oxidative stress and others.

It has been shown that upon cell cycle arrest, the ARE (AU-rich element) in tumor necrosis factor-α mRNA acts as a translation activation signal, recruiting AGO (argonaute RISC catalytic component) and fragile

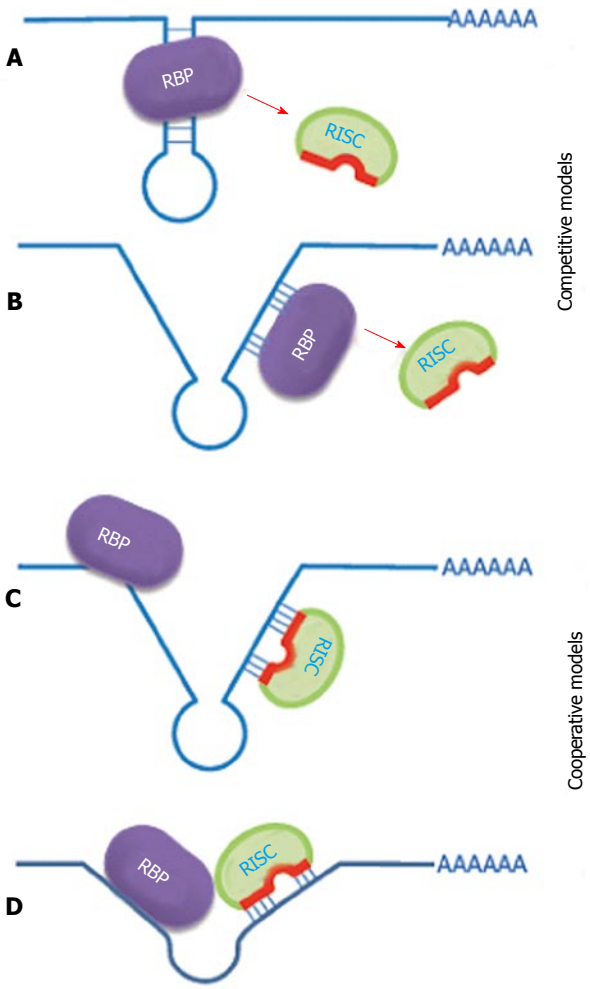

Figure 2 Models of RNA binding protein and miRNA interplay targeting the same mRNA. A, B: Competitive interaction. A: RBP stabilizes the secondary structure in 3'UTR, and prevents miRNA binding; B: RBP competes with miRNA for the same binding site; C, D: Cooperative interaction; C: RBP facilitates miRNA function by opening secondary structure in 3'UTR and liberating its binding site; D: RBP directly interacts with RISC (RNA-induced silencing complex) complex, stabilizing binding of the latter. RBPs: RNA binding proteins; 3'UTR: 3'-untranslated region.

X mental retardation-related protein 1 factors associated with miRNPs. Human miRNA mir-369-3 directs the association of these proteins with the AREs, leading to the activation of translation[7]. Moreover, two well-studied miRNAs, let-7 and the synthetic miRNA miRcxcr4, also induce translational up-regulation of target mRNA upon cell cycle arrest. However, they repress translation in proliferating cells. It has been proposed that translation regulation by miRNPs oscillates between repression and activation during the cell cycle[7].

Another example of inactivation, storage and reactivation is calcium transport protein (CAT)-1 mRNA targeting by mir-122 under stress conditions. The derepression of CAT-1 mRNA is accompanied by its release from cytoplasmic P-bodies and its recruitment to polysomes. Derepression requires binding of HuR, an AU-rich-element-binding protein, to the 3'UTR of CAT-1 mRNA[175]. Thus, interaction with RNA binding proteins can change the miRNA mode of action directed to the same target, according to conditions.

Some difficulty in understanding the affiliation of certain RBPs for oncogenes or tumor suppressors came from the observation that the same RBP interacting with different miRNAs in the regulation of different targets could lead to enhanced or suppressed cancer transformation, according to the nature of the target. The stimulation effect of Pomelia on miRNA function, most probably through mRNA remodeling, is directed towards the targets acting in opposite ways, as oncogene or tumor suppressor. It was shown by Kedde and co-workers that Pomelia RBP pumilio RNA-binding family member 1 (PUM1) and PUM2 promote the regulation of miR-221/222 on the $p27^{kip1}$ check-point protein and tumor suppressor mRNAs by opening of the secondary structure of the p27 3'UTR and exposing the binding sequence to miR-221/222. This causes down-regulation of $p27^{kip1}$ accumulation and stimulates cell proliferation and breast cancer development[176]. On the other hand, Pomelia collaborates with some miRNAs to repress E2F3, transcription factor and strong oncogene. This prevents cell proliferation and down-regulates bladder cancer development[177].

Another example of miRNA and RBP collaboration was shown by Nairismägi *et al*[73] who showed that miR-580 and CPEB1/2 down-regulate TWIST1 expression, one of the main inductors of EMT in a cooperative way. On the other hand, Dnd1 is an example of RBP that prevents binding of miRNA to their target sequences in a few genes, such as $p27^{kip1}$ and LATS2, and suppresses formation of germ cell tumor[178]. It also prevents miR-21 function on its MutS protein homolog 2 target, which suppresses tumorigenesis in skin[179]. Thus by preventing miRNA down-regulation of tumor suppressors, Dnd1 inhibits the development of certain tumors.

The same RNA binding protein can cooperate or antagonize miRNA functions, according to the mRNA-target. One of the most investigated examples is HuR[147], which was found to recruit let-7 to suppress c-MYC mRNA translation[8] but competes with miR-494 and miR-548-3p for the regulation of nucleolin and TOP2A mRNA, respectively[180,181].

Some RBPs not working alone but in complex with other RNA binding proteins can prevent miRNA actions. IMP1 in complex with heterogeneous nuclear ribonucleoprotein U, synaptotagmin binding, cytoplasmic RNA interacting protein, YXB1 (transcriptional regulator) and DHX9 [DEAH (Asp-Glu-Ala-His) box helicase 9] is able to stabilize the mRNA of MYC, possibly by inhibiting its translation-coupled degradation[182]. However, some studies showed that MYC is repressed by members of the let-7 microRNA family, suggesting a possible function of IMP1 in protecting MYC mRNAs from microRNA silencing. This was previously proposed as a mechanism for the stabilization of the BTRC (beta-transducin repeat containing E3 ubiquitin protein ligase) mRNA by IMP1[183,184].

Not only RNA-binding protein can influence miRNA function, but reciprocal action has already also been

documented in the literature. Some miRNAs can affect the function of RNA binding protein. For example, interaction of mir-16 (a member of the mir-15/16 family of miRNPs) and an ARE-binding protein TTP (tristetraprolin) has been shown to occur through association with AGO/eiF2C family members. Mir-16 assists TTP in targeting ARE, which appears to be an essential step in ARE-mediated mRNA degradation[185].

From all of these examples, one can see that interaction among factors binding to 3'UTR brings a new level of complexity to the mechanisms of action of these factors and their influences on cancer transformation. It is becoming clear that to understand the true picture of the post-transcriptional control of certain genes *via* 3'UTR, especially that involved in cancer transformation, one needs to take into account all proteins and miRNAs binding to their 3'UTRs.

APPLICATION OF 3'UTR-BINDING FACTORS TO CANCER DIAGNOSIS AND TREATMENT

From the very early investigations that suggested miRNA involvement in cancer, scientists began to think about using it as a tool for cancer diagnosis and therapy. A number of studies have been initiated utilizing miRNA expression profiling to determine markers for diseases. An early study comparing a limited number of available miRNAs in cancer and normal tissues drove the conclusions that miRNAs expression signatures are able to classify tumors based on the development lineage and the differentiation state, suggesting miRNAs as a potential biomarker[14]. Following works used the miRNA expression profile to define a number of normal and cancerous tissues from thyroid, kidney, bladder, liver *etc*[186-193]. Furthermore, miRNA profiling has also been used to classify tumor subtypes in breast cancer in development[194,195]. Mir-342 is differentially expressed in breast cancer subtypes with high expression in Luminal B-type tumors and decreased expression in therapeutically difficult estrogen receptor/human epidermal growth factor receptor 2-negative tumors[196]. This observation suggested that select miRNAs expression could differentiate tumor subtypes that can be more sensitive or resistant to particular treatments.

Radiation therapy (RT) is one of the most often used procedures in cancer treatment; however, not all patients respond well to it. So, it is very important to develop markers that can predict a patient's response to RT. MiRNA profiling has a big potential for this type of diagnosis.

One of the first reports identifies the let-7 family for its role in modulating sensitivity for RT in lung cancer[197]. It has been demonstrated that over-expression of let-7 promotes radio-sensitivity while knockdown increases resistance both *in vitro* and *in vivo*. Mir-181a has been identified as an important miRNA for radio-sensitivity in glioma cells. Transient over-expression of miR-181a prevented radio-sensitivity that correlated with decreased quantities of Bcl-2, an anti-apoptotic protein[198]. Similarly, over-expression of mir-451 in colorectal cancer cell lines decreases proliferation and increases RT sensitivity of colorectal cancer cells[199].

Chemotherapy is another widely used treatment in cancer therapy. The miRNA profile also has a big potential as a marker for chemo-sensitivity. Inhibition or introduction of some miRNAs to certain cancers can improve their chemo-sensitivity. Inhibition of mir-21 sensitizes U251 glioma cells to etoposide and glioma in mice to tumor necrosis factor-related apoptosis, inducing the ligand S-TRAIL (TNF-related apoptosis-inducing ligand)[200-202]. Mir-451 is downregulated in the glioblastoma stem cell population. Reintroduction of mir-451 in combination with the frequently used glioblastoma treatment imatinib inhibits the growth of glioblastoma stem cells and the formation of neurospheres[203].

Mir-122 was shown to be downregulated in hepatocellular carcinoma (HCC) cells, which promotes RT resistance as well as growth, proliferation and metastasis[204]. Insulin growth factor 1 tyrosine kinase receptor is targeted and suppressed by miR-122 in normal liver cells. However, depletion of mir-122 in HCC increases the IgfIR level. Reintroduction of mir-122 in HCC promotes sensitivity to the tyrosine kinase inhibitor sorafenib[204].

In colorectal cancer, a number of miRNAs have been associated with predicting the response to nucleoside analogs. Mir-143 is downregulated in colon cancer. It targets NF-κB, Bxl-2 and ERK5 and has been shown to increase sensitivity to fluorouracil in HCT-166 colon cancer cell lines[205]. In rectal cancer, mir-125b and mir-137 were associated with poor response to capecitabine, a prodrug that is enzymatically converted to fluorouracil[206]. In colon cancer, mir-519c targets and suppresses ATP-binding cassette sub-family G member 2 (ABCG2) in cell lines that are sensitive to mitoxantrone, whereas mir-519c inhibition increases the ABCG2 level and chemoresistance. In the ABCG2 resistant cell line, mRNA possess a shortened 3'UTR, which results in the loss of a mir-519c target site and a high-level of ABCG2 protein[207].

All these examples clearly show that miRNA profiling of each cancer could provide useful information for choosing the right treatment strategy. Few bio-pharmaceutical companies are working on developing miRNA-profiling platforms for more detailed identification of cancer subtypes that could improve recommendation of treatment. There are more than 100 ongoing trials incorporating miRNA as biomarkers underway in various bio-pharmacological companies.

Direct miRNA therapeutics, the fundamental principle of miRNA therapy, involves either directed silencing or reduction in tumor-promoting miRNAs versus enrichment of tumor suppressive miRNAs. *In vivo*, these approaches include genetically engineered animals and different ways of delivery, such as viral vectors, nanoparticle-based delivery, mimics and antimiRs. Targeting miRNA for suppression through the use of antimiRs is perhaps the most promising model. Through complementary binding to the target miRNA (working strand),

these molecules can repress the action of select miRNAs.

To improve stability and target specificity, investigators have developed various modifications. Three types of modification currently give the most promising results: replacement of 2-OH residues by 2'-O-methyl modified oligonucleotides, 2'-O-methoxyethyl and locked nucleic acid. In addition, conjugation of cholesterol may be used to improve target specificity[208].

Sponge is another tool for RNA-silencing. By having multiple target binding sites, sponges essentially compete with target mRNA for miRNA occupancy, thus decreasing binding miRNA to its real target[209].

To target a few miRNAs involved in the same cancer formation, investigators started using tiny 8-mer locked nucleic acids with a phosphorothioate backbone to enhance the stability level[210]. They were shown to inhibit families of miR-221/222 and let-7 with high specificity.

Viral vector-based delivery systems, including adenoviral, retroviral and lentiviral systems provide some advantages. For example, lentiviral let-7 delivery has been successfully used in murine models of lung cancer[32]. Several nanoparticles with lipid-based formulations were perhaps the most effective in delivery while minimizing toxicity. Lipid emulsions have been used to deliver miRNAs in lung cancer and lymphoma[211-213].

In spite of big efforts, only mir-122 has successfully reached the clinical trial in targeted therapy[214,215]. The systematic delivery of antimiR-122 could reduce the hepatitis C virus (HCV) viral load chimpanzee model of chronic HCV infection with minimal toxicity[216]. Santaris Pharma conducted a human phase Ⅱa trial safety antiviral function using miravirsen (a locked nucleic acid-modified miR-122 antagonist).

RNA binding proteins similarly can be used as markers for proper cancer diagnostics, leading to better treatment selection. For example, IMP3 over-expression has been associated with distinct cancer types. Several studies have suggested IMP3 as an important marker for poor prognosis in cancer[217,218]. Moreover, it was demonstrated that IMP3 promotes cell growth, proliferation and resistance to ionic irradiation in an IGF2-dependent manner[219,220]. Since CPEB4 was found to be a key protein for pancreatic cancer and glioblastoma development, one can try to apply siRNA-dependent direct down-regulation of CPEB4 protein in this type of tumor using delivery methods that are discussed in this chapter.

In conclusion, 3'UTRs of human mRNAs contained many cis-elements that bind trans factors and are important for the development of various diseases, including cancer. Additional work is required to identify the complete set of 3'UTR cis-elements and the trans-regulatory factors that interact with them and to determine functional consequences of these interactions and their role in cancer transformation. Powerful transcriptome-wide computational and experimental methods are now being used to address these questions. Along with lower-throughput reductionist approaches, they should move us closer to a system biology understanding of how 3'UTRs contribute to gene regulation during cancer transformation. This will allow developing new, more powerful drugs in cancer therapy.

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REFERENCES

1 **Baltz AG**, Munschauer M, Schwanhäusser B, Vasile A, Murakawa Y, Schueler M, Youngs N, Penfold-Brown D, Drew K, Milek M, Wyler E, Bonneau R, Selbach M, Dieterich C, Landthaler M. The mRNA-bound proteome and its global occupancy profile on protein-coding transcripts. *Mol Cell* 2012; **46**: 674-690 [PMID: 22681889 DOI: 10.1016/j.molcel.2012.05.021]

2 **Ortiz-Zapater E**, Pineda D, Martínez-Bosch N, Fernández-Miranda G, Iglesias M, Alameda F, Moreno M, Eliscovich C, Eyras E, Real FX, Méndez R, Navarro P. Key contribution of CPEB4-mediated translational control to cancer progression. *Nat Med* 2012; **18**: 83-90 [PMID: 22138752 DOI: 10.1038/nm.2540]

3 **D'Ambrogio A**, Nagaoka K, Richter JD. Translational control of cell growth and malignancy by the CPEBs. *Nat Rev Cancer* 2013; **13**: 283-290 [PMID: 23446545 DOI: 10.1038/nrc3485]

4 **Rivera Vargas T**, Boudoukha S, Simon A, Souidi M, Cuvellier S, Pinna G, Polesskaya A. Post-transcriptional regulation of cyclins D1, D3 and G1 and proliferation of human cancer cells depend on IMP-3 nuclear localization. *Oncogene* 2013 Jul; Epub ahead of print [PMID: 23812426 DOI: 10.1038/onc.2013.252]

5 **Vikesaa J**, Hansen TV, Jønson L, Borup R, Wewer UM, Christiansen J, Nielsen FC. RNA-binding IMPs promote cell adhesion and invadopodia formation. *EMBO J* 2006; **25**: 1456-1468 [PMID: 16541107 DOI: 10.1038/sj.emboj.7601039]

6 **Yisraeli JK**. RNA localization and cell polarity: a cellular 'pas de deux'. *Biol Cell* 2005; **97**: 3 [PMID: 15601253 DOI: 10.1042/BC20040152]

7 **Vasudevan S**, Tong Y, Steitz JA. Cell-cycle control of microRNA-mediated translation regulation. *Cell Cycle* 2008; **7**: 1545-1549 [PMID: 18469529]

8 **Kim VN**, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* 2009; **10**: 126-139 [PMID: 19165215 DOI: 10.1038/nrm2632]

9 **Fabian MR**, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem* 2010; **79**: 351-379 [PMID: 20533884 DOI: 10.1146/annurev-biochem-060308-103103]

10 **Bushati N**, Cohen SM. microRNA functions. *Annu Rev Cell Dev Biol* 2007; **23**: 175-205 [PMID: 17506695 DOI: 10.1146/annurev.cellbio.23.090506.123406]

11 **Ghildiyal M**, Zamore PD. Small silencing RNAs: an expanding universe. *Nat Rev Genet* 2009; **10**: 94-108 [PMID: 19148191 DOI: 10.1038/nrg2504]

12 **Lee HJ**. Exceptional stories of microRNAs. *Exp Biol Med* (Maywood) 2013; **238**: 339-343 [PMID: 23759998 DOI: 10.1258/ebm.2012.012251]

13 **Calin GA**, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 2002; **99**: 15524-15529 [PMID: 12434020 DOI: 10.1073/pnas.242606799]

14 **Lu J**, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck

D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834-838 [PMID: 15944708 DOI: 10.1038/nature03702]

15 **Volinia S**, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA* 2006; **103**: 2257-2261 [PMID: 16461460 DOI: 10.1073/pnas.0510565103]

16 **Calin GA**, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006; **6**: 857-866 [PMID: 17060945 DOI: 10.1038/nrc1997]

17 **Esquela-Kerscher A**, Slack FJ. Oncomirs - microRNAs with a role in cancer. *Nat Rev Cancer* 2006; **6**: 259-269 [PMID: 16557279 DOI: 10.1038/nrc1840]

18 **Bandi N**, Zbinden S, Gugger M, Arnold M, Kocher V, Hasan L, Kappeler A, Brunner T, Vassella E. miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer. *Cancer Res* 2009; **69**: 5553-5559 [PMID: 19549910 DOI: 10.1158/0008-5472.CAN-08-4277]

19 **Dyrskjøt L**, Ostenfeld MS, Bramsen JB, Silahtaroglu AN, Lamy P, Ramanathan R, Fristrup N, Jensen JL, Andersen CL, Zieger K, Kauppinen S, Ulhøi BP, Kjems J, Borre M, Orntoft TF. Genomic profiling of microRNAs in bladder cancer: miR-129 is associated with poor outcome and promotes cell death in vitro. *Cancer Res* 2009; **69**: 4851-4860 [PMID: 19487295 DOI: 10.1158/0008-5472.CAN-08-4043]

20 **Veerla S**, Lindgren D, Kvist A, Frigyesi A, Staaf J, Persson H, Liedberg F, Chebil G, Gudjonsson S, Borg A, Månsson W, Rovira C, Höglund M. MiRNA expression in urothelial carcinomas: important roles of miR-10a, miR-222, miR-125b, miR-7 and miR-452 for tumor stage and metastasis, and frequent homozygous losses of miR-31. *Int J Cancer* 2009; **124**: 2236-2242 [PMID: 19127597 DOI: 10.1002/ijc.24183]

21 **Voorhoeve PM**, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, Liu YP, van Duijse J, Drost J, Griekspoor A, Zlotorynski E, Yabuta N, De Vita G, Nojima H, Looijenga LH, Agami R. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* 2006; **124**: 1169-1181 [PMID: 16564011 DOI: 10.1016/j.cell.2006.02.037]

22 **Ventura A**, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, Newman J, Bronson RT, Crowley D, Stone JR, Jaenisch R, Sharp PA, Jacks T. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell* 2008; **132**: 875-886 [PMID: 18329372 DOI: 10.1016/j.cell.2008.02.019]

23 **Tetzlaff MT**, Liu A, Xu X, Master SR, Baldwin DA, Tobias JW, Livolsi VA, Baloch ZW. Differential expression of miRNAs in papillary thyroid carcinoma compared to multinodular goiter using formalin fixed paraffin embedded tissues. *Endocr Pathol* 2007; **18**: 163-173 [PMID: 18058265 DOI: 10.1007/s12022-007-0023-7]

24 **Magrelli A**, Azzalin G, Salvatore M, Viganotti M, Tosto F, Colombo T, Devito R, Di Masi A, Antoccia A, Lorenzetti S, Maranghi F, Mantovani A, Tanzarella C, Macino G, Taruscio D. Altered microRNA Expression Patterns in Hepatoblastoma Patients. *Transl Oncol* 2009; **2**: 157-163 [PMID: 19701500]

25 **Mendell JT**. miRiad roles for the miR-17-92 cluster in development and disease. *Cell* 2008; **133**: 217-222 [PMID: 18423194 DOI: 10.1016/j.cell.2008.04.001]

26 **Mott JL**, Kobayashi S, Bronk SF, Gores GJ. mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* 2007; **26**: 6133-6140 [PMID: 17404574 DOI: 10.1038/sj.onc.1210436]

27 **Henson BJ**, Bhattacharjee S, O'Dee DM, Feingold E, Gollin SM. Decreased expression of miR-125b and miR-100 in oral cancer cells contributes to malignancy. *Genes Chromosomes Cancer* 2009; **48**: 569-582 [PMID: 19396866 DOI: 10.1002/gcc.20666]

28 **Lerner M**, Harada M, Lovén J, Castro J, Davis Z, Oscier D, Henriksson M, Sangfelt O, Grandér D, Corcoran MM. DLEU2, frequently deleted in malignancy, functions as a critical host gene of the cell cycle inhibitory microRNAs miR-15a and miR-16-1. *Exp Cell Res* 2009; **315**: 2941-2952 [PMID: 19591824 DOI: 10.1016/j.yexcr.2009.07.001]

29 **Guled M**, Lahti L, Lindholm PM, Salmenkivi K, Bagwan I, Nicholson AG, Knuutila S. CDKN2A, NF2, and JUN are dysregulated among other genes by miRNAs in malignant mesothelioma -A miRNA microarray analysis. *Genes Chromosomes Cancer* 2009; **48**: 615-623 [PMID: 19396864 DOI: 10.1002/gcc.20669]

30 **Ferdin J**, Kunej T, Calin GA. Non-coding RNAs: identification of cancer-associated microRNAs by gene profiling. *Technol Cancer Res Treat* 2010; **9**: 123-138 [PMID: 20218735]

31 **Esquela-Kerscher A**, Trang P, Wiggins JF, Patrawala L, Cheng A, Ford L, Weidhaas JB, Brown D, Bader AG, Slack FJ. The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle* 2008; **7**: 759-764 [PMID: 18344688]

32 **Kumar MS**, Erkeland SJ, Pester RE, Chen CY, Ebert MS, Sharp PA, Jacks T. Suppression of non-small cell lung tumor development by the let-7 microRNA family. *Proc Natl Acad Sci USA* 2008; **105**: 3903-3908 [PMID: 18308936 DOI: 10.1073/pnas.0712321105]

33 **Legesse-Miller A**, Elemento O, Pfau SJ, Forman JJ, Tavazoie S, Coller HA. let-7 Overexpression leads to an increased fraction of cells in G2/M, direct down-regulation of Cdc34, and stabilization of Wee1 kinase in primary fibroblasts. *J Biol Chem* 2009; **284**: 6605-6609 [PMID: 19126550 DOI: 10.1074/jbc.C900002200]

34 **Slack F**. let-7 microRNA reduces tumor growth. *Cell Cycle* 2009; **8**: 1823 [PMID: 19377282]

35 **Johnson SM**, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ. RAS is regulated by the let-7 microRNA family. *Cell* 2005; **120**: 635-647 [PMID: 15766527 DOI: 10.1016/j.cell.2005.01.014]

36 **Lee YS**, Dutta A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes Dev* 2007; **21**: 1025-1030 [PMID: 17437991 DOI: 10.1101/gad.1540407]

37 **Mayr C**, Hemann MT, Bartel DP. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science* 2007; **315**: 1576-1579 [PMID: 17322030 DOI: 10.1126/science.1137999]

38 **Park SM**, Shell S, Radjabi AR, Schickel R, Feig C, Boyerinas B, Dinulescu DM, Lengyel E, Peter ME. Let-7 prevents early cancer progression by suppressing expression of the embryonic gene HMGA2. *Cell Cycle* 2007; **6**: 2585-2590 [PMID: 17957144]

39 **Sampson VB**, Rong NH, Han J, Yang Q, Aris V, Soteropoulos P, Petrelli NJ, Dunn SP, Krueger LJ. MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. *Cancer Res* 2007; **67**: 9762-9770 [PMID: 17942906 DOI: 10.1158/0008-5472.CAN-07-2462]

40 **Müller DW**, Bosserhoff AK. Integrin beta 3 expression is regulated by let-7a miRNA in malignant melanoma. *Oncogene* 2008; **27**: 6698-6706 [PMID: 18679415 DOI: 10.1038/onc.2008.282]

41 **Boyerinas B**, Park SM, Shomron N, Hedegaard MM, Vinther J, Andersen JS, Feig C, Xu J, Burge CB, Peter ME. Identification of let-7-regulated oncofetal genes. *Cancer Res* 2008; **68**: 2587-2591 [PMID: 18413726 DOI: 10.1158/0008-5472.CAN-08-0264]

42 **Tokumaru S**, Suzuki M, Yamada H, Nagino M, Takahashi T. let-7 regulates Dicer expression and constitutes a negative feedback loop. *Carcinogenesis* 2008; **29**: 2073-2077 [PMID: 18700235 DOI: 10.1093/carcin/bgn187]

43 **Schultz J**, Lorenz P, Gross G, Ibrahim S, Kunz M. MicroR-

NA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. *Cell Res* 2008; **18**: 549-557 [PMID: 18379589 DOI: 10.1038/cr.2008.45]

44 **Tanaka N**, Toyooka S, Soh J, Kubo T, Yamamoto H, Maki Y, Muraoka T, Shien K, Furukawa M, Ueno T, Asano H, Tsukuda K, Aoe K, Miyoshi S. Frequent methylation and oncogenic role of microRNA-34b/c in small-cell lung cancer. *Lung Cancer* 2012; **76**: 32-38 [PMID: 22047961 DOI: 10.1016/j.lungcan.2011.10.002]

45 **Vogt M**, Munding J, Grüner M, Liffers ST, Verdoodt B, Hauk J, Steinstraesser L, Tannapfel A, Hermeking H. Frequent concomitant inactivation of miR-34a and miR-34b/c by CpG methylation in colorectal, pancreatic, mammary, ovarian, urothelial, and renal cell carcinomas and soft tissue sarcomas. *Virchows Arch* 2011; **458**: 313-322 [PMID: 21225432 DOI: 10.1007/s00428-010-1030-5]

46 **Corney DC**, Hwang CI, Matoso A, Vogt M, Flesken-Nikitin A, Godwin AK, Kamat AA, Sood AK, Ellenson LH, Hermeking H, Nikitin AY. Frequent downregulation of miR-34 family in human ovarian cancers. *Clin Cancer Res* 2010; **16**: 1119-1128 [PMID: 20145172 DOI: 10.1158/1078-0432.CCR-09-2642]

47 **Fabbri M**, Bottoni A, Shimizu M, Spizzo R, Nicoloso MS, Rossi S, Barbarotto E, Cimmino A, Adair B, Wojcik SE, Valeri N, Calore F, Sampath D, Fanini F, Vannini I, Musuraca G, Dell'Aquila M, Alder H, Davuluri RV, Rassenti LZ, Negrini M, Nakamura T, Amadori D, Kay NE, Rai KR, Keating MJ, Kipps TJ, Calin GA, Croce CM. Association of a microRNA/TP53 feedback circuitry with pathogenesis and outcome of B-cell chronic lymphocytic leukemia. *JAMA* 2011; **305**: 59-67 [PMID: 21205967 DOI: 10.1001/jama.2010.1919]

48 **Xu Y**, Liu L, Liu J, Zhang Y, Zhu J, Chen J, Liu S, Liu Z, Shi H, Shen H, Hu Z. A potentially functional polymorphism in the promoter region of miR-34b/c is associated with an increased risk for primary hepatocellular carcinoma. *Int J Cancer* 2011; **128**: 412-417 [PMID: 20309940 DOI: 10.1002/ijc.25342]

49 **He L**, He X, Lowe SW, Hannon GJ. microRNAs join the p53 network--another piece in the tumour-suppression puzzle. *Nat Rev Cancer* 2007; **7**: 819-822 [PMID: 17914404 DOI: 10.1038/nrc2232]

50 **Rokhlin OW**, Scheinker VS, Taghiyev AF, Bumcrot D, Glover RA, Cohen MB. MicroRNA-34 mediates AR-dependent p53-induced apoptosis in prostate cancer. *Cancer Biol Ther* 2008; **7**: 1288-1296 [PMID: 18497571]

51 **Siemens H**, Jackstadt R, Hünten S, Kaller M, Menssen A, Götz U, Hermeking H. miR-34 and SNAIL form a double-negative feedback loop to regulate epithelial-mesenchymal transitions. *Cell Cycle* 2011; **10**: 4256-4271 [PMID: 22134354 DOI: 10.4161/cc.10.24.18552]

52 **Kim NH**, Kim HS, Li XY, Lee I, Choi HS, Kang SE, Cha SY, Ryu JK, Yoon D, Fearon ER, Rowe RG, Lee S, Maher CA, Weiss SJ, Yook JI. A p53/miRNA-34 axis regulates Snail1-dependent cancer cell epithelial-mesenchymal transition. *J Cell Biol* 2011; **195**: 417-433 [PMID: 22024162 DOI: 10.1083/jcb.201103097]

53 **Kim NH**, Kim HS, Kim NG, Lee I, Choi HS, Li XY, Kang SE, Cha SY, Ryu JK, Na JM, Park C, Kim K, Lee S, Gumbiner BM, Yook JI, Weiss SJ. p53 and microRNA-34 are suppressors of canonical Wnt signaling. *Sci Signal* 2011; **4**: ra71 [PMID: 22045851 DOI: 10.1126/scisignal.2001744]

54 **Yamakuchi M**, Ferlito M, Lowenstein CJ. miR-34a repression of SIRT1 regulates apoptosis. *Proc Natl Acad Sci USA* 2008; **105**: 13421-13426 [PMID: 18755897 DOI: 10.1073/pnas.0801613105]

55 **Cittelly DM**, Finlay-Schultz J, Howe EN, Spoelstra NS, Axlund SD, Hendricks P, Jacobsen BM, Sartorius CA, Richer JK. Progestin suppression of miR-29 potentiates dedifferentiation of breast cancer cells via KLF4. *Oncogene* 2013; **32**: 2555-2564 [PMID: 22751119 DOI: 10.1038/onc.2012.275]

56 **Pekarsky Y**, Santanam U, Cimmino A, Palamarchuk A, Efanov A, Maximov V, Volinia S, Alder H, Liu CG, Rassenti L, Calin GA, Hagan JP, Kipps T, Croce CM. Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181. *Cancer Res* 2006; **66**: 11590-11593 [PMID: 17178851 DOI: 10.1158/0008-5472.CAN-06-3613]

57 **Xiong Y**, Fang JH, Yun JP, Yang J, Zhang Y, Jia WH, Zhuang SM. Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. *Hepatology* 2010; **51**: 836-845 [PMID: 20041405 DOI: 10.1002/hep.23380]

58 **Zhao JJ**, Lin J, Lwin T, Yang H, Guo J, Kong W, Dessureault S, Moscinski LC, Rezania D, Dalton WS, Sotomayor E, Tao J, Cheng JQ. microRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. *Blood* 2010; **115**: 2630-2639 [PMID: 20086245 DOI: 10.1182/blood-2009-09-243147]

59 **Fabbri M**, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C, Volinia S, Guler G, Morrison CD, Chan KK, Marcucci G, Calin GA, Huebner K, Croce CM. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci USA* 2007; **104**: 15805-15810 [PMID: 17890317 DOI: 10.1073/pnas.0707628104]

60 **Tanzer A**, Stadler PF. Molecular evolution of a microRNA cluster. *J Mol Biol* 2004; **339**: 327-335 [PMID: 15136036 DOI: 10.1016/j.jmb.2004.03.065]

61 **Ota A**, Tagawa H, Karnan S, Tsuzuki S, Karpas A, Kira S, Yoshida Y, Seto M. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. *Cancer Res* 2004; **64**: 3087-3095 [PMID: 15126345]

62 **He L**, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM. A microRNA polycistron as a potential human oncogene. *Nature* 2005; **435**: 828-833 [PMID: 15944707 DOI: 10.1038/nature03552]

63 **Kan T**, Sato F, Ito T, Matsumura N, David S, Cheng Y, Agarwal R, Paun BC, Jin Z, Olaru AV, Selaru FM, Hamilton JP, Yang J, Abraham JM, Mori Y, Meltzer SJ. The miR-106b-25 polycistron, activated by genomic amplification, functions as an oncogene by suppressing p21 and Bim. *Gastroenterology* 2009; **136**: 1689-1700 [PMID: 19422085]

64 **Petrocca F**, Vecchione A, Croce CM. Emerging role of miR-106b-25/miR-17-92 clusters in the control of transforming growth factor beta signaling. *Cancer Res* 2008; **68**: 8191-8194 [PMID: 18922889 DOI: 10.1158/0008-5472.CAN-08-1768]

65 **Iorio MV**, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Ménard S, Croce CM. MicroRNA signatures in human ovarian cancer. *Cancer Res* 2007; **67**: 8699-8707 [PMID: 17875710 DOI: 10.1158/0008-5472.CAN-07-1936]

66 **Schetter AJ**, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 2008; **299**: 425-436 [PMID: 18230780 DOI: 10.1001/jama.299.4.425]

67 **Seike M**, Goto A, Okano T, Bowman ED, Schetter AJ, Horikawa I, Mathe EA, Jen J, Yang P, Sugimura H, Gemma A, Kudoh S, Croce CM, Harris CC. MiR-21 is an EGFR-regulated anti-apoptotic factor in lung cancer in never-smokers. *Proc Natl Acad Sci USA* 2009; **106**: 12085-12090 [PMID: 19597153 DOI: 10.1073/pnas.0905234106]

68 **Asangani IA**, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4

and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene* 2008; **27**: 2128-2136 [PMID: 17968323 DOI: 10.1038/sj.onc.1210856]

69 **Zhu S**, Si ML, Wu H, Mo YY. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem* 2007; **282**: 14328-14336 [PMID: 17363372 DOI: 10.1074/jbc.M611393200]

70 **Meng F**, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* 2007; **133**: 647-658 [PMID: 17681183 DOI: 10.1053/j.gastro.2007.05.022]

71 **Koch U**, Krause M, Baumann M. Cancer stem cells at the crossroads of current cancer therapy failures--radiation oncology perspective. *Semin Cancer Biol* 2010; **20**: 116-124 [PMID: 20219680 DOI: 10.1016/j.semcancer.2010.02.003]

72 **Kalluri R**, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009; **119**: 1420-1428 [PMID: 19487818 DOI: 10.1172/JCI39104]

73 **Nairismägi ML**, Vislovukh A, Meng Q, Kratassiouk G, Beldiman C, Petretich M, Groisman R, Füchtbauer EM, Harel-Bellan A, Groisman I. Translational control of TWIST1 expression in MCF-10A cell lines recapitulating breast cancer progression. *Oncogene* 2012; **31**: 4960-4966 [PMID: 22266852 DOI: 10.1038/onc.2011.650]

74 **Gregory PA**, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 2008; **10**: 593-601 [PMID: 18376396 DOI: 10.1038/ncb1722]

75 **Park SM**, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* 2008; **22**: 894-907 [PMID: 18381893 DOI: 10.1101/gad.1640608]

76 **Paterson EL**, Kazenwadel J, Bert AG, Khew-Goodall Y, Ruszkiewicz A, Goodall GJ. Down-regulation of the miRNA-200 family at the invasive front of colorectal cancers with degraded basement membrane indicates EMT is involved in cancer progression. *Neoplasia* 2013; **15**: 180-191 [PMID: 23441132]

77 **Tao ZH**, Wan JL, Zeng LY, Xie L, Sun HC, Qin LX, Wang L, Zhou J, Ren ZG, Li YX, Fan J, Wu WZ. miR-612 suppresses the invasive-metastatic cascade in hepatocellular carcinoma. *J Exp Med* 2013; **210**: 789-803 [PMID: 23478189 DOI: 10.1084/jem.20120153]

78 **Bornachea O**, Santos M, Martínez-Cruz AB, García-Escudero R, Dueñas M, Costa C, Segrelles C, Lorz C, Buitrago A, Saiz-Ladera C, Agirre X, Grande T, Paradela B, Maraver A, Ariza JM, Prosper F, Serrano M, Sánchez-Céspedes M, Paramio JM. EMT and induction of miR-21 mediate metastasis development in Trp53-deficient tumours. *Sci Rep* 2012; **2**: 434 [PMID: 22666537 DOI: 10.1038/srep00434]

79 **Zavadil J**, Narasimhan M, Blumenberg M, Schneider RJ. Transforming growth factor-beta and microRNA: mRNA regulatory networks in epithelial plasticity. *Cells Tissues Organs* 2007; **185**: 157-161 [PMID: 17587821 DOI: 10.1159/000101316]

80 **Ma L**, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* 2007; **449**: 682-688 [PMID: 17898713 DOI: 10.1038/nature06174]

81 **Yau WL**, Lam CS, Ng L, Chow AK, Chan ST, Chan JY, Wo JY, Ng KT, Man K, Poon RT, Pang RW. Over-expression of miR-106b promotes cell migration and metastasis in hepatocellular carcinoma by activating epithelial-mesenchymal transition process. *PLoS One* 2013; **8**: e57882 [PMID: 23483935 DOI: 10.1371/journal.pone.0057882]

82 **Dews M**, Homayouni A, Yu D, Murphy D, Sevignani C, Wentzel E, Furth EE, Lee WM, Enders GH, Mendell JT, Thomas-Tikhonenko A. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nat Genet* 2006; **38**: 1060-1065 [PMID: 16878133 DOI: 10.1038/ng1855]

83 **Lee DY**, Deng Z, Wang CH, Yang BB. MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. *Proc Natl Acad Sci USA* 2007; **104**: 20350-20355 [PMID: 18077375 DOI: 10.1073/pnas.0706901104]

84 **Linsley PS**, Schelter J, Burchard J, Kibukawa M, Martin MM, Bartz SR, Johnson JM, Cummins JM, Raymond CK, Dai H, Chau N, Cleary M, Jackson AL, Carleton M, Lim L. Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. *Mol Cell Biol* 2007; **27**: 2240-2252 [PMID: 17242205 DOI: 10.1128/MCB.02005-06]

85 **Furuta M**, Kozaki K, Tanimoto K, Tanaka S, Arii S, Shimamura T, Niida A, Miyano S, Inazawa J. The tumor-suppressive miR-497-195 cluster targets multiple cell-cycle regulators in hepatocellular carcinoma. *PLoS One* 2013; **8**: e60155 [PMID: 23544130 DOI: 10.1371/journal.pone.0060155]

86 **Luo Q**, Li X, Gao Y, Long Y, Chen L, Huang Y, Fang L. MiRNA-497 regulates cell growth and invasion by targeting cyclin E1 in breast cancer. *Cancer Cell Int* 2013; **13**: 95 [PMID: 24112607 DOI: 10.1186/1475-2867-13-95]

87 **Creevey L**, Ryan J, Harvey H, Bray IM, Meehan M, Khan AR, Stallings RL. MicroRNA-497 increases apoptosis in MYCN amplified neuroblastoma cells by targeting the key cell cycle regulator WEE1. *Mol Cancer* 2013; **12**: 23 [PMID: 23531080 DOI: 10.1186/1476-4598-12-23]

88 **Calin GA**, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* 2004; **101**: 2999-3004 [PMID: 14973191 DOI: 10.1073/pnas.0307323101]

89 **Mathelier A**, Carbone A. Large scale chromosomal mapping of human microRNA structural clusters. *Nucleic Acids Res* 2013; **41**: 4392-4408 [PMID: 23444140 DOI: 10.1093/nar/gkt112]

90 **Bartova E**, Galiova G, Legartova S, Stixova L, Jugova A, Kozubek S. Genome instability in the context of chromatin structure and fragile sites. *Crit Rev Eukaryot Gene Expr* 2010; **20**: 181-194 [PMID: 21175410]

91 **Cimmino A**, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc Natl Acad Sci USA* 2005; **102**: 13944-13949 [PMID: 16166262 DOI: 10.1073/pnas.0506654102]

92 **Mavrakis KJ**, Wolfe AL, Oricchio E, Palomero T, de Keersmaecker K, McJunkin K, Zuber J, James T, Khan AA, Leslie CS, Parker JS, Paddison PJ, Tam W, Ferrando A, Wendel HG. Genome-wide RNA-mediated interference screen identifies miR-19 targets in Notch-induced T-cell acute lymphoblastic leukaemia. *Nat Cell Biol* 2010; **12**: 372-379 [PMID: 20190740 DOI: 10.1038/ncb2037]

93 **Huse JT**, Brennan C, Hambardzumyan D, Wee B, Pena J, Rouhanifard SH, Sohn-Lee C, le Sage C, Agami R, Tuschl T, Holland EC. The PTEN-regulating microRNA miR-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. *Genes Dev* 2009; **23**: 1327-1337 [PMID: 19487573 DOI: 10.1101/gad.1777409]

94 **An J**, Pan Y, Yan Z, Li W, Cui J, Yuan J, Tian L, Xing R, Lu Y. MiR-23a in amplified 19p13.13 loci targets metallothionein 2A and promotes growth in gastric cancer cells. *J Cell Biochem* 2013; **114**: 2160-2169 [PMID: 23553990 DOI: 10.1002/jcb.24565]

95 **Linhares JJ**, Azevedo M, Siufi AA, de Carvalho CV, Wolgien Mdel C, Noronha EC, Bonetti TC, da Silva ID. Evaluation of single nucleotide polymorphisms in microRNAs (hsa-miR-196a2 rs11614913 C/T) from Brazilian women with breast

cancer. *BMC Med Genet* 2012; **13**: 119 [PMID: 23228090 DOI: 10.1186/1471-2350-13-119]
96 **Srivastava K**, Srivastava A. Comprehensive review of genetic association studies and meta-analyses on miRNA polymorphisms and cancer risk. *PLoS One* 2012; **7**: e50966 [PMID: 23226435 DOI: 10.1371/journal.pone.0050966]
97 **Wynendaele J**, Böhnke A, Leucci E, Nielsen SJ, Lambertz I, Hammer S, Sbrzesny N, Kubitza D, Wolf A, Gradhand E, Balschun K, Braicu I, Sehouli J, Darb-Esfahani S, Denkert C, Thomssen C, Hauptmann S, Lund A, Marine JC, Bartel F. An illegitimate microRNA target site within the 3' UTR of MDM4 affects ovarian cancer progression and chemosensitivity. *Cancer Res* 2010; **70**: 9641-9649 [PMID: 21084273 DOI: 10.1158/0008-5472.CAN-10-0527]
98 **Mishra PJ**, Mishra PJ, Banerjee D, Bertino JR. MiRSNPs or MiR-polymorphisms, new players in microRNA mediated regulation of the cell: Introducing microRNA pharmacogenomics. *Cell Cycle* 2008; **7**: 853-858 [PMID: 18414050]
99 **Preskill C**, Weidhaas JB. SNPs in microRNA binding sites as prognostic and predictive cancer biomarkers. *Crit Rev Oncog* 2013; **18**: 327-340 [PMID: 23614619]
100 **Emmrich S**, Pützer BM. Checks and balances: E2F-microRNA crosstalk in cancer control. *Cell Cycle* 2010; **9**: 2555-2567 [PMID: 20581444 DOI: 10.4161/cc.9.13.12061]
101 **Krol J**, Loedige I, Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* 2010; **11**: 597-610 [PMID: 20661255 DOI: 10.1038/nrg2843]
102 **Woods K**, Thomson JM, Hammond SM. Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. *J Biol Chem* 2007; **282**: 2130-2134 [PMID: 17135268 DOI: 10.1074/jbc.C600252200]
103 **Sylvestre Y**, De Guire V, Querido E, Mukhopadhyay UK, Bourdeau V, Major F, Ferbeyre G, Chartrand P. An E2F/miR-20a autoregulatory feedback loop. *J Biol Chem* 2007; **282**: 2135-2143 [PMID: 17135249 DOI: 10.1074/jbc.M608939200]
104 **Yang X**, Feng M, Jiang X, Wu Z, Li Z, Aau M, Yu Q. miR-449a and miR-449b are direct transcriptional targets of E2F1 and negatively regulate pRb-E2F1 activity through a feedback loop by targeting CDK6 and CDC25A. *Genes Dev* 2009; **23**: 2388-2393 [PMID: 19833767 DOI: 10.1101/gad.1819009]
105 **de Souza Rocha Simonini P**, Breiling A, Gupta N, Malekpour M, Youns M, Omranipour R, Malekpour F, Volinia S, Croce CM, Najmabadi H, Diederichs S, Sahin O, Mayer D, Lyko F, Hoheisel JD, Riazalhosseini Y. Epigenetically deregulated microRNA-375 is involved in a positive feedback loop with estrogen receptor alpha in breast cancer cells. *Cancer Res* 2010; **70**: 9175-9184 [PMID: 20978187 DOI: 10.1158/0008-5472.CAN-10-1318]
106 **Heng HH**, Bremer SW, Stevens JB, Ye KJ, Liu G, Ye CJ. Genetic and epigenetic heterogeneity in cancer: a genome-centric perspective. *J Cell Physiol* 2009; **220**: 538-547 [PMID: 19441078 DOI: 10.1002/jcp.21799]
107 **Weber B**, Stresemann C, Brueckner B, Lyko F. Methylation of human microRNA genes in normal and neoplastic cells. *Cell Cycle* 2007; **6**: 1001-1005 [PMID: 17457051]
108 **Saito Y**, Jones PA. Epigenetic activation of tumor suppressor microRNAs in human cancer cells. *Cell Cycle* 2006; **5**: 2220-2222 [PMID: 17012846]
109 **Liu ZY**, Zhang GL, Wang MM, Xiong YN, Cui HQ. MicroRNA-663 targets TGFB1 and regulates lung cancer proliferation. *Asian Pac J Cancer Prev* 2011; **12**: 2819-2823 [PMID: 22393947]
110 **Tili E**, Michaille JJ, Adair B, Alder H, Limagne E, Taccioli C, Ferracin M, Delmas D, Latruffe N, Croce CM. Resveratrol decreases the levels of miR-155 by upregulating miR-663, a microRNA targeting JunB and JunD. *Carcinogenesis* 2010; **31**: 1561-1566 [PMID: 20622002 DOI: 10.1093/carcin/bgq143]
111 **Vislovukh A**, Kratassiouk G, Porto E, Gralievska N, Beldiman C, Pinna G, El'skaya A, Harel-Bellan A, Negrutskii B, Groisman I. Proto-oncogenic isoform A2 of eukaryotic translation elongation factor eEF1 is a target of miR-663 and miR-744. *Br J Cancer* 2013; **108**: 2304-2311 [PMID: 23695020 DOI: 10.1038/bjc.2013.243]
112 **Lehmann U**, Hasemeier B, Christgen M, Müller M, Römermann D, Länger F, Kreipe H. Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer. *J Pathol* 2008; **214**: 17-24 [PMID: 17948228 DOI: 10.1002/path.2251]
113 **Potapova A**, Albat C, Hasemeier B, Haeussler K, Lamprecht S, Suerbaum S, Kreipe H, Lehmann U. Systematic cross-validation of 454 sequencing and pyrosequencing for the exact quantification of DNA methylation patterns with single CpG resolution. *BMC Biotechnol* 2011; **11**: 6 [PMID: 21235780 DOI: 10.1186/1472-6750-11-6]
114 **Yan-Fang T**, Jian N, Jun L, Na W, Pei-Fang X, Wen-Li Z, Dong W, Li P, Jian W, Xing F, Jian P. The promoter of miR-663 is hypermethylated in Chinese pediatric acute myeloid leukemia (AML). *BMC Med Genet* 2013; **14**: 74 [PMID: 23870168 DOI: 10.1186/1471-2350-14-74]
115 **Yang Y**, Wang LL, Li YH, Gao XN, Liu Y, Yu L. Effect of CpG island methylation on microRNA expression in the k-562 cell line. *Biochem Genet* 2012; **50**: 122-134 [PMID: 22089542 DOI: 10.1007/s10528-011-9478-9]
116 **Wong KY**, Yim RL, Kwong YL, Leung CY, Hui PK, Cheung F, Liang R, Jin DY, Chim CS. Epigenetic inactivation of the MIR129-2 in hematological malignancies. *J Hematol Oncol* 2013; **6**: 16 [PMID: 23406679 DOI: 10.1186/1756-8722-6-16]
117 **Li Z**, Qi CF, Shin DM, Zingone A, Newbery HJ, Kovalchuk AL, Abbott CM, Morse HC. Eef1a2 promotes cell growth, inhibits apoptosis and activates JAK/STAT and AKT signaling in mouse plasmacytomas. *PLoS One* 2010; **5**: e10755 [PMID: 20505761 DOI: 10.1371/journal.pone.0010755]
118 **The Cancer Genome Atlas Research Network**. Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature* 2013; **499**: 43-49 [PMID: 23792563 DOI: 10.1038/nature12222]
119 **Poliseno L**, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* 2010; **465**: 1033-1038 [PMID: 20577206 DOI: 10.1038/nature09144]
120 **Salmena L**, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? *Cell* 2011; **146**: 353-358 [PMID: 21802130 DOI: 10.1016/j.cell.2011.07.014]
121 **Hansen TB**, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. *Nature* 2013; **495**: 384-388 [PMID: 23446346 DOI: 10.1038/nature11993]
122 **Okuda H**, Xing F, Pandey PR, Sharma S, Watabe M, Pai SK, Mo YY, Iiizumi-Gairani M, Hirota S, Liu Y, Wu K, Pochampally R, Watabe K. miR-7 suppresses brain metastasis of breast cancer stem-like cells by modulating KLF4. *Cancer Res* 2013; **73**: 1434-1444 [PMID: 23384942 DOI: 10.1158/0008-5472.CAN-12-2037]
123 **Xu L**, Wen Z, Zhou Y, Liu Z, Li Q, Fei G, Luo J, Ren T. MicroRNA-7-regulated TLR9 signaling-enhanced growth and metastatic potential of human lung cancer cells by altering the phosphoinositide-3-kinase, regulatory subunit 3/Akt pathway. *Mol Biol Cell* 2013; **24**: 42-55 [PMID: 23135998 DOI: 10.1091/mbc.E12-07-0519]
124 **McGrew LL**, Dworkin-Rastl E, Dworkin MB, Richter JD. Poly(A) elongation during Xenopus oocyte maturation is required for translational recruitment and is mediated by a short sequence element. *Genes Dev* 1989; **3**: 803-815 [PMID: 2568313]
125 **Paris J**, Richter JD. Maturation-specific polyadenylation and translational control: diversity of cytoplasmic polyadenylation elements, influence of poly(A) tail size, and formation of stable polyadenylation complexes. *Mol Cell Biol* 1990; **10**: 5634-5645 [PMID: 1700272]
126 **Stebbins-Boaz B**, Hake LE, Richter JD. CPEB controls the

cytoplasmic polyadenylation of cyclin, Cdk2 and c-mos mRNAs and is necessary for oocyte maturation in Xenopus. *EMBO J* 1996; **15**: 2582-2592 [PMID: 8665866]
127 **Hake LE**, Richter JD. CPEB is a specificity factor that mediates cytoplasmic polyadenylation during Xenopus oocyte maturation. *Cell* 1994; **79**: 617-627 [PMID: 7954828]
128 **Alarcon JM**, Hodgman R, Theis M, Huang YS, Kandel ER, Richter JD. Selective modulation of some forms of schaffer collateral-CA1 synaptic plasticity in mice with a disruption of the CPEB-1 gene. *Learn Mem* 2004; **11**: 318-327 [PMID: 15169862 DOI: 10.1101/lm.72704]
129 **Costa-Mattioli M**, Sonenberg N, Richter JD. Translational regulatory mechanisms in synaptic plasticity and memory storage. *Prog Mol Biol Transl Sci* 2009; **90**: 293-311 [PMID: 20374745 DOI: 10.1016/S1877-1173(09)90008-4]
130 **Groisman I**, Huang YS, Mendez R, Cao Q, Richter JD. Translational control of embryonic cell division by CPEB and maskin. *Cold Spring Harb Symp Quant Biol* 2001; **66**: 345-351 [PMID: 12762037]
131 **Groisman I**, Ivshina M, Marin V, Kennedy NJ, Davis RJ, Richter JD. Control of cellular senescence by CPEB. *Genes Dev* 2006; **20**: 2701-2712 [PMID: 17015432 DOI: 10.1101/gad.1438906]
132 **Burns DM**, Richter JD. CPEB regulation of human cellular senescence, energy metabolism, and p53 mRNA translation. *Genes Dev* 2008; **22**: 3449-3460 [PMID: 19141477 DOI: 10.1101/gad.1697808]
133 **Novoa I**, Gallego J, Ferreira PG, Mendez R. Mitotic cell-cycle progression is regulated by CPEB1 and CPEB4-dependent translational control. *Nat Cell Biol* 2010; **12**: 447-456 [PMID: 20364142 DOI: 10.1038/ncb2046]
134 **Rouhana L**, Wang L, Buter N, Kwak JE, Schiltz CA, Gonzalez T, Kelley AE, Landry CF, Wickens M. Vertebrate GLD2 poly(A) polymerases in the germline and the brain. *RNA* 2005; **11**: 1117-1130 [PMID: 15987818 DOI: 10.1261/rna.2630205]
135 **Barnard DC**, Ryan K, Manley JL, Richter JD. Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. *Cell* 2004; **119**: 641-651 [PMID: 15550246 DOI: 10.1016/j.cell.2004.10.029]
136 **Mendez R**, Hake LE, Andresson T, Littlepage LE, Ruderman JV, Richter JD. Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. *Nature* 2000; **404**: 302-307 [PMID: 10749216 DOI: 10.1038/35005126]
137 **Sarkissian M**, Mendez R, Richter JD. Progesterone and insulin stimulation of CPEB-dependent polyadenylation is regulated by Aurora A and glycogen synthase kinase-3. *Genes Dev* 2004; **18**: 48-61 [PMID: 14724178 DOI: 10.1101/gad.1136004]
138 **Kwak JE**, Wang L, Ballantyne S, Kimble J, Wickens M. Mammalian GLD-2 homologs are poly(A) polymerases. *Proc Natl Acad Sci USA* 2004; **101**: 4407-4412 [PMID: 15070731 DOI: 10.1073/pnas.0400779101]
139 **Kahvejian A**, Svitkin YV, Sukarieh R, M'Boutchou MN, Sonenberg N. Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. *Genes Dev* 2005; **19**: 104-113 [PMID: 15630022 DOI: 10.1101/gad.1262905]
140 **Stöhr N**, Köhn M, Lederer M, Glass M, Reinke C, Singer RH, Hüttelmaier S. IGF2BP1 promotes cell migration by regulating MK5 and PTEN signaling. *Genes Dev* 2012; **26**: 176-189 [PMID: 22279049 DOI: 10.1101/gad.177642.111]
141 **Liao B**, Hu Y, Herrick DJ, Brewer G. The RNA-binding protein IMP-3 is a translational activator of insulin-like growth factor II leader-3 mRNA during proliferation of human K562 leukemia cells. *J Biol Chem* 2005; **280**: 18517-18524 [PMID: 15753088 DOI: 10.1074/jbc.M500270200]
142 **Bell JL**, Wächter K, Mühleck B, Pazaitis N, Köhn M, Lederer M, Hüttelmaier S. Insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs): post-transcriptional drivers of cancer progression? *Cell Mol Life Sci* 2013; **70**: 2657-2675 [PMID: 23069990 DOI: 10.1007/s00018-012-1186-z]
143 **Boudoukha S**, Cuvellier S, Polesskaya A. Role of the RNA-binding protein IMP-2 in muscle cell motility. *Mol Cell Biol* 2010; **30**: 5710-5725 [PMID: 20956565 DOI: 10.1128/MCB.00665-10]
144 **Oleynikov Y**, Singer RH. Real-time visualization of ZBP1 association with beta-actin mRNA during transcription and localization. *Curr Biol* 2003; **13**: 199-207 [PMID: 12573215]
145 **Hüttelmaier S**, Zenklusen D, Lederer M, Dictenberg J, Lorenz M, Meng X, Bassell GJ, Condeelis J, Singer RH. Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. *Nature* 2005; **438**: 512-515 [PMID: 16306994 DOI: 10.1038/nature04115]
146 **Pan F**, Hüttelmaier S, Singer RH, Gu W. ZBP2 facilitates binding of ZBP1 to beta-actin mRNA during transcription. *Mol Cell Biol* 2007; **27**: 8340-8351 [PMID: 17893325 DOI: 10.1128/MCB.00972-07]
147 **Srikantan S**, Tominaga K, Gorospe M. Functional interplay between RNA-binding protein HuR and microRNAs. *Curr Protein Pept Sci* 2012; **13**: 372-379 [PMID: 22708488]
148 **Abdelmohsen K**, Gorospe M. Posttranscriptional regulation of cancer traits by HuR. *Wiley Interdiscip Rev RNA* 2010; **1**: 214-229 [PMID: 21935886 DOI: 10.1002/wrna.4]
149 **López de Silanes I**, Lal A, Gorospe M. HuR: post-transcriptional paths to malignancy. *RNA Biol* 2005; **2**: 11-13 [PMID: 17132932]
150 **Abdelmohsen K**, Srikantan S, Kuwano Y, Gorospe M. miR-519 reduces cell proliferation by lowering RNA-binding protein HuR levels. *Proc Natl Acad Sci USA* 2008; **105**: 20297-20302 [PMID: 19088191 DOI: 10.1073/pnas.0809376106]
151 **Hinman MN**, Lou H. Diverse molecular functions of Hu proteins. *Cell Mol Life Sci* 2008; **65**: 3168-3181 [PMID: 18581050 DOI: 10.1007/s00018-008-8252-6]
152 **Gratacós FM**, Brewer G. The role of AUF1 in regulated mRNA decay. *Wiley Interdiscip Rev RNA* 2010; **1**: 457-473 [PMID: 21956942 DOI: 10.1002/wrna.26]
153 **Chen CY**, Gherzi R, Ong SE, Chan EL, Raijmakers R, Pruijn GJ, Stoecklin G, Moroni C, Mann M, Karin M. AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* 2001; **107**: 451-464 [PMID: 11719186]
154 **Raineri I**, Wegmueller D, Gross B, Certa U, Moroni C. Roles of AUF1 isoforms, HuR and BRF1 in ARE-dependent mRNA turnover studied by RNA interference. *Nucleic Acids Res* 2004; **32**: 1279-1288 [PMID: 14976220 DOI: 10.1093/nar/gkh282]
155 **Vázquez-Chantada M**, Fernández-Ramos D, Embade N, Martínez-Lopez N, Varela-Rey M, Woodhoo A, Luka Z, Wagner C, Anglim PP, Finnell RH, Caballería J, Lairde-Offringa IA, Gorospe M, Lu SC, Mato JM, Martínez-Chantar ML. HuR/methyl-HuR and AUF1 regulate the MAT expressed during liver proliferation, differentiation, and carcinogenesis. *Gastroenterology* 2010; **138**: 1943-1953 [PMID: 20102719 DOI: 10.1053/j.gastro.2010.01.032]
156 **Kim HS**, Headey SJ, Yoga YM, Scanlon MJ, Gorospe M, Wilce MC, Wilce JA. Distinct binding properties of TIAR RRMs and linker region. *RNA Biol* 2013; **10**: 579-589 [PMID: 23603827 DOI: 10.4161/rna.24341]
157 **Gilks N**, Kedersha N, Ayodele M, Shen L, Stoecklin G, Dember LM, Anderson P. Stress granule assembly is mediated by prion-like aggregation of TIA-1. *Mol Biol Cell* 2004; **15**: 5383-5398 [PMID: 15371533 DOI: 10.1091/mbc.E04-08-0715]
158 **Kuwano Y**, Pullmann R, Marasa BS, Abdelmohsen K, Lee EK, Yang X, Martindale JL, Zhan M, Gorospe M. NF90 selectively represses the translation of target mRNAs bearing an AU-rich signature motif. *Nucleic Acids Res* 2010; **38**: 225-238 [PMID: 19850717 DOI: 10.1093/nar/gkp861]
159 **Ogilvie RL**, Abelson M, Hau HH, Vlasova I, Blackshear PJ, Bohjanen PR. Tristetraprolin down-regulates IL-2 gene ex-

pression through AU-rich element-mediated mRNA decay. *J Immunol* 2005; **174**: 953-961 [PMID: 15634918]
160 **Stoecklin G**, Tenenbaum SA, Mayo T, Chittur SV, George AD, Baroni TE, Blackshear PJ, Anderson P. Genome-wide analysis identifies interleukin-10 mRNA as target of tristetraprolin. *J Biol Chem* 2008; **283**: 11689-11699 [PMID: 18256032 DOI: 10.1074/jbc.M709657200]
161 **Briata P**, Chen CY, Giovarelli M, Pasero M, Trabucchi M, Ramos A, Gherzi R. KSRP, many functions for a single protein. *Front Biosci* (Landmark Ed) 2011; **16**: 1787-1796 [PMID: 21196264]
162 **Suswam EA**, Nabors LB, Huang Y, Yang X, King PH. IL-1beta induces stabilization of IL-8 mRNA in malignant breast cancer cells via the 3' untranslated region: Involvement of divergent RNA-binding factors HuR, KSRP and TIAR. *Int J Cancer* 2005; **113**: 911-919 [PMID: 15514971 DOI: 10.1002/ijc.20675]
163 **Ishimaru D**, Zuraw L, Ramalingam S, Sengupta TK, Bandyopadhyay S, Reuben A, Fernandes DJ, Spicer EK. Mechanism of regulation of bcl-2 mRNA by nucleolin and A+U-rich element-binding factor 1 (AUF1). *J Biol Chem* 2010; **285**: 27182-27191 [PMID: 20571027 DOI: 10.1074/jbc.M109.098830]
164 **Lee JH**, Jeon MH, Seo YJ, Lee YJ, Ko JH, Tsujimoto Y, Lee JH. CA repeats in the 3'-untranslated region of bcl-2 mRNA mediate constitutive decay of bcl-2 mRNA. *J Biol Chem* 2004; **279**: 42758-42764 [PMID: 15294893 DOI: 10.1074/jbc.M407357200]
165 **Lossi L**, Gambino G, Ferrini F, Alasia S, Merighi A. Post-translational regulation of BCL2 levels in cerebellar granule cells: A mechanism of neuronal survival. *Dev Neurobiol* 2009; **69**: 855-870 [PMID: 19672954 DOI: 10.1002/dneu.20744]
166 **Ishimaru D**, Ramalingam S, Sengupta TK, Bandyopadhyay S, Dellis S, Tholanikunnel BG, Fernandes DJ, Spicer EK. Regulation of Bcl-2 expression by HuR in HL60 leukemia cells and A431 carcinoma cells. *Mol Cancer Res* 2009; **7**: 1354-1366 [PMID: 19671677 DOI: 10.1158/1541-7786.MCR-08-0476]
167 **Lal A**, Abdelmohsen K, Pullmann R, Kawai T, Galban S, Yang X, Brewer G, Gorospe M. Posttranscriptional derepression of GADD45alpha by genotoxic stress. *Mol Cell* 2006; **22**: 117-128 [PMID: 16600875 DOI: 10.1016/j.molcel.2006.03.016]
168 **Lal A**, Mazan-Mamczarz K, Kawai T, Yang X, Martindale JL, Gorospe M. Concurrent versus individual binding of HuR and AUF1 to common labile target mRNAs. *EMBO J* 2004; **23**: 3092-3102 [PMID: 15257295]
169 **Campbell ZT**, Menichelli E, Friend K, Wu J, Kimble J, Williamson JR, Wickens M. Identification of a conserved interface between PUF and CPEB proteins. *J Biol Chem* 2012; **287**: 18854-18862 [PMID: 22496444 DOI: 10.1074/jbc.M112.352815]
170 **Budkevich TV**, Timchenko AA, Tiktopulo EI, Negrutskii BS, Shalak VF, Petrushenko ZM, Aksenov VL, Willumeit R, Kohlbrecher J, Serdyuk IN, El'skaya AV. Extended conformation of mammalian translation elongation factor 1A in solution. *Biochemistry* 2002; **41**: 15342-15349 [PMID: 12484773]
171 **Kinzy TG**, Merrick WC. Characterization of a limited trypsin digestion form of eukaryotic elongation factor 1 alpha. *J Biol Chem* 1991; **266**: 4099-4105 [PMID: 1999404]
172 **Petrushenko ZM**, Negrutskii BS, Ladokhin AS, Budkevich TV, Shalak VF, El'skaya AV. Evidence for the formation of an unusual ternary complex of rabbit liver EF-1alpha with GDP and deacylated tRNA. *FEBS Lett* 1997; **407**: 13-17 [PMID: 9141472]
173 **Hussey GS**, Chaudhury A, Dawson AE, Lindner DJ, Knudsen CR, Wilce MC, Merrick WC, Howe PH. Identification of an mRNP complex regulating tumorigenesis at the translational elongation step. *Mol Cell* 2011; **41**: 419-431 [PMID: 21329880 DOI: 10.1016/j.molcel.2011.02.003]
174 **Friend K**, Campbell ZT, Cooke A, Kroll-Conner P, Wickens MP, Kimble J. A conserved PUF-Ago-eEF1A complex attenuates translation elongation. *Nat Struct Mol Biol* 2012; **19**: 176-183 [PMID: 22231398 DOI: 10.1038/nsmb.2214]
175 **Bhattacharyya SN**, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* 2006; **125**: 1111-1124 [PMID: 16777601 DOI: 10.1016/j.cell.2006.04.031]
176 **Kedde M**, van Kouwenhove M, Zwart W, Oude Vrielink JA, Elkon R, Agami R. A Pumilio-induced RNA structure switch in p27-3' UTR controls miR-221 and miR-222 accessibility. *Nat Cell Biol* 2010; **12**: 1014-1020 [PMID: 20818387 DOI: 10.1038/ncb2105]
177 **Miles WO**, Tschöp K, Herr A, Ji JY, Dyson NJ. Pumilio facilitates miRNA regulation of the E2F3 oncogene. *Genes Dev* 2012; **26**: 356-368 [PMID: 22345517 DOI: 10.1101/gad.182568.111]
178 **Kedde M**, Strasser MJ, Boldajipour B, Oude Vrielink JA, Slanchev K, le Sage C, Nagel R, Voorhoeve PM, van Duijse J, Ørom UA, Lund AH, Perrakis A, Raz E, Agami R. RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell* 2007; **131**: 1273-1286 [PMID: 18155131 DOI: 10.1016/j.cell.2007.11.034]
179 **Bhandari A**, Gordon W, Dizon D, Hopkin AS, Gordon E, Yu Z, Andersen B. The Grainyhead transcription factor Grhl3/Get1 suppresses miR-21 expression and tumorigenesis in skin: modulation of the miR-21 target MSH2 by RNA-binding protein DND1. *Oncogene* 2013; **32**: 1497-1507 [PMID: 22614019 DOI: 10.1038/onc.2012.168]
180 **Tominaga K**, Srikantan S, Lee EK, Subaran SS, Martindale JL, Abdelmohsen K, Gorospe M. Competitive regulation of nucleolin expression by HuR and miR-494. *Mol Cell Biol* 2011; **31**: 4219-4231 [PMID: 21859890 DOI: 10.1128/MCB.05955-11]
181 **Srikantan S**, Abdelmohsen K, Lee EK, Tominaga K, Subaran SS, Kuwano Y, Kulshrestha R, Panchakshari R, Kim HH, Yang X, Martindale JL, Marasa BS, Kim MM, Wersto RP, Indig FE, Chowdhury D, Gorospe M. Translational control of TOP2A influences doxorubicin efficacy. *Mol Cell Biol* 2011; **31**: 3790-3801 [PMID: 21768308 DOI: 10.1128/MCB.05639-11]
182 **Köbel M**, Weidensdorfer D, Reinke C, Lederer M, Schmitt WD, Zeng K, Thomssen C, Hauptmann S, Hüttelmaier S. Expression of the RNA-binding protein IMP1 correlates with poor prognosis in ovarian carcinoma. *Oncogene* 2007; **26**: 7584-7589 [PMID: 17546046 DOI: 10.1038/sj.onc.1210563]
183 **Elcheva I**, Goswami S, Noubissi FK, Spiegelman VS. CRD-BP protects the coding region of betaTrCP1 mRNA from miR-183-mediated degradation. *Mol Cell* 2009; **35**: 240-246 [PMID: 19647520 DOI: 10.1016/j.molcel.2009.06.007]
184 **Mongroo PS**, Noubissi FK, Cuatrecasas M, Kalabis J, King CE, Johnstone CN, Bowser MJ, Castells A, Spiegelman VS, Rustgi AK. IMP-1 displays cross-talk with K-Ras and modulates colon cancer cell survival through the novel proapoptotic protein CYFIP2. *Cancer Res* 2011; **71**: 2172-2182 [PMID: 21252116 DOI: 10.1158/0008-5472.CAN-10-3295]
185 **Jing Q**, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Di Padova F, Lin SC, Gram H, Han J. Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* 2005; **120**: 623-634 [PMID: 15766526 DOI: 10.1016/j.cell.2004.12.038]
186 **Cohn DE**, Fabbri M, Valeri N, Alder H, Ivanov I, Liu CG, Croce CM, Resnick KE. Comprehensive miRNA profiling of surgically staged endometrial cancer. *Am J Obstet Gynecol* 2010; **202**: 656.e1-656.e8 [PMID: 20400061 DOI: 10.1016/j.ajog.2010.02.051]
187 **Cosmopoulos K**, Pegtel M, Hawkins J, Moffett H, Novina C, Middeldorp J, Thorley-Lawson DA. Comprehensive profiling of Epstein-Barr virus microRNAs in nasopharyngeal carcinoma. *J Virol* 2009; **83**: 2357-2367 [PMID: 19091858 DOI: 10.1128/JVI.02104-08]
188 **Patnaik SK**, Kannisto E, Knudsen S, Yendamuri S. Evalu-

ation of microRNA expression profiles that may predict recurrence of localized stage I non-small cell lung cancer after surgical resection. *Cancer Res* 2010; **70**: 36-45 [PMID: 20028859 DOI: 10.1158/0008-5472.CAN-09-3153]
189 **Schepeler T**, Reinert JT, Ostenfeld MS, Christensen LL, Silahtaroglu AN, Dyrskjøt L, Wiuf C, Sørensen FJ, Kruhøffer M, Laurberg S, Kauppinen S, Ørntoft TF, Andersen CL. Diagnostic and prognostic microRNAs in stage II colon cancer. *Cancer Res* 2008; **68**: 6416-6424 [PMID: 18676867 DOI: 10.1158/0008-5472.CAN-07-6110]
190 **Katada T**, Ishiguro H, Kuwabara Y, Kimura M, Mitui A, Mori Y, Ogawa R, Harata K, Fujii Y. microRNA expression profile in undifferentiated gastric cancer. *Int J Oncol* 2009; **34**: 537-542 [PMID: 19148490]
191 **Varnholt H**, Drebber U, Schulze F, Wedemeyer I, Schirmacher P, Dienes HP, Odenthal M. MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. *Hepatology* 2008; **47**: 1223-1232 [PMID: 18307259 DOI: 10.1002/hep.22158]
192 **Yan LX**, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, Zeng YX, Shao JY. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA* 2008; **14**: 2348-2360 [PMID: 18812439 DOI: 10.1261/rna.1034808]
193 **Teplyuk NM**, Mollenhauer B, Gabriely G, Giese A, Kim E, Smolsky M, Kim RY, Saria MG, Pastorino S, Kesari S, Krichevsky AM. MicroRNAs in cerebrospinal fluid identify glioblastoma and metastatic brain cancers and reflect disease activity. *Neuro Oncol* 2012; **14**: 689-700 [PMID: 22492962 DOI: 10.1093/neuonc/nos074]
194 **Lee YS**, Dutta A. MicroRNAs in cancer. *Annu Rev Pathol* 2009; **4**: 199-227 [PMID: 18817506 DOI: 10.1146/annurev.pathol.4.110807.092222]
195 **Yang R**, Schlehe B, Hemminki K, Sutter C, Bugert P, Wappenschmidt B, Volkmann J, Varon R, Weber BH, Niederacher D, Arnold N, Meindl A, Bartram CR, Schmutzler RK, Burwinkel B. A genetic variant in the pre-miR-27a oncogene is associated with a reduced familial breast cancer risk. *Breast Cancer Res Treat* 2010; **121**: 693-702 [PMID: 19921425 DOI: 10.1007/s10549-009-0633-5]
196 **Lowery AJ**, Miller N, Devaney A, McNeill RE, Davoren PA, Lemetre C, Benes V, Schmidt S, Blake J, Ball G, Kerin MJ. MicroRNA signatures predict oestrogen receptor, progesterone receptor and HER2/neu receptor status in breast cancer. *Breast Cancer Res* 2009; **11**: R27 [PMID: 19432961 DOI: 10.1186/bcr2257]
197 **Weidhaas JB**, Babar I, Nallur SM, Trang P, Roush S, Boehm M, Gillespie E, Slack FJ. MicroRNAs as potential agents to alter resistance to cytotoxic anticancer therapy. *Cancer Res* 2007; **67**: 11111-11116 [PMID: 18056433 DOI: 10.1158/0008-5472.CAN-07-2858]
198 **Chen G**, Zhu W, Shi D, Lv L, Zhang C, Liu P, Hu W. MicroRNA-181a sensitizes human malignant glioma U87MG cells to radiation by targeting Bcl-2. *Oncol Rep* 2010; **23**: 997-1003 [PMID: 20204284]
199 **Bandres E**, Bitarte N, Arias F, Agorreta J, Fortes P, Agirre X, Zarate R, Diaz-Gonzalez JA, Ramirez N, Sola JJ, Jimenez P, Rodriguez J, Garcia-Foncillas J. microRNA-451 regulates macrophage migration inhibitory factor production and proliferation of gastrointestinal cancer cells. *Clin Cancer Res* 2009; **15**: 2281-2290 [PMID: 19318487 DOI: 10.1158/1078-0432.CCR-08-1818]
200 **Corsten MF**, Miranda R, Kasmieh R, Krichevsky AM, Weissleder R, Shah K. MicroRNA-21 knockdown disrupts glioma growth in vivo and displays synergistic cytotoxicity with neural precursor cell delivered S-TRAIL in human gliomas. *Cancer Res* 2007; **67**: 8994-9000 [PMID: 17908999 DOI: 10.1158/0008-5472.CAN-07-1045]
201 **Li Y**, Li W, Yang Y, Lu Y, He C, Hu G, Liu H, Chen J, He J, Yu H. MicroRNA-21 targets LRRFIP1 and contributes to VM-26 resistance in glioblastoma multiforme. *Brain Res* 2009; **1286**: 13-18 [PMID: 19559015 DOI: 10.1016/j.brainres.2009.06.053]
202 **Ren Y**, Zhou X, Mei M, Yuan XB, Han L, Wang GX, Jia ZF, Xu P, Pu PY, Kang CS. MicroRNA-21 inhibitor sensitizes human glioblastoma cells U251 (PTEN-mutant) and LN229 (PTEN-wild type) to taxol. *BMC Cancer* 2010; **10**: 27 [PMID: 20113523 DOI: 10.1186/1471-2407-10-27]
203 **Arnetoli M**, Vooijs R, ten Bookum W, Galardi F, Gonnelli C, Gabbrielli R, Schat H, Verkleij JA. Arsenate tolerance in Silene paradoxa does not rely on phytochelatin-dependent sequestration. *Environ Pollut* 2008; **152**: 585-591 [PMID: 17707110 DOI: 10.1016/j.envpol.2007.07.002]
204 **Bai S**, Nasser MW, Wang B, Hsu SH, Datta J, Kutay H, Yadav A, Nuovo G, Kumar P, Ghoshal K. MicroRNA-122 inhibits tumorigenic properties of hepatocellular carcinoma cells and sensitizes these cells to sorafenib. *J Biol Chem* 2009; **284**: 32015-32027 [PMID: 19726678 DOI: 10.1074/jbc.M109.016774]
205 **Borralho PM**, Kren BT, Castro RE, da Silva IB, Steer CJ, Rodrigues CM. MicroRNA-143 reduces viability and increases sensitivity to 5-fluorouracil in HCT116 human colorectal cancer cells. *FEBS J* 2009; **276**: 6689-6700 [PMID: 19843160 DOI: 10.1111/j.1742-4658.2009.07383.x]
206 **Svoboda M**, Izakovicova Holla L, Sefr R, Vrtkova I, Kocakova I, Tichy B, Dvorak J. Micro-RNAs miR125b and miR137 are frequently upregulated in response to capecitabine chemoradiotherapy of rectal cancer. *Int J Oncol* 2008; **33**: 541-547 [PMID: 18695884]
207 **To KK**, Robey RW, Knutsen T, Zhan Z, Ried T, Bates SE. Escape from hsa-miR-519c enables drug-resistant cells to maintain high expression of ABCG2. *Mol Cancer Ther* 2009; **8**: 2959-2968 [PMID: 19825807 DOI: 10.1158/1535-7163.MCT-09-0292]
208 **Bijsterbosch MK**, Rump ET, De Vrueh RL, Dorland R, van Veghel R, Tivel KL, Biessen EA, van Berkel TJ, Manoharan M. Modulation of plasma protein binding and in vivo liver cell uptake of phosphorothioate oligodeoxynucleotides by cholesterol conjugation. *Nucleic Acids Res* 2000; **28**: 2717-2725 [PMID: 10908328]
209 **Hebert C**, Norris K, Scheper MA, Nikitakis N, Sauk JJ. High mobility group A2 is a target for miRNA-98 in head and neck squamous cell carcinoma. *Mol Cancer* 2007; **6**: 5 [PMID: 17222355 DOI: 10.1186/1476-4598-6-5]
210 **Obad S**, dos Santos CO, Petri A, Heidenblad M, Broom O, Ruse C, Fu C, Lindow M, Stenvang J, Straarup EM, Hansen HF, Koch T, Pappin D, Hannon GJ, Kauppinen S. Silencing of microRNA families by seed-targeting tiny LNAs. *Nat Genet* 2011; **43**: 371-378 [PMID: 21423181 DOI: 10.1038/ng.786]
211 **Trang P**, Wiggins JF, Daige CL, Cho C, Omotola M, Brown D, Weidhaas JB, Bader AG, Slack FJ. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Mol Ther* 2011; **19**: 1116-1122 [PMID: 21427705 DOI: 10.1038/mt.2011.48]
212 **Babar IA**, Cheng CJ, Booth CJ, Liang X, Weidhaas JB, Saltzman WM, Slack FJ. Nanoparticle-based therapy in an in vivo microRNA-155 (miR-155)-dependent mouse model of lymphoma. *Proc Natl Acad Sci U S A* 2012; **109**: E1695-E1704 [PMID: 22685206 DOI: 10.1073/pnas.1201516109]
213 **Wu DW**, Cheng YW, Wang J, Chen CY, Lee H. Paxillin predicts survival and relapse in non-small cell lung cancer by microRNA-218 targeting. *Cancer Res* 2010; **70**: 10392-10401 [PMID: 21159652 DOI: 10.1158/0008-5472.CAN-10-2341]
214 **Elmén J**, Lindow M, Schütz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedtjärn M, Hansen HF, Berger U, Gullans S, Kearney P, Sarnow P, Straarup EM, Kauppinen S. LNA-mediated microRNA silencing in non-human primates. *Nature* 2008; **452**: 896-899 [PMID: 18368051 DOI: 10.1038/nature06783]

215 **Elmén J**, Lindow M, Silahtaroglu A, Bak M, Christensen M, Lind-Thomsen A, Hedtjärn M, Hansen JB, Hansen HF, Straarup EM, McCullagh K, Kearney P, Kauppinen S. Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic Acids Res* 2008; **36**: 1153-1162 [PMID: 18158304 DOI: 10.1093/nar/gkm1113]

216 **Lanford RE**, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, Kauppinen S, Ørum H. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 2010; **327**: 198-201 [PMID: 19965718 DOI: 10.1126/science.1178178]

217 **Kapoor S**. IMP3: a new and important biomarker of systemic malignancies. *Clin Cancer Res* 2008; **14**: 5640; author reply 5640-5641 [PMID: 18765560 DOI: 10.1158/1078-0432.CCR-08-0813]

218 **Findeis-Hosey JJ**, Xu H. The use of insulin like-growth factor II messenger RNA binding protein-3 in diagnostic pathology. *Hum Pathol* 2011; **42**: 303-314 [PMID: 20970161 DOI: 10.1016/j.humpath.2010.06.003]

219 **Suvasini R**, Shruti B, Thota B, Shinde SV, Friedmann-Morvinski D, Nawaz Z, Prasanna KV, Thennarasu K, Hegde AS, Arivazhagan A, Chandramouli BA, Santosh V, Somasundaram K. Insulin growth factor-2 binding protein 3 (IGF2BP3) is a glioblastoma-specific marker that activates phosphatidylinositol 3-kinase/mitogen-activated protein kinase (PI3K/MAPK) pathways by modulating IGF-2. *J Biol Chem* 2011; **286**: 25882-25890 [PMID: 21613208 DOI: 10.1074/jbc.M110.178012]

220 **Liao B**, Hu Y, Brewer G. RNA-binding protein insulin-like growth factor mRNA-binding protein 3 (IMP-3) promotes cell survival via insulin-like growth factor II signaling after ionizing radiation. *J Biol Chem* 2011; **286**: 31145-31152 [PMID: 21757716 DOI: 10.1074/jbc.M111.263913]

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REVIEW

Roles and mechanisms of the CD38/cyclic adenosine diphosphate ribose/Ca^{2+} signaling pathway

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Abstract

Mobilization of intracellular Ca^{2+} stores is involved in many diverse cell functions, including: cell proliferation; differentiation; fertilization; muscle contraction; secretion of neurotransmitters, hormones and enzymes; and lymphocyte activation and proliferation. Cyclic adenosine diphosphate ribose (cADPR) is an endogenous Ca^{2+} mobilizing nucleotide present in many cell types and species, from plants to animals. cADPR is formed by ADP-ribosyl cyclases from nicotinamide adenine dinucleotide. The main ADP-ribosyl cyclase in mammals is CD38, a multi-functional enzyme and a type Ⅱ membrane protein. It has been shown that many extracellular stimuli can induce cADPR production that leads to calcium release or influx, establishing cADPR as a second messenger. cADPR has been linked to a wide variety of cellular processes, but the molecular mechanisms regarding cADPR signaling remain elusive. The aim of this review is to summarize the CD38/cADPR/Ca^{2+} signaling pathway, focusing on the recent advances involving the mechanism and physiological functions of cADPR-mediated Ca^{2+} mobilization.

Key words: Cyclic adenosine diphosphate ribose; CD38; Ca^{2+}; Ryanodine receptors; Nicotinamide adenine dinucleotide

Core tip: This is a comprehensive review regarding the role and mechanism of the CD38/Cyclic adenosine diphosphate ribose (cADPR)/Ca^{2+} signaling pathway in various cellular processes. We introduce the structure and function of cADPR, together with its production and degradation pathways. We also describe CD38, the main enzyme that is responsible for synthesis of cADPR, through its structure and topology. Finally, we summarize the functions of the CD38/cADPR/Ca^{2+} signaling pathway under both physiological and pathological conditions.

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INTRODUCTION

Discovered more than two decades ago, cyclic adenosine diphosphate ribose (cADPR) has been established as a second messenger, according to criteria first proposed by Sutherland and co-workers[1]. Together with inositol 1,4,5-trisphosphate (IP_3) and nicotinic acid adenine dinucleotide phosphate (NAADP), cADPR has been recognized as a principal second messenger involved in cellular Ca^{2+} mobilization. Extracellular stimuli can induce cADPR production, which leads to Ca^{2+} mobilization from intracellular stores as well as Ca^{2+} entry from the extracellular compartment to initiate diverse cellular responses. cADPR is synthesized by ADP-ribosyl cyclases and the major ADP-ribosyl cyclase in mammals is CD38 (Figure 1). In this review, we will first introduce the structure and

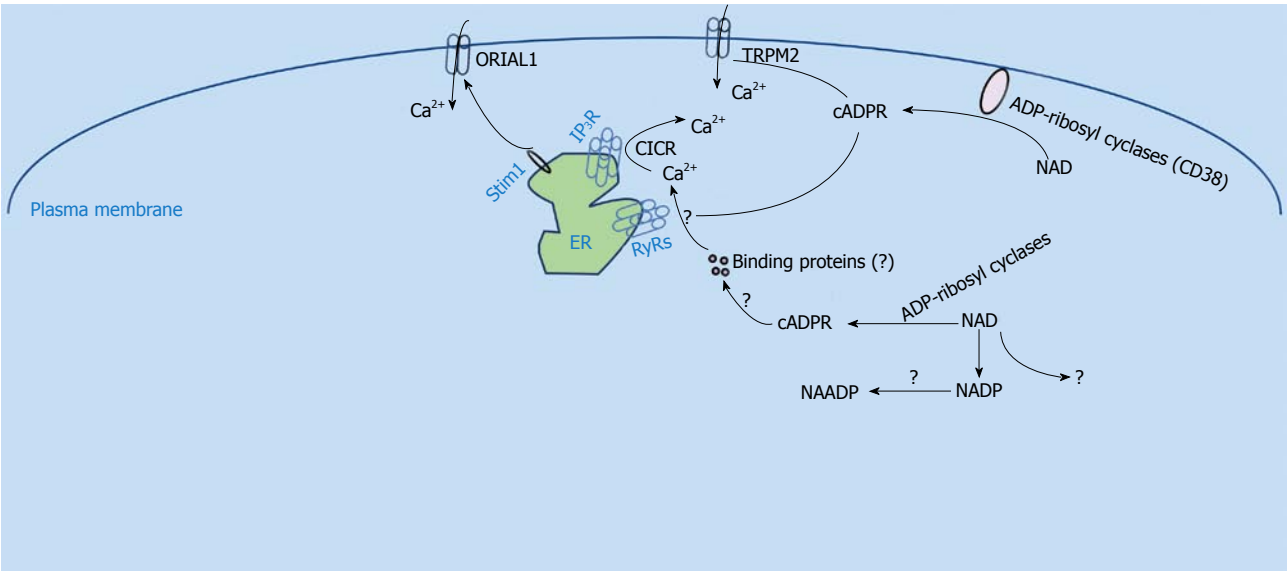

Figure 1 Cyclic adenosine diphosphate ribose mediated Ca^{2+} signaling. TRPM2: Transient receptor potential cation channel M2; cADPR: Cyclic adenosine diphosphate ribose; NAADP: Nicotinic acid adenine dinucleotide phosphate; NAD: Nicotinamide adenine dinucleotide; ER: Endoplasmic reticulum.

function of cADPR. Next, the structure and topology of CD38 will be reviewed. Finally, the physiological functions of CD38/cADPR/Ca^{2+} signaling and their involvement in pathological processes will be summarized.

THE STRUCTURE AND FUNCTION OF CADPR

A suitable model system is the foundation of any novel finding and this concept is also true for the discovery of cADPR. Sea urchin eggs are large and amenable for microinjection studies so that Ca^{2+} mobilizing activities during fertilization can be readily observed, and it is easy to isolate endoplasmic reticulum (ER) from sea urchin eggs, making them the perfect system to investigate mechanisms of intracellular Ca^{2+} mobilization[2]. Taking advantage of the sea urchin homogenate preparation and use of the fluorescent Ca^{2+} indicator Fura 2, Lee *et al*[3] and Clapper *et al*[4] found that the pyridine nucleotide nicotinamide adenine dinucleotide (NAD) can invoke a delayed Ca^{2+} release from ER independent of IP_3. They then determined that this delay was due to enzymatic conversion of NAD to cADPR by the homogenate. Later, Lee *et al*[5] solved the structure of cADPR by x-ray crystallography and showed that it is a novel cyclic nucleotide formed by the covalent linkage of the N1 nitrogen of the adenine ring to the anomeric carbon of the terminal ribose to become a closed cyclic structure (Figure 2). Benefiting from the identified structure, multiple cADPR analogs have been synthesized, which greatly promoted research on the role and mechanism of cADPR-mediated Ca^{2+} signaling[6-9].

From the very beginning of research on cADPR, several pharmacological studies have clearly shown that the mechanism of cADPR-induced Ca^{2+} release is different from that of IP_3. For example, desensitization experiments demonstrated that the sea urchin homogenates which were desensitized to IP_3 would still respond to cADPR[4], and the IP_3 inhibitor heparin had no effect on the cADPR-induced Ca^{2+} release[10]. Using the sea urchin homogenate as the model, Galione *et al*[11] proposed that calcium-induced calcium release (CICR) may be modulated by cADPR, since concentrations of cADPR in the nanomolar range could greatly increase the sensitivity to Ca^{2+} during the CICR process. Thus, ryanodine receptors (RyRs) were proposed to be the cADPR receptors through which the CICR functions, and this idea was supported by several subsequent studies. For example, cADPR was shown to directly activate RyR2 that was incorporated into lipid bilayers[12]. In HEK293 cells transfected with an islet type RyR, which is a splice variant of the *RyR2* gene by alternative splicing of exons 4 and 75, Ca^{2+} release was enhanced in the presence of 100 μmol/L cADPR, and the effect could be reversed by pre-incubating with a cADPR antagonist, 8-bromo-cADPR (8-Br-cADPR)[13]. Similarly, cADPR triggered a marked Ca^{2+} transient in HEK293 cells that stably expressed RyR1 and RyR3, and this Ca^{2+} transient was abolished by dantrolene, an RyR antagonist[14]. In summary, all these results suggested that RyRs might serve as cADPR receptors (Figure 1).

However, further experiments argued that the action of cADPR on ryanodine receptors might require the assistance of additional protein factors (Figure 1). For example, both calmodulin and FK506 binding protein (FKBP) have been shown to be required for cADPR action[15-20]. These data suggested that cADPR does not directly bind to the ryanodine receptors, but acts through some intermediate proteins, whose definitive identities remain to be established. Zheng *et al*[21] demonstrated in mouse bladder smooth muscle that Ca^{2+} release induced by cADPR is actually mediated by FKBP12.6 proteins. Nevertheless, additional research such as genome-wide

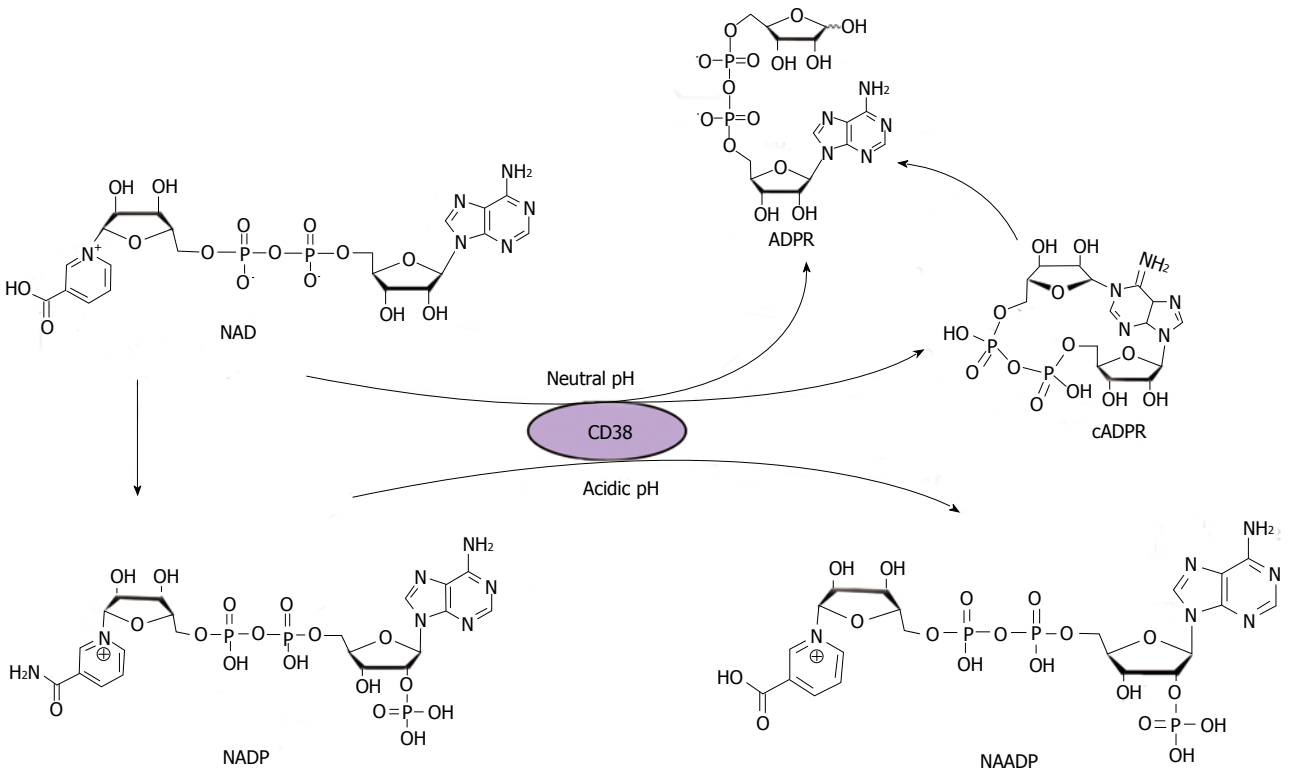

Figure 2 Schematic of the structure and synthesis of cyclic adenosine diphosphate ribose. cADPR: Cyclic adenosine diphosphate ribose; NAADP: Nicotinic acid adenine dinucleotide phosphate; NAD: Nicotinamide adenine dinucleotide.

RNAi screening is needed to elucidate the direct receptor of cADPR.

In addition, growing evidence has shown that cADPR also evokes Ca^{2+} influx (Figure 1)[22]. It has been shown that cADPR can significantly potentiate the transient receptor potential cation channel M2 (TRPM2) channel activity in a temperature dependent manner[23]. Similarly, we recently synthesized a novel fluorescent caged cADPR analogue, coumarin caged isopropylidene-protected cIDPRE (Co-*i*-cIDPRE), and found that it is a potent and controllable cell permeant cADPR agonist. Moreover, we demonstrated that uncaging of Co-*i*-cIDPRE activates RyRs for Ca^{2+} mobilization and triggers Ca^{2+} influx *via* TRPM2[24]. Yet, another experiment showed that TRPM2 is not involved in the effect of another membrane-permeant cADPR agonist, 8-bromo-cyclic IDP-ribose (8-Br-N^1-cIDPR), which induced Ca^{2+} entry in T cells[25]. Thus, the channel that mediates the cADPR induced Ca^{2+} influx still needs to be elucidated.

ENZYMATIC PATHWAY OF CADPR SYNTHESIS AND DEGRADATION

As mentioned above, the effect of NAD to induce Ca^{2+} release in sea urchin eggs was shown to result from its enzymatic conversion to cADPR. Subsequently, a similar enzymatic activity was shown to exist in a wide variety of mammalian tissues[26]. The first purified enzyme shown to produce cADPR from NAD was identified in *Aplysia* and was later named ADP-ribosyl cyclase[27]. Surprisingly, the amino acid sequence of *Aplysia* ADP-ribosyl cyclase, a soluble 30 kDa protein, showed overall about 68% homology with human CD38, a lymphocyte antigen[28,29]. CD38 was indeed able to catalyze the cyclization of NAD to cADPR in pancreatic beta-cells[30]. Moreover, purified murine CD38 was able to convert NAD to cADPR in an *in vitro* assay[28]. Later, CD157, a GPI-anchored antigen that shared 30% homology with CD38, was found to have ADP-ribosyl cyclase activity as well[31].

Overall, these ADP-ribosyl cyclases share about 25%-30% sequence identity[32], and this family is likely to grow since researchers have continued to find ADP-ribosyl cyclase activity that is undefined. In addition, it appears that these unknown cyclases function differently in different tissues. For example, an unidentified cardiac ADPR cyclase can be inhibited by micromolar concentrations of Zn^{2+}, which is different from the effects of this cation on CD38 and CD157[33,34]. A similar ADP-ribosyl cyclase that can be inhibited by the divalent cations Zn^{2+} and Cu^{2+} has also been found in the disks of bovine retinal rod outer segments[35]. Specific inhibitor based analysis confirmed the existence of a distinct ADP-ribosyl cyclase in the kidney since it responded differently to the inhibitor 4,4'-dihydroxy azobenzene (DHAB) treatment than CD38[36].

So far, CD38 is still considered to be the main mammalian ADP-ribosyl cyclase, as shown by the fact that extracts of tissues from CD38 knockout mice have little if any ADP-ribosyl activity compared to those from wild type mice. When incubated with NAD *in vitro*, CD38 only produced a small portion of cADPR, while the major-

ity of the product is ADP-ribose; thus CD38 possesses both cyclase and NADase activities. In addition, CD38 can hydrolyze cADPR to ADP-ribose and, other than CD157, it remains the only ADP-ribosyl cyclase that has been identified in mammals[28]. Moreover, CD38 shows another bifunctional character in that it catalyzes the synthesis and hydrolysis of another secondary messenger, NAADP. In this reaction, CD38 catalyzes the exchange of the nicotinamide group of NADP with nicotinic acid under acidic conditions to generate NAADP; furthermore, NAADP can also be hydrolyzed by CD38 to ADPRP (Figure 2)[37,38]. Understanding the structure and function of CD38 is a crucial part of cADPR/Ca^{2+} signaling research.

STRUCTURE AND ENZYMATIC FUNCTION OF CD38

CD38 is a transmembrane protein, containing a short 21 amino acid residue N-terminal cytoplasmic tail, a 23 amino acid residue hydrophobic transmembrane domain, and a large 256 amino acid residue carboxyl-terminal extracellular domain with four putative glycosylation sites[39]. The extracellular domain of human CD38 with the glycosylation sites removed has been expressed in yeast and purified. Structural analysis of the recombinant CD38 by X-ray crystallography showed that the secondary structure of CD38 is similar to that of the *Aplysia* cyclase. Overall, both CD38 and the cyclase have similar topology although the cyclase forms dimers in the crystals whereas CD38 does not. The middle cleft of both proteins forms a deep pocket as the active site, with a TLEDTL conserved sequence sitting in the bottom of the pocket[40,41]. Site-directed mutagenesis studies identified Glu226 as the catalytic residue of CD38[42]. Two other residues, Glu146 and Thr221, were found to be essential for the cyclization and hydrolysis activity of CD38, respectively[43]. Upon binding of NAD to the active site, the nicotinamide ring interacts with Trp189 by hydrophobic ring stacking, the 2' and 3' hydroxyls of the northern ribose form hydrogen bonds with Glu226, and the ribose diphosphate moiety interacts with amino acids Trp125, Ser126, Arg127, Thr221 and Phe222. Upon cleavage of the nicotinamide ring, the N1 nitrogen of the adenine ring gains access to the anomeric carbon to form a covalent bond and produce cADPR. Alternatively, a water molecule, rather than the adenine ring, attacks the intermediate to form ADP-ribose[44]. In contrast to the formation of cyclic ADP-ribose from NAD, CD38 also catalyzes the formation of NAADP from NADP. Under acidic pH and in the presence of nicotinic acid, the acidic residues in the active site of CD38 are protonated, thereby facilitating the nucleophilic attack of the intermediate of NADP by nicotinic acid to generate NAADP[44].

TOPOLOGY OF CD38

Structurally, CD38 is predicted to be a type-II transmembrane protein with its catalytic C-terminal domain located outside of the cell[39]. This circumstance presents a dilemma because the NAD substrate is located intracellularly whereas the enzyme is positioned extracellularly. If so, cytosolic NAD must be transported out of cells first and then cyclized by CD38 to produce cADPR in the extracellular space. Subsequently, the cADPR product must be transported back into the cytosol to induce Ca^{2+} release from the ER. This scenario obviously presents a "topological paradox" for the cADPR/Ca^{2+} signaling cascade. Two general hypotheses have been proposed to solve this puzzle (Figure 3). The first proposal is based on the presence of transporters, such as connexin 43 hemichannels, which allow intracellular NAD to move to the extracellular space so that it is available for access to the catalytic domain of CD38 to be converted to cADPR[45]. The cADPR product is then transferred back to cells *via* either CD38 or nucleoside transporters[46]. Besides this direct transport model *via* transporters, Zocchi *et al*[47] also suggested that CD38 undergoes an extensive internalization through invaginations of the plasma membrane to form endocytotic vesicles, which makes the active site of CD38 intravesicular and able to convert cytosolic NAD into cADPR. CD38 itself is a unidirectional transmembrane transporter of cADPR that mediates the cADPR efflux into the cytoplasm to reach the Ca^{2+} store, while influx of the cytosolic NAD^+ substrate into the endocytotic CD38-containing vesicles is mediated by other transmembrane transporters, such as connexin 43 hemichannels[48]. The internalization of CD38 has been supported by several studies. For example, the internalization of CD38 can be induced by NADP in Chinese hamster ovary (CHO) cells[49] and hemin treatment can induce internalization of CD38 in K562 cells[50]. Rah *et al*[51] have also demonstrated that association of phospho-nonmuscle myosin heavy chain ⅡA with tyrosine kinase Lck and CD38 is critical for the internalization and activation of CD38. However, mechanisms regarding the transporter mediated CD38 activation process remain elusive. For example, connexin 43 hemichannels are opened for NAD export only when the cellular Ca^{2+} is 100 nmol/L; thus this system is unlikely to operate when Ca^{2+} is elevated above basal levels[45].

The second proposal offered to explain the topological paradox involves a consideration of the orientation of CD38. Bruzzone and coworkers have shown that treatment of granulocytes with 8-Br-cyclic adenosine monophosphate (cAMP), a cell-permeant analog of cAMP, induced serine phosphorylation of CD38, correlating with a cAMP-dependent intracellular cADPR synthesis[52]. Although the exact location of the phosphorylation sites is unknown, it was predicted to be in the catalytic C-terminal domain that contains multiple serine residues. However, if the catalytic domain of CD38 is phosphorylated by protein kinase A (PKA), this domain should be in the cytosol to directly cyclize NAD, thereby synthesizing cADPR intracellularly. This suggests that although CD38 is believed to be a type-Ⅱ protein, at least a portion of the total CD38 is expressed as a type-Ⅲ membrane protein with its C-terminal catalytic domain sitting in the cyto-

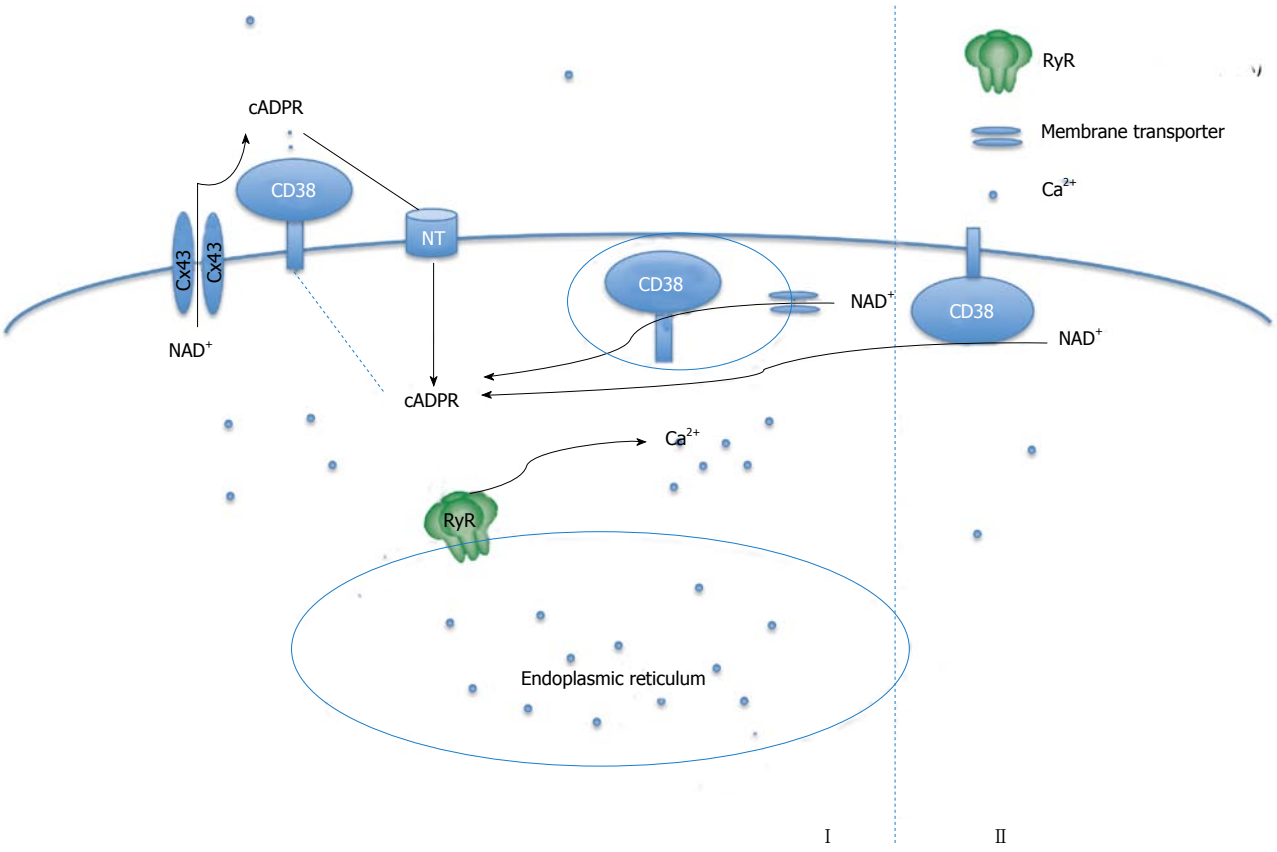

Figure 3 Models of CD38 topology. cADPR: Cyclic adenosine diphosphate ribose; NAD: Nicotinamide adenine dinucleotide; RyR: Ryanodine receptor.

sol[53]. Since the number of positive charges that determine the polarity of membrane protein is equal on each side of the CD38 transmembrane segment, studies from protease digestion[54] and electron microscopy[55] showed that the nuclear CD38 might be a type-Ⅲ membrane protein. Most recently, Zhao *et al*[56] reported that expression of a cytosolic CD38 protein with deletion of both the N-terminal tail and transmembrane domain results in intact disulfides as well as active enzyme in spite of the cytosolic reductive environment; this result appears to solve the fundamental need of the six disulfides for CD38 enzymatic activity. Based on this finding, they consequently proved the co-expression of type Ⅱ and type Ⅲ CD38 on the surface of leukemia HL-60 cells during retinoic acid-induced differentiation and on interferon Y-activated natural human monocytes and U937 cells[57]. They proposed that the type-Ⅲ structure may take part in fast cellular responses, while the type-Ⅱ structure may be more suitable for slower and long term responses (Figure 3)[58].

PHYSIOLOGICAL FUNCTIONS OF THE CD38/CADPR/CA^{2+} PATHWAY

In addition to its role in cADPR production, another function of CD38 is to regulate the NAD level inside cells. It has been well established that NAD plays an essential role in energy metabolism and is involved in diverse signal transduction pathways. A rather surprising finding is that CD38 has a dramatic role in intracellular NAD metabolism. NAD levels in CD38 knockout mice are 10 to 20-fold higher than that in wild-type animals. These results suggest that CD38 is a major regulator of NAD levels in mammalian cells[59].

CD38 was originally identified as a lymphocyte antigen; thus it is not surprising that the CD38/cADPR/Ca^{2+} pathway plays an important role in inflammatory processes. In an ischemic stroke study, $CD38^{-/-}$ mice produced less monocyte chemoattractant protein-1 (MCP-1) after temporary middle cerebral artery occlusion and had fewer infiltrating macrophages and lymphocytes in the ischemic hemisphere than the wild type mice, whereas the amount of resident microglia was unaltered. The same study also demonstrated that CD38 affected immune cell migration as well as activation, two crucial postischemic inflammatory responses in secondary brain damage, suggesting that CD38 might be a therapeutic target to modulate the inflammatory mechanisms after cerebral ischemia[60]. Recently, Ng *et al*[61] used intravital multi-photon microscopy to observe the neutrophil granulocyte traffic into the injury site in the dermis of mice and found that the amplification phase, which is the attraction of more neutrophils toward the damage focus after the initial phase of migration by scouting neutrophils, was mediated by cADPR. cADPR and CD38 were also involved in the regulation of leukocyte adhesion and chemotaxis and were required for the deletion of T regulatory cells during inflammation as well[62]. In addition, 8-Br-cADPR, a

cADPR antagonist, inhibited the MCP-1 induced Ca^{2+} increase, reactive oxygen species (ROS) production and apoptosis in human retinal pigment epithelium, suggesting that cADPR is also involved in the inflammatory responses of age-related macular degeneration (AMD)[63].

Recently, we demonstrated that cADPR is important for regulating cell proliferation and neuronal differentiation in PC12 cells. We found that acetylcholine (Ach) activates the CD38/cADPR pathway to induce Ca^{2+} release and the CD38/cADPR/Ca^{2+} signaling pathway is required for Ach-stimulated cell proliferation in PC12 cells. Interestingly, inhibition of the cADPR pathway accelerated nerve growth factor (NGF)-induced neuronal differentiation in PC12 cells. On the other hand, CD38 overexpression increased cell proliferation but delayed NGF-induced differentiation. Taken together, we demonstrated that cADPR plays a dichotomic role in regulating proliferation and neuronal differentiation of PC12 cells[64].

Abscisic acid (ABA) is an endogenous stimulator of insulin secretion in human and murine pancreatic beta cells. ABA triggered activation of CD38 and production of cADPR before insulin release, suggesting that CD38 is a regulator of insulin release[65]. Also, CD38 expression and cADPR production induced by ABA were required for ABA-induced upregulation of COX-2 and prostaglandin E2 in human mesenchymal stem cells (MSC) and for chemokinesis of MSC[66].

Since cADPR can activate RyRs for Ca^{2+} release from ER and can modulate the CICR process, the CD38/cADPR/Ca^{2+} pathway is predicted to participate in the regulation of cardiac activities, including cardiogenesis and the function of adult cardiac tissue. In fact, ever since the discovery of cADPR, researchers have vigorously explored its role in cardiac tissues. Galione *et al*[67] showed that application of cADPR through a patch electrode resulted in an increase in Ca^{2+} transients with a concomitant increase of the magnitude of contraction in guinea-pig cardiac ventricular myocytes, whereas application of the inhibitor 8-amino-cADPR resulted in a significant reduction in contractions and Ca^{2+} release from the SR. Similarly, in rat cardiac ventricular myocytes, cADPR increased the frequency of Ca^{2+} "sparks", which may contribute to the increase in subsequent whole-cell Ca^{2+} transients[68]. In addition, Prakash *et al*[69] found that microinjection of cADPR into adult rat ventricular myocytes not only induced sustained Ca^{2+} responses in a concentration dependent manner but also increased the frequency and amplitude of spontaneous Ca^{2+} waves, which were completely blocked by 8-amino-cADPR, a cADPR antagonist.

Interestingly, cardiac hypertrophy developed only in CD38 knockout male mice. The expression of RyR protein was increased only in female CD38 knockout mice compared with wild type, suggesting that the CD38/cADPR signaling plays an important role in intracellular Ca^{2+} homeostasis in cardiac myocytes *in vivo*, although its deficiency was compensated differentially according to gender[70].

cADPR was also shown to be involved in angiotensin Ⅱ-induced cardiac hypertrophy[71]. In rat cardiomyocytes, angiotensin Ⅱ evoked a Ca^{2+} increase *via* IP_3R to activate PKC, which then activated the NAD(P)H oxidase to initiate ROS generation. The ROS together with Ca^{2+} then activated the ADP-ribose cyclase to synthesize cADPR, which induced a sustained increase of both Ca^{2+} and ROS and finally led to cardiac hypertrophy[72]. Most recently, Xu *et al*[73] demonstrated that CD38/cADPR was involved in the regulation of superoxide ($O_2^{\bullet-}$) production in mouse coronary arterial myocytes (CAMs). NAD(P)H oxidase is responsible for $O_2^{\bullet-}$ production. Since CD38 can use NAD, an NAD(P)H oxidase product, to produce cADPR and cADPR production can result in an increase in NAD(P)H oxidase activity, the system contains a positive feedback loop. Xu *et al*[73] found that oxotremorine, a muscarinic type 1 receptor agonist, stimulated intracellular $O_2^{\bullet-}$ production in CAMs that was inhibited in CD38 knockout, CD38 knockdown, or nicotinamide-treated (a CD38 inhibitor) cells. On the other hand, direct application of cADPR into CAMs increased intracellular Ca^{2+} and $O_2^{\bullet-}$ production in $CD38^{-/-}$ CAMs. Moreover, CD38 knockout, Nox1 knockdown or Nox4 knockdown blocked oxotremorine-induced contraction in the isolated perfused coronary arteries in mice. Taken together, these data indicate that the CD38/cADPR pathway is an important regulator of Nox-mediated intracellular $O_2^{\bullet-}$ production.

The CD38/cADPR/Ca^{2+} pathway has also been shown to regulate the cardiogenesis process. We recently studied the role of CD38/cADPR/Ca^{2+} in the cardiomyogenesis of mouse embryonic stem (ES) cells. We found that beating cells appeared earlier and were more abundant in CD38 knockdown embryoid bodies (EBs) than control EBs, and the expression of several cardiac markers was increased significantly in CD38 knockdown EBs than control EBs. Similarly, more cardiomyocytes (CMs) existed in CD38 knockdown or cADPR antagonist-treated EBs compared to control EBs. Conversely, CD38 overexpression in mouse ES cells markedly inhibited CM differentiation. Surprisingly, CD38 knockdown ES cell derived CMs possess the functional properties characteristic of normal ES cell derived CMs. In addition, we found that the CD38/cADPR pathway inhibited the Erk1/2 cascade during CM differentiation of ES cells, and transient inhibition of Erk1/2 blocked the enhancive effects of CD38 knockdown on the differentiation of CM from ES cells. Taken together, we demonstrated that the CD38/cADPR/Ca^{2+} signaling pathway inhibits the CM differentiation of mouse ES cells[74].

The mechanism underlying cADPR regulation of Ca^{2+} sparks in cardiomyocyte remains elusive. Zhang *et al*[19]. showed that cADPR markedly increased the Ca^{2+} spark frequency in cardiomyocytes isolated from wild type mice, whereas cADPR failed to initiate Ca^{2+} sparks in cardiomyocytes isolated from FK506 binding protein 12.6 (FKBP12.6) knockout mice. They further demonstrated that cADPR induced FKBP12.6 dissociation from RyRs in a phosphorylation-dependent manner. Yet, another study showed that cAMP signaling is required for the

role of cADPR in the beta-adrenergic receptor induced Ca^{2+}increase in rat cardiomyocytes. They found that the isoproterenol-mediated increase of Ca^{2+} was blocked by pretreatment with 8-Br-cADPR, PKA inhibitor H89 or a high concentration of ryanodine. Moreover, incubation of ventricular lysates with isoproterenol, forskolin or cAMP resulted in activation of ADP-ribosyl cyclase of the ventricular lysates[34]. Interestingly, for comparison, estrogen increased CD38 expression and its cyclase activity, but did not affect its hydrolase activity, while progesterone eliminated the effects of estrogen on CD38 in the rat myometrium[75]. Nevertheless, the mechanism of how the CD38/cADPR is involved in the regulation of cardiac function is still unclear.

CD38/CADPR/CA^{2+} PATHWAY IN PATHOLOGICAL PROCESSES

The CD38/cADPR/Ca^{2+} pathway has been suggested to be involved in various pathological processes. For example, CD38 deficiency accelerated diabetes in a non-obese diabetic (NOD) mice model[76]. It has also been shown that both the specific kidney ADP-ribosyl cyclase activity and cADPR production were increased in the kidneys of diabetic mice, suggesting that cADPR plays a role in the renal pathogenesis of diabetes[77]. Down-regulation of CD38 has also been shown to mediate the intermittent hypoxia induced impairment of glucose-induced insulin secretion, suggesting that CD38 plays a role in type 2 diabetes progression[78]. Numerous studies have been attempted to dissect the molecular mechanism of the role of CD38/cADPR/Ca^{2+} pathway in mediating diabetes in order to identify an alternative therapeutic tool. Tian *et al*[79] found that the content of cADPR was elevated with concomitant enhanced activity of RyR2 in ventricular myocytes isolated from a type 1 diabetic rat model, suggesting that cADPR mediates type 1 diabetes through regulating the function of RyR2. Chen *et al*[80] demonstrated that the ATP-gated ion channel P2X7 was required for the acceleration of type 1 diabetes induced by CD38 deficiency. Taken together, knowledge about the role of the CD38/cADPR/Ca^{2+} pathway in diabetes is accumulating rapidly and there is hope that understanding this pathway will facilitate the development of novel therapeutics for the disease.

The CD38/cADPR/Ca^{2+} pathway has been associated with inflammatory airway disorders. In human airway smooth muscle (ASM) cells, increased ASM contractility in inflammatory diseases such as asthma was due to enhanced Ca^{2+} sensitivity to cytokines, which was correlated with the increase of CD38 expression and cADPR level[81]. This increase of CD38 was induced by TNFα *via* NFκB and could be inhibited by glucocorticoids[82]. In addition, the CD38/cADPR/Ca^{2+} pathway also mediated the 2-arachidonoylglycerol induced rapid actin rearrangement during differentiation of HL-60 cells into macrophage-like cells[83], and extracellular NAD^{+} induced stimulation and recruitment of human granulocytes during the inflammation process[84]. In addition, CD38 was involved in a neuroinflammatory disorder where CD38 expression level was markedly increased in IL-1beta- or HIV-1-activated human astrocytes, whereas CD38 knockdown significantly reduced proinflammatory cytokine and chemokine production in astrocytes[85]. Considering these results, the CD38/cADPR/Ca^{2+} pathway plays important roles in multiple inflammatory processes.

CONCLUSION

The CD38/cADPR/Ca^{2+} pathway modulates various processes of cells, including inflammation, insulin secretion, cardiogenesis, cardiac regulation *etc*. With further investigation, it is likely that other physiological roles of the CD38/cADPR/Ca^{2+} pathway will be revealed. For example, Yue *et al*[64] have shown that the CD38/cADPR/Ca^{2+} pathway delayed the nerve growth factor induced differentiation of PC12 cells; thus it is reasonable to predict that this pathway might also be involved in the regulation of neurogenesis. Using the mouse embryonic stem cell *in vitro* differentiation model, our preliminary results showed that the CD38/cADPR/Ca^{2+} pathway does play a role in neural differentiation of mES (unpublished data); however, further research is needed to decipher the underlying mechanism. A comprehensive understanding of the physiological and pathological roles of the CD38/cADPR/Ca^{2+} pathway in various cellular processes will undoubtedly be helpful for exploiting new molecular therapy targets. In addition, it still remains to be determined whether cADPR binds directly to RyRs or through some unknown proteins. Recently, the long-sought-after store-operated Ca^{2+} entry proteins were identified using a genome-wide RNAi screen by several groups[86-88]. A similar strategy could be applied to identify novel cADPR-interacting proteins or regulators.

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REFERENCES

1 **Sutherland EW**, Robison GA, Butcher RW. Some Aspects of the Biological Role of Adenosine 3′,5′-monophosphate (Cyclic AMP). *Circulation* 1968; **37**: 279-306 [DOI: 10.1161/01.CIR.37.2.279]
2 **Clapper DL**, Lee HC. Inositol trisphosphate induces calcium release from nonmitochondrial stores i sea urchin egg homogenates. *J Biol Chem* 1985; **260**: 13947-13954 [PMID: 2414285]
3 **Lee HC**, Walseth TF, Bratt GT, Hayes RN, Clapper DL. Structural determination of a cyclic metabolite of NAD+ with intracellular Ca2+-mobilizing activity. *J Biol Chem* 1989; **264**: 1608-1615 [PMID: 2912976]
4 **Clapper DL**, Walseth TF, Dargie PJ, Lee HC. Pyridine nucleotide metabolites stimulate calcium release from sea urchin egg microsomes desensitized to inositol trisphosphate. *J Biol Chem* 1987; **262**: 9561-9568 [PMID: 3496336]
5 **Lee HC**, Aarhus R, Levitt D. The crystal structure of cyclic

ADP-ribose. *Nat Struct Biol* 1994; **1**: 143-144 [PMID: 7656029 DOI: 10.1038/nsb0394-143]
6 **Shuto S**, Fukuoka M, Manikowsky A, Ueno Y, Nakano T, Kuroda R, Kuroda H, Matsuda A. Total synthesis of cyclic ADP-carbocyclic-ribose, a stable mimic of Ca2+-mobilizing second messenger cyclic ADP-ribose. *J Am Chem Soc* 2001; **123**: 8750-8759 [PMID: 11535079 DOI: 10.1021/ja010756d]
7 **Dong M**, Si YQ, Sun SY, Pu XP, Yang ZJ, Zhang LR, Zhang LH, Leung FP, Lam CM, Kwong AK, Yue J, Zhou Y, Kriksunov IA, Hao Q, Lee HC. Design, synthesis and biological characterization of novel inhibitors of CD38. *Org Biomol Chem* 2011; **9**: 3246-3257 [PMID: 21431168 DOI: 10.1039/c0ob00768d]
8 **Zhou Y**, Yu P, Jin H, Yang Z, Yue J, Zhang L, Zhang L. Synthesis and calcium mobilization activity of cADPR analogues which integrate nucleobase, northern and southern ribose modifications. *Molecules* 2012; **17**: 4343-4356 [PMID: 22491682 DOI: 10.3390/molecules17044343]
9 **Rosen D**, Bloor-Young D, Squires J, Parkesh R, Waters G, Vasudevan SR, Lewis AM, Churchill GC. Synthesis and use of cell-permeant cyclic ADP-ribose. *Biochem Biophys Res Commun* 2012; **418**: 353-358 [PMID: 22274607 DOI: 10.1016/j.bbrc.2012.01.025]
10 **Dargie PJ**, Agre MC, Lee HC. Comparison of Ca2+ mobilizing activities of cyclic ADP-ribose and inositol trisphosphate. *Cell Regul* 1990; **1**: 279-290 [PMID: 2100201]
11 **Galione A**, Lee HC, Busa WB. Ca(2+)-induced Ca2+ release in sea urchin egg homogenates: modulation by cyclic ADP-ribose. *Science* 1991; **253**: 1143-1146 [PMID: 1909457 DOI: 10.1126/science.1909457]
12 **Mészáros LG**, Bak J, Chu A. Cyclic ADP-ribose as an endogenous regulator of the non-skeletal type ryanodine receptor Ca2+ channel. *Nature* 1993; **364**: 76-79 [PMID: 8391127 DOI: 10.1038/364076a0]
13 **Takasawa S**, Kuroki M, Nata K, Noguchi N, Ikeda T, Yamauchi A, Ota H, Itaya-Hironaka A, Sakuramoto-Tsuchida S, Takahashi I, Yoshikawa T, Shimosegawa T, Okamoto H. A novel ryanodine receptor expressed in pancreatic islets by alternative splicing from type 2 ryanodine receptor gene. *Biochem Biophys Res Commun* 2010; **397**: 140-145 [PMID: 20471962 DOI: 10.1016/j.bbrc.2010.05.051]
14 **Ogunbayo OA**, Zhu Y, Rossi D, Sorrentino V, Ma J, Zhu MX, Evans AM. Cyclic adenosine diphosphate ribose activates ryanodine receptors, whereas NAADP activates two-pore domain channels. *J Biol Chem* 2011; **286**: 9136-9140 [PMID: 21216967 DOI: 10.1074/jbc.m110.2020002]
15 **Lee HC**. Physiological functions of cyclic ADP-ribose and NAADP as calcium messengers. *Annu Rev Pharmacol Toxicol* 2001; **41**: 317-345 [PMID: 11264460 DOI: 10.1146/annurev.pharmtox.41.1.317]
16 **Guse AH**. Biochemistry, biology, and pharmacology of cyclic adenosine diphosphoribose (cADPR). *Curr Med Chem* 2004; **11**: 847-855 [PMID: 15078169 DOI: 10.2174/0929867043455602]
17 **Galione A**, Churchill GC. Cyclic ADP ribose as a calcium-mobilizing messenger. *Sci STKE* 2000; **2000**: pe1 [PMID: 11752598 DOI: 10.1126/stke.2000.41.pe1]
18 **Noguchi N**, Takasawa S, Nata K, Tohgo A, Kato I, Ikehata F, Yonekura H, Okamoto H. Cyclic ADP-ribose binds to FK506-binding protein 12.6 to release Ca2+ from islet microsomes. *J Biol Chem* 1997; **272**: 3133-3136 [PMID: 9013543]
19 **Zhang X**, Tallini YN, Chen Z, Gan L, Wei B, Doran R, Miao L, Xin HB, Kotlikoff MI, Ji G. Dissociation of FKBP12.6 from ryanodine receptor type 2 is regulated by cyclic ADP-ribose but not beta-adrenergic stimulation in mouse cardiomyocytes. *Cardiovasc Res* 2009; **84**: 253-262 [PMID: 19578067 DOI: 10.1093/cvr/cvp212]
20 **Thomas JM**, Summerhill RJ, Fruen BR, Churchill GC, Galione A. Calmodulin dissociation mediates desensitization of the cADPR-induced Ca2+ release mechanism. *Curr Biol* 2002; **12**: 2018-2022 [PMID: 12477390 DOI: 10.1016/s0960-9822(02)01335-0]
21 **Zheng J**, Wenzhi B, Miao L, Hao Y, Zhang X, Yin W, Pan J, Yuan Z, Song B, Ji G. Ca(2+) release induced by cADP-ribose is mediated by FKBP12.6 proteins in mouse bladder smooth muscle. *Cell Calcium* 2010; **47**: 449-457 [PMID: 20451249 DOI: 10.1016/j.ceca.2010.03.006]
22 **Guse AH**, Berg I, da Silva CP, Potter BV, Mayr GW. Ca2+ entry induced by cyclic ADP-ribose in intact T-lymphocytes. *J Biol Chem* 1997; **272**: 8546-8550 [PMID: 9079684 DOI: 10.1074/jbc.272.13.8546]
23 **Togashi K**, Hara Y, Tominaga T, Higashi T, Konishi Y, Mori Y, Tominaga M. TRPM2 activation by cyclic ADP-ribose at body temperature is involved in insulin secretion. *EMBO J* 2006; **25**: 1804-1815 [PMID: 16601673 DOI: 10.1038/sj.ebmoj.7601083]
24 **Yu PL**, Zhang ZH, Hao BX, Zhao YJ, Zhang LH, Lee HC, Zhang L, Yue J. A novel fluorescent cell membrane-permeable caged cyclic ADP-ribose analogue. *J Biol Chem* 2012; **287**: 24774-24783 [PMID: 22661714 DOI: 10.1074.jbc.M111.329854]
25 **Kirchberger T**, Moreau C, Wagner GK, Fliegert R, Siebrands CC, Nebel M, Schmid F, Harneit A, Odoardi F, Flügel A, Potter BV, Guse AH. 8-Bromo-cyclic inosine diphosphoribose: towards a selective cyclic ADP-ribose agonist. *Biochem J* 2009; **422**: 139-149 [PMID: 19492987 DOI: 10.1042/bj20082308]
26 **Rusinko N**, Lee HC. Widespread occurrence in animal tissues of an enzyme catalyzing the conversion of NAD+ into a cyclic metabolite with intracellular Ca2+-mobilizing activity. *J Biol Chem* 1989; **264**: 11725-11731 [PMID: 2745413]
27 **Lee HC**, Aarhus R. ADP-ribosyl cyclase: an enzyme that cyclizes NAD+ into a calcium-mobilizing metabolite. *Cell Regul* 1991; **2**: 203-209 [PMID: 1830494]
28 **Howard M**, Grimaldi JC, Bazan JF, Lund FE, Santos-Argumedo L, Parkhouse RM, Walseth TF, Lee HC. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science* 1993; **262**: 1056-1059 [PMID: 8235624 DOI: 10.1126/science.8235624]
29 **States DJ**, Walseth TF, Lee HC. Similarities in amino acid sequences of Aplysia ADP-ribosyl cyclase and human lymphocyte antigen CD38. *Trends Biochem Sci* 1992; **17**: 495 [PMID: 1471258 DOI: 10.1016/0968-0004(92)90337-9]
30 **Takasawa S**, Tohgo A, Noguchi N, Koguma T, Nata K, Sugimoto T, Yonekura H, Okamoto H. Synthesis and hydrolysis of cyclic ADP-ribose by human leukocyte antigen CD38 and inhibition of the hydrolysis by ATP. *J Biol Chem* 1993; **268**: 26052-26054 [PMID: 8253715]
31 **Itoh M**, Ishihara K, Tomizawa H, Tanaka H, Kobune Y, Ishikawa J, Kaisho T, Hirano T. Molecular cloning of murine BST-1 having homology with CD38 and Aplysia ADP-ribosyl cyclase. *Biochem Biophys Res Commun* 1994; **203**: 1309-1317 [PMID: 7916574 DOI: 10.1006/bbrc.1994.2325]
32 **Lee HC**. Structure and enzymatic functions of human CD38. *Mol Med* 2006; **12**: 317-323 [PMID: 17380198]
33 **Kannt A**, Sicka K, Kroll K, Kadereit D, Gögelein H. Selective inhibitors of cardiac ADPR cyclase as novel anti-arrhythmic compounds. *Naunyn Schmiedebergs Arch Pharmacol* 2012; **385**: 717-727 [PMID: 22526470 DOI: 10.1007/s00210-012-0750-2]
34 **Xie GH**, Rah SY, Kim SJ, Nam TS, Ha KC, Chae SW, Im MJ, Kim UH. ADP-ribosyl cyclase couples to cyclic AMP signaling in the cardiomyocytes. *Biochem Biophys Res Commun* 2005; **330**: 1290-1298 [PMID: 15823583]
35 **Fabiano A**, Panfoli I, Calzia D, Bruschi M, Ravera S, Bachi A, Cattaneo A, Morelli A, Candiano G. Catalytic properties of the retinal rod outer segment disk ADP-ribosyl cyclase. *Vis Neurosci* 2011; **28**: 121-128 [PMID: 21269544]
36 **Nam TS**, Choi SH, Rah SY, Kim SY, Jang W, Im MJ, Kwon HJ, Kim UH. Discovery of a small-molecule inhibitor for kidney ADP-ribosyl cyclase: Implication for intracellular calcium signal mediated by cyclic ADP-ribose. *Exp Mol Med* 2006; **38**: 718-726 [PMID: 17202848]

37 **Aarhus R**, Graeff RM, Dickey DM, Walseth TF, Lee HC. ADP-ribosyl cyclase and CD38 catalyze the synthesis of a calcium-mobilizing metabolite from NADP. *J Biol Chem* 1995; **270**: 30327-30333 [PMID: 8530456]

38 **Graeff R**, Liu Q, Kriksunov IA, Hao Q, Lee HC. Acidic residues at the active sites of CD38 and ADP-ribosyl cyclase determine nicotinic acid adenine dinucleotide phosphate (NAADP) synthesis and hydrolysis activities. *J Biol Chem* 2006; **281**: 28951-28957 [PMID: 16861223]

39 **Jackson DG**, Bell JI. Isolation of a cDNA encoding the human CD38 (T10) molecule, a cell surface glycoprotein with an unusual discontinuous pattern of expression during lymphocyte differentiation. *J Immunol* 1990; **144**: 2811-2815 [PMID: 2319135]

40 **Prasad GS**, McRee DE, Stura EA, Levitt DG, Lee HC, Stout CD. Crystal structure of Aplysia ADP ribosyl cyclase, a homologue of the bifunctional ectozyme CD38. *Nat Struct Biol* 1996; **3**: 957-964 [PMID: 8901875 DOI: 10.1038/nsb1196-957]

41 **Liu Q**, Kriksunov IA, Graeff R, Munshi C, Lee HC, Hao Q. Crystal structure of human CD38 extracellular domain. *Structure* 2005; **13**: 1331-1339 [PMID: 16154090 DOI: 10.1016/j.str.2005.05.012]

42 **Munshi C**, Aarhus R, Graeff R, Walseth TF, Levitt D, Lee HC. Identification of the enzymatic active site of CD38 by site-directed mutagenesis. *J Biol Chem* 2000; **275**: 21566-21571 [PMID: 10781610 DOI: 10.1074/jbc.M909365199]

43 **Graeff R**, Munshi C, Aarhus R, Johns M, Lee HC. A single residue at the active site of CD38 determines its NAD cyclizing and hydrolyzing activities. *J Biol Chem* 2001; **276**: 12169-12173 [PMID: 11278881 DOI: 10.1074/jbc.M011299200]

44 **Liu Q**, Kriksunov IA, Graeff R, Lee HC, Hao Q. Structural basis for formation and hydrolysis of the calcium messenger cyclic ADP-ribose by human CD38. *J Biol Chem* 2007; **282**: 5853-5861 [PMID: 17182614]

45 **Bruzzone S**, Franco L, Guida L, Zocchi E, Contini P, Bisso A, Usai C, De Flora A. A self-restricted CD38-connexin 43 cross-talk affects NAD+ and cyclic ADP-ribose metabolism and regulates intracellular calcium in 3T3 fibroblasts. *J Biol Chem* 2001; **276**: 48300-48308 [PMID: 11602597 DOI: 10.1074/jbc.M107308200]

46 **Guida L**, Bruzzone S, Sturla L, Franco L, Zocchi E, De Flora A. Equilibrative and concentrative nucleoside transporters mediate influx of extracellular cyclic ADP-ribose into 3T3 murine fibroblasts. *J Biol Chem* 2002; **277**: 47097-47105 [PMID: 12368285 DOI: 10.1074/jbc.M207793200]

47 **Zocchi E**, Usai C, Guida L, Franco L, Bruzzone S, Passalacqua M, De Flora A. Ligand-induced internalization of CD38 results in intracellular Ca2+ mobilization: role of NAD+ transport across cell membranes. *FASEB J* 1999; **13**: 273-283 [PMID: 9973315]

48 **Bruzzone S**, Guida L, Zocchi E, Franco L. Connexin 43 hemi channels mediate Ca2+-regulated transmembrane NAD+ fluxes in intact cells. *FASEB J* 2001; **15**: 10-12 [PMID: 11099492 DOI: 10.1096/fj.00-0566fje]

49 **Chidambaram N**, Chang CF. NADP+-Dependent internalization of recombinant CD38 in CHO cells. *Arch Biochem Biophys* 1999; **363**: 267-272 [PMID: 10068448 DOI: 10.1006/abbi.1999.1103]

50 **Yalcintepe L**, Ercelen S, Adin-Cinar S, Badur S, Tiryaki D, Bermek E. Hemin-dependent induction and internalization of CD38 in K562 cells. *J Cell Biochem* 2003; **90**: 379-386 [PMID: 14505353 DOI: 10.1002/jcb.10637]

51 **Rah SY**, Park KH, Nam TS, Kim SJ, Kim H, Im MJ, Kim UH. Association of CD38 with nonmuscle myosin heavy chain IIA and Lck is essential for the internalization and activation of CD38. *J Biol Chem* 2007; **282**: 5653-5660 [PMID: 17182620 DOI: 10.1074/jbc.M609478200]

52 **Bruzzone S**, Moreschi I, Usai C, Guida L, Damonte G, Salis A, Scarfì S, Millo E, De Flora A, Zocchi E. Abscisic acid is an endogenous cytokine in human granulocytes with cyclic ADP-ribose as second messenger. *Proc Natl Acad Sci USA* 2007; **104**: 5759-5764 [PMID: 17389374 DOI: 10.1073/pnas.0609379104]

53 **Lee HC**. Cyclic ADP-ribose and NAADP: fraternal twin messengers for calcium signaling. *Sci China Life Sci* 2011; **54**: 699-711 [PMID: 21786193 DOI: 10.1007/s11427-011-4197-3]

54 **Adebanjo OA**, Anandatheerthavarada HK, Koval AP, Moonga BS, Biswas G, Sun L, Sodam BR, Bevis PJ, Huang CL, Epstein S, Lai FA, Avadhani NG, Zaidi M. A new function for CD38/ADP-ribosyl cyclase in nuclear Ca2+ homeostasis. *Nat Cell Biol* 1999; **1**: 409-414 [PMID: 10559984 DOI: 10.1038/15640]

55 **Khoo KM**, Han MK, Park JB, Chae SW, Kim UH, Lee HC, Bay BH, Chang CF. Localization of the cyclic ADP-ribose-dependent calcium signaling pathway in hepatocyte nucleus. *J Biol Chem* 2000; **275**: 24807-24817 [PMID: 10818108 DOI: 10.1074/jbc.M908231199]

56 **Zhao YJ**, Zhang HM, Lam CM, Hao Q, Lee HC. Cytosolic CD38 protein forms intact disulfides and is active in elevating intracellular cyclic ADP-ribose. *J Biol Chem* 2011; **286**: 22170-22177 [PMID: 21524995]

57 **Zhao YJ**, Lam CM, Lee HC. The membrane-bound enzyme CD38 exists in two opposing orientations. *Sci Signal* 2012; **5**: ra67 [PMID: 22969159]

58 **Lee HC**. Cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP) as messengers for calcium mobilization. *J Biol Chem* 2012; **287**: 31633-31640 [PMID: 22822066 DOI: 10.1074/jbc.R112.349464]

59 **Aksoy P**, White TA, Thompson M, Chini EN. Regulation of intracellular levels of NAD: a novel role for CD38. *Biochem Biophys Res Commun* 2006; **345**: 1386-1392 [PMID: 16730329 DOI: 10.1126/scisignal.2002700]

60 **Choe CU**, Lardong K, Gelderblom M, Ludewig P, Leypoldt F, Koch-Nolte F, Gerloff C, Magnus T. CD38 exacerbates focal cytokine production, postischemic inflammation and brain injury after focal cerebral ischemia. *PLoS One* 2011; **6**: e19046 [PMID: 21625615 DOI: 10.1371/journal.pone.0019046]

61 **Ng LG**, Qin JS, Roediger B, Wang Y, Jain R, Cavanagh LL, Smith AL, Jones CA, de Veer M, Grimbaldeston MA, Meeusen EN, Weninger W. Visualizing the neutrophil response to sterile tissue injury in mouse dermis reveals a three-phase cascade of events. *J Invest Dermatol* 2011; **131**: 2058-2068 [PMID: 21697893 DOI: 10.1038/jid.2011.179]

62 **Deaglio S**, Robson SC. Ectonucleotidases as regulators of purinergic signaling in thrombosis, inflammation, and immunity. *Adv Pharmacol* 2011; **61**: 301-332 [PMID: 21586363 DOI: 10.1016/B978-0-12-385526-8.00010-2]

63 **Yang D**, Elner SG, Chen X, Field MG, Petty HR, Elner VM. MCP-1-activated monocytes induce apoptosis in human retinal pigment epithelium. *Invest Ophthalmol Vis Sci* 2011; **52**: 6026-6034 [PMID: 21447688 DOI: 10.1167/iovs.10-7023]

64 **Yue J**, Wei W, Lam CM, Zhao YJ, Dong M, Zhang LR, Zhang LH, Lee HC. CD38/cADPR/Ca2+ pathway promotes cell proliferation and delays nerve growth factor-induced differentiation in PC12 cells. *J Biol Chem* 2009; **284**: 29335-29342 [PMID: 19696022 DOI: 10.1074/jbc.M109.049767]

65 **Bruzzone S**, Bodrato N, Usai C, Guida L, Moreschi I, Nano R, Antonioli B, Fruscione F, Magnone M, Scarfì S, De Flora A, Zocchi E. Abscisic acid is an endogenous stimulator of insulin release from human pancreatic islets with cyclic ADP ribose as second messenger. *J Biol Chem* 2008; **283**: 32188-32197 [PMID: 18784081 DOI: 10.1074/jbc.M802603200]

66 **Scarfì S**, Ferraris C, Fruscione F, Fresia C, Guida L, Bruzzone S, Usai C, Parodi A, Millo E, Salis A, Burastero G, De Flora A, Zocchi E. Cyclic ADP-ribose-mediated expansion and stimulation of human mesenchymal stem cells by the plant hormone abscisic acid. *Stem Cells* 2008; **26**: 2855-2864 [PMID: 18687991 DOI: 10.1634/stemcells.2008-0488]

67 **Galione A**, Cui Y, Empson R, Iino S, Wilson H, Terrar D. Cyclic ADP-ribose and the regulation of calcium-induced

calcium release in eggs and cardiac myocytes. *Cell Biochem Biophys* 1998; **28**: 19-30 [PMID: 9386890]
68 **Cui Y**, Galione A, Terrar DA. Effects of photoreleased cADP-ribose on calcium transients and calcium sparks in myocytes isolated from guinea-pig and rat ventricle. *Biochem J* 1999; **342** (Pt 2): 269-273 [PMID: 10455010]
69 **Prakash YS**, Kannan MS, Walseth TF, Sieck GC. cADP ribose and [Ca(2+)](i) regulation in rat cardiac myocytes. *Am J Physiol Heart Circ Physiol* 2000; **279**: H1482-H1489 [PMID: 11009432]
70 **Takahashi J**, Kagaya Y, Kato I, Ohta J, Isoyama S, Miura M, Sugai Y, Hirose M, Wakayama Y, Ninomiya M, Watanabe J, Takasawa S, Okamoto H, Shirato K. Deficit of CD38/cyclic ADP-ribose is differentially compensated in hearts by gender. *Biochem Biophys Res Commun* 2003; **312**: 434-440 [PMID: 14637156]
71 **Gul R**, Kim SY, Park KH, Kim BJ, Kim SJ, Im MJ, Kim UH. A novel signaling pathway of ADP-ribosyl cyclase activation by angiotensin II in adult rat cardiomyocytes. *Am J Physiol Heart Circ Physiol* 2008; **295**: H77-H88 [PMID: 18456728 DOI: 10.1152/ajpheart.01355.2007]
72 **Gul R**, Shawl AI, Kim SH, Kim UH. Cooperative interaction between reactive oxygen species and Ca2+ signals contributes to angiotensin II-induced hypertrophy in adult rat cardiomyocytes. *Am J Physiol Heart Circ Physiol* 2012; **302**: H901-H909 [PMID: 22140048 DOI: 10.1152/ajpheart.00250.2011]
73 **Xu M**, Zhang Y, Xia M, Li XX, Ritter JK, Zhang F, Li PL. NAD(P)H oxidase-dependent intracellular and extracellular O2•- production in coronary arterial myocytes from CD38 knockout mice. *Free Radic Biol Med* 2012; **52**: 357-365 [PMID: 22100343 DOI: 10.1016/j.freeradbiomed.2011.10.485]
74 **Wei WJ**, Sun HY, Ting KY, Zhang LH, Lee HC, Li GR, Yue J. Inhibition of cardiomyocytes differentiation of mouse embryonic stem cells by CD38/cADPR/Ca2+ signaling pathway. *J Biol Chem* 2012; **287**: 35599-35611 [PMID: 22908234 DOI: 10.1074/jbc.M112.392530]
75 **Dogan S**, Deshpande DA, White TA, Walseth TF, Kannan MS. Regulation of CD 38 expression and function by steroid hormones in myometrium. *Mol Cell Endocrinol* 2006; **246**: 101-106 [PMID: 16388888 DOI: 10.1016/j.mce.2005.11.014]
76 **Chen J**, Chen YG, Reifsnyder PC, Schott WH, Lee CH, Osborne M, Scheuplein F, Haag F, Koch-Nolte F, Serreze DV, Leiter EH. Targeted disruption of CD38 accelerates autoimmune diabetes in NOD/Lt mice by enhancing autoimmunity in an ADP-ribosyltransferase 2-dependent fashion. *J Immunol* 2006; **176**: 4590-4599 [PMID: 16585549]
77 **Kim SY**, Park KH, Gul R, Jang KY, Kim UH. Role of kidney ADP-ribosyl cyclase in diabetic nephropathy. *Am J Physiol Renal Physiol* 2009; **296**: F291-F297 [PMID: 19073639 DOI: 10.1152/ajprenal.90381.2008]
78 **Ota H**, Tamaki S, Itaya-Hironaka A, Yamauchi A, Sakuramoto-Tsuchida S, Morioka T, Takasawa S, Kimura H. Attenuation of glucose-induced insulin secretion by intermittent hypoxia via down-regulation of CD38. *Life Sci* 2012; **90**: 206-211 [PMID: 22154909 DOI: 10.1016/j.lfs.2011.11.011]
79 **Tian C**, Shao CH, Moore CJ, Kutty S, Walseth T, DeSouza C, Bidasee KR. Gain of function of cardiac ryanodine receptor in a rat model of type 1 diabetes. *Cardiovasc Res* 2011; **91**: 300-309 [PMID: 21421556 DOI: 10.1093/cvr/cvr076]
80 **Chen YG**, Scheuplein F, Driver JP, Hewes AA, Reifsnyder PC, Leiter EH, Serreze DV. Testing the role of P2X7 receptors in the development of type 1 diabetes in nonobese diabetic mice. *J Immunol* 2011; **186**: 4278-4284 [PMID: 21357538 DOI: 10.4049/jimmunol.1003733]
81 **Deshpande DA**, Walseth TF, Panettieri RA, Kannan MS. CD38/cyclic ADP-ribose-mediated Ca2+ signaling contributes to airway smooth muscle hyper-responsiveness. *FASEB J* 2003; **17**: 452-454 [PMID: 12514117]
82 **Kang BN**, Tirumurugaan KG, Deshpande DA, Amrani Y, Panettieri RA, Walseth TF, Kannan MS. Transcriptional regulation of CD38 expression by tumor necrosis factor-alpha in human airway smooth muscle cells: role of NF-kappaB and sensitivity to glucocorticoids. *FASEB J* 2006; **20**: 1000-1002 [PMID: 16571778]
83 **Gokoh M**, Kishimoto S, Oka S, Mori M, Waku K, Ishima Y, Sugiura T. 2-arachidonoylglycerol, an endogenous cannabinoid receptor ligand, induces rapid actin polymerization in HL-60 cells differentiated into macrophage-like cells. *Biochem J* 2005; **386**: 583-589 [PMID: 15456404]
84 **Bruzzone S**, Moreschi I, Guida L, Usai C, Zocchi E, De Flora A. Extracellular NAD+ regulates intracellular calcium levels and induces activation of human granulocytes. *Biochem J* 2006; **393**: 697-704 [PMID: 16225456]
85 **Kou W**, Banerjee S, Eudy J, Smith LM, Persidsky R, Borgmann K, Wu L, Sakhuja N, Deshpande MS, Walseth TF, Ghorpade A. CD38 regulation in activated astrocytes: implications for neuroinflammation and HIV-1 brain infection. *J Neurosci Res* 2009; **87**: 2326-2339 [PMID: 19365854 DOI: 10.1002/jnr.22060]
86 **Liou J**, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE, Meyer T. STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr Biol* 2005; **15**: 1235-1241 [PMID: 16005298]
87 **Zhang SL**, Yeromin AV, Zhang XH, Yu Y, Safrina O, Penna A, Roos J, Stauderman KA, Cahalan MD. Genome-wide RNAi screen of Ca(2+) influx identifies genes that regulate Ca(2+) release-activated Ca(2+) channel activity. *Proc Natl Acad Sci USA* 2006; **103**: 9357-9362 [PMID: 16751269]
88 **Roos J**, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Veliçelebi G, Stauderman KA. STIM1, an essential and conserved component of store-operated Ca2+ channel function. *J Cell Biol* 2005; **169**: 435-445 [PMID: 15866891]

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MINIREVIEWS

Thioredoxin and glutaredoxin-mediated redox regulation of ribonucleotide reductase

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Abstract

Ribonucleotide reductase (RNR), the rate-limiting enzyme in DNA synthesis, catalyzes reduction of the different ribonucleotides to their corresponding deoxyribonucleotides. The crucial role of RNR in DNA synthesis has made it an important target for the development of antiviral and anticancer drugs. Taking account of the recent developments in this field of research, this review focuses on the role of thioredoxin and glutaredoxin systems in the redox reactions of the RNR catalysis.

Key words: Ribonucleotide reductase; Thioredoxin; Glutaredoxin; DNA synthesis; Thiol disulfides; Replication

Core tip: Thioredoxin and glutaredoxin-mediated redox regulations of ribonucleotide reductase (RNR) catalysis play a vital role as the RNR catalysis involves different redox active thiol functions, thiyl radicals and thiol proteins. The in depth knowledge of the whole redox catalysis will contribute significantly to designing and developing new RNR inhibitors for improved cancer chemotherapy, antibiotic development and antiviral treatments.

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INTRODUCTION

Ribonucleotide reductase (RNR) catalyzes the rate limiting step of the DNA synthesis where the reduction of ribonucleotides (NTPs) results in the formation of corresponding deoxyribonucleotides (dNTPs)[1-4]. The RNR catalysis involves protein free radicals, redox-active thiols and proteins of the thioredoxin (Trx) superfamily. In the RNR complex, the R1 subunit contains the active site, allosteric sites and redox active thiols/disulfides required for the RNR catalysis; while the R2 subunit provides a dinuclear metal cluster and a tyrosyl free radical essential for the catalytic cycle. The RNR activity can be regulated by expression of different subunits, subcellular localization, post-translational modifications and allosteric regulation involving both activity and substrate specificity[3-8]. The DNA replication is coordinated with the cell growth by different regulatory mechanisms. Development of malignancy and cancer are found to be associated with an increased expression and activity of RNR. In cells, an imbalance in the levels of dNTPs will cause mutagenesis and carcinogenesis. On the other hand, blockage of RNR activity can inhibit DNA synthesis and repair which results in apoptosis. In recent years, several RNR inhibitors have entered clinical trials. Recent developments in the field will provide a new basis for the discovery of more

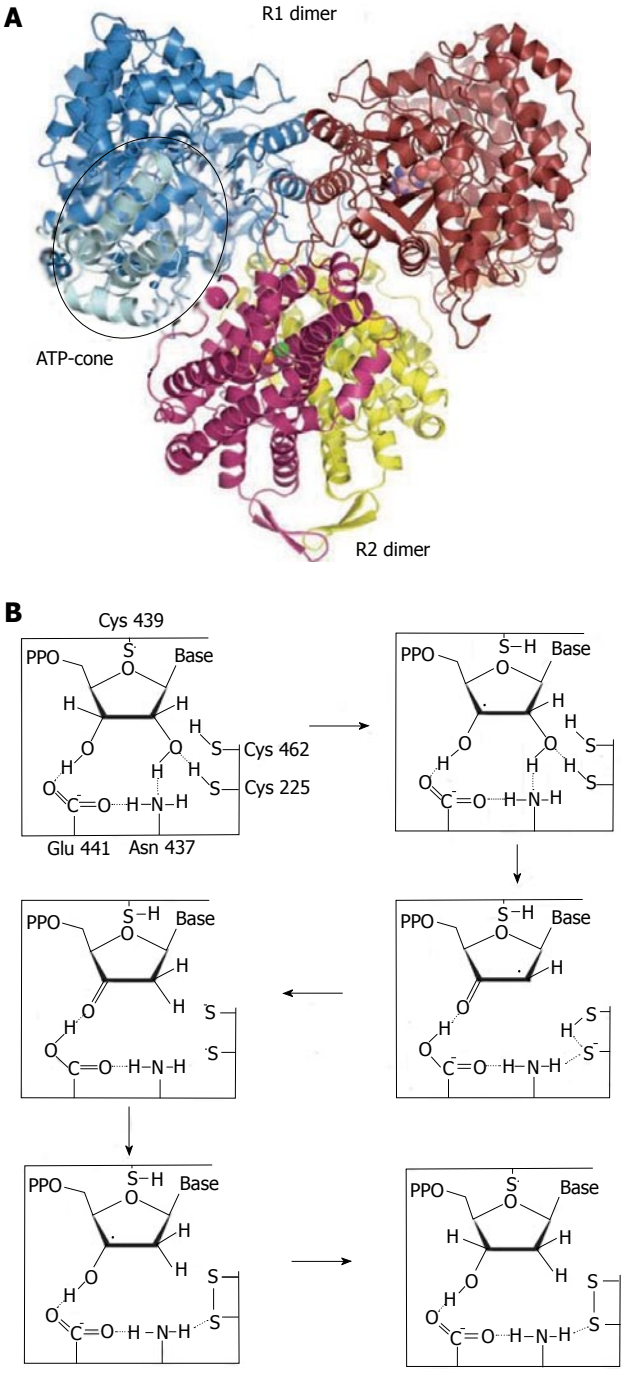

Figure 1 Crystal structure of Class I aerobic ribonucleotide reductase complex (A), and proposed reaction mechanism of ribonucleotide reductase catalysis (B). A: This is based on the crystal structures of the R1 and R2 proteins (Protein Data Bank ID: 1RLR and 1RIB). The figure shows the presence of substrate in R1 subunit and dinuclear iron center in R2 subunit. The ribonucleotide reductase complex (RNR) complex is a tetramer with the dimer of R1 subunit and the dimer of R2 subunit. The allosteric regulatory domain of R1 subunit (ATP-cone) binds either ATP or dATP to regulate the enzymatic activity (adapted from Logan *et al*[9]); B: The figure describes the reduction of nucleoside diphosphate (NDP) to deoxyribonucleoside diphosphates (dNDP) by class I RNR (*E. coli*). The reduction is initiated by a thiyl radical (Cys 439) by abstracting the 3'-hydrogen from the NDP. A water molecule is lost and the two cysteines (Cys 225 and Cys 462) then deliver the required reducing equivalents, generating a 3'-ketodeoxynucleotide which is subsequently reduced to give dNDP (adapted from Holmgren *et al*[4]).

effective RNR inhibitors for cancer therapy. Taking account of the recent progress in this field of research, this review focuses on the role of Trx and glutaredoxin (Grx) systems in the redox regulations of the RNR catalysis.

RNR: CLASSIFICATION AND CATALYSIS

Based on the pathways of radical initiation and requirements of metal cofactors, the RNRs have been divided in to three classes[2-5]. The active site of all three classes of RNR has a very similar structure (Figure 1)[2-5,9]. A conserved cysteine residue plays the vital role for the generation of a thiyl radical in all the classes of RNR (Figures 1 and 2). The reaction mechanisms of different classes of RNR are similar due to the structural similarities of the catalytic domains. The RNR catalysis starts with the generation of a thiyl radical close to the bound substrate[2-5]. Then the abstraction of hydrogen from the C3' of the ribose ring and generation of a substrate radical occur. During the RNR catalysis, a cysteinyl radical, required for the abstraction of hydrogen at the C3' ribose substrate, is derived from a tyrosyl radical for class I or cobalamin cofactor for class II or a glycyl radical for class III. For class I and II enzymes, electrons required for the reduction of the ribonucleotides aare provided by reduced nicotinamide adenine dinucleotide phosphate (NADPH) through Trx or Grx systems. However, for class III enzymes, the electrons are supplied by formate.

The class I RNR occurs in eukaryotes, eubacteria, bacteriophages and viruses. The complex, in its simple form, is a tetramer with the dimer of larger subunit (R1) and the dimer of R2 subunit (Figures 1 and 2)[2-5,9]. Oxygen is required for the generation of a tyrosyl radical (Tyr 122) in the R2 subunit. As described above, during catalysis, the radical is continuously transferred to a cysteine (Cys 439) residue of the R1 subunit and generates a thiyl radical to activate the substrate. The R1 subunit carries the catalytic site, allosteric effector binding sites and redox-active thiol groups required for the catalysis. p53R2 is an additional mammalian RNR protein which functions as a catalytic partner of the R1 subunit[10-12]. The expression of the p53R2 subunit is induced by DNA damage which is mediated by the tumor suppressor protein p53. Both R2 and p53R2 subunits use a diferric iron center generating a tyrosyl free radical required for the RNR catalysis. The R1-p53R2 complex is suggested to be required for basal DNA repair and the R1–R2 complex is suggested to be associated with DNA replication[11]. Moreover, the R1-p53R2 complex has been found to play a significant role in dNTP supply for mitochondrial DNA synthesis.

For class II RNR (archaebacteria, eubacteria), a cofactor (5'-deoxyadenosylcobalamin) replaces the presence of a separate subunit for storage of radicals[2-5]. The cleavage of 5'-deoxyadenosylcobalamin generates a deoxyadenosyl radical which abstracts hydrogen from the active site cysteine residue. Trx system can reduce the C-terminal pair of redox-active cysteines which, in turn, can reduce the active site to continue the RNR catalysis. For anaerobic class III RNR (archaebacteria, eubacteria, bacteriophages), a glycyl radical is generated by the action of activase, S-adenosylmethionine and a reducing

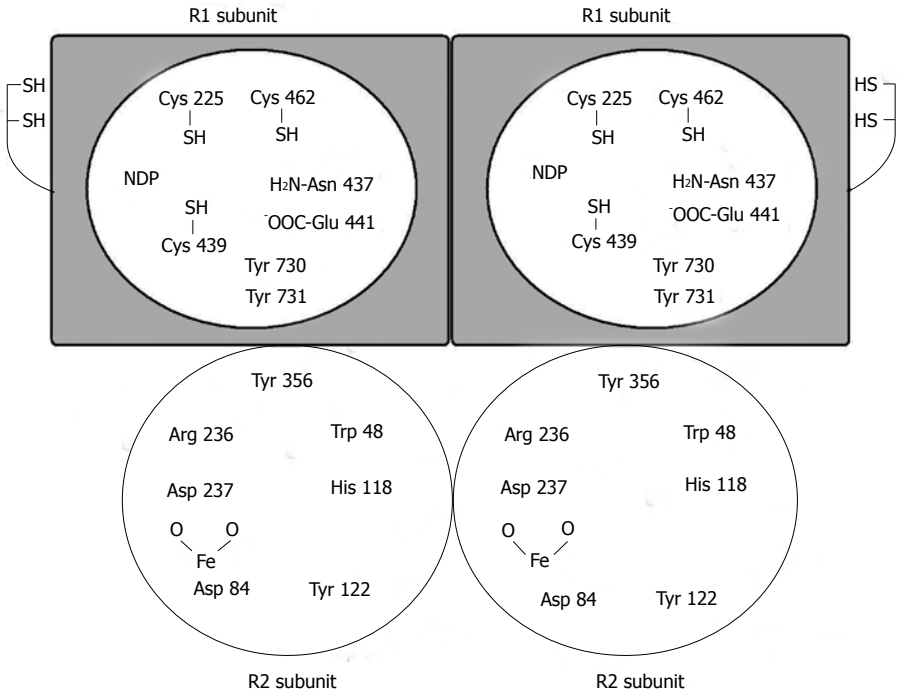

Figure 2 Subunit organization of ribonucleotide reductase complex. Amino acids are shown with *E. coli* numbering which are crucial for the radical transfer and ribonucleotide reductase (RNR) catalysis. The R2 subunit contains the iron-oxygen cluster (Fe-O-Fe) which reacts with dioxygen to generate a stable tyrosyl radical in Tyr 122 required for the RNR catalysis. The radical transfer pathway from Tyr 122 to the active-site Cys 439 in R1 subunit involves the network of Asp 84, His 118, Asp 237, Trp 48, Tyr 356 in R2 subunit and Tyr 730, Tyr 731 in R1 subunit[2-5]. The Cys 225, Cys 462, Asn 437 and Glu 441 residues are involved in binding the substrate nucleoside diphosphate (NDP) in R1 subunit. During the catalysis, the disulfide bond between Cys 225 and Cys 462 is reduced by the C-terminal shuttle dithiols[2-5]. The figure is adapted and modified from Holmgren *et al*[4].

system containing flavoredoxin, NADPH and NADPH-flavoredoxin reductase. Then the glycyl radical generates a thiyl radical required for the catalysis.

TRX AND GRX SYSTEM

Trx is a class of 12 kDa ubiquitous redox proteins found primarily in the cytosol[13]. Trxs possess a catalytically active dithiol function in a Cys-Gly-Pro-Cys motif and are present in all organisms. The complete mammalian Trx system comprising Trx, the selenoenzyme thioredoxin reductase (TrxR) and NADPH also plays a crucial role in redox signaling and thiol homeostasis of cells. Cytosolic Trx1 and mitochondrial Trx2 regulate several metabolic pathways, oxidative/nitrosative stress defence, apoptosis and DNA synthesis[4,13-15]. On the other hand, Grxs are small redox enzymes of approximately 10 kDa and they participate in thiol-disulfide exchange reactions in the presence of glutathione (GSH), glutathione reductase (GR) and NADPH. In the Grx system, Grx is reduced *via* GSH[16]. The glutathione disulfide formed is then reduced by GR and NADPH. Grxs are involved in redox signaling and maintenance of cellular redox environment. Moreover, the maintenance of cytosolic and mitochondrial iron homeostasis have been found to be linked to Grxs[16,17].

Several findings support the redundancy between cytosolic Trx and Grx systems to provide a backup for each other. Yeast and bacterial strains can survive in the absence of either of the disulphide reductase pathways[18]. Moreover, GSH synthesis was found to be essential for mouse development[19], whereas the deletion of Trx gene was reported to be lethal for mouse embryo[20]. Loss of TrxR1 showed no effect on the normal replicative potential[21]. However, the survival of TrxR1-deficient tumor cells was found to be very much dependent on GSH[22]. In a recent study, it has also been shown that the GSH/Grx system can reduce Trx1 in TrxR1-deficient HeLa cells[23].

ROLE OF TRX AND GRX AS EXTERNAL ELECTRON DONORS FOR RNR

Trxs and Grxs belong to related families of low molecular weight redox enzymes catalyzing thiol-disulfide exchange reactions with catalytically active cysteine thiols in a CXXC active site[13,16]. For class I and II RNR enzymes, the electrons are supplied by NADPH through Trx or Grx systems[2-5]. Reduction of ribonucleotide in the RNR catalysis involves the formation of a disulfide in the active site of R1 subunit. Structural studies with *E. coli* RNR show that the active site cleft of the R1 subunit is not very wide to permit the direct reduction by the external redoxin system(s)[3,24]. However, the reduction of active site disulfide is performed by a pair of shuttle cysteine residues in the C-terminal mobile tail of R1 subunit (Figure 3)[4,25,26]. The C-terminal shuttle dithiols of *E. coli* R1 subunit has the CXXXXC sequence; whereas yeast and mammalian R1 has a CXXC sequence. *In vitro*

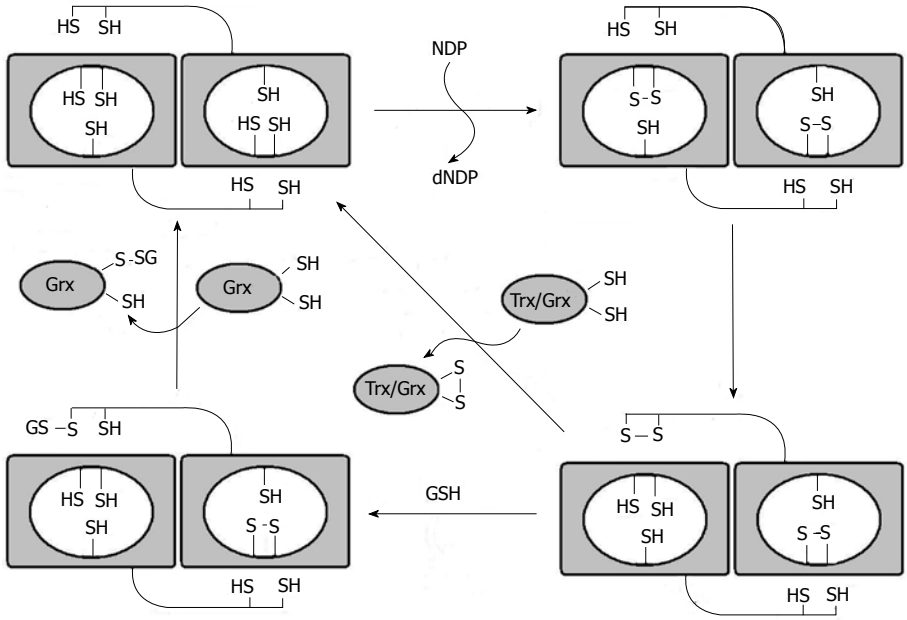

Figure 3 The mechanistic model for the role of thioredoxins and glutaredoxins for the ribonucleotide reductase catalysis. After the completion of one turnover cycle of ribonucleotide reductase (RNR) catalysis, a disulfide bond is formed between the conserved cysteine pair at the active site (shown in the circle). Shuttle dithiol function present at the C-terminal CXXC motif of the neighboring subunit reduces the disulfide bond through disulfide-exchange. Then, the resulting disulfide bond at the C-terminal tail is reduced by the thioredoxin/glutaredoxin (Trx/Grx) systems resulting in an active R1 to continue the next cycle of RNR catalysis. The Grx system can also reduce the C-terminal thiols by the glutathionylation mechanism[4,25,26]. For simplicity, only the reduction of active site of one subunit by the C-terminal shuttle dithiols of the neighboring subunit is shown in the diagram. The figure is adapted and modified from Holmgren *et al*[4].

mutagenesis and kinetic studies support a critical role for the C-terminal cysteine pair of R1 in regeneration of the active site[27,28]. The disulfide exchange reaction results in the formation of a disulfide in the C-terminal tail of R1. Then, the external redoxin systems reduce the disulfide bond to continue the next cycle of RNR catalysis.

Trx and Grx systems were found to act as dithiol electron donors of *E. coli* RNR[29,30]. Recently, Gustafsson *et al*[31] characterized the Trx1 system as the physiologically relevant electron donor for RNR in *Bacillus anthracis*. In *E. coli*, the class I a enzyme requires the dithiol form of at least one of Grx1, Trx1 or Trx2 to be viable[32-34]. The Grx1 system showed 10-fold lower k_m value compared to that of Trx1 system, while both of the redoxins had similar v_{max} (Table 1). This makes Grx1 the most efficient electron donor for the *E. coli* enzyme. However, there is a mechanistic difference between the *E. coli* and the mammalian RNR catalysis involving Trx1 and Grx1 systems as electron donors[26]. Trx1 and Grx1 system showed similar catalytic efficiencies (k_{cat}/k_m) with recombinant mouse RNR complex (Figure 4 and Table 1). In the presence of 4 mmol/L GSH, the Grx1 system showed a higher affinity compared to Trx1 and displayed a higher apparent k_{cat}. The RNR activity with the Grx system was found to be very much dependent on the concentrations of GSH. Here, it is noteworthy to mention that mammalian cells have significantly high concentrations of GSH (5-20 mmol/L)[35]. Moreover, the ability of the monothiol mutant of Grx2 to maintain RNR catalysis demonstrates a glutathionylation mechanism for Grx catalysis in contrast to the dithiol mechanism for the Trx system[26]. However, the *E. coli* RNR complex showed no activity with the monothiol mutant of bacterial Grx1 suggesting the involvement of a dithiol-disulfide mechanism for the catalysis[36].

The advantage of a glutathionylation mechanism may be with the very low levels of R1 involved in the repair and production of dNTPs for mitochondrial DNA synthesis. Trx is present at low levels in many resting postmitotic cells. The sigmoidal curve of Trx activity showed that reduced Trx could not be efficient with a low concentration of R1 in postmitotic cells[26]. The high concentration of GSH[35] would ensure that there is glutathionylated R1 and then monothiol/dithiol Grx should be able to catalyze reduction of the C-terminal disulfide. Several studies reported that the rapidly proliferating cells have increased GSH concentration, while a decrease in GSH concentration limits cell proliferation. GSH acts as a key regulator of cell proliferation and thus the colocalization of GSH with nuclear DNA was observed in proliferating cells[37]. In mammary carcinoma cells, the depletion of glutathione was found to inhibit DNA synthesis[38]. A similar study with 3T3 fibroblast cells showed a significant correlation between progression of cell cycle and the distribution of nuclear GSH[39]. Moreover, accumulation of DNA damage was found in liver, kidney and lung of mice deficient in γ-glutamyl transpeptidase, the enzyme responsible for initiating the catabolism of GSH[40]. In another study, down-regulation of TrxR showed no effects on the dNTP pools in malignant mouse cells[41]. This suggests the role of the GSH/Grx system as an alternative pathway used by the RNR in tumor cells. Moreover, a study in mouse hepatocytes, suggested the importance of a TrxR-independent pathway for the supply of electrons

Table 1 Kinetic parameters of thioredoxin 1, glutaredoxins (1, 2, 2C40S) for reduction of mouse ribonucleotide reductase complex

Electron donor system	V_{max} (nmol/L per second per/ microgram of R1)	k_m (μmol/L)
Trx1	22.2	1.90
Grx1	2.3	0.18
Grx2	1.5	0.30
Grx2C40S	1.5	0.36

Data adapted from Zahedi Avval *et al*[26]. Trx: Thioredoxin; Grx: Glutaredoxins.

to RNR[21]. These studies clearly show the crucial role of the GSH/Grx system in DNA repair *via* RNR catalysis.

ROLE OF TRX FOR CLASS Ⅲ RNR

The class Ⅲ RNR (present in strict and facultative anaerobes) forms an inactive $\alpha_2\beta_2$ complex in resting state[2-5]. The cysteine residues present in the C-terminus of protein α were found to be responsible for the formation of glycyl radical and to participate in radical transfer reactions during enzyme activation. Under the reducing condition, the small β subunit can activate several α proteins. The Trx system was found to activate the enzyme with the same efficiency as dithiothreitol (DTT)[42]. The data suggests that the Trx system keeps the conserved cysteines of the C-terminus of the α-polypeptide in a reduced form which is required for radical generation. Therefore, Trx acts only for the activation of the class Ⅲ RNR. Later, a structural study of the homologous enzyme from bacteriophage T4 revealed the presence of zinc bound to four conserved cysteine residues[43]. It was also shown that the Trx system or DTT is dispensable for the formation of the glycyl radical with the fully Zn-loaded RNR. The radical transfer from glycine to the active-site cysteine to the substrate is controlled by a crucial hydrogen-bond network. Thus, the suggested role of the Trx system (or DTT) was to facilitate the recognition of the network and allow efficient radical transfer.

CONCLUSION

For several years, the RNR inhibitors have been used to treat cancers and viral infections[6,7,44,45]. Most of the RNR inhibitors are either radical scavengers (hydroxyurea) or metal chelators (triapine) which specifically inactivate the R2 subunit[46,47]. On the other hand, several nucleoside analogs and sulfhydryl group inhibitors (such as cisplatin, caracemide, chlorambucil, *etc*.) are used as R1-specific inhibitors[48-50]. Gene expression silencers and R1-R2 polymerization inhibitors (oligopeptides) have also been used to block RNR activity[51-55]. In recent years, many new strategies have emerged in the designing of subunit-specific and more effective RNR inhibitors. Redox regulation of RNR catalysis plays a vital role as the RNR catalysis involves different redox active thiol functions, thiyl radicals and thiol proteins of the Trx superfamily.

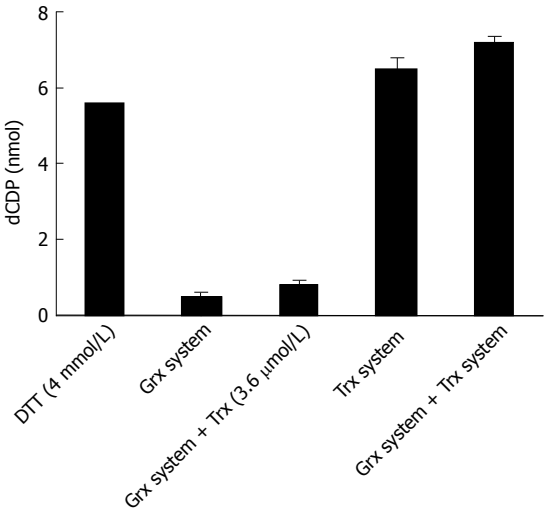

Figure 4 Activity profile of mouse ribonucleotide reductase in the presence of the thioredoxin and glutaredoxins system. Mouse R1 (120 μg/mL) and R2 (40 μg/mL) were assayed with dithiothreitol, thioredoxin (Trx) and glutaredoxin (Grx) systems. The Trx system contained 3.6 μmol/L Trx1, NADPH and TrxR. The Grx system contained 1 μmol/L Grx1, 4 mmol/L glutathione , NADPH and glutathione reductase. Combinations of 3.6 μmol/L Trx1 or the whole Trx system with the Grx system were also monitored (data adapted from Zahedi Avval *et al*[26]).

Therefore, further investigations on the Trx/Grx-mediated redox regulation of RNR catalysis will contribute significantly to design and develop new RNR inhibitors for improved cancer chemotherapy and antiviral treatments.

REFERENCES

1 **Holmgren A**, Reichard P, Thelander L. Enzymatic synthesis of deoxyribonucleotides, 8. The effects of ATP and dATP in the CDP reductase system from E. coli. *Proc Natl Acad Sci USA* 1965; **54**: 830-836 [PMID: 5324398 DOI: 10.1073/pnas.54.3.830]
2 **Jordan A**, Reichard P. Ribonucleotide reductases. *Annu Rev Biochem* 1998; **67**: 71-98 [PMID: 9759483 DOI: 10.1146/annurev.biochem.67.1.71]
3 **Nordlund P**, Reichard P. Ribonucleotide reductases. *Annu Rev Biochem* 2006; **75**: 681-706 [PMID: 16756507 DOI: 10.1146/annurev.biochem.75.103004.142443]
4 **Holmgren A**, Sengupta R. The use of thiols by ribonucleotide reductase. *Free Radic Biol Med* 2010; **49**: 1617-1628 [PMID: 20851762 DOI: 10.1016/j.freeradbiomed.2010.09.005]
5 **Kolberg M**, Strand KR, Graff P, Andersson KK. Structure, function, and mechanism of ribonucleotide reductases. *Biochim Biophys Acta* 2004; **1699**: 1-34 [PMID: 15158709]
6 **Kwon NS**, Stuehr DJ, Nathan CF. Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *J Exp Med* 1991; **174**: 761-767 [PMID: 1717630]
7 **Shao J**, Liu X, Zhu L, Yen Y. Targeting ribonucleotide reductase for cancer therapy. *Expert Opin Ther Targets* 2013; **17**: 1423-1437 [PMID: 24083455 DOI: 10.1517/14728222.2013.840293]
8 **Håkansson P**, Hofer A, Thelander L. Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem* 2006; **281**: 7834-7841 [PMID: 16436374]
9 **Logan DT**. Closing the circle on ribonucleotide reductases. *Nat Struct Mol Biol* 2011; **18**: 251-253 [PMID: 21372851 DOI: 10.1038/nsmb0311-251]
10 **Tanaka H**, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K, Takei Y, Nakamura Y. A ribonucleotide reductase

gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* 2000; **404**: 42-49 [PMID: 10716435]
11 **Thelander L**. Ribonucleotide reductase and mitochondrial DNA synthesis. *Nat Genet* 2007; **39**: 703-704 [PMID: 17534360]
12 **Bourdon A**, Minai L, Serre V, Jais JP, Sarzi E, Aubert S, Chrétien D, de Lonlay P, Paquis-Flucklinger V, Arakawa H, Nakamura Y, Munnich A, Rötig A. Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. *Nat Genet* 2007; **39**: 776-780 [PMID: 17486094]
13 **Lillig CH**, Holmgren A. Thioredoxin and related molecules--from biology to health and disease. *Antioxid Redox Signal* 2007; **9**: 25-47 [PMID: 17115886]
14 **Sengupta R**, Holmgren A. Thioredoxin and thioredoxin reductase in relation to reversible S-nitrosylation. *Antioxid Redox Signal* 2013; **18**: 259-269 [PMID: 22702224 DOI: 10.1089/ars.2012.4716]
15 **Sengupta R**, Holmgren A. The role of thioredoxin in the regulation of cellular processes by S-nitrosylation. *Biochim Biophys Acta* 2012; **1820**: 689-700 [PMID: 21878369 DOI: 10.1016/j.bbagen.2011.08.012]
16 **Lillig CH**, Berndt C, Holmgren A. Glutaredoxin systems. *Biochim Biophys Acta* 2008; **1780**: 1304-1317 [PMID: 18621099 DOI: 10.1016/j.bbagen.2008.06.003]
17 **Johansson C**, Roos AK, Montano SJ, Sengupta R, Filippakopoulos P, Guo K, von Delft F, Holmgren A, Oppermann U, Kavanagh KL. The crystal structure of human GLRX5: iron-sulfur cluster co-ordination, tetrameric assembly and monomer activity. *Biochem J* 2011; **433**: 303-311 [PMID: 21029046 DOI: 10.1042/BJ20101286]
18 **Trotter EW**, Grant CM. Non-reciprocal regulation of the redox state of the glutathione-glutaredoxin and thioredoxin systems. *EMBO Rep* 2003; **4**: 184-188 [PMID: 12612609]
19 **Shi ZZ**, Osei-Frimpong J, Kala G, Kala SV, Barrios RJ, Habib GM, Lukin DJ, Danney CM, Matzuk MM, Lieberman MW. Glutathione synthesis is essential for mouse development but not for cell growth in culture. *Proc Natl Acad Sci USA* 2000; **97**: 5101-5106 [PMID: 10805773]
20 **Matsui M**, Oshima M, Oshima H, Takaku K, Maruyama T, Yodoi J, Taketo MM. Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Dev Biol* 1996; **178**: 179-185 [PMID: 8812119]
21 **Rollins MF**, van der Heide DM, Weisend CM, Kundert JA, Comstock KM, Suvorova ES, Capecchi MR, Merrill GF, Schmidt EE. Hepatocytes lacking thioredoxin reductase 1 have normal replicative potential during development and regeneration. *J Cell Sci* 2010; **123**: 2402-2412 [PMID: 20571049 DOI: 10.1242/jcs.068106]
22 **Mandal PK**, Schneider M, Kölle P, Kuhlencordt P, Förster H, Beck H, Bornkamm GW, Conrad M. Loss of thioredoxin reductase 1 renders tumors highly susceptible to pharmacologic glutathione deprivation. *Cancer Res* 2010; **70**: 9505-9514 [PMID: 21045148 DOI: 10.1158/0008-5472.CAN-10-1509]
23 **Du Y**, Zhang H, Lu J, Holmgren A. Glutathione and glutaredoxin act as a backup of human thioredoxin reductase 1 to reduce thioredoxin 1 preventing cell death by aurothioglucose. *J Biol Chem* 2012; **287**: 38210-38219 [PMID: 22977247 DOI: 10.1074/jbc.M112.392225]
24 **Uhlin U**, Eklund H. Structure of ribonucleotide reductase protein R1. *Nature* 1994; **370**: 533-539 [PMID: 8052308]
25 **Zhang Z**, Yang K, Chen CC, Feser J, Huang M. Role of the C terminus of the ribonucleotide reductase large subunit in enzyme regeneration and its inhibition by Sml1. *Proc Natl Acad Sci USA* 2007; **104**: 2217-2222 [PMID: 17277086]
26 **Zahedi Avval F**, Holmgren A. Molecular mechanisms of thioredoxin and glutaredoxin as hydrogen donors for Mammalian s phase ribonucleotide reductase. *J Biol Chem* 2009; **284**: 8233-8240 [PMID: 19176520 DOI: 10.1074/jbc.M809338200]
27 **Aberg A**, Hahne S, Karlsson M, Larsson A, Ormö M, Ahgren A, Sjöberg BM. Evidence for two different classes of redox-active cysteines in ribonucleotide reductase of Escherichia coli. *J Biol Chem* 1989; **264**: 12249-12252 [PMID: 2663852]
28 **Lin AN**, Ashley GW, Stubbe J. Location of the redox-active thiols of ribonucleotide reductase: sequence similarity between the Escherichia coli and Lactobacillus leichmannii enzymes. *Biochemistry* 1987; **26**: 6905-6909 [PMID: 3322391]
29 **Laurent TC**, Moore ec, reichard p. enzymatic synthesis of deoxyribonucleotides. IV. Isolation and characterization of thioredoxin, the hydrogen donor from escherichia coli b. *J Biol Chem* 1964; **239**: 3436-3444 [PMID: 14245400]
30 **Holmgren A**. Hydrogen donor system for Escherichia coli ribonucleoside-diphosphate reductase dependent upon glutathione. *Proc Natl Acad Sci USA* 1976; **73**: 2275-2279 [PMID: 7783]
31 **Gustafsson TN**, Sahlin M, Lu J, Sjöberg BM, Holmgren A. Bacillus anthracis thioredoxin systems, characterization and role as electron donors for ribonucleotide reductase. *J Biol Chem* 2012; **287**: 39686-39697 [PMID: 23012357 DOI: 10.1074/jbc.M112.413427]
32 **Gallardo-Madueño R**, Leal JF, Dorado G, Holmgren A, López-Barea J, Pueyo C. In vivo transcription of nrdAB operon and of grxA and fpg genes is triggered in Escherichia coli lacking both thioredoxin and glutaredoxin 1 or thioredoxin and glutathione, respectively. *J Biol Chem* 1998; **273**: 18382-18388 [PMID: 9660805]
33 **Prieto-Alamo MJ**, Jurado J, Gallardo-Madueno R, Monje-Casas F, Holmgren A, Pueyo C. Transcriptional regulation of glutaredoxin and thioredoxin pathways and related enzymes in response to oxidative stress. *J Biol Chem* 2000; **275**: 13398-13405 [PMID: 10788450]
34 **Gon S**, Faulkner MJ, Beckwith J. In vivo requirement for glutaredoxins and thioredoxins in the reduction of the ribonucleotide reductases of Escherichia coli. *Antioxid Redox Signal* 2006; **8**: 735-742 [PMID: 16771665]
35 **Meister A**. Glutathione metabolism and its selective modification. *J Biol Chem* 1988; **263**: 17205-17208 [PMID: 3053703]
36 **Bushweller JH**, Billeter M, Holmgren A, Wüthrich K. The nuclear magnetic resonance solution structure of the mixed disulfide between Escherichia coli glutaredoxin(C14S) and glutathione. *J Mol Biol* 1994; **235**: 1585-1597 [PMID: 8107093]
37 **Vivancos PD**, Dong Y, Ziegler K, Markovic J, Pallardó FV, Pellny TK, Verrier PJ, Foyer CH. Recruitment of glutathione into the nucleus during cell proliferation adjusts whole-cell redox homeostasis in Arabidopsis thaliana and lowers the oxidative defence shield. *Plant J* 2010; **64**: 825-838 [PMID: 21105929 DOI: 10.1111/j.1365-313X.2010.04371.x]
38 **Dethlefsen LA**, Lehman CM, Biaglow JE, Peck VM. Toxic effects of acute glutathione depletion by buthionine sulfoximine and dimethylfumarate on murine mammary carcinoma cells. *Radiat Res* 1988; **114**: 215-224 [PMID: 3375425]
39 **Markovic J**, Mora NJ, Broseta AM, Gimeno A, de-la-Concepción N, Viña J, Pallardó FV. The depletion of nuclear glutathione impairs cell proliferation in 3t3 fibroblasts. *PLoS One* 2009; **4**: e6413 [PMID: 19641610 DOI: 10.1371/journal.pone.0006413]
40 **Rojas E**, Valverde M, Kala SV, Kala G, Lieberman MW. Accumulation of DNA damage in the organs of mice deficient in gamma-glutamyltranspeptidase. *Mutat Res* 2000; **447**: 305-316 [PMID: 10751614]
41 **Yoo MH**, Xu XM, Carlson BA, Patterson AD, Gladyshev VN, Hatfield DL. Targeting thioredoxin reductase 1 reduction in cancer cells inhibits self-sufficient growth and DNA replication. *PLoS One* 2007; **2**: e1112 [PMID: 17971875]
42 **Padovani D**, Mulliez E, Fontecave M. Activation of class III ribonucleotide reductase by thioredoxin. *J Biol Chem* 2001; **276**: 9587-9589 [PMID: 11266436]
43 **Logan DT**, Mulliez E, Larsson KM, Bodevin S, Atta M, Garnaud PE, Sjoberg BM, Fontecave M. A metal-binding site in the catalytic subunit of anaerobic ribonucleotide reductase. *Proc Natl Acad Sci USA* 2003; **100**: 3826-3831 [PMID:

12655046]
44 **Cerqueira NM**, Fernandes PA, Ramos MJ. Ribonucleotide reductase: a critical enzyme for cancer chemotherapy and antiviral agents. *Recent Pat Anticancer Drug Discov* 2007; **2**: 11-29 [PMID: 18221051]
45 **Shao J**, Zhou B, Chu B, Yen Y. Ribonucleotide reductase inhibitors and future drug design. *Curr Cancer Drug Targets* 2006; **6**: 409-431 [PMID: 16918309]
46 **Osterman Golkar S**, Czene S, Gokarakonda A, Haghdoost S. Intracellular deoxyribonucleotide pool imbalance and DNA damage in cells treated with hydroxyurea, an inhibitor of ribonucleotide reductase. *Mutagenesis* 2013; **28**: 653-660 [PMID: 24078015 DOI: 10.1093/mutage/get044]
47 **Aye Y**, Long MJ, Stubbe J. Mechanistic studies of semicarbazone triapine targeting human ribonucleotide reductase in vitro and in mammalian cells: tyrosyl radical quenching not involving reactive oxygen species. *J Biol Chem* 2012; **287**: 35768-35778 [PMID: 22915594 DOI: 10.1074/jbc.M112.396911]
48 **Piulats JM**, Jiménez L, García del Muro X, Villanueva A, Viñals F, Germà-Lluch JR. Molecular mechanisms behind the resistance of cisplatin in germ cell tumours. *Clin Transl Oncol* 2009; **11**: 780-786 [PMID: 20045784]
49 **Larsen IK**, Cornett C, Karlsson M, Sahlin M, Sjöberg BM. Caracemide, a site-specific irreversible inhibitor of protein R1 of Escherichia coli ribonucleotide reductase. *J Biol Chem* 1992; **267**: 12627-12631 [PMID: 1618768]
50 **Hurta RA**, Wright JA. Alterations in the activity and regulation of mammalian ribonucleotide reductase by chlorambucil, a DNA damaging agent. *J Biol Chem* 1992; **267**: 7066-7071 [PMID: 1551913]
51 **Lee Y**, Vassilakos A, Feng N, Jin H, Wang M, Xiong K, Wright J, Young A. GTI-2501, an antisense agent targeting R1, the large subunit of human ribonucleotide reductase, shows potent anti-tumor activity against a variety of tumors. *Int J Oncol* 2006; **28**: 469-478 [PMID: 16391803]
52 **Zhang M**, Wang J, Yao R, Wang L. Small interfering RNA (siRNA)-mediated silencing of the M2 subunit of ribonucleotide reductase: a novel therapeutic strategy in ovarian cancer. *Int J Gynecol Cancer* 2013; **23**: 659-666 [PMID: 23466567 DOI: 10.1097/IGC.0b013e318287e2b3]
53 **Ferdinandi ES**, Vassilakos A, Lee Y, Lightfoot J, Fitsialos D, Wright JA, Young AH. Preclinical toxicity and toxicokinetics of GTI-2040, a phosphorothioate oligonucleotide targeting ribonucleotide reductase R2. *Cancer Chemother Pharmacol* 2011; **68**: 193-205 [PMID: 20886212 DOI: 10.1007/s00280-010-1473-z]
54 **Ohrström M**, Popović-Bijelić A, Luo J, Stenmark P, Högbom M, Gräslund A. Inhibition of chlamydial class Ic ribonucleotide reductase by C-terminal peptides from protein R2. *J Pept Sci* 2011; **17**: 756-762 [PMID: 21976435 DOI: 10.1002/psc.1399]
55 **Cooperman BS**, Gao Y, Tan C, Kashlan OB, Kaur J. Peptide inhibitors of mammalian ribonucleotide reductase. *Adv Enzyme Regul* 2005; **45**: 112-125 [PMID: 16054677]

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

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Books

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Conference paper

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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REVIEW

Deubiquitinating enzyme regulation of the p53 pathway: A lesson from Otub1

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Abstract

Deubiquitination has emerged as an important mechanism of p53 regulation. A number of deubiquitinating enzymes (DUBs) from the ubiquitin-specific protease family have been shown to regulate the p53-MDM2-MDMX networks. We recently reported that Otub1, a DUB from the OTU-domain containing protease family, is a novel p53 regulator. Interestingly, Otub1 abrogates p53 ubiquitination and stabilizes and activates p53 in cells independently of its deubiquitinating enzyme activity. Instead, it does so by inhibiting the MDM2 cognate ubiquitin-conjugating enzyme (E2) UbcH5. Otub1 also regulates other biological signaling through this non-canonical mechanism, suppression of E2, including the inhibition of DNA-damage-induced chromatin ubiquitination. Thus, Otub1 evolves as a unique DUB that mainly suppresses E2 to regulate substrates. Here we review the current progress made towards the understanding of the complex regulation of the p53 tumor suppressor pathway by DUBs, the biological function of Otub1 including its positive regulation of p53, and the mechanistic insights into how Otub1 suppresses E2.

Key words: p53; MDM2; Ubiquitination; Deubiquitinating enzymes; Otub1; Cell cycle; Apoptosis

Core tip: p53 is tightly regulated by dynamic ubiquitination and deubiquitination. A number of deubiquitinating enzymes (DUBs) have been shown to regulate p53 stability and activity by either directly deubiquitinating p53 or indirectly deubiquitinating its regulators. We recently discovered that Otub1, an OTU family DUB, stabilizes and activates p53 *via* distinct and non-canonical mechanism wherein it suppresses the MDM2 cognate ubiquitin-conjugating enzymes UbcH5. Here we review the current progress made towards the understanding of the Otub1 functions as a potent E2 inhibitor and the underlying mechanisms.

Sun XX, Dai MS. Deubiquitinating enzyme regulation of the p53 pathway: A lesson from Otub1. *World J Biol Chem* 2014; 5(2): 75-84 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i2/75.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i2.75

MDM2 AND MDMX: KEEPING P53 UNDER CONTROL

The p53 tumor suppressor plays a central role in maintaining the genomic stability and preventing the organism from cancer[1-3]. Loss of p53 function, either through direct mutations in the *p53* gene or indirectly through alterations in the p53 regulatory networks, is associated with most, if not all, human cancers[4,5]. Germline mutations of *p53* result in the cancer-prone Li-Fraumeni

syndrome in human[6] and deletion of the *p53* gene leads to spontaneous tumors in mice[7,8]. p53 is a stress-induced transcription factor that activates or represses the expression of many target genes, thereby executing its anti-proliferative activity by inducing cell cycle arrest, apoptosis, or senescence[1,2,9-11]. Under normal circumstances, p53 is tightly controlled at low levels mainly by its negative regulator MDM2[12-14]. As a RING-finger-containing ubiquitin ligase (E3)[15,16] MDM2 mediates p53 ubiquitination and degradation through the proteasomal system[17,18]. MDM2 also directly suppresses p53 transactivation activity by binding and concealing the N-terminal transactivation domain of p53[19-21]. The centrality of the MDM2-mediated p53 suppression has been demonstrated by mouse genetic studies showing that deletion of the *mdm2* gene caused embryonic lethal phenotype, which is completely rescued by concomitant deletion of *p53*[22,23]. This essential function of MDM2 requires its E3 activity, as mice with homozygous knock-in of the E3 inactivation mutant, $MDM2^{C464A}$, are also embryonic lethal, which can be rescued by deleting p53 as well[24]. Consistently, MDM2 is overexpressed in a number of human cancers, most of which contain wild-type p53[25-29].

The MDM2 homolog MDMX has emerged as an equally important p53 regulator as MDM2[30]. MDMX shares high homology with MDM2 in their C-terminal RING-finger domain and the N-terminal p53-binding domain. Like MDM2, MDMX binds to the N-terminal transactivation domain of p53 and suppresses its activity. However, MDMX does not have appreciable ubiquitin ligase activity towards p53[31,32], yet it assists MDM2 to suppress p53 function. MDMX directly binds to MDM2 *via* their RING domains[33-35] and renders MDM2 sufficiently stable to ubiquitinate and degrade p53[33,36-38]. Also, MDMX suppresses p53 function by specifically promoting p53-induced MDM2 transcription following DNA damage[39]. MDM2, in turn, ubiquitinates and degrades MDMX in response to DNA damage[40-42]. Thus, the mutual regulation between MDM2 and MDMX ensures a proper cellular level and activity of p53. Supporting the indispensible role of MDMX towards p53, deleting the *p53* gene also rescues the lethal phenotype of knocking out the *mdmx* gene in mice[43-45]. Like MDM2, MDMX is also overexpressed or amplified in several types of human cancers that harbor wild-type p53[46-49]. Recent studies have provided further molecular insights into the non-redundant and indispensible role for MDMX in MDM2-mediated p53 degradation. First, like MDM2, the RING domain of MDMX and resulting MDM2-MDMX heterodimerization are required for the regulation of MDM2, as deletion of the RING-finger domain of MDMX or knock-in of the MDM2-binding defective MDMX mutant (C462A) resulted in embryonic lethal phenotype, which was completely rescued by deletion of *p53*[50,51]. Second, The extreme C-terminal short sequences outside of the RING domain of both MDM2 and MDMX contribute to the MDM2 E3 activity, owing to their role in the formation of MDM2-MDMX heterodimer and perhaps the E3 holoenzyme mediating p53 polyubiquitination[37,38,52]. Third, a recent *in vitro* study has shown that while MDM2 alone is sufficient to mediate multi-monoubiquitination of p53, the MDM2-MDMX complex is required for p53 polyubiquitination[53]. Thus, the stoichiometry of the p53-MDM2-MDMX complex is critical for the determination of whether targeting p53 for polyubiquitination or monoubiquitination.

The p53-MDM2-MDMX axis is among the most highly regulated pathways. Enormous molecules regulate the interplay among the three proteins in response to diverse stressors, leading to p53 stabilization and consequent activation. These include various post-translational modifications of all three proteins. Ubiquitination plays a key role in controlling the protein stability and activity of all three proteins. Under stress conditions, p53 ubiquitination mediated by MDM2/MDMX is crippled as a result of either dissociation of MDM2/MDMX from p53 or suppression of MDM2/MDMX activity towards p53. For example, DNA damage-mediated phosphorylation of both p53 and MDM2 disrupts their interaction, resulting in p53 stabilization[54-57]. DNA damage also triggers phosphorylation and degradation of MDMX, alleviating its suppressive effect on p53[58-63]. Oncogenic stress induces p53 *via* suppression of MDM2 by ARF[64-68], whereas ribosomal stress induces p53 *via* suppression of MDM2 by a number of ribosomal proteins[69-85]. Again, ARF also promotes MDM2-mediated MDMX degradation[40] and ribosomal stress-induced p53 activation requires MDM2-mediated MDMX degradation[86]. Thus, barricading the inhibition of p53 imposed by MDM2 and MDMX is centrally important for p53 activation in response to most, if not all, stressors. Indeed, both MDM2 and MDMX bind to p53 at its target gene promoters and suppress its transactivation activity[87-89]. Thus, p53 activation is thought to involve the release of such repression, called anti-repression under stress conditions, through diverse posttranslational modifications[90]. In addition, p53 is also ubiquitinated by a number of other ubiquitin ligases such as ARF-BP1[91], PIRH2[92], COP1[93], *etc.*[94,95]. For example, p53, under certain cellular levels, is thought no longer regulated by the MDM2/MDMX complex. Instead, the basal level of p53 is mainly regulated by ARF-BP1. Deletion of ARF-BP1 completely activates p53 in the presence of MDM2[91]. Adding to the complexity of the ubiquitination regulation of the p53 pathway, deubiquitination regulation has recently emerged as an equally important mechanism for p53 control.

REGULATION OF THE P53-MDM2-MDMX PATHWAY BY DEUBIQUITINATING ENZYMES

Like other posttranslational modifications, ubiquitination of p53, MDM2 and MDMX can be reversed through a process called deubiquitination, which is catalyzed by a different class of enzymes called deubiquitinating enzymes (DUBs). The human genome encodes approximately 95 predicted DUBs that are classified into

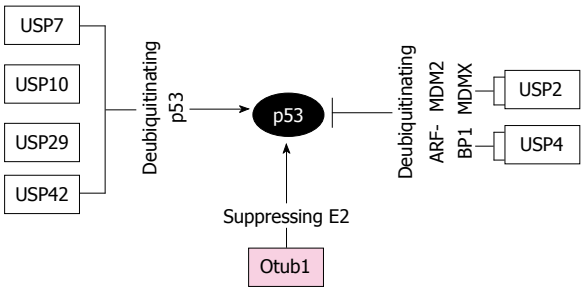

Figure 1 Diagram of the regulation of the p53 pathway by deubiquitinating enzymes. Arrows indicate activation and bars indicate inhibition. USP7, USP10, USP29, and USP42 deubiquitinate and activate p53, whereas USP2 destabilizes p53 by deubiquitinating MDM2 and MDMX and USP4 destabilizes p53 by deubiquitinating and stabilizing ARF-BP1. Otub1 stabilizes and activates p53 *via* non-canonical suppression of the MDM2 cognate E2 UbcH5, thereby inhibiting MDM2-mediated p53 ubiquitination and degradation. USP: Ubiquitin-specific protease

5 families: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumor associated proteases (OTUs), Machado-Joseph disease (or Josephin domain) proteins (MJDs), and JAB1/MPN/MOV34 proteins (JAMMs). Except that the JAMMs are zinc metalloproteases, all other DUBs are cysteine proteases[96,97].

Recently, several DUBs from the USP family have been shown to regulate the p53-MDM2-MDMX loop (Figure 1). USP7, also called herpesvirus associated USP (HAUSP), is the first DUB reported to be a bona fide p53 deubiquitinase[98-100]. Overexpression of USP7 stabilizes and activates p53[99]. Intriguingly, MDM2 seems to be a better substrate of USP7 compared to p53 under physiological circumstances, as substantial knockdown of USP7 results in destabilization of MDM2 and activation of p53[98,101]. Further, USP7 also deubiquitinates MDMX in cells and *in vitro* and depletion of USP7 results in destabilization of the otherwise stable MDMX[100]. DNA damage triggers ATM-dependent phosphorylation of MDMX, which disrupts its binding to USP7 and leads to the consequent increase of ubiquitination and degradation of MDMX[100], whereas the interaction between p53 and USP7 is increased following DNA damage. Thus USP7 scrutinizes the homeostatic levels of p53, MDM2, and MDMX under both normal and stress conditions. The second p53 DUB, USP10, has also been shown to play a critical role in p53 activation following DNA damage[102]. Unlike USP7, USP10 is a cytoplasmic DUB and specifically deubiquitinates p53, but not MDM2 and MDMX[102], reversing MDM2-mediated ubiquitination, nuclear export, and cytoplasmic degradation of p53. Following DNA damage, ATM phosphorylates USP10 at Thr42 and Ser337, resulting in not only the stabilization of USP10, but also the translocation of a fraction of USP10 into the nucleus to deubiquitinate and activate p53. Consistent with its function in regulating p53, USP10 expression is down-regulated in high percentage of clear cell carcinomas[102]. Recently, USP42 was reported to be another DUB that positively regulates p53 stability and activity. Interestingly, USP42 deubiquitinates p53 only during the early stages of stress response, without significant effect on p53 regulation under unstressed conditions. Despite of this, it has been shown that USP42 is required for rapid p53 activation and cell cycle arrest in response to mild or transient DNA damage stress[103]. In addition, Liu *et al*[104] has shown that USP29 positively regulates p53 stability and function following oxidative stress. This is achieved by the increased transcription of USP29 induced by oxidative stress, which in turn cleaves polyubiquitinated p53, leading to p53-dependent apoptosis in cells.

In contrast to above USPs positively regulating p53, USP2a and USP4 were reported to destabilize p53 and suppress p53 function, albeit *via* targeting different p53 E3s. USP2a destabilizes p53 by deubiquitinating and stabilizing both MDM2[105] and MDMX[106], whereas USP4 destabilizes p53 by deubiquitinating and stabilizing ARF-BP1[107]. Consistently, USP2a is overexpressed in a subset of prostate cancers[108,109], whereas USP4 is overexpressed in a broad range of human cancers[107]. Thus, USP2a and USP4 are likely oncogenic DUBs.

Together, these studies demonstrate that deubiquitination plays a crucial role in finely tuning the normal homeostasis of the p53-MDM2-MDMX loop as well as its response to stress. They also imply that different DUBs could regulate the p53 pathway *via* different mechanisms within different cellular compartments following different stress. However, whether p53 is regulated by DUBs other than USP family members is previously unknown. We recently identified that the OTU domain-containing ubiquitin aldehyde-binding proteins 1 (Otubain 1, Otub1 thereafter), an OTU family DUB, controls p53 stability and activity *via* a novel non-canonical mechanism[110].

OTUB1: A UNIQUE MEMBER OF OTU DUB FAMILY

Otub1 was identified along with its close homolog Otub2 by affinity purification using the DUB-specific inhibitor, Ub aldehyde[111]. Subsequent studies, including our own, revealed that Otub1 possesses *in vitro* deubiquitinating enzyme activity preferentially towards K48-linked polyubiquitin chains[110,112,113]. Like other cysteine proteases, Otub1 contains a catalytic triad consisting of Cys (C) 91, His (H) 265, and Asp (D) 268[112]. However, crystal structure studies demonstrated that Otub1 possesses unique structure features wherein H265 is located distantly from the catalytic C91 and D268 and the access of C91 to ubiquitin is blocked by Glu (E) 214 residue, forming a conformation incompatible with catalysis by typical cysteine proteases[112], implying that the activity of Otub1 may be highly regulated in cells and its activation may be subjected to conformational change (See below). Otub1 is ubiquitously expressed in tested human tissues. A longer isoform called Otub1 ARF (alternative reading frame)-1, resulting from alternative splicing and start codon, is predominantly expressed in peripheral blood mononuclear cells, lymph nodes, spleen, and the tonsils[114]. The function of Otub1 ARF-1 is thought to antagonize the function of Otub1 in cells[114].

Functionally, Otub1 has been implicated in the regula-

tion of immune response, estrogen signaling, DNA damage response, as well as pathogen biology. Soares *et al*[114] first reported that Otub1 regulates $CD4^+$ T cell clonal anergy by enhancing degradation of the ubiquitin ligase called GRAIL (gene related to anergy in lymphocytes) and promoting interleukin 2 production following antigenic stimulation, whereas the Otub1 ARF-1 has an opposite effect. Interestingly, the effect of Otub1 does not depend on its catalytic activity. As a matter of fact, the role of Otub1 in degrading GRAIL is opposite to its predicted role as a DUB[114]. A possible explanation is that Otub1 forms a ternary complex with GRAIL and USP8, another USP family DUB, thereby suppressing the deubiquitination of GRAIL by USP8. In this case, Otub1 may act as an ubiquitin editing protease[114]. Li *et al*[115] reported that Otub1 (and Otub2) mediate virus-induced deubiquitination of TNF receptor-associated factor 3 (TRAF3) and TRAF6, two ubiquitin ligases required for virus-induced Interferon regulatory factor 3 (IRF3) and NF-kB activation, leading to the inhibition of viral-induced production of INFβ. However, whether this effect requires the DUB enzymatic activity of Otub1 is not clear[115]. Further, Otub1 has recently been shown to enhance TGFβ signaling by inhibiting ubiquitination and degradation of SMAD2/3[116]. Otub1 also plays a role in pathogen invasion of the host cells. The *Yersinia*-encoded virulence factor YpkA interacts with and phosphorylates Otub1[117] and recruits the small GTPase RhoA, leading to the stabilization of the active RhoA[118]. Consequently, overexpression of wild-type, but not the C91S mutant, Otub1 increased the susceptibility of host cells to the *Yersinia* evasion[118]. Otub1 has been shown to deubiquitinate and stabilize ERα in chromatin[119], albeit this stabilization results in the inhibition of ERα-mediated transcription. Adding to the complexity, the catalytic mutant Otub1, C91S in which the catalytic C91 is mutated to S, did not abolish Otub1-mediated suppression of ERα activity[119]. Otub1 has been shown to inhibit DNA-damage-induced chromatin ubiquitination, which is also independent of its DUB activity. Instead, Otub1 suppresses RNF168-dependent chromatin polyubiquitination by binding to and inhibiting the RNF168 cognate E2 enzyme UBC13[120]. Recently, Otub1 has been shown to regulate apoptosis by deubiquitinating the cellular inhibitor of apoptosis (c-IAP1)[121].

Together, Otub1 has been implicated in multiple biological processes. In most cases, the effects of Otub1 do not require its DUB activity, such as the regulation of DNA damage-induced chromatin ubiquitination[120], T-cell anergy[114], ERα[119], and SMAD2/3[116], implying a unique model of ubiquitination regulation by a DUB: suppression of the ubiquitin-conjugating enzyme (E2) (see below). Because of this and the fact that it is expressed in most tissues, Otub1 may have a broad function in cells.

OTUB1 IS A NOVEL POSITIVE P53 REGULATOR

We recently found that Otub1 positively regulates the stability and activity of p53[110]. Overexpression of Otub1, but not its close homolog Otub2, markedly stabilizes and activates p53 and induces p53-dependent apoptosis and cell growth inhibition. Interestingly, Otub1 regulation of p53 does not require its catalytic activity, as mutating C91 to either A or S did not abolish the activity of Otub1 to block MDM2-mediated p53 ubiquitination and degradation, to stabilize and activate p53, and to induce p53-dependent cell growth inhibition[110]. Mechanistically, Otub1 suppresses MDM2-mediated p53 ubiquitination by binding to and inhibiting the MDM2 cognate E2 enzyme UbcH5s[110]. This is consistent with the non-canonical role for Otub1 in suppressing DNA damage-induced chromatin ubiquitination by inhibiting UBC13[120]. Therefore, our study further supports that the suppression of substrate ubiquitination through inhibiting cognate E2s by Otub1 represents a unique noncanonical mode of DUB regulation compared to classical cysteine proteases and this may be a general mechanism for Otub1 to regulate the substrate protein ubiquitination and stability.

Consistent with the noncanonical mode of regulation, mutating C91 to either A or S did not abolish the activity of Otub1 to bind to and suppress UbcH5[110]. However, a point mutation of Asp 88 to Ala ($Otub1^{D88A}$) abolished the function of Otub1 to suppress p53 ubiquitination and degradation and this mutant interacts with p53 stronger than wild-type Otub1, indicating this mutation might create a dominant-negative effect. D88 is located closely to the donor ubiquitin-binding surface and thus its mutation would affect the binding of Otub1 to donor ubiquitin conjugated to UbcH5. Although D88 is not located directly in the E2 binding surface, our experimental data revealed that this mutation clearly disrupted the Otub1-E2 interaction in cells[110]. This might be due to the overall structure change after D88 mutation. Supporting this conformational change is that D88A mutant also results in the loss of Otub1's DUB activity.

Our functional studies of the endogenous Otub1 suggest that Otub1 plays an important role in p53 stabilization and activation following DNA damage induced by diverse agents. This is consistent, but not completely, with the observation that Otub1 suppresses DNA damage-induced chromatin ubiquitination, thereby suppressing DNA repair pathway[120]. One explanation is that upon DNA damage, Otub1 might target UbcH5-MDM2 to stabilize p53, while it may dissociate from the RNF168-Ubc13 complex, allowing RNF168 to catalyze K63-linked chromatin ubiquitination and subsequent DNA repair response. Whether DNA damage-induced posttranslational modification plays a role in this functional switch remains unclear. However, phosphorylation of Otub1 has been observed at several residues such as T134. Further, it has been shown that the phosphorylation mimicking Otub1 mutant T134E, but not T134A, failed to rescue the DNA damage response in Otub1-depleted cells[122]. Thus it is interesting to examine the signaling pathways involved in the phosphorylation of Otub1 and how this phosphorylation plays a role in regulating Otub1 function in

response to DNA damage stress.

MECHANISTIC INSIGHTS INTO THE NON-CANONICAL SUPPRESSION OF E2 BY OTUB1

Recent biochemical and structural studies have shed a light on how Otub1 suppresses E2s[122-124]. It has been shown that Otub1 preferentially binds to ubiquitin-charged E2[120,122]. Otub1 contains two ubiquitin-binding motifs: a distal site that binds to free ubiquitin and a proximal site that binds to donor ubiquitin conjugated to the active site of an E2 (*e.g.,* Ubc13 or UbcH5). The structure of two ubiquitin binding to Otub1 is reminiscent of that of K48-linked di-ubiquitin[122]. Interestingly, the binding of a free ubiquitin to the distal site allosterically causes the conformational change of Otub1, allowing the formation of a N-terminal ubiquitin-binding helix where the E2-charged donor ubiquitin then binds[122,124]. Consequently, this binding limits the donor ubiquitin interaction with the backside of another E2 and the attack on the thioester bond by an acceptor ubiquitin, a step important for ubiquitin transfer[122,124]. On the other hand, Otub1 also makes contacts with E2 and the Otub1-binding surface in E2 (UbcH5 and Ubc13) overlaps with the E3-binding surface. Thus this Otub1-E2 interaction may also attenuate the E2-E3 engagement[122,124]. Collectively, Otub1 is a potential inhibitor of the E2 enzymes. Further supporting this notion, Otub1 has recently been shown to be a major DUB that interacts with the D and E classes of E2 as well as UbcE2N[125]. Thus disruption of the Otub1-E2 interaction or donor ubiquitin-Otub1 interaction would theoretically abolish Otub1's activity to suppress E2. This could distinguish Otub1's E2 suppressing activity from its DUB enzyme activity. Indeed, several mutants involved in the E2-contacting surface of the Otub1, such as F133A, T134R, F138A, have been shown to lack the E2-suppressing activity but retain the DUB activity[122,124]. Therefore, it is interesting to examine whether these mutants could fail to stabilize and activate p53 in cells. On another note, we recently found that Otub1 can be monoubiquitinated by UbcH5 and this monoubiquitination in turn plays a critical role in the Otub1's E2 suppressing activity. We further found that UbcH5 preferentially binds to monoubiquitinated Otub1, through the ubiquitin interaction with the backside ubiquitin-interacting surface of E2[126]. This binding could potentially disrupt the formation of self-assembled ubiquitin-charged UbcH5 (UbcH5-Ub) conjugates that is critical for ubiquitin transfer, polyubiquitin chain formation and efficient polyubiquitination of substrates[127,128], suggesting another novel mechanism of Otub1 suppression of E2.

CONCLUSION

Recent studies have convincingly demonstrated Otub1 as a unique DUB that executes diverse biology functions by non-canonically suppressing E2 enzymes. Therefore it is expected that Otub1 may play broad functions in cells. One question would be how these broad functions coordinate with each other in cells. We also do not know how Otub1's activity is regulated in cells. Interestingly, a recent observation showed that Otub1 DUB activity can be regulated by UbcH5, which stimulates the binding of the Lys48-linked polyubiquitin substrate by stabilizing the folding of the N-terminal ubiquitin-binding helix of Otub1, thereby promoting its deubiquitinating enzyme activity[129]. It is interesting to know how these mutually regulatory functions are controlled in cells. It is also important to test how Otub1's activity and levels are regulated in cells under physiologic and stress conditions. As Otub1 is a potent activator of p53[110] and plays a role in DNA damage repair[120], Otub1 may act as a tumor suppressor. Thus it is important to determine whether Otub1 is deregulated in human cancers. Gene targeting in mice could provide further information regarding the function of Otub1 and whether Otub1 indeed possesses tumor suppression function *in vivo*. Further characterization of mechanistic insights into the Otub1 suppression of E2 could also be useful for developing strategies that target the E2 enzymes for cancer therapy, *e.g.,* small molecule compounds that resemble Otub1 interaction with E2.

Together, p53 is ubiquitinated by MDM2/MDMX and several other E3s whereas it is deubiquitinated by a number of DUBs, including USP7, USP10, USP29 and USP42. One obvious question is how these multiple DUBs are coordinated to ensure the tight, precise, and dynamic control of p53 stability and activity. Different DUBs may regulate the p53 pathway in response to different cellular stress (*e.g.,* USP29 deubiquitinates p53 in response to oxidative stress[104] whereas USP10 deubiquitinates p53 following DNA damage[102]). Different DUBs may also regulate p53 in different cellular compartments (*e.g.,* USP7 regulates p53 in the nucleus whereas Otub1 regulates p53 in the cytoplasm[110] and USP10 relocates from the cytoplasm to the nucleus to regulate p53 in response to DNA damage[102]). It is interesting to examine whether different DUBs may cooperate with each other to synergistically regulate p53 stability and activity in future studies.

Nevertheless, efforts have been made towards targeting the ubiquitin-proteasome system (UPS) for reactivating p53 in cancer therapy. For example, compounds have been developed to target the p53-MDM2 interaction such as Nutlin-3s[130], the p53-MDMX interaction such as WK298[131], or both such as RO-2443[132]. Targeting DUBs has promising potential as well. For example, the cyano-indenopyrazine derivatives small molecule compounds HBX 41108, HBX 19818, and HBX 28258[133] and P22077[134] were discovered as USP7 inhibitors. For further details about targeting the UPS for cancer therapy, please refer our recent review[135]. Future directions will aim to discover more potent and specific DUB inhibitors that can be used for cancer treatment.

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REFERENCES

1 **Oren M**. Decision making by p53: life, death and cancer. *Cell Death Differ* 2003; **10**: 431-442 [PMID: 12719720]
2 **Vogelstein B**, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000; **408**: 307-310 [PMID: 11099028 DOI: 10.1038/35042675]
3 **Levine AJ**. p53, the cellular gatekeeper for growth and division. *Cell* 1997; **88**: 323-331 [PMID: 9039259]
4 **Hollstein M**, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sørlie T, Hovig E, Smith-Sørensen B, Montesano R, Harris CC. Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res* 1994; **22**: 3551-3555 [PMID: 7937055]
5 **Soussi T**, Dehouche K, Béroud C. p53 website and analysis of p53 gene mutations in human cancer: forging a link between epidemiology and carcinogenesis. *Hum Mutat* 2000; **15**: 105-113 [PMID: 10612830]
6 **Malkin D**. Li-fraumeni syndrome. *Genes Cancer* 2011; **2**: 475-484 [PMID: 21779515 DOI: 10.1177/1947601911413466]
7 **Donehower LA**, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Butel JS, Bradley A. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 1992; **356**: 215-221 [PMID: 1552940 DOI: 10.1038/356215A0]
8 **Donehower LA**, Lozano G. 20 years studying p53 functions in genetically engineered mice. *Nat Rev Cancer* 2009; **9**: 831-841 [PMID: 19776746 DOI: 10.1038/nrc2731]
9 **Levine AJ**, Oren M. The first 30 years of p53: growing ever more complex. *Nat Rev Cancer* 2009; **9**: 749-758 [PMID: 19776744 DOI: 10.1038/nrc2723]
10 **Vousden KH**, Prives C. Blinded by the Light: The Growing Complexity of p53. *Cell* 2009; **137**: 413-431 [PMID: 19410540 DOI: 10.1016/j.cell.2009.04.037]
11 **Vousden KH**, Ryan KM. p53 and metabolism. *Nat Rev Cancer* 2009; **9**: 691-700
12 **Barak Y**, Juven T, Haffner R, Oren M. mdm2 expression is induced by wild type p53 activity. *EMBO J* 1993; **12**: 461-468 [PMID: 8440237]
13 **Wu X**, Bayle JH, Olson D, Levine AJ. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev* 1993; **7**: 1126-1132 [PMID: 8319905]
14 **Picksley SM**, Lane DP. The p53-mdm2 autoregulatory feedback loop: a paradigm for the regulation of growth control by p53? *Bioessays* 1993; **15**: 689-690 [PMID: 7506024]
15 **Fang S**, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem* 2000; **275**: 8945-8951 [PMID: 10722742 DOI: 10.1074/jbc.275.12.8945]
16 26 **Honda R**, Tanaka H, Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* 1997; **420**: 25-27 [PMID: 9450543]
17 **Haupt Y**, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997; **387**: 296-299 [PMID: 9153395]
18 **Kubbutat MH**, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. *Nature* 1997; **387**: 299-303 [PMID: 9153396]
19 **Chen J**, Marechal V, Levine AJ. Mapping of the p53 and mdm-2 interaction domains. *Mol Cell Biol* 1993; **13**: 4107-4114 [PMID: 7686617]
20 **Oliner JD**, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW, Vogelstein B. Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* 1993; **362**: 857-860 [PMID: 8479525]
21 **Momand J**, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992; **69**: 1237-1245 [PMID: 1535557]
22 **Jones SN**, Roe AE, Donehower LA, Bradley A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* 1995; **378**: 206-208 [PMID: 7477327 DOI: 10.1038/378206a0]
23 **Montes de Oca Luna R**, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* 1995; **378**: 203-206 [PMID: 7477326 DOI: 10.1038/378203a0]
24 **Itahana K**, Mao H, Jin A, Itahana Y, Clegg HV, Lindström MS, Bhat KP, Godfrey VL, Evan GI, Zhang Y. Targeted inactivation of Mdm2 RING finger E3 ubiquitin ligase activity in the mouse reveals mechanistic insights into p53 regulation. *Cancer Cell* 2007; **12**: 355-366 [PMID: 17936560]
25 **Bueso-Ramos CE**, Yang Y, deLeon E, McCown P, Stass SA, Albitar M. The human MDM-2 oncogene is overexpressed in leukemias. *Blood* 1993; **82**: 2617-2623 [PMID: 8219216]
26 **Cordon-Cardo C**, Latres E, Drobnjak M, Oliva MR, Pollack D, Woodruff JM, Marechal V, Chen J, Brennan MF, Levine AJ. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res* 1994; **54**: 794-799 [PMID: 8306343]
27 **Dworakowska D**, Jassem E, Jassem J, Peters B, Dziadziuszko R, Zylicz M, Jakóbkiewicz-Banecka J, Kobierska-Gulida G, Szymanowska A, Skokowski J, Roessner A, Schneider-Stock R. MDM2 gene amplification: a new independent factor of adverse prognosis in non-small cell lung cancer (NSCLC). *Lung Cancer* 2004; **43**: 285-295 [PMID: 15165086]
28 **Momand J**, Jung D, Wilczynski S, Niland J. The MDM2 gene amplification database. *Nucleic Acids Res* 1998; **26**: 3453-3459 [PMID: 9671804]
29 **Deb SP**. Cell cycle regulatory functions of the human oncoprotein MDM2. *Mol Cancer Res* 2003; **1**: 1009-1016 [PMID: 14707284]
30 **Shvarts A**, Steegenga WT, Riteco N, van Laar T, Dekker P, Bazuine M, van Ham RC, van der Houven van Oordt W, Hateboer G, van der Eb AJ, Jochemsen AG. MDMX: a novel p53-binding protein with some functional properties of MDM2. *EMBO J* 1996; **15**: 5349-5357 [PMID: 8895579]
31 **Stad R**, Little NA, Xirodimas DP, Frenk R, van der Eb AJ, Lane DP, Saville MK, Jochemsen AG. Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms. *EMBO Rep* 2001; **2**: 1029-1034 [PMID: 11606419 DOI: 10.1093/embo-reports/kve227]
32 **Jackson MW**, Berberich SJ. MdmX protects p53 from Mdm2-mediated degradation. *Mol Cell Biol* 2000; **20**: 1001-1007 [PMID: 10629057 DOI: 10.1128/MCB.20.3.1001-1007.2000]
33 **Gu J**, Kawai H, Nie L, Kitao H, Wiederschain D, Jochemsen AG, Parant J, Lozano G, Yuan ZM. Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. *J Biol Chem* 2002; **277**: 19251-19254 [PMID: 11953423 DOI: 10.1074/jbc.C200150200]
34 **Sharp DA**, Kratowicz SA, Sank MJ, George DL. Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein. *J Biol Chem* 1999; **274**: 38189-38196 [PMID: 10608892 DOI: 10.1074/jbc.274.53.38189]
35 **Tanimura S**, Ohtsuka S, Mitsui K, Shirouzu K, Yoshimura A, Ohtsubo M. MDM2 interacts with MDMX through their RING finger domains. *FEBS Lett* 1999; **447**: 5-9 [PMID: 10218570]
36 **Linares LK**, Hengstermann A, Ciechanover A, Müller S, Scheffner M. HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. *Proc Natl Acad Sci USA* 2003; **100**: 12009-12014 [PMID: 14507994 DOI: 10.1073/pnas.2030930100]
37 **Poyurovsky MV**, Priest C, Kentsis A, Borden KL, Pan ZQ, Pavletich N, Prives C. The Mdm2 RING domain C-terminus is required for supramolecular assembly and ubiquitin li-

gase activity. *EMBO J* 2007; **26**: 90-101 [PMID: 17170710 DOI: 10.1038/sj.emboj.7601465]
38 **Uldrijan S**, Pannekoek WJ, Vousden KH. An essential function of the extreme C-terminus of MDM2 can be provided by MDMX. *EMBO J* 2007; **26**: 102-112 [PMID: 17159902 DOI: 10.1038/sj.emboj.7601469]
39 **Biderman L**, Poyurovsky MV, Assia Y, Manley JL, Prives C. MdmX is required for p53 interaction with and full induction of the Mdm2 promoter after cellular stress. *Mol Cell Biol* 2012; **32**: 1214-1225 [PMID: 22290440 DOI: 10.1128/MCB.06150-11]
40 **Pan Y**, Chen J. MDM2 promotes ubiquitination and degradation of MDMX. *Mol Cell Biol* 2003; **23**: 5113-5121 [PMID: 12860999 DOI: 10.1128/MCB.23.15.5113-5121.2003]
41 **de Graaf P**, Little NA, Ramos YF, Meulmeester E, Letteboer SJ, Jochemsen AG. Hdmx protein stability is regulated by the ubiquitin ligase activity of Mdm2. *J Biol Chem* 2003; **278**: 38315-38324 [PMID: 12874296 DOI: 10.1074/jbc.M213034200]
42 **Kawai H**, Wiederschain D, Yuan ZM. Critical contribution of the MDM2 acidic domain to p53 ubiquitination. *Mol Cell Biol* 2003; **23**: 4939-4947 [PMID: 12832479 DOI: 10.1128/MCB.23.14.4939-4947.2003]
43 **Migliorini D**, Lazzerini Denchi E, Danovi D, Jochemsen A, Capillo M, Gobbi A, Helin K, Pelicci PG, Marine JC. Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. *Mol Cell Biol* 2002; **22**: 5527-5538 [PMID: 12101245 DOI: 10.1128/MCB.22.15.5527-5538.2002]
44 **Parant J**, Chavez-Reyes A, Little NA, Yan W, Reinke V, Jochemsen AG, Lozano G. Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. *Nat Genet* 2001; **29**: 92-95 [PMID: 11528400 DOI: 10.1038/ng714]
45 **Finch RA**, Donoviel DB, Potter D, Shi M, Fan A, Freed DD, Wang CY, Zambrowicz BP, Ramirez-Solis R, Sands AT, Zhang N. mdmx is a negative regulator of p53 activity in vivo. *Cancer Res* 2002; **62**: 3221-3225 [PMID: 12036937]
46 **Danovi D**, Meulmeester E, Pasini D, Migliorini D, Capra M, Frenk R, de Graaf P, Francoz S, Gasparini P, Gobbi A, Helin K, Pelicci PG, Jochemsen AG, Marine JC. Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. *Mol Cell Biol* 2004; **24**: 5835-5843 [PMID: 15199139 DOI: 10.1128/MCB.24.13.5835-5843.2004]
47 **Ramos YF**, Stad R, Attema J, Peltenburg LT, van der Eb AJ, Jochemsen AG. Aberrant expression of HDMX proteins in tumor cells correlates with wild-type p53. *Cancer Res* 2001; **61**: 1839-1842 [PMID: 11280734]
48 **Riemenschneider MJ**, Büschges R, Wolter M, Reifenberger J, Boström J, Kraus JA, Schlegel U, Reifenberger G. Amplification and overexpression of the MDM4 (MDMX) gene from 1q32 in a subset of malignant gliomas without TP53 mutation or MDM2 amplification. *Cancer Res* 1999; **59**: 6091-6096 [PMID: 10626796]
49 **Riemenschneider MJ**, Knobbe CB, Reifenberger G. Refined mapping of 1q32 amplicons in malignant gliomas confirms MDM4 as the main amplification target. *Int J Cancer* 2003; **104**: 752-757 [PMID: 12640683 DOI: 10.1002/ijc.11023]
50 **Huang L**, Yan Z, Liao X, Li Y, Yang J, Wang ZG, Zuo Y, Kawai H, Shadfan M, Ganapathy S, Yuan ZM. The p53 inhibitors MDM2/MDMX complex is required for control of p53 activity in vivo. *Proc Natl Acad Sci USA* 2011; **108**: 12001-12006 [PMID: 21730163 DOI: 10.1073/pnas.1102309108]
51 **Pant V**, Xiong S, Iwakuma T, Quintás-Cardama A, Lozano G. Heterodimerization of Mdm2 and Mdm4 is critical for regulating p53 activity during embryogenesis but dispensable for p53 and Mdm2 stability. *Proc Natl Acad Sci USA* 2011; **108**: 11995-12000 [PMID: 21730132 DOI: 10.1073/pnas.1102241108]
52 **Linke K**, Mace PD, Smith CA, Vaux DL, Silke J, Day CL. Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. *Cell Death Differ* 2008; **15**: 841-848 [PMID: 18219319 DOI: 10.1038/sj.cdd.4402309]
53 **Wang X**, Wang J, Jiang X. MdmX protein is essential for Mdm2 protein-mediated p53 polyubiquitination. *J Biol Chem* 2011; **286**: 23725-23734 [PMID: 21572037 DOI: 10.1074/jbc.M110.213868]
54 **Banin S**, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, Smorodinsky NI, Prives C, Reiss Y, Shiloh Y, Ziv Y. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* 1998; **281**: 1674-1677 [PMID: 9733514 DOI: 10.1126/science.281.5383.1674]
55 **Canman CE**, Lim DS, Cimprich KA, Taya Y, Tamai K, Sakaguchi K, Appella E, Kastan MB, Siliciano JD. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science* 1998; **281**: 1677-1679 [PMID: 9733515 DOI: 10.1126/science.281.5383.1677]
56 **Maya R**, Balass M, Kim ST, Shkedy D, Leal JF, Shifman O, Moas M, Buschmann T, Ronai Z, Shiloh Y, Kastan MB, Katzir E, Oren M. ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes Dev* 2001; **15**: 1067-1077 [PMID: 11331603 DOI: 10.1101/gad.886901]
57 **Siliciano JD**, Canman CE, Taya Y, Sakaguchi K, Appella E, Kastan MB. DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev* 1997; **11**: 3471-3481 [PMID: 9407038 DOI: 10.1101/gad.11.24.3471]
58 **Chen L**, Gilkes DM, Pan Y, Lane WS, Chen J. ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage. *EMBO J* 2005; **24**: 3411-3422 [PMID: 16163388 DOI: 10.1038/sj.emboj.7600812]
59 **Jin Y**, Dai MS, Lu SZ, Xu Y, Luo Z, Zhao Y, Lu H. 14-3-3gamma binds to MDMX that is phosphorylated by UV-activated Chk1, resulting in p53 activation. *EMBO J* 2006; **25**: 1207-1218 [PMID: 16511572 DOI: 10.1038/sj.emboj.7601010]
60 **LeBron C**, Chen L, Gilkes DM, Chen J. Regulation of MDMX nuclear import and degradation by Chk2 and 14-3-3. *EMBO J* 2006; **25**: 1196-1206 [PMID: 16511560 DOI: 10.1038/sj.emboj.7601032]
61 **Okamoto K**, Kashima K, Pereg Y, Ishida M, Yamazaki S, Nota A, Teunisse A, Migliorini D, Kitabayashi I, Marine JC, Prives C, Shiloh Y, Jochemsen AG, Taya Y. DNA damage-induced phosphorylation of MdmX at serine 367 activates p53 by targeting MdmX for Mdm2-dependent degradation. *Mol Cell Biol* 2005; **25**: 9608-9620 [PMID: 16227609 DOI: 10.1128/MCB.25.21.9608-9620.2005]
62 **Pereg Y**, Lam S, Teunisse A, Biton S, Meulmeester E, Mittelman L, Buscemi G, Okamoto K, Taya Y, Shiloh Y, Jochemsen AG. Differential roles of ATM- and Chk2-mediated phosphorylations of Hdmx in response to DNA damage. *Mol Cell Biol* 2006; **26**: 6819-6831 [PMID: 16943424 DOI: 10.1128/MCB.00562-06]
63 **Pereg Y**, Shkedy D, de Graaf P, Meulmeester E, Edelson-Averbukh M, Salek M, Biton S, Teunisse AF, Lehmann WD, Jochemsen AG, Shiloh Y. Phosphorylation of Hdmx mediates its Hdm2- and ATM-dependent degradation in response to DNA damage. *Proc Natl Acad Sci USA* 2005; **102**: 5056-5061 [PMID: 15788536 DOI: 10.1073/pnas.0408595102]
64 **Zhang Y**, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* 1998; **92**: 725-734 [PMID: 9529249]
65 **Tao W**, Levine AJ. P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. *Proc Natl Acad Sci USA* 1999; **96**: 6937-6941 [PMID: 10359817 DOI: 10.1073/pnas.96.12.6937]
66 **Honda R**, Yasuda H. Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J* 1999; **18**: 22-27 [PMID: 9878046 DOI:

10.1093/emboj/18.1.22]
67 **Llanos S**, Clark PA, Rowe J, Peters G. Stabilization of p53 by p14ARF without relocation of MDM2 to the nucleolus. *Nat Cell Biol* 2001; **3**: 445-452 [PMID: 11331871 DOI: 10.1038/35074506]
68 **Midgley CA**, Desterro JM, Saville MK, Howard S, Sparks A, Hay RT, Lane DP. An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. *Oncogene* 2000; **19**: 2312-2323 [PMID: 10822382]
69 **Bhat KP**, Itahana K, Jin A, Zhang Y. Essential role of ribosomal protein L11 in mediating growth inhibition-induced p53 activation. *EMBO J* 2004; **23**: 2402-2412 [PMID: 15152193 DOI: 10.1038/sj.emboj.7600247]
70 **Chen D**, Zhang Z, Li M, Wang W, Li Y, Rayburn ER, Hill DL, Wang H, Zhang R. Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. *Oncogene* 2007; **26**: 5029-5037 [PMID: 17310983 DOI: 10.1038/sj.onc.1210327]
71 **Dai MS**, Lu H. Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. *J Biol Chem* 2004; **279**: 44475-44482 [PMID: 15308643 DOI: 10.1074/jbc.M403722200]
72 **Dai MS**, Zeng SX, Jin Y, Sun XX, David L, Lu H. Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. *Mol Cell Biol* 2004; **24**: 7654-7668 [PMID: 15314173 DOI: 10.1128/MCB.24.17.7654-7668.2004]
73 **Jin A**, Itahana K, O'Keefe K, Zhang Y. Inhibition of HDM2 and activation of p53 by ribosomal protein L23. *Mol Cell Biol* 2004; **24**: 7669-7680 [PMID: 15314174 DOI: 10.1128/MCB.24.17.7669-7680.2004]
74 **Lohrum MA**, Ludwig RL, Kubbutat MH, Hanlon M, Vousden KH. Regulation of HDM2 activity by the ribosomal protein L11. *Cancer Cell* 2003; **3**: 577-587 [PMID: 12842086]
75 **Ofir-Rosenfeld Y**, Boggs K, Michael D, Kastan MB, Oren M. Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26. *Mol Cell* 2008; **32**: 180-189 [PMID: 18951086 DOI: 10.1016/j.molcel.2008.08.031]
76 **Sun XX**, DeVine T, Challagundla KB, Dai MS. Interplay between ribosomal protein S27a and MDM2 protein in p53 activation in response to ribosomal stress. *J Biol Chem* 2011; **286**: 22730-22741 [PMID: 21561866 DOI: 10.1074/jbc.M111.223651]
77 **Xiong X**, Zhao Y, He H, Sun Y. Ribosomal protein S27-like and S27 interplay with p53-MDM2 axis as a target, a substrate and a regulator. *Oncogene* 2011; **30**: 1798-1811 [PMID: 21170087 DOI: 10.1038/onc.2010.569]
78 **Zhang X**, Wang W, Wang H, Wang MH, Xu W, Zhang R. Identification of ribosomal protein S25 (RPS25)-MDM2-p53 regulatory feedback loop. *Oncogene* 2013; **32**: 2782-2791 [PMID: 22777350 DOI: 10.1038/onc.2012.289]
79 **Zhang Y**, Wang J, Yuan Y, Zhang W, Guan W, Wu Z, Jin C, Chen H, Zhang L, Yang X, He F. Negative regulation of HDM2 to attenuate p53 degradation by ribosomal protein L26. *Nucleic Acids Res* 2010; **38**: 6544-6554 [PMID: 20542919 DOI: 10.1093/nar/gkq536]
80 **Zhang Y**, Wolf GW, Bhat K, Jin A, Allio T, Burkhart WA, Xiong Y. Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. *Mol Cell Biol* 2003; **23**: 8902-8912 [PMID: 14612427 DOI: 10.1128/MCB.23.23.8902-8912.2003]
81 **Zhou X**, Hao Q, Liao J, Zhang Q, Lu H. Ribosomal protein S14 unties the MDM2-p53 loop upon ribosomal stress. *Oncogene* 2013; **32**: 388-396 [PMID: 22391559 DOI: 10.1038/onc.2012.63]
82 **Zhu Y**, Poyurovsky MV, Li Y, Biderman L, Stahl J, Jacq X, Prives C. Ribosomal protein S7 is both a regulator and a substrate of MDM2. *Mol Cell* 2009; **35**: 316-326 [PMID: 19683495 DOI: 10.1016/j.molcel.2009.07.014]
83 **Bai D**, Zhang J, Xiao W, Zheng X. Regulation of the HDM2-p53 pathway by ribosomal protein L6 in response to ribosomal stress. *Nucleic Acids Res* 2014; **42**: 1799-1811 [PMID: 24174547 DOI: 10.1093/nar/gkt971]
84 **Cui D**, Li L, Lou H, Sun H, Ngai SM, Shao G, Tang J. The ribosomal protein S26 regulates p53 activity in response to DNA damage. *Oncogene* 2014; **33**: 2225-2235 [PMID: 23728348 DOI: 10.1038/onc.2013.170]
85 **Daftuar L**, Zhu Y, Jacq X, Prives C. Ribosomal proteins RPL37, RPS15 and RPS20 regulate the Mdm2-p53-MdmX network. *PLoS One* 2013; **8**: e68667 [PMID: 23874713 DOI: 10.1371/journal.pone.0068667]
86 **Gilkes DM**, Chen L, Chen J. MDMX regulation of p53 response to ribosomal stress. *EMBO J* 2006; **25**: 5614-5625 [PMID: 17110929 DOI: 10.1038/sj.emboj.7601424]
87 **Jin Y**, Zeng SX, Dai MS, Yang XJ, Lu H. MDM2 inhibits PCAF (p300/CREB-binding protein-associated factor)-mediated p53 acetylation. *J Biol Chem* 2002; **277**: 30838-30843 [PMID: 12068014 DOI: 10.1074/jbc.M204078200]
88 **Minsky N**, Oren M. The RING domain of Mdm2 mediates histone ubiquitylation and transcriptional repression. *Mol Cell* 2004; **16**: 631-639 [PMID: 15546622]
89 **Tang Y**, Zhao W, Chen Y, Zhao Y, Gu W. Acetylation is indispensable for p53 activation. *Cell* 2008; **133**: 612-626 [PMID: 18485870 DOI: 10.1016/j.cell.2008.03.025]
90 **Kruse JP**, Gu W. Modes of p53 regulation. *Cell* 2009; **137**: 609-622 [PMID: 19450511 DOI: 10.1016/j.cell.2009.04.050]
91 **Chen D**, Kon N, Li M, Zhang W, Qin J, Gu W. ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. *Cell* 2005; **121**: 1071-1083 [PMID: 15989956]
92 **Leng RP**, Lin Y, Ma W, Wu H, Lemmers B, Chung S, Parant JM, Lozano G, Hakem R, Benchimol S. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. *Cell* 2003; **112**: 779-791 [PMID: 12654245]
93 **Dornan D**, Wertz I, Shimizu H, Arnott D, Frantz GD, Dowd P, O'Rourke K, Koeppen H, Dixit VM. The ubiquitin ligase COP1 is a critical negative regulator of p53. *Nature* 2004; **429**: 86-92 [PMID: 15103385 DOI: 10.1038/nature02514]
94 **Brooks CL**, Gu W. p53 ubiquitination: Mdm2 and beyond. *Mol Cell* 2006; **21**: 307-315 [PMID: 16455486]
95 **Dai MS**, Jin Y, Gallegos JR, Lu H. Balance of Yin and Yang: ubiquitylation-mediated regulation of p53 and c-Myc. *Neoplasia* 2006; **8**: 630-644 [PMID: 16925946]
96 **Komander D**, Clague MJ, Urbé S. Breaking the chains: structure and function of the deubiquitinases. *Nat Rev Mol Cell Biol* 2009; **10**: 550-563 [PMID: 19626045 DOI: 10.1038/nrm2731]
97 **Nijman SM**, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, Bernards R. A genomic and functional inventory of deubiquitinating enzymes. *Cell* 2005; **123**: 773-786 [PMID: 16325574]
98 **Li M**, Brooks CL, Kon N, Gu W. A dynamic role of HAUSP in the p53-Mdm2 pathway. *Mol Cell* 2004; **13**: 879-886 [PMID: 15053880]
99 **Li M**, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, Gu W. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* 2002; **416**: 648-653 [PMID: 11923872 DOI: 10.1038/nature737]
100 **Meulmeester E**, Maurice MM, Boutell C, Teunisse AF, Ovaa H, Abraham TE, Dirks RW, Jochemsen AG. Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. *Mol Cell* 2005; **18**: 565-576 [PMID: 15916963]
101 **Cummins JM**, Rago C, Kohli M, Kinzler KW, Lengauer C, Vogelstein B. Tumour suppression: disruption of HAUSP gene stabilizes p53. *Nature* 2004; **428**: 1 p following 486 [PMID: 15058298 DOI: 10.1038/nature02501]
102 **Yuan J**, Luo K, Zhang L, Cheville JC, Lou Z. USP10 regulates p53 localization and stability by deubiquitinating p53. *Cell* 2010; **140**: 384-396 [PMID: 20096447 DOI: 10.1016/

j.cell.2009.12.032]
103 **Hock AK**, Vigneron AM, Carter S, Ludwig RL, Vousden KH. Regulation of p53 stability and function by the deubiquitinating enzyme USP42. *EMBO J* 2011; **30**: 4921-4930 [PMID: 22085928 DOI: 10.1038/emboj.2011.419]
104 **Liu J**, Chung HJ, Vogt M, Jin Y, Malide D, He L, Dundr M, Levens D. JTV1 co-activates FBP to induce USP29 transcription and stabilize p53 in response to oxidative stress. *EMBO J* 2011; **30**: 846-858 [PMID: 21285945 DOI: 10.1038/emboj.2011.11]
105 **Stevenson LF**, Sparks A, Allende-Vega N, Xirodimas DP, Lane DP, Saville MK. The deubiquitinating enzyme USP2a regulates the p53 pathway by targeting Mdm2. *EMBO J* 2007; **26**: 976-986 [PMID: 17290220]
106 **Allende-Vega N**, Sparks A, Lane DP, Saville MK. MdmX is a substrate for the deubiquitinating enzyme USP2a. *Oncogene* 2010; **29**: 432-441 [PMID: 19838211 DOI: 10.1038/onc.2009.330]
107 **Zhang X**, Berger FG, Yang J, Lu X. USP4 inhibits p53 through deubiquitinating and stabilizing ARF-BP1. *EMBO J* 2011; **30**: 2177-2189 [PMID: 21522127 DOI: 10.1038/emboj.2011.125]
108 **Benassi B**, Flavin R, Marchionni L, Zanata S, Pan Y, Chowdhury D, Marani M, Strano S, Muti P, Blandino G, Loda M. MYC is activated by USP2a-mediated modulation of microRNAs in prostate cancer. *Cancer Discov* 2012; **2**: 236-247 [PMID: 22585994 DOI: 10.1158/2159-8290.CD-11-0219]
109 **Priolo C**, Tang D, Brahamandan M, Benassi B, Sicinska E, Ogino S, Farsetti A, Porrello A, Finn S, Zimmermann J, Febbo P, Loda M. The isopeptidase USP2a protects human prostate cancer from apoptosis. *Cancer Res* 2006; **66**: 8625-8632 [PMID: 16951176 DOI: 10.1158/0008-5472.CAN-06-1374]
110 **Sun XX**, Challagundla KB, Dai MS. Positive regulation of p53 stability and activity by the deubiquitinating enzyme Otubain 1. *EMBO J* 2012; **31**: 576-592 [PMID: 22124327 DOI: 10.1038/emboj.2011.434]
111 **Balakirev MY**, Tcherniuk SO, Jaquinod M, Chroboczek J. Otubains: a new family of cysteine proteases in the ubiquitin pathway. *EMBO Rep* 2003; **4**: 517-522 [PMID: 12704427]
112 **Edelmann MJ**, Iphöfer A, Akutsu M, Altun M, di Gleria K, Kramer HB, Fiebiger E, Dhe-Paganon S, Kessler BM. Structural basis and specificity of human otubain 1-mediated deubiquitination. *Biochem J* 2009; **418**: 379-390 [PMID: 18954305 DOI: 10.1042/BJ20081318]
113 **Wang T**, Yin L, Cooper EM, Lai MY, Dickey S, Pickart CM, Fushman D, Wilkinson KD, Cohen RE, Wolberger C. Evidence for bidentate substrate binding as the basis for the K48 linkage specificity of otubain 1. *J Mol Biol* 2009; **386**: 1011-1023 [PMID: 19211026 DOI: 10.1016/j.jmb.2008.12.085]
114 **Soares L**, Seroogy C, Skrenta H, Anandasabapathy N, Lovelace P, Chung CD, Engleman E, Fathman CG. Two isoforms of otubain 1 regulate T cell anergy via GRAIL. *Nat Immunol* 2004; **5**: 45-54 [PMID: 14661020 DOI: 10.1038/ni1017]
115 **Li S**, Zheng H, Mao AP, Zhong B, Li Y, Liu Y, Gao Y, Ran Y, Tien P, Shu HB. Regulation of virus-triggered signaling by OTUB1- and OTUB2-mediated deubiquitination of TRAF3 and TRAF6. *J Biol Chem* 2010; **285**: 4291-4297 [PMID: 19996094 DOI: 10.1074/jbc.M109.074971]
116 **Herhaus L**, Al-Salihi M, Macartney T, Weidlich S, Sapkota GP. OTUB1 enhances TGFβ signalling by inhibiting the ubiquitylation and degradation of active SMAD2/3. *Nat Commun* 2013; **4**: 2519 [PMID: 24071738 DOI: 10.1038/ncomms3519]
117 **Juris SJ**, Shah K, Shokat K, Dixon JE, Vacratsis PO. Identification of otubain 1 as a novel substrate for the Yersinia protein kinase using chemical genetics and mass spectrometry. *FEBS Lett* 2006; **580**: 179-183 [PMID: 16364312]
118 **Edelmann MJ**, Kramer HB, Altun M, Kessler BM. Post-translational modification of the deubiquitinating enzyme otubain 1 modulates active RhoA levels and susceptibility to Yersinia invasion. *FEBS J* 2010; **277**: 2515-2530 [PMID: 20553488 DOI: 10.1111/j.1742-4658.2010.07665.x]
119 **Stanisić V**, Malovannaya A, Qin J, Lonard DM, O'Malley BW. OTU Domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) deubiquitinates estrogen receptor (ER) alpha and affects ERalpha transcriptional activity. *J Biol Chem* 2009; **284**: 16135-16145 [PMID: 19383985 DOI: 10.1074/jbc.M109.007484]
120 **Nakada S**, Tai I, Panier S, Al-Hakim A, Iemura S, Juang YC, O'Donnell L, Kumakubo A, Munro M, Sicheri F, Gingras AC, Natsume T, Suda T, Durocher D. Non-canonical inhibition of DNA damage-dependent ubiquitination by OTUB1. *Nature* 2010; **466**: 941-946 [PMID: 20725033 DOI: 10.1038/nature09297]
121 **Goncharov T**, Niessen K, de Almagro MC, Izrael-Tomasevic A, Fedorova AV, Varfolomeev E, Arnott D, Deshayes K, Kirkpatrick DS, Vucic D. OTUB1 modulates c-IAP1 stability to regulate signalling pathways. *EMBO J* 2013; **32**: 1103-1114 [PMID: 23524849 DOI: 10.1038/emboj.2013.62]
122 **Juang YC**, Landry MC, Sanches M, Vittal V, Leung CC, Ceccarelli DF, Mateo AR, Pruneda JN, Mao DY, Szilard RK, Orlicky S, Munro M, Brzovic PS, Klevit RE, Sicheri F, Durocher D. OTUB1 co-opts Lys48-linked ubiquitin recognition to suppress E2 enzyme function. *Mol Cell* 2012; **45**: 384-397 [PMID: 22325355 DOI: 10.1016/j.molcel.2012.01.011]
123 **Sato Y**, Yamagata A, Goto-Ito S, Kubota K, Miyamoto R, Nakada S, Fukai S. Molecular basis of Lys-63-linked polyubiquitination inhibition by the interaction between human deubiquitinating enzyme OTUB1 and ubiquitin-conjugating enzyme UBC13. *J Biol Chem* 2012; **287**: 25860-25868 [PMID: 22679021 DOI: 10.1074/jbc.M112.364752]
124 **Wiener R**, Zhang X, Wang T, Wolberger C. The mechanism of OTUB1-mediated inhibition of ubiquitination. *Nature* 2012; **483**: 618-622 [PMID: 22367539 DOI: 10.1038/nature10911]
125 **Zulkifle N.** Systemmatic yeast two-hybrid analysis of human E2 Ubiquitin-conjugating enzyme and deubiquitin [DUB] protein interaction. *Int J Biol Chem* 2013; **7**: 14 [DOI: 10.3923/ijbc.2013.1.14]
126 **Li Y**, Sun XX, Elferich J, Shinde U, David LL, Dai MS. Monoubiquitination is critical for ovarian tumor domain-containing ubiquitin aldehyde binding protein 1 (Otub1) to suppress UbcH5 enzyme and stabilize p53 protein. *J Biol Chem* 2014; **289**: 5097-5108 [PMID: 24403071 DOI: 10.1074/jbc.M113.533109]
127 **Brzovic PS**, Lissounov A, Christensen DE, Hoyt DW, Klevit RE. A UbcH5/ubiquitin noncovalent complex is required for processive BRCA1-directed ubiquitination. *Mol Cell* 2006; **21**: 873-880 [PMID: 16543155]
128 **Sakata E**, Satoh T, Yamamoto S, Yamaguchi Y, Yagi-Utsumi M, Kurimoto E, Tanaka K, Wakatsuki S, Kato K. Crystal structure of UbcH5b~ubiquitin intermediate: insight into the formation of the self-assembled E2~Ub conjugates. *Structure* 2010; **18**: 138-147 [PMID: 20152160 DOI: 10.1016/j.str.2009.11.007]
129 **Wiener R**, DiBello AT, Lombardi PM, Guzzo CM, Zhang X, Matunis MJ, Wolberger C. E2 ubiquitin-conjugating enzymes regulate the deubiquitinating activity of OTUB1. *Nat Struct Mol Biol* 2013; **20**: 1033-1039 [PMID: 23955022 DOI: 10.1038/nsmb.2655]
130 **Vassilev LT**, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004; **303**: 844-848 [PMID: 14704432 DOI: 10.1126/science.1092472]
131 **Popowicz GM**, Czarna A, Wolf S, Wang K, Wang W, Dömling A, Holak TA. Structures of low molecular weight inhibitors bound to MDMX and MDM2 reveal new approaches for p53-MDMX/MDM2 antagonist drug discovery. *Cell Cycle* 2010; **9**: 1104-1111 [PMID: 20237429]
132 **Graves B**, Thompson T, Xia M, Janson C, Lukacs C, Deo D, Di Lello P, Fry D, Garvie C, Huang KS, Gao L, Tovar C, Lovey A, Wanner J, Vassilev LT. Activation of the p53 pathway by small-molecule-induced MDM2 and MDMX dimer-

ization. *Proc Natl Acad Sci USA* 2012; **109**: 11788-11793 [PMID: 22745160 DOI: 10.1073/pnas.1203789109]

133 **Reverdy C**, Conrath S, Lopez R, Planquette C, Atmanene C, Collura V, Harpon J, Battaglia V, Vivat V, Sippl W, Colland F. Discovery of specific inhibitors of human USP7/HAUSP deubiquitinating enzyme. *Chem Biol* 2012; **19**: 467-477 [PMID: 22520753 DOI: 10.1016/j.chembiol.2012.02.007]

134 **Altun M**, Kramer HB, Willems LI, McDermott JL, Leach CA, Goldenberg SJ, Kumar KG, Konietzny R, Fischer R, Kogan E, Mackeen MM, McGouran J, Khoronenkova SV, Parsons JL, Dianov GL, Nicholson B, Kessler BM. Activity-based chemical proteomics accelerates inhibitor development for deubiquitylating enzymes. *Chem Biol* 2011; **18**: 1401-1412 [PMID: 22118674 DOI: 10.1016/j.chembiol.2011.08.018]

135 **Devine T**, Dai MS. Targeting the ubiquitin-mediated proteasome degradation of p53 for cancer therapy. *Curr Pharm Des* 2013; **19**: 3248-3262 [PMID: 23151129 DOI: 10.2174/13816128 11319180009]

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REVIEW

Oxidation of KCNB1 K^+ channels in central nervous system and beyond

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Abstract

KCNB1, a voltage-gated potassium (K^+) channel that conducts a major delayed rectifier current in the brain, pancreas and cardiovascular system is a key player in apoptotic programs associated with oxidative stress. As a result, this protein represents a *bona fide* drug target for limiting the toxic effects of oxygen radicals. Until recently the consensus view was that reactive oxygen species trigger a pro-apoptotic surge in KCNB1 current *via* phosphorylation and SNARE-dependent incorporation of KCNB1 channels into the plasma membrane. However, new evidence shows that KCNB1 can be modified by oxidants and that oxidized KCNB1 channels can directly activate pro-apoptotic signaling pathways. Hence, a more articulated picture of the pro-apoptotic role of KCNB1 is emerging in which the protein induces cell's death through distinct molecular mechanisms and activation of multiple pathways. In this review article we discuss the diverse functional, toxic and protective roles that KCNB1 channels play in the major organs where they are expressed.

Key words: Apoptosis; Kv2.1; Aging; Reactive oxygen species; Alzheimer's disease

Core tip: KCNB1 is a K^+ channel that plays a key role in the brain, pancreas and cardiovascular system. KCNB1 is unique in that it induces apoptosis in association with oxidative stress. In this review article we discuss the diverse roles of this channel in the organs where it is expressed including recent advances in the molecular mechanisms through which KCNB1 causes cytotoxicity.

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KCNB1 IS A PROMINENT MEMBER OF THE SHAB-RELATED FAMILY OF VOLTAGE-GATED K^+ CHANNELS

KCNB1 (HUGO nomenclature), formerly DRK1 and Kv2.1, is a *Shab* delayed rectifier voltage-gated K^+ channel which was cloned by Frech *et al*[1] using size-fractionated mRNA extracted from rat brain. KCNB1 is expressed in the central nervous system, pancreas, pulmonary arteries, heart, auditory outer hair cells, stem cells and retina[2-21]. As in other voltage-gated K^+ channels, KCNB1 spatial and temporal expressions are both developmentally regulated. For example, three distinct (4.4, 9.0, 11.5 kb) mRNA transcripts are expressed in the rat brain, with the 4.4 kb transcript being predominant in embryos and the 11.5 kb transcript being predominant in adults[15]. Accordingly, multiple KCNB1 isoforms are detected which differ in

their developmental expression. Functional KCNB1 channels can result from the assembly of four identical pore-forming subunits along a symmetry axis[22]. However, this simple stoichiometry is not likely to be observed in nature. In order to serve the specific requisites of the tissues in which the channel is expressed, heterogeneity of KCNB1 current can be achieved by formation of heterometric complexes containing non-conducting, pore-forming subunits of the KCNG and KCNS families as well as by assembly with accessory subunits of the KCNAB and KCNE families[16,17,23-28]. KCNB1 exhibits an unusual large number of consensus sites for phosphorylation. Accordingly, the channel is a substrate for protein kinases of different families and is constitutively phosphorylated in native cells[29-32]. KCNB1 can also be SUMOylated and acetylated in nervous system and pancreas even though the physiological role of these regulations awaits elucidation[33-35]. Finally, mature KCNB1 channels are not glycosylated despite the presence of consensus sites in the N-terminus[36].

Because of the potential therapeutic implications, the pharmacology of KCNB1 to a variety of toxins and drugs has been extensively investigated. Thus, KCNB1 is blocked by tarantula toxins that belong to the same structural family of inhibitor cystine knot spider peptides reticulated by disulfide bridges. Hanatoxin from *G. spatulata*, was the first toxin to be shown to interact with KCNB1, followed in more recent years by heteroscordratoxin and stromatoxin 1 from *H. maculata* and *S. calceata* and jingzhaotoxin (JZTX- Ⅰ, -Ⅲ, and -Ⅴ) and guangxitoxin (GxTx-1E), isolated from the venoms of the Chinese tarantulas *C. jingzhao* and *P. guangxiensis*[37-40]. All these structurally related toxins exhibit variable affinities for the channel in the nanomolar to micromolar range and act to alter its gating by interacting with the voltage sensor[41,42]. KCNB1 is susceptible to inhibition by a number of compounds of different classes including classic K^+ channel blockers tetraethylammonium (TEA) and 4-aminopyridine and antipsychotic, anesthetic and antiarrhythmic compounds[43-53]. Of particular relevance to the topic of this review is the fact that acetylcholinesterase inhibitor Donepezil, a drug used in the treatment of Alzheimer's disease and vascular dementia, protects neurons from apoptosis by inhibiting KCNB1[54]. The exact mechanism awaits elucidation but recent findings showing that KCNB1 is subject to direct oxidative modification may suggest that the protective effect of the drug may stem from its ability to prevent the oxidation of KCNB1[55].

In summary, toxins and synthetic drugs have significantly contributed to the effort of dissecting native KCNB1 currents in various tissues and probing channel's structure and functional mechanisms of gating.

KCNB1 IS A CRITICAL MEDIATOR OF HIPPOCAMPAL AND CORTICAL EXCITABILITY

KCNB1 is broadly expressed in the brain and is a major contributor to the delayed rectifier somatodendritic K^+ current in hippocampal and cortical neurons[14,56]. In electrically quiescent neurons, KCNB1 is mostly localized in microdomains in the membranes of dendrites and cell bodies where it is constitutively phosphorylated and poorly conducting[20,21,29-32,57-60]. Upon the onset of neuronal activity, a series of cellular events are initiated that lead to de-phosphorylation of the channel. This transition is associated with two major changes in channel's status: (1) its threshold for voltage activation is lowered; and (2) KCNB1 is released from the microdomains and begins to diffuse in the membrane[30]. The net effect of these changes is that KCNB1 conducts a delayed rectifier K^+ current that acts to slow down and/or terminate periods of high frequency firing. Activity-dependent phosphorylation/de-phosphorylation of KCNB1 is partly mediated by cyclin-dependent kinase 5 and the phosphatase calcineurin. The latter is activated by a calcium influx driven by the electrical activity of the neuron[29-32]. Using mass spectrometry, Trimmer and colleagues identified 16 phosphorylation sites in KCNB1, of which roughly half provided a substrate for calcineurin[32]. This indicates that modulation of KCNB1 by protein kinases is graded to reflect dynamic regulation of neuron firing properties. However, KCNB1 can also terminate periods of neuronal activity by being directly phosphorylated. For example, AMP-activated protein kinase (AMPK, which is activated by ATP depletion) can phosphorylate KCNB1 at residue S440 and induce hyperpolarizing shifts in the current-voltage relationship for activation, shifts that make the channel more conductive at negative voltages[61].

KCNB1 PROMOTES APOPTOSIS IN RESPONSE TO OXIDATIVE STRESS

KCNB1 is a specific mediator of apoptosis in a variety of neuronal cell types including hippocampal, cortical and granule neurons[62-65]. For example, a study investigating the molecular correlate of the apoptotic K^+ current in hippocampal neurons found that among nine alpha and 3 beta Kv subunits screened, KCNB1 was the primary correlate[63]. Several groups have demonstrated that the key event triggering KCNB1-induced apoptosis is an increase in reactive oxygen species (ROS), either following acute oxidation, or as a consequence of cellular stresses such as serum deprivation and excitotoxicity[55,62-65]. It is currently accepted that dysregulated K^+ homeostasis causes apoptosis by inducing mitochondrial swelling and depolarization, ROS generation, deficient energy production and cell volume decrease[66]. Accordingly, augmented insertion of KCNB1 channels into the plasma membrane is observed in neurons subjected to oxidative challenges[67]. The accompanying increase in KCNB1 current is thought to be a key step in the apoptotic program. The execution of the latter requires phosphorylation of KCNB1 by multiple types of protein kinases a fact that should not surprise considering the primary role that phosphorylation plays for the function of KCNB1. Zhou *et al*[68] investigated apoptosis induced by lack of serum

in granular neurons and found that this was associated with upregulation of KCNB1 *via* the activation of a signaling pathway involving cAMP, protein kinase A and cAMP response element-binding protein (CREB). Aras *et al*[69] have identified several kinases including apoptosis signal-regulating kinase 1 (ASK1), p38 MAPK-dependent kinase, c-Src tyrosine kinase, and $Ca(2^+)$/calmodulin-dependent protein kinase Ⅱ (CaMKII) that interact with KCNB1 in response to oxidative stress[70-73]. Their studies have provided a model that predicts that oxygen radicals induce simultaneous increases in cytosolic levels of Zn^{2+} and Ca^{2+}. These increases activate the previously listed kinases and accelerate KCNB1 forward trafficking by modulating and facilitating its interaction with SNARE family protein syntaxin. This apoptotic program is tightly regulated: knock down of just a single phosphorylation site (S800 for p-38, Y124 for c-Src) is sufficient to suppress the pro-apoptotic influence of KCNB1[70]. However, Src tyrosine kinases and protein tyrosine phosphatase epsilon (PTP epsilon) also play a role in the physiological modulation of KCNB1. In the Schwann cells of mice, Src-mediated phosphorylation of Y124, (the same residue responsible for Zn^{2+}/Ca^{2+} induced apoptosis), causes specific augmentation of KCNB1 current which appears to be critical for Schwann cell proliferation and myelination[74,75]. In fact, de-phosphorylation of KCNB1 at Y124 by PTP epsilon reduces KCNB1 activity and stops KCNB1-induced myelination[76,77]. Accordingly, mice lacking PTP epsilon exhibit hypomyelination of sciatic nerve axons at an early post-natal age, an effect due to constitutive activation of KCNB1 by Src tyrosine kinases[78]. Moreover, a number of K^+ channels can cause apoptosis *via* dysregulated K^+ homeostasis in a variety of cell types[66]. Therefore, increased K^+ current may not be the key feature responsible for the specific ability of KCNB1 to promote apoptosis, but rather a consequence of it. Recent work from our laboratory may shed light on this issue. Cotella *et al*[55] showed that oxygen radicals directly modify KCNB1 channels, leading to the formation of oligomers held together by disulfide bridges[55]. A KCNB1 variant which does not form oligomers, obtained by mutating an N-terminal cysteine (C73A), fails to increase apoptosis in mammalian cells. Cotella *et al*[55] further showed that in inside-out patches, oxidants inhibit KCNB1 current. These findings imply that the formation of oligomers, rather than KCNB1 current, is the event that triggers an initial pro-apoptotic stimulus. To answer this question, Wu *et al*[79] have investigated the fate of the KCNB1 oligomers. They found that they accumulate in the plasma membrane as a result of defective internalization. Notably, accumulation is transient, and normal endocytosis/surface expression are mostly restored within one hour post-oxidation. The transient accumulation of KCNB1 oligomers is associated with activation of c-Src and JNK kinases coupled to a steady increase in the levels of free radicals. Thus, oligomer-induced activation of a "death pathway" appears to trigger the initial pro-apoptotic stimulus. As apoptosis progresses and ROS levels increase in the cell, the surge of KCNB1 current follows to further execute the apoptotic program (Figure 1).

INHIBITION OF KCNB1 MAY REPRESENT A VALID ANTI-APOPTOTIC STRATEGY

Pharmacological inhibition of KCNB1 current may represent a valid approach to preventing apoptosis. Accordingly, Peers and colleagues have shown that carbon monoxide (CO) can provide neuronal protection against an increase in KCNB1 current *via* regulating ROS and protein kinase G activity[80]. The same group has further proposed that the anti-apoptotic effect of CO may also be partially responsible for the etiology of cancer, as many oncogenic cells constitutively express heme oxygenase-1 (HO-1), which generates CO as a by product of its catalytic activity[81]. Chronic viruses, which establish a state of persistent infection by rendering infected cells resistant to apoptosis also appear to exploit inhibition of KCNB1 current. In human hepatocytes infected with hepatitis C virus (HCV), oxidative insults fail to initiate apoptosis because the HCV NS5A protein inhibits phosphorylation of KCNB1 by p38 MAPK and thus suppresses the current surge[82,83]. Furthermore, a neuronal NS5A isoform from HCV genotype 1b, NS5A1b, protects rat neurons against apoptosis by inhibiting KCNB1[73]. However, while NS5A acts on tyrosine kinase phosphorylation at residue Y124, NS5A1b inhibits p38-MAPK at residue S800 suggesting that the actions of these viral proteins are genotype-selective probably reflecting the characteristic of these viruses to target specific tissues.

VASOCONSTRICTION OF SMALL PULMONARY ARTERIES MAY PROCEED THROUGH DIRECT INHIBITION OF KCNB1 CURRENT BY ROS

Hypoxic pulmonary vasoconstriction is a physiological response to alveolar hypoxia, in which blood flow is redirected to better ventilated lobes *via* constriction of small pulmonary arteries. The mechanical force leading to vasoconstriction is exerted by pulmonary arteries smooth muscle cells (PASMCs). Hypoxia initially promotes PASMCs depolarization *via* inhibition of an oxygen-sensitive K^+ current. This leads to the activation of L-type Ca^{2+} channels, which elevate cytosolic calcium thereby triggering PASMCs contraction. Biochemical, pharmacological, electrophysiological and genetic evidence designates KCNB1 - alone or mixed with KCNS3 silent subunits-as one of the major molecular correlates of the oxygen-sensitive K^+ current in PASMCs[16-18,84,85]. Studies using the human ductus arteriosus as model system have provided a detailed picture of the cellular and molecular events leading to vasoconstriction during hypoxia[86-88]. Changes in O_2 levels are translated to the mitochondrial electron transport chain (KCNB1 is insensitive to O_2[89])

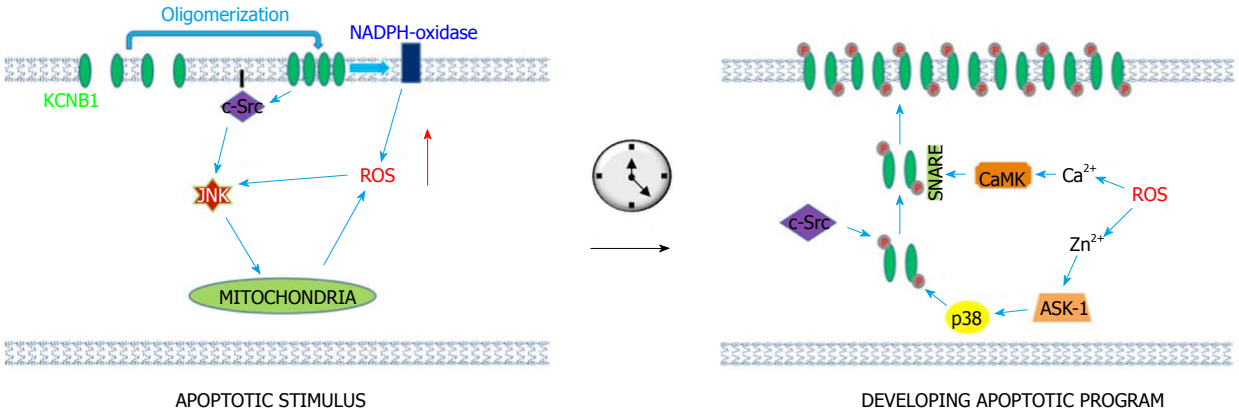

Figure 1 A two-step model for the pro-apoptotic actions of KCNB1. Upon exposure to oxidants, KCNB1 oligomers are formed. They accumulate in the plasma membrane thereby perturbing the organization of lipid rafts. This results in activation of an apoptotic stimulus mediated by c-Src and downstream, JNK kinases. As result of activation of c-Src and JNK kinases and in part of NADPH-oxidase (Xilong Wu, private communication) which is localized in the plasma membrane, ROS levels increase in the cell. ROS induce a raise in cytosolic Ca^{2+} and Zn^{2+} that initiate a phosphorylation-mediated surge of KCNB1 channels that further drives apoptosis. The signaling pathway activated by Zn^{2+} proceeds through activation of p38 by ASK-1 and independently, of c-Src tyrosine kinases (Zn^{2+} inhibits the activity of the tyrosine phosphatase PTP epsilon) which phosphorylate KCNB1 at S800 and Y124 thereby allowing interaction with SNARE family protein syntaxin. The Ca^{2+} signaling pathway results in activation of CaMKII kinase which in turn acts to modulate the interaction of KCNB1 with syntaxin. It is not known whether Src and p38 phosphorylation directly act to increase KCNB1 current. ROS: Reactive oxygen species.

which responds by speeding the synthesis of diffusible hydrogen peroxide (H_2O_2). H_2O_2 causes smooth muscle cell depolarization, *via* inhibition of K^+ current which further results in influx of calcium through L-type channels. The molecular details of the mechanism that links H_2O_2 to K^+ current inhibition were not known previously but the fact that KCNB1 can be directly oxidized by H_2O_2 and most importantly, that its current is suppressed by oxidants may now provide a natural explanation for this mechanism of inhibition. It is worth noticing that chronic hypoxia is characterized by depolarized resting potential and elevated cytosolic Ca^{2+}. Chronic depolarization is achieved by downregulation of KCNB1 protein[90-93] through mechanisms not completely understood, even though studies have implicated 15-lipoxygenase catalysis of arachidonic acid and hypoxia-inducible factor 1 in the mechanism[94,95]. Thus, different regulations of KCNB1 appear to mediate acute versus chronic conditions of hypoxia.

CONCLUSION

KCNB1 is a channel with a double-hedged sword nature: it is essential to the physiology of multiple organs, including the brain, pancreas and cardiovascular system and further acts as a mediator of apoptosis in response to oxidative stresses[2-21]. Dysregulated K^+ homeostasis is a well established mechanism through which K^+ channels contribute to an apoptotic program with a great deal of evidence implicating that KCNB1 do indeed work in this mechanism[55,62-65,67]. However recent findings have unveiled new ways through which KCNB1 mediates cell death: by giving rise to cytotoxic protein aggregates that result from direct oxidation of the protein[79]. The accumulation of these KCNB1 oligomers in the plasma membrane is transient but sufficient to trigger a pro-apoptotic signal *via* activation of a c-Src/JNK kinases pathway. As the apoptotic program progresses, a surge of KCNB1 current follows to induce mitochondrial destabilization, ROS generation, deficient energy production and cell volume decrease. Hence, KCNB1 plays a double role as both initiator and later executor of the apoptotic program.

Aging pathologies pose new challenges to health care, because even as advances in medicine are increasing lifespan, health problems become more prevalent as people age. A recent survey done by Harvard University School of Public Health and the Alzheimer's Europe Consortium suggests that senile dementia is the second leading health concern after cancer[96]. Aging is also the most important risk factor in neurodegenerative conditions such as Alzheimer's disease, the third most costly disease in the United Sates[97]. It is projected that the number of Western elders suffering from dementia and related neurodegenerative disease will increase by 350% by the midcentury[98,99]. Therefore, because of the impact of increasing lifespan on global human health issues, it is important to elucidate the cellular and molecular processes involved in aging. Oxidative modifications of KCNB1 are pervasive in the aging nervous system[55]. Hence, KCNB1 oxidation has the potential to impact all those conditions characterized by an imbalance in the redox status of the cell, from normal senescence to neuropathies such as Alzheimer's disease. Understanding how oxidation of KCNB1 influences the function of the brain during aging may provide the insight necessary to design better pharmacological strategies; these include targeting KCNB1 for the potential therapeutic use of antioxidants in neurological treatments or targeting other components of the signaling pathways activated by oxidation of KCNB1.

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REFERENCES

1 **Frech GC**, VanDongen AM, Schuster G, Brown AM, Joho RH. A novel potassium channel with delayed rectifier properties isolated from rat brain by expression cloning. *Nature* 1989; **340**: 642-645 [PMID: 2770868 DOI: 10.1038/340642a0]

2 **Dixon JE**, McKinnon D. Quantitative analysis of potassium channel mRNA expression in atrial and ventricular muscle of rats. *Circ Res* 1994; **75**: 252-260 [PMID: 8033339 DOI: 10.1161/01.RES.75.2.252]

3 **Barry DM**, Trimmer JS, Merlie JP, Nerbonne JM. Differential expression of voltage-gated K+ channel subunits in adult rat heart. Relation to functional K+ channels? *Circ Res* 1995; **77**: 361-369 [PMID: 7614722 DOI: 10.1161/01.RES.77.2.361]

4 **Kyle B**, Bradley E, Ohya S, Sergeant GP, McHale NG, Thornbury KD, Hollywood MA. Contribution of Kv2.1 channels to the delayed rectifier current in freshly dispersed smooth muscle cells from rabbit urethra. *Am J Physiol Cell Physiol* 2011; **301**: C1186-C1200 [PMID: 21813710 DOI: 10.1152/ajpcell.00455.2010]

5 **Li X**, Surguchev A, Bian S, Navaratnam D, Santos-Sacchi J. Extracellular chloride regulation of Kv2.1, contributor to the major outward Kv current in mammalian outer hair cells. *Am J Physiol Cell Physiol* 2012; **302**: C296-C306 [PMID: 21940671 DOI: 10.1152/ajpcell.00177.2011]

6 **You MH**, Song MS, Lee SK, Ryu PD, Lee SY, Kim DY. Voltage-gated K+ channels in adipogenic differentiation of bone marrow-derived human mesenchymal stem cells. *Acta Pharmacol Sin* 2013; **34**: 129-136 [PMID: 23222271 DOI: 10.1038/aps.2012.142]

7 **Yazulla S**, Studholme KM. Differential distribution of Shaker-like and Shab-like K+-channel subunits in goldfish retina and retinal bipolar cells. *J Comp Neurol* 1998; **396**: 131-140 [PMID: 9623892]

8 **Tamarina NA**, Kuznetsov A, Fridlyand LE, Philipson LH. Delayed-rectifier (KV2.1) regulation of pancreatic beta-cell calcium responses to glucose: inhibitor specificity and modeling. *Am J Physiol Endocrinol Metab* 2005; **289**: E578-E585 [PMID: 16014354 DOI: 10.1152/ajpendo.00054.2005]

9 **Yan L**, Figueroa DJ, Austin CP, Liu Y, Bugianesi RM, Slaughter RS, Kaczorowski GJ, Kohler MG. Expression of voltage-gated potassium channels in human and rhesus pancreatic islets. *Diabetes* 2004; **53**: 597-607 [PMID: 14988243 DOI: 10.2337/diabetes.53.3.597]

10 **MacDonald PE**, Ha XF, Wang J, Smukler SR, Sun AM, Gaisano HY, Salapatek AM, Backx PH, Wheeler MB. Members of the Kv1 and Kv2 voltage-dependent K(+) channel families regulate insulin secretion. *Mol Endocrinol* 2001; **15**: 1423-1435 [PMID: 11463864 DOI: 10.1210/mend.15.8.0685]

11 **Su J**, Yu H, Lenka N, Hescheler J, Ullrich S. The expression and regulation of depolarization-activated K+ channels in the insulin-secreting cell line INS-1. *Pflugers Arch* 2001; **442**: 49-56 [PMID: 11374068 DOI: 10.1007/s004240000508]

12 **Roe MW**, Worley JF, Mittal AA, Kuznetsov A, DasGupta S, Mertz RJ, Witherspoon SM, Blair N, Lancaster ME, McIntyre MS, Shehee WR, Dukes ID, Philipson LH. Expression and function of pancreatic beta-cell delayed rectifier K+ channels. Role in stimulus-secretion coupling. *J Biol Chem* 1996; **271**: 32241-32246 [PMID: 8943282 DOI: 10.1074/jbc.271.50.32241]

13 **Du J**, Tao-Cheng JH, Zerfas P, McBain CJ. The K+ channel, Kv2.1, is apposed to astrocytic processes and is associated with inhibitory postsynaptic membranes in hippocampal and cortical principal neurons and inhibitory interneurons. *Neuroscience* 1998; **84**: 37-48 [PMID: 9522360 DOI: 10.1016/S0306-4522(97)00519-8]

14 **Murakoshi H**, Trimmer JS. Identification of the Kv2.1 K+ channel as a major component of the delayed rectifier K+ current in rat hippocampal neurons. *J Neurosci* 1999; **19**: 1728-1735 [PMID: 10024359]

15 **Trimmer JS**. Expression of Kv2.1 delayed rectifier K+ channel isoforms in the developing rat brain. *FEBS Lett* 1993; **324**: 205-210 [PMID: 8508921 DOI: 10.1016/0014-5793(93)81394-F]

16 **Patel AJ**, Lazdunski M, Honoré E. Kv2.1/Kv9.3, a novel ATP-dependent delayed-rectifier K+ channel in oxygen-sensitive pulmonary artery myocytes. *EMBO J* 1997; **16**: 6615-6625 [PMID: 9362476 DOI: 10.1093/emboj/16.22.6615]

17 **Yuan XJ**, Wang J, Juhaszova M, Golovina VA, Rubin LJ. Molecular basis and function of voltage-gated K+ channels in pulmonary arterial smooth muscle cells. *Am J Physiol* 1998; **274**: L621-L635 [PMID: 9575881]

18 **Archer SL**, Souil E, Dinh-Xuan AT, Schremmer B, Mercier JC, El Yaagoubi A, Nguyen-Huu L, Reeve HL, Hampl V. Molecular identification of the role of voltage-gated K+ channels, Kv1.5 and Kv2.1, in hypoxic pulmonary vasoconstriction and control of resting membrane potential in rat pulmonary artery myocytes. *J Clin Invest* 1998; **101**: 2319-2330 [PMID: 9616203 DOI: 10.1172/JCI333]

19 **Archer SL**, Weir EK, Reeve HL, Michelakis E. Molecular identification of O2 sensors and O2-sensitive potassium channels in the pulmonary circulation. *Adv Exp Med Biol* 2000; **475**: 219-240 [PMID: 10849663]

20 **Trimmer JS**. Immunological identification and characterization of a delayed rectifier K+ channel polypeptide in rat brain. *Proc Natl Acad Sci USA* 1991; **88**: 10764-10768 [PMID: 1961744 DOI: 10.1073/pnas.88.23.10764]

21 **Hwang PM**, Fotuhi M, Bredt DS, Cunningham AM, Snyder SH. Contrasting immunohistochemical localizations in rat brain of two novel K+ channels of the Shab subfamily. *J Neurosci* 1993; **13**: 1569-1576 [PMID: 8463836]

22 **MacKinnon R**. Determination of the subunit stoichiometry of a voltage-activated potassium channel. *Nature* 1991; **350**: 232-235 [PMID: 1706481 DOI: 10.1038/350232a0]

23 **Zhong XZ**, Abd-Elrahman KS, Liao CH, El-Yazbi AF, Walsh EJ, Walsh MP, Cole WC. Stromatoxin-sensitive, heteromultimeric Kv2.1/Kv9.3 channels contribute to myogenic control of cerebral arterial diameter. *J Physiol* 2010; **588**: 4519-4537 [PMID: 20876197 DOI: 10.1113/jphysiol.2010.196618]

24 **Hristov KL**, Chen M, Soder RP, Parajuli SP, Cheng Q, Kellett WF, Petkov GV. KV2.1 and electrically silent KV channel subunits control excitability and contractility of guinea pig detrusor smooth muscle. *Am J Physiol Cell Physiol* 2012; **302**: C360-C372 [PMID: 21998137 DOI: 10.1152/ajpcell.00303.2010]

25 **Bocksteins E**, Labro AJ, Snyders DJ, Mohapatra DP. The electrically silent Kv6.4 subunit confers hyperpolarized gating charge movement in Kv2.1/Kv6.4 heterotetrameric channels. *PLoS One* 2012; **7**: e37143 [PMID: 22615922 DOI: 10.1371/journal.pone.0037143]

26 **Aimond F**, Kwak SP, Rhodes KJ, Nerbonne JM. Accessory Kvbeta1 subunits differentially modulate the functional expression of voltage-gated K+ channels in mouse ventricular myocytes. *Circ Res* 2005; **96**: 451-458 [PMID: 15662035 DOI: 10.1161/01.RES.0000156890.25876.63]

27 **McCrossan ZA**, Lewis A, Panaghie G, Jordan PN, Christini DJ, Lerner DJ, Abbott GW. MinK-related peptide 2 modulates Kv2.1 and Kv3.1 potassium channels in mammalian brain. *J Neurosci* 2003; **23**: 8077-8091 [PMID: 12954870]

28 **Bocksteins E**, Labro AJ, Mayeur E, Bruyns T, Timmermans JP, Adriaensen D, Snyders DJ. Conserved negative charges in the N-terminal tetramerization domain mediate efficient assembly of Kv2.1 and Kv2.1/Kv6.4 channels. *J Biol Chem* 2009; **284**: 31625-31634 [PMID: 19717558 DOI: 10.1074/jbc.M109.039479]

29 **Cerda O**, Trimmer JS. Activity-dependent phosphorylation of neuronal Kv2.1 potassium channels by CDK5. *J Biol Chem* 2011; **286**: 28738-28748 [PMID: 21712386 DOI: 10.1074/jbc.M111.251942]

30 **Misonou H**, Mohapatra DP, Park EW, Leung V, Zhen D, Misonou K, Anderson AE, Trimmer JS. Regulation of ion channel localization and phosphorylation by neuronal activity. *Nat Neurosci* 2004; **7**: 711-718 [PMID: 15195093 DOI: 10.1038/nn1260]

31 **Misonou H**, Mohapatra DP, Menegola M, Trimmer JS. Calcium- and metabolic state-dependent modulation of the voltage-dependent Kv2.1 channel regulates neuronal excitability in response to ischemia. *J Neurosci* 2005; **25**: 11184-11193 [PMID: 16319318 DOI: 10.1523/JNEUROSCI.3370-05.2005]

32 **Park KS**, Mohapatra DP, Misonou H, Trimmer JS. Graded regulation of the Kv2.1 potassium channel by variable phosphorylation. *Science* 2006; **313**: 976-979 [PMID: 16917065 DOI: 10.1126/science.1124254]

33 **Plant LD**, Dowdell EJ, Dementieva IS, Marks JD, Goldstein SA. SUMO modification of cell surface Kv2.1 potassium channels regulates the activity of rat hippocampal neurons. *J Gen Physiol* 2011; **137**: 441-454 [PMID: 21518833 DOI: 10.1085/jgp.201110604]

34 **Dai XQ**, Kolic J, Marchi P, Sipione S, Macdonald PE. SUMOylation regulates Kv2.1 and modulates pancreatic beta-cell excitability. *J Cell Sci* 2009; **122**: 775-779 [PMID: 19223394 DOI: 10.1242/jcs.036632]

35 **Kim SJ**, Widenmaier SB, Choi WS, Nian C, Ao Z, Warnock G, McIntosh CH. Pancreatic β-cell prosurvival effects of the incretin hormones involve post-translational modification of Kv2.1 delayed rectifier channels. *Cell Death Differ* 2012; **19**: 333-344 [PMID: 21818121 DOI: 10.1038/cdd.2011.102]

36 **Shi G**, Trimmer JS. Differential asparagine-linked glycosylation of voltage-gated K+ channels in mammalian brain and in transfected cells. *J Membr Biol* 1999; **168**: 265-273 [PMID: 10191360 DOI: 10.1007/s002329900515]

37 **Yuan C**, Yang S, Liao Z, Liang S. Effects and mechanism of Chinese tarantula toxins on the Kv2.1 potassium channels. *Biochem Biophys Res Commun* 2007; **352**: 799-804 [PMID: 17150181 DOI: 10.1016/j.bbrc.2006.11.086]

38 **Swartz KJ**, MacKinnon R. An inhibitor of the Kv2.1 potassium channel isolated from the venom of a Chilean tarantula. *Neuron* 1995; **15**: 941-949 [PMID: 7576642 DOI: 10.1016/0896-6273(95)90184-1]

39 **Escoubas P**, Diochot S, Célérier ML, Nakajima T, Lazdunski M. Novel tarantula toxins for subtypes of voltage-dependent potassium channels in the Kv2 and Kv4 subfamilies. *Mol Pharmacol* 2002; **62**: 48-57 [PMID: 12065754 DOI: 10.1124/mol.62.1.48]

40 **Herrington J**, Zhou YP, Bugianesi RM, Dulski PM, Feng Y, Warren VA, Smith MM, Kohler MG, Garsky VM, Sanchez M, Wagner M, Raphaelli K, Banerjee P, Ahaghotu C, Wunderler D, Priest BT, Mehl JT, Garcia ML, McManus OB, Kaczorowski GJ, Slaughter RS. Blockers of the delayed-rectifier potassium current in pancreatic beta-cells enhance glucose-dependent insulin secretion. *Diabetes* 2006; **55**: 1034-1042 [PMID: 16567526 DOI: 10.2337/diabetes.55.04.06.db05-0788]

41 **Chen R**, Robinson A, Chung SH. Binding of hanatoxin to the voltage sensor of Kv2.1. *Toxins* (Basel) 2012; **4**: 1552-1564 [PMID: 23250329 DOI: 10.3390/toxins4121552]

42 **Lee S**, Milescu M, Jung HH, Lee JY, Bae CH, Lee CW, Kim HH, Swartz KJ, Kim JI. Solution structure of GxTX-1E, a high-affinity tarantula toxin interacting with voltage sensors in Kv2.1 potassium channels . *Biochemistry* 2010; **49**: 5134-5142 [PMID: 20509680 DOI: 10.1021/bi100246u]

43 **Lopatin AN**, Nichols CG. Block of delayed rectifier (DRK1) K+ channels by internal 2,3-butanedione monoxime in Xenopus oocytes. *Receptors Channels* 1993; **1**: 279-286 [PMID: 8081725]

44 **Taglialatela M**, Vandongen AM, Drewe JA, Joho RH, Brown AM, Kirsch GE. Patterns of internal and external tetraethylammonium block in four homologous K+ channels. *Mol Pharmacol* 1991; **40**: 299-307 [PMID: 1875913]

45 **Wible B**, Murawsky MK, Crumb WJ, Rampe D. Stable expression and characterization of the human brain potassium channel Kv2.1: blockade by antipsychotic agents. *Brain Res* 1997; **761**: 42-50 [PMID: 9247064 DOI: 10.1016/S0006-8993(97)00315-6]

46 **Zhang ZH**, Lee YT, Rhodes K, Wang K, Argentieri TM, Wang Q. Inhibitory effects of pimozide on cloned and native voltage-gated potassium channels. *Brain Res Mol Brain Res* 2003; **115**: 29-38 [PMID: 12824052 DOI: 10.1016/S0169-328X(03)00175-X]

47 **Kulkarni RS**, Zorn LJ, Anantharam V, Bayley H, Treistman SN. Inhibitory effects of ketamine and halothane on recombinant potassium channels from mammalian brain. *Anesthesiology* 1996; **84**: 900-909 [PMID: 8638845 DOI: 10.1097/00000542-199604000-00018]

48 **Madeja M**, Steffen W, Mesic I, Garic B, Zhorov BS. Overlapping binding sites of structurally different antiarrhythmics flecainide and propafenone in the subunit interface of potassium channel Kv2.1. *J Biol Chem* 2010; **285**: 33898-33905 [PMID: 20709754 DOI: 10.1074/jbc.M110.159897]

49 **Frolov RV**, Bondarenko VE, Singh S. Mechanisms of Kv2.1 channel inhibition by celecoxib--modification of gating and channel block. *Br J Pharmacol* 2010; **159**: 405-418 [PMID: 20015088 DOI: 10.1111/j.1476-5381.2009.00539.x]

50 **Kirsch GE**, Drewe JA. Gating-dependent mechanism of 4-aminopyridine block in two related potassium channels. *J Gen Physiol* 1993; **102**: 797-816 [PMID: 8301258 DOI: 10.1085/jgp.102.5.797]

51 **Gordon E**, Cohen JL, Engel R, Abbott GW. 1,4-Diazabicyclo[2.2.2]octane derivatives: a novel class of voltage-gated potassium channel blockers. *Mol Pharmacol* 2006; **69**: 718-726 [PMID: 16317109]

52 **Salvador-Recatala V**, Kim Y, Zaks-Makhina E, Levitan ES. Voltage-gated k+ channel block by catechol derivatives: defining nonselective and selective pharmacophores. *J Pharmacol Exp Ther* 2006; **319**: 758-764 [PMID: 16880198 DOI: 10.1124/jpet.106.107607]

53 **Yoshikawa H**, Ma Z, Björklund A, Grill V. Short-term intermittent exposure to diazoxide improves functional performance of beta-cells in a high-glucose environment. *Am J Physiol Endocrinol Metab* 2004; **287**: E1202-E1208 [PMID: 15292032 DOI: 10.1152/ajpendo.00255.2004]

54 **Yuan H**, Wang WP, Feng N, Wang L, Wang XL. Donepezil attenuated oxygen-glucose deprivation insult by blocking Kv2.1 potassium channels. *Eur J Pharmacol* 2011; **657**: 76-83 [PMID: 21300054 DOI: 10.1016/j.ejphar.2011.01.054]

55 **Cotella D**, Hernandez-Enriquez B, Wu X, Li R, Pan Z, Leveille J, Link CD, Oddo S, Sesti F. Toxic role of K+ channel oxidation in mammalian brain. *J Neurosci* 2012; **32**: 4133-4144 [PMID: 22442077 DOI: 10.1523/JNEUROSCI.6153-11.2012]

56 **Du J**, Haak LL, Phillips-Tansey E, Russell JT, McBain CJ. Frequency-dependent regulation of rat hippocampal somato-dendritic excitability by the K+ channel subunit Kv2.1. *J Physiol* 2000; **522** Pt 1: 19-31 [PMID: 10618149 DOI: 10.1111/j.1469-7793.2000.t01-2-00019.xm]

57 **Scannevin RH**, Murakoshi H, Rhodes KJ, Trimmer JS. Identification of a cytoplasmic domain important in the polarized expression and clustering of the Kv2.1 K+ channel. *J Cell Biol* 1996; **135**: 1619-1632 [PMID: 8978827 DOI: 10.1083/jcb.135.6.1619]

58 **Fox PD**, Loftus RJ, Tamkun MM. Regulation of Kv2.1 K(+) conductance by cell surface channel density. *J Neurosci* 2013; **33**: 1259-1270 [PMID: 23325261 DOI: 10.1523/JNEUROSCI.3008-12.2013]

59 **Deutsch E**, Weigel AV, Akin EJ, Fox P, Hansen G, Haberkorn CJ, Loftus R, Krapf D, Tamkun MM. Kv2.1 cell surface clusters are insertion platforms for ion channel delivery to the plasma membrane. *Mol Biol Cell* 2012; **23**: 2917-2929 [PMID: 22648171 DOI: 10.1091/mbc.E12-01-0047]

60 **O'Connell KM**, Rolig AS, Whitesell JD, Tamkun MM. Kv2.1 potassium channels are retained within dynamic cell surface microdomains that are defined by a perimeter fence. *J Neurosci* 2006; **26**: 9609-9618 [PMID: 16988031 DOI: 10.1523/JNEU-

ROSCI.1825-06.2006]
61 **Ikematsu N**, Dallas ML, Ross FA, Lewis RW, Rafferty JN, David JA, Suman R, Peers C, Hardie DG, Evans AM. Phosphorylation of the voltage-gated potassium channel Kv2.1 by AMP-activated protein kinase regulates membrane excitability. *Proc Natl Acad Sci USA* 2011; **108**: 18132-18137 [PMID: 22006306 DOI: 10.1073/pnas.1106201108]
62 **Yao H**, Zhou K, Yan D, Li M, Wang Y. The Kv2.1 channels mediate neuronal apoptosis induced by excitotoxicity. *J Neurochem* 2009; **108**: 909-919 [PMID: 19077057 DOI: 10.1111/j.1471-4159.2008.05834.x]
63 **Shen QJ**, Zhao YM, Cao DX, Wang XL. Contribution of Kv channel subunits to glutamate-induced apoptosis in cultured rat hippocampal neurons. *J Neurosci Res* 2009; **87**: 3153-3160 [PMID: 19472219 DOI: 10.1002/jnr.22136]
64 **Jiao S**, Liu Z, Ren WH, Ding Y, Zhang YQ, Zhang ZH, Mei YA. cAMP/protein kinase A signalling pathway protects against neuronal apoptosis and is associated with modulation of Kv2.1 in cerebellar granule cells. *J Neurochem* 2007; **100**: 979-991 [PMID: 17156132 DOI: 10.1111/j.1471-4159.2006.04261.x]
65 **Pal S**, Hartnett KA, Nerbonne JM, Levitan ES, Aizenman E. Mediation of neuronal apoptosis by Kv2.1-encoded potassium channels. *J Neurosci* 2003; **23**: 4798-4802 [PMID: 12832499]
66 **Yu SP**. Regulation and critical role of potassium homeostasis in apoptosis. *Prog Neurobiol* 2003; **70**: 363-386 [PMID: 12963093 DOI: 10.1016/S0301-0082(03)00090-X]
67 **Pal SK**, Takimoto K, Aizenman E, Levitan ES. Apoptotic surface delivery of K+ channels. *Cell Death Differ* 2006; **13**: 661-667 [PMID: 16273079 DOI: 10.1038/sj.cdd.4401792]
68 **Zhou MH**, Yang G, Jiao S, Hu CL, Mei YA. Cholesterol enhances neuron susceptibility to apoptotic stimuli via cAMP/PKA/CREB-dependent up-regulation of Kv2.1. *J Neurochem* 2012; **120**: 502-514 [PMID: 22118516 DOI: 10.1111/j.1471-4159.2011.07593.x]
69 **Aras MA**, Aizenman E. Obligatory role of ASK1 in the apoptotic surge of K+ currents. *Neurosci Lett* 2005; **387**: 136-140 [PMID: 16006035 DOI: 10.1016/j.neulet.2005.06.024]
70 **Redman PT**, Hartnett KA, Aras MA, Levitan ES, Aizenman E. Regulation of apoptotic potassium currents by coordinated zinc-dependent signalling. *J Physiol* 2009; **587**: 4393-4404 [PMID: 19622611 DOI: 10.1113/jphysiol.2009.176321]
71 **Redman PT**, He K, Hartnett KA, Jefferson BS, Hu L, Rosenberg PA, Levitan ES, Aizenman E. Apoptotic surge of potassium currents is mediated by p38 phosphorylation of Kv2.1. *Proc Natl Acad Sci USA* 2007; **104**: 3568-3573 [PMID: 17360683 DOI: 10.1073/pnas.0610159104]
72 **McCord MC**, Aizenman E. Convergent Ca2+ and Zn2+ signaling regulates apoptotic Kv2.1 K+ currents. *Proc Natl Acad Sci USA* 2013; **110**: 13988-13993 [PMID: 23918396 DOI: 10.1073/pnas.1306238110]
73 **Norris CA**, He K, Springer MG, Hartnett KA, Horn JP, Aizenman E. Regulation of neuronal proapoptotic potassium currents by the hepatitis C virus nonstructural protein 5A. *J Neurosci* 2012; **32**: 8865-8870 [PMID: 22745487 DOI: 10.1523/JNEUROSCI.0937-12.2012]
74 **Sobko A**, Peretz A, Attali B. Constitutive activation of delayed-rectifier potassium channels by a src family tyrosine kinase in Schwann cells. *EMBO J* 1998; **17**: 4723-4734 [PMID: 9707431 DOI: 10.1093/emboj/17.16.4723]
75 **Peretz A**, Sobko A, Attali B. Tyrosine kinases modulate K+ channel gating in mouse Schwann cells. *J Physiol* 1999; **519** Pt 2: 373-384 [PMID: 10457056 DOI: 10.1111/j.1469-7793.1999.0373m.x]
76 **Tiran Z**, Peretz A, Attali B, Elson A. Phosphorylation-dependent regulation of Kv2.1 Channel activity at tyrosine 124 by Src and by protein-tyrosine phosphatase epsilon. *J Biol Chem* 2003; **278**: 17509-17514 [PMID: 12615930 DOI: 10.1074/jbc.M212766200]
77 **Tiran Z**, Peretz A, Sines T, Shinder V, Sap J, Attali B, Elson A. Tyrosine phosphatases epsilon and alpha perform specific and overlapping functions in regulation of voltage-gated potassium channels in Schwann cells. *Mol Biol Cell* 2006; **17**: 4330-4342 [PMID: 16870705 DOI: 10.1091/mbc.E06-02-0151]
78 **Peretz A**, Gil-Henn H, Sobko A, Shinder V, Attali B, Elson A. Hypomyelination and increased activity of voltage-gated K(+) channels in mice lacking protein tyrosine phosphatase epsilon. *EMBO J* 2000; **19**: 4036-4045 [PMID: 10921884 DOI: 10.1093/emboj/19.15.4036]
79 **Wu X**, Hernandez-Enriquez B, Banas M, Xu R, Sesti F. Molecular mechanisms underlying the apoptotic effect of KCNB1 K+ channel oxidation. *J Biol Chem* 2013; **288**: 4128-4134 [PMID: 23275378 DOI: 10.1074/jbc.M112.440933]
80 **Dallas ML**, Boyle JP, Milligan CJ, Sayer R, Kerrigan TL, McKinstry C, Lu P, Mankouri J, Harris M, Scragg JL, Pearson HA, Peers C. Carbon monoxide protects against oxidant-induced apoptosis via inhibition of Kv2.1. *FASEB J* 2011; **25**: 1519-1530 [PMID: 21248240 DOI: 10.1096/fj.10-173450]
81 **Al-Owais MM**, Scragg JL, Dallas ML, Boycott HE, Warburton P, Chakrabarty A, Boyle JP, Peers C. Carbon monoxide mediates the anti-apoptotic effects of heme oxygenase-1 in medulloblastoma DAOY cells via K+ channel inhibition. *J Biol Chem* 2012; **287**: 24754-24764 [PMID: 22593583 DOI: 10.1074/jbc.M112.357012]
82 **Mankouri J**, Dallas ML, Hughes ME, Griffin SD, Macdonald A, Peers C, Harris M. Suppression of a pro-apoptotic K+ channel as a mechanism for hepatitis C virus persistence. *Proc Natl Acad Sci USA* 2009; **106**: 15903-15908 [PMID: 19717445 DOI: 10.1073/pnas.0906798106]
83 **Amako Y**, Igloi Z, Mankouri J, Kazlauskas A, Saksela K, Dallas M, Peers C, Harris M. Hepatitis C virus NS5A inhibits mixed lineage kinase 3 to block apoptosis. *J Biol Chem* 2013; **288**: 24753-24763 [PMID: 23857585 DOI: 10.1074/jbc.M113.491985]
84 **Hulme JT**, Coppock EA, Felipe A, Martens JR, Tamkun MM. Oxygen sensitivity of cloned voltage-gated K(+) channels expressed in the pulmonary vasculature. *Circ Res* 1999; **85**: 489-497 [PMID: 10488051 DOI: 10.1161/01.RES.85.6.489]
85 **McDaniel SS**, Platoshyn O, Yu Y, Sweeney M, Miriel VA, Golovina VA, Krick S, Lapp BR, Wang JY, Yuan JX. Anorexic effect of K+ channel blockade in mesenteric arterial smooth muscle and intestinal epithelial cells. *J Appl Physiol* (1985) 2001; **91**: 2322-2333 [PMID: 11641377]
86 **Michelakis ED**, Rebeyka I, Wu X, Nsair A, Thébaud B, Hashimoto K, Dyck JR, Haromy A, Harry G, Barr A, Archer SL. O2 sensing in the human ductus arteriosus: regulation of voltage-gated K+ channels in smooth muscle cells by a mitochondrial redox sensor. *Circ Res* 2002; **91**: 478-486 [PMID: 12242265 DOI: 10.1161/01.RES.0000035057.63303.D1]
87 **Thébaud B**, Michelakis ED, Wu XC, Moudgil R, Kuzyk M, Dyck JR, Harry G, Hashimoto K, Haromy A, Rebeyka I, Archer SL. Oxygen-sensitive Kv channel gene transfer confers oxygen responsiveness to preterm rabbit and remodeled human ductus arteriosus: implications for infants with patent ductus arteriosus. *Circulation* 2004; **110**: 1372-1379 [PMID: 15353504 DOI: 10.1161/01.CIR.0000141292.28616.65]
88 **Archer SL**, Wu XC, Thébaud B, Moudgil R, Hashimoto K, Michelakis ED. O2 sensing in the human ductus arteriosus: redox-sensitive K+ channels are regulated by mitochondria-derived hydrogen peroxide. *Biol Chem* 2004; **385**: 205-216 [PMID: 15134333 DOI: 10.1515/BC.2004.014]
89 **Conforti L**, Takimoto K, Petrovic M, Pongs O, Millhorn D. The pore region of the Kv1.2alpha subunit is an important component of recombinant Kv1.2 channel oxygen sensitivity. *Biochem Biophys Res Commun* 2003; **306**: 450-456 [PMID: 12804584 DOI: 10.1016/S0006-291X(03)00989-6]
90 **Michelakis ED**, Dyck JR, McMurtry MS, Wang S, Wu XC, Moudgil R, Hashimoto K, Puttagunta L, Archer SL. Gene transfer and metabolic modulators as new therapies for pulmonary hypertension. Increasing expression and activity of

potassium channels in rat and human models. *Adv Exp Med Biol* 2001; **502**: 401-418 [PMID: 11950153 DOI: 10.1007/978-1-4757-3401-0_26]
91 **Hong Z**, Weir EK, Nelson DP, Olschewski A. Subacute hypoxia decreases voltage-activated potassium channel expression and function in pulmonary artery myocytes. *Am J Respir Cell Mol Biol* 2004; **31**: 337-343 [PMID: 15151918 DOI: 10.1165/rcmb.2003-0386OC]
92 **Wang J**, Weigand L, Wang W, Sylvester JT, Shimoda LA. Chronic hypoxia inhibits Kv channel gene expression in rat distal pulmonary artery. *Am J Physiol Lung Cell Mol Physiol* 2005; **288**: L1049-L1058 [PMID: 15665041 DOI: 10.1152/ajplung.00379.2004]
93 **Platoshyn O**, Yu Y, Golovina VA, McDaniel SS, Krick S, Li L, Wang JY, Rubin LJ, Yuan JX. Chronic hypoxia decreases K(V) channel expression and function in pulmonary artery myocytes. *Am J Physiol Lung Cell Mol Physiol* 2001; **280**: L801-L812 [PMID: 11238022]
94 **Dong Q**, Zhao N, Xia CK, Du LL, Fu XX, Du YM. Hypoxia induces voltage-gated K+ (Kv) channel expression in pulmonary arterial smooth muscle cells through hypoxia-inducible factor-1 (HIF-1). *Bosn J Basic Med Sci* 2012; **12**: 158-163 [PMID: 22938542]
95 **Guo L**, Qiu Z, Zhang L, Chen S, Zhu D. Hypoxia suppresses Kv 2.1 channel expression through endogenous 15-hydroxyeicosatetraenoic acid in rat pulmonary artery. *J Physiol Sci* 2010; **60**: 373-381 [PMID: 20680544 DOI: 10.1007/s12576-010-0105-z]
96 **Swaninathan N.** How to save your brain. *Psych Today* 2012; 45: 74-79
97 **Alzheimer's Drug Discovery Foundation.** Annual Report, 2005. Available from: URL: http: //www.alzdiscovery.org/assets/content/publications/ADDF_AR_3-22B.pdf
98 **Alzheimer's Association.** 2010 Alzheimer's disease facts and figures. *Alzheimers Dement* 2010; **6**: 158-194 [PMID: 20298981 DOI: 10.1016/j.jalz.2010.01.009]
99 **Alzheimer's Association.** Diagniostic Center for Alzheimer's Disease. Available from: URL: http: //www.alz.org/alzheimers_disease_diagnosis.asp

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REVIEW

Regulation of cell survival and death during *Flavivirus* infections

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Abstract

Flaviviruses, ss(+) RNA viruses, include many of mankind's most important pathogens. Their pathogenicity derives from their ability to infect many types of cells including neurons, to replicate, and eventually to kill the cells. *Flaviviruses* can activate tumor necrosis factor α and both intrinsic (Bax-mediated) and extrinsic pathways to apoptosis. Thus they can use many approaches for activating these pathways. Infection can lead to necrosis if viral load is extremely high or to other types of cell death if routes to apoptosis are blocked. Dengue and Japanese Encephalitis Virus can also activate autophagy. In this case the autophagy temporarily spares the infected cell, allowing a longer period of reproduction for the virus, and the autophagy further protects the cell against other stresses such as those caused by reactive oxygen species. Several of the viral proteins have been shown to induce apoptosis or autophagy on their own, independent of the presence of other viral proteins. Given the versatility of these viruses to adapt to and manipulate the metabolism, and thus to control the survival of, the infected cells, we need to understand much better how the specific viral proteins affect the pathways to apoptosis and autophagy. Only in this manner will we be able to minimize the pathology that they cause.

Key words: *Flavivirus*; Dengue virus; West Nile virus; Japanese encephalitis virus; Programmed cell death; Apoptosis; Extrinsic pathway; Intrinsic pathway; Autophagy; Necrosis

Core tip: The pathogenicity of *Flaviviruses* derives from their ability to infect many types of cells. They can activate both intrinsic and extrinsic pathways of apoptosis, by many means. Dengue and Japanese encephalitis virus can also activate autophagy, whereby autophagy temporarily spares the infected cell, allowing longer reproduction of virus and protecting the cell against other stresses. Given the versatility of these viruses, we need to understand much better how the specific viral proteins affect the pathways to apoptosis and autophagy. Only in this manner will we be able to minimize the pathology that they cause.

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INTRODUCTION

The aim of a virus is to infect and propagate and in doing so, affect the cell survival pathways. A wide range of viruses from different families (Poxviridae, Adenoviridae, Retroviridae, Picornoviridae, Flaviviridae, Orthomyxoviridae) have life cycles that intertwine with critical pathways involved in cell death and survival[1]. In this review we

focus our attention on *Flavivirus* (Flaviviridae).

Flaviviridae, a family of small and enveloped ss(+)-RNA virus, consists some of the worst pathogens known to mankind and mammals. The family is grouped into three genera, namely, *Flavivirus*, *Hepacivirus* and *Pestivirus* with each genus harboring potent killers, *viz*., dengue (DEN), hepatitis C (HCV) and classical swine fever virus (CSFV), respectively[2]. The largest and clinically the most relevant of three, *Flavivirus* contains almost 70 members, most of them transmitted to humans by mosquitos or ticks. Among the mosquito-borne are the most virulent viruses like dengue (DEN)[3,4], West Nile (WNV)[5], Japanese encephalitis (JEV) and Yellow fever (YFV)[6].

Although a few reviews address the role of cell death pathways during viral infection in general[1,7,8], there are none solely addressing *Flavivirus*. Here we summarize the most recent findings on survival and cell death pathways triggered by key members of *Flavivirus*. We focus on flaviviruses widely studied in relation to cell death - dengue, West Nile and Japanese encephalitis virus. We conclude that the viruses affect different parts of the apoptotic pathways in different cell types, and that dengue and JEV especially can protect cells by activating autophagy. Antiviral therapeutics will have to address these issues.

CELL DEATH AND ITS PATHWAYS

The ascendance of programmed cell death (PCD) as a theme of modern biology has followed an exciting trail from the mid-19th century until the present[9]. The idea of a cell programming its death had few takers during the early half of 20th century, though evidence was gathering since 1842, when Carl Vogt observed loss of notochord in amphibian metamorphosis[10]. Since then, evidence of programmed cell death has surfaced in various organisms as diverse as Dictyostelium[11], insects[12], and chicken[13]. Recognition of *apoptosis* as the primary form of programmed cell death, in the early 1970's[14] as well as recognition that apoptosis is conserved from *C. elegans* to humans)[15,16] has fueled interest among biologists. Moreover, association of apoptosis and other forms of cell death, notably the lysosomal (autophagic) cell death, with AIDS[17], cancer[18,19], Alzheimer's[20], and viral infection[1] has catapulted cell death to the forefront of biomedical research.

The importance of cell death was not fully appreciated until the late 1960's. This delay was partly due to the difficulty in documenting dying cells, as compared to dividing ones, as it was possible to monitor and finally trace a cell's duplication into daughter cells. While cells that have undergone mitosis can be traced considerably thereafter, an apoptotic cell in an organism is visible only up to 20 min after death[12].

Programmed cell death contributes to the sculpting of digits (prenatal disappearance of interdigital epidermis), removal of unnecessary tissues (involution of mammary glands during post-lactation) or irrelevant (wolffian/mullerian ducts after sex determination) organs, elimination of toxic and harmful cells (self-reactive thymocytes, UV-irradiated cells), and winnowing to only a properly integrated cell population (as in the case of differentiated neurons)[21,22]. A cell may trigger its own death (intrinsic/cell autonomous) or it may be brought upon by signals from the microenvironment (extrinsic). Deregulation of the cell death machinery can inflict upon the organism severe consequences like anomalous or stalled development, tumor formation, autoimmune disorder or neurological disorders (Huntington, Parkinson). In contrast, the vestiges of dead cells in some plants may serve important functions[22,23].

Most biologists make a clear distinction between "programmed" physiological (beneficial) and "accidental" (hazardous) cell death. The former denotes death of cells essential for physiological events (development, organogenesis, homeostasis, and defense) whereas the latter may be used for loss of cells during tissue damage. Apart from this functional distinction, cell death can also be classified based on morphology (apoptosis, autophagy, necrosis, and cornification) and enzyme involvement (proteases like calpains, caspases, and endonucleases). The Nomenclature Committee on Cell Death (NCCD) encourages researchers to clearly distinguish between "dying cells" and "dead cells", and by using the latter term, they should denote cells that have gone past the threshold "point-of-no-return" into a state of irreversibility. The NCCD has also revised the defining hallmarks for a dead cell: dissolution of the plasma membrane and complete fragmentation and engulfment by phagocytosis, since the traditional parameters like activation of caspases, mitochondrial trans membrane permeabilization and flipping of phosphatidylserine (PS) have been associated with non-lethal events[24].

APOPTOSIS

The most studied form of programmed cell death (PCD), *apoptosis* (Greek: falling of leaves), was first reported by Walter Flemming[10]. Kerr *et al*[14] characterized apoptosis (later described by Majno and Joris as PCD type I) and described it as a general process mistakenly previously identified as an arcane form of death called "shrinkage necrosis". While undergoing apoptosis, the cell separates from its neighboring cells, shrinks, undergoes chromatin condensation and DNA fragmentation, and is finally engulfed by a phagocyte (macrophage).

Apoptosis follows two distinct pathways, the extrinsic (death receptor) and intrinsic (mitochondrial) pathway[25]. The extrinsic branch of PCD is activated by external death signals. The cytotoxic effect is mediated by the binding of ligands [tumor necrosis factor-α (TNF-α), FasL, TRAIL] to the death receptors (TNF RI, Fas/CD95, DR3, TRAIL R1/DR4, or TRAIL R2/DR5) on the cell surface[26-28]. This binding leads to the trimerization of the membrane receptor, followed by the downstream activation of the DISC protein complex. The multi-protein complex initiates cleavage and activation of caspase-8, which in turn cleaves downstream zymogens (caspase-7, 10) and this sets forth a chain of reactions fi-

nally leading to activation of caspase-3 and cell death[25,28]. The caspase proteins (*C*ysteine-dependent *Asp*artate-directed Prote*ases* = C-A-S-PASES) are central to the entire apoptotic machinery within the cell. They are also integral to the intrinsic pathway, are synthesized as inactive zymogens that are activated by cleavage.

Intrinsic apoptosis is activated proximately by damage to mitochondria, which releases cytochrome C and apoptosis-activating factor from mitochondria. These latter, together with pro-caspase-9, bind together into an apoptosome, in which caspase-9 is activated. By means of this complex, caspase-3 is activated and, as in extrinsically-activated apoptosis, caspases 3 and 7 destroy the substructure of the cell.

Like caspases, Bcl-2 family members are also essential for carrying out intrinsic apoptosis. Based on domain structure and function, the members are grouped into anti-apoptotic guardians (Bcl-2, Bcl-xL, MCL-1), pro-apoptotic effectors (Bax, Bak) and sensors (Bad/Bim/Bid/Noxa)[29-31]. The intrinsic pathway is initiated by intracellular stress signals like ER stress, oxidative stress, DNA damage, growth factor withdrawals, and loss of contact with the extracellular matrix. Once the decision to die is made, the effectors are set free from their negative interaction with guardians by the sensors. They insert into and disintegrate the mitochondrial membrane, a phenomenon known as the mitochondrial outer membrane permeabilization (MOMP). This releases pro-apoptotic factors (cytochrome C, Smac/Diablo, HTRA2/Omi, apoptosis-inducing factor, and endonuclease G) into the cytoplasm. Cytochrome C interacts with the APAF-1, recruiting pro-caspase-9 (zymogen) to form the *apoptosome,* where the latter is cleaved and activated. This event triggers cleavage and activation of downstream caspases (2, 3, 7, 8) and accomplishes the death of cell[32]. Certain cell death regulators like inhibitor of apoptosis (IAP) can bind and suppress the apoptotic function of caspases[33].

AUTOPHAGY

Autophagy or PCD type II, literally meaning "self-eating", is a highly conserved catabolic process that is thought to precede apoptosis in evolution[34]. It is a surveillance process that is involved in the recycling of basic biomolecules. It oversees the entire cell homeostasis, packaging degraded/misfolded proteins or organelles in specialized bilayer membranes (autophagosomes) which fuse with the lysosome for digestion. This process is induced under conditions of high stress like starvation, growth factor withdrawal, viral invasion and ER stress. Deregulation of the autophagy pathway has been observed in pathogenic conditions like cancer or Parkinson's[35].

The induction of autophagy involves a set of multiprotein complexes, some of which have ubiquitin-like properties. mTORC1, a versatile signaling complex, strictly inhibits induction of autophagy by imposing an inhibitory phosphorylation on Unc-51-like kinase (ULK1). Under stress conditions, this block is removed by several factors, such as PTEN, AMPK, and TSC2. Activation of ULK1, which forms a complex with ATG13/FIP200/ATG101, leads to the nucleation of the pre-autophagosomal structure (PAS). This involves the phosphatidylinositol-3-kinase class Ⅲ (PI3K Ⅲ)-Vps34-Beclin 1 (ATG6) complex[36,37]. The subsequent elongation of the autophagosome is dependent on two ubiquitin-like conjugation systems. E1-like enzyme autophagy related gene 7 (*ATG7*) and E2-like enzymes ATG3, ATG10 are involved in the conjugation of ATG12-ATG5 and LC3 (ATG8)-phosphatidylethanolamine (PE). ATG12-ATG5 acts like an E3-like protein for the LC3-PE conjugation system, and then forms a complex with ATG16. These coordinated and combined steps accomplish the formation of a mature autophagosome which then fuses with a lysosome through a canonical endocytic pathway[25,38-40].

NECROSIS

Some forms of necrosis are programmed and controlled through a specific set of signal transduction pathways and degradative mechanisms. Cell death by specific necrosis can also contribute to embryonic development and adult tissue homeostasis[41]. Necrosis can be triggered by the same death signals that induce apoptosis[42]. The difference between apoptosis and specific necrosis lies in the rapid cytoplasmic swelling and release of extracellular components, seen in specific necrosis, which is often due to extreme physiochemical stress, osmotic shock, mechanical stress and high concentration of hydrogen peroxide[43]. When a cell is under such conditions, which can be produced by physiological or developmental situations, cell death occurs accidentally and uncontrolled. Necrosis signaling complex forms by interaction of receptor interacting protein 1 (RIPK1) with the receptor interacting protein 3 (RIPK3). This signaling complex forms by introducing death receptors either by inhibiting caspases or genotoxic stress[43]. In this type of cell death, unlike apoptosis, death is accidental and not programmed. Necrosis does not depend on caspase activation. In a study done by Nikoletopoulou *et al*[42], two different cell lines were treated with a tumor necrosis factor-α. In one cell line, apoptosis was triggered, whereas in another cell line it induced necrosis. In addition, necrosis can be in the form of regulated and programmed form of cell death. This phenomenon is referred to as necroptosis. Various death receptors associated with apoptosis, such as FAS, TNFR2, TRAILR1 and TRAILR2, have been shown to induce necroptosis in different cell types. Furthermore, necroptosis can be instigated by the members of the pathogen recognition receptor that are responsible for sensing pathogen-associated molecular patterns.

FLAVIVIRUS-STRUCTURE, INFECTIVITY, REPLICATION AND CELL SURVIVAL

Flaviviridae is a medically important family of animal virus, with members responsible for serious pathological conditions in human and other important mammals. This

group IV family (positive sense RNA) consists of three genera: *Flavivirus*, *Hepacivirus* and *Pestivirus*. The largest of them, *Flavivirus* (with approximately 70 members), includes some of the deadliest arthropod-transmitted virus. They are icosahedral, enveloped (+)-ssRNA virus measuring approximately 500Å in diameter. The typical *Flavivirus* (Latin *flavus* - yellow, indicating Yellow Fever) virion is composed of the genetic material surrounded by the capsid protein and 180 copies of two glycoproteins. The average genome size of the *Flavivirus* is 11kb, coding for a single polyprotein. The amino terminal accounts for the structural proteins: capsid (C), membrane precursor (prM) and envelope (E), and the remaining genome gives rise to the non-structural proteins (NS1, 2A, 2B, 3, 4A, 4B, and 5) which form the viral replication complex (RC)[2,44].

Infection starts as virions bind to the cell membrane through receptor-mediated endocytosis, aided by primary receptors (DC-SIGN, Grp78/BiP, CD-14 associated molecules) and low-affinity co-receptors (heparin, glycosaminoglycan). Acidification of the vesicle triggers disassembly of virus, releasing the genetic material into the cytoplasm. The resultant polyprotein undergoes co- and post-translational processing by viral and host proteases to give rise to the individual proteins. The structural proteins then assemble on the ER surface along with the RNA which is replicated on intracellular membranes. The assembly of virus in the ER lumen is followed by the movement of these immature viral particles through the trans-Golgi network. These are cleaved by the host protease furin to form mature virions, and are subsequently released by exocytosis[45-51].

Dengue virus

Among the members of *Flavivirus* family, Dengue is transmitted to human (in urban areas) and primates (in forests) by the urban-adapted mosquito strain *Aedes aegypti* (primary vector) and the emerging *Aedes albopictus*[52]. Dengue has been declared endemic in approximately 100 countries with 40% of the global population susceptible to infection. Dengue infection has doubled over the last two decades, and current annual figures have risen to 50-100 million humans affected[53].

Dengue has a genome of 10.7 kb positive sense single strand RNA that contains a type I cap at its 5' terminus[54]. The enveloped icosahedral virion measures 50 nm in diameter. The RNA is translated by the host cell machinery into a 3391-amino acid polyprotein that undergoes co- and post-translational processing by viral (NS2B-3) and cellular proteases[55-57]. The first quarter of the viral genome from the 5' end codes for the structural proteins C (capsid), prM (membrane), and E (lipopolysaccharide envelope), thus leaving the rest to code for eight non-structural proteins (NS1, 2A, 2B, 3, 4A, 2K peptide, 4B, 5) which are expressed only inside the host cell[58].

Dengue from different regions of the globe show four antigenically distinct serotypes (DENV 1-4), each having multiple phenotypes[59]. The distribution of these serotypes has spread alarmingly throughout the globe since 1970, when only South Asia had all four[60]. This spread has added to the complexity of dengue-induced pathogenesis since very little cross-immunity has been recorded between these serotypes, leading to multiple sequential infections and overwhelmed immune response[61]. Outcomes of dengue infection may lead to diverse pathogenic conditions, ranging from the mild-flu like febrile syndrome (dengue fever) to the very serious conditions resulting from infection with a second serotype, the lethal hemorrhagic condition dengue hemorrhagic fever (DHF) or the dengue shock syndrome (DSS)[62]. Dengue fever, the most important arboviral disease in humans, features rapid onset of fever, accompanied by headache, retro-orbital pain, myalgia, gastrointestinal irritation[63,64]. DHF, which claims more lives (5% mortality) than any other hemorrhagic fever, is characterized by bleeding, thrombocytopenia, increased vascular permeability beyond the usual dengue fever symptoms[65]. An equally lethal condition DSS is also characterized by vascular leakage, which is more pronounced in young children, and very low blood pressure[66]. Autopsies conducted on patients (predominantly children) dying from DSS have revealed a broad range of dengue susceptible tissue as shown by virus infecting skin, liver, spleen, lymph node, kidney, bone marrow, lung, thymus and brain[67-70].

Cell death and survival after infection with dengue

Dengue has been shown to derive pathogenic effect from apoptotic cell death in several types of mammalian cells. The role of apoptosis in dengue infection has been seriously studied since the mid-1990s, along with the identification of Bcl-2 superfamily members. Dengue-induced apoptosis has been observed in cells from the nervous system (human and mice neuroblastoma, murine cortical and hippocampal neurons, human cerebral cells); liver (human hepatoma); immune system(human peripheral blood mononuclear cells like $CD8^+$-T lymphocytes, monocyte-derived macrophages, human mast cells like KU812, HMC-1, and primary murine macrophages); vascular system (human umbilical cord vein endothelial cells/EA.hy296, human microvascular endothelial cells, pulmonary microvascular endothelial cells/MECs) and, digestive tract (intestinal cells); and kidney cells (human embryonic kidney HEK 293, green monkey kidney Vero). Of the four antigenically distinct serotypes infection with variants of dengue 1 (human isolates of dengue type 1 virus FGA/89 and BR/90, neurovirulent variant FGA/NA d1d), 2 (strain NGC, 16681) and 3 (DENV3/5532) lead to cell death and apoptosis within 25-36 h post infection.

Apoptosis is triggered by live virus or dengue proteins through components of both extrinsic and intrinsic apoptotic signaling (Figure 1). Death ligands and receptors participate in dengue-induced apoptosis. Increased levels of pro-apoptotic proinflammatory cytokines (TNF-α and interleukin-10) and Apo2L/TRAIL are observed after infection, which the virus possibly induces in a TNF-α-fashion[71]. Profiling of genes reveal the activation of death receptors FAS/CD 95, TNFR superfamily member

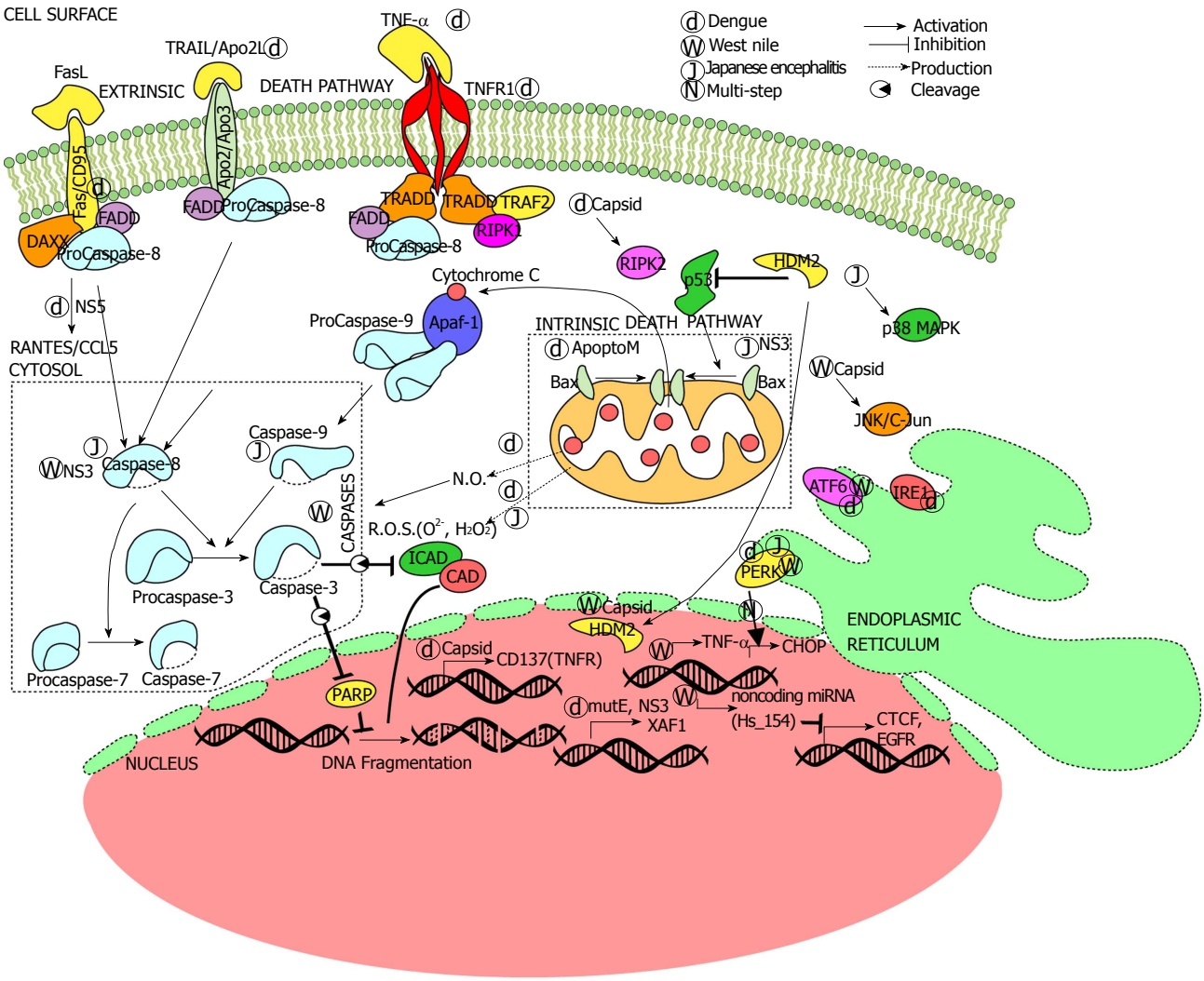

Figure 1 Flaviviruses target cell death and survival pathways. Extrinsic and intrinsic cell death pathways are activated during viral infection (d, w, and j are for dengue, west nile and japanese encephalitis live viruses respectively) or expression of specific viral proteins (d, w and j with viral protein). Expression of multiple genes including non-coding microRNAs (miRNA) also induced during flavivirus infections. FLaviviruses also activate ER stress signaling and increase metabolism related products (ROS and NO). TNF: Tumor necrosis factor; EGFR: Epidermal growth factor receptor.

9/CD 137, TNFRI/TNF-α (caspase-independent) and IL-1β/ NFκB (caspase-dependent) pathways[72,73].

Viral protein NS5 interacts with death protein 6 (Daxx), which among other functions interacts with death receptor FAS, to activate RANTES (CCL5), a cytokine closely associated with DHF[74,75]. Moreover, transfection with wild type capsid protein increased the expression of CD137, a member of the TNFR family. Receptor-interacting serine/threonine protein kinase 2 (RIPK2), a master regulator of stress pathways[76], is also necessary for capsid-induced apoptosis[76]. In addition to capsid protein modulation of death receptor expression, infection with live dengue virus leads to differential expression of several interferon-inducible genes, the most important being XAF1. XAF1 upregulates caspase 3 36 h after infection and mediates apoptosis[77]. The activation of caspases leads to the characteristic nuclear fragmentation and cytoplasmic blebbing of apoptosis.

Mitochondria-mediated or intrinsic apoptosis signaling also occurs after dengue infection. The reactive oxygen species (ROS) O_2 and H_2O_2, which are predominantly produced in the mitochondria, increase during infection. Toxic levels of ROS can activate calpains and lead to apoptosis. Secondary messenger oxides like nitric oxide (NO) also mediate in dengue-triggered apoptosis in a caspase dependent manner[78]. Other dengue structural proteins are also involved in apoptosis. Intracellular production of the M protein from all dengue strains activated the intrinsic pathway apoptosis in mouse neuroblastoma (Neuro2a) and human hepatoma (HepG2) cells. *ApoptoM*, a nine-residue sequence (M-32 to -40) from the M ectodomain (M-1 to -40), is instrumental in the cytopathic effect of the flavivirus[79].

The activation of apoptosis at different levels of the extrinsic and intrinsic pathways by several variants of dengue virus implies an important role in the life cycle of the virus. As infected cells undergo apoptosis by multiple means the extrinsic and intrinsic apoptotic pathways converge at the activation of phosphatidylserine (PS) for phagocytic clearance during secondary dengue infec-

tion[80].

Apoptosis, supposedly an innate immune response, is often manipulated by the viruses like dengue to act against the immune system itself, as shown by the more numerous apoptotic peripheral blood mononuclear cells (PBMC) in dengue infected children. The proportion of apoptosis and its mediators (CD95) in the circulating PBMCs was much higher in individuals progressing towards hemorrhage (DHF) than those developing febrile symptoms (Dengue Fever), indicating a higher viral load in the former. A fact that most of the apoptotic PBMCs were $CD8^+$-T lymphocytes bears testimony to the deranged immune machinery in infected individuals. The immune response to increased dengue-induced apoptosis does not curb virus proliferation. Apoptosis, in the context of dengue infection, fails to arrest viral reproduction and even correlates with increased virus production[72,73].

Unlike lytic viruses that indiscriminately trigger cell death, pro-apoptotic variants of dengue can lose their pathogenic ability in certain cells. For example the neurovirulent variant FGA/NA d1d, developed from the apoptosis inducing dengue 1 human isolate FGA/89, kills neuroblastoma but not hepatoma cells[81]. Apoptosis seen during infection of human umbilical cord vein endothelial cells (ECV304) and Swiss Webster primary macrophages by Dengue-2 virus strain 16681 is lost in MDCK, HeLa, HEK 293T, Vero and Swiss Webster primary mouse embryo fibroblasts (MEF) even after 144 h (6 d) post infection[82,83].

The differences in dengue outbreaks are partly explained by differences in cell killing by clinical isolates of virus from a fatal case (Paraguay 2007; DENV3/5532) had higher replication rate in monocyte-derived human dendritic cells (mdDCs) than isolates of virus from a non-fatal breakout (Brazil 2002; DENV3/290). The former also induced more proinflammatory cytokines associated with apoptosis[71]. Moreover, differences in cell toxicity among dengue variants have been attributed to mutations in the E and NS3[81]. Although adequate to explain certain differences in cell killing these mechanisms fail to explain the attenuated pathogenicity of immune/endothelial toxic dengue against other cells even in the presence of apoptotic agents like staurosporine, cycloheximide, camptothecin and influenza virus[83].

Involvement of autophagy in dengue infection is a relatively new finding, shown first in 2008. DENV2 caused ATG5-dependent autophagy in hepatic (Huh7) and fibroblast (MEF) cells. The virus' ability to induce autophagy correlated positively with viral replication without a direct role in infectivity, as its downregulation did not increase amounts of intracellular virus[84,85]. Denv2-mediated autophagy protects from toxic stimuli canine kidney epithelial (MDCK) and mouse embryo fibroblast (MEF) cells but not murine macrophages, where infection leads to apoptotic cell death. Expression of dengue NS4A protein, like infection with live virus, induces PI3K-mediated autophagy and protects these cells against death from toxins[83]. Specific inhibitors of autophagy like *spautin-1* have revealed the role autophagy plays in maturation of dengue virion. Blocking autophagy in Huh7.a.1, BHK21 cell lines and AG129 mice resulted in a heat-sensitive and non-infectious dengue virion[86].

West Nile virus

West Nile virus (WNV), first encountered in the New World in New York City (1999), has been the cause of three major arboviral neuroinvasive outbreaks in the United States[87]. It belongs to the same Flavivirus serocomplex as the Japanese encephalitis virus (JEV) and St. Louis encephalitis virus 15, following a bird-mosquito-bird transmission cycle. In the United States, *Culex pipiens* serves as the major arthropod vector. The human is a "dead-end host" for WNV due to low levels of serum viremia[88]. WNV consists of five phylogenetic lineages, of which 1, 2 have been associated with significant outbreaks. The primary targets are keratinocytes and dendritic cells, which upon infection migrate to visceral organs and the central nervous system. The neurovirulence of WNV is dependent on varying factors-its ability to cross the endothelium of blood-brain barrier (helped by cytokine mediated increased vascular permeability), import of infected macrophages into the CNS (Trojan horse mechanism) and viral retrograde transport from peripheral neurons to CNS[89-91]. Like dengue, outcome of infection varies from mild fever (WNV fever), accompanied by headache and diarrhea, to neurological symptoms (WNV neuroinvasive disease). While only 1% of infected individuals develop the latter, mild fever can be seen in 25%. However, neuroinvasive infections have a 10% fatality, which makes it extremely lethal. The serious pathological conditions (meningitis, encephalitis, acute flaccid paralysis) are also accompanied by chills, rash and visual disturbance. The severity is higher in elder patients, as is evident from the higher death rate (17%) in individuals aged at least 70 from those (0.8%) in their mid-40s[88,92,93]. Complete recovery following acute infection is extremely rare, and fatigue, cognitive difficulties, depression and muscle aches have been reported even after a year[94-97]. Diagnosis is dependent on detection of IgM levels in the cerebrospinal fluid by MAC-ELISA, although false positive results have been reported during infection with related Flavivirus[98,99]. To date, treatment has been supportive, relying on vector control, and no vaccine is licensed for human use. Human being the "dead-end host", future vaccinations will not prevent spreading of the virus in nature either[100-102]. It is extremely important that molecular mechanisms adopted by the virus, like manipulation of the cell survival pathway, be studied. This would help in developing an effective antiviral therapy.

Cell death and survival after infection with WNV

The relationship between WNV infectivity and cell survival pathways has been studied for more than a decade. WNV-mediated cell death and cytotoxicity depend on the severity of the initial infection. Vero cells infected with many virus particles (multiplicity of infection, *moi* > 10) showed signs of necrosis (leakage of HMGB1 and high LDH activity) within 8 h of infection. In contrast, cells infected with a lower load (*moi* < 10) showed signs

of apoptotic cell death at a later stage (32 *hpi*)[103]. Very similar to dengue, WNV induces apoptotic cell death in several cell types, such as, immune cells (human leukemic -K562), neuronal cells (mouse neuroblastoma - Neuro 2a, brain tumors), epithelial cells (Vero, A549), fibroblasts (MEF, BHK21), and embryonic cells (HEK293T)[104-106].

The upstream events leading to apoptotic death in WNV infected cells include endoplasmic reticulum (ER) stress pathways. Infection of human neuroblastoma (SK-N-MC) cells and primary rat hippocampal neurons led to activation of two branches of ER stress-mediated unfolded protein response (UPR). ATF6 and PERK pathways were induced during infection, resulting in CHOP activation and downstream apoptosis[107]. A different effect on the UPR pathways has been observed. The West Nile virus Kunjin strain (WNV$_{KUN}$) shuts off PERK pathway and interferon-mediated STAT phosphorylation in wild type MEFs. However, it activates the remaining two UPR (ATF6, IRE1) pathways. Studies with *ATF6*$^{-/-}$, *IRE1*$^{-/-}$ MEFs point to the synergetic role these pathways play in WNV$_{KUN}$ pathogenesis. They contribute to increased cell viability and viral load, by restricting apoptotic cell death[108].

WNV can regulate both extrinsic and intrinsic pathways to launch pathogenesis (Figure 1). The virus induces Bax-dependent intracellular apoptosis in human leukemic (K562) and mouse neuroblastoma (Neuro 2a) cells. Strains that did not possess the ability to induce apoptosis, due to UV-inactivation, could not establish infectivity in cells[104]. WNV encephalitis in CNS-derived mouse neurons was highly dependent on the activation of caspase-3, and infection in the permissive T98G (brain-derived tumor) cells involved both extrinsic and intrinsic apoptotic pathways[105,106]. Tetracyclines are well established antiviral compounds, and minocycline strongly inhibited WNV infection in three CNS-derived human cell types (HBN, HRPE, and T98G).The antibiotic blocked viral replication, apoptosis and the viral activation of JNK/c-jun pathway, establishing a link among them[109]. Kobayashi *et al*[110] proposed that the presence of ubiquitinated proteins had functional implication in apoptosis of WNV-infected mouse neuroblastoma (Neuro-2a) cells. Migration of CD8^{+}- T lymphocytes to drained lymph nodes (dLNs) was hindered in the CNS of *Cd22*$^{-/-}$ mice, which had a higher viral load than the wild type. This finding suggests a role for the B-cell marker, also an important component in cell survival, in modulating cellular immunity during infection[111].

Apoptosis often restricts viral replication and infection. Shrestha *et al*[112] showed the beneficial role of TNF-α related apoptosis inducing ligand (TRAIL), produced by CD8^{+}- T cells, in limiting WNV infection in mouse central nervous system. CD8^{+}- T cells in *TRAIL*$^{-/-}$ mice encountered difficulty in clearing the viral particles from the neurons. Zhang *et al*[113] demonstrated, using mouse neuron as an infection model, rise in the levels of TNF-α during infection. The rise served to downregulate the chemokine CXCR3, which would otherwise bind antiviral CXCL10 circulating in the central nervous system (CNS). This interaction results in calcium transients that lead to caspase-3 mediated apoptosis in the neurons, an adaptive mechanism to prevent cell death. Smith *et al*[114] showed an important aspect of WNV infection in human cell culture (HEK293, SK-N-MC) and mouse neuronal tissues - regulation of non-coding microRNAs (miRNAs). Among several miRNAs, Hs_154 is significantly up regulated in infection. Two of its targets, CCCTC-binding factor (CTCF) and epidermal growth factor receptor (EGFR), are associated with cell survival; this accounts for the role of Hs_154 in mediating apoptosis. While this activation has been found to lower viral replication, apoptotic cell death is also the basis for WNV pathogenesis.

As in dengue, both structural and non-structural proteins play a role in cellular survival after infection. WNV capsid (Cp) protein triggers a caspase-dependent apoptosis, leading to inflammation, in mouse brain and muscle[115]. WNV capsid is dependent upon p53 for its apoptotic effects. It has been shown to sequester HDM2, a negative regulator of p53, into the nucleolus. This results in a higher stability of p53, which can then target Bax to induce apoptosis in MEF cells[116]. Inhibitor-based studies on four types of mammalian cells (A549, HEK293T, Vero-76, BHK-21) suggest a role for WNV capsid (C) protein in the inhibition of apoptosis through Phosphatidylinositol-3-kinase (PI3K)- Akt prosurvival pathway[117]. The helicase and protease domains of NS3 protein are instrumental in inducing a caspase-8 dependent apoptosis in three types (Neuro 2a, HeLa, and Vero) of mammalian cells[118].

Our present knowledge does not suggest any significant role of autophagy in WNV pathogenesis, distinguishing it from dengue and Japanese encephalitis virus. Though infection induced autophagy in mice brain slice and several mammalian cells, it was actually PI3K that was involved in viral replication[119,120].

Japanese encephalitis virus

Japanese encephalitis virus (JEV) is extremely important as it is spreading throughout Asia, China, India, Australia, and Pakistan and is responsible for between 12500 to 17500 deaths reported annually. JEV is transmitted by a primary mosquito vector (*Culex tritaeniorhynchus)* and secondary mosquito vector (*Culex gelidus*, *Culex fuscocephala* and *Culex vishnui*) that primarily target domestic animals and human host[121]. Humans are "dead end host", since they cannot infect the feeding mosquitoes because of low viremia. Children are at higher risk for an infection with Japanese encephalitis than adults, especially in rural areas. They are also at higher risk for death due to their weaker immune system as compared to the adults. In addition, people who visit Asia and Indonesia are particularly prone to this viral infection since they lack the protective antibodies. Asymptomatic infection depends on host's age, immunity, general make-up and current health status. Symptoms include headache, fever, tremor, gadtrointestinal comfort as well as severe conditions of encephalitis

and Parkinson-like seizures[122].

The means of the entry of the virus entry into the system plays an important role on the progress of the infection. If the carrier, the mosquito, bites directly into the blood vessel, it is easier for the virus to spread directly to the central nervous system.

There have been efforts to make a vaccine against JEV, although its successful implementation has been impeded by frequent climate changes. The spread of Japanese encephalitis virus is assisted by wind-blown mosquitoes, bird migration and people traveling with infected virus, which further spread the disease. Programs in underdeveloped countries are established in order to prevent the increasing number of yearly deaths caused by Japanese encephalitis virus. These programs include mosquito control by using pesticide, mosquito nets, cattle segregation and vaccination of cattle as well as humans[121,123].

Cell death and survival after infection with japanese encephalitis virus

As shown in Figure 1, JEV-induced apoptotic cell death is reliant on endoplasmic reticulum (ER) stress and production of reactive oxygen species (ROS). ER stress-induced activation of UPR factors (CHOP-p38MAPK) is essential for triggering the apoptotic response in fibroblasts (BHK-21) and neuronal cells (N18, NT-2)[124]. Even replication-incompetent strains (UV-JEV), as shown by Lin *et al*[125], retain their ability to kill neuronal cells (N18, NT-2) by inducing ROS production and activating NF-κB. The structural E protein from JEV-YL induces apoptotic cytotoxicity in HepG2and Vero cells[126]. Earlier studies had pointed to a link between non-structural NS3 protein and induction of apoptosis. Transfection of pEGFP-NS3 1-619 plasmid (whole NS3 protein) into Vero cells caused apoptotic cell death. The same study also evaluated the role of caspases where it was found that NS3 only activates the intrinsic branch (casp -9,-3) of apoptosis[127,128].

Bcl-2 proteins can prevent apoptosis by controlling the release of cytochrome C. Overexpression of bcl-2, however, did not block viral replication and distribution in mouse neuroblastoma N18 cells, though it delayed cell death in BHK-21 cells. Moreover, in BHK-21 and CHO cells, bcl-2 overexpression established persistent infection by virtue of its antiapoptotic property. Thus, bcl-2 was not a fruitful target for preventing infection. It was due to the ability of this virus to activate complex pathways of caspase-dependent apoptosis in some cells. Though JEV induced classical intrinsic pathway in N18 neuroblastoma cells, it activated both caspase-8 (part of the extrinsic pathway) and caspase-9 in a predominantly mitochondria-dependent pathway in MCF cells[129-131].

Japanese Encephalitis virus causes autophagy to facilitate viral replication in certain cell types. Li *et al*[132] showed induction of autophagy by virulent (RP-9) and attenuated (RP-2ms) JEV strain in human NT-2 cells. They also showed the positive effect of rapamycin induced autophagy on viral infection, and the reversal of that effect on blocking autophagy. Infection with Japanese encephalitis virus triggers innate immune response (through RIG-1/IRF-3 and P13K/NF signaling pathway) and activates inflammatory cytokines, chemokines and IFN-inducible proteins[133]. JEV Infection also induces autophagy in human microglial (CHME-5) cell line, leading to pro-inflammatory cytokine response.

CONCLUSION

Dengue is the worst arboviral human disease and most lethal among all Flavivirus members. It is remarkable how it manipulates the cell survival pathway in many types of cells, ultimately increasing viral load. From the literature, it is evident that dengue triggers different responses in different mammalian cells. Most of the dengue proteins (NS2, NS3, NS5, C, and E) have been reported to trigger extrinsic apoptosis pathway in many cells, including neurons, hepatocytes, immune cells, and endothelial cells. TNF-α and interleukins (IL-1β, 10) play a key role in this mechanism. However, M protein domains induce intrinsic apoptosis in neurons and hepatocytes. The virus may have alternate strategies to kill the cell, in case one of the cell death pathways is nonfunctional. In some cases, the virus has been able to induce different kinds of stress (ER, ROS, NO) conditions that lead to apoptotic cell death (Figure 1). Recent discoveries have shown that dengue can also activate autophagy in epithelial cells, fibroblasts and hepatocytes. It even uses this pathway to increase energy production, which would facilitate viral replication. Nonstructural proteins (NS2, 3, 4) have been involved in this process. The ability of dengue to use cell death or protective autophagy for virus replication in specific cell types is crucial in dengue's versatility. Antivirals addressing the vast repertoire of the virus will contribute to counteracting dengue pathogenesis.

West Nile virus, though not as versatile as dengue, can trigger apoptosis in the central nervous system (CNS) to establish neuroinvasiveness. With a higher initial WNV dose, necrosis has been observed. An interesting aspect of infection with different strains lies in the differential regulation of ER stress-UPR pathways to achieve increased viral burden. The capsid protein positively interacts with p53 *in vivo*, activating the intrinsic pathway; however, in mammalian cells, it blocks apoptosis through PI3KI-Akt pathway. NS3 is involved in extrinsic apoptosis in neuroblastoma and cervical cancer cells. However, we need to know more about the effects of individual WNV proteins. A promising facet of WNV research is the attention focused on miRNA regulation, which needs to be extended to the other members of Flavivirus. This approach holds promise for antiviral therapy.

Japanese encephalitis virus, though pathogenetically similar to WNV, manipulates both intrinsic and extrinsic pathways to its advantage (Figure 1). JEV induces apoptosis in many neuronal cells by inducing upstream stress (UPR response, ROS production) events. JEV NS3, in contrast to DENV and WNV, induces the intrinsic pathway of apoptosis. There is also evidence that the virus

can infect and replicate even in the absence of caspase-3, as it can induce caspase-6 and activate caspase-8 and -9 in a mitochondria dependent pathway. Moreover, caspase inhibition does not block viral production. Thus this Flavivirus appears to rely more on mitochondrial apoptosis for its pathogenesis. To add to the severity, it also utilizes autophagy to mediate pro-inflammatory cytokine response in neuronal cells.

Under these circumstances, we postulate that the Flavivirus has the ability to manipulate cell survival and innate immune response. The aftermath of viral invasion is dependent on initial dose and cell type. It can also switch to different mechanisms to exert its pathogenic effect in different cells of our body. The current understanding of cell death and survival during Flavivirus infection has not addressed many critical and complicated issues like the role of apoptosis and autophagy in killing infected cells or helping them to survive. Future studies should be aimed at finding out the function of individual viral proteins and the regulation of non-coding RNAs in viral infection. More emphasis needs to be put on studying the signaling pathways by which viruses regulate the cell survival pathways.

REFERENCES

1 **McLean JE**, Ruck A, Shirazian A, Pooyaei-Mehr F, Zakeri ZF. Viral manipulation of cell death. *Curr Pharm Des* 2008; **14**: 198-220 [PMID: 18220831 DOI: 10.2174/138161208783413329]

2 **Kuno G**, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB. Phylogeny of the genus Flavivirus. *J Virol* 1998; **72**: 73-83 [PMID: 9420202]

3 **Gubler DJ**. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiol* 2002; **10**: 100-103 [PMID: 11827812 DOI: 10.1016/S0966-842X(01)02288-0]

4 WHO Dengue factsheet. Available from: URL: http: //www.who.int/mediacentre/factsheets/fs117/en/

5 West Nile virus factsheet. Available from: URL: http: //www.cdc.gov/westnile/index.html

6 WHO Yellow Fever factsheet. Available from: URL: http: //www.who.int/mediacentre/factsheets/fs100/en/

7 **Clarke P**, Debiasi RL, Goody R, Hoyt CC, Richardson-Burns S, Tyler KL. Mechanisms of reovirus-induced cell death and tissue injury: role of apoptosis and virus-induced perturbation of host-cell signaling and transcription factor activation. *Viral Immunol* 2005; **18**: 89-115 [PMID: 15802955 DOI: 10.1089/vim.2005.18.89]

8 **Fazakerley JK**, Allsopp TE. Programmed cell death in virus infections of the nervous system. *Curr Top Microbiol Immunol* 2001; **253**: 95-119 [PMID: 11417141 DOI: 10.1007/978-3-662-10356-2_5]

9 **Lockshin RA**, Zakeri Z. Programmed cell death and apoptosis: origins of the theory. *Nat Rev Mol Cell Biol* 2001; **2**: 545-550 [PMID: 11433369 DOI: 10.1038/35080097]

10 **Clarke PG**, Clarke S. Nineteenth century research on naturally occurring cell death and related phenomena. *Anat Embryol* (Berl) 1996; **193**: 81-99 [PMID: 8742050 DOI: 10.1007/BF00214700]

11 **Levraud JP**, Adam M, Luciani MF, de Chastellier C, Blanton RL, Golstein P. Dictyostelium cell death: early emergence and demise of highly polarized paddle cells. *J Cell Biol* 2003; **160**: 1105-1114 [PMID: 12654899 DOI: 10.1083/jcb.200212104]

12 **Maghsoudi N**, Zakeri Z, Lockshin RA. Programmed cell death and apoptosis--where it came from and where it is going: from Elie Metchnikoff to the control of caspases. *Exp Oncol* 2012; **34**: 146-152 [PMID: 23069998]

13 **Saunders JW**. Death in embryonic systems. *Science* 1966; **154**: 604-612 [PMID: 5332319 DOI: 10.1126/science.154.3749.604]

14 **Kerr JF**, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972; **26**: 239-257 [PMID: 4561027 DOI: 10.1038/bjc.1972.33]

15 **Yuan J**, Shaham S, Ledoux S, Ellis HM, Horvitz HR. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* 1993; **75**: 641-652 [PMID: 8242740 DOI: 10.1016/0092-8674(93)90485-9]

16 **Vaux DL**, Weissman IL, Kim SK. Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2. *Science* 1992; **258**: 1955-1957 [PMID: 1470921 DOI: 10.1126/science.1470921]

17 **Ameisen JC**, Capron A. Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis. *Immunol Today* 1991; **12**: 102-105 [PMID: 1676268 DOI: 10.1016/0167-5699(91)90092-8]

18 **Buttyan R**, Zakeri Z, Lockshin R, Wolgemuth D. Cascade induction of c-fos, c-myc, and heat shock 70K transcripts during regression of the rat ventral prostate gland. *Mol Endocrinol* 1988; **2**: 650-657 [PMID: 3137456 DOI: 10.1210/mend-2-7-650]

19 **Evan GI**, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC. Induction of apoptosis in fibroblasts by c-myc protein. *Cell* 1992; **69**: 119-128 [PMID: 1555236 DOI: 10.1016/0092-8674(92)90123-T]

20 **Orr ME,** Oddo S. Autophagic/lysosomal dysfunction in Alzheimer's disease. *Alzheimers Res Ther* 2013; **5**: 53 [PMID: 24171818 DOI: 10.1186/alzrt217]

21 **Nossal GJ**. Negative selection of lymphocytes. *Cell* 1994; **76**: 229-239 [PMID: 8293461 DOI: 10.1016/0092-8674(94)90331-X]

22 **Vaux DL**, Korsmeyer SJ. Cell death in development. *Cell* 1999; **96**: 245-254 [PMID: 9988219 DOI: 10.1016/S0092-8674(00)80564-4]

23 **Greenberg JT**. Programmed cell death: a way of life for plants. *Proc Natl Acad Sci USA* 1996; **93**: 12094-12097 [PMID: 8901538 DOI: 10.1073/pnas.93.22.12094]

24 **Kroemer G**, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nuñez G, Peter ME, Tschopp J, Yuan J, Piacentini M, Zhivotovsky B, Melino G. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* 2009; **16**: 3-11 [PMID: 18846107 DOI: 10.1038/cdd.2008.150]

25 **Rubinstein AD**, Kimchi A. Life in the balance - a mechanistic view of the crosstalk between autophagy and apoptosis. *J Cell Sci* 2012; **125**: 5259-5268 [PMID: 23377657 DOI: 10.1242/jcs.115865]

26 **Thomas LR**, Johnson RL, Reed JC, Thorburn A. The C-terminal tails of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas receptors have opposing functions in Fas-associated death domain (FADD) recruitment and can regulate agonist-specific mechanisms of receptor activation. *J Biol Chem* 2004; **279**: 52479-52486 [PMID: 15452120 DOI: 10.1074/jbc.M409578200]

27 **Dempsey PW**, Doyle SE, He JQ, Cheng G. The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev* 2003; **14**: 193-209 [PMID: 12787559 DOI: 10.1016/S1359-6101(03)00021-2]

28 **Barnhart BC**, Lee JC, Alappat EC, Peter ME. The death effector domain protein family. *Oncogene* 2003; **22**: 8634-8644 [PMID: 14634625 DOI: 10.1038/sj.onc.1207103]

29 **Colombel M**, Symmans F, Gil S, O'Toole KM, Chopin D, Benson M, Olsson CA, Korsmeyer S, Buttyan R. Detection

of the apoptosis-suppressing oncoprotein bc1-2 in hormone-refractory human prostate cancers. *Am J Pathol* 1993; **143**: 390-400 [PMID: 7688182]
30 **Youle RJ**, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008; **9**: 47-59 [PMID: 18097445 DOI: 10.1038/nrm2308]
31 **Shamas-Din A**, Brahmbhatt H, Leber B, Andrews DW. BH3-only proteins: Orchestrators of apoptosis. *Biochim Biophys Acta* 2011; **1813**: 508-520 [PMID: 21146563 DOI: 10.1016/j.bbamcr.2010.11.024]
32 **Caro-Maldonado A**, Tait SW, Ramírez-Peinado S, Ricci JE, Fabregat I, Green DR, Muñoz-Pinedo C. Glucose deprivation induces an atypical form of apoptosis mediated by caspase-8 in Bax-, Bak-deficient cells. *Cell Death Differ* 2010; **17**: 1335-1344 [PMID: 20203689 DOI: 10.1038/cdd.2010.21]
33 **Salvesen GS**, Duckett CS. IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* 2002; **3**: 401-410 [PMID: 12042762 DOI: 10.1038/nrm830]
34 **Meijer WH**, van der Klei IJ, Veenhuis M, Kiel JA. ATG genes involved in non-selective autophagy are conserved from yeast to man, but the selective Cvt and pexophagy pathways also require organism-specific genes. *Autophagy* 2007; **3**: 106-116 [PMID: 17204848]
35 **Levine B**, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 2008; **132**: 27-42 [PMID: 18191218 DOI: 10.1016/j.cell.2007.12.018]
36 **Lee J**, Yang KH, Joe CO, Kang SS. Formation of distinct inclusion bodies by inhibition of ubiquitin-proteasome and autophagy-lysosome pathways. *Biochem Biophys Res Commun* 2011; **404**: 672-677 [PMID: 21147067 DOI: 10.1016/j.bbrc.2010.12.040]
37 **Tripathi DN**, Chowdhury R, Trudel LJ, Tee AR, Slack RS, Walker CL, Wogan GN. Reactive nitrogen species regulate autophagy through ATM-AMPK-TSC2-mediated suppression of mTORC1. *Proc Natl Acad Sci USA* 2013; **110**: E2950-E2957 [PMID: 23878245 DOI: 10.1073/pnas.1307736110]
38 **Kuma A**, Mizushima N, Ishihara N, Ohsumi Y. Formation of the approximately 350-kDa Apg12-Apg5.Apg16 multimeric complex, mediated by Apg16 oligomerization, is essential for autophagy in yeast. *J Biol Chem* 2002; **277**: 18619-18625 [PMID: 11897782 DOI: 10.1074/jbc.M111889200]
39 **Tanida I**, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol* 2004; **36**: 2503-2518 [PMID: 15325588 DOI: 10.1016/j.biocel.2004.05.009]
40 **Geng J**, Klionsky DJ. The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. *EMBO Rep* 2008; **9**: 859-864 [PMID: 18704115 DOI: 10.1038/embor.2008.163]
41 **Kaminskyy V**, Zhivotovsky B. To kill or be killed: how viruses interact with the cell death machinery. *J Intern Med* 2010; **267**: 473-482 [PMID: 20433575 DOI: 10.1111/j.1365-2796.2010.02222.x]
42 **Nikoletopoulou V**, Markaki M, Palikaras K, Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. *Biochim Biophys Acta* 2013; **1833**: 3448-3459 [PMID: 23770045 DOI: 10.1016/j.bbamcr.2013.06.001]
43 **Vanden Berghe T**, Grootjans S, Goossens V, Dondelinger Y, Krysko DV, Takahashi N, Vandenabeele P. Determination of apoptotic and necrotic cell death in vitro and in vivo. *Methods* 2013; **61**: 117-129 [PMID: 23473780 DOI: 10.1016/j.ymeth.2013.02.011]
44 **Lorenz IC**, Allison SL, Heinz FX, Helenius A. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. *J Virol* 2002; **76**: 5480-5491 [PMID: 11991976 DOI: 10.1128/JVI.76.11.5480-5491.2002]
45 **Stadler K**, Allison SL, Schalich J, Heinz FX. Proteolytic activation of tick-borne encephalitis virus by furin. *J Virol* 1997; **71**: 8475-8481 [PMID: 9343204]
46 **Allison SL**, Schalich J, Stiasny K, Mandl CW, Heinz FX. Mutational evidence for an internal fusion peptide in flavivirus envelope protein E. *J Virol* 2001; **75**: 4268-4275 [PMID: 11287576 DOI: 10.1128/JVI.75.9.4268-4275.2001]
47 **Allison SL**, Schalich J, Stiasny K, Mandl CW, Kunz C, Heinz FX. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. *J Virol* 1995; **69**: 695-700 [PMID: 7529335]
48 **Corver J**, Ortiz A, Allison SL, Schalich J, Heinz FX, Wilschut J. Membrane fusion activity of tick-borne encephalitis virus and recombinant subviral particles in a liposomal model system. *Virology* 2000; **269**: 37-46 [PMID: 10725196 DOI: 10.1006/viro.1999.0172]
49 **Lindenbach BD**, Rice CM. Molecular biology of flaviviruses. *Adv Virus Res* 2003; **59**: 23-61 [PMID: 14696326 DOI: 10.1016/S0065-3527(03)59002-9]
50 **Brinton MA**. The molecular biology of West Nile Virus: a new invader of the western hemisphere. *Annu Rev Microbiol* 2002; **56**: 371-402 [PMID: 12142476 DOI: 10.1146/annurev.micro.56.012302.160654]
51 **Mukhopadhyay S**, Kuhn RJ, Rossmann MG. A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol* 2005; **3**: 13-22 [PMID: 15608696 DOI: 10.1038/nrmicro1067]
52 **La Ruche G**, Souarès Y, Armengaud A, Peloux-Petiot F, Delaunay P, Desprès P, Lenglet A, Jourdain F, Leparc-Goffart I, Charlet F, Ollier L, Mantey K, Mollet T, Fournier JP, Torrents R, Leitmeyer K, Hilairet P, Zeller H, Van Bortel W, Dejour-Salamanca D, Grandadam M, Gastellu-Etchegorry M. First two autochthonous dengue virus infections in metropolitan France, September 2010. *Euro Surveill* 2010; **15**: 19676 [PMID: 20929659]
53 **Guha-Sapir D**, Schimmer B. Dengue fever: new paradigms for a changing epidemiology. *Emerg Themes Epidemiol* 2005; **2**: 1 [PMID: 15743532 DOI: 10.1186/1742-7622-2-1]
54 **Cleaves GR**, Dubin DT. Methylation status of intracellular dengue type 2 40 S RNA. *Virology* 1979; **96**: 159-165 [PMID: 111410 DOI: 10.1016/0042-6822(79)90181-8]
55 **Amberg SM**, Nestorowicz A, McCourt DW, Rice CM. NS2B-3 proteinase-mediated processing in the yellow fever virus structural region: in vitro and in vivo studies. *J Virol* 1994; **68**: 3794-3802 [PMID: 8189517]
56 **Cahour A**, Falgout B, Lai CJ. Cleavage of the dengue virus polyprotein at the NS3/NS4A and NS4B/NS5 junctions is mediated by viral protease NS2B-NS3, whereas NS4A/NS4B may be processed by a cellular protease. *J Virol* 1992; **66**: 1535-1542 [PMID: 1531368]
57 **Falgout B**, Pethel M, Zhang YM, Lai CJ. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *J Virol* 1991; **65**: 2467-2475 [PMID: 2016768]
58 **Chambers TJ**, Hahn CS, Galler R, Rice CM. Flavivirus genome organization, expression, and replication. *Annu Rev Microbiol* 1990; **44**: 649-688 [PMID: 2174669 DOI: 10.1146/annurev.mi.44.100190.003245]
59 **Martina BE**, Koraka P, Osterhaus AD. Dengue virus pathogenesis: an integrated view. *Clin Microbiol Rev* 2009; **22**: 564-581 [PMID: 19822889 DOI: 10.1128/CMR.00035-09]
60 **Gubler DJ**. Dengue and dengue hemorrhagic fever. *Clin Microbiol Rev* 1998; **11**: 480-496 [PMID: 9665979]
61 **Guzmán MG**, Kouri GP, Bravo J, Soler M, Vazquez S, Morier L. Dengue hemorrhagic fever in Cuba, 1981: a retrospective seroepidemiologic study. *Am J Trop Med Hyg* 1990; **42**: 179-184 [PMID: 2316788]
62 **Harris E**, Videa E, Pérez L, Sandoval E, Téllez Y, Pérez ML, Cuadra R, Rocha J, Idiaquez W, Alonso RE, Delgado MA, Campo LA, Acevedo F, Gonzalez A, Amador JJ, Balmaseda A. Clinical, epidemiologic, and virologic features of dengue in the 1998 epidemic in Nicaragua. *Am J Trop Med Hyg* 2000; **63**: 5-11 [PMID: 11357995]

63 **Guilarde AO**, Turchi MD, Siqueira JB, Feres VC, Rocha B, Levi JE, Souza VA, Boas LS, Pannuti CS, Martelli CM. Dengue and dengue hemorrhagic fever among adults: clinical outcomes related to viremia, serotypes, and antibody response. *J Infect Dis* 2008; **197**: 817-824 [PMID: 18269315 DOI: 10.1086/528805]

64 **Kittigul L**, Pitakarnjanakul P, Sujirarat D, Siripanichgon K. The differences of clinical manifestations and laboratory findings in children and adults with dengue virus infection. *J Clin Virol* 2007; **39**: 76-81 [PMID: 17507286 DOI: 10.1016/j.jcv.2007.04.006]

65 **Rothman AL**. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. *Nat Rev Immunol* 2011; **11**: 532-543 [PMID: 21760609 DOI: 10.1038/nri3014]

66 **Wills BA**, Oragui EE, Stephens AC, Daramola OA, Dung NM, Loan HT, Chau NV, Chambers M, Stepniewska K, Farrar JJ, Levin M. Coagulation abnormalities in dengue hemorrhagic Fever: serial investigations in 167 Vietnamese children with Dengue shock syndrome. *Clin Infect Dis* 2002; **35**: 277-285 [PMID: 12115093 DOI: 10.1086/341410]

67 **Balsitis SJ**, Coloma J, Castro G, Alava A, Flores D, McKerrow JH, Beatty PR, Harris E. Tropism of dengue virus in mice and humans defined by viral nonstructural protein 3-specific immunostaining. *Am J Trop Med Hyg* 2009; **80**: 416-424 [PMID: 19270292]

68 **Basílio-de-Oliveira CA**, Aguiar GR, Baldanza MS, Barth OM, Eyer-Silva WA, Paes MV. Pathologic study of a fatal case of dengue-3 virus infection in Rio de Janeiro, Brazil. *Braz J Infect Dis* 2005; **9**: 341-347 [PMID: 16270128 DOI: 10.1590/S1413-86702005000400012]

69 **Guzmán MG**, Alvarez M, Rodríguez R, Rosario D, Vázquez S, Vald s L, Cabrera MV, Kourí G. Fatal dengue hemorrhagic fever in Cuba, 1997. *Int J Infect Dis* 1999; **3**: 130-135 [PMID: 10460923 DOI: 10.1016/S1201-9712(99)90033-4]

70 **Jessie K**, Fong MY, Devi S, Lam SK, Wong KT. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. *J Infect Dis* 2004; **189**: 1411-1418 [PMID: 15073678 DOI: 10.1086/383043]

71 **Silveira GF**, Meyer F, Delfraro A, Mosimann AL, Coluchi N, Vasquez C, Probst CM, Báfica A, Bordignon J, Dos Santos CN. Dengue virus type 3 isolated from a fatal case with visceral complications induces enhanced proinflammatory responses and apoptosis of human dendritic cells. *J Virol* 2011; **85**: 5374-5383 [PMID: 21450836 DOI: 10.1128/JVI.01915-10]

72 **Myint KS**, Endy TP, Mongkolsirichaikul D, Manomuth C, Kalayanarooj S, Vaughn DW, Nisalak A, Green S, Rothman AL, Ennis FA, Libraty DH. Cellular immune activation in children with acute dengue virus infections is modulated by apoptosis. *J Infect Dis* 2006; **194**: 600-607 [PMID: 16897658 DOI: 10.1086/506451]

73 **Jaiyen Y**, Masrinoul P, Kalayanarooj S, Pulmanausahakul R, Ubol S. Characteristics of dengue virus-infected peripheral blood mononuclear cell death that correlates with the severity of illness. *Microbiol Immunol* 2009; **53**: 442-450 [PMID: 19659928 DOI: 10.1111/j.1348-0421.2009.00148.x]

74 **Nagila A**, Netsawang J, Srisawat C, Noisakran S, Morchang A, Yasamut U, Puttikhunt C, Kasinrerk W, Malasit P, Yenchitsomanus PT, Limjindaporn T. Role of CD137 signaling in dengue virus-mediated apoptosis. *Biochem Biophys Res Commun* 2011; **410**: 428-433 [PMID: 21669186 DOI: 10.1016/j.bbrc.2011.05.151]

75 **Khunchai S**, Junking M, Suttitheptumrong A, Yasamut U, Sawasdee N, Netsawang J, Morchang A, Chaowalit P, Noisakran S, Yenchitsomanus PT, Limjindaporn T. Interaction of dengue virus nonstructural protein 5 with Daxx modulates RANTES production. *Biochem Biophys Res Commun* 2012; **423**: 398-403 [PMID: 22664104 DOI: 10.1016/j.bbrc.2012.05.137]

76 **Morchang A**, Yasamut U, Netsawang J, Noisakran S, Wongwiwat W, Songprakhon P, Srisawat C, Puttikhunt C, Kasinrerk W, Malasit P, Yenchitsomanus PT, Limjindaporn T. Cell death gene expression profile: role of RIPK2 in dengue virus-mediated apoptosis. *Virus Res* 2011; **156**: 25-34 [PMID: 21195733 DOI: 10.1016/j.virusres.2010.12.012]

77 **Long X**, Li Y, Qi Y, Xu J, Wang Z, Zhang X, Zhang D, Zhang L, Huang J. XAF1 contributes to dengue virus-induced apoptosis in vascular endothelial cells. *FASEB J* 2013; **27**: 1062-1073 [PMID: 23207547 DOI: 10.1096/fj.12-213967]

78 **Lin CF**, Lei HY, Shiau AL, Liu HS, Yeh TM, Chen SH, Liu CC, Chiu SC, Lin YS. Endothelial cell apoptosis induced by antibodies against dengue virus nonstructural protein 1 via production of nitric oxide. *J Immunol* 2002; **169**: 657-664 [PMID: 12097367]

79 **Catteau A**, Kalinina O, Wagner MC, Deubel V, Courageot MP, Desprès P. Dengue virus M protein contains a proapoptotic sequence referred to as ApoptoM. *J Gen Virol* 2003; **84**: 2781-2793 [PMID: 13679613 DOI: 10.1099/vir.0.19163-0]

80 **Alonzo MT**, Lacuesta TL, Dimaano EM, Kurosu T, Suarez LA, Mapua CA, Akeda Y, Matias RR, Kuter DJ, Nagata S, Natividad FF, Oishi K. Platelet apoptosis and apoptotic platelet clearance by macrophages in secondary dengue virus infections. *J Infect Dis* 2012; **205**: 1321-1329 [PMID: 22383677 DOI: 10.1093/infdis/jis180]

81 **Duarte dos Santos CN**, Frenkiel MP, Courageot MP, Rocha CF, Vazeille-Falcoz MC, Wien MW, Rey FA, Deubel V, Desprès P. Determinants in the envelope E protein and viral RNA helicase NS3 that influence the induction of apoptosis in response to infection with dengue type 1 virus. *Virology* 2000; **274**: 292-308 [PMID: 10964773 DOI: 10.1006/viro.2000.0457]

82 **Avirutnan P**, Malasit P, Seliger B, Bhakdi S, Husmann M. Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis. *J Immunol* 1998; **161**: 6338-6346 [PMID: 9834124]

83 **McLean JE**, Wudzinska A, Datan E, Quaglino D, Zakeri Z. Flavivirus NS4A-induced autophagy protects cells against death and enhances virus replication. *J Biol Chem* 2011; **286**: 22147-22159 [PMID: 21511946 DOI: 10.1074/jbc.M110.192500]

84 **Lee YR**, Lei HY, Liu MT, Wang JR, Chen SH, Jiang-Shieh YF, Lin YS, Yeh TM, Liu CC, Liu HS. Autophagic machinery activated by dengue virus enhances virus replication. *Virology* 2008; **374**: 240-248 [PMID: 18353420 DOI: 10.1016/j.virol.2008.02.016]

85 **Panyasrivanit M**, Greenwood MP, Murphy D, Isidoro C, Auewarakul P, Smith DR. Induced autophagy reduces virus output in dengue infected monocytic cells. *Virology* 2011; **418**: 74-84 [PMID: 21813150 DOI: 10.1016/j.virol.2011.07.010]

86 **Mateo R**, Nagamine CM, Spagnolo J, Méndez E, Rahe M, Gale M, Yuan J, Kirkegaard K. Inhibition of cellular autophagy deranges dengue virion maturation. *J Virol* 2013; **87**: 1312-1321 [PMID: 23175363 DOI: 10.1128/JVI.02177-12]

87 **Petersen LR**, Brault AC, Nasci RS. West Nile virus: review of the literature. *JAMA* 2013; **310**: 308-315 [PMID: 23860989 DOI: 10.1001/jama.2013.8042]

88 **Zou S**, Foster GA, Dodd RY, Petersen LR, Stramer SL. West Nile fever characteristics among viremic persons identified through blood donor screening. *J Infect Dis* 2010; **202**: 1354-1361 [PMID: 20874087 DOI: 10.1086/656602]

89 **Lim PY**, Behr MJ, Chadwick CM, Shi PY, Bernard KA. Keratinocytes are cell targets of West Nile virus in vivo. *J Virol* 2011; **85**: 5197-5201 [PMID: 21367890 DOI: 10.1128/JVI.02692-10]

90 **Schneider BS**, Higgs S. The enhancement of arbovirus transmission and disease by mosquito saliva is associated with modulation of the host immune response. *Trans R Soc Trop Med Hyg* 2008; **102**: 400-408 [PMID: 18342898 DOI: 10.1016/j.trstmh.2008.01.024]

91 **Cho H**, Diamond MS. Immune responses to West Nile virus infection in the central nervous system. *Viruses* 2012; **4**: 3812-3830 [PMID: 23247502 DOI: 10.3390/v4123812]

92 **Lindsey NP**, Staples JE, Lehman JA, Fischer M. Surveillance

for human West Nile virus disease - United States, 1999-2008. *MMWR Surveill Summ* 2010; **59**: 1-17 [PMID: 20360671]
93 **Bode AV**, Sejvar JJ, Pape WJ, Campbell GL, Marfin AA. West Nile virus disease: a descriptive study of 228 patients hospitalized in a 4-county region of Colorado in 2003. *Clin Infect Dis* 2006; **42**: 1234-1240 [PMID: 16586381 DOI: 10.1086/503038]
94 **Klee AL**, Maidin B, Edwin B, Poshni I, Mostashari F, Fine A, Layton M, Nash D. Long-term prognosis for clinical West Nile virus infection. *Emerg Infect Dis* 2004; **10**: 1405-1411 [PMID: 15496241 DOI: 10.3201/eid1008.030879]
95 **Cook RL**, Xu X, Yablonsky EJ, Sakata N, Tripp JH, Hess R, Piazza P, Rinaldo CR. Demographic and clinical factors associated with persistent symptoms after West Nile virus infection. *Am J Trop Med Hyg* 2010; **83**: 1133-1136 [PMID: 21036852 DOI: 10.4269/ajtmh.2010.09-0717]
96 **Haaland KY**, Sadek J, Pergam S, Echevarria LA, Davis LE, Goade D, Harnar J, Nofchissey RA, Sewel CM, Ettestad P. Mental status after West Nile virus infection. *Emerg Infect Dis* 2006; **12**: 1260-1262 [PMID: 16965710 DOI: 10.3201/eid1708.060097]
97 **Sadek JR**, Pergam SA, Harrington JA, Echevarria LA, Davis LE, Goade D, Harnar J, Nofchissey RA, Sewell CM, Ettestad P, Haaland KY. Persistent neuropsychological impairment associated with West Nile virus infection. *J Clin Exp Neuropsychol* 2010; **32**: 81-87 [PMID: 19513920 DOI: 10.1080/13803390902881918]
98 **Tilley PA**, Fox JD, Jayaraman GC, Preiksaitis JK. Nucleic acid testing for west nile virus RNA in plasma enhances rapid diagnosis of acute infection in symptomatic patients. *J Infect Dis* 2006; **193**: 1361-1364 [PMID: 16619182 DOI: 10.1086/503577]
99 **Busch MP**, Kleinman SH, Tobler LH, Kamel HT, Norris PJ, Walsh I, Matud JL, Prince HE, Lanciotti RS, Wright DJ, Linnen JM, Caglioti S. Virus and antibody dynamics in acute west nile virus infection. *J Infect Dis* 2008; **198**: 984-993 [PMID: 18729783 DOI: 10.1086/591467]
100 **Beasley DW**. Vaccines and immunotherapeutics for the prevention and treatment of infections with West Nile virus. *Immunotherapy* 2011; **3**: 269-285 [PMID: 21322763 DOI: 10.2217/imt.10.93]
101 **Diamond MS**. Progress on the development of therapeutics against West Nile virus. *Antiviral Res* 2009; **83**: 214-227 [PMID: 19501622 DOI: 10.1016/j.antiviral.2009.05.006]
102 **Zohrabian A**, Hayes EB, Petersen LR. Cost-effectiveness of West Nile virus vaccination. *Emerg Infect Dis* 2006; **12**: 375-380 [PMID: 16704772 DOI: 10.3201/eid1203.050782]
103 **Chu JJ**, Ng ML. The mechanism of cell death during West Nile virus infection is dependent on initial infectious dose. *J Gen Virol* 2003; **84**: 3305-3314 [PMID: 14645911 DOI: 10.1099/vir.0.19447-0]
104 **Parquet MC**, Kumatori A, Hasebe F, Morita K, Igarashi A. West Nile virus-induced bax-dependent apoptosis. *FEBS Lett* 2001; **500**: 17-24 [PMID: 11434919 DOI: 10.1016/S0014-5793(01)02573-X]
105 **Samuel MA**, Morrey JD, Diamond MS. Caspase 3-dependent cell death of neurons contributes to the pathogenesis of West Nile virus encephalitis. *J Virol* 2007; **81**: 2614-2623 [PMID: 17192305 DOI: 10.1128/JVI.02311-06]
106 **Kleinschmidt MC**, Michaelis M, Ogbomo H, Doerr HW, Cinatl J. Inhibition of apoptosis prevents West Nile virus induced cell death. *BMC Microbiol* 2007; **7**: 49 [PMID: 17535425 DOI: 10.1186/1471-2180-7-49]
107 **Medigeshi GR**, Lancaster AM, Hirsch AJ, Briese T, Lipkin WI, Defilippis V, Früh K, Mason PW, Nikolich-Zugich J, Nelson JA. West Nile virus infection activates the unfolded protein response, leading to CHOP induction and apoptosis. *J Virol* 2007; **81**: 10849-10860 [PMID: 17686866 DOI: 10.1128/JVI.01151-07]
108 **Ambrose RL**, Mackenzie JM. ATF6 signaling is required for efficient West Nile virus replication by promoting cell survival and inhibition of innate immune responses. *J Virol* 2013; **87**: 2206-2214 [PMID: 23221566 DOI: 10.1128/JVI.02097-12]
109 **Michaelis M**, Kleinschmidt MC, Doerr HW, Cinatl J. Minocycline inhibits West Nile virus replication and apoptosis in human neuronal cells. *J Antimicrob Chemother* 2007; **60**: 981-986 [PMID: 17872917 DOI: 10.1093/jac/dkm307]
110 **Kobayashi S**, Orba Y, Yamaguchi H, Kimura T, Sawa H. Accumulation of ubiquitinated proteins is related to West Nile virus-induced neuronal apoptosis. *Neuropathology* 2012; **32**: 398-405 [PMID: 22129084 DOI: 10.1111/j.1440-1789.2011.01275.x]
111 **Ma DY**, Suthar MS, Kasahara S, Gale M, Clark EA. CD22 is required for protection against West Nile virus Infection. *J Virol* 2013; **87**: 3361-3375 [PMID: 23302871 DOI: 10.1128/JVI.02368-12]
112 **Shrestha B**, Pinto AK, Green S, Bosch I, Diamond MS. CD8+ T cells use TRAIL to restrict West Nile virus pathogenesis by controlling infection in neurons. *J Virol* 2012; **86**: 8937-8948 [PMID: 22740407 DOI: 10.1128/JVI.00673-12]
113 **Zhang B**, Patel J, Croyle M, Diamond MS, Klein RS. TNF-alpha-dependent regulation of CXCR3 expression modulates neuronal survival during West Nile virus encephalitis. *J Neuroimmunol* 2010; **224**: 28-38 [PMID: 20579746 DOI: 10.1016/j.jneuroim.2010.05.003]
114 **Smith JL**, Grey FE, Uhrlaub JL, Nikolich-Zugich J, Hirsch AJ. Induction of the cellular microRNA, Hs_154, by West Nile virus contributes to virus-mediated apoptosis through repression of antiapoptotic factors. *J Virol* 2012; **86**: 5278-5287 [PMID: 22345437 DOI: 10.1128/JVI.06883-11]
115 **Yang JS**, Ramanathan MP, Muthumani K, Choo AY, Jin SH, Yu QC, Hwang DS, Choo DK, Lee MD, Dang K, Tang W, Kim JJ, Weiner DB. Induction of inflammation by West Nile virus capsid through the caspase-9 apoptotic pathway. *Emerg Infect Dis* 2002; **8**: 1379-1384 [PMID: 12498651 DOI: 10.3201/eid0812.020224]
116 **Yang MR**, Lee SR, Oh W, Lee EW, Yeh JY, Nah JJ, Joo YS, Shin J, Lee HW, Pyo S, Song J. West Nile virus capsid protein induces p53-mediated apoptosis via the sequestration of HDM2 to the nucleolus. *Cell Microbiol* 2008; **10**: 165-176 [PMID: 17697133]
117 **Urbanowski MD**, Hobman TC. The West Nile virus capsid protein blocks apoptosis through a phosphatidylinositol 3-kinase-dependent mechanism. *J Virol* 2013; **87**: 872-881 [PMID: 23115297 DOI: 10.1128/JVI.02030-12]
118 **Ramanathan MP**, Chambers JA, Pankhong P, Chattergoon M, Attatippaholkun W, Dang K, Shah N, Weiner DB. Host cell killing by the West Nile Virus NS2B-NS3 proteolytic complex: NS3 alone is sufficient to recruit caspase-8-based apoptotic pathway. *Virology* 2006; **345**: 56-72 [PMID: 16243374 DOI: 10.1016/j.virol.2005.08.043]
119 **Beatman E**, Oyer R, Shives KD, Hedman K, Brault AC, Tyler KL, Beckham JD. West Nile virus growth is independent of autophagy activation. *Virology* 2012; **433**: 262-272 [PMID: 22939285 DOI: 10.1016/j.virol.2012.08.016]
120 **Vandergaast R**, Fredericksen BL. West Nile virus (WNV) replication is independent of autophagy in mammalian cells. *PLoS One* 2012; **7**: e45800 [PMID: 23029249 DOI: 10.1371/journal.pone.0045800]
121 **Unni SK**, Růžek D, Chhatbar C, Mishra R, Johri MK, Singh SK. Japanese encephalitis virus: from genome to infectome. *Microbes Infect* 2011; **13**: 312-321 [PMID: 21238600 DOI: 10.1016/j.micinf.2011.01.002]
122 **Misra UK**, Kalita J. Overview: Japanese encephalitis. *Prog Neurobiol* 2010; **91**: 108-120 [PMID: 20132860 DOI: 10.1016/j.pneurobio.2010.01.008]
123 **Impoinvil DE**, Ooi MH, Diggle PJ, Caminade C, Cardosa MJ, Morse AP, Baylis M, Solomon T. The effect of vaccination coverage and climate on Japanese encephalitis in Sarawak, Malaysia. *PLoS Negl Trop Dis* 2013; **7**: e2334 [PMID: 23951373 DOI: 10.1371/journal.pntd.0002334]

124 **Su HL**, Liao CL, Lin YL. Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfolded protein response. *J Virol* 2002; **76**: 4162-4171 [PMID: 11932381 DOI: 10.1128/JVI.76.9.4162-4171.2002]
125 **Lin RJ**, Liao CL, Lin YL. Replication-incompetent virions of Japanese encephalitis virus trigger neuronal cell death by oxidative stress in a culture system. *J Gen Virol* 2004; **85**: 521-533 [PMID: 14769909 DOI: 10.1099/vir.0.19496-0]
126 **Chen SO**, Chang TJ, Stone G, Chen CH, Liu JJ. Programmed cell death induced by Japanese encephalitis virus YL vaccine strain or its recombinant envelope protein in varied cultured cells. *Intervirology* 2006; **49**: 346-351 [PMID: 16926547 DOI: 10.1159/000095154]
127 **Yang TC**, Shiu SL, Chuang PH, Lin YJ, Wan L, Lan YC, Lin CW. Japanese encephalitis virus NS2B-NS3 protease induces caspase 3 activation and mitochondria-mediated apoptosis in human medulloblastoma cells. *Virus Res* 2009; **143**: 77-85 [PMID: 19463724 DOI: 10.1016/j.virusres.2009.03.007]
128 **Yiang GT**, Chen YH, Chou PL, Chang WJ, Wei CW, Yu YL. The NS3 protease and helicase domains of Japanese encephalitis virus trigger cell death via caspase-dependent and -independent pathways. *Mol Med Rep* 2013; **7**: 826-830 [PMID: 23291778]
129 **Liao CL**, Lin YL, Wang JJ, Huang YL, Yeh CT, Ma SH, Chen LK. Effect of enforced expression of human bcl-2 on Japanese encephalitis virus-induced apoptosis in cultured cells. *J Virol* 1997; **71**: 5963-5971 [PMID: 9223486]
130 **Liao CL**, Lin YL, Shen SC, Shen JY, Su HL, Huang YL, Ma SH, Sun YC, Chen KP, Chen LK. Antiapoptotic but not antiviral function of human bcl-2 assists establishment of Japanese encephalitis virus persistence in cultured cells. *J Virol* 1998; **72**: 9844-9854 [PMID: 9811720]
131 **Tsao CH**, Su HL, Lin YL, Yu HP, Kuo SM, Shen CI, Chen CW, Liao CL. Japanese encephalitis virus infection activates caspase-8 and -9 in a FADD-independent and mitochondrion-dependent manner. *J Gen Virol* 2008; **89**: 1930-1941 [PMID: 18632964 DOI: 10.1099/vir.0.2008/000182-0]
132 **Li JK**, Liang JJ, Liao CL, Lin YL. Autophagy is involved in the early step of Japanese encephalitis virus infection. *Microbes Infect* 2012; **14**: 159-168 [PMID: 21946213 DOI: 10.1016/j.micinf.2011.09.001]
133 **Jin R,** Zhu W, Cao S, Chen R, Jin H, Liu Y, Wang S, Wang W, Xiao G. Japanese encephalitis virus activates autophagy as a viral immune evasion strategy. *PLoS One* 2013; **8**: e52909 [PMID: 23320079 DOI: 10.1371/journal.pone.0052909]

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REVIEW

Review of application of mass spectrometry for analyses of anterior eye proteome

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Abstract

Proteins have important functional roles in the body, which can be altered in disease states. The eye is a complex organ rich in proteins; in particular, the anterior eye is very sophisticated in function and is most commonly involved in ophthalmic diseases. Proteomics, the large scale study of proteins, has greatly impacted our knowledge and understanding of gene function in the post-genomic period. The most significant breakthrough in proteomics has been mass spectrometric identification of proteins, which extends analysis far beyond the mere display of proteins that classical techniques provide. Mass spectrometry functions as a "mass analyzer" which simplifies the identification and quantification of proteins extracted from biological tissue. Mass spectrometric analysis of the anterior eye proteome provides a differential display for protein comparison of normal and diseased tissue. In this article we present the key proteomic findings in the recent literature related to the cornea, aqueous humor, trabecular meshwork, iris, ciliary body and lens. Through this we identified unique proteins specific to diseases related to the anterior eye.

Key words: Mass spectrometry; Proteomics; Ocular; Glaucoma

Core tip: Mass spectrometric based proteomics has been an indispensable tool for molecular and cellular biology. The ability of mass spectrometry to identify and precisely quantify thousands of proteins from complex samples has contributed greatly to biology and medicine. Through this we have studied protein-protein interactions *via* affinity-based isolations on a small and proteome-wide scale, the mapping of numerous organelles, and the generation of quantitative protein profiles from diverse species. The anterior segment of the eye is one of the most complicated parts of the human body with over 5000 proteins identified. Proteomic analyses of different parts of the eye, in particular the anterior eye structures, involve high throughput methods that help identify proteins and their posttranslational modifications. In this article we review the current state of advancement in the identification of anterior chamber proteins. We will present our findings in the following order: cornea, aqueous humor, trabecular meshwork, ciliary body, iris and lens.

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INTRODUCTION

Each organ in the human body has unique specialized

structures responsible for specific functions. Two investigational approaches are revealing the importance of the organization of molecular constituents in protein structure and function. The first approach focuses on one specific molecule at a time, the structure of the molecule, and the function the molecule is responsible for delivering. The second approach uses a high throughput analyses, capturing molecules in specific locations, performing experiments that enables us to determine their roles, and functions at these locations. The overarching goal of such high throughput experiments is a faster as well as greater understanding of composition, structure, and function. Proteomic analyses of different parts of the eye, in particular the anterior eye structures, involve high throughput methods that help identify proteins and their posttranslational modifications. Proteomics involves all methods that help identify proteins in the anterior eye chamber. The mass spectrometric methods to identify proteins in different locations in the anterior chamber use relatively older techniques and do not properly portray our current state of understanding. We aim to review the current state of advancement in identification of anterior chamber proteins, compared to the data gathered in the earliest era of proteomic mass spectrometry. We will present information on the following areas: cornea, aqueous humor, trabecular meshwork, ciliary body, iris, and lens. As each section of the anterior eye is uniquely different in protein, function and pathology, we have written the review specific to, what we believe, are the key relevant findings in the literature.

MASS SPECTROMETRIC PROTEOMIC ANALYSES OF THE CORNEA

The human cornea is a transparent, avascular, and highly specialized connective tissue which reflects and absorbs light into the lens and retina, and contributes two thirds of the eye's refractive power. It is the most densely innervated tissue in the body and acts to protect the eye from infection as well as UV light[1]. The cornea also acts as a structural barrier providing the eye with biomechanical stability[2]. It is approximately 530 μm in thickness and is composed of five layers: the epithelium, Bowman's layer, the stroma, Descemet's membrane, and the endothelium[3]. The stroma contributes 90% of corneal volume[3]. Diseases of the cornea are commonly infectious, traumatic or genetic in nature and have a tendency to affect certain layers of the cornea[4]. Especially in developing countries, corneal disease often contributes to blindness. The most common etiologies of corneal blindness globally include infectious trachoma (C. trachomatis), oncherciasis (O. volvulus), leprosy (M. lepromatosis), and hypovitaminosis D (xerophthalmia)[5]. Keratoconus and Fuch's dystrophy, diseases of the stroma and endothelium respectively, are the most common causes of corneal disease resulting in blindness in developed countries[5].

In recent years, our understanding of the identities and functions of the various proteins involved in the cornea has grown immensely. In 2005 just over 140 proteins were identified in the cornea[3]. Since then, over 3000 proteins have been characterized[4]. We have chosen here to focus on a narrow set of 12 proteins that have been identified in multiple studies, and which have important cellular functions.

Transforming growth factor-beta-induced protein (TGFβIp) has been identified in multiple corneal proteome studies[3,6,7] and has been implicated in corneal disease[8]. Numerous isoforms of TGFβIp have been found in the human cornea with 29 isoforms being found in earlier mass spectrometric studies in the mid-2000s[3]. This protein group's most frequently described isoform, TGFβIp ig-h3, is 683 amino acids in length and has been described in several cellular compartments[7]. These include the membrane, Golgi apparatus, cytoplasm, endoplasmic reticulum, extracellular matrix/space, and the mitochondria[7,8]. Its molecular functions include catalysis, binding of nucleotides, signal transduction, regulation of enzyme activity, protein binding, and cell adhesion[7,8]. The relative abundance of this protein has been shown to be especially high in the stroma and endothelium[4]. In the stroma, it has been characterized as the second most abundant protein (17.6% abundance), and in the endothelium it has been described as the most abundant protein (36.8% abundance)[4]. As mentioned previously, this protein has been implicated in several disease states, including Fuch's endothelial corneal dystrophy[8]. Simply put, this disease involves the progressive loss of endothelial cells, which is associated with impaired vision[5]. Increased expression and accumulation of TGFβIp ig-h3 has also been associated with other corneal and lattice dystrophies[5]. Overall, more than 50 mutations of this protein have been noted to be involved in disease states[5].

Peroxiredoxins are a group of redox associated proteins[6] which play a role in oxidative stress response in the cornea[9]. These proteins decompose peroxide molecules[10]. It is thought that decreased expression of these and other antioxidant proteins may play a role in Fuch's dystrophy and keratoconus[8,9]. Peroxiredoxins 1, 2, and 6 have consistently been identified in corneal samples by mass spectrometry[3,6,7]. Peroxiredoxin 1 is 199 amino acids in length, and is found in the membrane, cytoplasm, nucleus, extracellular space, and mitochondria. It is involved in functions such as catalysis, DNA and protein binding, and inhibition of oxidation[7]. Peroxiredoxin 2 is 198 amino acids in length, found in the cytoplasm, nucleus, cytosol, mitochondria, organelle lumena, and chromosomes. It is also involved in catalysis, protein binding, and inhibition of oxidation, as well as metallic ion binding[7]. Peroxiredoxin 6 is 224 amino acids, and is found in similar cellular compartments as Peroxiredoxins 1 and 2, as well as in vacuoles; it also has similar cellular activities as its predecessors[7].

Transketolase is an enzyme involved in the pentose phosphate pathway and is involved in cell transparency[11]. It has been shown to be downregulated in keratoconus[12]. This protein is 623 amino acids in length and is found in the cytoplasm and cytosol. In addition to catalysis, it is

involved in protein and metallic ion binding[7].

Mitochondrial ATP synthase subunit alpha has also been found in multiple mass spectrometric corneal proteomic investigations. It is made up of 553 amino acids, and is found in the membrane, cytoplasm, extracellular space, mitochondria, and organelle lumena. In addition to its catalytic function, it also binds proteins, metals, and nucleotides and has transporter actions[7].

At a cellular level, L-lactate dehydrogenase is involved in fermentation of pyruvate to lactate. The protein is up-regulated in keratoconus[12]. The beta chain of this protein is 334 amino acids and is found in the cytoplasm, cytosol, nucleus, extracellular space, and mitochondri. It has been found in several corneal proteomics investigations, and in addition to its catalytic activity, it plays a role in transcription regulation, binding of nucleotides and metal ions, and transporter activity. It also regulates other enzymes[7].

F-actin-capping protein subunit alpha-1 is part of a protein which interacts with the fast-growing ends of actin filaments to prevent subunit exchange[13]. Its role in the cornea is not well characterized but it may play some role in colon cancer[14]. The protein is 286 amino acids in length and exists in a wide variety of cellular spaces. In addition to its catalytic activity, it is a structural protein, binds proteins and metals, regulates enzyme activity, and plays a role in redox reactions[7].

Vimentin is a class Ⅲ intermediate filament protein[15]. It is composed of 466 amino acids and is seen in the cytoskeleton, membrane, cytoplasm, cytosol, and extracellular space. It functions in catalysis, DNA and protein binding, motor and transportation activities, and is involved in structural activities[7]. It has been found to be increased in the epithelium of corneas with keratoconus. As this protein is generally found in mesenchymal cells, it is thought that epithelial to mesenchymal transformation may be a possible characteristic of keratoconus[15].

Annexin A5 is a blood/plasma protein[6] which is thought to be involved in cellular apoptosis and its expression is used to determine cytotoxicity[16]. This protein is found in the cytoplasm and extracellular space. It is 320 amino acids long, and functions in metal and protein binding, as well as in the regulation of enzymes[7].

Keratin, type Ⅱ cytoskeletal 4 is a protein found in the cytoskeleton. It is 534 amino acids in length, and functions in a wide array of cellular roles including catalysis, binding of nucleotides and proteins, and motor and structural molecular activities[7]. Epidermal fatty acid-binding protein is a small cytoplasmic protein of 135 amino acids, which is primarily involved in catalysis, protein binding, and transporter activity[7].

Understanding cornea proteomics has helped identify key proteins which in turn increased bimolecular understanding of disease and functions of proteins in wound healing[17,18].

MASS SPECTROMETRIC PROTEOMIC ANALYSES OF THE AQUEOUS HUMOR

The aqueous humor plays a substantial role in maintaining homeostasis within the eye. The pigmented and non-pigmented ciliary epithelium is responsible for production of aqueous humor, which is secreted into the posterior chamber. From the posterior chamber a majority of the aqueous humor traverses the trabecular meshwork, (a filter like structure), and flows into the Schlemm's canal where it continues on to bathe the cornea. A small amount of the aqueous humor follows a less conventional pathway, the uveosceral pathway. The aqueous humor distributes through many sections of the anterior eye and is thus a key component in looking for proteomic biomarkers. A complication in the investigation of these biomarkers is that there is only 150-200 μL of aqueous humor in an average age individual and this amount decreases with age. There is also a low overall protein concentration present in the aqueous humor. These obstacles can make protein analysis in the aqueous humor challenging and with time, specialized techniques have evolved to provide more accurate analysis. Through the evolution of these specialized techniques, different groups have used specific techniques to analyze the protein make-up of the aqueous humor.

The aqueous humor is abundant in numerous proteins such as antioxidant proteins, immunoregulatory proteins, and anti-angiogenic proteins. These proteins were identified using Multidimensional Protein Identification Technology (MudPIT)[19]. Protein composition of the aqueous humor is intricate as it is a key regulatory component of the eye. Up to 676 nonredundant proteins have been identified in the aqueous humor of patients with no disease. These proteins were identified using nanoflow liquid chromatography electrospray ionization tandem mass spectrometry (nano-LC-ESI-MS/MS). An issue that complicates this type of identification is the high prevalence of albumin, a protein that which makes up 50% of the proteins in the aqueous humor. Its abundance result in the masking of less abundant proteins during analysis. In order to overcome this issue, immunodepletion of several aqueous humor samples of albumin, transferrin, antitrypsin, haploglobin, fibrinogen, IgG, and IgA is commonly performed[13]. The presence of complement regulatory molecules, specifically 23 complement proteins, demonstrates the importance of the aqueous humor in maintaining a healthy environment and protecting against autoimmune disease. Catalytic enzymes crucial for respiratory pathways are also present in the aqueous humor, specifically aldolase and ketolase. Angiogenin, and angiogenic inducer were present along with angiogenic inhibitors, specifically PEDF, type Ⅳ collagen, and vitamin D binding protein. Finally, members of the transforming growth factor β (TGFβ), tumor necrosis factor (TNF), fibroblast growth factor, interleukin, and growth differentiation families were also present in the aqueous humor[20]. Taken together, the numerous components present in the aqueous humor make it a powerful regulatory mechanism for maintaining homeostasis in the eye.

The identification of aqueous humor proteins in normal samples provided a baseline for further investigation

to take part in diseased counterparts. The study of protein levels in the aqueous humor in diseased individuals provides substantial information for potential biomarkers to possibly identify disease earlier. Analyzing these protein levels also assists in further profiling the protein composition of the aqueous humor. Glaucoma refers to a family of eye optic nerve disorders, some of which are associated with increased intraocular pressure (IOP). The most common form of glaucoma is primary open angle glaucoma. Research has been carried out to analyze alterations in the protein composition of the aqueous humor in patients with increased IOP. Endothelial leukocyte adhesion molecule 1 (ELAM 1) plays a key role in inflammation and is significantly increased in glaucomatous aqueous humor. Interestingly, apolipoprotein B and E are present in increased amounts. Typically, these proteins are responsible for in the delivery of cholesterol to cells. Another set of proteins present are responsible for muscle cell differentiation and function, specifically, myotrophin, myoblast determination protein 1, myogenin, vasodilator-stimulated phosphoprotein, and ankyrin-2. Presence of stress response proteins such as heat shock 60 kilodaltons (kDa) and 90 kDa proteins as well as ubiquitin fusion degradation 1-like are responsible for the removal of damaged protein. Finally, phospholipase C, β, and γ are shown to take part in signal transduction as well as neural development[21]. Similarly, in an investigation performed in patients with primary congenital glaucoma, a select set of proteins was shown to be upregulated and downregulated. Apolipoprotein A-Ⅳ (APOA-Ⅳ) is a plasma protein commonly involved in lipid absorption and transport. This specific protein is increased in glaucomatous samples. Albumin was also increased in these samples. This protein is crucial for maintenance of colloid osmotic pressure of plasma, antioxidant activity, regulation of normal microvascular permeability as well as fatty acid, and hormone transport. Another protein increased in glaucomatous aqueous humor is antithrombin 3 (ANT3 or SERPINC1), a protease inhibitor belonging to the serpin family. There were several proteins downregulated in glaucomatous samples including Transthyretin (TTR), Glutathione independent prostaglandin D synthase (PTGDS), opticin (OPT), and Retinol binding protein 3 IRBP. TTR is the main iodothyronine-binding protein that transfers T4 from the blood in the brain across the blood-choroid plexus barrier and tends to decrease in serum when acute inflammation is taking place. PTGDS is responsible for converting prostaglandin H2 (PGH2) to prostaglandin D2 (PGD2), common in smooth muscle contraction/relaxation as well as platelet aggregation inhibition. This protein has been demonstrated to bind to retinal and retinoic acid, key players in tissue development/maintenance. OPT, a member of the small leucine-rich repeats proteoglycan (SLRP) gene family is believed to be anti-angiogenic, is present in normal aqueous humor. IRBP is a glycoprotein synthesized by rods and cones. This protein binds to retinoids as well as fatty acids and may act as a retinoid transporter[22]. The presence of these proteins further supports the idea of necessary equilibrium between different elements in the eye that needs to take place in order to maintain a healthy environment.

The profiling of the proteins in the aqueous humor has given insight to its importance as a regulator in many aspects of the eye. Investigating these proteins in the normal state has been as important as investigating those in the diseased state. Overall, the investigations carried out in this area further supports underline the importance of maintaining specific protein levels in the aqueous humor.

MASS SPECTROMETRIC PROTEOMIC ANALYSES OF THE TRABECULAR MESHWORK

The trabecular meshwork (TM) plays a fundamental role in the regulation of intraocular pressure (IOP) and is pathophysiologically involved in the development of glaucoma. The TM can be divided into the uveal, corneoscleral and juxtacanalicular meshworks. It consists of collagen beams, covered by endothelial cells and surrounded by extracellular matrix (ECM)[23,24]. Until recently, the pathogenesis of outflow resistance at the TM was largely unknown. Understanding the pathogenesis that contributes to outflow resistance has recently increased. We now know that TM cell gene expression alters with IOP and mechanical stress[25] which can induce changes in cell proteins. This can lead to altered cell behavior including the increased tendency of the TM to contract with raised IOP[26,27], alterations in metabolic processes, cell adhesion, signal transduction, regulation of transcription, increased stretch activated channels[28], and the remodeling of extracellular matrix of TM in POAG[29-32].

Proteomic analysis of the TM has played a major role in understanding the mechanisms involved in outflow obstruction. Over 850 proteins have been identified in the TM[32] and multiple studies have found alterations in the expression of proteins when IOP is raised[32-34]. Multiple proteins are altered in location and quantity with glaucoma. We previously discovered that cochlin, a protein of unknown function is present in conjunction with stretch activated channels, in glaucomatous TM in human eyes but absent in normal samples[35]. Cochlin was also uniquely found in DBA/2J mice with hypertensive IOP but absent in DBA2J with a normal IOP[36]. A study by Yu *et al*[32] used 2-DE protein-expression, combined gel-spot to identify proteins in the TM of human donors, some of which were cultured in dexamethasone. This study found 877 proteins in human TM, several of which were previously associated with glaucoma. Several proteins belonged to cytoskeletal protein families/extracellular matrix proteins, such as vimentin, lamin, actin, and annexin. The highest proportion of proteins found were involved in metabolic processes (13%), and similar percentages of proteins were involved in anti-apoptosis, motility, carbo-

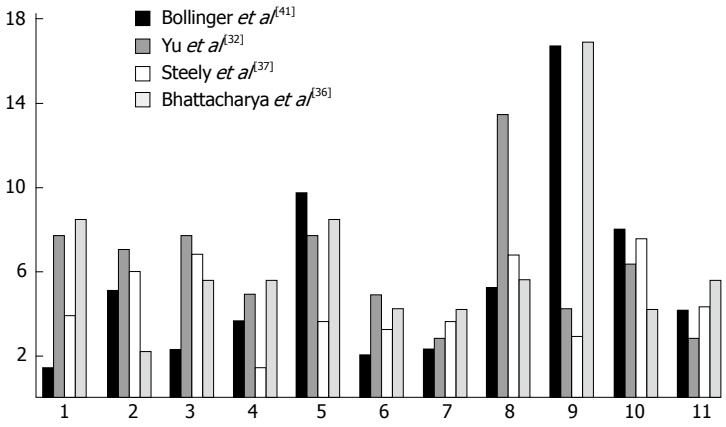

Figure 1 Comparing common protein functions in trabecular meshwork of eyes between fours students. 1: Anti-apoptosis; 2: Carbohydrate metabolic process; 3: Cell adhesion; 4: Cell cycle; 5: Cell motility; 6: Cell proliferation; 7: Lipid metabolic process; 8: Metabolic process; 9: Protein folding/metabolism; 10: Signal transduction; 11: Transport.

hydrate metabolism (10%-11%) (Figure 1). In contrast, few proteins were found to play roles in cell division and cell to cell signaling. Another study which grouped protein by their function found the largest number group were in protein folding (16.8%) which was significantly more than what we and Yu *et al*[32] found (2.9% and 4.2%).

Myocilin is a protein found in the TM; mutations in this protein have been associated with glaucoma[38-40]. Myocilin a prominent component of TM exosomes, suggesting that exosomes could contribute to aqueous humour outflow from the trabecular meshwork. As there are few studies which have examined TM exosome proteomics and exosome protein mutation is involved in disease, this is an area of which deserves further investigation.

Transforming growth factor beta 2 (TGFβ2) is often elevated in the TM of patients with POAG. Bollinger *et al*[41] examined TGFβ2-induced proteomic changes from four donors who were treated with or without TGFβ2. Cellular proteins in the TM were then analyzed by liquid chromatography-mass spectrometry iTRAQ. This study found that TGFβ2 significantly altered 47 proteins. More than half of the elevated proteins induced extracellular matrix remodeling and cytoskeleton interaction. Thirty proteins were elevated and 17 decreased after TGFβ2 treatment. CD9 antigen and mitochondrial superoxide dismutase 2 (SOD2) were the most significantly reduced proteins 64% and 46%, respectively. Interestingly the proteins most greatly decreased were from the mitochondria (40%). Downregulation of mitochondrial proteins may result in mitochondrial dysfunction and reduced ATP production, which may lead to disruption of outflow dynamics.

Overall TM proteomic studies have identified multiple proteins alterations associated with hypertensive IOP. Modulated protein patterns in glaucomatous eyes have emerged through proteomic studies. Future studies may look further into the gene expression of these altered proteins for a better understanding of their occurrence.

MASS SPECTROMETRIC PROTEOMIC ANALYSES OF THE CILIARY BODY

The ciliary body is a circumfirential layer of tissue behind the iris in the anterior chamber of the eye. Its epithelium serves as the main production center of aqueous humor. In recent years, literature regarding the proteome of the ciliary body has been sparse and had utilized immunohistochemistry, immunofluorescence, and Western blot technology, resulting in the characterization of fewer than 50 discrete proteins[42]. However, in 2013 Goel *et al*[42] profiled the ciliary body proteome utilizing MS/MS analysis on an LTQ-Orbitrap Velos ETD mass spectrometer. In this study, samples from the human ciliary body were processed and run on an SDS-PAGE. The bands were subsequently excised and digested with trypsin prior to LC-MS/MS analysis. MS data was then searched against the NCBI protein database, and 2815 proteins were characterized. Included in these data were proteins previously identified using the aforementioned techniques, including collagen type XVIII alpha 1 (COL18A1), cytochrome P450 family 1 subfamily B polypeptide 1 (CYP1B1), Opticin (OPTC), and aquaporin 1 (AQP1). Several of these proteins have possible implications in ocular disease. OPTC has been investigated as a possible target for primary open angle glaucoma. AQP1 is involved in the production of aqueous humor and its movement into the anterior chamber[42].

Goel *et al*[42] also identified a large number (> 2000) of proteins which were unknown to exist in the ciliary body. Some of these novel molecules include proteins involved in metabolism and energy pathways such as Neutrophil cytosol factor 2, Myosin-11, Pyruvate kinase isozymes M1/M2, and Alpha-1-antitrypsin. Other proteins such as ER lumen protein retaining receptor 2, Tubulin beta-2A chain, Exportin-1 are involved in transport mechanisms. Exportin-1 is overexpressed in cancer cells. Leukocyte surface antigen CD47 and complement C3 are part of the immune response mechanism. Desmin is an intermediate filament, which when defective is involved in several myopathies.

The Goel *et al*[42] group further investigated the proteins that were common and disparate between the ciliary body and plasma, and the ciliary body and aqueous humor. The majority of proteins found in the ciliary body (1895 of 2791) were also found in the plasma, which contained a total of 9393 proteins and therefore had 7498 unique proteins. In the comparison of the ciliary body and aqueous humor, 211 of the 2891 ciliary body

proteins were also found in the aqueous humor, leaving 321 unique aqueous humor proteins. These comparisons are important to know which proteins are natively found in the ciliary body, and which of them may have originated from elsewhere. In the future, work regarding ciliary body proteomics may explore the proteins now known to be unique in order to investigate further therapeutic targets.

MASS SPECTROMETRIC PROTEOMIC ANALYSES OF THE IRIS

Mass spectrometric analyses of the human iris proteome have not been well-published. Other methods of proteomic analysis have been used on a small number of known iris proteins. One such example includes the immunohistochemical analysis of Opticin (OPTC)[43]. The is protein was identified using an antibody targeting its amino terminal[43,44]. OPTC is the ortholog of a cDNA sequence which has been shown to be expressed abundantly in the iris[42,44]. Mass spectrometric analyses of this and other iris proteins are required to better characterize the more complete human anterior chamber proteome.

Mass spectrometric proteomic analyses of the lens

The Human lens is responsible for the refractive properties of the eye. It is avascular and contains one layer of epithelium found in the anterior capsule and posterior capsule. The lens is mostly acellular, consisting mainly of crystalline proteins with some non-crystalline proteins also present[44]. Its main function is to change shape and thus allow for accommodation of vision. Another function of the lens is to maintain transparency. Loss of accommodation results in presbyopia and loss of transparency results in cataract. There are 3 main types of crystalline proteins in the human body, including type α, β, and γ. Type α-A is a heat shock and chaperone protein and is found mostly in the lens while α-B is ubiquitous throughout the human body. It was also known that the α-crystallines play a role as heat shock proteins and are chaperone proteins. Most recently protein analysis was performed in a mice mouse model in which the genes responsible for the α-crystallin wereas missing. This was carried out to determine what happens with the other proteins inside of the lens giving further insight into the development of cataracts[45]. Wild type and αA/αB knockout mice were compared using two-dimensional gel electrophoresis and mass spectrometry. There was a greater abundance of histones H2A,H4, and H2B fragment, and a low molecular weight β1-catenin in postnatal 2 d of the knockout mice. There was increased abundance of βB2-crystallin and vimentin in 30 d-old lenses of knockout mice. Gel permeation chromatography was able to demonstrate an aggregation of β-crystalline. Therefore, the absence of crystalline type αA and αB resulted in changes of protein expression indicating that lens proteins also result in interactive functions beyond just plain functions the. Aggregation of α crystalline was also found by recent Matrix-assisted laser desorption/ionization (MALDI) studies[4].

Type γ requires the use of post-translational modification in order to maintain its transparency. Given that crystallins are life-long proteins, post-translational modification may play a role in the development of cataracts[46]. Heat and deamidation (a chemical reaction in which an amide functional group is removed from an organic compound and damages the amide-containing side chains of asparagine and glutamine) may play a role in the change of the physical properties of the protein. This study used 2D LC-MS/MS to examine which major lens proteins undergo deamidation and the exact sites of deamidation. It was found that all of the major proteins found in the lens were deamidated. Each crystallin protein differed in the sites and extents of deamidation. Many of the areas of deamidatation were characterized by the presence of a basic amino acid one residue from the glutamine and asparagine.

Although the lens consists mostly of crystalline proteins, the advent of new analytical techniques allowed for analysis of proteins involved in lens besides crystalline. One of the first complete proteomics studies to address the protein inside of the lens was in 2008[47]. The lens from fetal, cataract, and normal lenses were evaluated by 2D LC-MS/MS and PANTHER was used for protein classification. This study identified a total of 231 proteins across all of the lens samples. Fetal samples showed the highest amount of unique proteins compared to cataract and normal lenses. A 5-mm core of lens was used in the adult some of which lacked epithelial and outer cortical fibers which play a role in the metabolic machinery of the lens. The fetal samples were all pooled together. While many studies have shown the crystallin class as the dominant protein, this study showed that many low abundance proteins also existed in the lens.

A more recent study[48] was performed, using MALDI, which concentrated on the major protein differences for identification in order to determine the variances between proteins in age-related cataracts and normal lens nuclei. Observers graded cataracts and total solubilized proteins were compared using gel electrophoresis. MALDI was used to identify the proteins that had different abundances. LC-MS/MS analyses determined the compositions of > 200 kDa molecular weight aggregates found in age related nuclear cataract lens nuclei. It was identified that α, β-A3, βA4, βB1, and γD-crystallin were involving with the higher molecular weight aggregates. An uncharacterized protein found and this protein, along with αA, αB, and γ-D crystallin, were more found to be more prone to aggregation. Therefore, aggregation of crystallins may account for the development of cataracts. Also, some enzymes may play a role in the protein aggregation and possibly accelerate the process.

Membrane proteins were purified from young mouse lenses and shotgun proteomics was employed in order to analyze the membrane proteins of the mouse lens cells[49]. These same techniques were then applied to analyze the

human lens protein of the membrane[50]. HPLC-mass spectrometry with multidimensional protein identification technology (MudPIT) with and without phosphopeptide enrichment was applied for the study of the proteome of the lens membrane. There were 951 proteins that were identified in which 379 were membrane and membrane-associated proteins. Many of these proteins are responsible for carbohydrate metabolism, proteasome, cell-cell signaling and communication, glutathione metabolism and actin regulation.

LOXL-1 protein and apolipoprotein E, both found in the extracellular matrix, were abnormal in pseudoexfoliation syndrome, a disease of the anterior lens capsule[51]. This study performed mass spectrometry on isolated surgically removed anterior capsules in patients with pseudoexfoliation syndrome. Direct analysis showed LOXL-1 protein and apoliprotein E which shows that these extracellular matrix proteins play a role in pseudoexfoliation[52]. This study employed MALDI imaging on the anterior capsule which showed presence of LOXL-1 protein was more abundant in the iris region and apolipoprotein E in the pseudoexfoliation deposits in anterior capsule in the pupillary area. There could also be significant post-translational modification involved in promoting the aggregation of proteins.

The lens is unique in that it contains many fibers that are acellular and proteins that exist for the lifetime of the individual. The advantage of studying the proteomics of the lens is that it may provide a powerful model for the rest of the human body with regard to understanding the changes involved in proteins that are maintained throughout a lifetime. It is essential that the proteins maintain transparency, and aggregation may result in lack of solubility resulting in cataracts. Proteomics studies have shown that α-crystallins play a role in preventing aggregation and serving as chaperone proteins. α-crystallins are present only in the lens while α-B crystallin is ubiquitous throughout the human body and dysfunction of the α-B protein has been implicated in many degenerative disorders. Post-translational modification also plays a role in the lens protein.

CONCLUSION

Identification of proteins in different regions of the anterior chamber including the: cornea, aqueous humor, trabecular meshwork, ciliary body, and lens has expanded in recent years. Among other proteomic methods, mass spectrometry has enabled rapid protein sequencing while simultaneously determining posttranslational modifications in the amino acid residues. Mass spectrometry has rapidly evolved since 1990, allowing improved identification of proteins. Although the advances in mass spectrometry have been rapid, the identification of proteins from tissue or cell samples often remains unsatisfactory. Currently approximately 5000 proteins from each anterior eye segment tissue or fluid is identified against a theoretical prediction of 20000 proteins. Thus at best approximately 25% of actual proteins are captured compared to theoretical estimates. Part of the reason why protein identification is relatively poor compared to mRNA is due to differences in the chemistry of RNA and proteins. The identification of posttranslational modifications of proteins, remains another frontier in mass spectrometry (or any other suitable high throughput method) that is yet to be conquered. One important issue remaining to be elucidated is the process of natural aging. Several age-related changes that can be easily quantified occur in eyes such as prebyopia and the progressive ability to form sharp images. Several eye diseases are also age associated such as age-related macular degeneration and glaucoma. Important insight into true age related changes, and the result of aging and disease on protein turnover. The Current methods do not allow the juxtaposition of mRNA and protein information together. Modern proteomic methods lack in their ability to juxtapose mRNA and protein information from inactive proteins, deactivated proteins, or proteins undergoing degradation. These are the avenues for future advancement which will expand our insight into how protein-drug interactions keep proteins in their active states. We presented an account of the current state of proteins in different regions of anterior eye chamber and what improvement has occurred compared to that in the previous decade. Further improvements will enable us to address the question of protein turnover in tissues and better enable us to distinguish active, inactive, partially degraded, and degraded states of proteins.

REFERENCES

1 **Goldberg RA**, Lufkin R, Farahani K, Wu JC, Jesmanowicz A, Hyde JS. Physiology of the lower eyelid retractors: tight linkage of the anterior capsulopalpebral fascia demonstrated using dynamic ultrafine surface coil MRI. *Ophthal Plast Reconstr Surg* 1994; **10**: 87-91 [PMID: 8086368 DOI: 10.1097/00002341-199406000-00003]

2 **Marfurt CF**, Cox J, Deek S, Dvorscak L. Anatomy of the human corneal innervation. *Exp Eye Res* 2010; **90**: 478-492 [PMID: 20036654 DOI: 10.1016/j.exer.2009.12.010]

3 **Karring H**, Thøgersen IB, Klintworth GK, Møller-Pedersen T, Enghild JJ. A dataset of human cornea proteins identified by Peptide mass fingerprinting and tandem mass spectrometry. *Mol Cell Proteomics* 2005; **4**: 1406-1408 [PMID: 15911533 DOI: 10.1074/mcp.D500003-MCP200]

4 **Dyrlund TF**, Poulsen ET, Scavenius C, Nikolajsen CL, Thøgersen IB, Vorum H, Enghild JJ. Human cornea proteome: identification and quantitation of the proteins of the three main layers including epithelium, stroma, and endothelium. *J Proteome Res* 2012; **11**: 4231-4239 [PMID: 22698189 DOI: 10.1021/pr300358k]

5 **Semba RD**, Enghild JJ, Venkatraman V, Dyrlund TF, Van Eyk JE. The Human Eye Proteome Project: perspectives on an emerging proteome. *Proteomics* 2013; **13**: 2500-2511 [PMID: 23749747 DOI: 10.1002/pmic.201300075]

6 **Meade ML**, Shiyanov P, Schlager JJ. Enhanced detection method for corneal protein identification using shotgun proteomics. *Proteome Sci* 2009; **7**: 23 [PMID: 19563675 DOI: 10.1186/1477-5956-7-23]

7 **Galiacy SD**, Froment C, Mouton-Barbosa E, Erraud A, Chaoui K, Desjardins L, Monsarrat B, Malecaze F, Burlet-Schiltz O. Deeper in the human cornea proteome using nanoLC-

Orbitrap MS/MS: An improvement for future studies on cornea homeostasis and pathophysiology. *J Proteomics* 2011; **75**: 81-92 [PMID: 21989269 DOI: 10.1016/j.jprot.2011.09.020]
8 **Jurkunas UV**, Bitar M, Rawe I. Colocalization of increased transforming growth factor-beta-induced protein (TGFBIp) and Clusterin in Fuchs endothelial corneal dystrophy. *Invest Ophthalmol Vis Sci* 2009; **50**: 1129-1136 [PMID: 19011008 DOI: 10.1167/iovs.08-2525]
9 **Wojcik KA**, Kaminska A, Blasiak J, Szaflik J, Szaflik JP. Oxidative stress in the pathogenesis of keratoconus and Fuchs endothelial corneal dystrophy. *Int J Mol Sci* 2013; **14**: 19294-19308 [PMID: 24065107 DOI: 10.3390/ijms140919294]
10 **Singh AK**, Shichi H. Peroxiredoxin in bovine ocular tissues: immunohistochemical localization and in situ hybridization. *J Ocul Pharmacol Ther* 2001; **17**: 279-286 [PMID: 11436947 DOI: 10.1089/108076801750295308]
11 **Stramer BM**, Fini ME. Uncoupling keratocyte loss of corneal crystallin from markers of fibrotic repair. *Invest Ophthalmol Vis Sci* 2004; **45**: 4010-4015 [PMID: 15505050 DOI: 10.1167/iovs.03-1057]
12 **Joseph R**, Srivastava OP, Pfister RR. Differential epithelial and stromal protein profiles in keratoconus and normal human corneas. *Exp Eye Res* 2011; **92**: 282-298 [PMID: 21281627 DOI: 10.1016/j.exer.2011.01.008]
13 **Weeds A,** Maciver S. F-actin capping proteins. *Current opinion in cell biology* 1993; **5**: 63-69 [DOI: 10.1016/S0955-0674(05)80009-2]
14 **Zou J**, Yu XF, Bao ZJ, Dong J. Proteome of human colon cancer stem cells: a comparative analysis. *World J Gastroenterol* 2011; **17**: 1276-1285 [PMID: 21455326 DOI: 10.3748/wjg.v17.i10.1276]
15 **Chaerkady R**, Shao H, Scott SG, Pandey A, Jun AS, Chakravarti S. The keratoconus corneal proteome: loss of epithelial integrity and stromal degeneration. *J Proteomics* 2013; **87**: 122-131 [PMID: 23727491 DOI: 10.1016/j.jprot.2013.05.023]
16 **Rusovici R**, Sakhalkar M, Chalam KV. Evaluation of cytotoxicity of bevacizumab on VEGF-enriched corneal endothelial cells. *Mol Vis* 2011; **17**: 3339-3346 [PMID: 22219629]
17 **Karring H**, Thøgersen IB, Klintworth GK, Enghild JJ, Møller-Pedersen T. Proteomic analysis of the soluble fraction from human corneal fibroblasts with reference to ocular transparency. *Mol Cell Proteomics* 2004; **3**: 660-674 [PMID: 15054125 DOI: 10.1074/mcp.M400016-MCP200]
18 **Carlsson DJ**, Li F, Shimmura S, Griffith M. Bioengineered corneas: how close are we? *Curr Opin Ophthalmol* 2003; **14**: 192-197 [PMID: 12888716 DOI: 10.1097/00055735-200308000-00004]
19 **Richardson MR**, Price MO, Price FW, Pardo JC, Grandin JC, You J, Wang M, Yoder MC. Proteomic analysis of human aqueous humor using multidimensional protein identification technology. *Mol Vis* 2009; **15**: 2740-2750 [PMID: 20019884]
20 **Chowdhury UR**, Madden BJ, Charlesworth MC, Fautsch MP. Proteome analysis of human aqueous humor. *Invest Ophthalmol Vis Sci* 2010; **51**: 4921-4931 [PMID: 20463327 DOI: 10.1167/iovs.10-5531]
21 **Saccà SC**, Centofanti M, Izzotti A. New proteins as vascular biomarkers in primary open angle glaucomatous aqueous humor. *Invest Ophthalmol Vis Sci* 2012; **53**: 4242-4253 [PMID: 22618596 DOI: 10.1167/iovs.11-8902]
22 **Bouhenni RA**, Al Shahwan S, Morales J, Wakim BT, Chomyk AM, Alkuraya FS, Edward DP. Identification of differentially expressed proteins in the aqueous humor of primary congenital glaucoma. *Exp Eye Res* 2011; **92**: 67-75 [PMID: 21078314 DOI: 10.1016/j.exer.2010.11.004]
23 **Tamm ER**. The trabecular meshwork outflow pathways: structural and functional aspects. *Exp Eye Res* 2009; **88**: 648-655 [PMID: 19239914 DOI: 10.1016/j.exer.2009.02.007]
24 **Tektas OY**, Lütjen-Drecoll E. Structural changes of the trabecular meshwork in different kinds of glaucoma. *Exp Eye Res* 2009; **88**: 769-775 [PMID: 19114037 DOI: 10.1016/j.exer.2008.11.025]
25 **Chow J**, Liton PB, Luna C, Wong F, Gonzalez P. Effect of cellular senescence on the P2Y-receptor mediated calcium response in trabecular meshwork cells. *Mol Vis* 2007; **13**: 1926-1933 [PMID: 17982416]
26 **Tian B**, Gabelt BT, Geiger B, Kaufman PL. The role of the actomyosin system in regulating trabecular fluid outflow. *Exp Eye Res* 2009; **88**: 713-717 [PMID: 18793636 DOI: 10.1016/j.exer.2008.08.008]
27 **Paulaviciute-Baikstiene D,** Barsauskaite R, Januleviciene I. New insights into pathophysiological mechanisms regulating conventional aqueous humor outflow. *Medicina* (Kaunas) 2013; **49**: 165-169
28 **Goel M**, Sienkiewicz AE, Picciani R, Wang J, Lee RK, Bhattacharya SK. Cochlin, intraocular pressure regulation and mechanosensing. *PLoS One* 2012; **7**: e34309 [PMID: 22496787 DOI: 10.1371/journal.pone.0034309]
29 **Kaufman PL**. Enhancing trabecular outflow by disrupting the actin cytoskeleton, increasing uveoscleral outflow with prostaglandins, and understanding the pathophysiology of presbyopia interrogating Mother Nature: asking why, asking how, recognizing the signs, following the trail. *Exp Eye Res* 2008; **86**: 3-17 [PMID: 18053986 DOI: 10.1016/j.exer.2007.10.007]
30 **Friedl P,** Brocker EB. The biology of cell locomotion within three-dimensional extracellular matrix. *Cell Mol Life Sci* 2000; **57**: 41-64 [DOI: 10.1007/s000180050498]
31 **Zhao X**, Ramsey KE, Stephan DA, Russell P. Gene and protein expression changes in human trabecular meshwork cells treated with transforming growth factor-beta. *Invest Ophthalmol Vis Sci* 2004; **45**: 4023-4034 [PMID: 15505052 DOI: 10.1167/iovs.04-0535]
32 **Yu M**, Sun J, Peng W, Chen Z, Lin X, Liu X, Li M, Wu K. Protein expression in human trabecular meshwork: downregulation of RhoGDI by dexamethasone in vitro. *Mol Vis* 2010; **16**: 213-223 [PMID: 20161819]
33 **Shinzato M**, Yamashiro Y, Miyara N, Iwamatsu A, Takeuchi K, Umikawa M, Bayarjargal M, Kariya K, Sawaguchi S. Proteomic analysis of the trabecular meshwork of rats in a steroid-induced ocular hypertension model: downregulation of type I collagen C-propeptides. *Ophthalmic Res* 2007; **39**: 330-337 [PMID: 18046086 DOI: 10.1159/000109989]
34 **Iragavarapu S**, Algeciras ME, Lee RK, Bhattacharya SK. ETX1 is over-expressed in the glaucomatous trabecular meshwork. *Mol Vis* 2009; **15**: 2061-2067 [PMID: 19862339]
35 **Picciani RG**, Diaz A, Lee RK, Bhattacharya SK. Potential for transcriptional upregulation of cochlin in glaucomatous trabecular meshwork: a combinatorial bioinformatic and biochemical analytical approach. *Invest Ophthalmol Vis Sci* 2009; **50**: 3106-3111 [PMID: 19098315 DOI: 10.1167/iovs.08-3106]
36 **Bhattacharya SK**, Annangudi SP, Salomon RG, Kuchtey RW, Peachey NS, Crabb JW. Cochlin deposits in the trabecular meshwork of the glaucomatous DBA/2J mouse. *Exp Eye Res* 2005; **80**: 741-744 [PMID: 15862180 DOI: 10.1016/j.exer.2005.01.028]
37 **Steely HT**, Dillow GW, Bian L, Grundstad J, Braun TA, Casavant TL, McCartney MD, Clark AF. Protein expression in a transformed trabecular meshwork cell line: proteome analysis. *Mol Vis* 2006; **12**: 372-383 [PMID: 16636656]
38 **Stamer WD**, Hoffman EA, Luther JM, Hachey DL, Schey KL. Protein profile of exosomes from trabecular meshwork cells. *J Proteomics* 2011; **74**: 796-804 [PMID: 21362503 DOI: 10.1016/j.jprot.2011.02.024]
39 **Allingham RR**, Liu Y, Rhee DJ. The genetics of primary open-angle glaucoma: a review. *Exp Eye Res* 2009; **88**: 837-844 [PMID: 19061886 DOI: 10.1016/j.exer.2008.11.003]
40 **Resch ZT**, Fautsch MP. Glaucoma-associated myocilin: a better understanding but much more to learn. *Exp Eye Res* 2009; **88**: 704-712 [PMID: 18804106 DOI: 10.1016/j.exer.2008.08.011]

41 **Bollinger KE**, Crabb JS, Yuan X, Putliwala T, Clark AF, Crabb JW. Quantitative proteomics: TGFβ$_2$ signaling in trabecular meshwork cells. *Invest Ophthalmol Vis Sci* 2011; **52**: 8287-8294 [PMID: 21917933 DOI: 10.1167/iovs.11-8218]

42 **Goel R**, Murthy KR, Srikanth SM, Pinto SM, Bhattacharjee M, Kelkar DS, Madugundu AK, Dey G, Mohan SS, Krishna V, Prasad TsK, Chakravarti S, Harsha H, Pandey A. Characterizing the normal proteome of human ciliary body. *Clin Proteomics* 2013; **10**: 9 [PMID: 23914977 DOI: 10.1186/1559-0275-10-9]

43 **Friedman JS**, Faucher M, Hiscott P, Biron VL, Malenfant M, Turcotte P, Raymond V, Walter MA. Protein localization in the human eye and genetic screen of opticin. *Hum Mol Genet* 2002; **11**: 1333-1342 [PMID: 12019215 DOI: 10.1093/hmg/11.11.1333]

44 **Ramesh S**, Bonshek RE, Bishop PN. Immunolocalisation of opticin in the human eye. *Br J Ophthalmol* 2004; **88**: 697-702 [PMID: 15090426 DOI: 10.1136/bjo.2003.031989]

45 **Andley UP**, Malone JP, Hamilton PD, Ravi N, Townsend RR. Comparative proteomic analysis identifies age-dependent increases in the abundance of specific proteins after deletion of the small heat shock proteins αA- and αB-crystallin. *Biochemistry* 2013; **52**: 2933-2948 [PMID: 23590631 DOI: 10.1021/bi400180d]

46 **Hains PG**, Truscott RJ. Age-dependent deamidation of lifelong proteins in the human lens. *Invest Ophthalmol Vis Sci* 2010; **51**: 3107-3114 [PMID: 20053973 DOI: 10.1167/iovs.09-4308]

47 **Hains PG**, Truscott RJ. Proteome analysis of human foetal, aged and advanced nuclear cataract lenses. *Proteomics Clin Appl* 2008; **2**: 1611-1619 [PMID: 21136811 DOI: 10.1002/prca.200800085]

48 **Su S**, Liu P, Zhang H, Li Z, Song Z, Zhang L, Chen S. Proteomic analysis of human age-related nuclear cataracts and normal lens nuclei. *Invest Ophthalmol Vis Sci* 2011; **52**: 4182-4191 [PMID: 21436267 DOI: 10.1167/iovs.10-7094]

49 **Bassnett S**, Wilmarth PA, David LL. The membrane proteome of the mouse lens fiber cell. *Mol Vis* 2009; **15**: 2448-2463 [PMID: 19956408]

50 **Wang Z**, Han J, David LL, Schey KL. Proteomics and phosphoproteomics analysis of human lens fiber cell membranes. *Invest Ophthalmol Vis Sci* 2013; **54**: 1135-1143 [PMID: 23349431 DOI: 10.1167/iovs.12-11168]

51 **Sharma S**, Chataway T, Burdon KP, Jonavicius L, Klebe S, Hewitt AW, Mills RA, Craig JE. Identification of LOXL1 protein and Apolipoprotein E as components of surgically isolated pseudoexfoliation material by direct mass spectrometry. *Exp Eye Res* 2009; **89**: 479-485 [PMID: 19442659 DOI: 10.1016/j.exer.2009.05.001]

52 **Ronci M,** Sharma S, Martin S, Craig JE, Voelcker NH. MALDI MS imaging analysis of apolipoprotein E and lysyl oxidase-like 1 in human lens capsules affected by pseudoexfoliation syndrome. *J Proteomics* 2013; **82**: 27-34 [PMID: 23411028 DOI: 10.1016/j.jprot.2013.01.008]

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REVIEW

Role of PRMTs in cancer: Could minor isoforms be leaving a mark?

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Abstract

Protein arginine methyltransferases (PRMTs) catalyze the methylation of a variety of protein substrates, many of which have been linked to the development, progression and aggressiveness of different types of cancer. Moreover, aberrant expression of PRMTs has been observed in several cancer types. While the link between PRMTs and cancer is a relatively new area of interest, the functional implications documented thus far warrant further investigations into its therapeutic potential. However, the expression of these enzymes and the regulation of their activity in cancer are still significantly understudied. Currently there are nine main members of the PRMT family. Further, the existence of alternatively spliced isoforms for several of these family members provides an additional layer of complexity. Specifically, PRMT1, PRMT2, CARM1 and PRMT7 have been shown to have alternative isoforms and others may be currently unrealized. Our knowledge with respect to the relative expression and the specific functions of these isoforms is largely lacking and needs attention. Here we present a review of the current knowledge of the known alternative PRMT isoforms and provide a rationale for how they may impact on cancer and represent potentially useful targets for the development of novel therapeutic strategies.

Key words: Protein arginine methyltransferase; Arginine methylation; Cancer, Alternative splicing; Isoforms

Core tip: This review focuses on the current knowledge regarding alternative protein arginine methyltransferases (PRMT) isoforms and evidence supporting their potential impact in cancer. Alternative PRMT isoforms have been identified for PRMT1, PRMT2, CARM1 and PRMT7 and more may exist for the other PRMT family members. The presence of these isoforms adds a layer of complexity to the functional roles PRMTs play in normal and disease contexts. These alternative isoforms have unique characteristics that may offer clarification to conflicting roles documented in the literature. Finally, understanding the specific functions of these isoforms is crucial for fully characterizing the therapeutic potential of PRMTs in cancer.

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INTRODUCTION

Cancer is a leading cause of death worldwide. As we improve our understanding of the complex biologic processes behind this devastating disease we are able to develop improved treatments and increase patient survival. The biology of human tumours has been characterized as

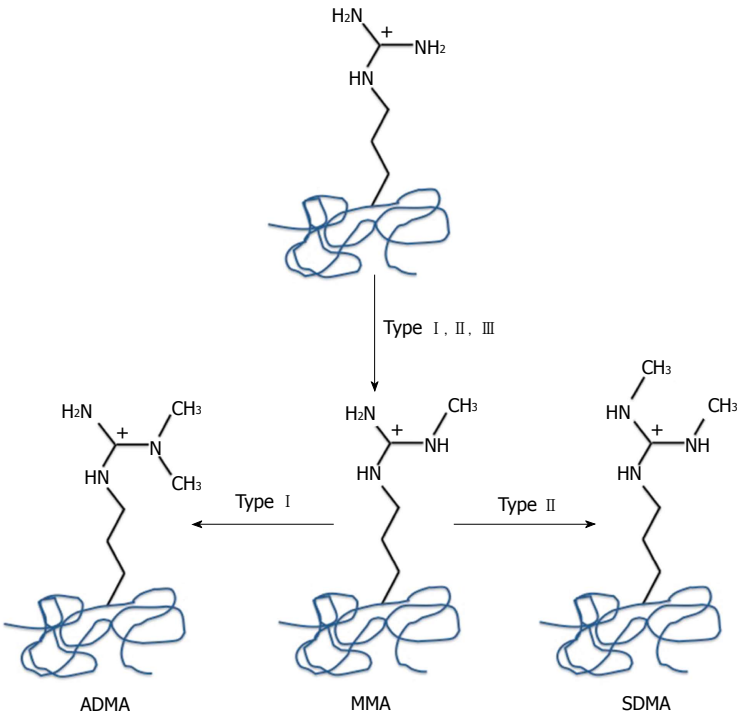

Figure 1 Arginine methylation reactions catalyzed by protein arginine methyltransferases. Type Ⅰ protein arginine methyltransferases catalyze the asymmetric dimethylation of arginine residues, Type Ⅱ symmetrically dimethylated arginine and Type Ⅲ monomethylated arginine residues.

having six key hallmarks: sustained proliferative capacity, evasion of growth suppressors, resisting death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis[1]. Each of these features is distinct, but they all cooperate to promote tumour development, growth and aggressiveness. Identifying key molecular regulators of one or more of these characteristics is essential in understanding cancer and potentially discovering new and better therapeutic strategies.

Arginine methylation is a common posttranslational modification that is known to have a role in several cellular processes, including signal transduction, DNA repair, transcription, protein subcellular localization and RNA processing[2,3]. Arginine methylation, in mammalian cells, is catalyzed by a family of enzymes called protein arginine methyltransferases (PRMTs). This family currently consists of nine characterized members in higher eukaryotes. These enzymes are subdivided into three categories based on the type of methyl mark produced on the arginine residue. These methylation reactions are depicted in Figure 1. Type Ⅰ [PRMT1, 3, 4 (CARM1), 6, and 8] generate ω-N^G,N^G-asymmetric dimethylarginine. Type Ⅱ (PRMT 5 and potentially PRMT9) generate ω-N^G,N^G-symmetric dimethylarginine. Finally, Type Ⅲ generate ω-N^G-monomethylarginine residues. Recently, it has been demonstrated that PRMT7 is the only bona fide type Ⅲ methyltransferase[4,5]. The majority of arginine methylation is catalyzed by PRMT1 (asymmetric) and PRMT5 (symmetric), and loss of expression of either of these enzymes is not compatible with life[6,7]. Currently, there is more that 120 known arginine methylated proteins, including histone and non-histone proteins[8,9]. The list of arginine methylated protein substrates is constantly growing, and along with it the discovery of new functional roles and involvement in numerous regulatory pathways[8,10,11].

Accumulating evidence convincingly shows that arginine methylation may represent a driving force behind the development, progression and aggressiveness of several cancer types. While the link between arginine methylation and cancer is a relatively new area of interest, the roles that the PRMTs have been shown to play in cancer thus far demonstrate their importance. These roles and the cancer types that have been studied are highlighted in Table 1. Dysregulated PRMT expression has been observed in a number of human tumours, including lung, breast, prostate, colorectal, bladder and leukemia[12-19]. For a comprehensive review summarizing the roles of each PRMT family member in cancer see Yang and Bedford's review article in Nature Reviews: Cancer entitled, Protein arginine methyltransferases and cancer[20]. The primary focus of this review is to specifically highlight the current knowledge regarding alternatively spliced PRMT family members and the potentially distinct roles that they play in cancer. While a survey within the *Ensembl* database predicts the existence of alternatively spliced isoforms for all the *PRMT* gene family members, only the expression of PRMT1, PRMT2, CARM1 and PRMT7 isoforms has been characterized and confirmed in mammalian cells[21-27].

Interestingly, the majority of these alternative isoforms were found in cancer cells, suggesting they may have specific roles in cancer. Characterization of several of these alternative PRMT isoforms has shown that they are differentially expressed in various cell types and they possess distinct functional characteristics. However, the individual roles that these alternative isoforms play in cells remains poorly understood and understudied. There-

Table 1 Protein arginine methyltransferases in cancer cells

PRMT	Cancer type	Role(s) in cancer	Ref.
PRMT1	Breast cancer, Lung cancer, Colon cancer, Bladder cancer, Acute myeloid leukemia, Mixed lineage leukemia	Cell proliferation and survival, Transformation, Resistance to DNA damaging agents, Invasion	[13,15-17,19, 21,36-38]
PRMT2	Breast cancer	Cell proliferation and invasion	[22,72]
PRMT3	Breast cancer	Cell survival	[101,102]
CARM1/PRMT4	Breast cancer, Prostate cancer, Colorectal cancer	Cell proliferation	[12,14,77-79,88]
PRMT5	Lung cancer, Leukemia, Lymphoma, Melanoma, Gastric cancer, Colorectal cancer	Cell proliferation, Transformation, Invasion, Resistance to DNA damaging agents	[18,103-109]
PRMT6	Lung cancer, Bladder cancer	Cell proliferation	[17,110]
PRMT7	Breast cancer	Resistance to DNA damaging agents	[27,91,92,94]
PRMT8	ND	ND	
PRMT9	ND	ND	

ND: Not determined; PRMT: Protein arginine methyltransferase.

fore, more attention needs to be given to their individual functions under normal biological conditions, as well as their contribution to diseases such as cancer. PRMTs are thought to be potentially useful therapeutic targets for the treatment of diseases such as cancer[28]. Moreover, these alternative PRMT isoforms must be taken into account when designing and evaluating potential candidate therapeutic strategies or compounds. This is essential so there is a clear understanding of the precise mechanism of action. Although our knowledge of the specific roles of these isoforms is limited, there is evidence in the literature strongly suggesting that they are not redundant. While they may share some similar functions, they also have clearly distinct roles.

PRMT ISOFORMS AND CANCER

PRMT1

PRMT1 is a Type I arginine methyltransferase and is responsible for generating upwards of 85% of the asymmetrically dimethylated proteins within cells[29]. PRMT1 is the most well characterized protein within this family of enzymes. While the PRMT1 protein is mainly described in the literature as a single entity, it has been identified, that at least seven distinct PRMT1 isoforms are generated by complex alternative splicing in the 5' region of its pre-mRNA[21,30,31]. The exon structure for the identified PRMT1 isoforms is summarized in Figure 2 and detailed in Goulet *et al*[21] 2007. Each of these isoforms, named PRMT1v1-v7, has distinct characteristics in terms of expression. PRMT1v1 is the most abundantly expressed isoform and likely represents the isoform that is described as PRMT1 in most reports. The expression levels of PRMT1v1, v2 and v3 have all been shown to be ubiquitous across tissues[21,30,31]. Interestingly, a higher level of PRMT1v1 mRNA expression is observed in the kidney, liver, lung, skeletal muscle and spleen[21]. PRMT1v2 mRNA was found to be elevated in the kidney, liver and pancreas, while, PRMT1v3 mRNA expression was observed at similar levels in all tissues examined (brain, heart, kidney, liver, lung, pancreas, skeletal muscle and spleen), however at low levels compared to PRMT1v1 and PRMT1v2. The mRNA expression levels of PRMT1v4 to v7 showed a more tissue specific profile, with v4 being detected only in the heart, v5 mainly in the pancreas, and v7 observed in the heart and skeletal muscle. PRMT1v6 mRNA was not detected in any normal tissues examined[21]. Further studies would need to be performed to determine if this differential expression has any correlation with the development of cancer from a particular tissue of origin.

While tissue specific expression of PRMT1 isoforms is observed, at the cellular level there are also differences in their subcellular localization (Table 2). PRMT1v3, v4, v5 and v6 all show an equal distribution of nuclear and cytoplasmic expression[21]. In contrast, PRMT1v1, v2 and v7 display a more compartmentalized expression profile within cells. PRMT1v1 and v7 display a more intense nuclear expression, while PRMT1v2 is expressed predominantly in the cytoplasm, however this may vary depending on cell type and methylation status of substrates as it was clarified by the Fackelmayer lab[32,33]. The cytoplasmic expression of PRMT1v2 is due to the retention of exon 2 within the N-terminal coding sequence. This short exon contains a leucine-rich nuclear export sequence (NES). Careful analysis showed that this NES does in fact control the nuclear export of PRMT1v2 and that its export is dependent on the nuclear export receptor CRM1[21].

A comparison of the PRMT1 isoforms revealed they have dstinct enzymatic activity and substrate specificity profiles[21]. Additionally, stable isotope labeling by amino acids in cell culture (SILAC[34,35]) followed by immunopurification of PRMT1v1 and PRMT1v2 from cells has been used to identify their isoform-specific protein binding partners and/or substrates (Figure 3). In Figure 3 we show the full data set from this analysis comparing the SILAC ratios of PRMT1v1 and PRMT1v2 binding proteins (unpublished data). Each point represents an identified interacting protein. This clearly shows that there is a potential set of PRMT1v1-specific interacting proteins (lower right quadrant) and PRMT1v2-specific interacting proteins (upper left quadrant). Also, there are some com-

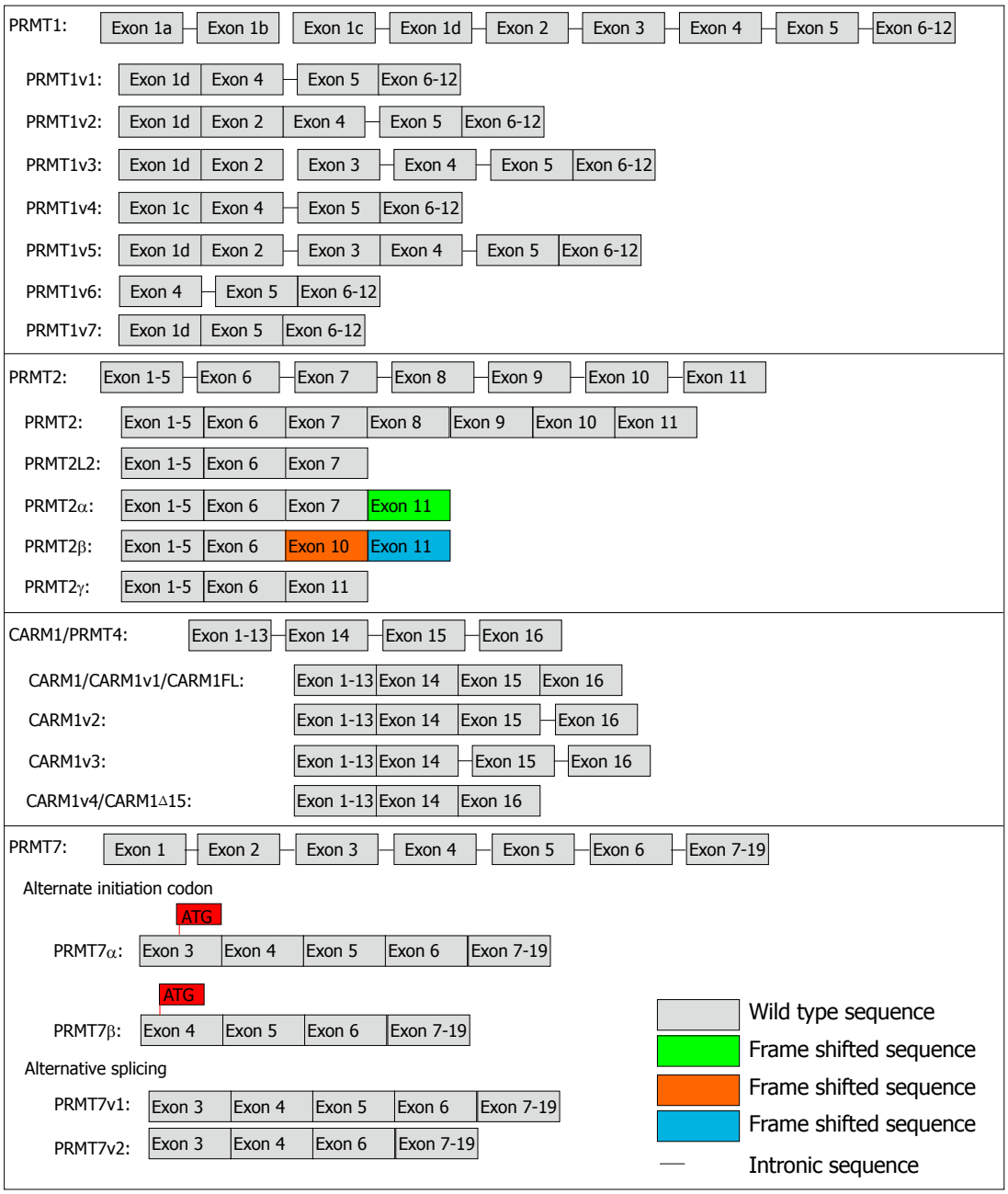

Figure 2 Protein arginine methyltransferase variant isoforms. Schematic representation of the identified variant isoforms of protein arginine methyltransferase (PRMT) 1, PRMT2, CARM1/PRMT4 and PRMT7. The PRMT1 sequence has 12 exons. Exon organization of the seven identified PRMT1 isoforms are shown. The intronic sequences (-) that have been shown to be included in several of these alternative PRMT1 isoform transcripts are due to the splicing sites[21]. PRMT2 is made up of 11 exons. The PRMT2L2 transcript is produced as a result of alternative polyadenylation[72]. This silences the 5' splice site on exon 7 and results in a transcript retains a significant portion of intron 7 and a premature termination codon. PRMT2α has a deletion of exons 8-10 with a frame shift that produces 12 new amino acids at the C-terminus (ν). The PRMT2β isoform has a deletion of exons 7, 8, 9 resulting in a frame shift that generates 83 alternate amino acids at the C-terminus (νν). PRMT2γ has an in frame deletion of exons 7 to 10. The full-length CARM1 gene, CARM1/CARM1v1/CARM1FL, consists of 16 exons. CARM1v2 is generated through retention of the intron 15 sequence; CARM1v3 is produced through the retention of introns 15 (-) and 16 (-). CARM1v4/CARM1Δ15 results from the skipping of exon 15[23,24]. The PRMT7 sequence consists of 19 exons. In Hamster cells, these two PRMT7 isoforms (α and β) are thought to be generated by the use of distinct 5' translation initiation codons within the primary transcript. The PRMT7β isoform sequence contains 37 extra amino acids at the N-terminus. Alternatively, at least 2 alternatively spliced PRMT7 isoforms can be produced from the human PRMT7 gene. These two isoforms have the same N- and C-terminal regions but variant 2 (PRMT7v2) has an in frame deletion of exon 5.

mon binding partners (upper left quadrant). This emphasizes the importance of understanding their individual functions. Conservation of these alternatively spliced isoforms of PRMT1 through evolution suggests they are likely to each have their own function(s) within cells and tissues.

Deregulated PRMT1 expression has been observed in a number of tumour types, which include those of the lung, breast, colon, bladder and leukemia[13,15-17,19,21,36-38]. The question is then, "What are the functions of these

Table 2 Protein arginine methyltransferase isoform specific subcellular localization and current cancer cell types in which they have been shown to be expressed

PRMT isoform	Molecular weight (kDa)	Subcellular localization	Cancer cell type	Ref.
PRMT1v1	40.5	Predominantly nuclear	Breast cancer cell lines and tumour samples, cervical cancers cells	[21]
PRMT1v2	42.5	Predominantly cytoplasmic	Breast cancer cell lines and tumour samples, cervical cancers cells	[21,39]
PRMT1v3	39.9	Cytoplasmic and nuclear	Breast cancer cell lines and tumour samples	[21]
PRMT1v4	40.1	Cytoplasmic and nuclear	Breast cancer cell lines	[21]
PRMT1v5	39.4	Cytoplasmic and nuclear	Breast cancer cell lines	[21]
PRMT1v6	37.7	Cytoplasmic and nuclear	Breast cancer cell lines	[21]
PRMT1v7	36.7	Predominantly nuclear	Breast cancer cell lines	[21]
PRMT2	48.5	Predominantly nuclear, excluding nucleoli	Breast cancer cell lines and tumour samples	[22,72]
PRMT2L2	32	Predominantly cytoplasmic	Breast cancer cell lines and tumour samples	[72]
PRMT2α	32.6	Predominantly nuclear, excluding nucleoli	Breast cancer cell lines and tumour samples	[22]
PRMT2β	34	Cytoplasmic and nuclear, including nucleoli	Breast cancer cell lines and tumour samples	[22]
PRMT2γ	25.8	Predominantly nuclear, excluding nucleoli	Breast cancer cell lines and tumour samples	[22]
CARM1/CARM1v1/ CARM1FL	66	ND	Breast cancer cell lines	[23,24]
CARM1v2	71	ND	Breast cancer cell lines	[23,24]
CARM1v3	63	ND	Breast cancer cell lines	[23,24]
CARM1v4/CARM1Δ15	64	ND	Breast cancer cell lines	[23,24]
PRMT7α	78	Cytoplasmic and nuclear	ND	[27]
PRMT7β	82	Predominantly cytoplasmic	ND	[27]

ND: Not determined; PRMT: Protein arginine methyltransferase.

isoforms and do they have specific roles in cancer?" To date our knowledge is limited as to the specific functions of each of these PRMT1 isoforms. However, there is evidence showing potential individual roles for them in cancer. In breast cancer, both the mRNA and protein expression of several alternative PRMT1 isoforms is elevated (Table 2)[21,31]. This is observed not only in breast cancer cell lines, but also in breast tumours. Specifically, the mRNA expression of PRMT1v1, v2, v3 and v7 is elevated across several breast cancer cell lines compared to a non-transformed mammary epithelial cell line[21]. In contrast, PRMT1v5 and v6 were upregulated only in a subset of breast cancer cell lines. Furthermore, PRMT1v1, v2 and v3 mRNA expression was increased in breast cancer tumour tissue compared to normal tissue. Interestingly, while this study concluded an overall upregulation of PRMT1 alternative isoforms in breast cancer, the cytoplasmically localized PRMT1v2 isoform had the greatest increase in expression in breast cancer compared to PRMT1v1, the most abundantly expressed isoform. It is difficult to assess the protein expression of each of these individual isoforms due to the sequence similarities between them. However, in the case of PRMT1v2, exploitation of the exon 2 sequence has allowed for a more specific examination. Indeed, results have shown that PRMT1v2 protein expression is elevated in breast cancer cells[21]. A recent clinical assessment of PRMT1v1, v2 and v3 expression within breast cancer tissues has identified that high PRMT1v1 mRNA expression correlates with poor patient prognosis and a reduced disease-free survival[16]. An examination of PRMT1 protein expression within breast tumours *via* immunohistochemistry demonstrated a predominantly cytoplasmic expression and only in rare cases nuclear expression. We, and others have shown that PRMT1v2 is predominantly localized to the cytoplasm[21,32,39]. Therefore, one could speculate that PRMT1v2 could represent a significant proportion of the cytoplasmic PRMT1 detected in these breast tumour samples. This evidence shows that the expression of the PRMT1v2 isoform is elevated in breast tumours and it may have its own unique contributions to breast cancer progression. This also emphasizes the need to study these alternative isoform individually, in order to determine their specific functions and contribution to disease. While this has been mainly assessed in breast cancer thus far, it does not rule out that these PRMT1 isoforms may be expressed in other cancer types as well and this should be explored further.

The involvement of PRMT1 in cancer is supported by evidence showing its involvement in pivotal oncogenic processes. PRMT1 plays an active role in MLL-mediated transformation of primary myeloid progenitor cells[13]. PRMT1 has also been shown to have a significant role in cell proliferation/viability and cell cycle progression. Depletion of PRMT1 resulted in a significant decrease in the proliferation of osteosarcoma, breast, bladder and lung cancer cell lines[6,17,37]. This reduction in cell proliferation was associated with cell cycle arrest at the G_0/G_1 phase. Additionally, breast cancer cells showed a loss of cyclin D1 and increase in $p21^{cip1}$ expression, indicative of a cell cycle arrest at this phase[37]. While these studies examined PRMT1 as a whole, PRMT1 isoform-specific contributions have also been investigated. The specific depletion of the PRMT1v2 isoform using RNA interference in breast cancer cells resulted in a significant reduction in cell viability and growth[40]. This decreased cell viability was attributed, at least in part, to an induction of apoptosis occurring with the suppression of PRMT1v2

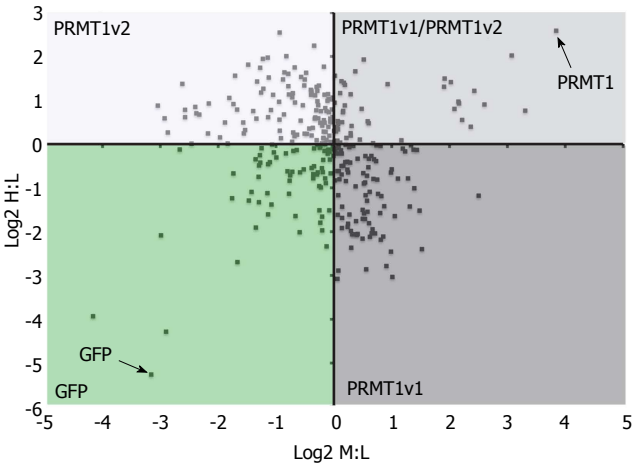

Figure 3 Protein arginine methyltransferase 1v1 and protein arginine methyltransferase 1v2 have potentially different interacting protein profiles. Stable isotope labeling by amino acids in cell culture (SILAC) and mass spectrometry was used to identify protein arginine methyltransferase (PRMT) 1v1 protein binding partners and PRMT1v2 protein binding partners. Cells stably expressing GFP alone, GFP-tagged PRMT1v1 or GFP-tagged PRMT1v2 were grown independently in media containing light (L), medium (M) and heavy (H) isotopes of arginine and lysine residues, respectively. Protein lysates were collected, immunoprecipitated for GFP (isolation of PRMT1v1 and PRMT1v2 interacting protein), and subjected to mass spectrometry for peptide identification. The Log2 of the SILAC ratios for the peptides identified from this experiment are plotted on the scatter plot. The x-axis is the Log2 of the H:L SILAC ratio or PRMT1v2 interacting proteins. The y-axis is the Log2 of the M:L SILAC ratio or PRMT1v1 interacting proteins. Each data point represents a single protein that was identified in this experiment. The greater this ratio is for a protein, the higher the probability of the interaction being real. This revealed a protein interacting profile identifying PRMT1v1-specific interacting proteins (PRMT1v1 quadrant), PRMT1v2-specific interacting proteins (PRMT1v2 quadrant) and common interacting proteins (PRMT1v1/PRMT1v2 quadrant; unpublished data). These results require further validation.

expression. Additionally, breast cancer cells overexpressing PRMT1v2 showed an increased growth rate, which was not observed upon PRMT1v1 overexpression and points to isoform specific effects. This evidence suggests that in these breast cancers cells PRMT1v2 may represent a key cell survival-promoting factor. Overall, this evidence links PRMT1 to the self-sustaining proliferative signaling acquired by cancer cells, enabling them to grow and survive.

The impact that PRMT1 has on the survival and aggressiveness of cancer cells is becoming increasingly evident with the identification of new intracellular substrates. It has been demonstrated that the asymmetric dimethylation of histone H4R3 is associated with active transcription and increased tumour grade in prostate cancer[41-43]. However, the downstream consequences of this methylation event are poorly understood in most cases[44]. Many of the recently identified PRMT1 substrates are key regulators of cancer cell growth, survival and invasion signaling. PRMT1 has been shown to influence receptor activation at the cell surface through direct methylation of the receptor or indirect methylation of a receptor associated protein. PRMT1 was shown to directly methylate the estrogen receptor α (ERα) at arginine (R) 260 and affects its downstream signaling[37,45]. This results in cytoplasmic retention of ERα and the interaction of ERα with Src, focal adhesion kinase (FAK) and the regulatory subunit of PI-3 kinase (p85). All three of which are involved in oncogenic intracellular signaling that promotes cancer cell survival and invasiveness[46-50]. Furthermore, loss of this methylation site on ERα, by point mutation, impaired downstream signaling, as evidenced by a loss of PKB/Akt phosphorylation. Recently, it was shown that PRMT1 is involved in the induction of transforming growth factor (TGF) β signaling in response to bone morphogenetic protein (BMP) binding its TGFβ receptors, R Ⅰ and R Ⅱ[51]. Activation of this receptor is achieved through the ligation and dimerization of the R Ⅰ and R Ⅱ receptors[52]. The R Ⅰ receptor is held in an inactive state by its association with Smad 6. Upon BMP ligation and dimerization of R Ⅰ and R Ⅱ, PRMT1 methylates Smad 6, causing its dissociation from RI and activation, thereby inducing BMP signaling which has a role in cancer stem cell proliferation and cancer cell invasion[53]. PRMT1 has also been shown to interact with PRMT8[54]. PRMT8 harbours a unique property, as it is tethered to the plasma membrane *via* an N-terminal myristoylation motif. Additionally, PRMT8 is specifically only expressed in brain tissue. This PRMT1-PRMT8 interaction effectively localizes PRMT1 activity at the plasma membrane and could potentially be affecting a distinct set of substrates. A specific role for PRMT8 in cancer has not been examined. These functions of PRMT1 occur in the cytoplasm of cells, and the RNA interference method used in these studies targeted all PRMT1 isoforms. Therefore, it would be of interest to assess whether specific PRMT1 isoforms might differentially contribute to the above-mentioned regulatory pathways. This would offer not only more functional understanding, but therapeutic insight as well.

PRMT1 has been shown to methylate key cytoplasmic proteins that are linked to apoptotic signaling pathways. Intriguingly, there have been conflicting roles presented for PRMT1 in apoptotic signal regulation. One study demonstrated that PRMT1 methylates apoptosis signal-regulating kinase 1 (ASK1) and this inhibits its activity[55]. This methylation promotes the interaction of ASK1 with its negative regulator, thioredoxin. As a consequence breast cancer cells were shown to be more resistant to treatment with paclitaxel. In contrast, the BCL-2 antagonist of cell death (BAD) has also been identified as a PRMT1 substrate in breast cancer cells[56]. This methylation prevents PKB/Akt mediated phosphorylation of BAD, thus preventing its inactivation, resulting in enhanced BAD-induced apoptosis. These conflicting roles highlight the complex role that methylation plays within cellular signaling pathways. These observations were seen in two distinct breast cancer cells, MDA-MB-231 and MCF7 respectively. Therefore, it is unknown whether these observations are due to cell specific behaviors or more interestingly the genetic differences between these two distinct breast cancer cells. Furthermore, they may also be influenced by differential expression of alternative PRMT1 isoforms, potentially reflecting differences in

function and substrate specificities within cancer cells.

A recent study identified Axin, a mainly cytoplasmic protein, as a PRMT1 substrate[57]. Importantly, it was shown that Axin could be methylated by two PRMT1 isoforms, PRMT1v1 and PRMT1v2 *in vitro*. However, this methylation analysis was not conducted within cells and would have been a very informative experiment, considering both Axin and PRMT1v2 share a cytoplasmic localization. Axin is a critical scaffolding protein that complexes with adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β), forming a degradation complex. This complex negatively regulates Wnt signaling and impacts actin cytoskeletal dynamics through the degradation of β-catenin[57,58]. Methylation of Axin by PRMT1 increases Axin protein stability, resulting in decreased β-catenin protein levels. Interestingly, isoform specific overexpression of PRMT1v1 or PRMT1v2 in a weakly invasive breast cancer cell line (MCF7) resulted in an increase in cell motility[40]. However, only the overexpression of the PRMT1v2 isoform increased cell invasion through a Matrigel barrier. Additionally, specific depletion of PRMT1v2 in an invasive breast cancer cell line, MDA-MB-231, resulted in decreased invasion through a Matrigel barrier. PRMT1v2 overexpression caused a decrease in β-catenin protein expression, which was not seen with the overexpression of PRMT1v1. This loss in β-catenin protein expression was directly linked to the PRMT1v2-induced invasion observed in breast cancer cells. Furthermore, PRMT1v2 enzymatic activities as well as proper subcellular localization were required for its ability to promote invasion. Therefore, it is conceivable that within cells Axin is preferentially methylated by PRMT1v2, thereby regulating β-catenin protein levels. This evidence has shown for the first time direct functional differences between PRMT1 isoforms in cancer, and identified a specific role for PRMT1v2 in promoting breast cancer cell invasion.

PRMT1 methylates several proteins within the nucleus that are involved in transcription, telomere stability and DNA repair. Similarly to the methylation of BAD, PRMT1 methylates the forkhead box protein 1 (FOXO1) at R248 and R250 blocking PKB/Akt-mediated phosphorylation of S253[59]. This methylation results in nuclear retention of FOXO1, increased transcriptional activity and increased oxidative-stress induced cell death. This evidence again supports a role for PRMT1 promoting cell death. PRMT1 also affects telomere length and stability, which impacts the replicative capacity of cancer cells[1,60]. PRMT1 methylates the telomeric repeat binding factor 2 (TRF2), thereby regulating its association with telomeres. TRF2 is a component of the sheltering complex that binds telomeric DNA and functions to protect telomeres and maintain their length. Depletion of PRMT1 in cancer cells increased the association of TRF2 with telomeres and promoted shortening. This supports a role for PRMT1 in dysregulated cancer cell replication. Additionally, PRMT1 is linked to the DNA damage response and DNA repair pathways through the methylation of MRE11 and p53 binding protein 1 (53BP1). PRMT1 has been shown to methylate MRE11 and 53BP1 within their GAR motif[61-63]. Methylation of MRE11 regulates its DNA exonuclease activity in response to DNA damage[61]. Similarly, methylation of 53BP1 is necessary for its DNA binding activity and localization to sites of DNA damage[63]. Mutation of this methylation motif in both MRE11 and 53BP1 disrupts the functions of these two key proteins in the DNA damage pathway. Finally, PRMT1 was shown to methylate the tumour suppressor gene BRCA1[36]. Methylation of BRCA1 had a significant impact on its ability to bind to different gene promoters, adding a level of complexity to the transcriptional regulating function of PRMT1. It would be interesting to determine if these effects are isoform specific, as it has been shown that the PRMT1v1 isoform is predominantly localized to the nucleus.

These studies demonstrate that PRMT1 has a significant impact on the vital processes and signaling that are involved in the development, progression and aggressiveness of cancer cells. The majority of these studies have examined PRMT1 as one single enzyme, however the existence of the distinct PRMT1 isoforms adds a level of complexity that requires further study and clarification. This evidence suggests that PRMT1 may be a potentially valuable therapeutic target for the treatment of several cancer types, however our knowledge of this target is limited due to our lack of understanding of the precise roles of the alternative isoforms that are present.

PRMT2

PRMT2, also known as HRMT1L1, was discovered through its sequence homology with the catalytic domain of PRMT1 (approximately 50%)[30]. Interestingly, within its sequence it contains an Src homology 3 (SH3) binding domain, which potentially links it to many intracellular processes. Initially, it had no characterized methyltransferease activity. However, more recent evidence has shown that it possesses Type I arginine methyltransferase activity, albeit much lower than that of PRMT1[64]. There is limited knowledge with regards to PRMT2 methyl substrates. Evidence has shown PRMT2 is recruited by β-catenin to histone H3 where it deposits an asymmetric dimethyl mark on R8 of target gene promoters[65]. However, further experiments are required in order to generate a more complete substrate repertoire for PRMT2. Nevertheless, it has been demonstrated that PRMT2 can affect the activation of several key receptors *via* a co-activator function within cells. PRMT2 has been shown to interact with and enhance the transactivation of ERα, progesterone receptor (PR), androgen receptor (AR), peroxisome proliferator-activated receptor γ (PPARγ) and the retinoic acid receptor α (RARα) in a ligand independent fashion[66]. Interestingly, the activation of these receptors within cells has both distinct and in some cases opposing effects. Activation of ERα, PR and AR has been implicated in tumour cell growth and progression, while PPARγ and RARα activation results in growth ar-

rest and apoptosis[67-71]. This suggests that the functional role PRMT2 plays within cells is quite diverse.

Recently, in two separate papers by Zhong *et al*[22,72], four alternatively spliced PRMT2 isoforms (PRMT2L2, PRMT2α, β, and γ) in addition to the original PRMT2 isoform were identified. The *PRMT2* gene consists of 11 exons and these alternative isoforms are generated through alterations in sequence that occur from exon 7 to exon 10 (Figure 2). The first report identified a novel PRMT2L2 transcript that is produced as a result of alternative polyadenylation[72]. This polyadenylation silences the 5' splice site on exon 7 and results in a transcript that retains a significant portion of intron 7 and a premature termination codon. Subsequently, they identified PRMT2α, β and γ and showed that these isoforms are generated through splicing events occurring in the 3' C-terminal region of the *PRMT2* pre-mRNA leading to exon exclusion[22]. PRMT2α has a deletion of exons 8-10 with a frame shift that produces 12 new amino acids at the C-terminus. The PRMT2β isoform has a deletion of exons 7, 8, 9 resulting in a frame shift that generates 83 alternate amino acids at the C-terminus, while PRMT2γ has an in frame deletion of exons 7 to 10. All of these deletions in the alternatively spliced isoforms result in the loss of conserved protein arginine methyltransferase motifs. They have each lost domain Ⅲ and the THW loop. The THW loop has been shown to form part to the AdoMet-binding pocket with domains I and post I [73], therefore these variant isoforms may lack arginine methylation activity. Methylation activity of these isoforms has not yet been examined. An examination of the subcellular localization of GFP tagged PRMT2 isoforms showed that PRMT2, PRMT2α and PRMT2γ have a predominantly nuclear localization, excluding the nucleolus (Table 2)[22]. The PRMT2β isoform showed a relatively even distribution throughout the nucleus, including the nucleolus, and also localized to the cytoplasm within cells. The PRMT2L2 had a predominantly cytoplasmic localization with concentrated perinuclear staining observed[72]. It is thought that the 3' sequence may impact the localization of these isoforms.

Characterization of these alternative isoforms showed differential expression across a panel of breast cancer cell lines (Table 2). Interestingly, mRNA and protein expression of all PRMT2 isoforms are elevated in ER, PR-positive cell lines (MCF7, T47D, BT474 and ZR-75-1) compare to double negative cell lines (MDA-MB-231, MDA-MB-453 and SK-BR-3)[22,72]. Furthermore, in breast tumour samples, the mRNA expression of all PRMT2 isoforms was shown to be significantly increased in breast tumour tissues compared to normal adjacent breast samples. Additionally, the expression of each isoform was shown to be slightly higher in ER-positive compared to ER-negative tumours. Moreover, an immunohistochemical analysis, which did not differentiate between isoforms, showed that PRMT2 protein expression is elevated in breast tumour samples compared to normal breast tissue[22]. Additionally, similar to the mRNA, PRMT2 protein expression was elevated to a greater extent in ER-positive tumours compared to ER-negative tumours.

A functional assessment of the PRMT2 isoforms showed that they are able to directly bind and enhance estrogen-mediated transactivation of ERα, and also enhance the promoter activity of the downstream target gene, snail[22,72]. Increased snail transcriptional activity is associated with an increased cancer cell invasive potential[74]. Interestingly, all the isoforms had a lower transcriptional activity compared to PRMT2. Additionally, PRMT2β also had the lowest estrogen stimulated transcriptional activity and showed the lowest interaction affinity for ERα. This demonstrates that these isoform may perform different functions within cells. This interaction with ERα occurs *via* the N-terminus of the PRMT2 isoforms. Each PRMT2 isoform was also shown to directly bind to the AR. Intriguingly, it was revealed that PRMT2 negatively impacts the proliferation of ERα positive breast cancer cells in response to estrogen stimulation[22]. Depletion of the PRMT2 isoforms caused an increase in estrogen-induced proliferation and an enhancement in E2F expression and downstream activity. This is consistent with results showing that PRMT2 can bind to retinoblastoma protein (RB), and this interaction causes repression of E2F transcriptional activity[75]. It should be highlighted that the increase in proliferation may be specific to the original PRMT2 isoform, as depletion of this specific isoform caused a result similar in magnitude to the depletion of all four isoforms (PRMT2, PRMT2α, PRMT2β, PRMT2γ) simultaneously. Therefore, the contribution of the PRMT2α, PRMT2β, PRMT2γ isoforms to this proliferation phenotype is unclear. Similar to PRMT1, further research is required into the specific functions of these newly identified PRMT2 isoforms in order to determine their exact contributions to cancer development and progression. Nevertheless, these results demonstrate that the expression of PRMT2 and its alternative isoforms are clearly positively correlated with ERα status in breast cancers, consistent with a regulatory role in this pathway.

PRMT4/CARM1

PRMT4, more commonly known as Co-activator-associated arginine methyltransferase 1 (CARM1), was originally identified through its binding to GRIP1, the p160 steroid receptor co-activator[76]. It is involved in the regulation of a number of cellular processes including, transcription, pre-mRNA splicing, cell cycle progression and the DNA damage response. CARM1 is a type I arginine methyltransferase. In contrast to other type I PRMTs, which generally recognize substrate GAR motifs, it has no known substrate methylation motif[8,44]. CARM1 is most well characterized for its co-activator role in transcription which it performs through its interaction and methylation of a diverse substrate repertoire, including both histone and non-histone proteins[77-81]. The activity of CARM1 has also been shown to be influenced by posttranslational modifications. Specifically, CARM1 can be phosphorylat-

ed at several sites that can inhibit both dimerization (S229) and AdoMet binding (S217)[82,83]. Alternatively, phosphorylation at another site (S448) facilitates association with the ERα and stimulates ligand-independent activation of ERα[84]. Recently, it was identified that CARM1 is also regulated by auto-methylation[85]. The auto-methylation site was mapped to R551 in exon 15 of the mouse homolog of CARM1. This site is conserved in all vertebrate CARM1 proteins. Mutation of this auto-methylation site did not affect the enzymatic activity of CARM1, however it significantly impaired both CARM1-activated ERα mediated transcription and CARM1 regulated pre-mRNA splicing. Furthermore, it has been shown that essentially 100% of CARM1 is auto-methylated at R551 in cells[24]. Therefore, the regulation of CARM1 activity appears to be complex.

The expression of CARM1 has been shown to be dysregulated in colorectal, prostate and breast cancer[12,14,15]. CARM1 was found to be overexpressed in a significant number of colorectal tumours[14]. In prostate cancer, CARM1 was found to be overexpressed not only in tumours, but also in prostatic intraepithelial neoplasia (PIN). PINs are thought to be a precursor to the development of prostate cancer[12,14]. Finally, CARM1 expression was also found to be upregulated in breast cancer[14,86]. Interestingly, in the study conducted by Kim *et al*[14], for both prostate and breast cancers the expression level of CARM1 was lower. In a more recent study by Cheng *et al*[86], CARM1 expression was observed to be increased in invasive breast cancer, correlating with high tumour grade and to a greater extent with HER2, p53 and Ki-67 expression. CARM1 expression showed a lower correlative rate with ER and PR expression. The results from these studies are surprising given the role that CARM1 plays in the association and co-activation of ERα and AR[87,88]. They suggest that CARM1 has a multifaceted contribution to the development and progression of cancers. Furthermore, it shows that CARM1 may be an informative prognostic marker for breast cancer.

Within tumour cells, CARM1 plays a role in regulating cell proliferation and survival through its interaction and cooperation with several critical cancer related proteins. CARM1 is recruited to the promoter of the *cyclin E1* gene, where it acts as a transcriptional co-activator in regulating cyclin E1 protein expression. Furthermore, both CARM1 and cyclin E1 were shown to be co-overexpressed and correlated with grade 3 breast tumours[78]. CARM1 has also been shown to be necessary for estrogen-stimulated proliferation of breast cancer cells[77]. This occurs *via* estrogen-stimulated methylation of H3R17 by CARM1, resulting in expression of the cell cycle regulator E2F1. Moreover, CARM1 is involved in the regulation of both the stability and activity of AIB1, a transcriptional co-activator that is often overexpressed in breast tumours. Additionally, it has been recently shown that CARM1 can promote breast cancer cell migration and metastasis through the methylation of BAF155, a component of the chromatin-remodeling complex[89].

While these studies define a role for CARM1 in promoting cancer progression, a study by Al-Dhaheri *et al*[79] showed some conflicting effects. Overexpression of CARM1 in MCF7 breast cancer cells, an ER+ cell line, inhibited estrogen-stimulated cell growth, while overexpression or depletion of CARM1 in MDA-MB-231 (ER-) breast cancer cells had no effect on their growth. Interestingly, the inhibited cell growth observed in MCF7 cells with CARM1 overexpression was accompanied by increased expression of cell cycle inhibitors, $p21^{cip1}$ and $p27^{kip1}$ and a change in cell morphology reminiscent of a more differentiated phenotype. Additionally, CARM1 was shown to repress the expression of approximately 16% of estrogen-activated target genes. An expression analysis in a set of ER+ tumours showed that CARM1 expression positively correlates with ERα expression. However, it inversely correlated with tumour grade. It should also be noted that a recent report suggested that only small proportion of endogenous CARM1 protein expression is required in order to perform its biological functions in cells[89]. Therefore, suppression of 100% of CARM1 protein expression is required in experimentation because it is thought that only a very small amount of CARM1 protein is necessary for its normal functioning. These reports suggest that a further understanding of CARM1 regulation and function is required in order to clarify its role and potential marker/therapeutic value in cancer.

A plausible explanation for these opposing results in breast cancer cells is the existence of alternatively spliced isoforms of CARM1. In the literature there are two papers that describe the presence of distinct alternatively spliced CARM1 isoforms. The first by Ohkura *et al*[23] describes, that in normal rat tissue, four isoforms are transcribed from the *CARM1* gene; the primary isoform CARM1 (CARM1v1) and three alternative isoforms, v2, v3 and v4 (Figure 2). All four contain the arginine methyltransferase domain and the GRIP1-binding domain. The primary *CARM1* isoform, CARM1v1, consists of 16 exons. CARM1v2 is generated through retention of the intron 15 sequence, CARM1v3 is produced through the retention of introns 15 and 16 and CARM1v4 results from the skipping of exon 15[23]. Each of these enzymes showed a distinct mRNA expression profile when examined across a panel of normal rat tissues. Functionally, the CARM1v3 isoform was shown to alter the splicing pattern of both E1A and CD44 reporters. This was not observed with the other isoforms suggesting they may have different functions. The splicing activity demonstrated for CARM1v3 was shown to be independent of the CARM1v3 methylation activity. In contrast to this, Cheng *et al*[90] showed that CARM1 enzymatic activity is required for its effect on alternative splicing of the CD44 pre-mRNA, which is thought to occur co-transcriptionally. They also suggest that while CARM1v3 is an alternative isoform, it may represent a very rare form not playing a major role in cells. Hence the precise biological roles of these CARM1 isoforms remains unclear.

Alternatively, in the second paper, Wang *et al*[24]

showed that in human cells and tissues, two CARM1 isoforms are present. These are designated CARM1 full length (CARM1FL) and CARM1Δ15 (Figure 2). The CARM1Δ15 is a transcript in which exon 15 is excluded by alternative splicing. This alternative isoform represents the CARM1v4 isoform described previously. The other two isoforms were not detected in human cells or tissues. Importantly, exclusion of exon 15 removes the auto-methylation site that can functionally regulate CARM1, however it does not impact the methylation activity. An examination of mRNA expression across a panel of normal human tissues revealed that the CARM1Δ15 isoform is the major isoform expressed, with the exception of the brain, heart, skeletal muscle and testis. The CARM1FL isoform is expressed highest in these tissues. Additionally, the CARM1FL isoform is predominantly auto-methylated in cells.

In breast cancer cells, the CARM1Δ15 was shown to be the predominant isoform expressed (Table 2)[24]. However, only a limited number of cancer cell lines were assessed. It would be interesting to know the expression profile in other cancer types as well. Specifically, an assessment of CARM1 isoform expression in a panel of breast cancer cell lines showed a greater percentage of the CARM1Δ15 isoform compared to the CARM1FL isoform. This is surprising due to the fact that the CARM1Δ15 isoform has impaired ERα co-activator activity and failed to stimulate ERα transcriptional activity. However, it may have distinct roles with respect to activity and functions within cells. The existence of these two isoforms may shed light on some of the conflicting reports in the literature with respect to the biological functions of CARM1 and potential roles in cancer. Further study of these isoforms is required to establish if they are responsible for the methylation of distinct substrates and their individual functions.

PRMT7

PRMT7 was originally identified from a screen of genetic suppressor elements (GSE) aimed at identifying genes conferring resistance to cytotoxic agents performed in Chinese Hamster cells[91]. This screen identified a gene that encoded two proteins, p77 and p82, that were highly homologous to the PRMT family and later designated PRMT7α and β, respectively[27,91]. In Hamster cells, these two isoforms are thought to be generated by the use of distinct 5' translation initiation codons within the primary transcript (Figure 2). The PRMT7β isoform sequence contains an extra 37 amino acids at the N-terminus. Both isoforms were shown to be active and have slightly different methylation profiles[27], though further analysis is required to clarify these differences between the isoforms. Each isoform has a distinct subcellular localization patterns (Table 2). PRMT7α localizes to the cytoplasm and nucleus, whereas PRMT7β is exclusively cytoplasmic[27]. In human tissues, only a single PRMT7 transcript is detected (approximately 3.6 kb) and in two human cell cancer cell lines, HeLa and HuH7, one protein at 78 kDa was detected. This transcript was shown to share the greatest homology to the PRMT7α isoform[25-27]. However, the limited subset of cell lines used cannot completely rule out the existence of PRMT7β isoform expression in human cells and a more comprehensive examination of expression in cells is required. Moreover, a survey within both NCBI and *Ensembl* databases predicts the existence of at least 2 alternatively spliced PRMT7 isoforms that can be produced from the human *PRMT7* gene (Figure 2). These two isoforms have the same N- and C-terminal regions but variant 2 (PRMT7v2) has an in frame deletion of exon 5. Importantly, this may affect methyltransferase activity because it removes the post I domain. Functionally, PRMT7 was initially characterized as a Type Ⅱ methyltransferase[26], but it has recently been deemed a Type Ⅲ and is thus the only PRMT enzyme known to catalyze predominantly this reaction in mammalian cells[4,5]. The generation of monomethylarginine is thought to represent a reaction intermediate for the other PRMTs.

There is limited knowledge into the precise biological functions of PRMT7, however evidence has shown it is linked to cancer. A gene expression analysis of independent data sets of more than 1200 breast tumours identified increased expression in the chromosomal region where the PRMT7 gene is located (16q22)[92]. Importantly, this was also correlated with an increased metastatic potential of breast cancer. The PRMT7 gene locus was also identified in an unbiased genome-wide study to confer resistance to etoposide-induced cytotoxicity in patients[93]. As previously mentioned, PRMT7 was originally identified by a screen for GSEs conferring resistance to cytotoxic agents (etoposide and 9-OH-E)[91]. This study showed that GSE-mediated repression of PRMT7 conferred resistance to topoisomerase Ⅱ inhibitors and also cisplatin. In contrast, in this same study, repression of PRMT7 caused increased sensitivity to other DNA-damaging agents, such as the topoisomerase I inhibitor, camptothecin, as well as UV-irradiation. Increased sensitivity to camptothecin was also observed when PRMT7 was depleted from HeLa cells[94]. Intriguingly, depletion of PRMT7 from NIH 3T3 cells conferred resistance to cisplatin, mytomycin C and chlorambucil[95]. Additionally, one of its only identified interacting protein partners, CTCFL, is a proposed proto-oncogene[96,97]. Further studies are required to identify additional PRMT7 substrates to better understand its role in cells. While these results strongly suggest that PRMT7 may play a key role in several cancer related processes, the opposing functions of PRMT7 in response to cytotoxic agents requires some attention. The reason for these differential effects is unclear; perhaps PRMT7 has distinct functions in different cell types. More interestingly, they could be the result of PRMT7 isoforms specific expression and function within cells.

CONCLUSION

The importance of PRMTs in cancer is only beginning to

be examined. There have been many key discoveries thus far that have demonstrated the potential impact that the PRMTs have in regulating critical effectors and pathways involved in the development and progression of cancer. In fact, the PRMTs have the potential of impacting the majority of the described hallmarks of cancer proposed by Hanahan *et al*[1,98]. Current research efforts aim to identify and characterize the precise mechanistic roles that these PRMTs play in cancer. Importantly, the functional contribution of PRMTs to different cancer types, as well as subtypes within the same cancer, requires further investigation. The significance of this requirement is highlighted by several of the conflicting findings describe in this review.

Here we have highlighted the existence of alternatively spliced PRMT isoforms that have been identified for PRMT1, PRMT2, CARM1 and PRMT7. While not currently realized, more PRMT isoforms for these and other PRMT family members may be present in cells. The presence of distinct PRMT alternative isoforms adds a further level of complexity to this family of enzymes. Additionally, the isoforms identified for PRMT1, PRMT2, CARM1 and PRMT7 have mainly been assessed in breast cancer cells and tissues as indicated in Table 2. A more extensive analysis of their expression in other tumour types has not been performed and could uncover more interesting results with respect to these PRMT isoforms. This fact requires more attention as it may provide possible explanations for the opposing functions identified within cells. Furthermore, while these isoforms may have overlapping functions, it is clear from the data presented here that they also possess distinct functions. Interestingly, while dysregulated PRMT expression has been observed in cancer, no genetic abnormalities have been identified, with one exception being PRMT8[99,100]. While there may be no obvious change at the genome level, a shift in the expression from one alternative PRMT isoform to another may be a crucial event that occurs in cancer cells, thereby affecting development, progression and aggressiveness. Interestingly, a particular PRMT isoform may not be expressed or is expressed at lower levels in normal tissues and as a consequence of the tumourigenic process cancer cells may preferentially upregulate a specific isoform due to its advantageous functions. Understanding both the shared and distinct functions of these alternative PRMT isoforms will not only improve our knowledge of their biological significance but also provide insight into their specific contributions to diseases, such as cancer.

The roles that the PRMTs play in cancer make them an attractive target for the development of drugs that could be used in treatment strategies. This increases the importance of gaining more knowledge about the alternative PRMT isoforms, so that there is a complete understanding of the therapeutic mechanism. This will enable the development of an optimal therapeutic strategy and an improved understanding of the resulting outcomes when targeting PRMT enzymes as a treatment in cancer.

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REFERENCES

1 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]
2 **Bedford MT**, Clarke SG. Protein arginine methylation in mammals: who, what, and why. *Mol Cell* 2009; **33**: 1-13 [PMID: 19150423]
3 **Bedford MT**, Richard S. Arginine methylation an emerging regulator of protein function. *Mol Cell* 2005; **18**: 263-272 [PMID: 15866169 DOI: 10.1016/j.molcel.2005.04.003]
4 **Zurita-Lopez CI**, Sandberg T, Kelly R, Clarke SG. Human protein arginine methyltransferase 7 (PRMT7) is a type III enzyme forming ω-NG-monomethylated arginine residues. *J Biol Chem* 2012; **287**: 7859-7870 [PMID: 22241471 DOI: 10.1074/jbc.M111.336271]
5 **Feng Y**, Maity R, Whitelegge JP, Hadjikyriacou A, Li Z, Zurita-Lopez C, Al-Hadid Q, Clark AT, Bedford MT, Masson JY, Clarke SG. Mammalian protein arginine methyltransferase 7 (PRMT7) specifically targets RXR sites in lysine- and arginine-rich regions. *J Biol Chem* 2013; **288**: 37010-37025 [PMID: 24247247 DOI: 10.1074/jbc.M113.525345]
6 **Yu Z**, Chen T, Hébert J, Li E, Richard S. A mouse PRMT1 null allele defines an essential role for arginine methylation in genome maintenance and cell proliferation. *Mol Cell Biol* 2009; **29**: 2982-2996 [PMID: 19289494 DOI: 10.1128/MCB.00042-09]
7 **Tee WW**, Pardo M, Theunissen TW, Yu L, Choudhary JS, Hajkova P, Surani MA. Prmt5 is essential for early mouse development and acts in the cytoplasm to maintain ES cell pluripotency. *Genes Dev* 2010; **24**: 2772-2777 [PMID: 21159818 DOI: 10.1101/gad.606110]
8 **Boisvert FM**, Chénard CA, Richard S. Protein interfaces in signaling regulated by arginine methylation. *Sci STKE* 2005; **2005**: re2 [PMID: 15713950 DOI: 10.1126/stke.2712005re2]
9 **Guo A**, Gu H, Zhou J, Mulhern D, Wang Y, Lee KA, Yang V, Aguiar M, Kornhauser J, Jia X, Ren J, Beausoleil SA, Silva JC, Vemulapalli V, Bedford MT, Comb MJ. Immunoaffinity enrichment and mass spectrometry analysis of protein methylation. *Mol Cell Proteomics* 2014; **13**: 372-387 [PMID: 24129315 DOI: 10.1074/mcp.O113.027870]
10 **Ong SE**, Mittler G, Mann M. Identifying and quantifying in vivo methylation sites by heavy methyl SILAC. *Nat Methods* 2004; **1**: 119-126 [PMID: 15782174 DOI: 10.1038/nmeth715]
11 **Boisvert FM**, Côté J, Boulanger MC, Richard S. A proteomic analysis of arginine-methylated protein complexes. *Mol Cell Proteomics* 2003; **2**: 1319-1330 [PMID: 14534352 DOI: 10.1074/mcp.M300088-MCP200]
12 **Hong H**, Kao C, Jeng MH, Eble JN, Koch MO, Gardner TA, Zhang S, Li L, Pan CX, Hu Z, MacLennan GT, Cheng L. Aberrant expression of CARM1, a transcriptional coactivator of androgen receptor, in the development of prostate carcinoma and androgen-independent status. *Cancer* 2004; **101**: 83-89 [PMID: 15221992 DOI: 10.1002/cncr.20327]
13 **Cheung N**, Chan LC, Thompson A, Cleary ML, So CW. Protein arginine-methyltransferase-dependent oncogenesis. *Nat Cell Biol* 2007; **9**: 1208-1215 [PMID: 17891136 DOI: 10.1038/ncb1642]
14 **Kim YR**, Lee BK, Park RY, Nguyen NT, Bae JA, Kwon DD, Jung C. Differential CARM1 expression in prostate and colorectal cancers. *BMC Cancer* 2010; **10**: 197 [PMID:

20462455 DOI: 10.1186/1471-2407-10-197]
15 **Mathioudaki K**, Papadokostopoulou A, Scorilas A, Xynopoulos D, Agnanti N, Talieri M. The PRMT1 gene expression pattern in colon cancer. *Br J Cancer* 2008; **99**: 2094-2099 [PMID: 19078953 DOI: 10.1038/sj.bjc.6604807]
16 **Mathioudaki K**, Scorilas A, Ardavanis A, Lymberi P, Tsiambas E, Devetzi M, Apostolaki A, Talieri M. Clinical evaluation of PRMT1 gene expression in breast cancer. *Tumour Biol* 2011; **32**: 575-582 [PMID: 21229402 DOI: 10.1007/s13277-010-0153-2]
17 **Yoshimatsu M**, Toyokawa G, Hayami S, Unoki M, Tsunoda T, Field HI, Kelly JD, Neal DE, Maehara Y, Ponder BA, Nakamura Y, Hamamoto R. Dysregulation of PRMT1 and PRMT6, Type I arginine methyltransferases, is involved in various types of human cancers. *Int J Cancer* 2011; **128**: 562-573 [PMID: 20473859 DOI: 10.1002/Ijc.25366]
18 **Pal S**, Baiocchi RA, Byrd JC, Grever MR, Jacob ST, Sif S. Low levels of miR-92b/96 induce PRMT5 translation and H3R8/H4R3 methylation in mantle cell lymphoma. *EMBO J* 2007; **26**: 3558-3569 [PMID: 17627275 DOI: 10.1038/sj.emboj.7601794]
19 **Papadokostopoulou A**, Mathioudaki K, Scorilas A, Xynopoulos D, Ardavanis A, Kouroumalis E, Talieri M. Colon cancer and protein arginine methyltransferase 1 gene expression. *Anticancer Res* 2009; **29**: 1361-1366 [PMID: 19414388]
20 **Pacold ME**, Suire S, Perisic O, Lara-Gonzalez S, Davis CT, Walker EH, Hawkins PT, Stephens L, Eccleston JF, Williams RL. Crystal structure and functional analysis of Ras binding to its effector phosphoinositide 3-kinase gamma. *Cell* 2000; **103**: 931-943 [PMID: 11136978]
21 **Goulet I**, Gauvin G, Boisvenue S, Côté J. Alternative splicing yields protein arginine methyltransferase 1 isoforms with distinct activity, substrate specificity, and subcellular localization. *J Biol Chem* 2007; **282**: 33009-33021 [PMID: 17848568 DOI: 10.1074/jbc.M704349200]
22 **Zhong J**, Cao RX, Zu XY, Hong T, Yang J, Liu L, Xiao XH, Ding WJ, Zhao Q, Liu JH, Wen GB. Identification and characterization of novel spliced variants of PRMT2 in breast carcinoma. *FEBS J* 2012; **279**: 316-335 [PMID: 22093364 DOI: 10.1111/j.1742-4658.2011.08426.x]
23 **Ohkura N**, Takahashi M, Yaguchi H, Nagamura Y, Tsukada T. Coactivator-associated arginine methyltransferase 1, CARM1, affects pre-mRNA splicing in an isoform-specific manner. *J Biol Chem* 2005; **280**: 28927-28935 [PMID: 15944154 DOI: 10.1074/jbc.M502173200]
24 **Wang L**, Charoensuksai P, Watson NJ, Wang X, Zhao Z, Coriano CG, Kerr LR, Xu W. CARM1 automethylation is controlled at the level of alternative splicing. *Nucleic Acids Res* 2013; **41**: 6870-6880 [PMID: 23723242 DOI: 10.1093/nar/gkt415]
25 **Miranda TB**, Miranda M, Frankel A, Clarke S. PRMT7 is a member of the protein arginine methyltransferase family with a distinct substrate specificity. *J Biol Chem* 2004; **279**: 22902-22907 [PMID: 15044439 DOI: 10.1074/Jbc.M312904200]
26 **Lee JH**, Cook JR, Yang ZH, Mirochnitchenko O, Gunderson SI, Felix AM, Herth N, Hoffmann R, Pestka S. PRMT7, a new protein arginine methyltransferase that synthesizes symmetric dimethylarginine. *J Biol Chem* 2005; **280**: 3656-3664 [PMID: 15494416 DOI: 10.1074/Jbc.M405295200]
27 **Gros L**, Renodon-Cornière A, de Saint Vincent BR, Feder M, Bujnicki JM, Jacquemin-Sablon A. Characterization of prmt7alpha and beta isozymes from Chinese hamster cells sensitive and resistant to topoisomerase II inhibitors. *Biochim Biophys Acta* 2006; **1760**: 1646-1656 [PMID: 17049166 DOI: 10.1016/j.bbagen.2006.08.026]
28 **Cha B**, Jho EH. Protein arginine methyltransferases (PRMTs) as therapeutic targets. *Expert Opin Ther Targets* 2012; **16**: 651-664 [PMID: 22621686 DOI: 10.1517/14728222.2012.688030]
29 **Tang J**, Frankel A, Cook RJ, Kim S, Paik WK, Williams KR, Clarke S, Herschman HR. PRMT1 is the predominant type I protein arginine methyltransferase in mammalian cells. *J Biol Chem* 2000; **275**: 7723-7730 [PMID: 10713084]
30 **Scott HS**, Antonarakis SE, Lalioti MD, Rossier C, Silver PA, Henry MF. Identification and characterization of two putative human arginine methyltransferases (HRMT1L1 and HRMT1L2). *Genomics* 1998; **48**: 330-340 [PMID: 9545638 DOI: 10.1006/geno.1997.5190]
31 **Scorilas A**, Black MH, Talieri M, Diamandis EP. Genomic organization, physical mapping, and expression analysis of the human protein arginine methyltransferase 1 gene. *Biochem Biophys Res Commun* 2000; **278**: 349-359 [PMID: 11097842 DOI: 10.1006/bbrc.2000.3807]
32 **Herrmann F**, Pably P, Eckerich C, Bedford MT, Fackelmayer FO. Human protein arginine methyltransferases in vivo--distinct properties of eight canonical members of the PRMT family. *J Cell Sci* 2009; **122**: 667-677 [PMID: 19208762 DOI: 10.1242/Jcs.039933]
33 **Herrmann F**, Lee J, Bedford MT, Fackelmayer FO. Dynamics of human protein arginine methyltransferase 1(PRMT1) in vivo. *J Biol Chem* 2005; **280**: 38005-38010 [PMID: 16159886]
34 **Ong SE**, Mann M. A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat Protoc* 2006; **1**: 2650-2660 [PMID: 17406521]
35 **Trinkle-Mulcahy L**, Andersen J, Lam YW, Moorhead G, Mann M, Lamond AI. Repo-Man recruits PP1 gamma to chromatin and is essential for cell viability. *J Cell Biol* 2006; **172**: 679-692 [PMID: 16492807 DOI: 10.1083/jcb.200508154]
36 **Guendel I**, Carpio L, Pedati C, Schwartz A, Teal C, Kashanchi F, Kehn-Hall K. Methylation of the tumor suppressor protein, BRCA1, influences its transcriptional cofactor function. *PLoS One* 2010; **5**: e11379 [PMID: 20614009 DOI: 10.1371/journal.pone.0011379]
37 **Le Romancer M**, Treilleux I, Leconte N, Robin-Lespinasse Y, Sentis S, Bouchekioua-Bouzaghou K, Goddard S, Gobert-Gosse S, Corbo L. Regulation of estrogen rapid signaling through arginine methylation by PRMT1. *Mol Cell* 2008; **31**: 212-221 [PMID: 18657504 DOI: 10.1016/j.molcel.2008.05.025]
38 **Shia WJ**, Okumura AJ, Yan M, Sarkeshik A, Lo MC, Matsuura S, Komeno Y, Zhao X, Nimer SD, Yates JR, Zhang DE. PRMT1 interacts with AML1-ETO to promote its transcriptional activation and progenitor cell proliferative potential. *Blood* 2012; **119**: 4953-4962 [PMID: 22498736 DOI: 10.1182/blood-2011-04-347476]
39 **Herrmann F**, Fackelmayer FO. Nucleo-cytoplasmic shuttling of protein arginine methyltransferase 1 (PRMT1) requires enzymatic activity. *Genes Cells* 2009; **14**: 309-317 [PMID: 19170758 DOI: 10.1111/j.1365-2443.2008.01266.x]
40 **Baldwin RM**, Morettin A, Paris G, Goulet I, Côté J. Alternatively spliced protein arginine methyltransferase 1 isoform PRMT1v2 promotes the survival and invasiveness of breast cancer cells. *Cell Cycle* 2012; **11**: 4597-4612 [PMID: 23187807 DOI: 10.4161/cc.22871]
41 **Strahl BD**, Briggs SD, Brame CJ, Caldwell JA, Koh SS, Ma H, Cook RG, Shabanowitz J, Hunt DF, Stallcup MR, Allis CD. Methylation of histone H4 at arginine 3 occurs in vivo and is mediated by the nuclear receptor coactivator PRMT1. *Curr Biol* 2001; **11**: 996-1000 [PMID: 11448779]
42 **Wang H**, Huang ZQ, Xia L, Feng Q, Erdjument-Bromage H, Strahl BD, Briggs SD, Allis CD, Wong J, Tempst P, Zhang Y. Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science* 2001; **293**: 853-857 [PMID: 11387442 DOI: 10.1126/science.1060781]
43 **Seligson DB**, Horvath S, Shi T, Yu H, Tze S, Grunstein M, Kurdistani SK. Global histone modification patterns predict risk of prostate cancer recurrence. *Nature* 2005; **435**: 1262-1266 [PMID: 15988529 DOI: 10.1038/nature03672]
44 **Pahlich S**, Zakaryan RP, Gehring H. Protein arginine methylation: Cellular functions and methods of analysis. *Biochim Biophys Acta* 2006; **1764**: 1890-1903 [PMID: 17010682 DOI: 10.1016/j.bbapap.2006.08.008]
45 **Poulard C**, Treilleux I, Lavergne E, Bouchekioua-Bouzaghou

K, Goddard-Léon S, Chabaud S, Trédan O, Corbo L, Le Romancer M. Activation of rapid oestrogen signalling in aggressive human breast cancers. *EMBO Mol Med* 2012; **4**: 1200-1213 [PMID: 23065768 DOI: 10.1002/emmm.201201615]

46 **Hernandez-Aya LF**, Gonzalez-Angulo AM. Targeting the phosphatidylinositol 3-kinase signaling pathway in breast cancer. *Oncologist* 2011; **16**: 404-414 [PMID: 21406469 DOI: 10.1634/theoncologist.2010-0402]

47 **Navarro-Tito N**, Robledo T, Salazar EP. Arachidonic acid promotes FAK activation and migration in MDA-MB-231 breast cancer cells. *Exp Cell Res* 2008; **314**: 3340-3355 [PMID: 18804105 DOI: 10.1016/j.yexcr.2008.08.018]

48 **Zheng S**, Huang J, Zhou K, Zhang C, Xiang Q, Tan Z, Wang T, Fu X. 17β-Estradiol enhances breast cancer cell motility and invasion via extra-nuclear activation of actin-binding protein ezrin. *PLoS One* 2011; **6**: e22439 [PMID: 21818323 DOI: 10.1371/journal.pone.0022439]

49 **Ohgaki H**, Kleihues P. Genetic pathways to primary and secondary glioblastoma. *Am J Pathol* 2007; **170**: 1445-1453 [PMID: 17456751 DOI: 10.2353/ajpath.2007.070011]

50 **Weber GL**, Parat MO, Binder ZA, Gallia GL, Riggins GJ. Abrogation of PIK3CA or PIK3R1 reduces proliferation, migration, and invasion in glioblastoma multiforme cells. *Oncotarget* 2011; **2**: 833-849 [PMID: 22064833]

51 **Xu J**, Wang AH, Oses-Prieto J, Makhijani K, Katsuno Y, Pei M, Yan L, Zheng YG, Burlingame A, Brückner K, Derynck R. Arginine Methylation Initiates BMP-Induced Smad Signaling. *Mol Cell* 2013; **51**: 5-19 [PMID: 23747011 DOI: 10.1016/j.molcel.2013.05.004]

52 **ten Dijke P**, Arthur HM. Extracellular control of TGFbeta signalling in vascular development and disease. *Nat Rev Mol Cell Biol* 2007; **8**: 857-869 [PMID: 17895899 DOI: 10.1038/nrm2262]

53 **Blanco Calvo M**, Bolós Fernández V, Medina Villaamil V, Aparicio Gallego G, Díaz Prado S, Grande Pulido E. Biology of BMP signalling and cancer. *Clin Transl Oncol* 2009; **11**: 126-137 [PMID: 19293049]

54 **Lee J**, Sayegh J, Daniel J, Clarke S, Bedford MT. PRMT8, a new membrane-bound tissue-specific member of the protein arginine methyltransferase family. *J Biol Chem* 2005; **280**: 32890-32896 [PMID: 16051612 DOI: 10.1074/jbc.M506944200]

55 **Cho JH**, Lee MK, Yoon KW, Lee J, Cho SG, Choi EJ. Arginine methylation-dependent regulation of ASK1 signaling by PRMT1. *Cell Death Differ* 2012; **19**: 859-870 [PMID: 22095282]

56 **Sakamaki J**, Daitoku H, Ueno K, Hagiwara A, Yamagata K, Fukamizu A. Arginine methylation of BCL-2 antagonist of cell death (BAD) counteracts its phosphorylation and inactivation by Akt. *Proc Natl Acad Sci USA* 2011; **108**: 6085-6090 [PMID: 21444773 DOI: 10.1073/pnas.1015328108]

57 **Cha B**, Kim W, Kim YK, Hwang BN, Park SY, Yoon JW, Park WS, Cho JW, Bedford MT, Jho EH. Methylation by protein arginine methyltransferase 1 increases stability of Axin, a negative regulator of Wnt signaling. *Oncogene* 2011; **30**: 2379-2389 [PMID: 21242974 DOI: 10.1038/onc.2010.610]

58 **Yonemura S**. Cadherin-actin interactions at adherens junctions. *Curr Opin Cell Biol* 2011; **23**: 515-522 [PMID: 21807490 DOI: 10.1016/J.Ceb.2011.07.001]

59 **Yamagata K**, Daitoku H, Takahashi Y, Namiki K, Hisatake K, Kako K, Mukai H, Kasuya Y, Fukamizu A. Arginine methylation of FOXO transcription factors inhibits their phosphorylation by Akt. *Mol Cell* 2008; **32**: 221-231 [PMID: 18951090 DOI: 10.1016/j.molcel.2008.09.013]

60 **Mitchell TR**, Glenfield K, Jeyanthan K, Zhu XD. Arginine methylation regulates telomere length and stability. *Mol Cell Biol* 2009; **29**: 4918-4934 [PMID: 19596784 DOI: 10.1128/MCB.00009-09]

61 **Boisvert FM**, Déry U, Masson JY, Richard S. Arginine methylation of MRE11 by PRMT1 is required for DNA damage checkpoint control. *Genes Dev* 2005; **19**: 671-676 [PMID: 15741314 DOI: 10.1101/Gad.1279805]

62 **Yu Z**, Vogel G, Coulombe Y, Dubeau D, Spehalski E, Hébert J, Ferguson DO, Masson JY, Richard S. The MRE11 GAR motif regulates DNA double-strand break processing and ATR activation. *Cell Res* 2012; **22**: 305-320 [PMID: 21826105 DOI: 10.1038/cr.2011.128]

63 **Boisvert FM**, Rhie A, Richard S, Doherty AJ. The GAR motif of 53BP1 is arginine methylated by PRMT1 and is necessary for 53BP1 DNA binding activity. *Cell Cycle* 2005; **4**: 1834-1841 [PMID: 16294045 DOI: 10.4161/Cc.4.12.2250]

64 **Lakowski TM**, Frankel A. Kinetic analysis of human protein arginine N-methyltransferase 2: formation of monomethyl- and asymmetric dimethyl-arginine residues on histone H4. *Biochem J* 2009; **421**: 253-261 [PMID: 19405910 DOI: 10.1042/BJ20090268]

65 **Blythe SA**, Cha SW, Tadjuidje E, Heasman J, Klein PS. beta-Catenin primes organizer gene expression by recruiting a histone H3 arginine 8 methyltransferase, Prmt2. *Dev Cell* 2010; **19**: 220-231 [PMID: 20708585 DOI: 10.1016/j.devcel.2010.07.007]

66 **Qi C**, Chang J, Zhu Y, Yeldandi AV, Rao SM, Zhu YJ. Identification of protein arginine methyltransferase 2 as a coactivator for estrogen receptor alpha. *J Biol Chem* 2002; **277**: 28624-28630 [PMID: 12039952 DOI: 10.1074/jbc.M201053200]

67 **Tan H**, Zhong Y, Pan Z. Autocrine regulation of cell proliferation by estrogen receptor-alpha in estrogen receptor-alpha-positive breast cancer cell lines. *BMC Cancer* 2009; **9**: 31 [PMID: 19171042 DOI: 10.1186/1471-2407-9-31]

68 **Faivre EJ**, Lange CA. Progesterone receptors upregulate Wnt-1 to induce epidermal growth factor receptor transactivation and c-Src-dependent sustained activation of Erk1/2 mitogen-activated protein kinase in breast cancer cells. *Mol Cell Biol* 2007; **27**: 466-480 [PMID: 17074804 DOI: 10.1128/MCB.01539-06]

69 **Heinlein CA**, Chang C. Androgen receptor in prostate cancer. *Endocr Rev* 2004; **25**: 276-308 [PMID: 15082523]

70 **Kersten S**, Desvergne B, Wahli W. Roles of PPARs in health and disease. *Nature* 2000; **405**: 421-424 [PMID: 10839530 DOI: 10.1038/35013000]

71 **Soprano KJ**, Soprano DR. Retinoic acid receptors and cancer. *J Nutr* 2002; **132**: 3809S-3813S [PMID: 12468629]

72 **Zhong J**, Cao RX, Hong T, Yang J, Zu XY, Xiao XH, Liu JH, Wen GB. Identification and expression analysis of a novel transcript of the human PRMT2 gene resulted from alternative polyadenylation in breast cancer. *Gene* 2011; **487**: 1-9 [PMID: 21820040 DOI: 10.1016/j.gene.2011.06.022]

73 **Zhang X**, Zhou L, Cheng X. Crystal structure of the conserved core of protein arginine methyltransferase PRMT3. *EMBO J* 2000; **19**: 3509-3519 [PMID: 10899106 DOI: 10.1093/emboj/19.14.3509]

74 **Barrallo-Gimeno A**, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* 2005; **132**: 3151-3161 [PMID: 15983400 DOI: 10.1242/dev.01907]

75 **Yoshimoto T**, Boehm M, Olive M, Crook MF, San H, Langenickel T, Nabel EG. The arginine methyltransferase PRMT2 binds RB and regulates E2F function. *Exp Cell Res* 2006; **312**: 2040-2053 [PMID: 16616919 DOI: 10.1016/j.yexcr.2006.03.001]

76 **Chen D**, Ma H, Hong H, Koh SS, Huang SM, Schurter BT, Aswad DW, Stallcup MR. Regulation of transcription by a protein methyltransferase. *Science* 1999; **284**: 2174-2177 [PMID: 10381882]

77 **Frietze S**, Lupien M, Silver PA, Brown M. CARM1 regulates estrogen-stimulated breast cancer growth through up-regulation of E2F1. *Cancer Res* 2008; **68**: 301-306 [PMID: 18172323 DOI: 10.1158/0008-5472.Can-07-1983]

78 **El Messaoudi S**, Fabbrizio E, Rodriguez C, Chuchana P, Fauquier L, Cheng D, Theillet C, Vandel L, Bedford MT, Sardet C. Coactivator-associated arginine methyltransferase 1 (CARM1) is a positive regulator of the Cyclin E1 gene. *Proc*

Natl Acad Sci USA 2006; **103**: 13351-13356 [PMID: 16938873 DOI: 10.1073/pnas.0605692103]
79 **Al-Dhaheri M**, Wu J, Skliris GP, Li J, Higashimato K, Wang Y, White KP, Lambert P, Zhu Y, Murphy L, Xu W. CARM1 is an important determinant of ERα-dependent breast cancer cell differentiation and proliferation in breast cancer cells. *Cancer Res* 2011; **71**: 2118-2128 [PMID: 21282336 DOI: 10.1158/0008-5472.CAN-10-2426]
80 **Yadav N**, Lee J, Kim J, Shen J, Hu MC, Aldaz CM, Bedford MT. Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice. *Proc Natl Acad Sci USA* 2003; **100**: 6464-6468 [PMID: 12756295 DOI: 10.1073/pnas.1232272100]
81 **Kuhn P**, Xu W. Protein arginine methyltransferases: nuclear receptor coregulators and beyond. *Prog Mol Biol Transl Sci* 2009; **87**: 299-342 [PMID: 20374708 DOI: 10.1016/S1877-1173(09)87009-9]
82 **Higashimoto K**, Kuhn P, Desai D, Cheng X, Xu W. Phosphorylation-mediated inactivation of coactivator-associated arginine methyltransferase 1. *Proc Natl Acad Sci USA* 2007; **104**: 12318-12323 [PMID: 17640894 DOI: 10.1073/pnas.0610792104]
83 **Feng Q**, He B, Jung SY, Song Y, Qin J, Tsai SY, Tsai MJ, O' Malley BW. Biochemical control of CARM1 enzymatic activity by phosphorylation. *J Biol Chem* 2009; **284**: 36167-36174 [PMID: 19843527 DOI: 10.1074/jbc.M109.065524]
84 **Carascossa S**, Dudek P, Cenni B, Briand PA, Picard D. CARM1 mediates the ligand-independent and tamoxifen-resistant activation of the estrogen receptor alpha by cAMP. *Genes Dev* 2010; **24**: 708-719 [PMID: 20360387 DOI: 10.1101/gad.568410]
85 **Kuhn P**, Chumanov R, Wang Y, Ge Y, Burgess RR, Xu W. Automethylation of CARM1 allows coupling of transcription and mRNA splicing. *Nucleic Acids Res* 2011; **39**: 2717-2726 [PMID: 21138967 DOI: 10.1093/nar/gkq1246]
86 **Cheng H**, Qin Y, Fan H, Su P, Zhang X, Zhang H, Zhou G. Overexpression of CARM1 in breast cancer is correlated with poorly characterized clinicopathologic parameters and molecular subtypes. *Diagn Pathol* 2013; **8**: 129 [PMID: 23915145 DOI: 10.1186/1746-1596-8-129]
87 **Teyssier C**, Le Romancer M, Sentis S, Jalaguier S, Corbo L, Cavaillès V. Protein arginine methylation in estrogen signaling and estrogen-related cancers. *Trends Endocrinol Metab* 2010; **21**: 181-189 [PMID: 20005732 DOI: 10.1016/j.tem.2009.11.002]
88 **Majumder S**, Liu Y, Ford OH, Mohler JL, Whang YE. Involvement of arginine methyltransferase CARM1 in androgen receptor function and prostate cancer cell viability. *Prostate* 2006; **66**: 1292-1301 [PMID: 16705743 DOI: 10.1002/pros.20438]
89 **Wang L**, Zhao Z, Meyer MB, Saha S, Yu M, Guo A, Wisinski KB, Huang W, Cai W, Pike JW, Yuan M, Ahlquist P, Xu W. CARM1 methylates chromatin remodeling factor BAF155 to enhance tumor progression and metastasis. *Cancer Cell* 2014; **25**: 21-36 [PMID: 24434208 DOI: 10.1016/j.ccr.2013.12.007]
90 **Cheng D**, Côté J, Shaaban S, Bedford MT. The arginine methyltransferase CARM1 regulates the coupling of transcription and mRNA processing. *Mol Cell* 2007; **25**: 71-83 [PMID: 17218272 DOI: 10.1016/j.molcel.2006.11.019]
91 **Gros L**, Delaporte C, Frey S, Decesse J, de Saint-Vincent BR, Cavarec L, Dubart A, Gudkov AV, Jacquemin-Sablon A. Identification of new drug sensitivity genes using genetic suppressor elements: protein arginine N-methyltransferase mediates cell sensitivity to DNA-damaging agents. *Cancer Res* 2003; **63**: 164-171 [PMID: 12517794]
92 **Thomassen M**, Tan Q, Kruse TA. Gene expression meta-analysis identifies chromosomal regions and candidate genes involved in breast cancer metastasis. *Breast Cancer Res Treat* 2009; **113**: 239-249 [PMID: 18293085 DOI: 10.1007/s10549-008-9927-2]
93 **Bleibel WK**, Duan S, Huang RS, Kistner EO, Shukla SJ, Wu X, Badner JA, Dolan ME. Identification of genomic regions contributing to etoposide-induced cytotoxicity. *Hum Genet* 2009; **125**: 173-180 [PMID: 19089452 DOI: 10.1007/s00439-008-0607-4]
94 **Verbiest V**, Montaudon D, Tautu MT, Moukarzel J, Portail JP, Markovits J, Robert J, Ichas F, Pourquier P. Protein arginine (N)-methyl transferase 7 (PRMT7) as a potential target for the sensitization of tumor cells to camptothecins. *FEBS Lett* 2008; **582**: 1483-1489 [PMID: 18381071 DOI: 10.1016/J.Febslet.2008.03.031]
95 **Karkhanis V**, Wang L, Tae S, Hu YJ, Imbalzano AN, Sif S. Protein arginine methyltransferase 7 regulates cellular response to DNA damage by methylating promoter histones H2A and H4 of the polymerase δ catalytic subunit gene, POLD1. *J Biol Chem* 2012; **287**: 29801-29814 [PMID: 22761421 DOI: 10.1074/jbc.M112.378281]
96 **Martin-Kleiner I**. BORIS in human cancers -- a review. *Eur J Cancer* 2012; **48**: 929-935 [PMID: 22019212 DOI: 10.1016/j.ejca.2011.09.009]
97 **Jelinic P**, Stehle JC, Shaw P. The testis-specific factor CTCFL cooperates with the protein methyltransferase PRMT7 in H19 imprinting control region methylation. *PLoS Biol* 2006; **4**: e355 [PMID: 17048991 DOI: 10.1371/journal.pbio.0040355]
98 **Hanahan D**, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57-70 [PMID: 10647931]
99 **Forbes SA**, Bindal N, Bamford S, Cole C, Kok CY, Beare D, Jia M, Shepherd R, Leung K, Menzies A, Teague JW, Campbell PJ, Stratton MR, Futreal PA. COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer. *Nucleic Acids Res* 2011; **39**: D945-D950 [PMID: 20952405 DOI: 10.1093/nar/gkq929]
100 **Yang Y**, Bedford MT. Protein arginine methyltransferases and cancer. *Nat Rev Cancer* 2013; **13**: 37-50 [PMID: 23235912 DOI: 10.1038/nrc3409]
101 **Singh V**, Miranda TB, Jiang W, Frankel A, Roemer ME, Robb VA, Gutmann DH, Herschman HR, Clarke S, Newsham IF. DAL-1/4.1B tumor suppressor interacts with protein arginine N-methyltransferase 3 (PRMT3) and inhibits its ability to methylate substrates in vitro and in vivo. *Oncogene* 2004; **23**: 7761-7771 [PMID: 15334060 DOI: 10.1038/sj.onc.1208057]
102 **Jiang W**, Newsham IF. The tumor suppressor DAL-1/4.1B and protein methylation cooperate in inducing apoptosis in MCF-7 breast cancer cells. *Mol Cancer* 2006; **5**: 4 [PMID: 16420693 DOI: 10.1186/1476-4598-5-4]
103 **Gu Z**, Gao S, Zhang F, Wang Z, Ma W, Davis RE, Wang Z. Protein arginine methyltransferase 5 is essential for growth of lung cancer cells. *Biochem J* 2012; **446**: 235-241 [PMID: 22708516 DOI: 10.1042/BJ20120768]
104 **Wang L**, Pal S, Sif S. Protein arginine methyltransferase 5 suppresses the transcription of the RB family of tumor suppressors in leukemia and lymphoma cells. *Mol Cell Biol* 2008; **28**: 6262-6277 [PMID: 18694959 DOI: 10.1128/MCB.00923-08]
105 **Nicholas C**, Yang J, Peters SB, Bill MA, Baiocchi RA, Yan F, Sif S, Tae S, Gaudio E, Wu X, Grever MR, Young GS, Lesinski GB. PRMT5 is upregulated in malignant and metastatic melanoma and regulates expression of MITF and p27(Kip1.). *PLoS One* 2013; **8**: e74710 [PMID: 24098663 DOI: 10.1371/journal.pone.0074710]
106 **Kim JM**, Sohn HY, Yoon SY, Oh JH, Yang JO, Kim JH, Song KS, Rho SM, Yoo HS, Kim YS, Kim JG, Kim NS. Identification of gastric cancer-related genes using a cDNA microarray containing novel expressed sequence tags expressed in gastric cancer cells. *Clin Cancer Res* 2005; **11**: 473-482 [PMID: 15701830]
107 **Cho EC**, Zheng S, Munro S, Liu G, Carr SM, Moehlenbrink J, Lu YC, Stimson L, Khan O, Konietzny R, McGouran J, Coutts AS, Kessler B, Kerr DJ, Thangue NB. Arginine methylation controls growth regulation by E2F-1. *EMBO J* 2012; **31**: 1785-1797 [PMID: 22327218 DOI: 10.1038/emboj.2012.17]

108 **Jansson M**, Durant ST, Cho EC, Sheahan S, Edelmann M, Kessler B, La Thangue NB. Arginine methylation regulates the p53 response. *Nat Cell Biol* 2008; **10**: 1431-1439 [PMID: 19011621 DOI: 10.1038/ncb1802]
109 **Durant ST**, Cho EC, La Thangue NB. p53 methylation--the Arg-ument is clear. *Cell Cycle* 2009; **8**: 801-802 [PMID: 19221494]
110 **Stein C**, Riedl S, Rüthnick D, Nötzold RR, Bauer UM. The arginine methyltransferase PRMT6 regulates cell proliferation and senescence through transcriptional repression of tumor suppressor genes. *Nucleic Acids Res* 2012; **40**: 9522-9533 [PMID: 22904088 DOI: 10.1093/nar/gks767]

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REVIEW

What have we learned about the kallikrein-kinin and renin-angiotensin systems in neurological disorders?

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Abstract

The kallikrein-kinin system (KKS) is an intricate endogenous pathway involved in several physiological and pathological cascades in the brain. Due to the pathological effects of kinins in blood vessels and tissues, their formation and degradation are tightly controlled. Their components have been related to several central nervous system diseases such as stroke, Alzheimer's disease, Parkinson's disease, multiple sclerosis, epilepsy and others. Bradykinin and its receptors (B1R and B2R) may have a role in the pathophysiology of certain central nervous system diseases. It has been suggested that kinin B1R is up-regulated in pathological conditions and has a neurodegenerative pattern, while kinin B2R is constitutive and can act as a neuroprotective factor in many neurological conditions. The renin angiotensin system (RAS) is an important blood pressure regulator and controls both sodium and water intake. Ang Ⅱ is a potent vasoconstrictor molecule and angiotensin converting enzyme is the major enzyme responsible for its release. Ang Ⅱ acts mainly on the AT1 receptor, with involvement in several systemic and neurological disorders. Brain RAS has been associated with physiological pathways, but is also associated with brain disorders. This review describes topics relating to the involvement of both systems in several forms of brain dysfunction and indicates components of the KKS and RAS that have been used as targets in several pharmacological approaches.

Key words: Kallikrein-kinin system; Renin-angiotensin system; Neurological disorders; Alzheimer's disease; Epilepsy; Parkinson's disease

Core tip: This review is a description of the involvement of the kallikrein-kinin and renin-angiotensin systems in neurological disorders. We describe all components of both systems, relating them to several brain diseases such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, blood brain barrier disruption, stroke and inflammation, including the involvement of each molecule, their receptor and specific enzymes in individual pathologies. We also show that brain homeostasis depends on a dynamic balance between the kallikrein-kinin and renin-angiotensin systems.

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KALLIKREIN-KININ SYSTEM IN NEUROLOGICAL DISORDERS

Components of the kallikrein-kinin system

The kallikrein-kinin system (KKS) is an intricate endogenous pathway involved in blood pressure regulation, inflammation, cardiovascular homeostasis, analgesic responses, pain-transmitting mechanisms, cytokines release, prostacyclin, nitric oxide and cell proliferation[1,2].

Initial studies on the importance of the KKS in mammals were performed at the beginning of the last century, when Abelous *et al*[3] verified that human urine injected into dogs induced a reduction in blood pressure. After that, several authors identified a great number of molecules, with biological activity, involved in this bioactive cascade[4-8]. Thus, since 1900 to date, all components of the KKS were sequentially identified in plasma and/or in tissue as part of a complex enzymatic process linked to several biological and pathological events.

Due to the effects of kinins in blood vessels and tissues, their formation and degradation are tightly controlled. In plasma, the coagulation factor XII (Hageman factor XII) is activated to XIIa by the negative surface and is then able to cleave prekallikrein into the active form of kallikrein. This latter enzyme hydrolyzes high molecular weight kininogen and releases bradykinin (BK) into the circulation, which is an important vasoactive nonapeptide (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9). After C-terminal arginine removal, by circulating and/or tissue kininases, BK is converted into Des-Arg^9BK, another potent peptide or to inactive peptides. BK has high affinity for the constitutive kinin B2 receptors (B2R), while Des-Arg^9BK shows preference for binding to inductive kinin B1 receptors (B1R)[8].

In tissues, prekallikrein is also converted into kallikrein, which hydrolyzes the low molecular weight kininogen, releasing Lys-BK, also known as kallidin. After the action of tissue kininases, Lys-Bk is converted into BK or Des-Arg^{10}-Lys-BK, which also have high affinity for B1R, while its precursor (kallidin) shows more affinity for B2R (Figure 1). All these enzymes involved in the KKS are serine-proteases. Plasma kallikrein and tissue kallikrein 1 (KK1) are the main enzymes involved in kinin release in blood and tissue, respectively.

KKS in the central nervous system

All components of the KKS have been localized in the cerebral cortex, brain stem, cerebellum, hypothalamus, hippocampus, and pineal gland, among others. They are found surrounding blood vessels, in neurons and glial cells[9-12]. Kinins are able to stimulate the production and release of inflammatory mediators such as eicosanoids, cytokines, nitric oxide (NO) and free radicals. Kinins also induce the release of excitatory amino acids, increasing intracellular (Ca^{2+})i levels and inducing brain excitotoxicity. These peptides are also involved in disruption of the blood-brain-barrier (BBB) and dilation of the parenchyma of cerebral arteries causing edema[13-15]. The mitogen-activated protein kinase pathway, which culminates in the transcription of many genes involved in later responses[16] is also activated by B1R. Stimulation of both B1R and B2R leads to classical G-protein activation with the generation of different second messengers (Figure 1).

In addition, plasma and tissue enzymes, other serinoproteases, similar to chymo/trypsin-like proteases, have been described and they are also known as kallikreins (KK1 to KK15). According to Sotiropoulou *et al*[17], this family of 15 enzymes has been related to diseases such as hypertension, renal dysfunction, inflammation, neurodegeneration and several types of cancer[18].

The KKS influences multiple players in the immune system acting on targets such as macrophages, dendritic cells, T and B lymphocytes modulating the activation, proliferation, migration and the effector function of these cells[19]. Thus, kallikreins have been associated with several pathologies, supporting new insights related to the KKS, which could be useful as targets in the treatment of pathological conditions.

KKS in inflammation

In neurodegenerative disorders, inflammation is considering a primary response to injury or to infection, repairing and healing the injured tissue[20]. Vascular permeability and blood flow increases in the first stage of inflammation and substances produced by mast cells and by platelets such as histamine, BK, leukotrienes, prostaglandins and serotonin are released during the initial inflammation process[20]. Blood vessel walls change their permeability allowing the entry of proteins and small molecules, which are important to the recruitment of defense cells. At this stage, leukocytes, adhesion molecules, cytokines and chemotactic factors are recruited to the injured site. Indeed, the release of BK may participate in this process and several authors have studied KKS targets to improve the delivery of drugs through the blood-tumor barrier[21-23].

KKS and cerebrovascular alterations

According to Kung *et al*[24], patients with traumatic brain injury, subarachnoid hemorrhage, intracerebral hemorrhage and ischemic stroke have increased BK levels in CSF and these high levels correlate with the intensity of edema formation. In addition, patients with aneurysmal subarachnoid hemorrhage have low levels of serum KK6 and KK6 levels in blood could predict early complications of this disease. Thus, Martinez-Morillo *et al*[25] suggested that KK6 could be a useful prognostic marker in this pathological condition. Similarly, cerebral hematoma expansion induced by hyperglycemia is mediated by plasma KK[26].

Kininogen-deficient mice show less severe BBB damage, edema and inflammation formation after thrombosis and ischemic stroke. According to some authors, kininogen deficiency is able to reduce thrombosis after stroke, without increasing the risk of intracerebral hemorrhage. In the absence of kininogen, mice are completely unable to produce BK. This lack of kininogen underlies

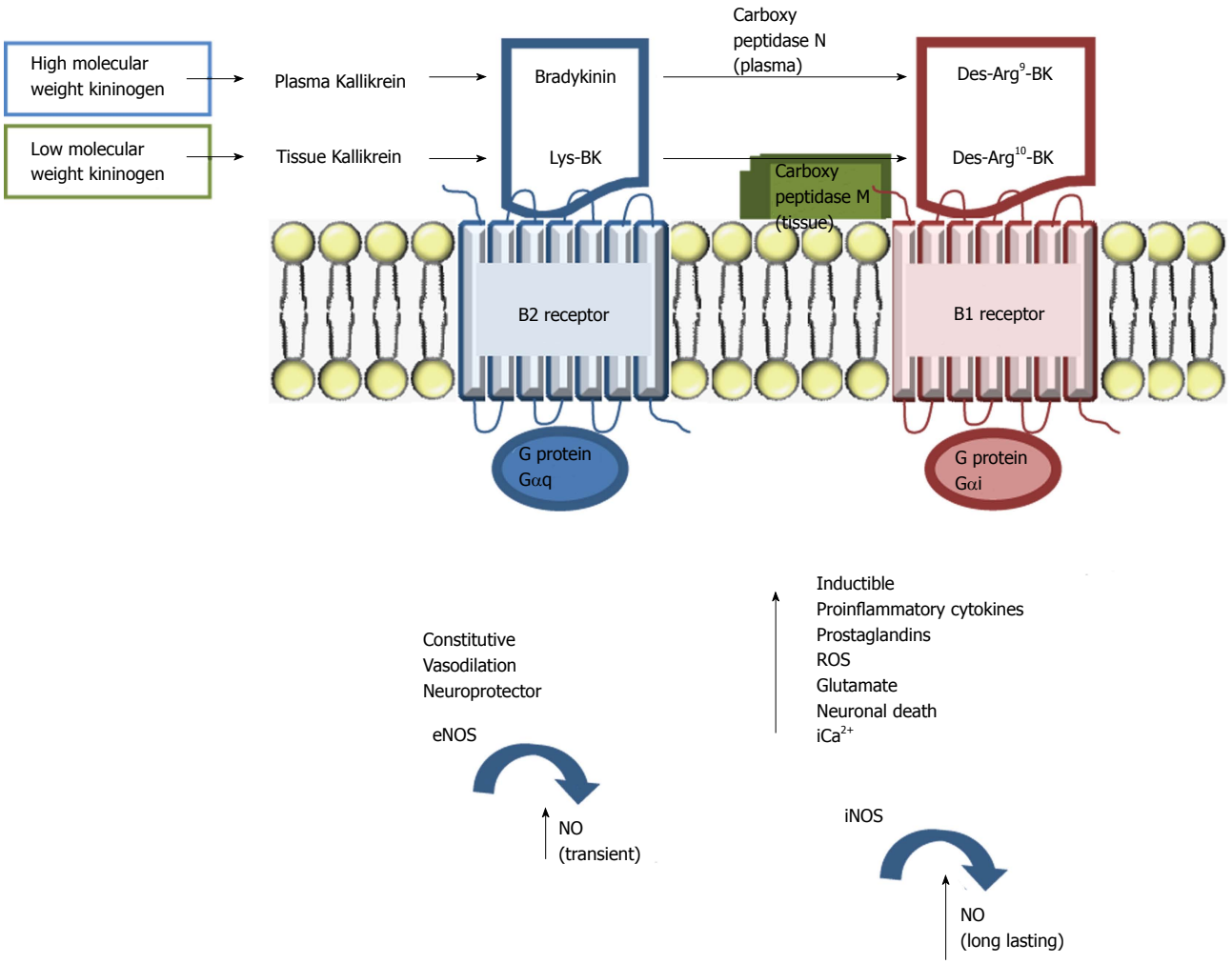

Figure 1 Schematic representation of the kallikrein-kinin system. Bradykinin and Lys-bradykinin (BK), generated by the action of plasma or tissue kallikrein on the precursor (high or low molecular weight kininogen) are the main bradykinin and its receptor (B2R) agonists. These peptides can be converted to B1R agonists after removal of C-terminal-Arg. Both peptidases, membrane-bound carboxypeptidase M, linked to B1R at the C-terminal domain or the soluble carboxypeptidase N are able to remove Arg from the C-terminal portion of BK. B2R is constitutively expressed, showing physiological effects such as vasodilation, transient nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS), whereas B1R expression is induced by injury or inflammatory conditions, with long-lasting NO production, resulting in a neurotoxic environment with reactive oxygen species (ROS) production and increased release of glutamate with excitoxicity-induced neuronal death.

the strong anti-inflammatory phenotype observed in the context of brain ischemia in these animals[27]. Moreover, genetic depletion of B1R improves functional outcome after focal head injury in mice. This effect is similar to that obtained by a pharmacological approach, using a selective B1R antagonist[8]. Thus, mice with B1R depletion show minor axonal damage, reduced apoptosis, astrocyte activation and less inflammation. In contrast, blockage of B2R had no effect on brain protection.

KKS and dementias

Decreased cerebral flow and BBB disruption are also features of Alzheimer's disease (AD)[28,29]. BK activity affects cerebrovascular tone and BBB permeability, both of which are abnormal in AD[30]. According to Farrall *et al*[30], the frontal cortex of patients with AD, the frontal and temporal cortex of patients with vascular dementia showed high levels of plasma kallikrein as well as its mRNA. In addition, this enzyme also had high activity showing that kinin production could influence cerebral blood flow and vascular permeability related to AD. Other types of KK are also modified in the CSF of patients with AD and with frontotemporal dementia. KK6, KK7 and KK10 were decreased in the CSF of patients with frontotemporal dementia, while KK10 increased in the CSF of subjects with AD. These differences could be useful in the diagnosis of both diseases[31]. Increased expression of KK6 was also observed in CSF, plasma and whole blood of patients with AD[32], showing a strong relationship between the KKS and brain degeneration. Furthermore, mice expressing human amyloid precursor protein (APP), carrying familial AD gene mutations, showed increased expression of B1R in astrocytes of the hippocampal formation. Similarly, blockage of this receptor, using specific antagonists, decreased amyloidosis plaque deposits in the somatosensory/cingulate cortex and dorsal hippocampus[33]. These authors also showed improvements in learning and memory after B1R block-

age in APP mice. Thus, according to Lemos *et al*[34] during the aging process, B1R could be involved in memory degeneration, while B2R could act as a neuroprotective factor.

Kallikrein 8 also known as neuropsin participates in extracellular proteolysis involved in long-term potentiation (LTP), necessary for the establishment of memory acquisition in the hippocampus[35]. According to these authors, KK8 knockout mice were impaired, failed memory tasks and showed the involvement of this enzyme in phosphorylation of the GluR1 subunit of AMPA receptors, linked with LTP and with memory acquisition. Taken together, these data show that the KKS participates in these degenerative diseases.

KKS and neuromuscular diseases

Kallikreins are also associated with secondary progressive multiple sclerosis and promote neurodegeneration[36]. According to these authors, high levels of KK1 and KK6 may serve as biomarkers of multiple sclerosis progression. KK1 levels correlate positively with expanded disability status scale (EDSS) scores and KK6 with future prognostic and worsening of the EDSS scale, in relapsing remitting patients. These authors also showed that exposure to kallikrein promoted neurite retraction and neuronal death in murine cortical neurons[36].

Recent work showed that deletion of the *KK6* gene affected the number of oligodendrocytes and the amount of myelin in the developing spinal cord, in particular the myelin basic protein[37]. These data suggest that KK6 has an important function in promoting oligodendrocyte development in the spinal cord as well as in damaged spinal cords. In addition, KK6 has also been associated with hypertrophic astrocytes in human pathological conditions, promoting astrocyte stellation, stimulating inflammatory cytokine (IL-6) secretion and suppressing GFAP mRNA expression[38]. Undoubtedly, KK6 seems to be very important for the homeostasis of CNS cells, participating in several events during physiological and pathological conditions.

KKS and epilepsy

It is already known that the brain inflammatory process is able to initiate seizures[39] and this event is accompanied by an immune-mediated leakage in the BBB. The first evidence linking the KKS with epilepsies was demonstrated by several authors around the 1970s[40,41]. Since then, a large number of studies have emerged localizing more specific targets in the KKS cascade that could help in understanding epilepsy physiopathology. In 1999, Bregola *et al*[42] showed changes in hippocampal and cortical B1R in two experimental models of epilepsy. These authors reported that Lys-des-Arg^{9}BK, an agonist of B1R, increased the overflow of glutamate after electrical stimulation, in hippocampal and cortical slices of rats submitted to kindling. This effect was also visualized in rats submitted to the kainate model of epilepsy, but to a lesser extent. The authors associated B1R with the condition of latent epileptic hyperexcitability[42]. These data were confirmed by Mazzuferi *et al*[43] when they showed the increased release of glutamate after B1R stimulation, induced by Lys-des-Arg^9-BK in kindled animals.

When studying the expression of B1R and B2R in the hippocampus of rats submitted to the pilocarpine model of epilepsy, our group[44] found increased expression of both receptors in the hippocampus. We also found[45] these alterations in knockout mice (B1KO and B2KO) in the pilocarpine model. This means that the absence of B1R (B1KO) decreases pyramidal cell death, decreases mossy fiber sprouting and decreases the number of spontaneous recurrent seizures, during the chronic phase, showing that B1R is proconvulsant. These data were confirmed by Silva *et al*[46]. However, using the model of audiogenic kindling with limbic recruitment, Pereira *et al*[47] found increased expression of B1R and B2R in the hippocampus of rats, but reported that this increase did not correlate with inflammatory levels as IL1β, COX2 and TNFα were not modified in this tissue.

We also showed[45] that B2R was linked to neuroprotection, as its absence is associated with decreased pyramidal cell survival and increased mossy fiber sprouting. Confirming these data, other authors have shown that BK triggers a neuroprotective cascade *via* B2R activation, which conferred protection against NMDA-induced excitotoxicity[48]. However, different data were recently reported concerning the role of B2R in epileptogenesis. Rodi *et al*[49] found that B2R was overexpressed in limbic areas and that slices prepared from B1R knockout mice (B1K0) were more excitable than those from wild-type mice. This effect was abolished using B2R antagonists. Due to this result, the authors concluded that this excitatory phenomenon was B2R dependent. In addition, these authors also demonstrated that kainic acid-induced seizures are attenuated by a B2R antagonist, supporting the hypothesis that B2R is involved in an early event that leads a normal brain to epileptic conditions.

When studying patients with temporal lobe epilepsy (TLE) and hippocampal sclerosis we also showed increased levels of B1R and B2R in the hippocampus[50], when compared with autopsy-control tissues. These receptors were visualized in pyramidal neurons of the hilus and in CA1 and CA3 regions of the hippocampal formation. The hippocampus of these patients also showed overexpression of KK1 by astrocytes, which were co-localized with GFAP protein, confirming participation of the KKS[51].

Together, these data show effective participation of the KKS system in TLE and Figure 2 shows our suggestion concerning a possible cross-talk between hippocampal neurons and astrocytes in the KKS in epileptic diseases.

RENIN-ANGIOTENSIN SYSTEM AND NEUROLOGICAL DISORDERS

Components of the renin-angiotensin system

The renin-angiotensin system (RAS) was initially consid-

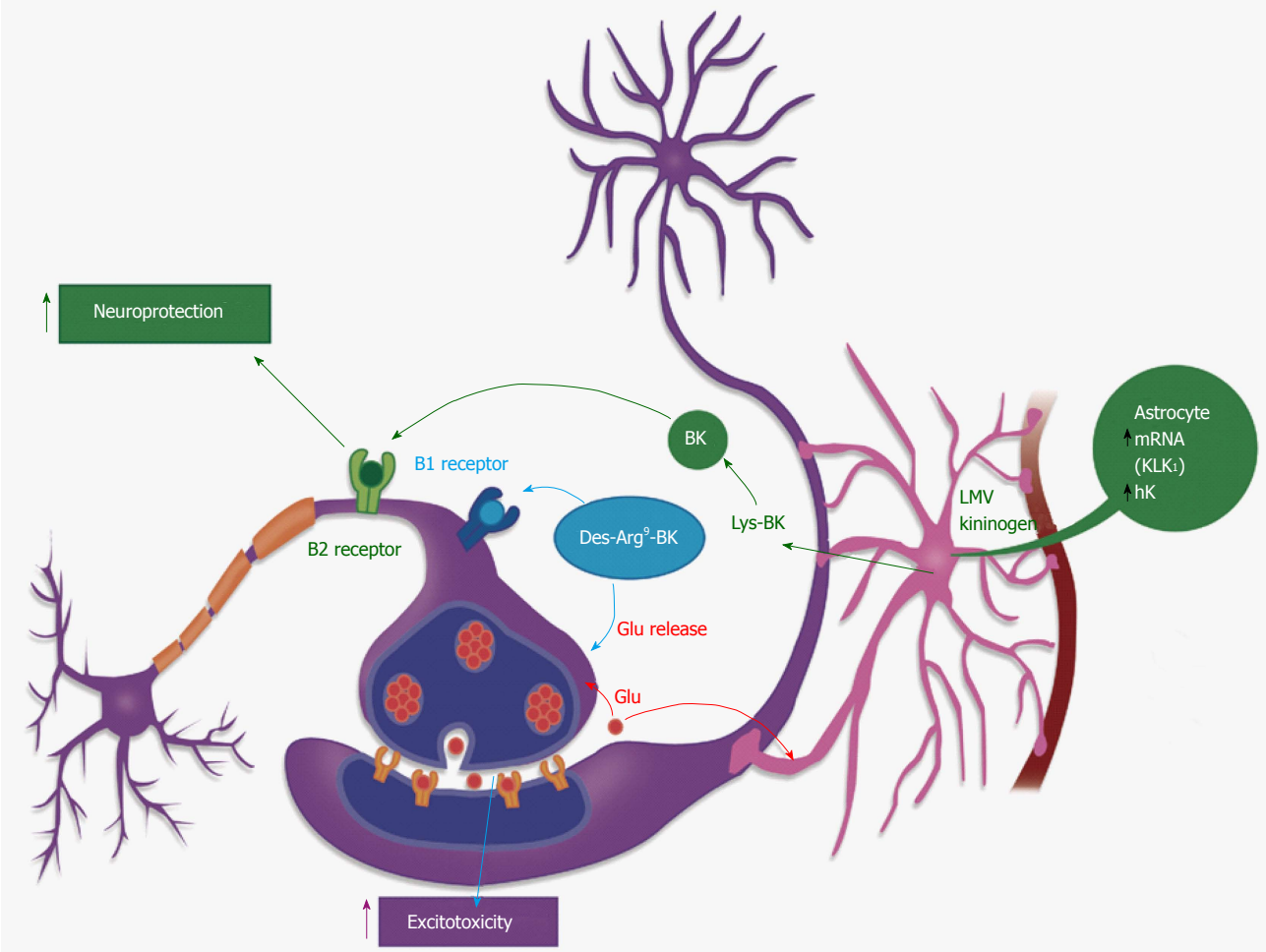

Figure 2 Cross-talk between glial and neural cells related to the kallikrein-kinin system. An adaptation based on the image found at the following site: http://learn.genetics.utah.edu/units/addiction/reward/images/neuronsAstrocyte.jpg. Kallikrein 1 (KK1) in the hippocampus, acts on its main substrate, the low molecular weight kininogen, to release Lys-bradykinin (BK) which can be hydrolyzed to BK, Des-Arg9BK or des-Arg10-Lys-BK by kininases, localized in astrocytes or at the extracellular matrix. These short-living peptides will act on the neuronal surface: binding to kinin B1R they will induce an increase in glutamate release, thus increasing neuronal excitability. Acting on kinin B2R these peptides will produce neuroprotection[42-45].

ered to be a circulating humoral system, involved in blood pressure regulation and the control of both sodium and water intake. Molecules formed by this system are associated with vasoconstriction and the release of aldosterone from the adrenal cortex and antidiuretic hormone from the neurohypophysis. RAS components act in the vasculature to promote vasoconstriction and at sites within the central nervous system to stimulate sympathetic outflow, impair the baroreflex sensitivity for heart rate control, promote release of catecholamines and aldosterone, and sodium retention, which have an important role in the development and maintenance of hypertension and insulin resistance during aging[52].

Renin is the rate-limiting enzyme of the RAS and acting on its precursor, angiotensinogen, releases angiotensin I , Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu (Ang I). After dipeptide His-Leu removal by angiotensin converting enzyme (ACE), Ang Ⅱ is produced (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Ang Ⅱ is the main effector peptide in this system. Binding to Ang Ⅱ type 1 receptor (AT1R), Ang Ⅱ stimulates vasoconstriction, aldosterone and steroid hormones release, which are involved in sodium reabsorption and water retention. AT1R activity is also related to hypertension, heart dysfunction, brain ischemia, abnormal stress responses, BBB breakdown and inflammation in several species[53]. The second receptor involved in Ang Ⅱ activity is AT2R. However, the function of AT2R is more elusive and controversial. AT2R is expressed during fetal development, decreasing after birth and remaining at a low concentration during adulthood. It has been linked to cell proliferation, differentiation, apoptosis and regeneration of several tissues[54] (Figure 3).

RAS in CNS

In addition to the well-known humoral RAS, in the last decades a tissue RAS has been described, particularly in the CNS. Thus, all components of the RAS have been found in the brain. However, as this tissue has a low level of renin, it remains controversial as to how Ang I is generated by this system. Recently[55], the presence of a prorenin receptor (PRR) was reported, which has a high

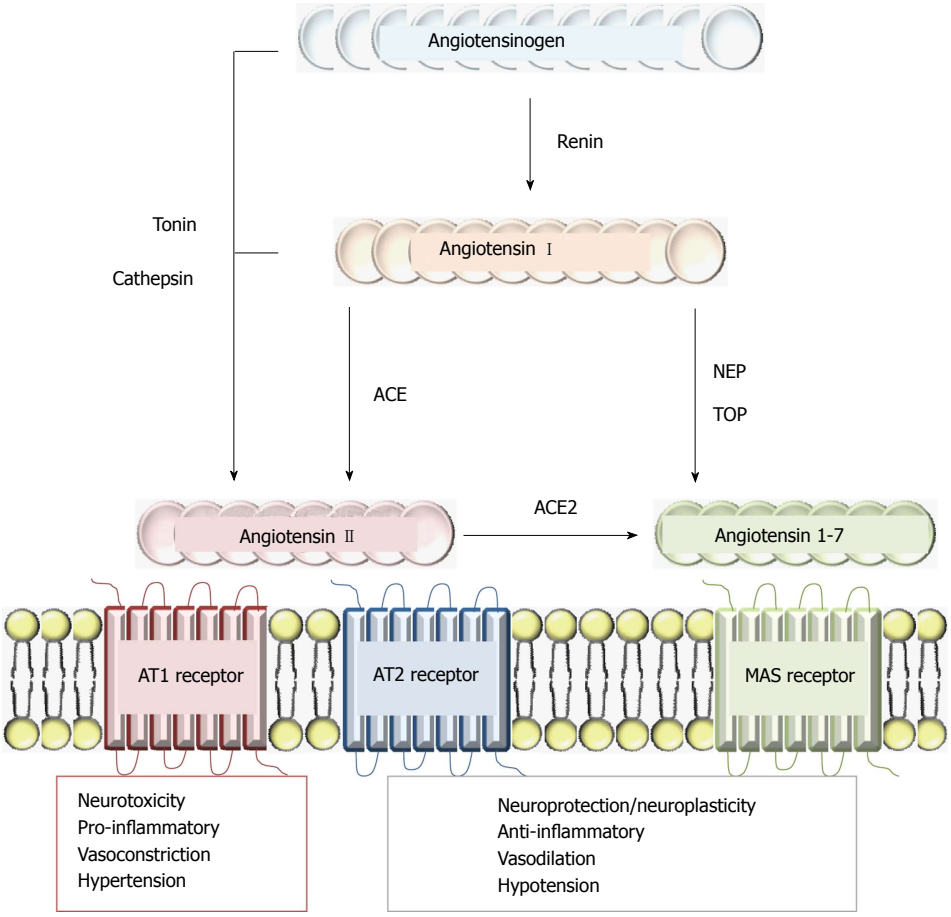

Figure 3 Schematic representation of the renin-angiotensin system and its physiopathological effects. Ang Ⅱ may be generated in the brain *via* the classical pathway, through renin and angiotensin converting enzyme (ACE) action (through Ang Ⅰ cleavage) or can be directly released from angiotensinogen by cathepsin G or tonin actions. Ang1-7 is active in several organs including the brain and several endopeptidases such as thimet oligopeptidase (TOP) or neutral endopeptidases (NEP) may metabolize Ang Ⅰ, generating Ang1-7. Ang Ⅱ may also be hydrolyzed by ACE2 to generate Ang1-7. Binding to Ang Ⅱ type 1 receptor (AT1R), Ang Ⅱ stimulates vasoconstriction, aldosterone and steroid hormones release, which are involved in sodium reabsorption and water retention. AT1R activity is also related to hypertension, heart dysfunction, brain ischemia, abnormal stress responses, blood-brain barrier breakdown and inflammation. The second receptor involved in Ang Ⅱ activity is AT2R and is expressed during fetal development, decreasing after birth and remaining at a low concentration during adulthood. It has been linked to cell proliferation, differentiation, apoptosis and the regeneration of several tissues. Ang1-7 is a Mas receptor agonist, which is related to neuronal plasticity and changes in cellular phenotype that are produced by neuronal activity such as synaptic rearrangements and mossy fiber sprouting in the hippocampus.

level of expression in the brain by neurons and astrocytes. Prorenin binds to its receptors without proteolytic activation and this binding initiates the rate-limiting step in angiotensin formation in the CNS. PRR also acts as an accessory protein for vesicular ATPase, linked to vesicular acidification.

Further to ACE, some homologue components of the RAS have been described such as ACE2 and chymase. Furthermore, peptides such as angiotensin 1-7 (Ang1-7), angiotensin Ⅲ (Ang Ⅲ) and Ang Ⅳ are involved in RAS function. Ang Ⅳ acts at AT4R and Ang1-7 at the Mas receptor. Another enzyme involved in Ang Ⅱ generation is Tonin, which is able to hydrolyze angiotensinogen releasing Ang Ⅱ in tissue, without ACE intervention (Figure 3).

Connection between the KKS and RAS

There is a connection between the KKS and RAS (Figure 4), which is produced by ACE linking both of these important systems. ACE is considered to be the most potent kininase in the blood and in several tissues, such as lung and liver. This enzyme, removes the dipeptide His-Leu from Ang Ⅰ, generates Ang Ⅱ, removes Phe-Arg dipeptide from BK, and inactivates this hypotensor peptide. This is a very important link and it is through the balance between RAS and KKS, that blood pressure can be controlled. This balance is also very important in the brain due to control of BBB permeability.

RAS and inflammation

Despite its action in important physiological processes, RAS has also been associated with pathological conditions. In a recent review[53], authors showed a relationship between the RAS and inflammatory brain disorders, focusing attention on the actions of AT1R in diseases such as stress-induced disorders, anxiety and depression, stroke, brain inflammation, traumatic brain injury and DA. These authors reported that AT1R activation up-regulates common pro-inflammatory mechanisms, activating transcription factors such as NF-κB, triggering an inflammatory cascade with the production of adhe-

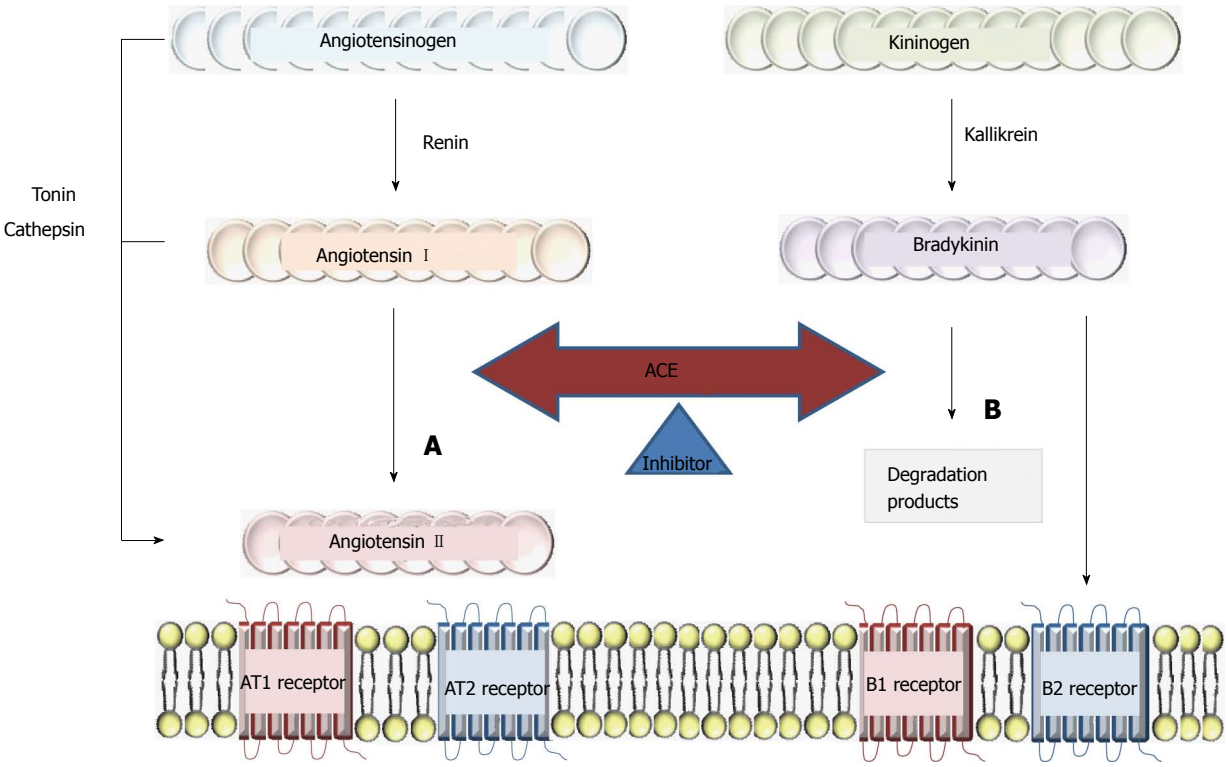

Figure 4 Schematic representation of the role of angiotensin converting enzyme in the renin-angiotensin and kallikrein-kinin systems. A: Conversion of Ang I into Ang II by angiotensin converting enzyme (ACE); B: Bradykinin (BK) degradation by ACE. Physiological effects on the renin-angiotensin system mediated by Ang II type 1 receptor (AT1R) include: vasoconstriction, neuroinflammation, and increased sympathetic nerve activity. Those mediated by Ang II type 2 receptor (AT2R) include cell differentiation and vasodilation. The effects on the kallikrein-kinin system, mediated by kinins, bradykinin and their receptor (B2R) also include vasodilation and hypotension, *via* the release of nitric oxide (NO), prostacyclins and endothelium-derived hyperpolarizing factor (EDHF). It is important to emphasize that in human pathological conditions, the use of ACE inhibitors results in downregulation of Ang II production. In this sense, the kallikrein-kinin system is upregulated and the physiological effects of kinins are potentiated, as all kinin-related peptides are less hydrolyzed by ACE inhibition.

sion molecules, cytokines, reactive oxygen species (ROS), prostaglandins and NO. It was also proposed that circulating Ang II stimulates brain vascular endothelial target cells, producing BBB breakdown, allowing macrophage infiltration into brain parenchyma, increasing microglia and astrocytes activation[53]. Ang II also induces C-reactive protein production by vascular cells as well as by macrophages in culture[56].

RAS and cerebrovascular alteration

Several authors have shown that captopril (ACE inhibitor) improves cerebrovascular structure and function in old hypertensive rats, attenuating eutrophic and hypertrophic inward, remodeling cerebral arterioles. In contrast, Tanahashi *et al*[57] showed that Ang II is related to stroke protection, mediated by AT2R, AT4R and Ang1-7/Mas receptor. However, these authors also indicated that recent clinical trials demonstrated that blockade of the RAS has a potential role in stroke prevention. These data show that the RAS may have dual function in the brain, depending on the action of different peptides and their receptors.

RAS in extrapyramidal diseases

RAS has been identified in the nigrostriatal system and, according to several authors, dopaminergic neurons have an intracellular/intracrine RAS[58,59]. As already mentioned, Ang II acts on the inflammatory cascade, *via* AT1R, producing high levels of ROS by activating the NADPH oxidase complex[60], which are the early processes leading to dopaminergic cell death, in the nigrostriatal system, in Parkinson's disease[61]. These data showed that AT1R blockage reduces dopaminergic neuron loss as well as lipid peroxidation in the Parkinson model (injection of 6-OHDA in rats). These authors also concluded that the RAS is present in dopaminergic neurons with high vulnerability in the nigrostriatal system. The interaction of dopamine/Ang II may be a major factor in age-related dopaminergic vulnerability, that could be the result of increased AT1R expression, decreased AT2R expression, enhanced levels of inflammatory mediators and ROS in dopaminergic pathways[61]. Thus, manipulation of RAS using AT1R antagonists or ACE inhibitors could be helpful in the treatment of Parkinson's disease. In addition, other authors[53,62] also advocate the use of AT1R blockers in the treatment of several inflammatory brain disorders.

RAS and dementias

Other brain pathologies such as AD have also been linked to the RAS. Longitudinal studies have suggested an association between high blood pressure and demen-

tia, showing that hypertension is a risk factor for the development of AD during aging. Patients treated with perindopril (ACE inhibitor) with previous stroke and/or ischemic events were followed for 4 years and dementia and/or cognitive decline were reduced in the treated group, showing a connection between these dual pathologies[63]. Captopril (ACE inhibitor) improves cerebrovascular structure in hypertensive subjects. Indeed, benefit was found when an ACE inhibitor was able to cross the BBB, showing that peripheral action is important, but the effect on cognition is not exclusively due to blood pressure control, but is related to the central action of these drugs[64]. Yamada *et al*[65] showed that perindopril ameliorated cognitive performance in rats submitted to AD models, through inhibition of brain ACE.

In contrast, other authors showed that ACE converts Aβ1-42 (amyloidogenic form) to Aβ1-40 (soluble form), decreasing the Aβ1-42/Aβ1-40 ratio. According to these authors, ACE is also able to degrade Aβ1-42 and Aβ1-40, thus reducing the risk of AD development. They also suggested that treatment with captopril promotes predominant Aβ1-42 deposit in the brain, increasing neuronal vulnerability and death, contradicting the data obtained in patients with hypertension and dementia, treated with this ACE inhibitor. These authors suggest that new strategies could be implemented to improve ACE activity, as novel targets in the treatment of AD[66].

RAS and epilepsy

Other ACE inhibitors such as fosinopril, zofenopril, enalapril and captopril have been associated with the potentiation of antiepileptic drugs[67]. These authors showed that the combination of carbamazepine, lamotrigine, topiramate and valproate with ACE inhibitors decrease audiogenic seizures. Captopril also potentiates the effect of carbamazepine and lamotrigine against electroshock seizures[68]. These data were confirmed in other models of epilepsy. According to Pereira *et al*[69], ACE inhibitor and/or AT1R antagonist were able to reduce the severity of audiogenic seizures. These data link the RAS with generalized seizures and with other types of epilepsies.

In 2008 our group showed, for the first time, an upregulation of AT1R as well as its messenger expression in the cortex and hippocampus of patients with temporal lobe epilepsy, associated with temporal mesial sclerosis[70]. Increased expression of AT2R was also found in the hippocampus showing that the RAS is inwardly associated with this brain disorder. AT1Rs were colocalized with NeuN protein, labeling pyramidal neurons in more vulnerable areas. We also found that a common mutation, which increases ACE activity, occurs in high frequency in the blood cells of patients with TLE and mesial sclerosis. Interestingly, in the hippocampus of these patients, ACE activity was down regulated. Investigating this contradictory data we found that carbamazepine, used to treat seizures was able to inhibit hippocampal ACE activity in these patients. The inhibition of ACE by carbamazepine occurred *in vitro* and *in vivo*, confirming a strong link between TLE and RAS. Patients not treated with carbamazepine showed increased ACE activity[71].

In trying to understand the alteration of RAS components in the epileptogenic process we studied Ang I, Ang II and Ang1-7 levels in the hippocampus of rats submitted to pilocarpine-induced TLE. We found decreased levels of Ang I in acute (status epilepticus), silent (seizure-free period) and chronic (spontaneous recurrent seizures) phases. In contrast, Ang II was increased in the chronic phase, while Ang1-7 was increased in acute and silent periods. These data showed that during the epileptogenic process Ang I was converted into Ang II or Ang1-7. However, ACE expression was decreased in all phases, showing that other enzymes in the RAS may participate in this event such as NEP and Tonin. Indeed, both enzymes were upregulated in the hippocampus of these rats[72]. Our results also showed an upregulation of AT1R during the spontaneous seizure period (chronic phase)[71], in accordance with data found in patients with TLE[70], supporting the involvement of this receptor in seizure generation. The silent phase was characterized by an increase in Ang1-7 levels as well as its Mas receptor. Interestingly, during the silent phase of this model, intense hippocampal reorganization occurs, which has been related to Ang1-7/Mas-induced plasticity.

CONCLUSION

In conclusion, peptides generated by the RAS or KKS are deeply involved in several neurological diseases and an improvement in the knowledge of their function and release in tissues and blood could be useful in the development of new targets and drugs to treat these pathologies.

REFERENCES

1 **Bhoola KD**, Figueroa CD, Worthy K. Bioregulation of kinins: kallikreins, kininogens, and kininases. *Pharmacol Rev* 1992; **44**: 1-80 [PMID: 1313585]

2 **Pesquero JB**, Bader M. Molecular biology of the kallikrein-kinin system: from structure to function. *Braz J Med Biol Res* 1998; **31**: 1197-1203 [PMID: 9876287 DOI: 10.1590/S0100-879X1998000900013]

3 **Abelous JE,** Bardier E. Les substances hypotensives de l`urine humaine normale. *CR Soc Biol* (Paris) 1909; **66**: 511-520

4 **Frey EK,** Kraut H. Ein neues Kreislaufhormon und seine wirkung. *Arch Exp Pathol Pharmakol* 1928; **133**: 1-56

5 **Werle E,** Gotze W, Keppler A. Über die Wirkung des Kallikreins auf den isolierten darm und uber eine neue darmkontrahierende Substang. *Biochem J* 1937; **289**: 217-233

6 **Werle E,** Berek U. Zur Kenntnis des Kallikreins. *Angew Chem* 1948; **60**: 53

7 **Rocha E Silva M**, Beraldo WT, Rosenfeld G. Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. *Am J Physiol* 1949; **156**: 261-273 [PMID: 18127230]

8 **Albert-Weißenberger C**, Sirén AL, Kleinschnitz C. Ischemic stroke and traumatic brain injury: the role of the kallikrein-kinin system. *Prog Neurobiol* 2013; **101-102**: 65-82 [PMID: 23274649 DOI: 10.1016/j.pneurobio.2012.11.004]

9 **Kitagawa A**, Kizuki K, Moriya H, Kudo M, Noguchi T. Localization of kallikrein in rat pineal glands. *Endocrinol Jpn* 1991; **38**: 109-112 [PMID: 1915108 DOI: 10.1507/endocrj1954.38.109]

10 **Hösli E**, Hösli L. Autoradiographic localization of binding sites for neuropeptide Y and bradykinin on astrocytes. *Neuroreport* 1993; **4**: 159-162 [PMID: 8453054 DOI: 10.1097/00001756-199302000-00011]

11 **Raidoo DM**, Bhoola KD. Pathophysiology of the kallikrein-kinin system in mammalian nervous tissue. *Pharmacol Ther* 1998; **79**: 105-127 [PMID: 9749879 DOI: 10.1016/S0163-7258(98)00011-4]

12 **Chao J**, Woodley C, Chao L, Margolius HS. Identification of tissue kallikrein in brain and in the cell-free translation product encoded by brain mRNA. *J Biol Chem* 1983; **258**: 15173-15178 [PMID: 6558077]

13 **Görlach C**, Wahl M. Bradykinin dilates rat middle cerebral artery and its large branches via endothelial B2 receptors and release of nitric oxide. *Peptides* 1996; **17**: 1373-1378 [PMID: 8971934 DOI: 10.1016/S0196-9781(96)00223-9]

14 **Regoli D**, Calo G, Rizzi A, Bogoni G, Gobeil F, Campobasso C, Mollica G, Beani L. Bradykinin receptors and receptor ligands (with special emphasis on vascular receptors). *Regul Pept* 1996; **65**: 83-89 [PMID: 8876040 DOI: 10.1016/0167-0115(96)00076-6]

15 **Sobey CG**, Heistad DD, Faraci FM. Mechanisms of bradykinin-induced cerebral vasodilatation in rats. Evidence that reactive oxygen species activate K+ channels. *Stroke* 1997; **28**: 2290-2294; discussion 2295 [PMID: 9368578 DOI: 10.1161/01STR.28.11.2290]

16 **Leeb-Lundberg LM**, Marceau F, Müller-Esterl W, Pettibone DJ, Zuraw BL. International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* 2005; **57**: 27-77 [PMID: 15734727 DOI: 10.1124/pr.57.1.2]

17 **Sotiropoulou G**, Pampalakis G, Diamandis EP. Functional roles of human kallikrein-related peptidases. *J Biol Chem* 2009; **284**: 32989-32994 [PMID: 19819870 DOI: 10.1074/jbc.R109.027946]

18 **Pathak M**, Wong SS, Dreveny I, Emsley J. Structure of plasma and tissue kallikreins. *Thromb Haemost* 2013; **110**: 423-433 [PMID: 23494059 DOI: 10.1160/TH12-11-0840]

19 **Schulze-Topphoff U**, Prat A, Bader M, Zipp F, Aktas O. Roles of the kallikrein/kinin system in the adaptive immune system. *Int Immunopharmacol* 2008; **8**: 155-160 [PMID: 18182219 DOI: 10.1016/j.intimp.2007.08.001]

20 **Guevara-Lora I**. Kinin-mediated inflammation in neurodegenerative disorders. *Neurochem Int* 2012; **61**: 72-78 [PMID: 22554400 DOI: 10.1016/j.neuint.2012.04.013]

21 **Liu LB**, Xue YX, Liu YH. Bradykinin increases the permeability of the blood-tumor barrier by the caveolae-mediated transcellular pathway. *J Neurooncol* 2010; **99**: 187-194 [PMID: 20146088 DOI: 10.1007/s11060-010-0124-x]

22 **Easton AS**. Regulation of permeability across the blood-brain barrier. *Adv Exp Med Biol* 2012; **763**: 1-19 [PMID: 23397617]

23 **Ikeda Y**, Ueno A, Naraba H, Matsuki N, Oh-Ishi S. Intracellular Ca2+ increase in neuro-2A cells and rat astrocytes following stimulation of bradykinin B2 receptor. *Jpn J Pharmacol* 2000; **84**: 140-145 [PMID: 11128036 DOI: 101254/jjp84140]

24 **Kunz M**, Nussberger J, Holtmannspötter M, Bitterling H, Plesnila N, Zausinger S. Bradykinin in blood and cerebrospinal fluid after acute cerebral lesions: correlations with cerebral edema and intracranial pressure. *J Neurotrauma* 2013; **30**: 1638-1644 [PMID: 23638655 DOI: 10.1089/neu.2012.2774]

25 **Martínez-Morillo E**, Diamandis A, Romaschin AD, Diamandis EP. Kallikrein 6 as a serum prognostic marker in patients with aneurysmal subarachnoid hemorrhage. *PLoS One* 2012; **7**: e45676 [PMID: 23049835 DOI: 10.1371/journal.pone.0045676]

26 **Liu J**, Gao BB, Clermont AC, Blair P, Chilcote TJ, Sinha S, Flaumenhaft R, Feener EP. Hyperglycemia-induced cerebral hematoma expansion is mediated by plasma kallikrein. *Nat Med* 2011; **17**: 206-210 [PMID: 21258336 DOI: 10.1038/nm.2295]

27 **Langhauser F**, Göb E, Kraft P, Geis C, Schmitt J, Brede M, Göbel K, Helluy X, Pham M, Bendszus M, Jakob P, Stoll G, Meuth SG, Nieswandt B, McCrae KR, Kleinschnitz C. Kininogen deficiency protects from ischemic neurodegeneration in mice by reducing thrombosis, blood-brain barrier damage, and inflammation. *Blood* 2012; **120**: 4082-4092 [PMID: 22936662 DOI: 10.1182/blood-2012-06-440057]

28 **Ashby EL**, Love S, Kehoe PG. Assessment of activation of the plasma kallikrein-kinin system in frontal and temporal cortex in Alzheimer's disease and vascular dementia. *Neurobiol Aging* 2012; **33**: 1345-1355 [PMID: 21074291 DOI: 10.1016/j.neurobiolaging.2010.09.024]

29 **Eberling JL**, Jagust WJ, Reed BR, Baker MG. Reduced temporal lobe blood flow in Alzheimer's disease. *Neurobiol Aging* 1992; **13**: 483-491 [PMID: 1508299 DOI: 10.1016/0197-4589(92)90076-A]

30 **Farrall AJ**, Wardlaw JM. Blood-brain barrier: ageing and microvascular disease--systematic review and meta-analysis. *Neurobiol Aging* 2009; **30**: 337-352 [PMID: 17869382 DOI: 10.1016/j.neurobiolaging.2007.07]

31 **Diamandis EP**, Scorilas A, Kishi T, Blennow K, Luo LY, Soosaipillai A, Rademaker AW, Sjogren M. Altered kallikrein 7 and 10 concentrations in cerebrospinal fluid of patients with Alzheimer's disease and frontotemporal dementia. *Clin Biochem* 2004; **37**: 230-237 [PMID: 14972646 DOI: 1016/j.clinbiochem.2003.11.012]

32 **Diamandis EP**, Yousef GM, Petraki C, Soosaipillai AR. Human kallikrein 6 as a biomarker of alzheimer's disease. *Clin Biochem* 2000; **33**: 663-667 [PMID: 11166014 DOI: 10.1016/S0009-9120(00)00185-5]

33 **Lacoste B**, Tong XK, Lahjouji K, Couture R, Hamel E. Cognitive and cerebrovascular improvements following kinin B1 receptor blockade in Alzheimer's disease mice. *J Neuroinflammation* 2013; **10**: 57 [PMID: 23642031 DOI: 10.1186/1742-2094-10-57]

34 **Lemos MT**, Amaral FA, Dong KE, Bittencourt MF, Caetano AL, Pesquero JB, Viel TA, Buck HS. Role of kinin B1 and B2 receptors in memory consolidation during the aging process of mice. *Neuropeptides* 2010; **44**: 163-168 [PMID: 20060587 DOI: 10.1016/j.npep.2009.12.006]

35 **Tamura H**, Ishikawa Y, Hino N, Maeda M, Yoshida S, Kaku S, Shiosaka S. Neuropsin is essential for early processes of memory acquisition and Schaffer collateral long-term potentiation in adult mouse hippocampus in vivo. *J Physiol* 2006; **570**: 541-551 [PMID: 16308352 DOI: 10.1113/jphysiol.2005.098715]

36 **Scarisbrick IA**, Linbo R, Vandell AG, Keegan M, Blaber SI, Blaber M, Sneve D, Lucchinetti CF, Rodriguez M, Diamandis EP. Kallikreins are associated with secondary progressive multiple sclerosis and promote neurodegeneration. *Biol Chem* 2008; **389**: 739-745 [PMID: 18627300 DOI: 10.1515/BC.2008.085]

37 **Murakami K**, Jiang YP, Tanaka T, Bando Y, Mitrovic B, Yoshida S. In vivo analysis of kallikrein-related peptidase 6 (KLK6) function in oligodendrocyte development and the expression of myelin proteins. *Neuroscience* 2013; **236**: 1-11 [PMID: 23376368 DOI: 10.1016/j.neuroscience.2012.12.073]

38 **Scarisbrick IA**, Radulovic M, Burda JE, Larson N, Blaber SI, Giannini C, Blaber M, Vandell AG. Kallikrein 6 is a novel molecular trigger of reactive astrogliosis. *Biol Chem* 2012; **393**: 355-367 [PMID: 22505518 DOI: 10.1515/hsz-2011-0241]

39 **Kleen JK**, Holmes GL. Brain inflammation initiates seizures. *Nat Med* 2008; **14**: 1309-1310 [PMID: 19057551 DOI: 10.1038/nm1208-1309]

40 **Wiśniewski K**, Mackiewicz W. Kinins and hypoglycemic

convulsions. *Pol J Pharmacol Pharm* 1974; **26**: 611-615 [PMID: 4548528]
41 **Shikimi T**, Kema R, Matsumoto M, Yamahata Y, Miyata S. Studies on kinin-like substances in brain. *Biochem Pharmacol* 1973; **22**: 567-573 [PMID: 4348114]
42 **Bregola G**, Varani K, Gessi S, Beani L, Bianchi C, Borea PA, Regoli D, Simonato M. Changes in hippocampal and cortical B1 bradykinin receptor biological activity in two experimental models of epilepsy. *Neuroscience* 1999; **92**: 1043-1049 [PMID: 10426544 DOI: 10.1016/S0306-4522(99)00075-5]
43 **Mazzuferi M**, Binaschi A, Rodi D, Mantovani S, Simonato M. Induction of B1 bradykinin receptors in the kindled hippocampus increases extracellular glutamate levels: a microdialysis study. *Neuroscience* 2005; **135**: 979-986 [PMID: 16125864 DOI: 10.1016.j.neuroscience.2005.06.070]
44 **Argañaraz GA**, Silva JA, Perosa SR, Pessoa LG, Carvalho FF, Bascands JL, Bader M, da Silva Trindade E, Amado D, Cavalheiro EA, Pesquero JB, da Graça Naffah-Mazzacoratti M. The synthesis and distribution of the kinin B1 and B2 receptors are modified in the hippocampus of rats submitted to pilocarpine model of epilepsy. *Brain Res* 2004; **1006**: 114-125 [PMID: 15047030 DOI: 10.1016.j.brainres.2003.12.050]
45 **Adolfo Argañaraz G**, Regina Perosa S, Cristina Lencioni E, Bader M, Abrão Cavalheiro E, da Graça Naffah-Mazzacoratti M, Pesquero JB, Antônio Silva J. Role of kinin B1 and B2 receptors in the development of pilocarpine model of epilepsy. *Brain Res* 2004; **1013**: 30-39 [PMID: 15196965 DOI: 10.1016.j.brainres.2004.03.046]
46 **Silva JA**, Goto EM, Perosa SR, Argañaraz GA, Cavalheiro EA, Naffah-Mazzacoratti MG, Pesquero JB. Kinin B1 receptors facilitate the development of temporal lobe epilepsy in mice. *Int Immunopharmacol* 2008; **8**: 197-199 [PMID: 18182226 DOI: 10.1016/j.intimp.2007.09.003]
47 **Pereira MG**, Gitaí DL, Paçó-Larson ML, Pesquero JB, Garcia-Cairasco N, Costa-Neto CM. Modulation of B1 and B2 kinin receptors expression levels in the hippocampus of rats after audiogenic kindling and with limbic recruitment, a model of temporal lobe epilepsy. *Int Immunopharmacol* 2008; **8**: 200-205 [PMID: 18182227 DOI: 10.1016/j.intimp.2007.07.028]
48 **Martins AH**, Alves JM, Perez D, Carrasco M, Torres-Rivera W, Eterović VA, Ferchmin PA, Ulrich H. Kinin-B2 receptor mediated neuroprotection after NMDA excitotoxicity is reversed in the presence of kinin-B1 receptor agonists. *PLoS One* 2012; **7**: e30755 [PMID: 22348022 DOI: 10.1371/journal.pone.0030755]
49 **Rodi D,** Buzzi A, Barbieri M, Zucchini S, Verlengia G, Binaschi A, Regoli D, Boschi A, Ongali B, Couture R, Simonato M. Bradykinin B2 receptors increase hippocampal excitability and susceptibility to seizures in mice. *Neuroscience* 2013; **248C**: 392-402 [PMID: 23811399 DOI: 10.1016/j.neuroscience.2013.06.038]
50 **Perosa SR**, Argañaraz GA, Goto EM, Costa LG, Konno AC, Varella PP, Santiago JF, Pesquero JB, Canzian M, Amado D, Yacubian EM, Carrete H, Centeno RS, Cavalheiro EA, Silva JA, Mazzacoratti Mda G. Kinin B1 and B2 receptors are overexpressed in the hippocampus of humans with temporal lobe epilepsy. *Hippocampus* 2007; **17**: 26-33 [PMID: 17094085 DOI: 10.1002/hipo.20239]
51 **Simões PS**, Perosa SR, Arganãraz GA, Yacubian EM, Carrete H, Centeno RS, Varella PP, Santiago JF, Canzian M, Silva JA, Mortara RA, Amado D, Cavalheiro EA, Mazzacoratti Mda G. Kallikrein 1 is overexpressed by astrocytes in the hippocampus of patients with refractory temporal lobe epilepsy, associated with hippocampal sclerosis. *Neurochem Int* 2011; **58**: 477-482 [PMID: 21211543 DOI: 10.1016/j.neuint.2010.12.021]
52 **Arnold AC**, Okamoto LE, Gamboa A, Shibao C, Raj SR, Robertson D, Biaggioni I. Angiotensin II, independent of plasma renin activity, contributes to the hypertension of autonomic failure. *Hypertension* 2013; **61**: 701-706 [PMID: 23266540 DOI: 10.1161/hypertensionaha.111.00377]
53 **Saavedra JM**. Angiotensin II AT(1) receptor blockers as treatments for inflammatory brain disorders. *Clin Sci* (Lond) 2012; **123**: 567-590 [PMID: 22827472 DOI: 10.1042/CS20120078]
54 **Labandeira-Garcia JL**, Rodriguez-Pallares J, Rodríguez-Perez AI, Garrido-Gil P, Villar-Cheda B, Valenzuela R, Guerra MJ. Brain angiotensin and dopaminergic degeneration: relevance to Parkinson's disease. *Am J Neurodegener Dis* 2012; **1**: 226-244 [PMID: 23383395]
55 **Li W**, Peng H, Seth DM, Feng Y. The Prorenin and (Pro)renin Receptor: New Players in the Brain Renin-Angiotensin System? *Int J Hypertens* 2012; **2012**: 290635 [PMID: 23316344 DOI: 10.1155/2012/290635]
56 **Li M**, Liu J, Han C, Wang B, Pang X, Mao J. Angiotensin II induces the expression of c-reactive protein via MAPK-dependent signal pathway in U937 macrophages. *Cell Physiol Biochem* 2011; **27**: 63-70 [PMID: 21325823 DOI: 10.1159/000325206]
57 **Tanahashi N**. [Effect of RAAS inhibition on stroke prevention]. *Nihon Rinsho* 2012; **70**: 1582-1587 [PMID: 23012807]
58 **Joglar B**, Rodriguez-Pallares J, Rodriguez-Perez AI, Rey P, Guerra MJ, Labandeira-Garcia JL. The inflammatory response in the MPTP model of Parkinson's disease is mediated by brain angiotensin: relevance to progression of the disease. *J Neurochem* 2009; **109**: 656-669 [PMID: 19245663 DOI: 10.1111/j.1471-4159.2009.05999.x]
59 **Garrido-Gil P**, Valenzuela R, Villar-Cheda B, Lanciego JL, Labandeira-Garcia JL. Expression of angiotensinogen and receptors for angiotensin and prorenin in the monkey and human substantia nigra: an intracellular renin-angiotensin system in the nigra. *Brain Struct Funct* 2013; **218**: 373-388 [PMID: 22407459 DOI: 10.1007/s00429-012-0402-9]
60 **Yanagitani Y**, Rakugi H, Okamura A, Moriguchi K, Takiuchi S, Ohishi M, Suzuki K, Higaki J, Ogihara T. Angiotensin II type 1 receptor-mediated peroxide production in human macrophages. *Hypertension* 1999; **33**: 335-339 [PMID: 9931126 DOI: 10.1161/01.HYP.33.1.335]
61 **Labandeira-Garcia JL**, Rodriguez-Pallares J, Dominguez-Meijide A, Valenzuela R, Villar-Cheda B, Rodríguez-Perez AI. Dopamine-angiotensin interactions in the basal ganglia and their relevance for Parkinson's disease. *Mov Disord* 2013; **28**: 1337-1342 [PMID: 23925977 DOI: 10.1002/mds.25614]
62 **Ohshima K**, Mogi M, Horiuchi M. Therapeutic approach for neuronal disease by regulating renin-angiotensin system. *Curr Hypertens Rev* 2013; **9**: 99-107 [PMID: 23971691 DOI: 10.2174/15734021113099990004]
63 **Hanon O**, Seux ML, Lenoir H, Rigaud AS, Forette F. Prevention of dementia and cerebroprotection with antihypertensive drugs. *Curr Hypertens Rep* 2004; **6**: 201-207 [PMID: 15128472 DOI: 10.1007/S11906-004-0070-0]
64 **Kehoe PG**, Wilcock GK. Is inhibition of the renin-angiotensin system a new treatment option for Alzheimer's disease? *Lancet Neurol* 2007; **6**: 373-378 [PMID: 17362841 DOI: 10.1016/S1474-4422(07)70077-7]
65 **Yamada K**, Uchida S, Takahashi S, Takayama M, Nagata Y, Suzuki N, Shirakura S, Kanda T. Effect of a centrally active angiotensin-converting enzyme inhibitor, perindopril, on cognitive performance in a mouse model of Alzheimer's disease. *Brain Res* 2010; **1352**: 176-186 [PMID: 20627092 DOI: 10.1016/j.brainres.2010.07.006]
66 **Zou K**, Maeda T, Watanabe A, Liu J, Liu S, Oba R, Satoh Y, Komano H, Michikawa M. Abeta42-to-Abeta40- and angiotensin-converting activities in different domains of angiotensin-converting enzyme. *J Biol Chem* 2009; **284**: 31914-31920 [PMID: 19773553 DOI: 10.1074/jbc.M109.011437]
67 **Sarro GD**, Paola ED, Gratteri S, Gareri P, Rispoli V, Siniscalchi A, Tripepi G, Gallelli L, Citraro R, Russo E. Fosinopril and zofenopril, two angiotensin-converting enzyme (ACE) inhibitors, potentiate the anticonvulsant activity of antiepileptic drugs against audiogenic seizures in DBA/2

mice. *Pharmacol Res* 2012; **65**: 285-296 [PMID: 22107891 DOI: 10.1016/j.phrs.2011.11.005]

68 **Lukawski K**, Jakubus T, Raszewski G, Czuczwar SJ. Captopril potentiates the anticonvulsant activity of carbamazepine and lamotrigine in the mouse maximal electroshock seizure model. *J Neural Transm* 2010; **117**: 1161-1166 [PMID: 20714908 DOI: 10.1007/S00702-010-0455-y]

69 **Pereira MG**, Becari C, Oliveira JA, Salgado MC, Garcia-Cairasco N, Costa-Neto CM. Inhibition of the renin-angiotensin system prevents seizures in a rat model of epilepsy. *Clin Sci* (Lond) 2010; **119**: 477-482 [PMID: 20533906 DOI: 10.1042/CS20100053]

70 **Argañaraz GA**, Konno AC, Perosa SR, Santiago JF, Boim MA, Vidotti DB, Varella PP, Costa LG, Canzian M, Porcionatto MA, Yacubian EM, Sakamoto AC, Carrete H, Centeno RS, Amado D, Cavalheiro EA, Junior JA, Mazzacoratti Mda G. The renin-angiotensin system is upregulated in the cortex and hippocampus of patients with temporal lobe epilepsy related to mesial temporal sclerosis. *Epilepsia* 2008; **49**: 1348-1357 [PMID: 18363708 DOI: 10.1111/j.1528-1167.2008.01581.x]

71 **Almeida SS**, Naffah-Mazzacoratti MG, Guimarães PB, Wasinski F, Pereira FE, Canzian M, Centeno RS, Carrete H, Yacubian EM, Carmona AK, Vieira RF, Nakaie CR, Sabatini RA, Perosa SR, Bacurau RF, Gouveia TL, Gallo G, Würtele M, Cavalheiro EA, Silva JA, Pesquero JB, Araujo RC. Carbamazepine inhibits angiotensin I-converting enzyme, linking it to the pathogenesis of temporal lobe epilepsy. *Transl Psychiatry* 2012; **2**: e93 [PMID: 22832858 DOI: 10.1038/tp.2012.21]

72 **Gouveia TL**, Frangiotti MI, de Brito JM, de Castro Neto EF, Sakata MM, Febba AC, Casarini DE, Amado D, Cavalheiro EA, Almeida SS, Manchini MT, Araújo RC, Silva JA, Naffah-Mazzacoratti Mda G. The levels of renin-angiotensin related components are modified in the hippocampus of rats submitted to pilocarpine model of epilepsy. *Neurochem Int* 2012; **61**: 54-62 [PMID: 22542773 DOI: 10.1016/j.neuint.2012.04.012]

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REVIEW

"Stop Ne(c)king around": How interactomics contributes to functionally characterize Nek family kinases

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Abstract

Aside from Polo and Aurora, a third but less studied kinase family involved in mitosis regulation is the never in mitosis-gene A (NIMA)-related kinases (Neks). The founding member of this family is the sole member NIMA of Aspergillus nidulans, which is crucial for the initiation of mitosis in that organism. All 11 human Neks have been functionally assigned to one of the three core functions established for this family in mammals: (1) centrioles/mitosis; (2) primary ciliary function/ciliopathies; and (3) DNA damage response (DDR). Recent findings, especially on Nek 1 and 8, showed however, that several Neks participate in parallel in at least two of these contexts: primary ciliary function and DDR. In the core section of this in-depth review, we report the current detailed functional knowledge on each of the 11 Neks. In the discussion, we return to the cross-connections among Neks and point out how our and other groups' functional and interactomics studies revealed that most Neks interact with protein partners associated with two if not all three of the functional contexts. We then raise the hypothesis that Neks may be the connecting regulatory elements that allow the cell to fine tune and synchronize the cellular events associated with these three core functions. The new and exciting findings on the Nek family open new perspectives and should allow the Neks to finally claim the attention they deserve in the field of kinases and cell cycle biology.

Key words: Cell cycle; Mitosis; DNA damage response; Protein interactions; Kinases

Core tip: Never in mitosis-gene A (NIMA)-related kinases (Neks) are a family of 11 human kinases involved in cell cycle regulation. This article represents an in-depth review of the current knowledge on the function of each of the 11 human Nek kinases. Furthermore, we present arguments in the discussion of how systems biology, especially interactomics, helped to uncover that the majority of Neks are involved in more than one of

the three Neks core functions: (1) centrioles/mitosis; (2) primary ciliary function/ciliopathies; and (3) the DNA damage response. Possibly, the Neks act on a higher regulatory level which may control the core functions.

Meirelles GV, Perez AM, de Souza EE, Basei FL, Papa PF, Melo Hanchuk TD, Cardoso VB, Kobarg J. "Stop Ne(c)king around": How interactomics contributes to functionally characterize Nek family kinases. *World J Biol Chem* 2014; 5(2): 141-160 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i2/141.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i2.141

INTRODUCTION

The never in mitosis-gene A (NIMA)-related kinases (Neks) represent, aside from the Polo and Aurora kinase families, a third family of mitotic kinases, but remain the least studied to date and hence least understood family of kinases involved in the regulation of the cell cycle. The founding member of this family of kinases is the *Aspergillus nidulans* NIMA, which exists as a single member in this fungus, is functionally involved in the initiation of mitosis and promotes the chromosome condensation by phosphorylation of histone H3[1]. Humans have 11 members of the Nek family which show highly conserved kinase domains but differ significantly in the composition and length of their N- and especially C-terminal regulatory and docking domains (Figure 1).

Although some protein interaction partners have been described for the majority of the human Neks (Figure 2), the domain of interaction at the side of Neks has been mapped only for a smaller subset of interacting proteins (Figure 1). As we can see, most interactors are assigned to specific regions in the regulatory domains, which represent in most cases classical protein-protein interaction modules, such as coiled coil regions. Identification of interaction with the kinases domains have been scarce due to the transient and weak nature of these interactions and therefore the discovery and characterization of true *bona fide in vivo* substrates of Nek kinases remain one of the main challenges in the field. Among the interacting proteins identified by our[2,3] and other groups, through both yeast two-hybrid screens and mass spectrometry analyses, there were hopefully not only those that regulate the Neks but maybe also candidate substrate proteins. The binding of these substrate proteins possibly contributes to "opening up" the Neks or to the activation of these kinases and then, as a consequence, these proteins may be phosphorylated by the Neks.

There has been a series of very good and concise reviews on NIMA and Neks in the past years[4-8]. However, due to scarce or absent knowledge on several family members, including Nek5, 10 and 11 for instance, most reviews opted to focus on a subset of Neks or grouped them according to phylogenetic or functional relatedness. Here, we try to discuss all 11 human Neks in some depth and to include all recent novelty on the least studied Neks as well as our own group's published and unpublished findings, with a special emphasis on the characterization of the functional context based on the identification of interacting proteins (interactomics). A point we would like to stress here is that most Neks interact with proteins of several of the classical functional contexts reported initially for a subset of specific Neks. In other words, we may characterize the following three areas as the main functional contexts of Neks: (1) centriolar function and mitosis regulation (Nek2, 6, 7 and 9); (2) primary ciliary function, ciliopathies and microtubule dynamics in general (Nek1, 4 and 8); and more recently (3) DDR and G_2/M checkpoint (Nek1, 4, 6, 8, 10 and 11)[8,9].

However, published interactome data (Figure 2), as well as our group's efforts to identify new interacting proteins for all Neks, showed some surprising cross-connections and novelties, which we would like to point out here. Most of the above mentioned Neks seem to interact with proteins that are functionally linked to two or even all three of the above mentioned areas, thereby raising the possibility that these are somehow connected on a higher regulatory level and that the Neks may be key elements to understand how the regulation of these functional contexts is performed. A typical recently published example is the role of Nek8 in both primary ciliary function and DNA repair mechanisms[10]. Our own studies revealed that Nek6, a kinase primarily associated with mitotic regulatory events[11,12], also interacts with proteins involved in the DNA damage response, such as putative DNA repair and recombination protein RAD26-like (RAD26L) and PHD finger protein 1 (PHF1) (Figure 2)[3]. In fact, for the majority of Neks we found interacting partners of the DDR or effector proteins of different DNA repair pathways, which clearly suggests a larger than initially imagined involvement of Neks in these biological processes. Other insights came from the identification of interacting proteins from the apoptosis regulatory pathways with several Neks (*e.g.*, Nek 1[13] and 5). This suggests that, aside the well established mitotic context, we must be open minded about additional new roles for Neks (Table 1). Before we go into details of new cross-connections and suggested additional functional contexts in the final discussion, we will present each of the 11 human Neks in detail in the following section of this review.

NEK1

Although Nek1 is only the third most studied Nek family member after Nek2 and aside from Nek6, it is in many ways a representative member of this family of protein kinases. Along this line, Nek1 started to draw the attention of the kinase and signaling research communities, not only to itself but to the Nek family after the publication of the seminal article of Upadhya *et al* in 2000[14]. It reported that deletion mutations in the Nek1 gene in mice caused polycystic kidney disease (PKD) among other pleiotropic effects, ranging from facial dysmorphism,

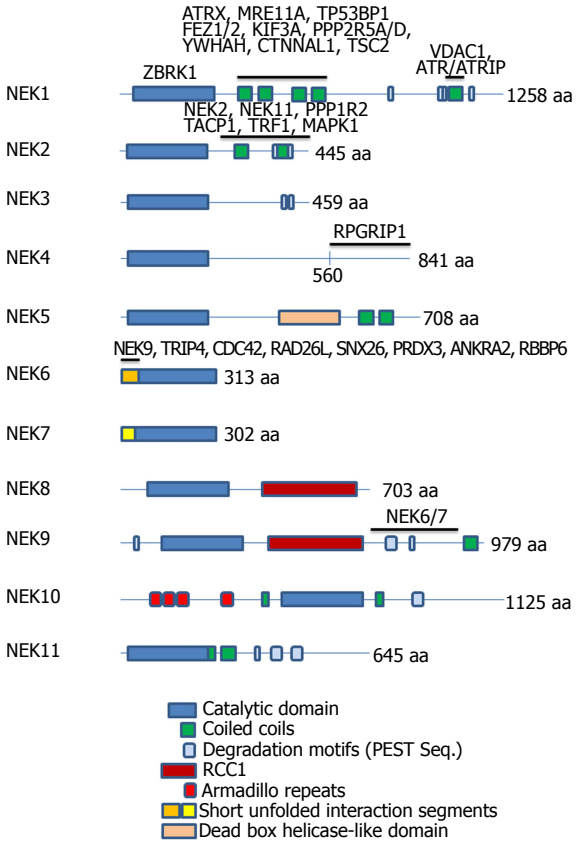

Figure 1 Representation of the domain organization of the eleven human Neks depicting the domain regions for selected protein interactions. The gene symbols corresponding to interacting proteins are shown above the Neks primary structure regions with which they have been found to interact. The list of interactors is not intended to be complete but is necessarily shorter than the list of all proteins known in the literature to interact with Neks (*e.g.*, see Figure 2), since, for the majority of interactors, the location of interaction in the Neks has not been reported. Different repeated domains have been indicated by the color code at the bottom of the figure. The lengths of the full proteins are indicated by number of amino acids (aa) at the C-terminal of the proteins. At least two isoforms of Nek1, 2, 3 and three of Nek4 and 11, all generated by alternative splicing, have been reported and known functional distinctions have been briefly discussed in the text, where feasible. References for the proteins and their mapped interactors: Nek1[2,13,25]; Nek2[116,121-124]; Nek4[53]; Nek6[3]; Nek9[66]. Nek: Never in mitosis-gene A-related kinases.

dwarfing, male sterility, anemia and cystic choroid plexus. The pleiotropic nature of these phenotypes suggested a role of Nek1 early on in basic cellular functions, possibly involved in signaling pathways associated with polycystin-1 and 2, whose mutations also cause PKD and signaling initiates at the renal epithelial cell's primary cilia[15].

Recently, another set of insertion, non-sense and splice site mutations in the Nek1 gene were reported in Majewski type short-rib polydactyl syndrome (SRPS), an autosomal-recessive familiar ciliopathy[16,17]. Ciliopathies have been associated with a series of defects of proteins involved in intra-flagellar transport (IFT), as well as cilia, basal body and centrosome maintenance, and in the case of Nek1, SRPS also presents a broad phenotypic spectrum, including reduced cilia number and cell cycle associated cilia morphogenesis. This results ultimately in severe or lethal embryonic malformations and especially osteochondrodysplasia, shortened ribs and tibias, polysyndactyly, fused kidneys, heart defects and mouth clefts, among others[17].

In terms of molecular functions, a first breakthrough came from a protein interactome study that shed light on the involvement of Nek1 in several pathways related to the above diseases, but also opened new avenues in the context of cell cycle regulation and DNA damage responses[2]. These findings were later not only confirmed by functional studies but also extended to other Nek family members, including Nek4, 6, 10 and 11[3,8,9,18]. The interactome study was a yeast two-hybrid assay using Nek1 as bait and a human fetal brain cDNA library as prey. Nek1 is a rather large, 1258 amino acids containing protein and interacts with these proteins mainly through the two N-terminals of its four coiled coil regions, which are located at the C-terminal of its kinase domain (Figure 1). Among the Nek1 interacting proteins were the kinesin-like protein KIF3A, tuberin and alpha-catulin, mutation in all three of these genes also have been reported to cause PKD. This suggests the existence of a multicomponent signaling or regulatory pathway, which regulates the kidney cell's proliferation and when affected by mutations may lead to PKD[19-21]. Evidence in support for a major role of Nek1 in primary ciliary function also came from other model organisms, including *Chlamydomonas*[22].

Surprising at that time was the discovery of interactions with several cell cycle regulatory proteins, 14-3-3 protein η (*eta*, YWHAH), tumor suppressor p53-binding protein 1 (TP53BP1), serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit alpha/delta isoform (PPP2R5A/D) and especially with proteins involved in the DNA damage response, such as the double-strand break repair protein MRE11A (MRE11A) and the transcriptional regulator ATRX (ATRX)[2]. Soon, additional experiments with the irradiation of wild-type and Nek1-/- cells revealed that Nek1 is overexpressed and activated in response to ionizing radiation (IR) and co-localizes to γ-H2AX positive DNA repair foci in the nucleus[23]. Cells without Nek1 died in response to sub-lethal doses of IR and knockdown of Nek1 also diminished their capacity to clear DNA damage caused by chemical genotoxic agents, such as cisplatin and methyl-metanesulfonate (MMS)[24]. This line of experiments culminated recently in a paper where the authors showed that Nek1 kinase is not only physically associated with ATR-ATRIP, but also required for ATR priming to allow an efficient DNA damage signaling[25]. Furthermore, Nek1 has been indicated to act in apoptosis signaling, especially by phosphorylation of key mitochondrial proteins such as the voltage-dependent anion-selective channel protein 1 (VDAC1)[13]. This is a pore complex that functions both as a voltage dependent anion channel and permeability pore that regulates cytochrome c leakage to the cytoplasm, which upon exit initiates apoptotic events[13]. Nek1's activity to maintain cells in homeostasis is mediated through phosphorylation of a specific external VDAC1 Ser residue. Upon apoptotic stimuli, Nek1 is degraded and the lack of

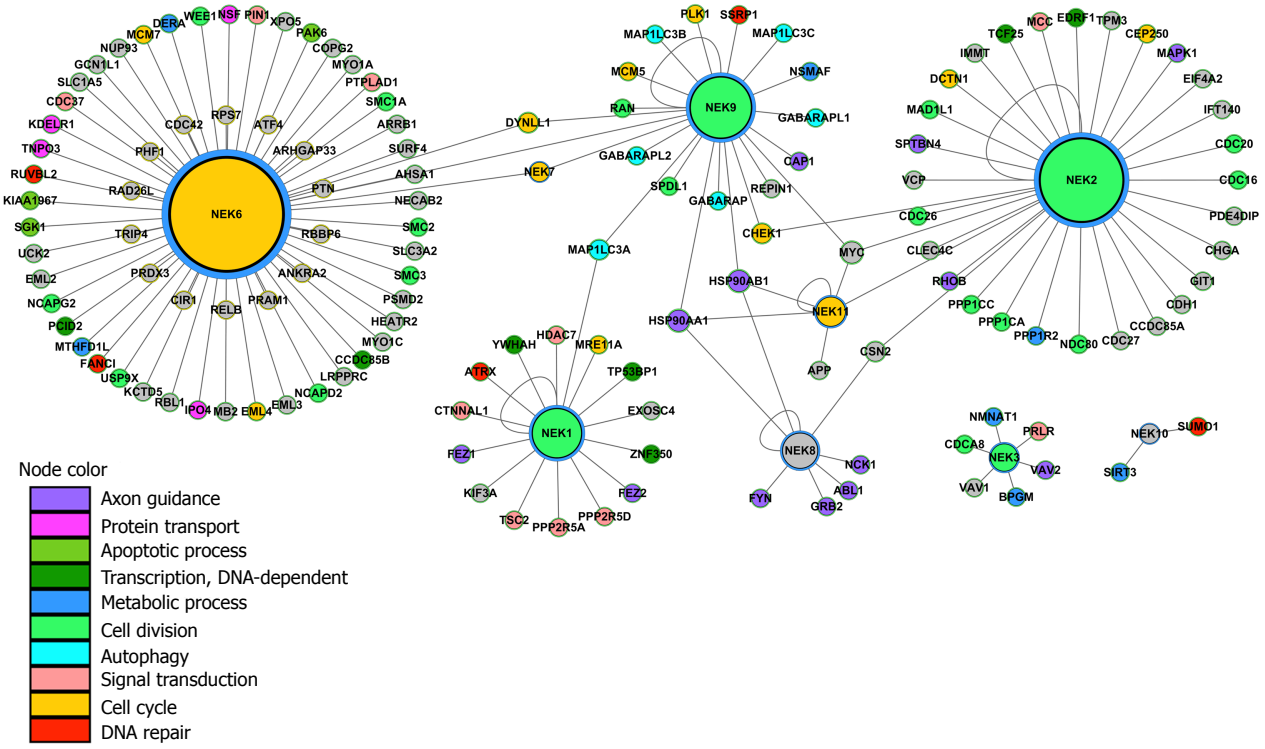

Figure 2 Global interactome of Nek1-11, involving their published interactors. The proteins color code refers to their main biological function given by the top enriched Gene Ontology[125] biological processes ($P \leqslant 0.05$). Common interactors establish crosslinks between Neks, thereby emphasizing their common functional contexts. The protein sizes are depicted proportional to their connectivity degree. The protein-protein interaction network was built for the first neighbors of Neks using the Integrated Interactome System (IIS) platform, developed at National Laboratory of Biosciences, Brazil (http://www.lge.ibi.unicamp.br/lnbio/IIS/) and visualized using the Cytoscape software[126]. Nek: Never in mitosis-gene A-related kinases.

VDAC1 phosphorylation causes opening of the channel, loss of the membrane potential and leakage of cytochrome c to the cytoplasm.

Finally, Nek1 has been implicated in gametogenesis due to its high expression levels in meiotic tissues[26]. In another interactome study, this time using a testicular tissue cDNA library, the protein Nurit was found to be an interactor of Nek1[27]. Nurit is expressed in the late phase of spermatogenesis, has structural resemblance with leucin zippers and contains additional super helix domains, possibly involved in its homo-multimerization. Furthermore, the structural maintenance of chromosomes protein 3 (SMC3) was found to interact with Nek1, further implying important functions in meiotic events such as spindle assembly checkpoints[28].

In summary, Nek1 has been functionally implied in three major functional contexts and their sub-functions: ciliogenesis (PKD, SRPS), DNA damage response in a wider sense, also including cell cycle checkpoints and centrosome functions and, finally, gametogenesis. Unpublished recent mass spectrometry studies of the Nek1 interactome after challenging cells with genotoxic drugs identified a number of nuclear proteins, the majority of which were associated with DNA repair, replication and transcription regulation. This, together with a very recent article which reports on Nek1 interaction with NHEJ (Non homologous end joining) repair protein Ku80, clearly establishes Nek1 as a key player in DDR signaling[29].

NEK2

Nek2 is the most studied and most well understood of the human Neks. In fact, it will be difficult to cover all of its aspects in the context of this review. Therefore, we focused on the most important features of Nek2 and would like to apologize to the many researchers whose work could not be covered here due to space restrictions.

Nek2 shares the highest sequence similarity with NIMA in its kinase domain and many biochemical, structural and functional features. This has led many researchers to believe that it may be the prototype NIMA among all vertebrate Neks and that Nek2 may maintain the primordial functions of NIMA in mitosis progression. For this reason, Nek2 became the most studied Nek family member in mammals[6]. However, care must be taken with such an interpretation since Nek2 cannot rescue NIMA defective mutants and Nek1 also shares many NIMA characteristics[30].

Nek2 expression varies during the cell cycle, being maximal between the S and G_2 phase, during which it localizes predominantly to the centrosome[31,32]. Nek2 is a component of the MTOC (microtubule organization center) at mitosis entry and a core component of the centrosome, where it phosphorylates the centrosomal key components C-Nap1 and rootletin, which form the intercentriolar linker that holds the pair of centrioles physically together. This event in turn promotes centro-

Table 1 Subcellular localization, established and possible additional functions of human and mammalian Neks

Nek	Gene/ protein synonyms	Subcellular localization	Established function	Possible additional functions (under investigation)
1	NY-REN-55 SRPS2, SRPS2A, KIAA1901	Cytoplasm, cilia, centrosome, γH2X positive DNA damage sites in nucleus	Stability and function of the primary cilium/polycystic kidney disease[14], DNA damage response to IR and chemical mutagens[2,23-25]	Meiosis[26-28], apoptosis mediated by mitochondria[13]
2	NEK2A, NLK1, RP67, HsPK21, SRPS2A	Centrosome	Regulation and promotion of centrosome segregation[33-35]	DNA damage response[127]
3	HSPK36, RP11-248G5.5	Cytoplasm	Regulation of prolactin response[41], microtubule deacetylation in neurons[47]	?
4	STK2, NRK2, pp12301	Cilia/basal bodies	Microtubule stability (silencing alters sensitivity to vincristine/taxol)[54]	DNA damage response[9], replicative senescence[9], primary cilia function[53]
5	-	?	Skeletal muscle differentiation[60], caspase-3 substrate/ apoptosis[60]	?
6	SID6-1512, RP11-101K10.6	Citotic spindle, centrosome	Mitotic spindle formation[11-12], centrosome separation[69-70]	DNA damage response[18], NF-kappa B signaling?[3,71]
7	-	Spindle poles	Mitotic spindle formation[12,88], centrosome separation[69-70]	DNA damage response?[1]
8	JCK, NEK12A, NPHP9, RHPD2	Centrosome, cilia, γH2X positive DNA damage sites in nucleus	Stability and function of the primary cilium/polycystic kidney disease[95], DNA damage response[10]	Integration of primary cilia function and DNA damage response[10]
9	NERCC, NERCC1, KIAA1995, (NEK8)	Spindle poles, centrosome, cytoplasm	Mitotic spindle formation[106], centrosome separation[100]	?
10	-	Possible centrosome/pericentriolar localization (?)	DNA damage response after UV induced damage[74]	Centrosome function?
11	-	Nucleus, nucleoli	DNA damage response induced by IR[73]	?

[1]Souza *et al*, unpublished observation.

some separation itself[33,34]. During the interphase, Nek2 is maintained in an inactive state by association with the protein kinase MST-2 and the phosphatase PP1, which keeps Nek2 dephosphorylated. After mitosis onset, polo-like kinase 1 (PLK1) phosphorylates MST-2, disrupting the trimeric complex and resulting in Nek2´s activation through auto-phosphorylation. In addition, the centrosomal proteins Nlp (ninein-like protein) and centrobin contain coiled coils and are dislocated from the centrosomes in Nek2 overexpression conditions. In contrast, the Nek2 knockdown or inhibition of its catalytic activity results in the inhibition of the centrosome separation[35].

A second important functional context for Nek2 is at the spindle assembly checkpoint, where through its interaction with the major kinetochore proteins Mad1/2 and the phosphorylation of the kinetochore core protein Hec1, Nek2 may be involved in the identification of unaligned sister chromatids[36]. Failure at this checkpoint may lead to aneuploidy and other chromosomal abnormalities and knockdown or knockout of other Neks, including Nek7, has been reported to cause aneuploidy, pointing to a potential major involvement of the Nek family in the spindle assembly checkpoint[37].

Another functional context for Nek2 is in the gametogenesis, where Nek2 acts in chromatin condensation reminiscent of the role of NIMA in *Aspergillus nidulans*. In spermatocytes, the architectural chromatin protein Hmga2 is under control through phosphorylation by mitogen-activated protein kinase (MAPK) and possibly also by Nek2[38].

Finally, in *Drosophila*, Nek2 was detected at the midbody in the late mitosis and overexpression of Nek2 led to actin and actin-binding protein dislocation and cytokinesis failure, among other phenotypic effects[39].

NEK3

Nek3 is a 506 amino acid serine/threonine kinase[40] and localizes both to the nucleus and cytoplasm[41,42]. It is highly expressed in testis, prostate, ovary and brain, and shows moderate to low expression in lung and liver[40]. Its gene localizes to chromosome 13q14.2 and its mRNA is expressed in tumor, normal prostate and blood control cell lines. Insertion/deletion polymorphisms were described, in which a stretch of adenines at the end of exon 9 leads to the introduction of a premature stop codon, resulting in a truncated protein that encodes only 298 or 299 of the protein amino acids. Interestingly, this polymorphism around 13q14 is a mutational hotspot for several cancer types[43-45]. Moreover, human Nek3 has an N-terminal catalytic domain and a C-terminal regulatory domain and shares high amino acid sequence identities with mouse Nek3 (56%), but not with other NIMA-related kinases due to the absence of coiled coil regions (Figure 1)[46]. This suggests that Nek3 and its orthologs constitute a separated sub-family of the Neks[40].

Nek3 is involved in the invasion and motility of T47D cells (a human ductal breast epithelial tumor cell

line) through interaction with the guanine nucleotide exchange factor VAV2, which promotes both p21-Rac1 and transforming protein RhoA activation. These interactions are mediated by prolactin-induced association of Nek3 with the human prolactin receptor (PRLR). The signaling pathway resulting from prolactin's binding to its receptor promotes phosphorylation of paxillin, a cell adhesion mediator, and is dependent on Nek3's association with VAV2[41,42].

In its C-terminal domain, Nek3 contains a PEST motif which contains Thr475, a residue that is phosphorylated upon activation. The Thr475 and the PEST domains are phylogenetically conserved, suggesting that they are important for Nek's regulation. Expression of mutants without the Thr475 or the PEST domain cause changes in cellular morphology and polarity of both epithelial and neuronal cells. Thus, Nek3 may also be crucial to the regulation of neuronal microtubules and in disorders which involve axonal degeneration, possibly through modification of its acetylation status[47].

Another functional involvement of Nek3 with cytoskeleton components is mediated through its interaction with the EH domain-containing protein 2 (EHD2). EHD2 interacts with plasma membrane phospholipids, associates with VAV1, and forms the complex VAV1-NEK3-EHD2, which modulates p21-Rac1 activity, causing actin reorganization close to the plasma membrane at the initial stages of endocytosis[48]. In summary, Nek3 plays a role in cytoskeleton organization and dynamics through actin re-organization and may be involved in the regulation of neuronal development, endocytosis, cell motility and invasiveness of breast cancer tumor cells.

NEK4

Nek4 was initially described as serine/threonine-protein kinase 2 (STK2) by Cance *et al*[49]. In a study of a kinase specific cDNA library in human breast cancer tumors or cell lines, they identified STK2 that showed homology to *Aspergillus nidulans* NIMA and expression levels that varied widely in human breast tumors. Later, Levedakou *et al*[50] showed that STK2 is highly expressed in the heart and that its mRNA level does not vary along the cell cycle. After studies characterizing the murine STK2 the nomenclature changed to Nek4[51,52].

The human Nek4 gene is located on chromosome 3p21.1 and is transcribed into about 4kb mRNA, which encodes an 841 amino acid residue protein[50]. It is constituted by a N-terminal kinase domain and a C-terminal regulatory domain (Figure 1). Hayashi *et al*(1999)[51] described a short and a long isoform for murine Nek4. The long mNek4 isoform differs from hNek4 due to the absence of a small fragment in the regulatory domain that corresponds to an *Alu* sequence[51,52]. To date, three isoforms have been described for human Nek4. The longest canonical sequence (isoform 1: UniProtAccession P51957-1, NCBI RefSeq NM_003157) was identified by the Cance and Levedakou groups[49,50] and used to compare it to mNek4. The isoform 2 (UniProt database (UniProt Accession P51957-2 , KJ592714), is identical to mNek4 and lacks the *Alu* sequence. The isoform 3 (UniProtAccession P51957-3 and NCBI RefSeq NM_001193533) is the shortest one, with a smaller alternative N-terminal region.

Hayashi *et al*[51], (1999) showed that two isoforms of mNek4 are expressed in most tissues, except in the liver and heart where only a short isoform is expressed[50]. Recently, hNek4 expression was also observed in ciliated tissues, such as the retina, kidney tubules, brain (specifically the ventricles), heart and testis[53]. Expression in testis suggests a role in meiosis, as has been already reported for mNek4[52]. Furthermore, these new functional studies demonstrated that hNek4 depletion does not alter the cell cycle[53,54]. Therefore, as shown for other Nek family members, roles other than the regulation of the cell cycle can be attributed to Nek4, including microtubule stabilization, primary cilium assembly and, more recently, replicative senescence entry and DNA damage response[9,53,54].

Interestingly, Nek4 activity is evidenced mainly in the presence of chemotherapeutic agents. For example, in lymphoma cells, a simple Nek4 knockdown is not enough to change cell cycle or microtubule dynamics, but Nek4 knockdown triggers taxol resistance and promotes sensibility to vincristine in these cells[54]. These results indicate that Nek4 has an effect on microtubule stability in the presence of these drugs and suggests that this particularity could be explored in therapies, depending on the patient's specific levels of Nek4 protein in the tumor cells.

Besides the direct role in microtubule polymerization, Nek4 is also important for primary cilium stabilization, as was already described for Nek1 and Nek8[14,55,56]. Nek4 interacts with RPGR-interacting protein 1 (RPGRIP1) and RPGRIP1-like protein (RPGRIP1L)[53], both associated with ciliopathies. Both the eye-restricted disease "Leber Congenital Amaurosis" and the "Joubert and Meckel syndrome", which affects multiple organs, are at the severe end of the ciliopathy spectrum. After Nek4 knockdown, the number of ciliated cells decreases, but this effect is apparently not related to RPGRIP1 and RPGRIP1L phosphorylation status. This suggests that Nek4 may act as a scaffold for other cilia signaling proteins[53] and, together with Nek1 and Nek8, may be important to other ciliopathies such as PKD[14,55,56].

More recently, the role of Nek4 was also connected to the DDR because Nek4 depleted cells were found to be resistant to DNA damaging agents, such as etoposide or bleomycin, and to γ-irradiation. Besides, Nek4 interacted with DNA-PKcs, Ku70 and Ku80, proteins that have important roles in the NHEJ (non-homologous end joining) repair pathway. Nek4 depleted cells also show a decrease of histone γ-H2AX activation, probably as a result of an impairment of the DNA-PKcs recruitment[9].

NEK5

Among all members of the Nek family, Nek5 is the kinase with the least amount of information. Although identified in different organisms such as *Homo sapiens,*

Mus musculus, Arabidopsis thaliana, among others, there is little information about its function and localization. In humans, Nek5 is a protein of 708 amino acids, whose kinase domain is located at its N-terminus[4,8]. According to Moniz *et al*[7], Nek5 is the only member of the Nek family that has a dead box domain (Figure 1). This domain is involved in cellular processes such as pre-mRNA processing, rearrangement of ribonucleoprotein (RNP) complexes and gene expression[57]. In *Arabidopsis thaliana*, during epidermal cell expansion, Nek5 interacts with Nek4 and 6 and these interactions are important to regulate microtubule organization, probably through the phosphorylation of beta-tubulins[58]. Therefore, Nek5 may be associated with the already established cascade consisting of Nek9, 6 and 7 (see details below). However, care must be taken because the evolutionary gap between mammals and flower-plants is too large to deduce direct conclusions and the functional information on Neks in plants is even scarcer than in mammals[59]. In human cells, Nek5 is able to interact with caspase-3 and this interaction is important for skeletal muscle differentiation[60]. Caspase-3 is a protease involved in mechanisms such as apoptosis and cell differentiation. It was proposed by Larsen *et al*[61] that caspase-3 activates caspase-activated DNase to promote and regulate DNA strand breaks introduced into promoter regions of genes encoding effector proteins such as p21 and that this process may represent a more general mechanism of genome alterations that occur during cell differentiation. Since Nek5 interacts with caspase-3 during cell differentiation, other members of this kinase family may also be involved in differentiation associated molecular events and this possibility should be explored in future experiments.

NEK6

Unlike the other Neks, Nek6 and Nek7 are the smallest and structurally the simplest Neks, consisting only of the catalytic domain with a relatively short N-terminal extension[8]. Although they share significant similarity with each other, being about 86% identical within their catalytic domains, their N-terminal extensions are not conserved and it has been suggested that they may play a role in the differential regulation of these kinases[3,62]. SAXS experiments, together with SEC-MALS and comparative molecular modeling performed by our group revealed that hNek6 is a monomeric kinase, slightly elongated, with a flexible and disordered N-terminal domain[63].

Nek6 was initially identified in a classic biochemical screen for kinases capable of phosphorylating the hydrophobic regulatory site of the p70 ribosomal S6 kinase (S6K). Nek6 phosphorylated the Thr412 residue of S6K and other sites, *in vitro* and *in vivo*, suggesting it to be a possible regulator of this kinase[64]. Subsequently, Nek6 was described as not seeming to be responsible for the physiological phosphorylation of S6K, SGK or PKB since it was characterized as having a high preference for a Leu three residues N-terminal to the phosphorylation site of the substrate[65], and more recent evidence supports a NIMA-like mitotic role for Nek6.

Both Nek6 and Nek7 co-purify with Nek9 as a result of specific interactions and strong binding to a region located between the RCC1 domain and coiled coil motif of Nek9[66] (Figure 1). The endogenous Nek6 is activated during mitosis, concomitant with an increase in its level of expression, but this requires phosphorylation at the Ser206 residue, which is mediated through Nek9. Nek7 too is phosphorylated by Nek9 at Ser195 and both phosphorylation sites are found in the activation loops of these kinases[67]. This information led to the construction of a model in which Neks 6, 7 and 9 act as partners of the same signaling cascade[67], with Nek6/7 being substrates of Nek9. However, Nek9 remains inactive during the interphase but is activated during mitosis, phosphorylating and activating Nek6/7, which, in turn, coordinates the organization and maintenance of the mitotic spindle[66].

Overexpression of a catalytically inactive mutant of Nek6 generates cells displaying high mitotic index, defects in mitotic spindle, nuclear abnormalities and apoptosis[11]. These phenotypes are also observed from the depletion of Nek6/7 in HeLa cells using siRNA, which causes retention of cells in metaphase, with a normal chromatin condensation and alignment, but an inability to complete the segregation of chromosomes. The activity of Nek6 and also 7, therefore, seems necessary for the progression of anaphase, where the cells are either retained at the spindle assembly checkpoint (SAC), or undergo apoptosis or complete mitosis, but with an elevated risk of acquiring chromosomal abnormalities during the process[11,12]. Moreover, treatment of these depleted cells with an Aurora B inhibitor to bypass the SAC led to a reduction in the frequency of metaphase arrest, concomitant with an increase in the frequency of cells blocked in cytokinesis. Cells expressing the hypoactive mutants, even in the absence of the SAC inhibitor, also accumulated in cytokinesis. Therefore, Nek6 and Nek7 seem to have independent, non-redundant roles in mitotic spindle formation and cytokinesis: one at metaphase that requires a certain level of kinase activity and one in late mitosis that requires a higher level of activity[12].

Intriguingly, using phospho specific antibodies that detect activated Nek6, Rapley *et al*[68] showed that Nek6 activity increased 2 h after release from a nocodazole arrest, when cells would be progressing through cytokinesis. In this same study, the kinesin-related motor protein Eg5, required for spindle bipolarity, has also been described as a substrate of Nek6. It phosphorylates Eg5 kinesin *in vitro* at several residues, including Ser1033, which is also phosphorylated *in vivo* during mitosis at the spindle poles[68]. A signaling cascade seems to occur where Nek2 first phosphorylates proteins at the intercentrosomal linker in G_2 phase, resulting in their dissociation, followed by activation of Nek9 by the cyclin-dependent kinase 1 (CDK1) and the polo-like kinase 1 (PLK1) in early mitosis and subsequent activation of Nek6 and Nek7. These

kinases, in turn, phosphorylate Eg5 (previously phosphorylated by CDK1), promoting the separation of the centrosomes by the motor activity of Eg5 accumulated in the centrosomes[69,70].

Apart from roles in mitosis, human Nek6 was recently reported by our group to have a broad set of protein partners involved in diverse biological processes[3]. The hNek6 interactome showed that it is a high confidence hub kinase possibly involved in several known and novel cellular pathways, through interactions with and phosphorylation of diverse proteins. Figure 3 depicts some of the main cellular pathways identified for hNek6 based on the interacting proteins retrieved by our screenings. The novel putative pathways shown are the non-canonical Wnt signaling, Notch signaling and the actin cytoskeleton regulation, whereas the other pathways were already suggested by other studies: the nuclear factor kappa B (NF-κB) signaling[71] and the DNA damage response[18]. In regard to the DNA damage response category identified in our work, many studies show its importance among the tasks triggered by Neks[2, 8-10,18,23-25,72-74].

On the other hand, Nek6 phosphorylates the transcription factor Oct-1 (POU2F1), a potent regulator of metabolism and tumorigenicity, at S335 in the DNA binding domain during mitosis, causing Oct-1 to dissociate from the chromatin and concentrate in the centrosomes, spindle poles, kinetochores and midbody[75]. Furthermore, Nek6 phosphorylates histones H1 and H3 *in vitro*, possibly contributing to mitotic chromatin condensation[76]. Nek6 finally also binds the BTB/POZ domain-containing protein KCTD5, which appears to have a role in cytokinesis[77] and apoptosis[78].

As the other human Neks, hNek6 was recently found to be linked to carcinogenesis. It shows an increased expression and activity in gastric cancer according to the progression of the disease[79] and up-regulation of Nek6 mRNA correlates with the Peptidyl-prolyl cis-trans isomerase Pin1 up-regulation in 70% of hepatic cell carcinomas[80]. The overexpression of a catalytically inactive Nek6 promotes cell cycle arrest in human breast cancer in metaphase and leads to apoptosis[11], while its knockdown induces senescence and also apoptosis[81]. In a large-scale screening of serine/threonine kinases on different types of human tumors, Nek6 was shown to be up-regulated in non-Hodgkin's lymphoma, breast, colorectal and lung tumors[82]. Moreover, NEK6 gene, besides AURKA, has its expression increased in esophagitis and esophageal adenocarcinoma, representing a promising candidate marker of these diseases[83]. Recently, it was demonstrated that transcript, protein and kinase activity levels of Nek6 were highly elevated in malignant tumors and human cancer cell lines compared with normal tissue and fibroblast cells, indicating an important role for Nek6 in tumorigenesis[84]. Its phosphorylation at Thr210 and Ser206 is critical for the phosphorylation of STAT3 (signal transducer and activator of transcription 3) at Ser727[85]. Furthermore, its overexpression suppresses p53-induced senescence in cancer cells: it inhibits the cell cycle arrest at both G_1 and G_2/M transition, the reduction in the Cdc2 and cyclin B levels and the increase in ROS levels induced by p53[86]. Its overexpression also makes cancer cells resistant to premature senescence induced by the anti-cancer drugs camptothecin and doxorubicin[87]. The inhibition of the Nek6 function sensitizes human tumor cells to premature senescence after anti-cancer drug treatment or serum depletion[81], suggesting Nek6 to be a potential therapeutic target for various types of human cancers.

NEK7

Human Nek7 was originally described as a possible regulator of the p70 ribosomal S6 kinase[64] and of important events in the mitotic progression[12,6,67,88] (see above for Nek6). These findings have led to studies on the regulatory effects of hNek7 in key functions of the cell cycle and in cancer. The siRNA-mediated down-regulation of hNek7 and expression of kinase inactive mutants reduced centrosomal γ-tubulin levels in interphase cells and caused prometaphase arrest with defects in mitotic spindles[6,88]. Nek7 overexpression in culture cells, on the other hand, resulted in multinucleated cells and a higher proportion of apoptotic cells[89]. In the same line, the Nek7 depletion also decreased microtubule stability, while its ectopic overexpression rescued this phenotype[90]. Furthermore, hNek7 deficient mice die early in development and, on a cellular level, lack of Nek7 led to decreased chromosome numbers, increased centrosome numbers, binucleation, micronuclei formation, cytokinesis failure, growth retardation or cell death[37]. The PCM (centrosomal pericentriolar material) proteins do not accumulate at the centrosome in Nek7-depleted cells in the G_1/S and G_2/M transitions[91], indicating that Nek7 is required for centriole duplication, centrosome maturation and mitotic spindle formation[88].

The direct interaction of Nek7 with the non-catalytic domain of Nek9 allosterically activates Nek7 by interruption of its autoinhibitory conformation[92]. Consistent with these findings, recent studies demonstrated that PLK1 and CDK1 control the centrosome separation through phosphorylation and activation of Nek9 during mitosis. This leads to the Nek6/7-dependent phosphorylation of kinesin Eg5, a key event for centrosome separation and mitosis[69]. Thus, as in the case of Nek6, it is not surprising that cancer cells express elevated levels of Nek7, suggesting a role in tumor progression. Higher expression levels of Nek7 were found in larynx, breast, colorectal[82] and gall bladder cancers[93]. Taken together, these findings suggest Nek7 as a potentially important regulator of the cell cycle and reveal it as an essential component for growth and survival of mammalian cells. Furthermore, the linkage with a failure in centrosome biogenesis, chromosomal stability and ploidy, as well as the observed disturbance of microtubule dynamics connects Nek7 to hallmark features of oncogenesis.

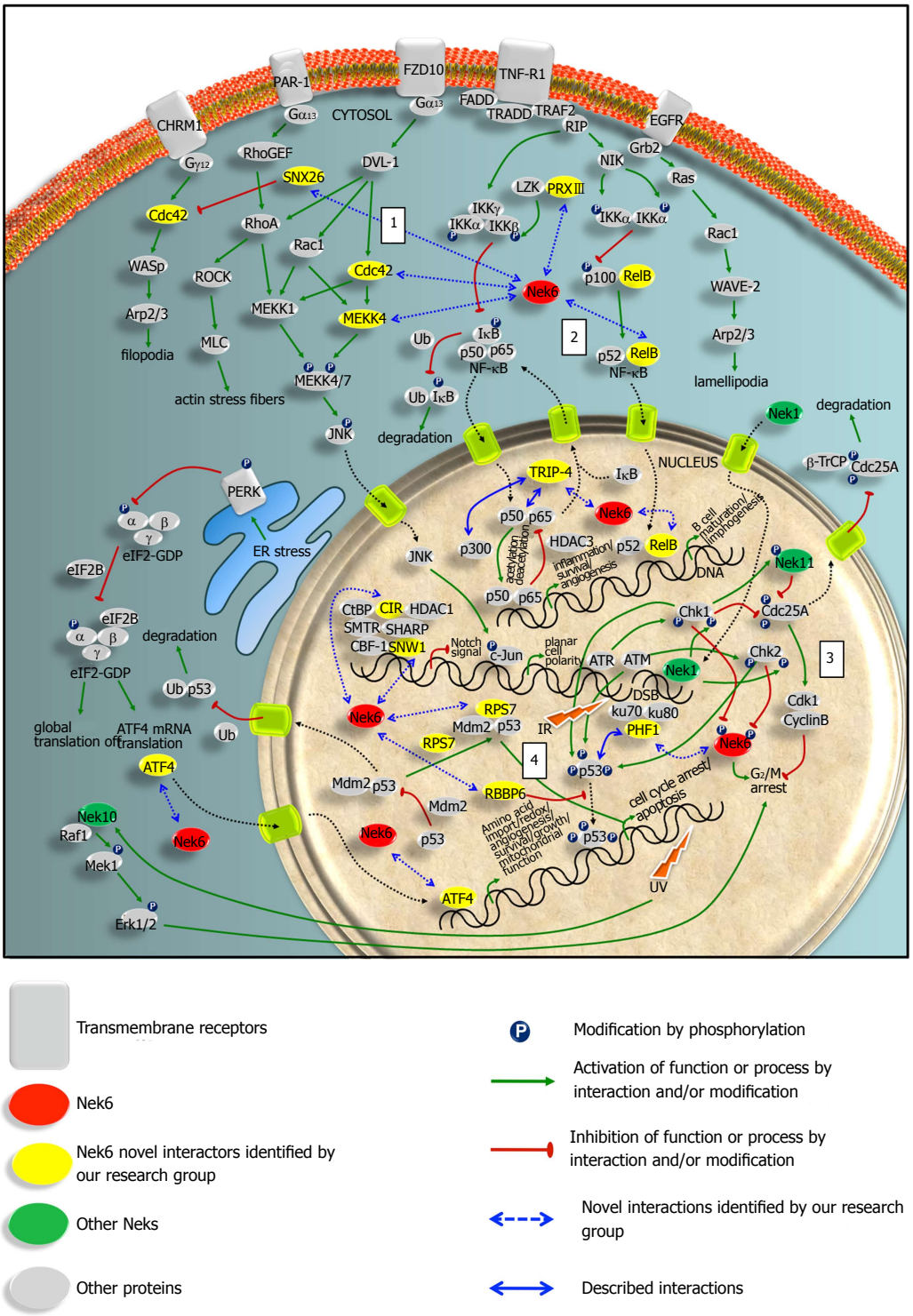

Figure 3 Nek6 interactome and the cellular functional contexts based on its interacting proteins. The four major pathways discussed in the text are: (1) actin cytoskeleton organization; (2) nuclear factor-κB signaling; (3) DNA damage response; (4) p53 signaling (according to Meirelles *et al*). See detailed legend for symbols at the bottom of the figure. IR: Ionizing radiation.

NEK8

Nek8 was first described as the mutated gene in murine autosomal recessive juvenile cystic kidney (*jck*) mice[55]. As observed for Nek1, these mutational changes found in Nek8 C-terminal domain can cause genetic kidney diseases, including polycystic kidney disease (PKD)[55]. PKD is one of the most frequent genetic kidney diseases and has a highly variable pathology, involving aberrant cell proliferation in the kidney and pleiotropic effects in multiple other organ systems, including the liver and the pancreas. Evidence that renal cyst formation is caused by defects in ciliogenesis or ciliary function is substantial[56]. In mouse cells, Nek8 localizes to the proximal region of

the primary cilium and is not observed in dividing cells[56]. In humans, Nek8 is overexpressed in primary breast tumors[94] and localizes to centrosomes and the proximal region of cilia in dividing and ciliated cells, respectively. The localization of Nek8 to centrosomes and cilia is dependent on both the kinase activity and the C-terminal non-catalytic domain homologous to RCC 1 (regulator of chromosome condensation). It is capable of autophosphorylation in the non-catalytic C-terminal region to regulate its localization or activation. Its activity is not cell cycle regulated but, in the same way as observed for Nek3, activity levels are higher in G_0-arrested cells. The kinase domain alone, although catalytically active, does not localize correctly, while a fragment containing only the RCC1 domain shows correct localization and can also be phosphorylated by Nek8[95].

Nek8 carries the causal mutations of two of the eight established mouse models of polycystic kidneys (*jck*). In these models, an abnormal interaction between Nek8 and the polycystin complex may give rise to PKD by disturbing microtubule dynamics, the mitotic spindle checkpoint and the cytoskeleton. Nek8 mutations cause overexpression of galectin-1, sorcin and vimentin and accumulation of the MUP (major urinary protein) in renal cysts of *jck* mice[96].

The role of the RCC1 domain in Nek8 is yet unknown. However, a single G448V substitution is responsible for the *jck* phenotype[55]. Overexpression of mutant forms of Nek8 (including G448V) in tissue culture cells leads to the formation of enlarged multinucleated cells and reduced numbers of actin stress fibers, although tubule cells in *jck* mice are not multinucleated, suggesting that the cellular role of Nek8 may be related to the regulation of the cytoskeleton[55].

Co-immunoprecipitation experiments demonstrated that Nek8 interacts with polycystin-2 (PKD2), a mechanosensing receptor protein, involved in the regulation of the cilium length. However, the *jck* mutation of Nek8 did not apparently affect this interaction directly. These data suggest that Nek8 interferes with the polycystic signal transduction pathways and/or the control of the targeting process of these ciliary proteins. Dysfunction of Nek8 may lead to cystogenesis by altering the structure and function of cilia in cells located at the distal nephron[97].

Recent results suggest that Nek8 has a function in the maintenance of genomic stability[10]. Loss of Nek8 leads to spontaneous DNA damage and a defect in the response of cells to replication stress. Furthermore, Nek8 interacts physically and functionally with components of the ATR-mediated DDR. The disease-related *jck* mutant of Nek8 fails to both interact with the ATR pathway proteins and to rescue the genome maintenance defects associated with Nek8 knockdown. Thus, Nek8 is a critical component of the DDR that links replication stress with primary ciliary functions and the related cystic kidney disorders[10].

NEK9

Nek9, also called Nercc1, is one of the largest Neks with 979 amino acids, with an extensive C-terminal regulatory domain, which contains seven RanGEF homology repeats, an RCC1 domain, a segment rich in Ser/Thr/Pro residues and, like in Nek2, a coiled coil dimerization motif (Figure 1)[66,98].

Nek9 was first described as Nek8 and isolated with a catalytic activity against beta-casein in rabbit lung extracts treated with IL-1, revealing the co-chromatography of a second protein homologous to the Drosophila bicaudal D protein, Bicd2, which is *in vitro* phosphorylated by Nek9 and resembles a cytoskeleton structure[99]. Moreover, Nek9 immunoprecipitation of *Xenopus laevis* egg extracts showed γ-tubulin and other members of the γ-tubulin ring complex (γ-TuRC), which are essential for the microtubule nucleating activity of the centrosome[98]. Centrosomal γ-tubulin recruitment depends on the adaptor protein NEDD1 and is controlled by PLK1. In a recent study by Sdelci *et al*[100], it was reported that PLK1 activates Nek9, which phosphorylates the Ser377 in NEDD1, promoting its recruitment together with γ-tubulin to the centrosomes of dividing cells (independently of Nek6/7). Furthermore, the microinjection of anti-Nek9 in human cells during prophase, after the chromosomes condensation, interferes in the organization of the spindles and the proper segregation of chromosomes, resulting in cell cycle arrest in prometaphase or aneuploidy[66].

Nek9 expression remains constant in different cell cycle phases (G_1/S, G_2, M, G_1); however, as observed for NIMA, there is a specific increase in its catalytic activity during mitosis, which was found to be triggered by *in vitro* and *in vivo* phosphorylation events[66]. The recombinant wild-type Nek9 shows reduced activity when extracted from exponentially growing cells, but its pre-incubation with ATP and Mg^{2+} induces its autophosphorylation at its activation loop Thr210 residue and its activation, whereas mutants lacking the coiled coil dimerization motif show significantly reduced activity[66,98]. Interestingly, the deletion of the RCC1 region leads to a catalytic hyperactivity, indicating that this region may be required for Nek9 autoinhibition[66]. Moreover, Nek9 binds to dynein light chain 1, cytoplasmic (DYNLL1), a highly conserved protein originally described as a component of the dynein complex, *via* its C-terminal (K/R) XTQT motif adjacent to Nek9 C-terminal coiled coil motif, resulting in Nek9 oligomerization, an increase in its autoactivation rate and a reduction in its binding to Nek6[101].

It is possible that Nek9 activation in mitosis involves a very small percentage (< 5%) of the total expressed protein, and in contrast with the vast majority of inactive protein, the active Nek9 (Thr210P) is first evident during prophase, concentrated at the centrosome, where it can be phosphorylated by CDK1/cyclin-B[102], until metaphase is reached. During the transition to anaphase, the immunoreactivity of Nek9 (Thr210P) decreases at the centrosomes and becomes detectable at the chromo-

somes, which is evident until telophase. Before disappearing, the active Nek9 is detected at the midbody as two points flanking the cleavage furrow during cytokinesis[98].

Due to its possible roles in the mitotic spindle organization and chromosome segregation through its activation during mitosis and interaction with Nek6/7, it is possible that most of the phenotypes observed with the microinjection of anti-Nek9 antibodies in human cells are caused by interference with Nek6/7 function[66]. Taken together, the data suggest that Nek9 is a positive upstream regulator of Nek6/7.

Among other kinases, Nek9 was recently identified by quantitative chemical proteomics as a possible marker for the diagnosis and therapy of head and neck tumors[103]. Moreover, Nek9 shows, along with other kinases implicated in cancer, its activity inhibited by the drug quercetin[104]. Its expression is increased in chronic myeloid leukemia cells resistant to imatinib[105], indicating that its up-regulation could be involved in chemotherapy resistance mechanisms. Depletion of Nek9 in glioblastoma (U1242) and renal carcinoma (Caki2) cells results in failures in cytokinesis and cell death in Caki2 cells, after overriding mitosis, and incorrect alignment of chromosomes and micronuclei formation. Therefore, it is suggested that inhibition of Nek9 is a potential anti-cancer therapeutic strategy by induction of mitotic catastrophe *via* reduced dynamics of the spindle, cytokinesis and mitotic checkpoint control[106].

NEK10

One of the most intriguing but less studied members of the Nek family is Nek10 since it has its catalytic domain flanked by two regulatory domains (Figure 1). Each of these two regulatory domains has their own peculiarities. As NIMA and Neks 1, 2, 5, 9 and 11, Nek10 also has coiled coil regions closely located to the kinase domains[8]. Furthermore, four repetitions of an armadillo repeat motif in its N-terminal regulatory domain may serve as an important region for protein-protein interactions, as has been reported for other proteins[107]. In the case of its C-terminus, a PEST region may be important to the proteolytic regulation of the protein's abundance. There are some contradictions and a debate about Nek10's full length since several different cDNAs have been deposited that differ in the C-terminal domain length.

Mutations in the Nek10 gene locus have been linked to breast cancer in different studies that were trying to find new polymorphisms in carriers of mutations in BRCA1/2 (breast cancer type 1/2 susceptibility protein)[108-110]. Moniz *et al*[74] have shown an important role for Nek10, comparing normal and tumor mammary gland cell lines. They found that Nek10 affects the ERK1/2 (extracellular signal-regulated kinase 1/2) signaling pathway, after activation with UV radiation. Nek10 has been shown to form a functional complex with RAF1 and MEK1 (dual specificity mitogen-activated protein kinase kinase 1). In this sense, cell cycle arrest in G_2/M was observed and Nek10 caused both MEK1 activation and the ERK1/2 phosphorylation. However, these preliminary data suggest a possible involvement of Nek10 in the DDR, as already demonstrated for Nek1, 4, 6, 8 and 11[2,8-10,18,23-25,72-73]. Moreover, like BRCA1 and BRCA2, Nek10 may be a therapeutic target in breast cancer.

NEK11

Nek11 is one of the least studied Nek family members and has the highest sequence similarity to Nek4. Its gene is present on the same chromosome as that of Nek4 but on the long arm (3q22-1). Nek11 was first identified by Noguchi *et al* (2002)[111] and shows a high sequence similarity with Nek4 and 3 in its kinase domain, but is more similar to Nek2 in its regulatory region (Figure 1). Interestingly, Noguchi *et al*[111] have not found Nek4/11-related kinases in *C. elegans* or *D. melanogaster*, suggesting that the Nek11-containing subfamily may have only appeared through gene or genome duplication after separation of the deuterostome branch in the animal kingdom[111].

Noguchi *et al*[111] (2002) described two isoforms for Nek11. The longer isoform (Nek11L) is composed of 645 residues, while the shorter one (Nek11S) contains only 470 residues. Nek11 shows a N-terminal kinase domain and a C-terminal regulatory domain with a coiled coil and three PEST sequences, suggesting a proteolytic, cell cycle specific regulation of its expression. Nek11, different from Nek1, 2 and 4, is not present in a higher quantity in the testis or ovary, but its mRNA is found in the brain's cerebellum, trachea, lung, appendix and uterus[111]. Another important difference to Nek4 is that Nek11 shows a timely cell cycle related expression pattern, relating it closer to Nek2, with both showing an expression peak at the G_2/M transition.

The first indication that Nek11 could be important in the regulation of cell cycle checkpoints was the identification of histones H1, H2A and H3 as Nek11 phosphorylation substrates. Furthermore, in the presence of genotoxic agents, Nek11 showed both an increased expression and activity at the G_2/M transition. Although this is decreased by caffeine, suggesting that Nek11 DDR may be associated with the ATM/ATR pathways, which also showed the same inhibition by caffeine[111].

Another common point between Nek11 and Nek2 is their localization to the nucleolus. In the study of Noguchi *et al*[112] (2004), it was observed that in U2OS cells Nek11L is present in the nucleolus during interphase and telophase and that it probably interacts with Nek2A in the nucleolus. Moreover, Noguchi *et al*[112]speculated that Nek2A could phosphorylate Nek11L C-terminal and, in this way, antagonize its auto-inhibitory function, which would cause Nek11 activation in G_1/S arrested cells[112].

Recently, some of Noguchi's results were followed up by Melixetian *et al*[73]. This study points to Nek11 as an important player in cancer development. Melixetian *et al*[73] observed that Nek11 depleted U2OS cells lose an important G_2/M checkpoint after IR. In this way, it was

verified that after IR Chk1 phosphorylates both M-phase inducer phosphatase 1 (CDC25A) and Nek11. Nek11 in turn also phosphorylates CDC25A, leading to its proteasomal degradation and subsequent inhibition of cyclins followed by a cell cycle arrest at the G2/M transition.

The studies involving Nek11 so far point to it as an important protein for the cell cycle regulation in the context of the DDR. However, more interactome studies are required to clarify other possible functions of Nek11 in the cell.

DISCUSSION

After knowing sufficient details on all of the eleven individual Neks, we will now return to a more general and integrative approach and try to find common functional contexts for the family as a whole in human cells. As pointed out in the introduction, Neks may be assigned to three major functional contexts: (1) centrioles and mitotic spindle functions; (2) primary ciliary function; and (3) G2/M phase associated DDR. Although most individual Neks have been associated with one main context, recent functional data as well as the identification of interaction partners for several Neks from two or even all three contexts may suggest that Neks have a broader function, possibly on a regulatory level, that consequently affects the three main functions. A first way of looking at this is by comparing the interaction profiles and functional contexts of the published interacting partners, summarized in Figure 2, which shows the Neks global interaction profile and the possible new biological processes in which they are involved due to their interaction with multiple proteins.

Several protein interactors with violet color interact with Nek1, 2, 3, 8, 9 and 11 and can be described as associated with the "axon guidance"/transport processes. They include, for example, fasciculation and elongation protein zeta (FEZ)-1 and 2 that interact with Nek1[2,113,114].

Several proteins associated with apoptotic processes interact with Nek6: serine/threonine-protein kinase PAK 6 (PAK6), serine/threonine-protein kinase Sgk1 (SGK1) and DBIRD complex subunit KIAA1967 (KIAA1967) (darker green color).

Nek9 interacts with several proteins from the autophagy-related protein 8 family (GABARAP, GABARAPL1, GABARAPL2, MAP1LC3A, MAP1LC3B and MAP1LC3C) (light blue).

Several proteins from DNA repair processes interact with either Nek1, 6, 9 or 10: RuvB-like 2 (RUVBL2), Fanconi anemia group I protein (FANCI), transcriptional regulator ATRX (ATRX), FACT complex subunit SSRP1 (SSRP1) and SUMO-1 (SUMO1) (red). The putative DNA repair and recombination protein RAD26-like (RAD26L), the PHD finger protein 1 (PHF1), and also the double-strand-break repair protein rad21 homolog (RAD21, not shown in Figure 2), all identified as Nek6 interactors in our yeast two-hybrid screens[3], are also possibly involved in the DDR[115,116].

In order to demonstrate the potential discovery of additional functional contexts through interactomics studies, we will now have a closer look at the Nek6 interactome as described by our group[3] (Figure 3). Novel Nek6 interacting partners are indicated by yellow ellipses and suggest the following new functional contexts: (1) Nek6 is possibly involved in actin cytoskeleton organization through its interaction with cell division control protein 42 homolog (CDC42) and sorting nexin-26 (SNX26)[3]. Since SNX26 has a negative regulatory role on CDC42 and Nek6 interacts with both of them, the final output of Nek6 must be addressed by future experiments. However, these findings are supported by the fact that for Nek3 a clear involvement in related processes has been reported (see Nek3 section above); (2) Nek6 may be involved in the activation of the NF-κB signaling on multiple layers, since it interacts with the transcription factor RelB, Prx-Ⅲ and/or TRIP-4[3,71]. Matsuda *et al*[71] found Nek6 as an activating protein in a siRNA knockdown screen to identify proteins that participate in the regulation of cellular survival transcription factor NF-κB[71]. The regulation may occur on several levels: through direct phosphorylation, interaction or regulation of the nuclear translocation of key components of the NF-κB complex, like RelB, or even on the transcriptional level. The latter seems likely, since Nek6 also interacts with SNW domain-containing protein 1 (SNW1) and a PHF domain containing protein (PHF1)[3], both of which have been recently identified as key components involved in the complex, multiprotein machinery involved in the transcriptional activation of the NF-κB gene[117]. Again, Nek6 regulatory role here may be mediated through interaction and/or phosphorylation; (3) the IR-induced DNA damage response is mediated by Nek1, 6 and 11, leading to cell cycle arrest[18,23,25,72,73]. The UV-induced DNA damage response is mediated by Nek10, also leading to cell cycle arrest[74]. This may suggest that different Neks may have specialized to mediate different forms of DNA damage responses; and (4) it is known that Nek6 can counteract p53 induced senescence[86]. As we can observe in Figure 3, this may occur indirectly through Nek6 modulation of p53 interactors 40S ribosomal protein S7 (RPS7) and/or E3 ubiquitin-protein ligase RBBP6 (RBBP6). It is worth noting here that Nek4 has the opposite effect of Nek6. Nek4 seems to be required for the cell to enter in senescence[9].

Another important point is the finding that certain functions first only described for isolated specific Neks have been later confirmed for most if not all other Neks. Nek1 was the first family member to be associated with DDR signaling events[23]. In our yeast two-hybrid screen to identify Nek1 interacting proteins, we identified proteins involved in the repair process itself (MRE11A) and in different signaling pathways associated with it (ATRX, PPP2R5 A/D, YWHAH, TP53BP1) (Figure 4).

Nek4, 6, 8, 10 and 11 have also been reported to physically interact with key members of DDR pathways or to interfere functionally in signaling cascades in a

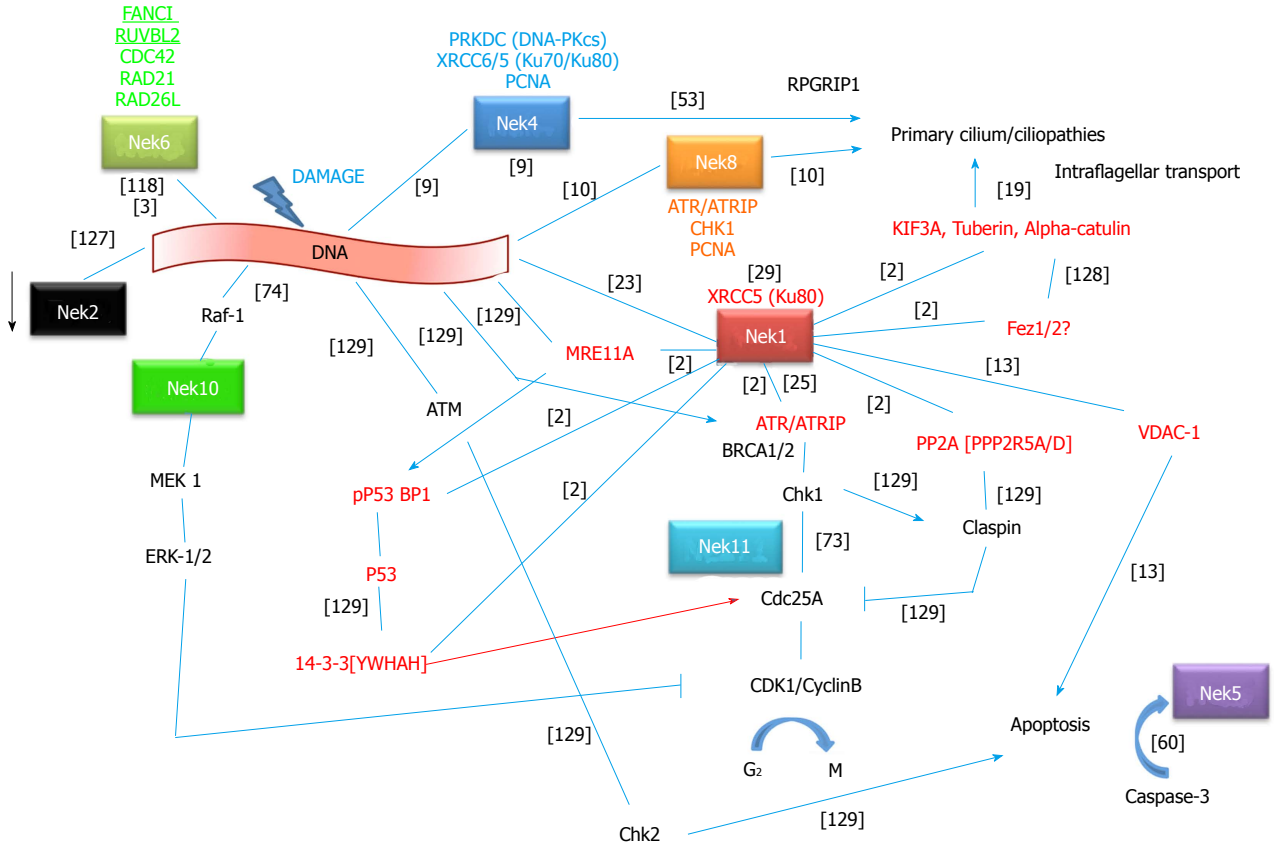

Figure 4 Nek1 interactome and crosstalk with other Neks and protein interactors in the context of the DNA damage response pathways. Interactions between proteins are depicted as simple lines, activation is depicted as an arrow and inhibition as an arrow with a line as arrowhead. A red arrow for 14-3-3 means that it causes activation by the transport of CDC25 to the nucleus. Nek1 interacted with a specific 14-3-3 isoform called YWHAH[2] (gene symbols inside brackets correspond to the isoforms of those proteins which were described to interact with Nek1). Not necessarily the same specific 14-3-3 protein promotes the indicated functions. Rather, a family characteristic is intended to be assigned. Nek2 kinase activity is inhibited after DNA damage (arrow)[127]. The red protein names are those that have been identified to directly interact with Nek1 as identified by the yeast two-hybrid system[2] or other as indicated in the figure. Gene symbols above/under protein names represent other interactors of those proteins. Nek4 interactors have been identified by mass spectrometry[9]. As can be seen, all but three Neks (Nek3, 7 and 9) seem to be directly linked to the DNA damage response. Most strikingly, we can see a direct connection for Nek8, 4 and 1 between DDR and primary cilium function and ciliopathies. New connections to apoptosis have been recently pointed out for Nek1 and 5. References for interactions are depicted in brackets: Nek6[3,118]; Nek1[2,13,25]; Nek4[9,53]; Nek8[10]; Nek11[73]; Nek10[74]; Nek2[127]; Nek5[60]; KIF3A[19]; Fez1/2[128], various known interactions[129].

broader context of the G2/M transition[8-10,18,73-74]. As described above for Nek6, the interactors RAD26L, PHF1, RAD21[3], FANCI and RUVBL2[118] are all associated with the DDR. Together with the relatively recent work by Lee *et al* (2008)[18], this suggests Nek6 may also interfere in DDR. However, the stimuli that activate such possible pathways *via* Nek6 are still unknown. In further yeast two-hybrid screens and mass spectrometry interactomics studies we found other DDR members interacting with Nek3, 4, 5, 7, 8, and 10 (unpublished data). Recent publications clearly confirmed part of those findings or went beyond them by characterizing this new involvement not only functionally, but also establishing possible cross-connections between primary cilia signaling and DDR in the case of Nek8[10]. For Nek4, an involvement in senescence signaling was established and in mass spectrometry experiments, several DDR proteins such as DNA-PKcs (PRKDC), Ku70/Ku80 (XRCC6/5) and PCNA were identified as Nek4 interacting proteins (Figure 4)[9]. Furthermore, Nek4 has been reported to interact with RPGRIP at the primary cilium[53], thereby establishing another link between DDR and primary cilium function.

A new role for Nek5 in differentiation and apoptosis signaling has been identified and characterized through its interaction with and proteolytic processing by caspase-3[60]. Evidently, apoptosis signaling is closely related to DDR and the G2/M checkpoint because cells unable to repair major DNA damage must either halt in the cycle or be dispatched by apoptosis. The link between Neks, DDR and apoptosis is not new as Chen *et al*[13] had also already reported an interaction of Nek1 with mitochondrial VDAC1. Nek1 phosphorylates VDAC1 and prevents apoptosis by avoiding VDAC1 opening and leakage of cytochrome c, which would activate apoptotic caspases. The down-regulation of Nek1 protein level or kinase activity through apoptosis signaling decreases VDAC1 phosphorylation and results in its opening and leakage of cytochrome c, thereby activating the apoptosis program.

For Nek1, the coexistence of functional roles in both DDR and ciliopathies and primary cilia function has been long established (Figure 4). Nek1 interacts with several proteins involved in the primary cilia function

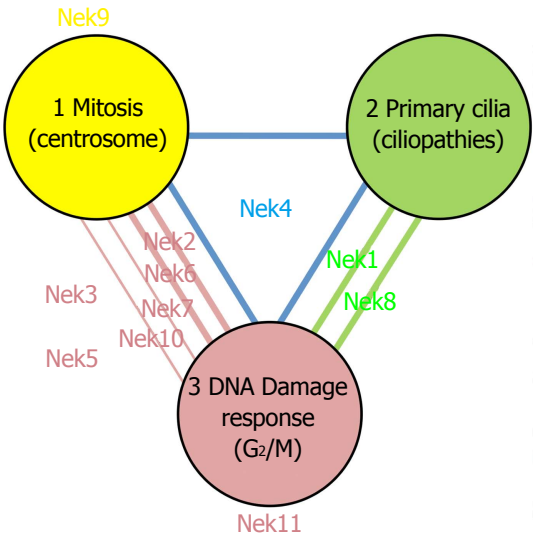

Figure 5 Functional overlap in the human Nek kinase family: seven of eleven Neks participate in two and one Nek in all three of the main core functions of the Nek family (centrosome-related mitosis, primary cilia and DNA damage response). The three corners of the triangle represent each a key concept function for the Nek family, *e.g.*, Nek9 and 11 sole involvement in mitosis[66,67] and DDR[73] respectively, has been well documented. The Nek names and bold lines represent cases where accumulated experimental evidence strongly suggests a regulatory role for that Nek in that context or in both of the contexts the line connects: Nek1[2,22,23]; Nek2[123]; Nek4[9,53] (Basei *et al* unpublished); Nek6[3]; Nek7[67]; Nek8[8,10]; Nek10[74]. The thinner lines represent our own group's preliminary or unpublished interaction data (both from yeast two-hybrid system and immunoprecipitation coupled to mass spectrometry analysis data), suggestive of a participation of that Nek in both connected functions (Nek7: Souza *et al*, unpublished).

and especially in kidney duct mechanosensing (KIF3A, tuberin, alpha-catulin, polycystin 1/2). Mutations in the genes that encode all of these proteins like those that cause expression of truncated non-functional Nek1 itself, cause PKD[14]. Since Nek8 is functionally and evolutionary most closely related to Nek1 among the Nek family, it came as no surprise that Nek8 mutations were also found to cause ciliopathies and cystic kidney disease. Moreover, Nek8 interacts with some key DDR proteins, including ATR, Chk1 and PCNA, just like Nek1[10]. What is new in these milestone discoveries, however, is the possibility that somehow these two pathways are causative or coincidentally connected. Choi *et al*[10] made the observation that mice cells with diminished Nek8 kinase activity, simulating a kidney ciliopathy, already show a constitutive activation of DDR pathways in the embryonic phase, as evidenced by repair foci in their kidney cells nuclei. This raises a couple of possibilities to consider: either the cilia have some function in the sensing of DNA damage or in transmitting downstream events, or otherwise, the cilia defects somehow transduce (*via* Nek8) to a possible lack of repair of replication defects. Of course a simpler explanation could be that both phenomena are affected simply because Nek8 participates in both of them simultaneously. However, an additional possibility is that Nek8 acts on a higher regulatory level that coordinates both pathways based on the necessity of the cell to coordinate these events closely during the course of the cell cycle. Clearly, further studies are necessary to evaluate these new possibilities. However, it seems to be clear now that the three central functions controlled by Neks, mitosis, primary cilia and DDR, are more connected than previously expected and that several if not all Neks participate in more than one of them.

A possibility exists that the Neks *per se* are the key regulatory elements that may connect these three functions. The seemingly functional redundancy may in fact rather represent connecting elements between hitherto non-connected regulatory circuits (Figure 5), *e.g.*, between primary ciliary function and DDR for Nek8[10] and Nek1[2,23,14]. Furthermore, these circuits may cooperate in a concerted one or two-directional fashion (Nek8).

Most interestingly, from a cilium perspective, recent evidence also indicates a strong link between cilia, stress responses and DNA damage repair processes. A recent study showed that environmental stresses, including UV and IR, result in altering the protein composition of centriolar satellites, thereby promoting de novo ciliogenesis[119]. Together with the recent findings that ciliopathy-associated mutations in DNA damage key regulators (*e.g.*, Mre, Znf423) also connect cilia and DDR[120-124], it is tempting to speculate that cilia may act as platforms for cell cycle checkpoints or the DDR.

CONCLUSION

Clearly, the past 10 years have provided new and exciting insights into the multifaceted functions of this interesting protein kinase family and the future promises to hold more surprises and the discovery of new functional connections. An exciting time has come to the field of Nek research and the Neks are ready to step out of the shade and take a main role along the other important cell cycle regulatory kinases: Polo-like kinases, Aurora kinases and Cyclin-dependent kinases. It is time to stop Ne(c)king around with them and allow them to enter the spot light in the field of cell cycle biology.

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REFERENCES

1 **De Souza CP**, Horn KP, Masker K, Osmani AS. The SNOB (NUP98) nucleoporin interact with the NIMA kinase in Aspergillusnidulans. *Genetics* 2003; **165**: 1071-1081 [PMID: 14668365]

2 **Surpili MJ**, Delben TM, Kobarg J. Identification of proteins that interact with the central coiled-coil region of the human protein kinase NEK1. *Biochemistry* 2003; **42**: 15369-15376 [PMID: 14690447 DOI: 10.1021/bi034575v]

3 **Vaz Meirelles G**, Ferreira Lanza DC, da Silva JC, Santana Bernachi J, Paes Leme AF, Kobarg J. Characterization of hNek6 interactome reveals an important role for its short N-terminal domain and colocalization with proteins at the centrosome. *J Proteome Res* 2010; **9**: 6298-6316 [PMID: 20873783 DOI: 10.1021/pr100562w]

4 **O'Connell MJ**, Krien MJ, Hunter T. Never say never. The NIMA-related protein kinases in mitotic control. *Trends Cell Biol* 2003; **13**: 221-228 [PMID: 12742165 DOI: 10.1016/S0962-8924(03)00056-4]

5 **Quarmby LM**, Mahjoub MR. Caught Nek-ing: cilia and centrioles. *J Cell Sci* 2005; **118**: 5161-5169 [PMID: 16280549 DOI: 10.1242/jcs.02681]

6 **O'regan L**, Blot J, Fry AM. Mitotic regulation by NIMA-related kinases. *Cell Div* 2007; **2**: 25 [PMID: 17727698 DOI: 10.1186/1747-1028-2-25]

7 **Moniz L**, Dutt P, Haider N, Stambolic V. Nek family of kinases in cell cycle, checkpoint control and cancer. *Cell Div* 2011; **6**: 18 [PMID: 22040655 DOI: 10.1186/1747-1028-6-18]

8 **Fry AM**, O'Regan L, Sabir SR, Bayliss R. Cell cycle regulation by the NEK family of protein kinases. *J Cell Sci* 2012; **125**: 4423-4433 [PMID: 23132929 DOI: 10.1242/jcs.111195]

9 **Nguyen CL**, Possemato R, Bauerlein EL, Xie A, Scully R, Hahn WC. Nek4 regulates entry into replicative senescence and the response to DNA damage in human fibroblasts. *Mol Cell Biol* 2012; **32**: 3963-3977 [PMID: 22851694 DOI: 10.1128/MCB.00436-12]

10 **Choi HJC**, Lin JR, Vannier JB, Slaats GG, Kile AC, Paulsen RD. NEK8 Links the ATR-Regulated Replication Stress Response and S Phase CDK Activity to Renal Ciliopathies. *Molecular Cell* 2013; **51**: 423-439 [DOI: 10.1016/j.molcel.2013.08.006]

11 **Yin MJ**, Shao L, Voehringer D, Smeal T, Jallal B. The serine/threonine kinase Nek6 is required for cell cycle progression through mitosis. *J Biol Chem* 2003; **278**: 52454-52460 [PMID: 14563848 DOI: 10.1074/jbc.M308080200]

12 **O'Regan L**, Fry AM. The Nek6 and Nek7 protein kinases are required for robust mitotic spindle formation and cytokinesis. *Mol Cell Biol* 2009; **29**: 3975-3990 [PMID: 19414596 DOI: 10.1128/MCB.01867-08]

13 **Chen Y**, Craigen WJ, Riley DJ. Nek1 regulates cell death and mitochondrial membrane permeability through phosphorylation of VDAC1. *Cell Cycle* 2009; **8**: 257-267 [PMID: 19158487 DOI: 104161/cc.8.2.7551]

14 **Upadhya P**, Birkenmeier EH, Birkenmeier CS, Barker JE. Mutations in a NIMA-related kinase gene, Nek1, cause pleiotropic effects including a progressive polycystic kidney disease in mice. *Proc Natl Acad Sci USA* 2000; **97**: 217-221 [PMID: 10618398 DOI: 10.1073/pnas.97.1.217]

15 **Wilson PD**. Polycystic kidney disease: new understanding in the pathogenesis. *Int J Biochem Cell Biol* 2004; **36**: 1868-1873 [PMID: 15203099]

16 **Thiel C**, Kessler K, Giessl A, Dimmler A, Shalev SA, von der Haar S, Zenker M, Zahnleiter D, Stöss H, Beinder E, Abou Jamra R, Ekici AB, Schröder-Kress N, Aigner T, Kirchner T, Reis A, Brandstätter JH, Rauch A. NEK1 mutations cause short-rib polydactyly syndrome type majewski. *Am J Hum Genet* 2011; **88**: 106-114 [PMID: 21211617]

17 **Chen CP**, Chang TY, Tzen CY, Wang W. Second-trimester sonographic detection of short rib-polydactyly syndrome type II (Majewski) following an abnormal maternal serum biochemical screening result. *Prenat Diagn* 2003; **23**: 353-355 [PMID: 12673646 DOI: 10.1002/pd.574]

18 **Lee MY**, Kim HJ, Kim MA, Jee HJ, Kim AJ, Bae YS, Park JI, Chung JH, Yun J. Nek6 is involved in G2/M phase cell cycle arrest through DNA damage-induced phosphorylation. *Cell Cycle* 2008; **7**: 2705-2709 [PMID: 18728393 DOI: 10.4161/cc.7.17.6551]

19 **Lin F**, Hiesberger T, Cordes K, Sinclair AM, Goldstein LS, Somlo S, Igarashi P. Kidney-specific inactivation of the KIF3A subunit of kinesin-II inhibits renal ciliogenesis and produces polycystic kidney disease. *Proc Natl Acad Sci USA* 2003; **100**: 5286-5291 [PMID: 12672950 DOI: 10.1073/pnas.0836980100]

20 **Kleymenova E**, Ibraghimov-Beskrovnaya O, Kugoh H, Everitt J, Xu H, Kiguchi K, Landes G, Harris P, Walker C. Tuberin-dependent membrane localization of polycystin-1: a functional link between polycystic kidney disease and the TSC2 tumor suppressor gene. *Mol Cell* 2001; **7**: 823-832 [PMID: 11336705 DOI: 10.1016/S1097-2765(01)00226-X]

21 **Huan Y**, van Adelsberg J. Polycystin-1, the PKD1 gene product, is in a complex containing E-cadherin and the catenins. *J Clin Invest* 1999; **104**: 1459-1468 [PMID: 10562308 DOI: 10.1172/JCI5111]

22 **Mahjoub MR**, Qasim Rasi M, Quarmby LM. A NIMA-related kinase, Fa2p, localizes to a novel site in the proximal cilia of Chlamydomonas and mouse kidney cells. *Mol Biol Cell* 2004; **15**: 5172-5186 [PMID: 15371535 DOI: 10.1091/mbc.E04-07-0571]

23 **Polci R**, Peng A, Chen PL, Riley DJ, Chen Y. NIMA-related protein kinase 1 is involved early in the ionizing radiation-induced DNA damage response. *Cancer Res* 2004; **64**: 8800-8803 [PMID: 15604234 DOI: 10.1158/0008-5472.CAN-04-2243]

24 **Pelegrini AL**, Moura DJ, Brenner BL, Ledur PF, Maques GP, Henriques JA, Saffi J, Lenz G. Nek1 silencing slows down DNA repair and blocks DNA damage-induced cell cycle arrest. *Mutagenesis* 2010; **25**: 447-454 [PMID: 20501547 DOI: 10.1093/mutage/geq026]

25 **Liu S**, Ho CK, Ouyang J, Zou L. Nek1 kinase associates with ATR-ATRIP and primes ATR for efficient DNA damage signaling. *Proc Natl Acad Sci USA* 2013; **110**: 2175-2180 [PMID: 23345434 DOI: 10.1073/pnas.1217781110]

26 **Letwin K**, Mizzen L, Motro B, Ben-David Y, Bernstein A, Pawson T. A mammalian dual specificity protein kinase, Nek1, is related to the NIMA cell cycle regulator and highly expressed in meiotic germ cells. *EMBO J* 1992; **11**: 3521-3531 [PMID: 1382974]

27 **Feige E**, Chen A, Motro B. Nurit, a novel leucine-zipper protein, expressed uniquely in the spermatid flower-like structure. *Mech Dev* 2002; **117**: 369-377 [PMID: 12204287 DOI: 10.1016/S0925-4773(02)00217-4]

28 **Holloway K,** Roberson EC, Corbett KL, Kolas NK, Nieves E, Cohen PE. NEK1 Facilitates Cohesin Removal during Mammalian Spermatogenesis. *Genes* (Basel) 2011; **2**: 260-279[PMID: 21931878]

29 **Patil M**, Pabla N, Ding HF, Dong Z. Nek1 interacts with Ku80 to assist chromatin loading of replication factors and S-phase progression. *Cell Cycle* 2013; **12**: 2608-2616 [PMID: 23851348 DOI: 10.4161/cc.25624]

30 **Feige E**, Shalom O, Tsuriel S, Yissachar N, Motro B. Nek1 shares structural and functional similarities with NIMA kinase. *Biochim Biophys Acta* 2006; **1763**: 272-281 [PMID: 16603261 DOI: 10.1016/j.bbamcr.2006.01.009]

31 **Fry AM**, Schultz SJ, Bartek J, Nigg EA. Substrate specificity and cell cycle regulation of the Nek2 protein kinase, a potential human homolog of the mitotic regulator NIMA of Aspergillus nidulans. *J Biol Chem* 1995; **270**: 12899-12905 [PMID: 7759549 DOI: 10.1074/jbc.270.21.12899]

32 **Fry AM**, Arnaud L, Nigg EA. Activity of the human centrosomal kinase, Nek2, depends on an unusual leucine zipper dimerization motif. *J Biol Chem* 1999; **274**: 16304-16310 [PMID: 10347187 DOI: 10.1074/jbc.274.23.16304]

33 **Fry AM**, Mayor T, Meraldi P, Stierhof YD, Tanaka K, Nigg EA. C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. *J Cell Biol* 1998; **141**: 1563-1574 [PMID: 9647649 DOI: 10.1083/jcb.141.7.1563]

34 **Yang J**, Adamian M, Li T. Rootletin interacts with C-Nap1 and may function as a physical linker between the pair of centrioles/basal bodies in cells. *Mol Biol Cell* 2006; **17**: 1033-1040 [PMID: 16339073 DOI: 10.1091/mbc.E05-10-0943]

35 **Rellos P**, Ivins FJ, Baxter JE, Pike A, Nott TJ, Parkinson DM, Das S, Howell S, Fedorov O, Shen QY, Fry AM, Knapp S,

Smerdon SJ. Structure and regulation of the human Nek2 centrosomal kinase. *J Biol Chem* 2007; **282**: 6833-6842 [PMID: 17197699 DOI: 10.1074/jbc.M609721200]
36 **Wei R**, Ngo B, Wu G, Lee WH. Phosphorylation of the Ndc80 complex protein, HEC1, by Nek2 kinase modulates chromosome alignment and signaling of the spindle assembly checkpoint. *Mol Biol Cell* 2011; **22**: 3584-3594 [PMID: 21832156 DOI: 10.1091/mbc.E11-01-0012]
37 **Salem H**, Rachmin I, Yissachar N, Cohen S, Amiel A, Haffner R, Lavi L, Motro B. Nek7 kinase targeting leads to early mortality, cytokinesis disturbance and polyploidy. *Oncogene* 2010; **29**: 4046-4057 [PMID: 20473324 DOI: 10.1038/onc.2010]
38 **Di Agostino S**, Fedele M, Chieffi P, Fusco A, Rossi P, Geremia R, Sette C. Phosphorylation of high-mobility group protein A2 by Nek2 kinase during the first meiotic division in mouse spermatocytes. *Mol Biol Cell* 2004; **15**: 1224-1232 [PMID: 14668482 DOI: 10.1091/mbc.E03-09-0638]
39 **Prigent C**, Glover DM, Giet R. Drosophila Nek2 protein kinase knockdown leads to centrosome maturation defects while overexpression causes centrosome fragmentation and cytokinesis failure. *Exp Cell Res* 2005; **303**: 1-13 [PMID: 15572022 DOI: 10.1016/j.yexcr.2004.04.052]
40 **Kimura M,** Okano Y. Molecular cloning and characterization of the human NIMA-related protein kinase 3 gene (NEK3). *Cytogenet Cell Genet* 2001; **95**: 177-182 [PMID: 12063396 DOI: 10.1074/jbc.274.19.13491]
41 **Miller SL**, DeMaria JE, Freier DO, Riegel AM, Clevenger CV. Novel association of Vav2 and Nek3 modulates signaling through the human prolactin receptor. *Mol Endocrinol* 2005; **19**: 939-949 [PMID: 15618286 DOI: 10.1210/me.2004-0443]
42 **Miller SL**, Antico G, Raghunath PN, Tomaszewski JE, Clevenger CV. Nek3 kinase regulates prolactin-mediated cytoskeletal reorganization and motility of breast cancer cells. *Oncogene* 2007; **26**: 4668-4678 [PMID: 17297458 DOI: 10.1038/sj.onc.1210264]
43 **Hernández M**, Almeida TA. Is there any association between nek3 and cancers with frequent 13q14 deletion? *Cancer Invest* 2006; **24**: 682-688 [PMID: 17118778 DOI: 10.1080/07357900600981364]
44 **Kytölä S**, Farnebo F, Obara T, Isola J, Grimelius L, Farnebo LO, Sandelin K, Larsson C. Patterns of chromosomal imbalances in parathyroid carcinomas. *Am J Pathol* 2000; **157**: 579-586 [PMID: 10934160 DOI: 10.1016/S0002-9440(10)64568-3]
45 **Shaughnessy J**, Tian E, Sawyer J, Bumm K, Landes R, Badros A, Morris C, Tricot G, Epstein J, Barlogie B. High incidence of chromosome 13 deletion in multiple myeloma detected by multiprobe interphase FISH. *Blood* 2000; **96**: 1505-1511 [PMID: 10942398]
46 **Schultz SJ**, Fry AM, Sütterlin C, Ried T, Nigg EA. Cell cycle-dependent expression of Nek2, a novel human protein kinase related to the NIMA mitotic regulator of Aspergillus nidulans. *Cell Growth Differ* 1994; **5**: 625-635 [PMID: 7522034]
47 **Chang J**, Baloh RH, Milbrandt J. The NIMA-family kinase Nek3 regulates microtubule acetylation in neurons. *J Cell Sci* 2009; **122**: 2274-2282 [PMID: 19509051 DOI: 10.1242/jcs.048975]
48 **Benjamin S**, Weidberg H, Rapaport D, Pekar O, Nudelman M, Segal D, Hirschberg K, Katzav S, Ehrlich M, Horowitz M. EHD2 mediates trafficking from the plasma membrane by modulating Rac1 activity. *Biochem J* 2011; **439**: 433-442 [PMID: 21756249 DOI: 10.1042/BJ20111010]
49 **Cance WG**, Craven RJ, Weiner TM, Liu ET. Novel protein kinases expressed in human breast cancer. *Int J Cancer* 1993; **54**: 571-577 [PMID: 8099900 DOI: 10.1002/ijc.2910540409]
50 **Levedakou EN**, He M, Baptist EW, Craven RJ, Cance WG, Welcsh PL, Simmons A, Naylor SL, Leach RJ, Lewis TB. Two novel human serine/threonine kinases with homologies to the cell cycle regulating Xenopus MO15, and NIMA kinases: cloning and characterization of their expression pattern. *Oncogene* 1994; **9**: 1977-1988 [PMID: 8208544]
51 **Hayashi K**, Igarashi H, Ogawa M, Sakaguchi N. Activity and substrate specificity of the murine STK2 Serine/Threonine kinase that is structurally related to the mitotic regulator protein NIMA of Aspergillus nidulans. *Biochem Biophys Res Commun* 1999; **264**: 449-456 [PMID: 10529384 DOI: 10.1006/bbrc.1999.1536]
52 **Chen A**, Yanai A, Arama E, Kilfin G, Motro B. NIMA-related kinases: isolation and characterization of murine nek3 and nek4 cDNAs, and chromosomal localization of nek1, nek2 and nek3. *Gene* 1999; **234**: 127-137 [PMID: 10393247 DOI: 10.1016/S0378-1119(99)00165-1]
53 **Coene KL**, Mans DA, Boldt K, Gloeckner CJ, van Reeuwijk J, Bolat E, Roosing S, Letteboer SJ, Peters TA, Cremers FP, Ueffing M, Roepman R. The ciliopathy-associated protein homologs RPGRIP1 and RPGRIP1L are linked to cilium integrity through interaction with Nek4 serine/threonine kinase. *Hum Mol Genet* 2011; **20**: 3592-605 [PMID: 21685204 DOI: 10.1093/hmg/ddr280]
54 **Doles J**, Hemann MT. Nek4 status differentially alters sensitivity to distinct microtubule poisons. *Cancer Res* 2010; **70**: 1033-1041 [PMID: 20103636 DOI: 10.1158/0008-5472/CAN-09-2113]
55 **Liu S**, Lu W, Obara T, Kuida S, Lehoczky J, Dewar K, Drummond IA, Beier DR. A defect in a novel Nek-family kinase causes cystic kidney disease in the mouse and in zebrafish. *Development* 2002; **129**: 5839-5846 [PMID: 12421721 DOI: 10.1242/dev.00173]
56 **Mahjoub MR**, Trapp ML, Quarmby LM. NIMA-related kinases defective in murine models of polycystic kidney diseases localize to primary cilia and centrosomes. *J Am Soc Nephrol* 2005; **16**: 3485-3489 [PMID: 16267153]
57 **Young CL**, Khoshnevis S, Karbstein K. Cofactor-dependent specificity of a DEAD-box protein. *Proc Natl Acad Sci USA* 2013; **110**: E2668-E2676 [PMID: 23630256 DOI: 10.1073/pnas.1302577110]
58 **Motose H**, Hamada T, Yoshimoto K, Murata T, Hasebe M, Watanabe Y, Hashimoto T, Sakai T, Takahashi T. NIMA-related kinases 6, 4, and 5 interact with each other to regulate microtubule organization during epidermal cell expansion in Arabidopsis thaliana. *Plant J* 2011; **67**: 993-1005 [PMID: 21605211 DOI: 10.1111/j.1365-313X.2011.04652.x]
59 **Vigneault F**, Lachance D, Cloutier M, Pelletier G, Levasseur C, Séguin A. Members of the plant NIMA-related kinases are involved in organ development and vascularization in poplar, Arabidopsis and rice. *Plant J* 2007; **51**: 575-588 [PMID: 17886359 DOI: 10.1111/j.1365-313X.2007.03161.x]
60 **Shimizu K**, Sawasaki T. Nek5, a novel substrate for caspase-3, promotes skeletal muscle differentiation by up-regulating caspase activity. *FEBS Lett* 2013; **587**: 2219-2225 [PMID: 23727203 DOI: 10.1016/j.febslet.2013.05.049]
61 **Larsen BD**, Rampalli S, Burns LE, Brunette S, Dilworth FJ, Megeney LA. Caspase 3/caspase-activated DNase promote cell differentiation by inducing DNA strand breaks. *Proc Natl Acad Sci USA* 2010; **107**: 4230-4235 [PMID: 20160104]
62 **Minoguchi S**, Minoguchi M, Yoshimura A. Differential control of the NIMA-related kinases, Nek6 and Nek7, by serum stimulation. *Biochem Biophys Res Commun* 2003; **301**: 899-906 [PMID: 12589797 DOI: 10.1016/S0006-291X(03)00049-4]
63 **Meirelles GV,** Silva JC, Mendonça Y de A, Ramos CH, Torriani IL, Kobarg J. Human Nek6 is a monomeric mostly globular kinase with an unfolded short N-terminal domain. *BMC Struct Biol* 2011; **11**: 12 [PMID: 21320329]
64 **Belham C**, Comb MJ, Avruch J. Identification of the NIMA family kinases NEK6/7 as regulators of the p70 ribosomal S6 kinase. *Curr Biol* 2001; **11**: 1155-1167 [PMID: 11516946 DOI: 10.1016/S0960-9822(01)00369-4]
65 **Lizcano JM**, Deak M, Morrice N, Kieloch A, Hastie CJ, Dong

L, Schutkowski M, Reimer U, Alessi DR. Molecular basis for the substrate specificity of NIMA-related kinase-6 (NEK6). Evidence that NEK6 does not phosphorylate the hydrophobic motif of ribosomal S6 protein kinase and serum- and glucocorticoid-induced protein kinase in vivo. *J Biol Chem* 2002; **277**: 27839-27849 [PMID: 12023960 DOI: 10.1074/jbc.M202042200]

66 **Roig J**, Mikhailov A, Belham C, Avruch J. Nercc1, a mammalian NIMA-family kinase, binds the Ran GTPase and regulates mitotic progression. *Genes Dev* 2002; **16**: 1640-1658 [PMID: 12101123 DOI: 10.1101/gad.972202]

67 **Belham C**, Roig J, Caldwell JA, Aoyama Y, Kemp BE, Comb M, Avruch J. A mitotic cascade of NIMA family kinases. Nercc1/Nek9 activates the Nek6 and Nek7 kinases. *J Biol Chem* 2003; **278**: 34897-34909 [PMID: 12840024 DOI: 10.1074/jbc.M303663200]

68 **Rapley J**, Nicolàs M, Groen A, Regué L, Bertran MT, Caelles C, Avruch J, Roig J. The NIMA-family kinase Nek6 phosphorylates the kinesin Eg5 at a novel site necessary for mitotic spindle formation. *J Cell Sci* 2008; **121**: 3912-3921 [PMID: 19001501 DOI: 10.1242/jcs.035360]

69 **Bertran MT**, Sdelci S, Regué L, Avruch J, Caelles C, Roig J. Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5. *EMBO J* 2011; **30**: 2634-2647 [PMID: 21642957 DOI: 10.1038/emboj.2011.179]

70 **Sdelci S**, Bertran MT, Roig J. Nek9, Nek6, Nek7 and the separation of centrosomes. *Cell Cycle* 2011; **10**: 3816-3817 [PMID: 22064517 DOI: 10.4161/cc.10.22.18226]

71 **Matsuda A**, Suzuki Y, Honda G, Muramatsu S, Matsuzaki O, Nagano Y, Doi T, Shimotohno K, Harada T, Nishida E, Hayashi H, Sugano S. Large-scale identification and characterization of human genes that activate NF-kappaB and MAPK signaling pathways. *Oncogene* 2003; **22**: 3307-3318 [PMID: 12761501 DOI: 10.1038/sj.onc.1206406]

72 **Chen Y**, Chen PL, Chen CF, Jiang X, Riley DJ. Never-in-mitosis related kinase 1 functions in DNA damage response and checkpoint control. *Cell Cycle* 2008; **7**: 3194-3201 [PMID: 18843199 DOI: 10.4161/cc.7.20.6815]

73 **Melixetian M**, Klein DK, Sørensen CS, Helin K. NEK11 regulates CDC25A degradation and the IR-induced G2/M checkpoint. *Nat Cell Biol* 2009; **11**: 1247-1253 [PMID: 19734889 DOI: 10.1038/ncb1969]

74 **Moniz LS**, Stambolic V. Nek10 mediates G2/M cell cycle arrest and MEK autoactivation in response to UV irradiation. *Mol Cell Biol* 2011; **31**: 30-42 [PMID: 20956560 DOI: 10.1128/MCB.00648-10]

75 **Kang J**, Goodman B, Zheng Y, Tantin D. Dynamic regulation of Oct1 during mitosis by phosphorylation and ubiquitination. *PLoS One* 2011; **6**: e23872 [PMID: 21897860 DOI: 10.1371/journal.pone.0023872]

76 **Hashimoto Y**, Akita H, Hibino M, Kohri K, Nakanishi M. Identification and characterization of Nek6 protein kinase, a potential human homolog of NIMA histone H3 kinase. *Biochem Biophys Res Commun* 2002; **293**: 753-758 [PMID: 12054534 DOI: 10.1016/S0006-291X(02)00297-8]

77 **Skoblov M**, Marakhonov A, Marakasova E, Guskova A, Chandhoke V, Birerdinc A, Baranova A. Protein partners of KCTD proteins provide insights about their functional roles in cell differentiation and vertebrate development. *Bioessays* 2013; **35**: 586-596 [PMID: 23592240 DOI: 10.1002/bies.201300002]

78 **Lee EJ**, Hyun SH, Chun J, Kang SS. Human NIMA-related kinase 6 is one of the Fe65 WW domain binding proteins. *Biochem Biophys Res Commun* 2007; **358**: 783-788 [PMID: 17512906 DOI: 10.1016/j.bbrc.2007.04.203]

79 **Takeno A**, Takemasa I, Doki Y, Yamasaki M, Miyata H, Takiguchi S, Fujiwara Y, Matsubara K, Monden M. Integrative approach for differentially overexpressed genes in gastric cancer by combining large-scale gene expression profiling and network analysis. *Br J Cancer* 2008; **99**: 1307-1315 [PMID: 18827816 DOI: 10.1038/sj.bjc.6604682]

80 **Chen J**, Li L, Zhang Y, Yang H, Wei Y, Zhang L, Liu X, Yu L. Interaction of Pin1 with Nek6 and characterization of their expression correlation in Chinese hepatocellular carcinoma patients. *Biochem Biophys Res Commun* 2006; **341**: 1059-1065 [PMID: 16476580 DOI: 10.1016/j.bbrc.2005.12.228]

81 **Jee HJ**, Kim HJ, Kim AJ, Song N, Kim M, Lee HJ, Yun J. The inhibition of Nek6 function sensitizes human cancer cells to premature senescence upon serum reduction or anticancer drug treatment. *Cancer Lett* 2013; **335**: 175-182 [PMID: 23416273 DOI: 10.1016/j.canlet.2013.02.012]

82 **Capra M**, Nuciforo PG, Confalonieri S, Quarto M, Bianchi M, Nebuloni M, Boldorini R, Pallotti F, Viale G, Gishizky ML, Draetta GF, Di Fiore PP. Frequent alterations in the expression of serine/threonine kinases in human cancers. *Cancer Res* 2006; **66**: 8147-8154 [PMID: 16912193 DOI: 10.1158/0008-5472.CAN-05-3489]

83 **Kasap E**, Boyacioglu SO, Korkmaz M, Yuksel ES, Unsal B, Kahraman E, Ozütemiz O, Yuceyar H. Aurora kinase A (AURKA) and never in mitosis gene A-related kinase 6 (NEK6) genes are upregulated in erosive esophagitis and esophageal adenocarcinoma. *Exp Ther Med* 2012; **4**: 33-42 [PMID: 23060919 DOI: 10.3892/etm.2012.561]

84 **Nassirpour R**, Shao L, Flanagan P, Abrams T, Jallal B, Smeal T, Yin MJ. Nek6 mediates human cancer cell transformation and is a potential cancer therapeutic target. *Mol Cancer Res* 2010; **8**: 717-728 [PMID: 20407017 DOI: 10.1158/1541-7786.MCR-09-0291]

85 **Jeon YJ**, Lee KY, Cho YY, Pugliese A, Kim HG, Jeong CH, Bode AM, Dong Z. Role of NEK6 in tumor promoter-induced transformation in JB6 C141 mouse skin epidermal cells. *J Biol Chem* 2010; **285**: 28126-28133 [PMID: 20595392 DOI: 10.1074/jbc.M110.137190]

86 **Jee HJ**, Kim AJ, Song N, Kim HJ, Kim M, Koh H, Yun J. Nek6 overexpression antagonizes p53-induced senescence in human cancer cells. *Cell Cycle* 2010; **9**: 4703-4710 [PMID: 21099361 DOI: 10.4161/cc.9.23.14059]

87 **Jee HJ**, Kim HJ, Kim AJ, Song N, Kim M, Yun J. Nek6 suppresses the premature senescence of human cancer cells induced by camptothecin and doxorubicin treatment. *Biochem Biophys Res Commun* 2011; **408**: 669-673 [PMID: 21539811 DOI: 10.1016/j.bbrc.2011.04.083]

88 **Kim S**, Lee K, Rhee K. NEK7 is a centrosomal kinase critical for microtubule nucleation. *Biochem Biophys Res Commun* 2007; **360**: 56-62 [PMID: 17586473 DOI: 10.1016/j.bbrc.2007.05.206]

89 **Yissachar N**, Salem H, Tennenbaum T, Motro B. Nek7 kinase is enriched at the centrosome, and is required for proper spindle assembly and mitotic progression. *FEBS Lett* 2006; **580**: 6489-6495 [PMID: 17101132 DOI: 10.1016/j.febslet.2006.10.069]

90 **Cohen S**, Aizer A, Shav-Tal Y, Yanai A, Motro B. Nek7 kinase accelerates microtubule dynamic instability. *Biochim Biophys Acta* 2013; **1833**: 1104-1113 [PMID: 23313050 DOI: 10.1016/j.bbamcr.2012.12.021]

91 **Kim S**, Kim S, Rhee K. NEK7 is essential for centriole duplication and centrosomal accumulation of pericentriolar material proteins in interphase cells. *J Cell Sci* 2011; **124**: 3760-3770 [PMID: 22100915 DOI: 10.1242/jcs.078089]

92 **Richards MW**, O'Regan L, Mas-Droux C, Blot JM, Cheung J, Hoelder S, Fry AM, Bayliss R. An autoinhibitory tyrosine motif in the cell-cycle-regulated Nek7 kinase is released through binding of Nek9. *Mol Cell* 2009; **36**: 560-570 [PMID: 19941817 DOI: 10.1016/j.molcel.2009.09.038]

93 **Wang R**, Song Y, Xu X, Wu Q, Liu C. The expression of Nek7, FoxM1, and Plk1 in gallbladder cancer and their relationships to clinicopathologic features and survival. *Clin Transl Oncol* 2013; **15**: 626-632 [PMID: 23359173 DOI: 10.1007/s12094-012-0978-9]

94 **Bowers AJ**, Boylan JF. Nek8, a NIMA family kinase mem-

ber, is overexpressed in primary human breast tumors. *Gene* 2004; **328**: 135-142 [PMID: 15019993 DOI: 10.1016/j.gene.2003.12.002]

95 **Zalli D**, Bayliss R, Fry AM. The Nek8 protein kinase, mutated in the human cystic kidney disease nephronophthisis, is both activated and degraded during ciliogenesis. *Human Molecular Genetics* 2012; **21**: 1155-1171 [DOI: 10.1093/hmg/ddr544]

96 **Valkova N**, Yunis R, Mak SK, Kang K, Kultz D. Nek8 Mutation Causes Overexpression of Galectin-1, Sorcin, and Vimentin and Accumulation of the Major Urinary Protein in Renal Cysts of jck Mice. *Molecular Cellular Proteomics* 2005; **4**: 1007-1009 [DOI: 10.1074/mcp.M500091 MCP200]

97 **Sohara E**, Luo Y, Zhang J, Manning DK, Beier DR, Zhou J. Nek8 Regulates the Expression and Localization of Polycystin-1 and Polycystin-2. *J Am Soc Nephrol* 2008; **19**: 469-476 [DOI: 10.1681/ASN.2006090985]

98 **Roig J**, Groen A, Caldwell J, Avruch J. Active Nercc1 protein kinase concentrates at centrosomes early in mitosis and is necessary for proper spindle assembly. *Mol Biol Cell* 2005; **16**: 4827-4840 [PMID: 16079175 DOI: 10.1091/mbc.E05-04-0315]

99 **Holland PM**, Milne A, Garka K, Johnson RS, Willis C, Sims JE, Rauch CT, Bird TA, Virca GD. Purification, cloning, and characterization of Nek8, a novel NIMA-related kinase, and its candidate substrate Bicd2. *J Biol Chem* 2002; **277**: 16229-16240 [PMID: 11864968 DOI: 10.1074/jbc.M108662200]

100 **Sdelci S**, Schütz M, Pinyol R, Bertran MT, Regué L, Caelles C, Vernos I, Roig J. Nek9 phosphorylation of NEDD1/GCP-WD contributes to Plk1 control of γ-tubulin recruitment to the mitotic centrosome. *Curr Biol* 2012; **22**: 1516-1523 [PMID: 22818914 DOI: 10.1016/j.cub.2012.06.027]

101 **Regué L**, Sdelci S, Bertran MT, Caelles C, Reverter D, Roig J. DYNLL/LC8 protein controls signal transduction through the Nek9/Nek6 signaling module by regulating Nek6 binding to Nek9. *J Biol Chem* 2011; **286**: 18118-18129 [PMID: 21454704 DOI: 10.1074/jbc.M110.209080]

102 **Jackman M**, Lindon C, Nigg EA, Pines J. Active cyclin B1-Cdk1 first appears on centrosomes in prophase. *Nat Cell Biol* 2003; **5**: 143-148 [PMID: 12524548 DOI: 10.1038/ncb918]

103 **Wu Z**, Doondeea JB, Gholami AM, Janning MC, Lemeer S, Kramer K, Eccles SA, Gollin SM, Grenman R, Walch A, Feller SM, Kuster B. Quantitative chemical proteomics reveals new potential drug targets in head and neck cancer. *Mol Cell Proteomics* 2011; **10**: M111.011635 [PMID: 21955398 DOI: 10.1074/mcp.M111.011635]

104 **Boly R**, Gras T, Lamkami T, Guissou P, Serteyn D, Kiss R, Dubois J. Quercetin inhibits a large panel of kinases implicated in cancer cell biology. *Int J Oncol* 2011; **38**: 833-842 [PMID: 21206969 DOI: 10.3892/ijo.2010.890]

105 **Cooper MJ**, Cox NJ, Zimmerman EI, Dewar BJ, Duncan JS, Whittle MC, Nguyen TA, Jones LS, Ghose Roy S, Smalley DM, Kuan PF, Richards KL, Christopherson RI, Jin J, Frye SV, Johnson GL, Baldwin AS, Graves LM. Application of multiplexed kinase inhibitor beads to study kinome adaptations in drug-resistant leukemia. *PLoS One* 2013; **8**: e66755 [PMID: 23826126 DOI: 10.1371/journal.pone.0066755]

106 **Kaneta Y**, Ullrich A. NEK9 depletion induces catastrophic mitosis by impairment of mitotic checkpoint control and spindle dynamics. *Biochem Biophys Res Commun* 2013; **442**: 139-146 [PMID: 23665325 DOI: 10.1016/j.bbrc.2013.04.105]

107 **Huber AH**, Nelson WJ, Weis WI. Three-dimensional structure of the armadillo repeat region of beta-catenin. *Cell* 1997; **90**: 871-882 [PMID: 9298899 DOI: 10.1016/S0092-8674(00)80352-9]

108 **Antoniou AC**, Beesley J, McGuffog L, Sinilnikova OM, Healey S, Neuhausen SL, Ding YC, Rebbeck TR, Weitzel JN, Lynch HT, Isaacs C, Ganz PA, Tomlinson G, Olopade OI, Couch FJ, Wang X, Lindor NM, Pankratz VS, Radice P, Manoukian S, Peissel B, Zaffaroni D, Barile M, Viel A, Allavena A, Dall'Olio V, Peterlongo P, Szabo CI, Zikan M, Claes K, Poppe B, Foretova L, Mai PL, Greene MH, Rennert G, Lejbkowicz F, Glendon G, Ozcelik H, Andrulis IL, Thomassen M, Gerdes AM, Sunde L, Cruger D, Birk Jensen U, Caligo M, Friedman E, Kaufman B, Laitman Y, Milgrom R, Dubrovsky M, Cohen S, Borg A, Jernström H, Lindblom A, Rantala J, Stenmark-Askmalm M, Melin B, Nathanson K, Domchek S, Jakubowska A, Lubinski J, Huzarski T, Osorio A, Lasa A, Durán M, Tejada MI, Godino J, Benitez J, Hamann U, Kriege M, Hoogerbrugge N, van der Luijt RB, van Asperen CJ, Devilee P, Meijers-Heijboer EJ, Blok MJ, Aalfs CM, Hogervorst F, Rookus M, Cook M, Oliver C, Frost D, Conroy D, Evans DG, Lalloo F, Pichert G, Davidson R, Cole T, Cook J, Paterson J, Hodgson S, Morrison PJ, Porteous ME, Walker L, Kennedy MJ, Dorkins H, Peock S, Godwin AK, Stoppa-Lyonnet D, de Pauw A, Mazoyer S, Bonadona V, Lasset C, Dreyfus H, Leroux D, Hardouin A, Berthet P, Faivre L, Loustalot C, Noguchi T, Sobol H, Rouleau E, Nogues C, Frénay M, Vénat-Bouvet L, Hopper JL, Daly MB, Terry MB, John EM, Buys SS, Yassin Y, Miron A, Goldgar D, Singer CF, Dressler AC, Gschwantler-Kaulich D, Pfeiler G, Hansen TV, Jønson L, Agnarsson BA, Kirchhoff T, Offit K, Devlin V, Dutra-Clarke A, Piedmonte M, Rodriguez GC, Wakeley K, Boggess JF, Basil J, Schwartz PE, Blank SV, Toland AE, Montagna M, Casella C, Imyanitov E, Tihomirova L, Blanco I, Lazaro C, Ramus SJ, Sucheston L, Karlan BY, Gross J, Schmutzler R, Wappenschmidt B, Engel C, Meindl A, Lochmann M, Arnold N, Heidemann S, Varon-Mateeva R, Niederacher D, Sutter C, Deissler H, Gadzicki D, Preisler-Adams S, Kast K, Schönbuchner I, Caldes T, de la Hoya M, Aittomäki K, Nevanlinna H, Simard J, Spurdle AB, Holland H, Chen X, Platte R, Chenevix-Trench G, Easton DF. Common breast cancer susceptibility alleles and the risk of breast cancer for BRCA1 and BRCA2 mutation carriers: implications for risk prediction. *Cancer Res* 2010; **70**: 9742-9754 [PMID: 21118973 DOI: 10.1158/0008-5472]

109 **Ahmed S**, Thomas G, Ghoussaini M, Healey CS, Humphreys MK, Platte R, Morrison J, Maranian M, Pooley KA, Luben R, Eccles D, Evans DG, Fletcher O, Johnson N, dos Santos Silva I, Peto J, Stratton MR, Rahman N, Jacobs K, Prentice R, Anderson GL, Rajkovic A, Curb JD, Ziegler RG, Berg CD, Buys SS, McCarty CA, Feigelson HS, Calle EE, Thun MJ, Diver WR, Bojesen S, Nordestgaard BG, Flyger H, Dörk T, Schürmann P, Hillemanns P, Karstens JH, Bogdanova NV, Antonenkova NN, Zalutsky IV, Bermisheva M, Fedorova S, Khusnutdinova E, Kang D, Yoo KY, Noh DY, Ahn SH, Devilee P, van Asperen CJ, Tollenaar RA, Seynaeve C, Garcia-Closas M, Lissowska J, Brinton L, Peplonska B, Nevanlinna H, Heikkinen T, Aittomäki K, Blomqvist C, Hopper JL, Southey MC, Smith L, Spurdle AB, Schmidt MK, Broeks A, van Hien RR, Cornelissen S, Milne RL, Ribas G, González-Neira A, Benitez J, Schmutzler RK, Burwinkel B, Bartram CR, Meindl A, Brauch H, Justenhoven C, Hamann U, Chang-Claude J, Hein R, Wang-Gohrke S, Lindblom A, Margolin S, Mannermaa A, Kosma VM, Kataja V, Olson JE, Wang X, Fredericksen Z, Giles GG, Severi G, Baglietto L, English DR, Hankinson SE, Cox DG, Kraft P, Vatten LJ, Hveem K, Kumle M, Sigurdson A, Doody M, Bhatti P, Alexander BH, Hooning MJ, van den Ouweland AM, Oldenburg RA, Schutte M, Hall P, Czene K, Liu J, Li Y, Cox A, Elliott G, Brock I, Reed MW, Shen CY, Yu JC, Hsu GC, Chen ST, Anton-Culver H, Ziogas A, Andrulis IL, Knight JA, Beesley J, Goode EL, Couch F, Chenevix-Trench G, Hoover RN, Ponder BA, Hunter DJ, Pharoah PD, Dunning AM, Chanock SJ, Easton DF. Newly discovered breast cancer susceptibility loci on 3p24 and 17q23.2. *Nat Genet* 2009; **41**: 585-590 [PMID: 19330027 DOI: 10.1038/ng.354]

110 **Mulligan AM**, Couch FJ, Barrowdale D, Domchek SM, Eccles D, Nevanlinna H, Ramus SJ, Robson M, Sherman M, Spurdle AB, Wappenschmidt B, Lee A, McGuffog L, Healey

S, Sinilnikova OM, Janavicius R, Hansen Tv, Nielsen FC, Ejlertsen B, Osorio A, Muñoz-Repeto I, Durán M, Godino J, Pertesi M, Benítez J, Peterlongo P, Manoukian S, Peissel B, Zaffaroni D, Cattaneo E, Bonanni B, Viel A, Pasini B, Papi L, Ottini L, Savarese A, Bernard L, Radice P, Hamann U, Verheus M, Meijers-Heijboer HE, Wijnen J, Gómez García EB, Nelen MR, Kets CM, Seynaeve C, Tilanus-Linthorst MM, van der Luijt RB, van Os T, Rookus M, Frost D, Jones JL, Evans DG, Lalloo F, Eeles R, Izatt L, Adlard J, Davidson R, Cook J, Donaldson A, Dorkins H, Gregory H, Eason J, Houghton C, Barwell J, Side LE, McCann E, Murray A, Peock S, Godwin AK, Schmutzler RK, Rhiem K, Engel C, Meindl A, Ruehl I, Arnold N, Niederacher D, Sutter C, Deissler H, Gadzicki D, Kast K, Preisler-Adams S, Varon-Mateeva R, Schoenbuchner I, Fiebig B, Heinritz W, Schäfer D, Gevensleben H, Caux-Moncoutier V, Fassy-Colcombet M, Cornelis F, Mazoyer S, Léoné M, Boutry-Kryza N, Hardouin A, Berthet P, Muller D, Fricker JP, Mortemousque I, Pujol P, Coupier I, Lebrun M, Kientz C, Longy M, Sevenet N, Stoppa-Lyonnet D, Isaacs C, Caldes T, de la Hoya M, Heikkinen T, Aittomäki K, Blanco I, Lazaro C, Barkardottir RB, Soucy P, Dumont M, Simard J, Montagna M, Tognazzo S, D'Andrea E, Fox S, Yan M, Rebbeck T, Olopade O, Weitzel JN, Lynch HT, Ganz PA, Tomlinson GE, Wang X, Fredericksen Z, Pankratz VS, Lindor NM, Szabo C, Offit K, Sakr R, Gaudet M, Bhatia J, Kauff N, Singer CF, Tea MK, Gschwantler-Kaulich D, Fink-Retter A, Mai PL, Greene MH, Imyanitov E, O'Malley FP, Ozcelik H, Glendon G, Toland AE, Gerdes AM, Thomassen M, Kruse TA, Jensen UB, Skytte AB, Caligo MA, Soller M, Henriksson K, Wachenfeldt vA, Arver B, Stenmark-Askmalm M, Karlsson P, Ding YC, Neuhausen SL, Beattie M, Pharoah PD, Moysich KB, Nathanson KL, Karlan BY, Gross J, John EM, Daly MB, Buys SM, Southey MC, Hopper JL, Terry MB, Chung W, Miron AF, Goldgar D, Chenevix-Trench G, Easton DF, Andrulis IL, Antoniou AC. Common breast cancer susceptibility alleles are associated with tumour subtypes in BRCA1 and BRCA2 mutation carriers: results from the Consortium of Investigators of Modifiers of BRCA1/2. *Breast Cancer Res* 2011; **13**: R110 [PMID: 22053997 DOI: 10.1186/bcr3052.]

111 **Noguchi K**, Fukazawa H, Murakami Y, Uehara Y. Nek11, a new member of the NIMA family of kinases, involved in DNA replication and genotoxic stress responses. *J Biol Chem* 2002; **277**: 39655-39665 [PMID: 12154088]

112 **Noguchi K**, Fukazawa H, Murakami Y, Uehara Y. Nucleolar Nek11 is a novel target of Nek2A in G1/S-arrested cells. *J Biol Chem* 2004; **279**: 32716-32727 [PMID: 15161910]

113 **Alborghetti MR**, Furlan AS, Kobarg J. FEZ2 has acquired additional protein interaction partners relative to FEZ1: functional and evolutionary implications. *PLoS One* 2011; **6**: e17426 [PMID: 21408165 DOI: 10.1371/journal.pone.0017426]

114 **Lanza DC**, Meirelles GV, Alborghetti MR, Abrile CH, Lenz G, Kobarg J.FEZ1 interacts with CLASP2 and NEK1 through coiled-coil regions and their cellular colocalization suggests centrosomal functions and regulation by PKC. *Mol Cell Biochem* 2010; **338**: 35-45 [PMID: 19924516 DOI: 10.1007/s11010-009-0317-9]

115 **Hong Z**, Jiang J, Lan L, Nakajima S, Kanno S, Koseki H, Yasui A. A polycomb group protein, PHF1, is involved in the response to DNA double-strand breaks in human cell. *Nucleic Acids Res* 2008; **36**: 2939-2947 [PMID: 18385154 DOI: 10.1093/nar/gkn146]

116 **Sonoda E**, Matsusaka T, Morrison C, Vagnarelli P, Hoshi O, Ushiki T, Nojima K, Fukagawa T, Waizenegger IC, Peters JM, Earnshaw WC, Takeda S. Scc1/Rad21/Mcd1 is required for sister chromatid cohesion and kinetochore function in vertebrate cells. *Dev Cell* 2001; **1**: 759-770 [PMID: 11740938 DOI: 10.1016/S1534-5807(01)00088-0]

117 **Ishizaka A**, Mizutani T, Kobayashi K, Tando T, Sakurai K, Fujiwara T, Iba H. Double plant homeodomain (PHD) finger proteins DPF3a and -3b are required as transcriptional co-activators in SWI/SNF complex-dependent activation of NF-κB RelA/p50 heterodimer. *J Biol Chem* 2012; **287**: 11924-11933 [PMID: 22334708 DOI: 10.1074/jbc.M111.322792]

118 **Ewing RM**, Chu P, Elisma F, Li H, Taylor P, Climie S, McBroom-Cerajewski L, Robinson MD, O'Connor L, Li M, Taylor R, Dharsee M, Ho Y, Heilbut A, Moore L, Zhang S, Ornatsky O, Bukhman YV, Ethier M, Sheng Y, Vasilescu J, Abu-Farha M, Lambert JP, Duewel HS, Stewart II, Kuehl B, Hogue K, Colwill K, Gladwish K, Muskat B, Kinach R, Adams SL, Moran MF, Morin GB, Topaloglou T, Figeys D. Large-scale mapping of human protein-protein interactions by mass spectrometry. *Mol Syst Biol* 2007; **3**: 89 [PMID: 17353931 DOI: 10.1038/msb4100134]

119 **Villumsen BH**, Danielsen JR, Povlsen L, Sylvestersen KB, Merdes A, Beli P, Yang YG, Choudhary C, Nielsen ML, Mailand N, Bekker-Jensen S. A new cellular stress response that triggers centriolar satellite reorganization and ciliogenesis. *EMBO J* 2013; **32**: 3029-3040 [PMID: 24121310 DOI: 10.1038/emboj.2013.223]

120 **Chaki M**, Airik R, Ghosh AK, Giles RH, Chen R, Slaats GG, Wang H, Hurd TW, Zhou W, Cluckey A, Gee HY, Ramaswami G, Hong CJ, Hamilton BA, Cervenka I, Ganji RS, Bryja V, Arts HH, van Reeuwijk J, Oud MM, Letteboer SJ, Roepman R, Husson H, Ibraghimov-Beskrovnaya O, Yasunaga T, Walz G, Eley L, Sayer JA, Schermer B, Liebau MC, Benzing T, Le Corre S, Drummond I, Janssen S, Allen SJ, Natarajan S, O'Toole JF, Attanasio M, Saunier S, Antignac C, Koenekoop RK, Ren H, Lopez I, Nayir A, Stoetzel C, Dollfus H, Massoudi R, Gleeson JG, Andreoli SP, Doherty DG, Lindstrad A, Golzio C, Katsanis N, Pape L, Abboud EB, Al-Rajhi AA, Lewis RA, Omran H, Lee EY, Wang S, Sekiguchi JM, Saunders R, Johnson CA, Garner E, Vanselow K, Andersen JS, Shlomai J, Nurnberg G, Nurnberg P, Levy S, Smogorzewska A, Otto EA, Hildebrandt F. Exome capture reveals ZNF423 and CEP164 mutations, linking renal ciliopathies to DNA damage response signaling. *Cell* 2012; **150**: 533-548 [PMID: 22863007 DOI: 10.1016/j.cell.2012.06.028]

121 **Zhu YY**, Lan JP, Yu J.Interaction between a novel centrosomal protein TACP1 and mitotic kinase Nek2A [Article in Chinese]. *Zhejiang Daxue Xuebao Yixueban* 2007; **36**: 337-42 [PMID: 17717823]

122 **Prime G**, Markie D. The telomere repeat binding protein Trf1 interacts with the spindle checkpoint protein Mad1 and Nek2 mitotic kinase. *Cell Cycle* 2005; **4**: 121-124 [PMID: 15611654]

123 **Lou Y**, Xie W, Zhang DF, Yao JH, Luo ZF, Wang YZ, Shi YY, Yao XB. Nek2A specifies the centrosomal localization of Erk2. *Biochem Biophys Res Commun* 2004; **321**: 495-501 [PMID: 15358203]

124 **Helps NR**, Luo X, Barker HM, Cohen PT. NIMA-related kinase 2 (Nek2), a cell-cycle-regulated protein kinase localized to centrosomes, is complexed to protein phosphatase 1. *Biochem J* 2000; **349**: 509-518 [PMID: 10880350]

125 **Ashburner M**, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 2000; **25**: 25-29 [PMID: 10802651 DOI: 10.1038/75556]

126 **Shannon P**, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003; **13**: 2498-2504 [PMID: 14597658]

127 **Mi J**, Guo C, Brautigan DL, Larner JM. Protein phosphatase-1alpha regulates centrosome splitting through Nek2. *Cancer Res* 2007; **67**: 1082-1089 [PMID: 17283141 DOI:

10.1158/0008-5472.CAN-06-3071]
128 **Sann S**, Wang Z, Brown H, Jin Y. Roles of endosomal trafficking in neurite outgrowth and guidance. *Trends Cell Biol* 2009; **19**: 317-324 [PMID: 19540123 DOI: 10.1016/j.tcb.2009.05.001]
129 **Pelengaris S**, Khan M. The Molecular Biology of Cancer: A Bridge from Bench to Bedside. 2nd ed. Oxford: Wiley-Blackwell, 2013: 324

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REVIEW

Value of a newly sequenced bacterial genome

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Abstract

Next-generation sequencing (NGS) technologies have made high-throughput sequencing available to medium- and small-size laboratories, culminating in a tidal wave of genomic information. The quantity of sequenced bacterial genomes has not only brought excitement to the field of genomics but also heightened expectations that NGS would boost antibacterial discovery and vaccine development. Although many possible drug and vaccine targets have been discovered, the success rate of genome-based analysis has remained below expectations. Furthermore, NGS has had consequences for genome quality, resulting in an exponential increase in draft (partial data) genome deposits in public databases. If no further interests are expressed for a particular bacterial genome, it is more likely that the sequencing of its genome will be limited to a draft stage, and the painstaking tasks of completing the sequencing of its genome and annotation will not be undertaken. It is important to know what is lost when we settle for a draft genome and to determine the "scientific value" of a newly sequenced genome. This review addresses the expected impact of newly sequenced genomes on antibacterial discovery and vaccinology. Also, it discusses the factors that could be leading to the increase in the number of draft deposits and the consequent loss of relevant biological information.

Key words: Next-generation sequencing; Drafts; Prokaryotic genomes; Computational tools; *Omics*

Core tip: Next-generation sequencing (NGS) technologies have made high-throughput sequencing available to medium- and small-size laboratories, culminating in a tidal wave of genomic information. The quantity of bacterial genomes has not only brought excitement to the field of genomics, it has also heightened expectations that NGS would boost antibacterial discovery and vaccine development. Although many possible drug and vaccine targets have been discovered, the success rate of genome-based analysis has remained below expectations. Furthermore, NGS has consequences for genome quality, resulting in an exponential increase in draft genome deposits in public databases. This review will address the expected impact of newly sequenced genomes on antibacterial discovery and vaccinology, as well as the impact of NGS on draft bacterial genomes.

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INTRODUCTION

Since its release in 2005, next-generation sequencing (NGS) has been responsible for a drastic reduction in the price of genome sequencing and for a tidal wave of genetic information[1]. NGS technologies have made high-throughput sequencing available to medium- and small-size laboratories. The new possibility of generating a large number of sequenced bacterial genomes not only brought excitement to the field of genomics but also heightened expectations that the development of vaccines and the search for new antibacterial targets would be boosted. Nevertheless, these expectations were shown to be naïve. The complexity of host-bacteria interactions and the large diversity of bacterial genetic products have been shown to play greater roles in vaccine development and antibacterial discovery[2-4].

Additionally, as with any methodology, NGS presents its own drawbacks. Among the new sequencing technologies the most consolidated in the market are the 454 GS FLX platform (Roche), Illumina (Genome Analyzer) and SOLiD (Life Technologies)[5,6]. These devices are capable of generating millions of reads, providing high coverage genomic but with a drawback, reads are considerably smaller than the ones produced by Sanger methodology[7,8]. While Sanger methodology produces reads ranging from 800 to 1000 bases, NGS platforms produces reads ranging from 50 (SOLiD V3) to 2 × 150 bases (Illumina)[9]. The small amount of information contained in each read makes it difficult to completely assemble a genome using exclusively computational tools[10,11]. Therefore small reads made the genome assembly process a quite more laborious task.

In recent years, approaches that use hybrid assemblies were developed to facilitate the assembly process. They take advantage of high read quality of second generation sequencers, *i.e.,* Illumina (Genome Analyzer), and longer read lengths from third generation sequencers, *i.e.*, SMRT sequencers (Pacific Biosciences) and Ion Torrent PGM[12,13]. Although empirically logical, this kind of approach wasn't facilitated due to the lack of integration between sequencers.

In order to improving and verifying quality genome is essential to know which combination of sequencing data, computer algorithms, and parameters can produce the highest quality assembly[14,15]. Also, it is necessary to know the more likely type of error data a sequencer platform will present. For instance, Illumina and SOLiD are more likely to present nucleotide substitution, while 454 GS FLX and Ion Torrent are more likely to present indels[16]. Nearly none bioinformatic system has been developed to integrate reads from different sequencers into a single assembly[12,17]. This new developed approaches aim to reduce the manual intervention in finishing genomes, since repetitive regions may be solved using an hybrid approach.

Although NGS is directly responsible for considerable growth in the size of genomic databases, it has also been indirectly responsible for a decrease in genome quality[1,10]. The number of draft genome (partial data) deposits in public databases has grown exponentially since 2005 (Figure 1). In general, if no further studies will be developed using a particular organism's genome, it is more likely to be deposited as a draft genome. Otherwise, the painstaking tasks of improving and finishing the genome (complete data) must be undertaken[18].

This review will address the "scientific value" of a newly sequenced genome and the amount of insight it can provide. We will address the factors that could be leading to the increase in the number of draft deposits and the consequent loss of relevant biological information. Additionally, we will summarize the expectations created by NGS technologies regarding vaccine development and antibacterial discovery.

OVERVIEW OF SEQUENCING AND ASSEMBLY

For 30 years, sequencing technologies based on Sanger chemistry dominated the market. Although sequencing had undergone numerous improvements over the years, gene cloning techniques were still necessary to obtain genomic DNA sequences. Therefore, the time and cost required to obtain a complete genome sequence remained high. Moreover, the capacity of parallel sequencing was quite limited[19-21]. NGS platforms made it possible to sequence complete prokaryotic genomes using massively parallel sequencing more rapidly and at a lower cost[20,22].

Although NGS has facilitated sequencing processes, its relatively smaller reads make the assembly process a computational challenge[10,11]. The main limitation of short-read assembly methods is their inability to resolve repetitive regions of the genome without paired libraries[11]. The assembly of repetitive regions was an important issue even before the introduction of NGS platforms; shorter reads only made the problem worse.

In 2001, Kececioglu *et al*[23] argued about the impossibility of correctly assembling regions of the genome that contain identical copies of a sequence. Usually, long DNA repeats are not exact copies. They contain small differences that could, in principle, permit their correct assembly. Nevertheless, a major difficulty arises from sequencing errors. Assembly software must accept imperfect sequencing alignments to avoid missing genuine connections between sequences[22]. With the small amount of information within each read adding to the inherent sequencing error, it is difficult to separate true differences within repeated sequences from sequencing errors.

A study by Phillippy *et al*[24] revealed that the majority of contig ends in draft genomes were associated with repeated regions. They concluded that it was possible to

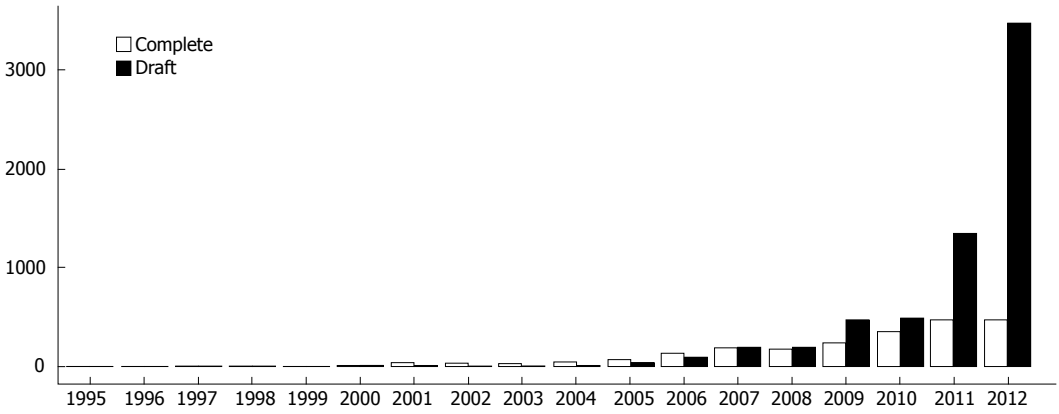

Figure 1 Number of complete genome and draft genome (partial data) deposits in public databases.

categorize the majority of mis-assembly events into two general classes: (1) repeat collapse or expansion; and (2) sequence rearrangement and inversion. Each of these classes exhibits specific mis-assembly signatures: the first class is the result of incorrect assembly in repetitive regions, including fewer or additional copies; the second class is the result of the rearrangement of multiple repeated copies, which is caused by the insertion of a read between them. The second class may be considered more influential because, if not fixed, it might be interpreted as a real biological rearrangement event[25,26]. If the assembler cannot resolve the region between two genomic fragments, a gap is formed. Gaps may occur due to: (1) an intrinsic characteristic of the sequencing platform that leads to incomplete or incorrect information; or (2) the inability of an assembly algorithm to handle regions of low complexity or repeated DNA[18,27,28]. The process of identifying and closing these gaps is quite laborious and requires additional manual intervention.

Gap closure processes usually involve the design of primers flanking the gap region to perform semi-automated sequencing of the unrepresented parts of the genome[28]. Several bioinformatics methodologies have been developed to facilitate gap closure. IMAGE is a tool that uses de Bruijn methodology to fill gaps with short reads that are aligned with flanking regions of the gap and were not used in the assembly[28]. In 2011, Cerdeira *et al*[29] generated a similar strategy by using CLC Genomics Workbench for the recursive alignment of unused short reads from the SOLiD platform. GapFiller is another tool that uses local alignment; its main advantage is the use of paired reads to estimate gap size and allows define the type of paired library: reverse-reverse, forward-forward, reverse-forward and forward-reverse[30].

From a purely practical standpoint, assembly tools are not required to produce a perfectly finished genome as an output. Their main function is to reduce the sequencing reads to a manageable number of contigs[26]. The process of finishing a genome, ensuring that gaps are closed and the gene order is correct, requires human decision-making. Therefore, the lack of fully automated processes constitutes a bottleneck in generating complete genomes.

"SCIENTIFIC VALUE" OF A NEWLY SEQUENCED GENOME

The value of a newly sequenced genome can be assessed using many different metrics. If publications are considered the main "currency" within the scientific community, there has been a considerable decrease in the value of new sequences over the last four decades.

The introduction of Sanger methodology in 1977 was one of the main landmarks in the early stages of the genomic era[31]. During the first years of using Sanger sequencing, a sequence of no more than 1000 nucleotides was sufficient for a work to be accepted in a journal such as Cell (current impact factor: 32.40) or Nature (current impact factor: 36.28)[32-34]. In 1980, the shotgun DNA sequencing methodology was introduced, enabling the sequencing of longer DNA fragments[35]. Complete bacterial operons were sequenced and published in journals such as Molecular Microbiology (current impact factor: 5.01) and Proceedings of the National Academy of Sciences (PNAS - current impact factor: 9.68)[36-38].

A combination of DNA sequencing improvements and the newly developed TIGR Assembler[39] culminated in the publication of the first complete bacterial genomes in 1995. Papers containing the complete nucleotide sequences of *Haemophilus influenzae* Rd (1830137 base pairs) and *Mycoplasma genitalium* (580070 base pairs) were both published in Science (current impact factor: 31.20)[40,41]. Almost 20 years later, a paper containing the sequence of a prokaryotic genome alone may be published in the Genome Announcement section of the Journal of Bacteriology (current impact factor: 3.82) or in Standards in Genomic Sciences (SIGS - has not been published sufficiently long to receive an impact factor). A recent article by Smith even refers to the not-so-distant "death" of the "genome paper", noting that the space for genome publication may come to an end soon[42].

The publication impact of newly sequenced genomes decreased following DNA sequencing improvements, and the reason is no mystery. High-impact journals only publish groundbreaking original scientific research or

results of outstanding scientific importance. To produce a higher-impact publication, more information must be extracted from genomes. For instance, several genomes may be examined in a comparative genomic analysis or pangenomic study[43,44], or an analysis may focus on the presence or absence of specific markers or on small differences between DNA sequences[26,45]. In this context, the genome becomes a stepping stone to the main goal, the comparative analysis. As the basis of the analysis, the genome sequence remains important. Nevertheless, it may not be of sufficient importance for one to undertake the painstaking task of completing the genome sequence.

WHAT IS LOST WHEN WE OPT FOR A DRAFT GENOME?

Over the years, arguments have been presented in favor both of complete genomes[41,46] and of the superior "tradeoff" that a draft genome represents[47]. The discussion has been centered around two main points: (1) to provide the greatest amount of useful data, sequences must be as complete as possible; and (2) draft genomes (partial data) are sufficient for most scientific contexts. The issue at stake is the extra money and manpower necessary to finish a genome. Is the additional information contained in a finished genome worth the investment? To answer this question, one must identify the information that is lost from a draft and analyze the quality of data that is generated using drafts. Furthermore, it is necessary to understand the limits of draft genome use.

The first issue to consider is whether it is possible to properly identify all of an organism's genes in a draft genome. Gene characterization consists of the following: (1) gene prediction with the identification of an open reading frame (ORF); and (2) the functional annotation of the gene product. The main gene identification problems in drafts are associated with the partial or complete loss of ORFs[10]. Such errors may lead either to over-annotation, due to the annotation of multiple fragments originating from the same ORF, or to under-annotation, possibly due to the absence of partial or entire domains from the ORF[10]. These problems affect genomic analyses, causing errors due to missing ORFs that are not annotated or due to multiple fragments that belong to the same ORF but are annotated separately. In other words, the mere absence of a gene from a draft cannot be considered definitive proof of its absence from the organism's genome[10,41].

The pangenomic approach is one type of analysis that may be impaired by reliance on draft genomes, because many genes in a draft may be misidentified due to fragmentation. Pangenomic projects attempt to characterize the gene pool of a bacterial species as the genes that are present in all strains (the "core genome") and the genes that are present in only a few species (the "dispensable genome")[43]. Horizontal gene transfer (HGT) analysis is another approach that cannot be performed using drafts. HGT is one of the main sources of variability among bacteria because it allows the acquisition of several new genes[36,37]. There is evidence that most gaps in genomic sequences are associated with transposases, insertion sequences and integrases, structures that usually flank a genomic island[48]. Another approach that may be impaired by reliance on drafts is phylogenomics, which aims to reconstruct both the vertical and lateral gene transfer processes of a bacterial species using a whole-genome analysis[49].

Although not strictly related to drafts, the functional annotation of genes is another feature that is usually neglected when we opt for a draft genome (Figure 2). Complete genomes may also present this problem because the quality of functional annotation is related to the amount of effort dedicated to a genome. DNA sequence is being generated much more rapidly than it can be analyzed; thus, a large proportion of the sequence information in databases has been annotated solely by automatic algorithms[50]. It is disturbing that although automatic annotation algorithms have improved over the years, misannotation has increased over time[50]. The misannotation of a reference strain is particularly harmful because the error will likely be propagated to other genomes. In our attempts to exploit the full potential of NGS, we risk having databases filled with incomplete and/or incorrect genomic data.

Because the purpose of many sequencing projects is to identify a small number of differences between a newly sequenced genome and the sequence of a closely related species, a large number of genomes are left as drafts[26]. Considering the constant evolution of organisms, a sequenced genome represents a snapshot in the biological history of a species. Therefore, a single finished genome might be useful for decades of future studies. By opting for draft genomes, we may be shutting down the full gamut of future scientific analysis.

VACCINE DEVELOPMENT

Genomic information was expected to boost vaccine discovery. In an attempt to measure the impact of genomic information on this field, Prachi *et al*[2] analyzed all the patent applications that contained genomic information. They observed that there was an enormous increase in such applications shortly after the first complete genomes were released, but since 2002, there has been a continuous decrease. The authors attributed this decrease to more stringent legal requirements, which call for empirical evidence to complement in silico data.

The initial increase in patent applications containing genomic information was related to the development of a new paradigm in vaccine development. In 2000, Rappuoli[51] described the "reverse vaccinology" (RV) concept, in which he proposed inverting the traditional process of antigen identification. Instead of identifying the antigenic components of a pathogenic organism using serological or biochemical methods, RV uses the organism's genome to predict all of its protein antigens. RV approaches mainly focus on secreted proteins because they are more likely to induce immune responses. Secreted proteins are involved in several processes that modulate

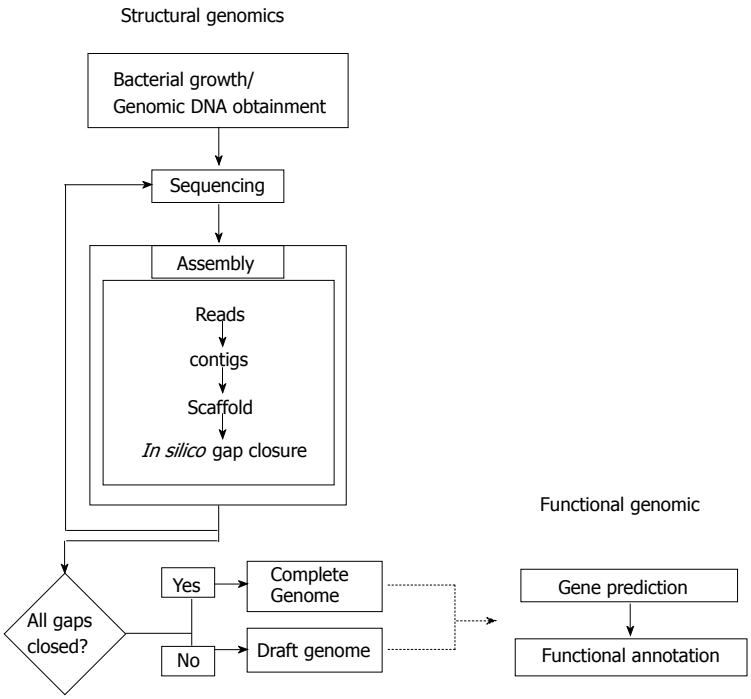

Figure 2 General workflow during sequencing process a bacterial genome.

the host-pathogen relationship, such as cell adhesion and invasion, as well as resistance to stress conditions[52-54]. Over the years, several methodologies have been developed to predict secreted proteins and to evaluate their potential immunological properties.

In 2010, Vaxign was released as the first vaccine design tool with a web interface (http://www.violinet.org/vaxign/). Vaxign allows users to submit their own sequences to perform vaccine target predictions. The Vaxign predictions have been consistent with existing reports for organisms such as *Mycobacterium tuberculosis* and *Neisseria meningitides*[55]. Another vaccine design tool is MED (Mature Epitope Density - http://med.mmci.uni-saarland.de/). MED attempts to select the more promising vaccine targets by identifying proteins with higher concentrations of epitopes[56]. There are also tools exclusively for protein epitope prediction, such as Immune Epitope Analysis (http://tools.immuneepitope.org/main/) and Vaxitope (http://www.violinet.org/vaxign/vaxitop/index.php).

Because a large number of bacterial genomes are already available, reverse vaccinology is quite accessible and inexpensive. Nevertheless, as has been previously discussed[57,58], the expectations for reverse vaccinology techniques do not correspond to reality, given the small number of vaccines have been developed using the bacterial genome sequences available[59]. This occurs because there are also several factors that are involved in the host response during infection, for example, the production of antibodies by the immune system.

ANTIBACTERIAL DISCOVERY

The period between the 1930s and the 1960s is known as the "golden age" of antibiotic discovery[11,60]. During this period, most of the known classes of antibiotics were discovered. These discoveries involved screening natural products regardless of their mechanisms of action. After most of the low-hanging fruits were harvested, the rate of antibacterial discovery decreased, culminating in a slowdown beginning in the 1990s[61].

Hopes for turning this void into a rapid acceleration accompanied the completion of the first bacterial genome sequences. The goal was to use comparative genomic analysis to identify potential targets present in a desirable spectrum (*e.g.*, the bacteria responsible for upper respiratory tract infections)[3,4,62]. It was naive to assume that having the genome sequences would be sufficient for this level of discovery; a possible drug target must undergo numerous stages, from discovery through human clinical tests, and it is not possible to develop drugs for all potential targets[3,62]. Nevertheless, the prospect of exploring hundreds of potential targets revived the interest of pharmaceutical companies.

After some years of trials, several companies ended their target-based programs because of a lack of productivity. Despite reports of multi-resistant bacterial strains, the efforts to discover new antibacterial targets were again reduced[63,64]. Although genomics has not been able to reverse the lack of new antibiotic development, it has significantly improved screening methodologies. Genomics has facilitated high-throughput drug campaigns, which are being used to determine the mechanisms of action of antibacterial compounds and bacterial resistance mechanisms[4].

CONCLUSION

Several next-generation platforms have been developed

in recent decades, as well as bioinformatics programs to an enhancement of performance and optimization omics techniques. Is not yet possible to integrate reads from different sequencers into a single assembly[17,23]. This newly developed approach aims to reduce the amount of manual intervention needed to complete a genome sequence by using a hybrid approach to resolve repetitive regions.

Improvements are expected not only in sequencing platforms but also in assemblers. Recently, two groups assessed the quality of the currently available assemblers. The 2011 Assemblathon was the first competition among assemblers[65]. For this competition, simulated data were generated and groups of assemblers were asked to blindly assemble it. The use of simulated data poses a problem in determining the applicability of the results to other data sets. The 2012 GAGE (Genome Assembly Gold-Standard Evaluations) competition for assembling real data resulted in the following conclusions: (1) the data quality has a greater influence on the final outcome than the assembler itself; and (2) the results do not support the current measures of correctness (related to contiguity)[26].

There is a large gap between the availability of genomic sequences in databases and the commercial production of vaccines and antibiotics in recent years, especially in the fields of investment and success ("expected return"). Drug development for all potential targets and effective vaccines has produced limited success. In contrast, there has been an acceleration in the discovery of new targets due to the refinement of bioinformatics tools for this purpose, such as epitope mapping and searching for secreted proteins. However, the major problems facing vaccine and antibiotic development, such as resistance mechanisms and host immune responses, remain unsolved.

Genome analysis constitutes a strategy for the expansion and diversification of the pharmacology and vaccinology sectors. This methodology can be used to explore a large number of targets and to reduce the costs of molecular and immunological tests. Finally, to improve the production of antibiotics and vaccines, it is necessary to know more about bacterial regulatory pathways. New interactome and microbiome studies must be implemented to assist this search.

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REFERENCES

1 **Zhang J**, Chiodini R, Badr A, Zhang G. The impact of next-generation sequencing on genomics. *J Genet Genomics* 2011; **38**: 95-109 [PMID: 21477781 DOI: 10.1016/j.jgg.2011.02.003]
2 **Prachi P**, Donati C, Masciopinto F, Rappuoli R, Bagnoli F. Deep sequencing in pre- and clinical vaccine research. *Public Health Genomics* 2013; **16**: 62-68 [PMID: 23548719 DOI: 10.1159/000345611]
3 **Pucci MJ**. Use of genomics to select antibacterial targets. *Biochem Pharmacol* 2006; **71**: 1066-1072 [PMID: 16412986 DOI: 10.1016/j.bcp.2005.12.004]
4 **Mills SD**. When will the genomics investment pay off for antibacterial discovery? *Biochem Pharmacol* 2006; **71**: 1096-1102 [PMID: 16387281 DOI: 10.1016/j.bcp.2005.11.025]
5 **Pareek CS**, Smoczynski R, Tretyn A. Sequencing technologies and genome sequencing. *J Appl Genet* 2011; **52**: 413-435 [PMID: 21698376 DOI: 10.1007/s13353-011-0057-x]
6 **Liu L**, Li Y, Li S, Hu N, He Y, Pong R, Lin D, Lu L, Law M. Comparison of next-generation sequencing systems. *J Biomed Biotechnol* 2012; **2012**: 251364 [PMID: 22829749 DOI: 10.1155/2012/251364]
7 **Metzker ML**. Sequencing technologies - the next generation. *Nat Rev Genet* 2010; **11**: 31-46 [PMID: 19997069 DOI: 10.1038/nrg2626]
8 **Magi A,** Benelli M, Gozzini A, Girolami F, Torricelli F, Brandi ML. Bioinformatics for Next Generation Sequencing Data. *Genes* 2010; **1**: 294-307 [DOI: 10.3390/genes1020294]
9 **Loman NJ**, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE, Wain J, Pallen MJ. Performance comparison of benchtop high-throughput sequencing platforms. *Nat Biotechnol* 2012; **30**: 434-439 [PMID: 22522955 DOI: 10.1038/nbt.2198]
10 **Klassen JL**, Currie CR. Gene fragmentation in bacterial draft genomes: extent, consequences and mitigation. *BMC Genomics* 2012; **13**: 14 [PMID: 22233127 DOI: 10.1186/1471-2164-13-14]
11 **Miller JR**, Koren S, Sutton G. Assembly algorithms for next-generation sequencing data. *Genomics* 2010; **95**: 315-327 [PMID: 20211242 DOI: 10.1016/j.ygeno.2010.03.001]
12 **Bashir A**, Klammer AA, Robins WP, Chin CS, Webster D, Paxinos E, Hsu D, Ashby M, Wang S, Peluso P, Sebra R, Sorenson J, Bullard J, Yen J, Valdovino M, Mollova E, Luong K, Lin S, LaMay B, Joshi A, Rowe L, Frace M, Tarr CL, Turnsek M, Davis BM, Kasarskis A, Mekalanos JJ, Waldor MK, Schadt EE. A hybrid approach for the automated finishing of bacterial genomes. *Nat Biotechnol* 2012; **30**: 701-707 [PMID: 22750883 DOI: 10.1038/nbt.2288]
13 **Ribeiro FJ**, Przybylski D, Yin S, Sharpe T, Gnerre S, Abouelleil A, Berlin AM, Montmayeur A, Shea TP, Walker BJ, Young SK, Russ C, Nusbaum C, MacCallum I, Jaffe DB. Finished bacterial genomes from shotgun sequence data. *Genome Res* 2012; **22**: 2270-2277 [PMID: 22829535 DOI: 10.1101/gr.141515.112]
14 **Baker M.** De novo genome assembly: what every biologist should know. *Nature Methods* 2012; **9**: 333-337 [DOI: 10.1038/nmeth.1935]
15 **Salzberg SL**, Phillippy AM, Zimin A, Puiu D, Magoc T, Koren S, Treangen TJ, Schatz MC, Delcher AL, Roberts M, Marçais G, Pop M, Yorke JA. GAGE: A critical evaluation of genome assemblies and assembly algorithms. *Genome Res* 2012; **22**: 557-567 [PMID: 22147368 DOI: 10.1101/gr.131383.111]
16 **Faircloth BC**, Glenn TC. Not all sequence tags are created equal: designing and validating sequence identification tags robust to indels. *PLoS One* 2012; **7**: e42543 [PMID: 22900027 DOI: 10.1371/journal.pone.0042543]
17 **Diguistini S**, Liao NY, Platt D, Robertson G, Seidel M, Chan SK, Docking TR, Birol I, Holt RA, Hirst M, Mardis E, Marra MA, Hamelin RC, Bohlmann J, Breuil C, Jones SJ. De novo

genome sequence assembly of a filamentous fungus using Sanger, 454 and Illumina sequence data. *Genome Biol* 2009; **10**: R94 [PMID: 19747388 DOI: 10.1186/gb-2009-10-9-r94]

18 **Chain PS**, Grafham DV, Fulton RS, Fitzgerald MG, Hostetler J, Muzny D, Ali J, Birren B, Bruce DC, Buhay C, Cole JR, Ding Y, Dugan S, Field D, Garrity GM, Gibbs R, Graves T, Han CS, Harrison SH, Highlander S, Hugenholtz P, Khouri HM, Kodira CD, Kolker E, Kyrpides NC, Lang D, Lapidus A, Malfatti SA, Markowitz V, Metha T, Nelson KE, Parkhill J, Pitluck S, Qin X, Read TD, Schmutz J, Sozhamannan S, Sterk P, Strausberg RL, Sutton G, Thomson NR, Tiedje JM, Weinstock G, Wollam A, Detter JC. Genomics. Genome project standards in a new era of sequencing. *Science* 2009; **326**: 236-237 [PMID: 19815760 DOI: 10.1126/science.1180614]

19 **Shendure J**, Mitra RD, Varma C, Church GM. Advanced sequencing technologies: methods and goals. *Nat Rev Genet* 2004; **5**: 335-344 [PMID: 15143316 DOI: 10.1038/nrg1325]

20 **Shendure J**, Porreca GJ, Reppas NB, Lin X, McCutcheon JP, Rosenbaum AM, Wang MD, Zhang K, Mitra RD, Church GM. Accurate multiplex polony sequencing of an evolved bacterial genome. *Science* 2005; **309**: 1728-1732 [PMID: 16081699 DOI: 10.1126/science.1117389]

21 **Richardson P.** Special Issue: Next Generation DNA Sequencing. *Genes* 2010; **1**: 385-387 [DOI: 10.3390/genes1030385]

22 **Munroe DJ**, Harris TJ. Third-generation sequencing fireworks at Marco Island. *Nat Biotechnol* 2010; **28**: 426-428 [PMID: 20458306 DOI: 10.1038/nbt0510-426]

23 **Kececioglu J,** Ju J. Separating repeats in DNA sequence assembly. Proceedings of the 5th International Conference on Computational Biology, 2001. New York: ACM, 2001: 176-183 [DOI: 10.1145/369133.36919]

24 **Phillippy AM**, Schatz MC, Pop M. Genome assembly forensics: finding the elusive mis-assembly. *Genome Biol* 2008; **9**: R55 [PMID: 18341692 DOI: 10.1186/gb-2008-9-3-r55]

25 **Soares SC**, Abreu VA, Ramos RT, Cerdeira L, Silva A, Baumbach J, Trost E, Tauch A, Hirata R, Mattos-Guaraldi AL, Miyoshi A, Azevedo V. PIPS: pathogenicity island prediction software. *PLoS One* 2012; **7**: e30848 [PMID: 22355329 DOI: 10.1371/journal.pone.0030848]

26 **Ricker N**, Qian H, Fulthorpe RR. The limitations of draft assemblies for understanding prokaryotic adaptation and evolution. *Genomics* 2012; **100**: 167-175 [PMID: 22750556 DOI: 10.1016/j.ygeno.2012.06.009]

27 **Pop M**. Genome assembly reborn: recent computational challenges. *Brief Bioinform* 2009; **10**: 354-366 [PMID: 19482960 DOI: 10.1093/bib/bbp026]

28 **Tsai IJ**, Otto TD, Berriman M. Improving draft assemblies by iterative mapping and assembly of short reads to eliminate gaps. *Genome Biol* 2010; **11**: R41 [PMID: 20388197 DOI: 10.1186/gb-2010-11-4-r41]

29 **Cerdeira LT**, Carneiro AR, Ramos RT, de Almeida SS, D' Afonseca V, Schneider MP, Baumbach J, Tauch A, McCulloch JA, Azevedo VA, Silva A. Rapid hybrid de novo assembly of a microbial genome using only short reads: Corynebacterium pseudotuberculosis I19 as a case study. *J Microbiol Methods* 2011; **86**: 218-223 [PMID: 21620904 DOI: 10.1016/j.mimet.2011.05.008]

30 **Boetzer M**, Pirovano W. Toward almost closed genomes with GapFiller. *Genome Biol* 2012; **13**: R56 [PMID: 22731987 DOI: 10.1186/gb-2012-13-6-r56]

31 **Sanger F**, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977; **74**: 5463-5467 [PMID: 271968 DOI: 10.1073/pnas.74.12.5463]

32 **de Boer HA**, Gilbert SF, Nomura M. DNA sequences of promoter regions for rRNA operons rrnE and rrnA in E. coli. *Cell* 1979; **17**: 201-209 [PMID: 378405 DOI: 10.1016/0092-8674(79)90308-8]

33 **Nakamura K**, Inouye M. DNA sequence of the gene for the outer membrane lipoprotein of E. coli: an extremely AT-rich promoter. *Cell* 1979; **18**: 1109-1117 [PMID: 391404 DOI: 10.1016/0092-8674(79)90224-1]

34 **Porter AG**, Barber C, Carey NH, Hallewell RA, Threlfall G, Emtage JS. Complete nucleotide sequence of an influenza virus haemagglutinin gene from cloned DNA. *Nature* 1979; **282**: 471-477 [PMID: 503226 DOI: 10.1038/282471a0]

35 **Messing J**, Crea R, Seeburg PH. A system for shotgun DNA sequencing. *Nucleic Acids Res* 1981; **9**: 309-321 [PMID: 6259625 DOI: 10.1093/nar/9.2.309]

36 **Brown NL**, Misra TK, Winnie JN, Schmidt A, Seiff M, Silver S. The nucleotide sequence of the mercuric resistance operons of plasmid R100 and transposon Tn501: further evidence for mer genes which enhance the activity of the mercuric ion detoxification system. *Mol Gen Genet* 1986; **202**: 143-151 [PMID: 3007931 DOI: 10.1007/BF00330531]

37 **Postle K**, Good RF. DNA sequence of the Escherichia coli tonB gene. *Proc Natl Acad Sci USA* 1983; **80**: 5235-5239 [PMID: 6310567 DOI: 10.1073/pnas.80.17.5235]

38 **Overduin P**, Boos W, Tommassen J. Nucleotide sequence of the ugp genes of Escherichia coli K-12: homology to the maltose system. *Mol Microbiol* 1988; **2**: 767-775 [PMID: 3062310 DOI: 10.1111/j.1365-2958.1988.tb00088.x]

39 **Sutton GG,** White O, Adams MD and Kerlavage A. TIGR Assembler: A new tool for assembling large shotgun sequencing projects. *Genome Sci Technol* 1995; **1** Suppl 1: S9-S19 [DOI: 10.1089/gst.1995.1.9]

40 **Fleischmann RD**, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM. Whole-genome random sequencing and assembly of Haemophilus influenzae Rd. *Science* 1995; **269**: 496-512 [PMID: 7542800 DOI: 10.1126/science.7542800]

41 **Fraser CM**, Eisen JA, Nelson KE, Paulsen IT, Salzberg SL. The value of complete microbial genome sequencing (you get what you pay for). *J Bacteriol* 2002; **184**: 6403-6405; discusion 6405 [PMID: 12426324 DOI: 10.1128/JB.184.23.6403-6405.2002]

42 **Smith DR**. Death of the genome paper. *Front Genet* 2013; **4**: 72 [PMID: 23653633 DOI: 10.3389/fgene.2013.00072]

43 **Medini D**, Donati C, Tettelin H, Masignani V, Rappuoli R. The microbial pan-genome. *Curr Opin Genet Dev* 2005; **15**: 589-594 [PMID: 16185861 DOI: 10.1016/j.gde.2005.09.006]

44 **Soares SC**, Silva A, Trost E, Blom J, Ramos R, Carneiro A, Ali A, Santos AR, Pinto AC, Diniz C, Barbosa EG, Dorella FA, Aburjaile F, Rocha FS, Nascimento KK, Guimarães LC, Almeida S, Hassan SS, Bakhtiar SM, Pereira UP, Abreu VA, Schneider MP, Miyoshi A, Tauch A, Azevedo V. The pan-genome of the animal pathogen Corynebacterium pseudotuberculosis reveals differences in genome plasticity between the biovar ovis and equi strains. *PLoS One* 2013; **8**: e53818 [PMID: 23342011 DOI: 10.1371/journal.pone.0053818]

45 **Jakobsen TH**, Hansen MA, Jensen PØ, Hansen L, Riber L, Cockburn A, Kolpen M, Rønne Hansen C, Ridderberg W, Eickhardt S, Hansen M, Kerpedjiev P, Alhede M, Qvortrup K, Burmølle M, Moser C, Kühl M, Ciofu O, Givskov M, Sørensen SJ, Høiby N, Bjarnsholt T. Complete genome sequence of the cystic fibrosis pathogen Achromobacter xylosoxidans NH44784-1996 complies with important pathogenic phenotypes. *PLoS One* 2013; **8**: e68484 [PMID: 23894309 DOI: 10.1371/journal.pone.0068484]

46 **Parkhill J**. In defense of complete genomes. *Nat Biotechnol* 2000; **18**: 493-494 [PMID: 10802612 DOI: 10.1038/75346]

47 **Branscomb E**, Predki P. On the high value of low standards. *J Bacteriol* 2002; **184**: 6406-6409; discussion 6409 [PMID: 12426325 DOI: 10.1128/JB.184.23.6406-6409.2002]

48 **Kingsford C**, Schatz MC, Pop M. Assembly complexity of prokaryotic genomes using short reads. *BMC Bioinformatics* 2010; **11**: 21 [PMID: 20064276 DOI: 10.1186/1471-2105-11-21]

49 **Dagan T**. Phylogenomic networks. *Trends Microbiol* 2011; **19**: 483-491 [PMID: 21820313 DOI: 10.1016/j.tim.2011.07.001]

50 **Schnoes AM**, Brown SD, Dodevski I, Babbitt PC. Annotation error in public databases: misannotation of molecular function in enzyme superfamilies. *PLoS Comput Biol*

2009; **5**: e1000605 [PMID: 20011109 DOI: 10.1371/journal.pcbi.1000605]
51 **Rappuoli R**. Reverse vaccinology. *Curr Opin Microbiol* 2000; **3**: 445-450 [PMID: 11050440 DOI: 10.1016/S1369-5274(00)00119-3]
52 **Wooldridge K.** Bacterial secreted proteins: secretory mechanisms and role in pathogenesis. Caister Academic Press, 2009: 300-315
53 **Simeone R**, Bottai D, Brosch R. ESX/type VII secretion systems and their role in host-pathogen interaction. *Curr Opin Microbiol* 2009; **12**: 4-10 [PMID: 19155186 DOI: 10.1016/j.mib.2008.11.003]
54 **Stavrinides J**, McCann HC, Guttman DS. Host-pathogen interplay and the evolution of bacterial effectors. *Cell Microbiol* 2008; **10**: 285-292 [PMID: 18034865 DOI: 10.1111/j.1462-5822.2007.01078.x]
55 **He Y**, Xiang Z, Mobley HL. Vaxign: the first web-based vaccine design program for reverse vaccinology and applications for vaccine development. *J Biomed Biotechnol* 2010; **2010**: 297505 [PMID: 20671958 DOI: 10.1155/2010/297505]
56 **Santos AR**, Pereira VB, Barbosa E, Baumbach J, Pauling J, Röttger R, Turk MZ, Silva A, Miyoshi A, Azevedo V. Mature Epitope Density--a strategy for target selection based on immunoinformatics and exported prokaryotic proteins. *BMC Genomics* 2013; **14** Suppl 6: S4 [PMID: 24564223 DOI: 10.1186/1471-2164-14-S6-S4]
57 **Seib KL**, Zhao X, Rappuoli R. Developing vaccines in the era of genomics: a decade of reverse vaccinology. *Clin Microbiol Infect* 2012; **18** Suppl 5: 109-116 [PMID: 22882709 DOI: 10.1111/j.1469-0691.2012.03939.x]
58 **Tettelin H**. The bacterial pan-genome and reverse vaccinology. *Genome Dyn* 2009; **6**: 35-47 [PMID: 19696492 DOI: 10.1159/000235761]
59 **Donati C**, Rappuoli R. Reverse vaccinology in the 21st century: improvements over the original design. *Ann N Y Acad Sci* 2013; **1285**: 115-132 [PMID: 23527566 DOI: 10.1111/nyas.12046]
60 **Walsh C**. Where will new antibiotics come from? *Nat Rev Microbiol* 2003; **1**: 65-70 [PMID: 15040181 DOI: 10.1038/nrmicro727]
61 **Silver LL**. Challenges of antibacterial discovery. *Clin Microbiol Rev* 2011; **24**: 71-109 [PMID: 21233508 DOI: 10.1128/CMR.00030-10]
62 **Payne DJ**, Gwynn MN, Holmes DJ, Pompliano DL. Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat Rev Drug Discov* 2007; **6**: 29-40 [PMID: 17159923 DOI: 10.1038/nrd2201]
63 **Projan SJ**. Why is big Pharma getting out of antibacterial drug discovery? *Curr Opin Microbiol* 2003; **6**: 427-430 [PMID: 14572532 DOI: 10.1016/j.mib.2003.08.003]
64 **Bush K**, Pucci MJ. New antimicrobial agents on the horizon. *Biochem Pharmacol* 2011; **82**: 1528-1539 [PMID: 21798250 DOI: 10.1016/j.bcp.2011.07.077]
65 **Earl D,** Bradnam K, St John J, Darling A, Lin D, Fass J, Yu HO, Buffalo V, Zerbino DR, Diekhans M, Nguyen N, Ariyaratne PN, Sung WK, Ning Z, Haimel M, Simpson JT, Fonseca NA, Birol İ, Docking TR, Ho IY, Rokhsar DS, Chikhi R, Lavenier D, Chapuis G, Naquin D, Maillet N, Schatz MC, Kelley DR, Phillippy AM, Koren S, Yang SP, Wu W, Chou WC, Srivastava A, Shaw TI, Ruby JG, Skewes-Cox P, Betegon M, Dimon MT, Solovyev V, Seledtsov I, Kosarev P, Vorobyev D, Ramirez-Gonzalez R, Leggett R, MacLean D, Xia F, Luo R, Li Z, Xie Y, Liu B, Gnerre S, MacCallum I, Przybylski D, Ribeiro FJ, Yin S, Sharpe T, Hall G, Kersey PJ, Durbin R, Jackman SD, Chapman JA, Huang X, DeRisi JL, Caccamo M, Li Y, Jaffe DB, Green RE, Haussler D, Korf I, Paten B. Assemblathon 1: a competitive assessment of de novo short read assembly methods. *Genome Res* 2011; **21**: 2224-2241 [PMID: 21926179 DOI: 10.1101/gr.126599]

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REVIEW

Activated protein C: A regulator of human skin epidermal keratinocyte function

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Abstract

Activated protein C (APC) is a physiological anticoagulant, derived from its precursor protein C (PC). Independent of its anticoagulation, APC possesses strong anti-inflammatory, anti-apoptotic and barrier protective properties which appear to be protective in a number of disorders including chronic wound healing. The epidermis is the outermost skin layer and provides the first line of defence against the external environment. Keratinocytes are the most predominant cells in the epidermis and play a critical role in maintaining epidermal barrier function. PC/APC and its receptor, endothelial protein C receptor (EPCR), once thought to be restricted to the endothelium, are abundantly expressed by skin epidermal keratinocytes. These cells respond to APC by upregulating proliferation, migration and matrix metalloproteinase-2 activity and inhibiting apoptosis/inflammation leading to a wound healing phenotype. APC also increases barrier function of keratinocyte monolayers by promoting the expression of tight junction proteins and re-distributing them to cell-cell contacts. These cytoprotective properties of APC are mediated through EPCR, protease-activated receptors, epidermal growth factor receptor or Tie2. Future preventive and therapeutic uses of APC in skin disorders associated with disruption of barrier function and inflammation look promising. This review will focus on APC's function in skin epidermis/keratinocytes and its therapeutical potential in skin inflammatory conditions.

Key words: Activated protein C; Endothelial protein C receptor; Protease-activated receptor; Keratinocyte; Proliferation; Junction protein; Barrier function

Core tip: The anti-inflammatory, barrier stabilisation and anti-apoptotic properties of APC make it an appealing treatment for skin conditions associated with inflammation, barrier disruption and keratinocyte dysfunction.

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INTRODUCTION

Protein C (PC) is a vitamin-K dependent glycoprotein that circulates in blood plasma in its zymogenic and activated forms [activated PC (APC)]. PC/APC was first characterised for its role in blood coagulation, but has a range of cytoprotective functions including anti-inflammation, anti-apoptosis and barrier stabilisation. Although originally thought to be synthesised almost exclusively by the liver and vascular endothelial cells, PC/APC has been found to be synthesised by skin epidermal keratinocytes. Keratinocytes are the major cell type in

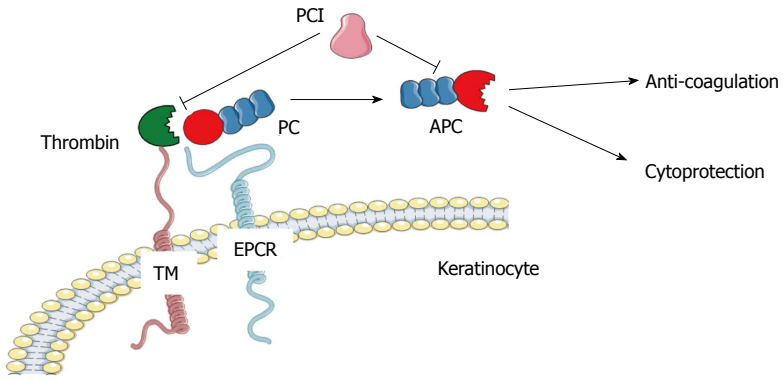

Figure 1 Schematic representation of protein C/activated protein C activation and cellular effects. APC: Activated protein C; EPCR: Endothelial protein C receptor; PC: Protein C; PCI: Protein C inhibitor; TM: Thrombomodulin. Figure was produced using Servier Medical Art - www.servier.com.

the skin epidermis, the most outer layer of human skin that provides a semi-impermeable barrier against injury from the external environment, including ultraviolet radiation, heat, water loss and infectious pathogens. On keratinocytes, PC/APC promotes cell proliferation, survival, migration, and the barrier function. This review will focus on the actions of APC on skin epidermis/keratinocytes and its therapeutical potential in the treatment of skin inflammatory conditions.

PC and APC

The PC pathway plays a key role in the regulation of blood coagulation. As a vitamin K-dependent zymogen, PC is activated to APC when thrombin binds to thrombomodulin and cleaves the activation peptide (Figure 1). This conversion is augmented by its specific receptor, endothelial cell protein C receptor (EPCR)[1]. In human plasma APC is present at relatively low levels approximation 40 pmol/L and has a short physiological half-life of approximation 20 min compared to PC at 70 nmol/L and approximation 10 h[2,3]. Thrombin is the only endogenous activator of PC. The importance of APC as an anticoagulant is reflected by findings that deficiencies in PC result in severe familial disorders of thrombosis[4]. Replenishment of PC in patients with systemic or local hypercoagulation can reverse the abnormality.

Independent of its effect on anti-coagulation, APC possesses strong anti-inflammatory and anti-apoptotic properties, as well as enhancing endothelial and epithelial barrier integrity (Figure 1).

Inhibiting inflammation: The anti-inflammatory effects of APC are associated with a decrease in pro-inflammatory cytokines and a reduction in leukocyte recruitment. APC inhibits neutrophil, monocyte and lymphocyte chemotaxis[5] and directly suppresses expression and activation of nuclear factor (NF)-κB[6]; a pathway that controls the expression of a wide range of inflammatory genes including tumour necrosis factor (TNF)-α and cell adhesion molecules. Acute inflammation is exacerbated in mice genetically predisposed to a severe PC deficiency[7]. *In vitro*, APC suppresses the activation of NF-κB and production of TNF-α, upregulates matrix metalloproteinase (MMP)-2 activity yet inhibits MMP-9 in rheumatoid synovial fibroblasts and monocytes[8]. In addition to the degradation of extracellular matrix, these MMPs can regulate inflammation by processing cytokines/chemokines with MMP-9 having stimulatory and MMP-2 having inhibitory effects on inflammation both *in vitro* and *in vivo*[9-11].

Promoting cell proliferation and inhibiting cell apoptosis: APC promotes cell proliferation in cultured human umbilical vein endothelial cells[12], smooth muscle cells[13], keratinocytes[14], neural stem and progenitor cells[15,16], neuroblasts[17], osteoblasts[18] and ovine tenocytes[19]. Consistent with the stimulatory effects on cell growth, APC displays strong anti-apoptotic properties in keratinocytes, endothelial cells and podocytes[14,20-22]. APC-dependent anti-apoptotic activity shows improved survival in human and various animal models of sepsis[23-28]. APC inhibits spontaneous monocyte apoptosis leading to increased lifespan and phagocytosis *in vivo*[29] and protects murine cortical neurons from N-methyl-D-aspartate and staurosporine excitotoxicity-induced apoptosis[30].

Stabilising endothelial and epithelial barrier: Endothelial cells normally form a dynamically regulated stable barrier at the blood-tissue interface. Breakdown of this barrier is a key pathogenic factor in inflammatory disorders. APC enhances endothelial barrier integrity by stabilising the cytoskeleton and reducing endothelial permeability[20,31-33]. Recently, APC has been shown to promote epithelial barrier function in human skin epidermal keratinocytes[34] and mouse intestine[35].

APC's signalling pathway: Many of the anti-inflammatory properties of APC are mediated through EPCR, which itself is anti-inflammatory[36]. APC bound to EPCR can activate protease-activated receptor (PAR)-1 and promote the anti-inflammatory actions of APC[37]. Cytoprotective effects of APC are also mediated by the other PAR receptors. Akin to PAR-1, APC can bind to PAR-2 and activate the Akt signaling pathway to promote keratinocyte proliferation[37]. Independent of EPCR, APC can inhibit podocyte apoptosis by activating PAR-3[38]. APC-mediated arrest of lymphocyte chemotaxis is dependent on epidermal growth factor receptor (EGFR)[39]. In addition, EGFR transactivation by APC/EPCR/PAR-1 supports cell motility and invasiveness of endothelial cells

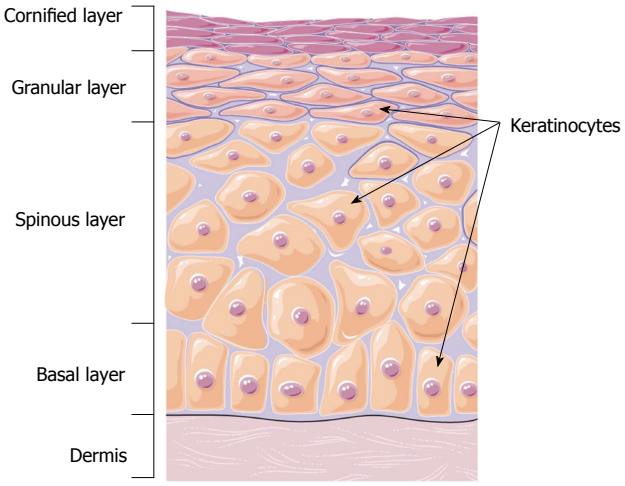

Figure 2 Schematic representation of the structure of skin showing the epidermal layers. Figure was produced using Servier Medical Art - www.servier.com.

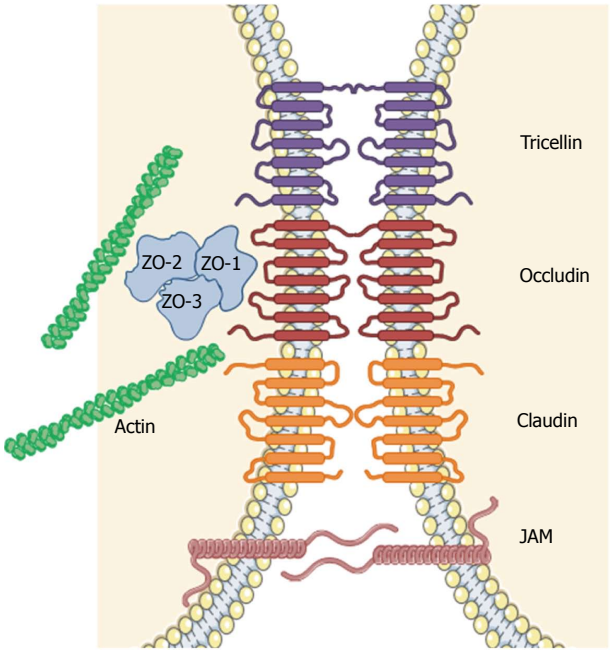

Figure 3 Schematic representation of epidermal tight junction complex. JAM: Junctional adhesion molecule; ZO: Zona occludin. Figure was produced using Servier Medical Art - www.servier.com.

and breast cancer cells[40]. APC utilises the angiopoietin/Tie2 axis to promote endothelial barrier function[33]. In addition other receptors such as integrins[41] and apolipoprotein E receptor-2[42] also mediate the effects of APC.

Skin function and keratinocytes

The skin forms an effective barrier between the human body and outside environment and protects the body from mechanical trauma, pathogens, radiation, dehydration, and dangerous temperature fluctuations[43]. Skin consists of two main layers, the outermost epidermis layer and the underlying dermis (Figure 2). The epidermis is a stratified epithelium composed of proliferating basal and differentiated suprabasal keratinocytes. The dermis provides the epidermis with mechanical support and nutrients. The barrier function of skin is provided by the epidermis. Defective epidermal barrier is responsible for many inflammatory and blistering skin disorders[43,44].

Keratinocytes are the most abundant cell type in the epidermis and are responsible for maintaining structure and homeostasis of the epidermal barrier. The epidermal barrier is generated by a sophisticated differentiation program[44] comprising stratified epithelium composed of basal, spinous, granular, and cornified layers (Figure 2)[45]. The basal layer consists of proliferating keratinocytes, that maintain the epidermis and post-mitotic basal keratinocytes which migrate out of the basal layer. This migration marks the start of epidermal differentiation that ends with the formation of the cornified layer, where keratinocytes end their lives and are sloughed off. The epidermis has complete self-renewal capacity with an estimated turnover time of approximately 40 d in humans[46].

The physical barrier of the epidermis is localised primarily in the upper layers of the epidermis (granular and cornified layers). The barrier properties of nucleated keratinocytes in the granular layer are largely dependent on the function and integrity of the tight junctions [involving the proteins tricellin, occludin, claudins and junctional adhesion molecule (JAM)] and their corresponding intracellular proteins, such as zona occludin (ZO)-1[44], which seal the intercellular space between neighbouring keratinocytes and control the pathway of molecules and liquid (Figure 3)[46].

Deregulation of these junction proteins perturbs this barrier[43] and is characteristic of many inflammatory skin diseases[47,48]. Psoriatic skin, characterised by small scaly plaques, has an over-expression of occludin and ZO-1, while claudin-1 and 3 are down-regulated[49,50]. Keratinocyte cytoskeletal elements are also important for maintaining the epidermal barrier. Among the genetic mutations in atopic dermatitis is the filaggrin gene (*FLG*)[51,52], which encodes a protein in the corneal epidermal layer and aids terminal differentiation of keratinocytes, water retention and barrier stabilisation[53]. Loss or mutation of this gene contribute to the red, dry, itchy skin that is hallmark of this condition.

In addition, keratinocytes provide an immunological barrier in response to injury or infection. Keratinocytes are a potent source of cytokines and chemokines[54]; freshly isolated and cultured keratinocytes express toll-like receptors[55] and inflammasomes[56]. This allows keratinocytes to elicit innate immune responses to microbial components when the epidermal barrier is breached, particularly through secretion of interleukin (IL)-1β and activation of leukocytes.

Upon activation, keratinocytes express a plethora of cytokines, chemokines and accessory molecules, which can transmit both positive and negative signals to cells of the innate and adaptive immune system. Dysregulation of the immune response of keratinocytes is implicated in the pathogenesis of chronic inflammatory skin diseases.

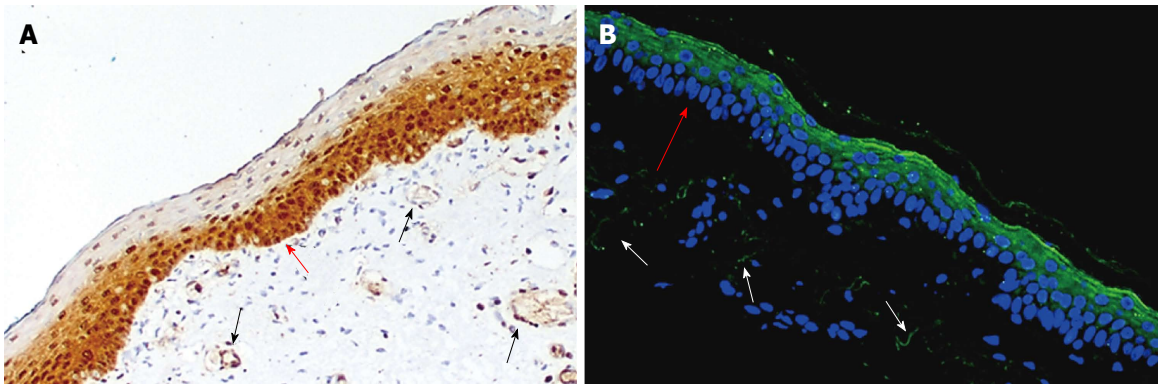

Figure 4 Immunostaining of protein C/activated protein C in human neonatal and adult skin epidermis. A: Neonatal; B: Adult. PC/APC indicated by brown and green staining in the epidermis (red arrow) and dermal blood vessels (arrow). APC: Activated protein C; PC: Protein C.

PC system on keratinocytes

Keratinocytes in the epidermis express all the components of the PC/APC pathway, including EPCR[57], thrombomodulin[58], thrombin and PC inhibitor[59], PAR-1, EGFR[60], and Tie2[34] which can regulate the activation of PC to APC and mediate the functions of APC on keratinocytes in skin epidermis.

PC/APC and its activation on keratinocytes

PC/APC: Since its discovery in 1960[61], PC has been characterised as the vitamin-K dependent protein precursor for the anticoagulant APC[62]. Thought to be exclusively synthesised by the liver and vascular endothelial cells, recent evidence shows that keratinocytes can also synthesise PC[60]. Cultured keratinocytes express PC mRNA and protein, and APC activity is presented on these cells[60]. In neonatal foreskin, PC is strongly expressed in the basal and suprabasal layers of the epidermis, with weaker expression in the outer cornified layer[60]. In the adult skin, however, the PC/APC is strongly stained in the upper layer of epidermis (Figure 4)

Thrombin: Thrombin is the only endogenous activator of PC. Keratinocytes express mRNA for the thrombin precursor, pro-thrombin[63]. Pro-thrombin and thrombin are expressed at low levels in normal epidermis, with thrombin markedly upregulated in scar tissue[63]. Thrombin activity is regulated by keratinocyte thrombomodulin at sites of cutaneous injury[64].

Thrombomodulin: Upon binding to thrombomodulin on surface of vascular endothelial cells, thrombin cleaves PC at the activation peptide between Arg^{211} and Leu^{212} and converts it to APC. Cultured human keratinocytes constitutively express thrombomodulin on their cell surface[58,64]. In normal epidermis thrombomodulin is present in spinous layer and on the outer root sheath of hair follicles[58,64].

PC inhibitor: PC inhibitor is a non-specific serpin that inhibits a variety of serine proteases, including PC and thrombin[65]. This inhibitor can inhibit the activation of PC to APC by inactivating thrombin and/or preventing thrombin binding to thrombomodulin[66,67]. It can also inactivate APC. PC inhibitor mRNA and protein is constitutively expressed by immortalised human keratinocytes (HaCaT) and epidermoid carcinoma cells (A431) in culture[59]. Normal skin from the trunk of adults show strong staining for PC inhibitor antigen throughout the epidermal layers[59].

In summary, epidermal keratinocytes express all aspects of the PC system to not only activate PC to APC, but regulate this activation process and APC activity (Figure 4).

PC/APC function and regulation

EPCR: EPCR is a type I transmembrane protein which exhibits significant homology with the major histocompatibility class 1/CD1 family of proteins. EPCR is the main receptor to regulate the function of PC/APC. Although first described as being restricted to the endothelium, EPCR is abundantly expressed by cultured human keratinocytes and is strongly expressed in the basal and suprabasal layers of the epidermis of neonatal foreskin[57].

EPCR has similar affinity for both PC and APC[1]. After binding to EPCR, APC cleaves PAR-1 to promote its cytoprotective functions in keratinocytes[57]. In addition PAR-1, EGFR and Tie2 are shown to mediate keratinocyte proliferation, migration and barrier stabilisation. In addition, EPCR enhances the rate of PC/APC activation by thrombin/thrombomodulin 3-4 fold[68]. Inhibition of EPCR reduces the level of circulating APC by more than 80% following thrombin infusion[69].

PAR-1: PARs are a family of G-protein coupled receptors which utilise G-protein and non-G-protein signaling pathways to mediate their cellular responses[70]. They are expressed by a wide range of cell types in the skin, including keratinocytes[57]. PARs are activated by a range of proteases through cleavage of an activation peptide. The most common endogenous activator is thrombin which activates PAR-1, PAR-3 and PAR-4, but not PAR-2. Other serine proteases including trypsin, mast cell tryptase

and factor Xa activate PAR-2. In keratinocytes, PAR-1 mediates APC's induction of cell proliferation, anti-inflammatory and barrier protective effects[34,57].

Cytoprotective effects of APC are also mediated by the other PAR receptors. APC can bind to PAR-2[37] and activate the Akt signaling pathway to promote keratinocyte proliferation[71]. Though only PAR-2 activity appears to be required for APC-mediated wound healing in a murine model[71].

EGFR: EGFR is a crucial receptor for autocrine growth of healthy epidermis. Its activation suppresses terminal differentiation, promotes cell proliferation and survival, and regulates cell migration during epidermal morphogenesis and wound healing[72]. Following tissue injury, EGFR is upregulated to promote re-epithelialisation of the wound by encouraging keratinocyte proliferation and migration. EGFR regulates cell adhesion, extracellular matrix degrading enzymes, and cell migration to contribute to the migratory and invasive potential of keratinocytes[72]. In human skin, EGFR and EPCR are expressed in the basal and suprabasal layers of the epidermis, consistent with the localisation of PC/APC[60]. Expression of EGFR by keratinocytes appears to be synchronised with the PC pathway. APC treatment increases EGFR expression while silencing of PC decreases EGFR levels[60].

Tie2: Tie2 is a protein-tyrosine kinase receptor expressed by endothelial and epithelial cells. Its major ligands are angiopoeitin 1 and 2 which bind with similar affinity[73,74]. Both Tie2 and its activated form phosphorylated (P)-Tie2 are present on neonatal foreskin and adult skin keratinocytes[34]. However, adult skin keratinocytes show less intensive staining for Tie-2 and P-Tie2 when compared with neonatal foreskin keratinocytes. Foreskin epidermis exhibits faint staining of Tie2 but strong staining for P-Tie2, which is mainly located in the uppermost layers of the epidermis (Figure 4). Similarly, P-Tie2 is expressed by normal adult skin epidermis, although the staining intensity is considerably lower than neonatal foreskin.

Functions of PC/APC in keratinocytes

APC promotes proliferation and inhibits apoptosis in keratinocytes: APC promotes cell proliferation in cultured human skin keratinocytes[14]. The replicative capacity of keratinocytes is mediated by EGFR, and acts to inhibit terminal differentiation and apoptosis. APC increases keratinocyte proliferation, while gene silencing of PC increases apoptosis in keratinocytes 3-fold[60]. Proliferation is mediated by APC's regulation of mitogen activated protein (MAP) kinase activity[12,14-16,18]. This family of highly conserved serine/threonine protein kinases enhances DNA synthesis, and regulates cell survival/apoptosis and differentiation[13]. In human skin keratinocytes, PC/APC-induced proliferation is mediated by EPCR, PAR-2, EGFR, activation of ERK1/2 and PI3K/Src/Akt signalling and suppression of p38[34,60,71].

Consistent with the stimulatory effects on cell growth, APC displays strong anti-apoptotic properties. APC prevents apoptosis of keratinocytes[14]. The molecular mechanism of APC's ability to protect cells from apoptosis is multi-faceted. APC regulates caspase activation, DNA degradation and the induction of anti-apoptotic mediators[25-28]. PC regulates the activation of apoptosis marker caspase-3, of which the inactive form is expressed in a wide range of tissues, including the epidermis[75]. In normal oral epithelium, cleaved caspase-3 distinguishes apoptotic keratinocytes from cells that are terminally differentiated[76]. Recent findings indicate that caspase-14, not caspase-3, is activated during normal keratinocyte differentiation[77]. Therefore caspase-3 activation appears to be restricted to keratinocytes undergoing apoptosis, and is increased by blocking PC by siRNA consistent with a role for PC in preventing keratinocyte apoptosis[60].

While additional anti-apoptotic pathways for APC have not yet been demonstrated in keratinocytes, in hypoxic retinal epithelia and photoreceptor cells APC reduces caspase-8 and 9[78]; decreases p21 and p53 proteins in murine model of sepsis-induced apoptosis[79]; and prevents glucose-induced apoptosis in endothelial cells and podocytes by reducing Bax induction and Bcl-2 suppression[21].

APC promotes migration of keratinocytes: Keratinocyte migration is a crucial step in stratification of the epidermis to form a protective barrier, and during re-epithelialisation of a wound site. EGF is a chemotactic factor for keratinocytes, as shown by phagokinetic track analysis[80]. In human skin, EGFR localises with PC/APC and EPCR in the basal and suprabasal layers of the epidermis[60]. Recombinant human (rh) APC treatment of keratinocytes increases EGFR activation and keratinocyte migration[57,60]. APC promotes keratinocyte migration at concentrations 5 μg/mL but had an inhibitory effect at 20 μg/mL[14]. At 5 μg/mL APC, the migration of keratinocytes was equivalent to that induced by 50 ng/mL EGF[14]. Gene silencing of PC inhibits EGFR expression and reduces keratinocyte migration by 20% using an *in vitro* scratch wounding assay[60].

MMP secretion appears to be are required for keratinocyte migration, as blockade of MMP's using GM6001, a broad spectrum MMP inhibitor, eliminated cell migration in a dose-dependent manner and delayed *in vitro* wound healing[60]. Full-thickness rat excisional skin wound healing model, a single topical application of rhAPC enhances wound healing compared to saline by stimulating re-epithelialisation[71,81]. This is also observed in human skin wound healing. In humans, topical application of 200 μg/mL rhAPC to chronic wounds of varying aetiology reduced wound area by 52%-95% over 16 wk[82]. A follow-up study of venous and diabetic ulcers treated with 400 μg/mL rhAPC showed a significant reduction in wound area and volume compared to baseline at 20 wk[83].

APC reduces inflammation of keratinocytes: APC regulates the expression of serine protease MMP-2. MMPs degrade tissue components and are commonly as-

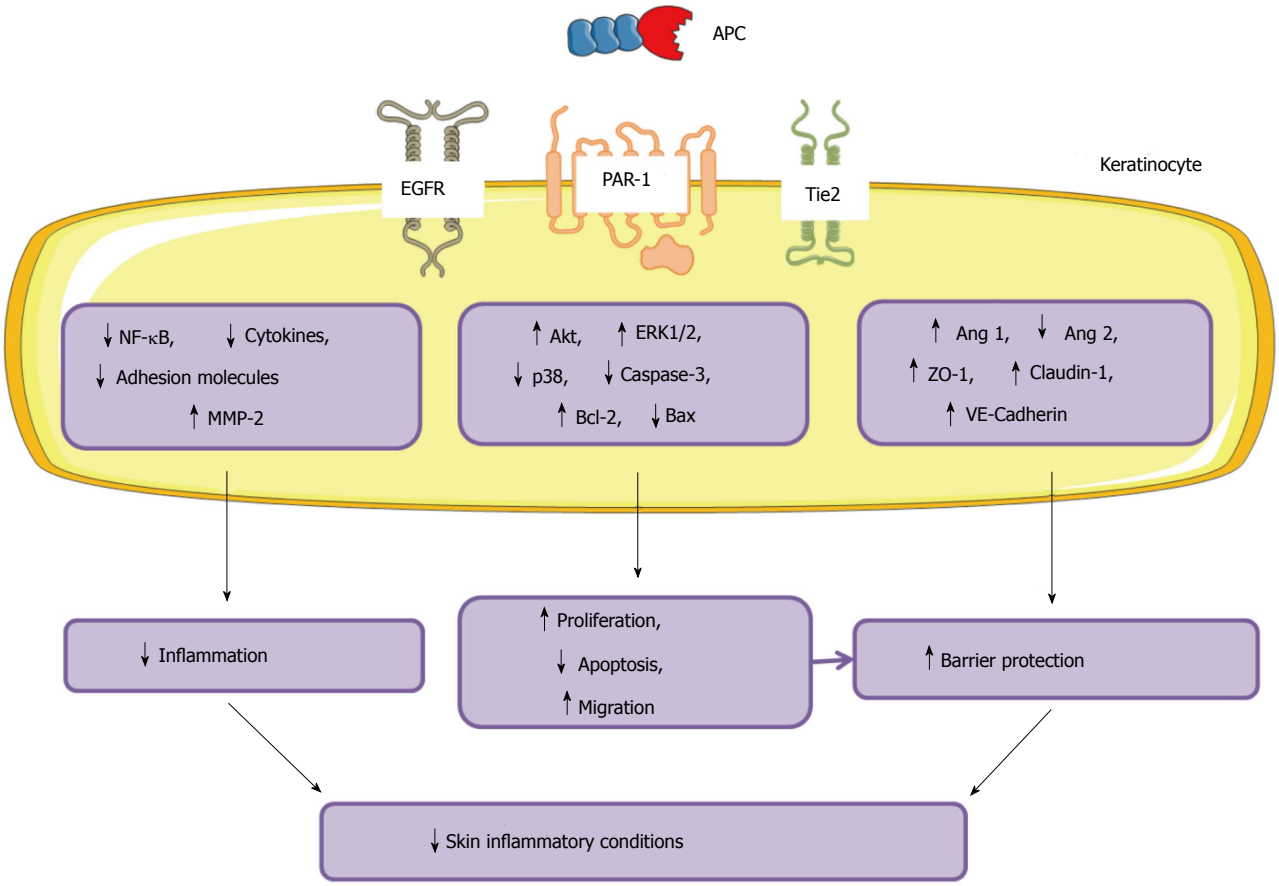

Figure 5 Schematic representation of protein C/activated protein C effects on skin epidermal keratinocyte function. APC: Activated protein C; EGFR: Epidermal growth factor receptor; PAR-1: Protease-activated receptor 1. Figure was produced using Servier Medical Art - www.servier.com.

sociated with skin inflammatory conditions[84]. In cultured human keratinocytes, APC enhances MMP-2 activity[14] which has anti-inflammatory properties[11,85] and plays a vital role in the tissue repair process by remodelling the extracellular matrix[86]. In contrast, MMP-9, which exhibits pro-inflammatory actions[11,87-89], is suppressed by APC[8,90].

Other indirect effects APC may have on suppressing cytokine production and activation is *via* inhibition of NF-κB subunits p50 and p52[28]. APC inhibits calcium- and lipopolysaccharide-stimulated activation of NF-κB in keratinocytes[14]. The NF-κB pathway is important for the expression of a wide variety of inflammatory genes including TNF-α and cell adhesion molecules, intercellular adhesion molecule-1, vascular cell adhesion molecule-1and E-selectin.

APC promotes barrier function of keratinocyte monolayers: The barrier protective effect of APC is relevant to skin epidermal keratinocytes[34]. Keratinocytes play a critical role in maintaining epidermal barrier function *via* tight junctions[43,91,92]. Dysregulation of tight junction proteins such as occludins, claudins and JAMs perturbs this barrier[43,91] and contributes to many skin inflammatory conditions[93].

APC enhances the barrier function of cultured human keratinocyte monolayers in a dose-dependent manner by up-regulating tight junction protein and redistributing them to cell-cell contacts *via* regulation of Tie2 and subsequent activation of Akt[34]. In response to APC treatment, Tie2 is activated within 30 min on keratinocyte monolayers, and relocates to cell-cell contacts where it impedes barrier permeability[34]. Expression of ZO-1, claudin-1 and vascular endothelial cadherin are subsequently increased. Interestingly, APC does not activate Tie2 through its major ligand, angiopoeitin-1, but binds directly to EPCR, cleaves PAR-1, and transactivates EGFR, then Tie2 which activates PI3K/Akt signalling to increase stabilisation of the keratinocyte barrier[34].

Prospective therapeutic potential of PC/APC

The skin, the body's largest organ, provides an epidermal barrier to protect the body from external insults, maintain temperature and control evaporation. Breaches of this barrier are common events. However, the inability to restore this barrier function can result in health problems, including inflammatory skin diseases, which are very common and have high morbidity. This group of diseases includes: acne, which affects 50% of teenagers (5% have severe acne); rosacea which affects 10% of the adult population; atopic dermatitis which affects up to 20% population; psoriasis which affects 2%-3% population[94,95]; chronic wounds which affect < 1% population and the devastating, often fatal, toxic epidermal necrolysis[96,97].

These diseases can be controlled to a certain extent, but no cure exists and they have high morbidity[98,99].

Management of most skin inflammatory conditions involves the use of emollients, phototherapy, topical corticosteroids, antibiotics, retinoids, immunomodulators (tacrolimus, pimecrolimus), or systemic treatments (ciclosporin, azathioprine). While targeted immunosuppressive drugs have been developed, including TNF-α inhibitors, antibodies and receptor blockers, in most studies they do not show improved outcome and their use for skin inflammatory conditions remains controversial[97]. For other conditions such as Stevens-Johnson syndrome and toxic epidermal necrolysis, to date no treatment has been identified to be capable of halting the progression of skin detachment[96].

APC is emerging as a critical regulator of keratinocyte and epidermal function. APC protects the epidermis by promoting keratinocyte proliferation, survival, reducing inflammation and maintaining barrier function. These keratinocyte cytoprotective functions are dependent on APC's interaction with EPCR, PARs, EGFR and Tie2.

Topical administration of rhAPC has shown promising results in the field of skin wound healing. Single or multiple topical applications of rhAPC to excisional wound sites reduced oedema and leukocyte infiltration, in addition to promoting angiogenesis and re-epithelialisation of wounds in rat models of skin wound healing[71,81]. These same APC-mediated benefits have been demonstrated in humans chronic wounds of venous and diabetic origin[82,83], as well as recalcitrant orthopaedic wounds[100].

The anti-inflammatory, barrier stabilisation and anti-apoptotic properties of APC make it an appealing treatment for skin diseases associated with inflammation, barrier disruption and keratinocyte dysfunction. A summary of the actions of APC on keratinocytes and skin inflammatory disorders is shown in Figure 5.

In late 2011, rhAPC (Xigris; drotrecogin alfa [activated]; Eli Lily) was withdrawn from the market after failure to significantly improve patient outcome in a clinical trial of septic shock[101], in an attempt to replicate earlier favourable results[102]. One concern was the observation of serious bleeding in patients, although there was no significant difference between patients treated with rhAPC and placebo[101,102]. Most *in vivo* studies, including our own, show that systemic rhAPC does not induce any bleeding side-effects[71,82,100,103-105]. Bleeding has occurred in a subset of near-death sepsis patients with recent surgery and although APC efficacy and safety is controversial in treatment of sepsis patients, it is beneficial and safe in clinical trials for chronic wound healing[82,100], acute lung injury[106,107], and solid organ transplantation[108]. Recently APC mutants (3K3A-APC and APC-2Cys) with minimal anticoagulant activity, but normal cytoprotective activity have been generated[109,110] and shown pre-clinically to be safe[12,111-116]. Although both variants are yet to be assessed in the field of skin inflammatory diseases. The notion that rhAPC may increase bleeding during wound healing could be circumvented by use of APC variants lacking anticoagulant activity.

Nevertheless, the future for utilising exogenous APC as a topical treatment for skin inflammatory conditions remains a novel and exciting avenue of investigation.

REFERENCES

1 **Fukudome K**, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem* 1994; **269**: 26486-26491 [PMID: 7929370]

2 **Okajima K**, Koga S, Kaji M, Inoue M, Nakagaki T, Funatsu A, Okabe H, Takatsuki K, Aoki N. Effect of protein C and activated protein C on coagulation and fibrinolysis in normal human subjects. *Thromb Haemost* 1990; **63**: 48-53 [PMID: 2140205]

3 **Gruber A**, Griffin JH. Direct detection of activated protein C in blood from human subjects. *Blood* 1992; **79**: 2340-2348 [PMID: 1571547]

4 **Baker WF**, Bick RL. Treatment of hereditary and acquired thrombophilic disorders. *Semin Thromb Hemost* 1999; **25**: 387-406 [PMID: 10548072 DOI: 10.1055/s-2007-994942]

5 **Esmon CT**. Crosstalk between inflammation and thrombosis. *Maturitas* 2004; **47**: 305-314 [PMID: 15063484 DOI: 10.1016/j.maturitas.2003.10.015]

6 **Yuksel M**, Okajima K, Uchiba M, Horiuchi S, Okabe H. Activated protein C inhibits lipopolysaccharide-induced tumor necrosis factor-alpha production by inhibiting activation of both nuclear factor-kappa B and activator protein-1 in human monocytes. *Thromb Haemost* 2002; **88**: 267-273 [PMID: 12195699]

7 **Lay AJ**, Donahue D, Tsai MJ, Castellino FJ. Acute inflammation is exacerbated in mice genetically predisposed to a severe protein C deficiency. *Blood* 2007; **109**: 1984-1991 [PMID: 17047151 DOI: 10.1182/blood-2006-07-037945]

8 **Xue M**, March L, Sambrook PN, Jackson CJ. Differential regulation of matrix metalloproteinase 2 and matrix metalloproteinase 9 by activated protein C: relevance to inflammation in rheumatoid arthritis. *Arthritis Rheum* 2007; **56**: 2864-2874 [PMID: 17763449 DOI: 10.1002/art.22844]

9 **Gearing AJ**, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature* 1994; **370**: 555-557 [PMID: 8052310 DOI: 10.1038/370555a0]

10 **McQuibban GA**, Gong JH, Tam EM, McCulloch CA, Clark-Lewis I, Overall CM. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 2000; **289**: 1202-1206 [PMID: 10947989 DOI: 10.1126/science.289.5482.1202]

11 **Itoh T**, Matsuda H, Tanioka M, Kuwabara K, Itohara S, Suzuki R. The role of matrix metalloproteinase-2 and matrix metalloproteinase-9 in antibody-induced arthritis. *J Immunol* 2002; **169**: 2643-2647 [PMID: 12193736]

12 **Uchiba M**, Okajima K, Oike Y, Ito Y, Fukudome K, Isobe H, Suda T. Activated protein C induces endothelial cell proliferation by mitogen-activated protein kinase activation in vitro and angiogenesis in vivo. *Circ Res* 2004; **95**: 34-41 [PMID: 15166095 DOI: 10.1161/01.RES.0000133680.87668.FA]

13 **Bretschneider E**, Uzonyi B, Weber AA, Fischer JW, Pape R, Lötzer K, Schrör K. Human vascular smooth muscle cells express functionally active endothelial cell protein C receptor. *Circ Res* 2007; **100**: 255-262 [PMID: 17170365 DOI: 10.1161/01.RES.0000255685.06922.c7]

14 **Xue M**, Thompson P, Kelso I, Jackson C. Activated protein C stimulates proliferation, migration and wound closure, inhibits apoptosis and upregulates MMP-2 activity in cultured human keratinocytes. *Exp Cell Res* 2004; **299**: 119-127 [PMID:

15302579 DOI: 10.1016/j.yexcr.2004.05.015]
15 **Guo H**, Zhao Z, Yang Q, Wang M, Bell RD, Wang S, Chow N, Davis TP, Griffin JH, Goldman SA, Zlokovic BV. An activated protein C analog stimulates neuronal production by human neural progenitor cells via a PAR1-PAR3-S1PR1-Akt pathway. *J Neurosci* 2013; **33**: 6181-6190 [PMID: 23554499 DOI: 10.1523/JNEUROSCI.4491-12.2013]
16 **Thiyagarajan M**, Fernández JA, Lane SM, Griffin JH, Zlokovic BV. Activated protein C promotes neovascularization and neurogenesis in postischemic brain via protease-activated receptor 1. *J Neurosci* 2008; **28**: 12788-12797 [PMID: 19036971 DOI: 10.1523/JNEUROSCI.3485-08.2008]
17 **Cerbák R**, Stětka F, Filkuka J, Utrata P, Rubácek M, Dominik J, Nicovský J, Bednarík M. [Type II atrial septal defects in adulthood]. *Vnitr Lek* 1989; **35**: 650-655 [PMID: 2800370 DOI: 10.1227/01.NEU.0000363148.49779.68]
18 **Kurata T**, Hayashi T, Yoshikawa T, Okamoto T, Yoshida K, Iino T, Uchida A, Suzuki K. Activated protein C stimulates osteoblast proliferation via endothelial protein C receptor. *Thromb Res* 2010; **125**: 184-191 [PMID: 19804899 DOI: 10.1016/j.thromres.2009.09.005]
19 **Xue M**, Smith MM, Little CB, Sambrook P, March L, Jackson CJ. Activated protein C mediates a healing phenotype in cultured tenocytes. *J Cell Mol Med* 2009; **13**: 749-757 [PMID: 18466356 DOI: 10.1111/j.1582-4934.2008.00359.x]
20 **Xue M**, Minhas N, Chow SO, Dervish S, Sambrook PN, March L, Jackson CJ. Endogenous protein C is essential for the functional integrity of human endothelial cells. *Cell Mol Life Sci* 2010; **67**: 1537-1546 [PMID: 20127387 DOI: 10.1007/s00018-010-0269-y]
21 **Isermann B**, Vinnikov IA, Madhusudhan T, Herzog S, Kashif M, Blautzik J, Corat MA, Zeier M, Blessing E, Oh J, Gerlitz B, Berg DT, Grinnell BW, Chavakis T, Esmon CT, Weiler H, Bierhaus A, Nawroth PP. Activated protein C protects against diabetic nephropathy by inhibiting endothelial and podocyte apoptosis. *Nat Med* 2007; **13**: 1349-1358 [PMID: 17982464 DOI: 10.1038/nm1667]
22 **Hemmer CJ**, Löbermann M, Unverricht M, Vogt A, Krause R, Reisinger EC. Activated protein C protects vascular endothelial cells from apoptosis in malaria and in sepsis. *Trop Med Int Health* 2011; **16**: 906-913 [PMID: 21615630 DOI: 10.1111/j.1365-3156.2011.02788.x]
23 **Hotchkiss RS**, Chang KC, Swanson PE, Tinsley KW, Hui JJ, Klender P, Xanthoudakis S, Roy S, Black C, Grimm E, Aspiotis R, Han Y, Nicholson DW, Karl IE. Caspase inhibitors improve survival in sepsis: a critical role of the lymphocyte. *Nat Immunol* 2000; **1**: 496-501 [PMID: 11101871 DOI: 10.1038/82741]
24 **Yen YT**, Yang HR, Lo HC, Hsieh YC, Tsai SC, Hong CW, Hsieh CH. Enhancing autophagy with activated protein C and rapamycin protects against sepsis-induced acute lung injury. *Surgery* 2013; **153**: 689-698 [PMID: 23434181 DOI: 10.1016/j.surg.2012.11.021]
25 **Cheng T**, Liu D, Griffin JH, Fernández JA, Castellino F, Rosen ED, Fukudome K, Zlokovic BV. Activated protein C blocks p53-mediated apoptosis in ischemic human brain endothelium and is neuroprotective. *Nat Med* 2003; **9**: 338-342 [PMID: 12563316 DOI: 10.1038/nm826]
26 **Liu D**, Cheng T, Guo H, Fernández JA, Griffin JH, Song X, Zlokovic BV. Tissue plasminogen activator neurovascular toxicity is controlled by activated protein C. *Nat Med* 2004; **10**: 1379-1383 [PMID: 15516929 DOI: doi:]
27 **Mosnier LO**, Griffin JH. Inhibition of staurosporine-induced apoptosis of endothelial cells by activated protein C requires protease-activated receptor-1 and endothelial cell protein C receptor. *Biochem J* 2003; **373**: 65-70 [PMID: 12683950 DOI: 10.1042/BJ20030341]
28 **Joyce DE**, Gelbert L, Ciaccia A, DeHoff B, Grinnell BW. Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. *J Biol Chem* 2001; **276**: 11199-11203 [PMID: 11278252 DOI: 10.1074/jbc.C100017200]
29 **Joyce DE**, Grinnell BW. Recombinant human activated protein C attenuates the inflammatory response in endothelium and monocytes by modulating nuclear factor-kappaB. *Crit Care Med* 2002; **30**: S288-S293 [PMID: 12004250]
30 **Guo H**, Liu D, Gelbard H, Cheng T, Insalaco R, Fernández JA, Griffin JH, Zlokovic BV. Activated protein C prevents neuronal apoptosis via protease activated receptors 1 and 3. *Neuron* 2004; **41**: 563-572 [PMID: 14980205 DOI: 10.1016/S0896-6273(04)00019-4]
31 **Feistritzer C**, Riewald M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. *Blood* 2005; **105**: 3178-3184 [PMID: 15626732 DOI: 10.1182/blood-2004-10-3985]
32 **Finigan JH**, Dudek SM, Singleton PA, Chiang ET, Jacobson JR, Camp SM, Ye SQ, Garcia JG. Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. *J Biol Chem* 2005; **280**: 17286-17293 [PMID: 15710622 DOI: 10.1074/jbc.M412427200]
33 **Minhas N**, Xue M, Fukudome K, Jackson CJ. Activated protein C utilizes the angiopoietin/Tie2 axis to promote endothelial barrier function. *FASEB J* 2010; **24**: 873-881 [PMID: 19858095 DOI: 10.1096/fj.09-134445]
34 **Xue M**, Chow SO, Dervish S, Chan YK, Julovi SM, Jackson CJ. Activated protein C enhances human keratinocyte barrier integrity via sequential activation of epidermal growth factor receptor and Tie2. *J Biol Chem* 2011; **286**: 6742-6750 [PMID: 21173154 DOI: 10.1074/jbc.M110.181388]
35 **Vetrano S**, Ploplis VA, Sala E, Sandoval-Cooper M, Donahue DL, Correale C, Arena V, Spinelli A, Repici A, Malesci A, Castellino FJ, Danese S. Unexpected role of anticoagulant protein C in controlling epithelial barrier integrity and intestinal inflammation. *Proc Natl Acad Sci USA* 2011; **108**: 19830-19835 [PMID: 22109555 DOI: 10.1073/pnas.1107140108]
36 **Esmon CT**. Structure and functions of the endothelial cell protein C receptor. *Crit Care Med* 2004; **32**: S298-S301 [PMID: 15118534 DOI: 10.1097/01.CCM.0000126128.64614.81]
37 **Riewald M**, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science* 2002; **296**: 1880-1882 [PMID: 12052963 DOI: 10.1126/science.1071699]
38 **Madhusudhan T**, Wang H, Straub BK, Gröne E, Zhou Q, Shahzad K, Müller-Krebs S, Schwenger V, Gerlitz B, Grinnell BW, Griffin JH, Reiser J, Gröne HJ, Esmon CT, Nawroth PP, Isermann B. Cytoprotective signaling by activated protein C requires protease-activated receptor-3 in podocytes. *Blood* 2012; **119**: 874-883 [PMID: 22117049 DOI: 10.1182/blood-2011-07-365973]
39 **Feistritzer C**, Mosheimer BA, Sturn DH, Riewald M, Patsch JR, Wiedermann CJ. Endothelial protein C receptor-dependent inhibition of migration of human lymphocytes by protein C involves epidermal growth factor receptor. *J Immunol* 2006; **176**: 1019-1025 [PMID: 16393989]
40 **Gramling MW**, Beaulieu LM, Church FC. Activated protein C enhances cell motility of endothelial cells and MDA-MB-231 breast cancer cells by intracellular signal transduction. *Exp Cell Res* 2010; **316**: 314-328 [PMID: 19891966 DOI: 10.1016/j.yexcr.2009.10.024]
41 **Cao C**, Gao Y, Li Y, Antalis TM, Castellino FJ, Zhang L. The efficacy of activated protein C in murine endotoxemia is dependent on integrin CD11b. *J Clin Invest* 2010; **120**: 1971-1980 [PMID: 20458145 DOI: 10.1172/JCI40380]
42 **White TC**, Berny MA, Tucker EI, Urbanus RT, de Groot PG, Fernández JA, Griffin JH, Gruber A, McCarty OJ. Protein C supports platelet binding and activation under flow: role of glycoprotein Ib and apolipoprotein E receptor 2. *J Thromb*

Haemost 2008; **6**: 995-1002 [PMID: 18489431 DOI: 10.1111/j.1538-7836.2008.02979.x]
43 **Proksch E**, Brandner JM, Jensen JM. The skin: an indispensable barrier. *Exp Dermatol* 2008; **17**: 1063-1072 [PMID: 19043850 DOI: 10.1111/j.1600-0625.2008.00786.x]
44 **Kalinin A**, Marekov LN, Steinert PM. Assembly of the epidermal cornified cell envelope. *J Cell Sci* 2001; **114**: 3069-3070 [PMID: 11590230]
45 **Elias PM**. Stratum corneum defensive functions: an integrated view. *J Invest Dermatol* 2005; **125**: 183-200 [PMID: 16098026 DOI: 10.1111/j.0022-202X.2005.23668.x]
46 **Weinstein GD**, McCullough JL, Ross P. Cell proliferation in normal epidermis. *J Invest Dermatol* 1984; **82**: 623-628 [PMID: 6725985]
47 **Kirschner N**, Bohner C, Rachow S, Brandner JM. Tight junctions: is there a role in dermatology? *Arch Dermatol Res* 2010; **302**: 483-493 [PMID: 20563589 DOI: 10.1007/s00403-010-1058-z]
48 **O'Neill CA**, Garrod D. Tight junction proteins and the epidermis. *Exp Dermatol* 2011; **20**: 88-91 [PMID: 21255086 DOI: 10.1111/j.1600-0625.2010.01206.x]
49 **Watson RE**, Poddar R, Walker JM, McGuill I, Hoare LM, Griffiths CE, O'neill CA. Altered claudin expression is a feature of chronic plaque psoriasis. *J Pathol* 2007; **212**: 450-458 [PMID: 17582238 DOI: 10.1002/path.2200]
50 **Peltonen S**, Riehokainen J, Pummi K, Peltonen J. Tight junction components occludin, ZO-1, and claudin-1, -4 and -5 in active and healing psoriasis. *Br J Dermatol* 2007; **156**: 466-472 [PMID: 17300235 DOI: 10.1111/j.1365-2133.2006.07642.x]
51 **van den Oord RA**, Sheikh A. Filaggrin gene defects and risk of developing allergic sensitisation and allergic disorders: systematic review and meta-analysis. *BMJ* 2009; **339**: b2433 [PMID: 19589816 DOI: 10.1136/bmj.b2433]
52 **Rodríguez E**, Baurecht H, Herberich E, Wagenpfeil S, Brown SJ, Cordell HJ, Irvine AD, Weidinger S. Meta-analysis of filaggrin polymorphisms in eczema and asthma: robust risk factors in atopic disease. *J Allergy Clin Immunol* 2009; **123**: 1361-1370. e7 [PMID: 19501237 DOI: 10.1016/j.jaci.2009.03.036]
53 **Palmer CN**, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, Goudie DR, Sandilands A, Campbell LE, Smith FJ, O'Regan GM, Watson RM, Cecil JE, Bale SJ, Compton JG, DiGiovanna JJ, Fleckman P, Lewis-Jones S, Arseculeratne G, Sergeant A, Munro CS, El Houate B, McElreavey K, Halkjaer LB, Bisgaard H, Mukhopadhyay S, McLean WH. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet* 2006; **38**: 441-446 [PMID: 16550169 DOI: 10.1038/ng1767]
54 **Luger TA**, Schwarz T. Epidermal cell-derived secretory regulins. In: Schuler G, editor. Boca Raton (FL): CRC Press, 1989: 217-253
55 **Köllisch G**, Kalali BN, Voelcker V, Wallich R, Behrendt H, Ring J, Bauer S, Jakob T, Mempel M, Ollert M. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology* 2005; **114**: 531-541 [PMID: 15804290 DOI: 10.1111/j.1365-2567.2005.02122.x]
56 **Watanabe H**, Gaide O, Pétrilli V, Martinon F, Contassot E, Roques S, Kummer JA, Tschopp J, French LE. Activation of the IL-1beta-processing inflammasome is involved in contact hypersensitivity. *J Invest Dermatol* 2007; **127**: 1956-1963 [PMID: 17429439 DOI: 10.1038/sj.jid.5700819]
57 **Xue M**, Campbell D, Sambrook PN, Fukudome K, Jackson CJ. Endothelial protein C receptor and protease-activated receptor-1 mediate induction of a wound-healing phenotype in human keratinocytes by activated protein C. *J Invest Dermatol* 2005; **125**: 1279-1285 [PMID: 16354200 DOI: 10.1111/j.0022-202X.2005.23952.x]
58 **Jackson DE**, Mitchell CA, Bird P, Salem HH, Hayman JA. Immunohistochemical localization of thrombomodulin in normal human skin and skin tumours. *J Pathol* 1995; **175**: 421-432 [PMID: 7790996 DOI: 10.1002/path.1711750410]
59 **Krebs M**, Uhrin P, Vales A, Prendes-Garcia MJ, Wojta J, Geiger M, Binder BR. Protein C inhibitor is expressed in keratinocytes of human skin. *J Invest Dermatol* 1999; **113**: 32-37 [PMID: 10417615 DOI: 10.1046/j.1523-1747.1999.00644.x]
60 **Xue M**, Campbell D, Jackson CJ. Protein C is an autocrine growth factor for human skin keratinocytes. *J Biol Chem* 2007; **282**: 13610-13616 [PMID: 17293597 DOI: 10.1074/jbc.M610740200]
61 **Mammen EF**, Thomas WR, Seegers WH. Activation of purified prothrombin to autoprothrombin I or autoprothrombin II (platelet cofactor II or autoprothrombin II-A). *Thromb Diath Haemorrh* 1960; **5**: 218-249 [PMID: 13765990]
62 **Kisiel W**, Canfield WM, Ericsson LH, Davie EW. Anticoagulant properties of bovine plasma protein C following activation by thrombin. *Biochemistry* 1977; **16**: 5824-5831 [PMID: 588557 DOI: 10.1021/bi00645a029]
63 **Artuc M**, Hermes B, Algermissen B, Henz BM. Expression of prothrombin, thrombin and its receptors in human scars. *Exp Dermatol* 2006; **15**: 523-529 [PMID: 16761961 DOI: 10.1111/j.1600-0625.2006.00444.x]
64 **Raife TJ**, Lager DJ, Madison KC, Piette WW, Howard EJ, Sturm MT, Chen Y, Lentz SR. Thrombomodulin expression by human keratinocytes. Induction of cofactor activity during epidermal differentiation. *J Clin Invest* 1994; **93**: 1846-1851 [PMID: 8163684 DOI: 10.1172/jci117171]
65 **Suzuki K**, Deyashiki Y, Nishioka J, Toma K. Protein C inhibitor: structure and function. *Thromb Haemost* 1989; **61**: 337-342 [PMID: 2552602]
66 **Li W**, Adams TE, Kjellberg M, Stenflo J, Huntington JA. Structure of native protein C inhibitor provides insight into its multiple functions. *J Biol Chem* 2007; **282**: 13759-13768 [PMID: 17337440 DOI: 10.1074/jbc.M701074200]
67 **Elisen MG**, von dem Borne PA, Bouma BN, Meijers JC. Protein C inhibitor acts as a procoagulant by inhibiting the thrombomodulin-induced activation of protein C in human plasma. *Blood* 1998; **91**: 1542-1547 [PMID: 9473218]
68 **Stearns-Kurosawa DJ**, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. *Proc Natl Acad Sci USA* 1996; **93**: 10212-10216 [PMID: 8816778]
69 **Taylor FB**, Peer GT, Lockhart MS, Ferrell G, Esmon CT. Endothelial cell protein C receptor plays an important role in protein C activation in vivo. *Blood* 2001; **97**: 1685-1688 [PMID: 11238108 DOI: 10.1182/blood.V97.6.1685]
70 **Coughlin SR**. Thrombin signalling and protease-activated receptors. *Nature* 2000; **407**: 258-264 [PMID: 11001069 DOI: 10.1038/35025229]
71 **Julovi SM**, Xue M, Dervish S, Sambrook PN, March L, Jackson CJ. Protease activated receptor-2 mediates activated protein C-induced cutaneous wound healing via inhibition of p38. *Am J Pathol* 2011; **179**: 2233-2242 [PMID: 21907694 DOI: 10.1016/j.ajpath.2011.07.024]
72 **Hudson LG**, McCawley LJ. Contributions of the epidermal growth factor receptor to keratinocyte motility. *Microsc Res Tech* 1998; **43**: 444-455 [PMID: 9858341 DOI: 10.1002/(SICI)1097-0029(19981201)43]
73 **Makinde T**, Agrawal DK. Intra and extravascular transmembrane signalling of angiopoietin-1-Tie2 receptor in health and disease. *J Cell Mol Med* 2008; **12**: 810-828 [PMID: 18266978 DOI: 10.1111/j.1582-4934.2008.00254.x]
74 **Fiedler U**, Augustin HG. Angiopoietins: a link between angiogenesis and inflammation. *Trends Immunol* 2006; **27**: 552-558 [PMID: 17045842 DOI: 10.1016/j.it.2006.10.004]
75 **Krajewska M**, Wang HG, Krajewski S, Zapata JM, Shabaik A, Gascoyne R, Reed JC. Immunohistochemical analysis of in vivo patterns of expression of CPP32 (Caspase-3), a cell death protease. *Cancer Res* 1997; **57**: 1605-1613 [PMID:

9108467]
76 **Hague A**, Eveson JW, MacFarlane M, Huntley S, Janghra N, Thavaraj S. Caspase-3 expression is reduced, in the absence of cleavage, in terminally differentiated normal oral epithelium but is increased in oral squamous cell carcinomas and correlates with tumour stage. *J Pathol* 2004; **204**: 175-182 [PMID: 15376256 DOI: 10.1002/path.1630]
77 **Lippens S**, Kockx M, Knaapen M, Mortier L, Polakowska R, Verheyen A, Garmyn M, Zwijsen A, Formstecher P, Huylebroeck D, Vandenabeele P, Declercq W. Epidermal differentiation does not involve the pro-apoptotic executioner caspases, but is associated with caspase-14 induction and processing. *Cell Death Differ* 2000; **7**: 1218-1224 [PMID: 11175259]
78 **Du ZJ**, Yamamoto T, Ueda T, Suzuki M, Tano Y, Kamei M. Activated protein C rescues the retina from ischemia-induced cell death. *Invest Ophthalmol Vis Sci* 2011; **52**: 987-993 [PMID: 20688738 DOI: 10.1167/iovs.10-5557]
79 **Sakar A**, Vatansever S, Sepit L, Ozbilgin K, Yorgancioglu A. Effect of recombinant human activated protein C on apoptosis-related proteins. *Eur J Histochem* 2007; **51**: 103-109 [PMID: 17664160]
80 **Ando Y**, Jensen PJ. Epidermal growth factor and insulin-like growth factor I enhance keratinocyte migration. *J Invest Dermatol* 1993; **100**: 633-639 [PMID: 8491986]
81 **Jackson CJ**, Xue M, Thompson P, Davey RA, Whitmont K, Smith S, Buisson-Legendre N, Sztynda T, Furphy LJ, Cooper A, Sambrook P, March L. Activated protein C prevents inflammation yet stimulates angiogenesis to promote cutaneous wound healing. *Wound Repair Regen* 2005; **13**: 284-294 [PMID: 15953048 DOI: 10.1111/j.1067-1927.2005.00130311.x]
82 **Whitmont K**, Reid I, Tritton S, March L, Xue M, Lee M, Fulcher G, Sambrook P, Slobedman E, Cooper A, Jackson C. Treatment of chronic leg ulcers with topical activated protein C. *Arch Dermatol* 2008; **144**: 1479-1483 [PMID: 19015423 DOI: 10.1001/archderm.144.11.1479]
83 **Whitmont K,** McKelvey KJ, Fulcher G, Reid I, March L, Xue M, Cooper A, Jackson CJ. Treatment of chronic diabetic lower leg ulcers with activated protein C: a randomised placebo-controlled, double-blind pilot clinical trial. *Int Wound J* 2013 Jul 15; Epub ahead of print [PMID: 23848141 DOI: 10.1111/iwj.12125]
84 **Starodubtseva NL**, Sobolev VV, Soboleva AG, Nikolaev AA, Bruskin SA. [Expression of genes for metalloproteinases (MMP-1, MMP-2, MMP-9, and MMP-12) associated with psoriasis]. *Genetika* 2011; **47**: 1254-1261 [PMID: 22117411 DOI: 10.1134/s102279541109016x]
85 **McQuibban GA**, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* 2002; **100**: 1160-1167 [PMID: 12149192]
86 **Ravanti L**, Kähäri VM. Matrix metalloproteinases in wound repair (review). *Int J Mol Med* 2000; **6**: 391-407 [PMID: 10998429]
87 **Ram M**, Sherer Y, Shoenfeld Y. Matrix metalloproteinase-9 and autoimmune diseases. *J Clin Immunol* 2006; **26**: 299-307 [PMID: 16652230 DOI: 10.1007/s10875-006-9022-6]
88 **Schönbeck U**, Mach F, Libby P. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. *J Immunol* 1998; **161**: 3340-3346 [PMID: 9759850]
89 **Yu Q**, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000; **14**: 163-176 [PMID: 10652271 DOI: 10.1101/gad.14.2.163]
90 **Cheng T**, Petraglia AL, Li Z, Thiyagarajan M, Zhong Z, Wu Z, Liu D, Maggirwar SB, Deane R, Fernández JA, LaRue B, Griffin JH, Chopp M, Zlokovic BV. Activated protein C inhibits tissue plasminogen activator-induced brain hemorrhage. *Nat Med* 2006; **12**: 1278-1285 [PMID: 17072311]
91 **Proksch E**, Brasch J. Abnormal epidermal barrier in the pathogenesis of contact dermatitis. *Clin Dermatol* 2012; **30**: 335-344 [PMID: 22507049 DOI: 10.1016/j.clindermatol.2011.08.019]
92 **Vandenbroucke E**, Mehta D, Minshall R, Malik AB. Regulation of endothelial junctional permeability. *Ann N Y Acad Sci* 2008; **1123**: 134-145 [PMID: 18375586 DOI: 10.1196/annals.1420.016]
93 **Koster MI**. Making an epidermis. *Ann N Y Acad Sci* 2009; **1170**: 7-10 [PMID: 19686098 DOI: 10.1111/j.1749-6632.2009.04363.x]
94 **Gutowska-Owsiak D**, Schaupp AL, Salimi M, Selvakumar TA, McPherson T, Taylor S, Ogg GS. IL-17 downregulates filaggrin and affects keratinocyte expression of genes associated with cellular adhesion. *Exp Dermatol* 2012; **21**: 104-110 [PMID: 22229441 DOI: 10.1111/j.1600-0625.2011.01412.x]
95 **Bangert C**, Brunner PM, Stingl G. Immune functions of the skin. *Clin Dermatol* 2011; **29**: 360-376 [PMID: 21679864 DOI: 10.1016/j.clindermatol.2011.01.006]
96 **Mockenhaupt M**. The current understanding of Stevens-Johnson syndrome and toxic epidermal necrolysis. *Expert Rev Clin Immunol* 2011; **7**: 803-813; quiz 814-815 [PMID: 22014021 DOI: 10.1586/eci.11.66]
97 **Gerull R**, Nelle M, Schaible T. Toxic epidermal necrolysis and Stevens-Johnson syndrome: a review. *Crit Care Med* 2011; **39**: 1521-1532 [PMID: 21358399 DOI: 10.1097/CCM.0b013e31821201ed]
98 **Menter A**, Gottlieb A, Feldman SR, Van Voorhees AS, Leonardi CL, Gordon KB, Lebwohl M, Koo JY, Elmets CA, Korman NJ, Beutner KR, Bhushan R. Guidelines of care for the management of psoriasis and psoriatic arthritis: Section 1. Overview of psoriasis and guidelines of care for the treatment of psoriasis with biologics. *J Am Acad Dermatol* 2008; **58**: 826-850 [PMID: 18423260 DOI: 10.1016/j.jaad.2008.02.039]
99 **Garcia-Valladares I**, Cuchacovich R, Espinoza LR. Comparative assessment of biologics in treatment of psoriasis: drug design and clinical effectiveness of ustekinumab. *Drug Des Devel Ther* 2011; **5**: 41-49 [PMID: 21267358 DOI: 10.2147/DDDT.S10494]
100 **Wijewardena A**, Vandervord E, Lajevardi SS, Vandervord J, Jackson CJ. Combination of activated protein C and topical negative pressure rapidly regenerates granulation tissue over exposed bone to heal recalcitrant orthopedic wounds. *Int J Low Extrem Wounds* 2011; **10**: 146-151 [PMID: 21807809 DOI: 10.1177/1534734611417342]
101 **Ranieri VM**, Thompson BT, Barie PS, Dhainaut JF, Douglas IS, Finfer S, Gårdlund B, Marshall JC, Rhodes A, Artigas A, Payen D, Tenhunen J, Al-Khalidi HR, Thompson V, Janes J, Macias WL, Vangerow B, Williams MD. Drotrecogin alfa (activated) in adults with septic shock. *N Engl J Med* 2012; **366**: 2055-2064 [PMID: 22616830 DOI: 10.1056/NEJMoa1202290]
102 **Bernard GR**, Vincent JL, Laterre PF, LaRosa SP, Dhainaut JF, Lopez-Rodriguez A, Steingrub JS, Garber GE, Helterbrand JD, Ely EW, Fisher CJ. Efficacy and safety of recombinant human activated protein C for severe sepsis. *N Engl J Med* 2001; **344**: 699-709 [PMID: 11236773 DOI: 10.1056/NEJM200103083441001]
103 **Shorr AF**, Janes JM, Artigas A, Tenhunen J, Wyncoll DL, Mercier E, Francois B, Vincent JL, Vangerow B, Heiselman D, Leishman AG, Zhu YE, Reinhart K. Randomized trial evaluating serial protein C levels in severe sepsis patients treated with variable doses of drotrecogin alfa (activated). *Crit Care* 2010; **14**: R229 [PMID: 21176144 DOI: 10.1186/cc9382]
104 **Sadaka F**, O'Brien J, Migneron M, Stortz J, Vanston A, Taylor RW. Activated protein C in septic shock: a propensity-matched analysis. *Crit Care* 2011; **15**: R89 [PMID: 21385410 DOI: 10.1186/cc10089]
105 **Xue M**, Dervish S, Harrison LC, Fulcher G, Jackson CJ. Activated protein C inhibits pancreatic islet inflammation, stimulates T regulatory cells, and prevents diabetes in non-obese diabetic (NOD) mice. *J Biol Chem* 2012; **287**: 16356-16364

[PMID: 22447930 DOI: 10.1074/jbc.M111.325951]
106 **Tuinman PR**, Dixon B, Levi M, Juffermans NP, Schultz MJ. Nebulized anticoagulants for acute lung injury - a systematic review of preclinical and clinical investigations. *Crit Care* 2012; **16**: R70 [PMID: 22546487 DOI: 10.1186/cc11325]
107 **Cornet AD**, Hofstra JJ, Vlaar AP, Tuinman PR, Levi M, Girbes AR, Schultz MJ, Groeneveld AB, Beishuizen A. Activated protein C attenuates pulmonary coagulopathy in patients with acute respiratory distress syndrome. *J Thromb Haemost* 2013; **11**: 894-901 [PMID: 23433188]
108 **Funk DJ**, Palma Vargas J, Tuttle-Newhall J, Moretti EW. The use of recombinant human activated protein C (drotrecogin alpha) in solid organ transplant recipients: case series and review of the literature. *Transpl Infect Dis* 2011; **13**: 592-597 [PMID: 21794040 DOI: 10.1111/j.1399-3062.2011.00636.x]
109 **Mosnier LO**, Gale AJ, Yegneswaran S, Griffin JH. Activated protein C variants with normal cytoprotective but reduced anticoagulant activity. *Blood* 2004; **104**: 1740-1744 [PMID: 15178575 DOI: 10.1182/blood-2004-01-0110]
110 **Bae JS**, Yang L, Manithody C, Rezaie AR. Engineering a disulfide bond to stabilize the calcium-binding loop of activated protein C eliminates its anticoagulant but not its protective signaling properties. *J Biol Chem* 2007; **282**: 9251-9259 [PMID: 17255099 DOI: 10.1074/jbc.M610547200]
111 **Williams PD**, Zlokovic BV, Griffin JH, Pryor KE, Davis TP. Preclinical safety and pharmacokinetic profile of 3K3A-APC, a novel, modified activated protein C for ischemic stroke. *Curr Pharm Des* 2012; **18**: 4215-4222 [PMID: 22632606 DOI: 10.2174/138161212802430413]
112 **Kerschen EJ**, Fernandez JA, Cooley BC, Yang XV, Sood R, Mosnier LO, Castellino FJ, Mackman N, Griffin JH, Weiler H. Endotoxemia and sepsis mortality reduction by non-anticoagulant activated protein C. *J Exp Med* 2007; **204**: 2439-2448 [PMID: 17893198 DOI: 10.1084/jem.20070404]
113 **Zhong Z**, Ilieva H, Hallagan L, Bell R, Singh I, Paquette N, Thiyagarajan M, Deane R, Fernandez JA, Lane S, Zlokovic AB, Liu T, Griffin JH, Chow N, Castellino FJ, Stojanovic K, Cleveland DW, Zlokovic BV. Activated protein C therapy slows ALS-like disease in mice by transcriptionally inhibiting SOD1 in motor neurons and microglia cells. *J Clin Invest* 2009; **119**: 3437-3449 [PMID: 19841542 DOI: 10.1172/JCI38476]
114 **Guo H**, Singh I, Wang Y, Deane R, Barrett T, Fernández JA, Chow N, Griffin JH, Zlokovic BV. Neuroprotective activities of activated protein C mutant with reduced anticoagulant activity. *Eur J Neurosci* 2009; **29**: 1119-1130 [PMID: 19302148 DOI: 10.1111/j.1460-9568.2009.06664.x]
115 **Wang J**, Yang L, Rezaie AR, Li J. Activated protein C protects against myocardial ischemic/reperfusion injury through AMP-activated protein kinase signaling. *J Thromb Haemost* 2011; **9**: 1308-1317 [PMID: 21535395 DOI: 10.1111/j.1538-7836.2011.04331.x]
116 **Costa R**, Morrison A, Wang J, Manithody C, Li J, Rezaie AR. Activated protein C modulates cardiac metabolism and augments autophagy in the ischemic heart. *J Thromb Haemost* 2012; **10**: 1736-1744 [PMID: 22738025 DOI: 10.1111/j.1538-7836.2012.04833.x]

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REVIEW

Endoglin in liver fibrogenesis: Bridging basic science and clinical practice

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Abstract

Endoglin, also known as cluster of differentiation CD105, was originally identified 25 years ago as a novel marker of endothelial cells. Later it was shown that endoglin is also expressed in pro-fibrogenic cells including mesangial cells, cardiac and scleroderma fibroblasts, and hepatic stellate cells. It is an integral membrane-bound disulfide-linked 180 kDa homodimeric receptor that acts as a transforming growth factor-β (TGF-β) auxiliary co-receptor. In humans, several hundreds of mutations of the endoglin gene are known that give rise to an autosomal dominant bleeding disorder that is characterized by localized angiodysplasia and arteriovenous malformation. This disease is termed hereditary hemorrhagic telangiectasia type Ⅰ and induces various vascular lesions, mainly on the face, lips, hands and gastrointestinal mucosa. Two variants of endoglin (*i.e.*, S- and L-endoglin) are formed by alternative splicing that distinguishes from each other in the length of their cytoplasmic tails. Moreover, a soluble form of endoglin, *i.e.*, sol-Eng, is shedded by the matrix metalloprotease-14 that cleaves within the extracellular juxtamembrane region. Endoglin interacts with the TGF-β signaling receptors and influences Smad-dependent and -independent effects. Recent work has demonstrated that endoglin is a crucial mediator during liver fibrogenesis that critically controls the activity of the different Smad branches. In the present review, we summarize the present knowledge of endoglin expression and function, its involvement in fibrogenic Smad signaling, current models to investigate endoglin function, and the diagnostic value of endoglin in liver disease.

Key words: Telangiectasia; Signalling; Transforming growth factor-β; Disease; Bleeding disorders

Core tip: Endoglin is an accessory receptor for transforming growth factor-β impacting various aspects of its signaling and biological functions. Endoglin mutations are inherited as autosomal dominant disorders and may cause severe defects in different organs, including brain, lung and liver. In the present review, we will highlight the pathogenesis of several of these disorders and give an overview about the important role of endoglin dysfunction in the pathology of liver fibrosis.

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INTRODUCTION

Endoglin (OMIM 131195) was originally identified 25 years ago by immunofluorescence staining of vascular endothelium with a monoclonal antibody (mAb 44G4) that

was produced against a human pre-B leukemia cell line[1]. It is composed as a homodimer of two subunits with an apparent molecular weight of 95 kDa that are linked by disulfide bonds[1]. Two years later, cDNA clones were isolated from an endothelial cell λgt11 expression library using a rabbit antibody prepared against endoglin purified from placenta[2]. Subsequent screening with an endoglin-specific cDNA probe resulted in the isolation of a different splice variant in which the encoded cytoplasmic tail contains only 14 amino acids (aa) as opposed to the stretch of 47 residues that was published previously[3]. The *ENG* gene was mapped to the long arm of human chromosome 9 (9q34→qter) by Southern blot analysis of DNA isolated from human-hamster somatic cell hybrids and by fluorescent *in situ* hybridization coupled with DAPI banding on human chromosomes[4]. The detailed chromosomal assignment was subsequently predicted from the fact that the mouse homolog is located on chromosome 2 directly in the close proximity of the adenylate kinase-1 gene that is syntenic to human chromosome subband 9q34.1[5,6].

Mutations within endoglin were first brought into context of hereditary hemorrhagic telangiectasia type I (HHT-1) in three affected individuals in whom nucleotide substitutions or deletions gave rise to premature termination codons[7]. Since that, several hundred independent mutations or variations have been identified in the *ENG* gene that most often show regional distribution[8-12]. The different mutations show different phenotype-genotype correlation with the severity of HHT-1[13]. Moreover, it has been shown that soluble endoglin (sol-Eng) is an anti-angiogenesis factor that contributes to the pathogenesis of pre-eclampsia that is associated with hypertension, proteinuria, premature labor, hemolysis, liver abnormalities, thrombocytopenia, seizures and death[14,15]. Increased levels of sol-Eng in vascular surgical specimens were also brought into context with the pathogenesis of arteriovenous malformations (AVM) of the brain and aberrant cerebral vascular remodelling[16]. Other reports propose sol-Eng as a marker in diabetic patients[17] for estimating progression or treatment efficacy of the atherosclerotic process[18,19], systemic lupus erythematosus[20], non-small cell lung cancer patients[21], hypertension[22], disturbed angiogenesis in systemic sclerosis[23], Alzheimer's disease[24], breast cancer[25], premalignant lesions of the colon mucosa[26], outcome of biliary atresia[27] and cystic fibrosis associated liver disease[28], unexplained fetal death[29], malaria pathogenesis[30], prostate cancer[31] and many other diseases. In addition, endoglin expression was found to be related to tumor size, aggressiveness and metastatic potential in patients with gastroenteropancreatic neuroendocrine tumors[32].

A similar phenotype, *i.e.,* HHT type 2, is observed when the activin-like kinase (ALK)-1 receptor is functionally altered[33]. Likewise, mutations in the gene encoding Smad4 (MADH4) can cause a syndrome called Juvenile Polyposis/Hereditary Hemorrhagic Telangiectasia Syndrome (JPHT), consisting of both juvenile polyposis and hereditary hemorrhagic telangiectasia phenotypes[34]. Also, the mutations of other yet unidentified genes on the long arm of chromosome 5[35] and on the short arm of chromosome 7[36] were linked to the formation of other HHT types.

Endoglin expression and dysregulation has been shown in a number of cell types, including mesangial cells, cardiac and scleroderma fibroblasts, and hepatic stellate cells (HSC), suggesting some important function in cell and organ homeostasis and disease formation[37-40]. In particular, many independent findings demonstrate that endoglin is a critical factor that orchestrates transforming growth factor-β (TGF-β) signaling in wound healing in the pathogenesis of fibrosis. In regard to hepatic fibrogenesis, it was shown that endoglin is expressed in HSC[41] representing the most pro-fibrogenic cell type within the liver. Interestingly, endoglin expression is up-regulated during liver damage and transiently induced in HSC by TGF-β1[40]. In this hepatic subpopulation, endoglin binds to the TGF-β type Ⅱ receptor (TβRⅡ), becomes phosphorylated by the activity of the TβRⅡ, and shows highest expression during maximal cell activation with a transdifferentiation-dependent cellular localisation and ligand affinity[40]. Interestingly, transient overexpression of endoglin results in a stronger activation of the Smad1/Smad5 signaling cascade and a prominent increase of α-smooth muscle actin expression, thereby promoting cellular activation and transdifferentiation[40], while contrarily the activity of the TGF-β1/Smad3 pathway is inhibited[42]. All these findings demonstrate that endoglin is one of the central switches controlling fibrotic and anti-fibrotic activities by producing different variant forms, adjusting ligand affinity, amending expression levels, and interacting with a versatile receptor network, thereby modulating the specific outcome of TGF-β-dependent and -independent pathways.

In the present review, we will summarize the actual knowledge of endoglin function and discuss the impact of this receptor on disease formation, hepatic fibrogenesis and its diagnostic value in initiation, progression and prognosis of various liver diseases.

MOLECULAR AND BIOCHEMICAL CHARACTERISTICS OF ENDOGLIN

The human endoglin gene contains 15 exons numbered 1 to 14, where exon 9 is split into 9a and 9b (Figure 1)[7]. Beside the full length endoglin (FL-Eng), a splice variant has been identified, *i.e.*, short-endoglin (S-Eng), that is characterized by the retention of intron 14 in the mature mRNA[3,40,43]. The expression of S-Eng is increased in senescent endothelial cells and alternative splicing is most likely performed by the alternative splicing factor or splicing factor-2 (ASF/SF2)[44,45]. However, the orthologous S-Eng mRNAs of men and mice give rise to different proteins (Figure 1) with either shortened and in part alternate C-termini[2,43] or a full length endoglin with a peptide insertion in rat[40]. Although the C-terminal domain of FL-Eng does not possess catalytic activity, it

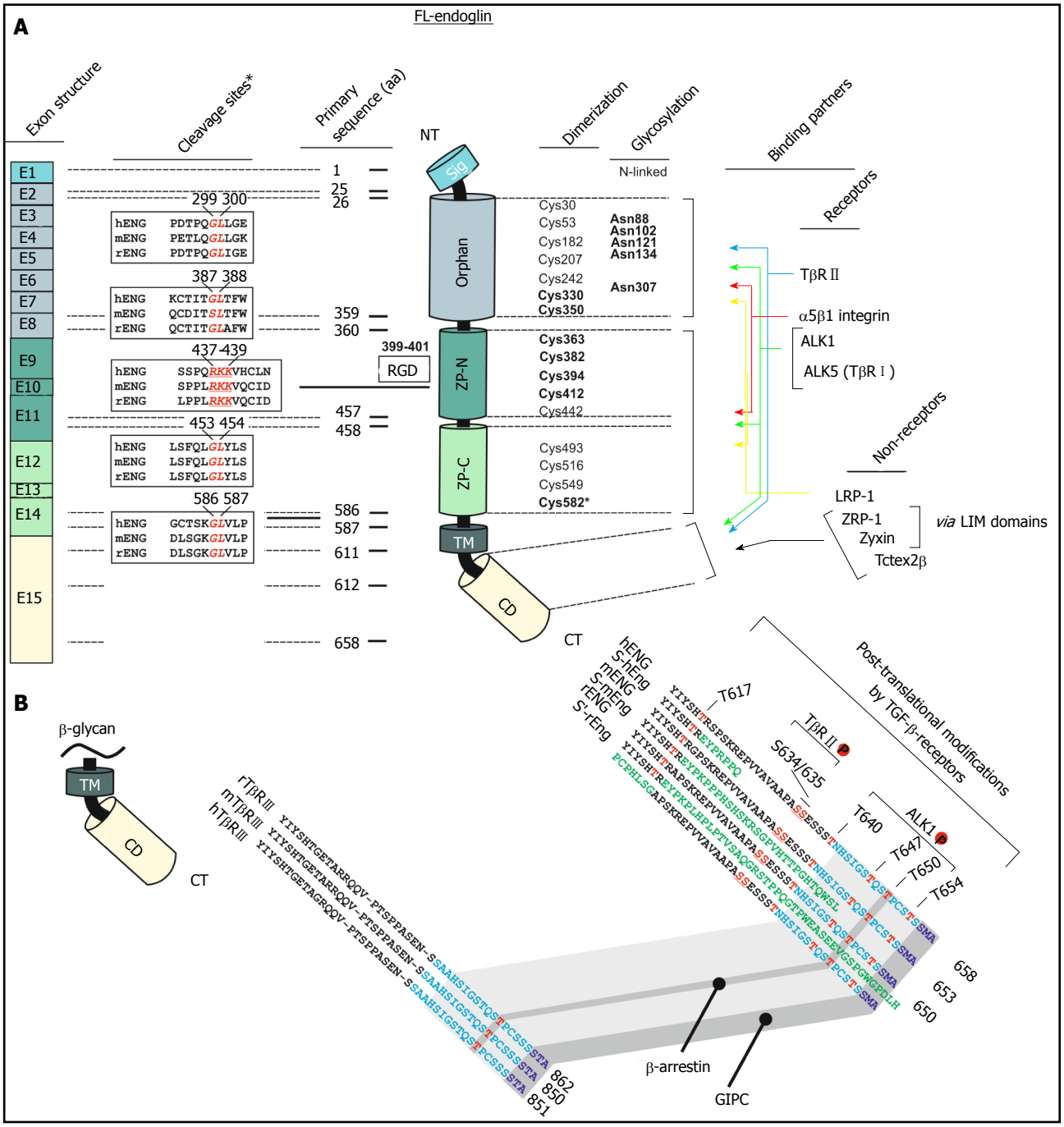

Figure 1 Schematic representation of the structural and functional modules of human endoglin. A: Left, first panel, Exon structure: Structure of the endoglin gene and assignment of respective protein modules. Left, second panel, cleavage sites: Predicted consensus proteolytical cleavage sites deduced from the primary sequence and experimentally confirmed matrix metalloproteinases-14 cleavage site between aa positions 586 and 587[59]. Middle panel, primary sequence positions: aa boundaries of the structural domains of human endoglin and location of the Arginine-Glycin-Aspartic acid (RGD) sequence that is only present in human endoglin. Right, first panel, dimerization: The cysteine residues of the extracellular domain of endoglin are depicted including the 8 highly conserved residues within the ZP-domain[47]. Cysteines that are involved in dimerization are shown in bold. Cysteine 582 that is involved in human endoglin dimerization is not present in the mouse and rat homologues[56]. Right, second panel, glycosylation: This figure part displays verified N-linked glycosylation sites within human endoglin. There is experimental evidence for O-linked glycosylation, but respective sites are not shown[1]. Right, third panel, binding partners: Depicted are interaction partners of endoglin as either receptor proteins (upper) or cytosolic non receptors proteins (lower). Colored arrows indicate interacting domains of endoglin with its binding partners. Zyxin and ZRP-1 bind to endoglin *via* their LIM-domains; B: Displayed is an aa alignment of betaglycan (left) and endoglin (right) of human, mouse and rat. Receptor kinase substrates (serine and threonines) are shown in red. Threonine 650 is essential for binding to β-arrestin2[66]. The C-termini of betaglycan and endoglin that are highly conserved are indicated in light blue. The PDZ-I domain which binds to GIPC is depicted in dark blue[67]. The alternative C-termini which results from differential splicing are shown in green.

is substrate for different kinases and comprises several protein-protein interaction domains (Figure 1)[46]. Therefore, structural alterations imposed by differential splicing of the mRNA that encodes the intracellular domain results in functional consequences for Eng in signaling (see below). In addition to splicing, Northern blot analy-

sis of mouse and rat transcripts revealed two mRNA species differing in molecular weight more than the size of the retained intron 14 of S-Eng. Analysis of the corresponding cDNA with 3'-RACE and inspection of the rat genomic DNA sequence confirmed a variation in the non-coding region of the mRNA and the presence of a second polyadenylation signal in the genomic DNA[40]. Whether this differential polyadenylation modulates mRNA stability or other features of the mRNA is currently not known. Since it has been realized that endoglin mutations are causative for HHT-1[7], a wealth of different mutations in the endoglin gene which lead to altered expression or formation of aberrant protein products has been identified (see below). Nevertheless, mutations are not spread randomly in the genomic sequence. A bias for mutations is found in the orphan domain and the N-terminal zona pellucida (ZP-N) subdomain in which three highly conserved cysteines (Cys363, Cys382 and Cys412) are exceptionally prone to mutations[47].

Biochemical characteristics

Endoglin, a type I transmembrane glycoprotein, is expressed as a disulfide-bound dimer at the cell surface[48]. Endoglin belongs structurally to the zona pellucida (ZP) family of sperm receptors sharing a ZP domain of approximately 260 aa in their extracellular part[49,50]. This domain is localized between Lys362-Asp561 (Figure 1) and contains eight highly conserved cysteine residues[47]. Common characteristics of ZP domain proteins are that they are: (1) shed to generate a soluble form; (2) membrane proteins with a hydrophobic region at their C-termini; (3) strongly glycosylated; and (4) finally highly expressed in the corresponding tissues in which they occur[50].

Among TGF-β-family receptors, endoglin and betaglycan constitute the TGF-β type Ⅲ receptor family. Both receptors share a high degree of similarity, especially in their intracellular domain (Figure 2) that is also the most conserved region between endoglin from different species (Figure 3), implying that this region has an important function, although lacking enzymatic activity[40].

In line, the signaling specificity of endoglin compared to betaglycan is at least for some specific functions determined by the extracellular domain (ECD)[51]. Since both of these receptors possess no enzymatic activity in their short C-terminal domain and are not obligatory for general signaling, they have been assigned an accessory/modulating function in signaling[52]. The primary sequence of FL-Eng comprises 658 aa in human[2,3], 650 aa in rat[41], and 653 aa in mouse (Figure 3)[53]. The ECD of human Eng harbors a Arginine-Glycin-Aspartic acid (RGD) peptide representing a potent binding site for integrins which is not present in the rat and mouse homologues[2,41,53]. Along with the FL-Eng, a splice variant designated S-Eng has been identified. The longer mRNA is due to the retained intron 14 (see above) and codes for a protein with a shortened C-terminus of 14 aa in human and 35 aa in mouse because of an in-frame stop codon present in the intron which is not found in rat resulting in a protein that contains a 49 aa insertion[3,41,53]. As outlined below, the shortening of the C-terminal domain of the splice variant in human and mouse have structural and functional consequences because specific modules are missing. The mentioned insertion in rat FL-Eng only causes minor effects which may be due to sterical alterations in the C-terminal domain[40]. In addition to these splice variants, two transcripts in mouse and rat occur which differ in the 3'-non-coding part and which arise from differential polyadenylation[40]. With respect to post-translational modifications, the primary Eng sequence contains several potential N- and O-dependent glycosylation sites. Initial enzymatic de-glycosylation studies confirmed the usage of both N- and O-dependent glycosylation consensus motives[48]. In a more detailed study, single N-dependent glycosylation sites (Asn88, Asn102, Asn121, Asn134 and Asn307) have been identified by mutational analysis[54]. Although the corresponding N-glycosylation sites seem to influence the stability of the corresponding domain, *e.g.,* Asn102 and Asn307[54], the removal of carbohydrates by peptide N-glycosidase F (PNGase F) was shown to be exiguous for function of the ECD[55].

In general, FL-Eng has a tripartide structure comprising a short intracellular region (47 aa), a single transmembranal portion (25 aa), a large ECD (561 aa) and a predicted signal peptide (25 aa)[3]. Preceding the ZP domain there is an orphan domain (Glu26-Ile359), sharing no similarity to other protein families/domains[47]. The ZP domain (Gln360-Gly586) is further subdivided in a ZP-N (Gln360-Ser457) and ZP-C (Pro458-Gly586) subdomain (Figure 1). Deletion and substitution studies revealed that at least Cys582 in human FL-Eng in the ECD is involved in intermolecular disulfide binding[56]. Additional work revealed that the six cysteines between Cys330 and Cys412 are necessary to mediate receptor dimerization[57], allowing the receptor to be expressed as a dimer at the cell surface, or in case of the soluble form as a secreted dimer[58]. A high resolution structure established for the ECD of endoglin revealed information about the sterical arrangement of the 3-dimensional protein fold[47]. These studies confirmed the three-modular-structure (orphan domain, ZP-N and ZP-C domain) and further raised the hypothesis of the occurrence of a putative cleavage site for a sheddase with specificity for the linker region between the folded domains of ZP-N and ZP-C at position Arg437-Lys438-Lys439 (RKK)[47]. However, the biochemical elucidation showed that the cleavage site is located closer to the membrane at position Gly586-Leu587. The executing enzyme was shown to be matrix metalloprotease-14 (MMP-14 or MT1-MMP)[59,60] promoting a shedding process that is similar to that described for betaglycan before[61]. On a functional level, endoglin is able to interact with the TGF-β signaling receptors (cf. Figure 1, Figure 4)[62] as well as other regulatory proteins[63-67]. These interactions are mediated by the different subdomains (or combinations). In general, FL-Eng is able to interact with ALK5 and TβR Ⅱ independent of ligand and the activation state of the signaling receptors[56]. In more detail it was

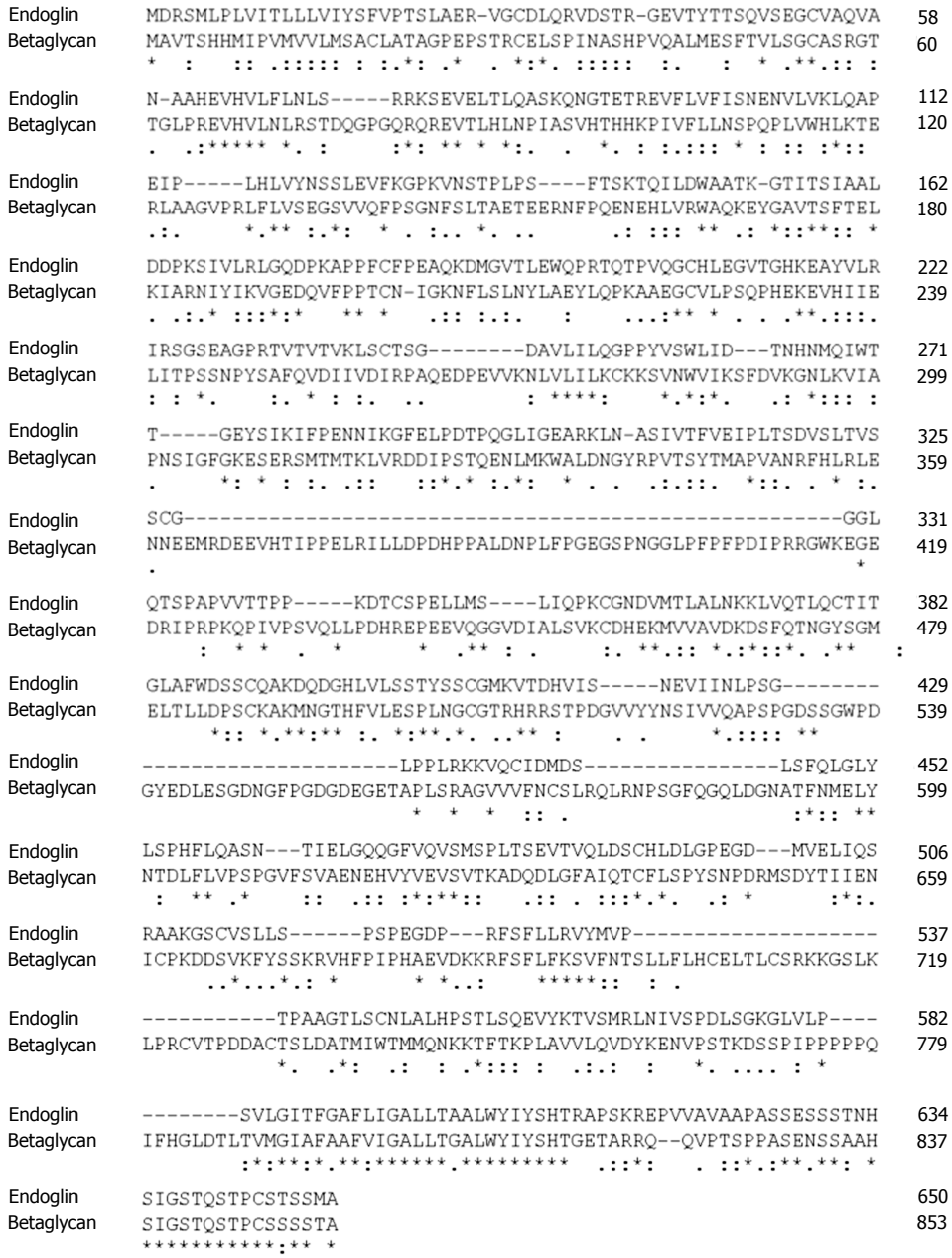

Figure 2 Sequence alignment of rat endoglin and betaglycan. The protein sequences of rat endoglin and betaglycan were aligned using the ClustalW2 algorithm. Respective sequences of rat endoglin (AAS67893) and betalycan (AAA42236.1) were taken from the GenBank. Please note the high degree of similarity of both proteins at their C-termini. Fully conserved aa in endoglin are marked by asterisk (*), positions that carry aa with strongly similar properties by a colon (:) and positions with weakly similar properties by a period (.), respectively. Please note that the highest degree of homology is found at the C-terminal regions that encompass the cytosolic part of endoglin

shown that TβR Ⅱ interacts with the region 437-558 (mainly ZP-C domain) of the endoglin ECD. In contrast to TβR Ⅱ, ALK5 contacts two regions, spanning aa 26-437 and aa 437-558 (orphan and ZP-C domains)[56]. Similarly, the second type I receptor ALK1 was shown to interact with the region Glu26-Gly586 of the ECD of endoglin[68].

Since the soluble variant of endoglin comprises all these ECD, it should in principal also be capable of mediating the same receptor interactions. Nevertheless, the binding of the soluble ECD to membrane bound endoglin could not be shown[57].

Whereas the binding to the ECD of FL-endoglin is independent of the signaling receptor activity, interaction of TβR Ⅱ, ALK5 and ALK1 with the intracellular domain of endoglin is regulated by the activation state of the signaling receptors since binding of the constitutive active ALK5/ALK1 could not be detected, while the binding of kinase dead and wild type ALK5/ALK1 could be demonstrated[56,68]. In line, the association of endoglin with the inactive form (kinase dead) of TβR Ⅱ was reported to be stronger when compared to wild type TβR Ⅱ[56].

It is known that FL-Eng is phosphorylated at serine and threonine residues[69,70] and both ALK5 and TβR Ⅱ use the C-terminus of endoglin as a substrate[56,70,71]. In

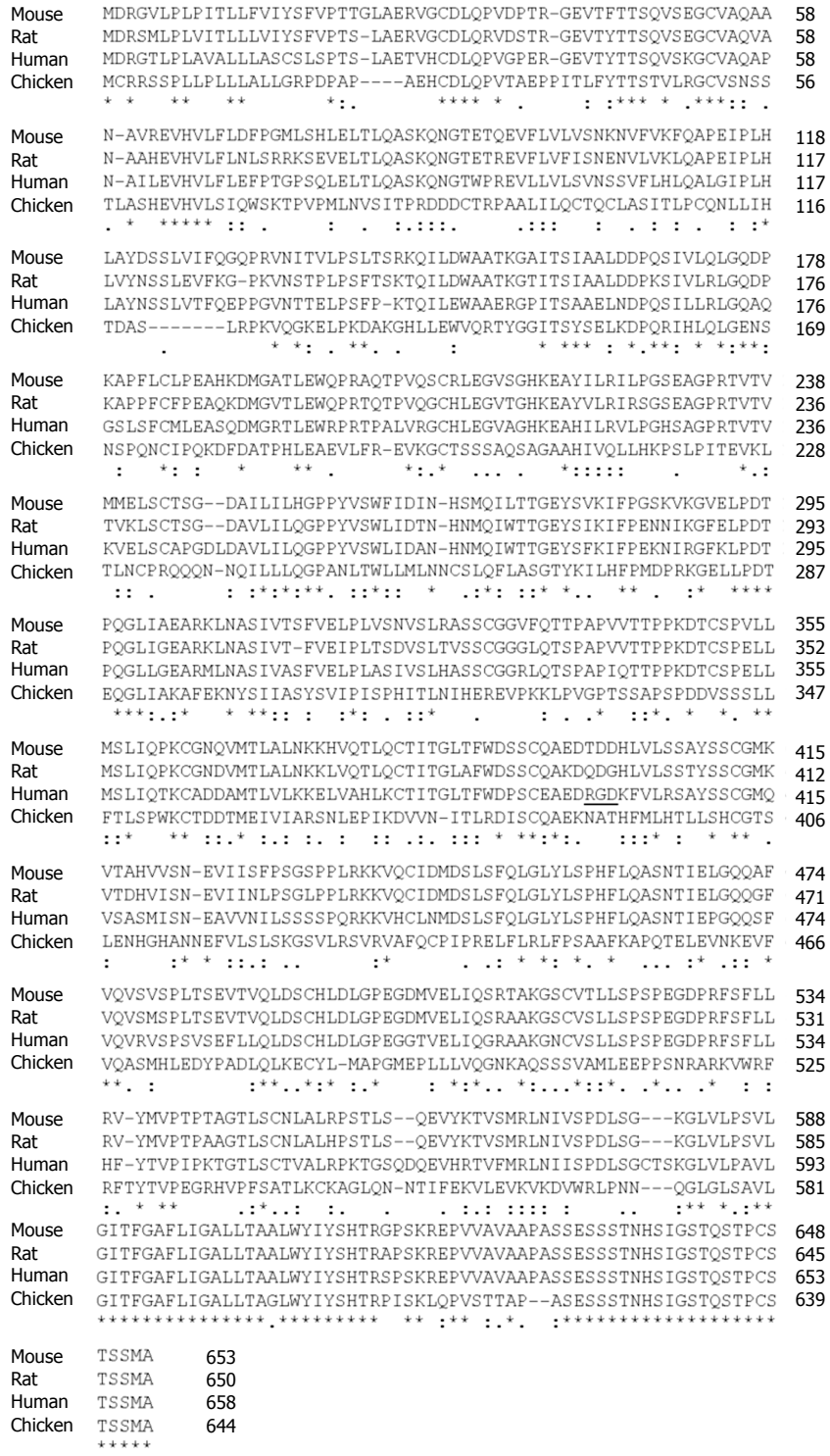

Figure 3 Sequence alignment of endoglin from different species. The protein sequences of rat, mouse, human, and chicken endoglin were aligned using the ClustalW2 tool (http://www.expasy.org/genomics/sequence_alignment). Sequences of mouse (NP_031958), rat (AAS67893), human (NP_001108225) and chicken (AAT84715) were taken from the GenBank (http://www.ncbi.nlm.nih.gov/). The Arginine-Glycin-Aspartic acid sequence in human endoglin (aa 399-aa 401) is underlined. Fully conserved aa in endoglin are marked by asterisk (*), positions that carry aa with strongly similar properties by a colon (:) and positions with weakly similar properties by a period (.), respectively. Please note that the highest degree of homology is found at the C-terminal regions that encompass the cytosolic part of endoglin.

turn, FL-Eng inhibits autophosphorylation of TβR Ⅱ but enhances phosphorylation of ALK5 by TβR Ⅱ leading to a stronger Smad2 transcriptional activity (see below)[56]. Aside from ALK5, ALK1 is also able to phosphorylate the FL-Eng C-terminus, but in contrast to ALK5, primarily on threonine residues[70]. Threonine phosphorylation by ALK1 (Thr654) necessitates serine phosphorylation by TβR Ⅱ which is enforced by removal of the C-terminal PDZ domain[70]. Moreover, ALK1 phosphorylation and binding of endoglin was observed only in the presence of TGF-β1 and this phosphorylation leads to loss of FL-Eng from focal adhesions (see below)[70]. This modulates

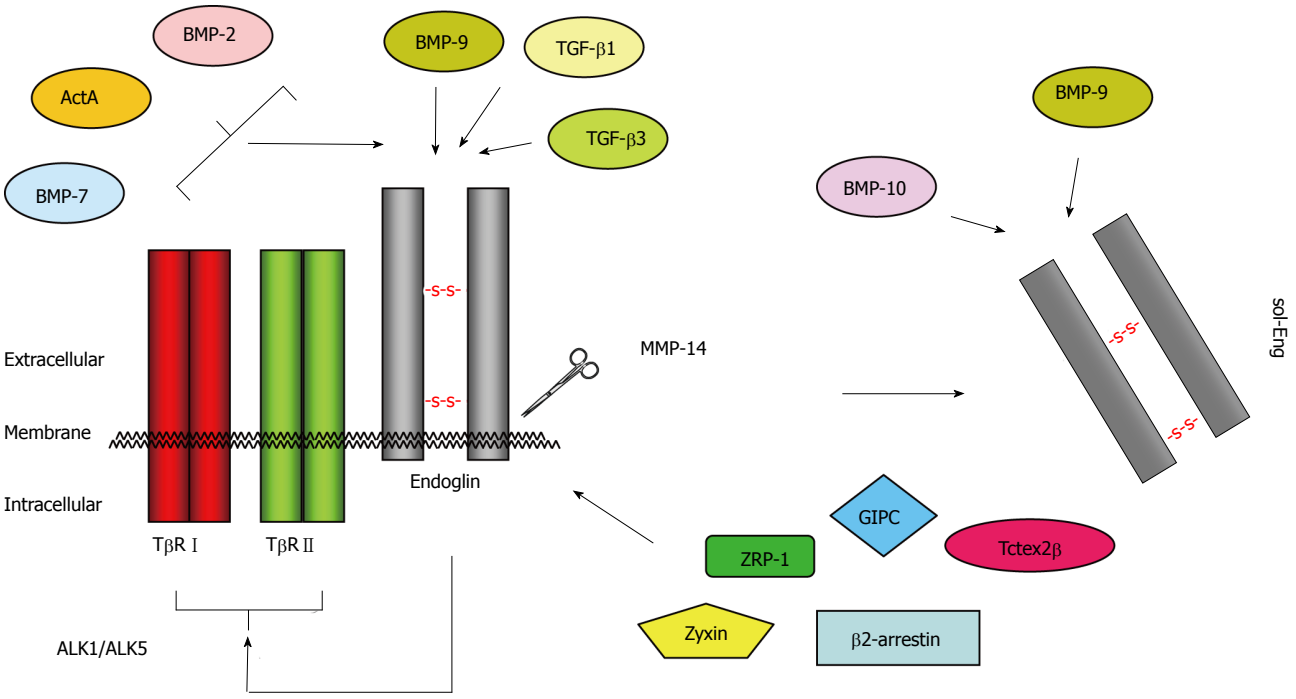

Figure 4 Binding partners of endoglin. Endoglin physically interacts *via* its extracellular domain with TGF-β1, TGF-β3 and BMP-9[110]. The short cytoplasmic domain has affinity for ZRP-1[63], Zyxin[64], GIPC[67], β-arrestin-2[66], and Tctex2β[65]. In conjunction with TβR Ⅰ and TβR Ⅱ, the binding spectrum is extended to BMP-2, BMP-7 and ActA[111]. After proteolytic cleavage (shedding) by MMP-14 (also known as membrane-type matrix metalloproteinase MT1-MMP), the soluble form of endoglin (sol-Eng) is released[59]. This form has capacity to bind BMP-9 and BMP-10[113]. TGF: Transforming growth factor; BMP: Bone morphogenetic protein.

proliferative and adhesive properties of endothelial cells. In more detail, it was shown that the exponentiation activity of endoglin on ALK1 signaling and Smad1 activity is located between residues 26-558 within the ECD of endoglin[68]. Another interaction with the ECD of endoglin is mediated by integrin α5β1, which contacts not only the RGD-peptide but several parts of the ECD of endoglin. Clustering of α5β1/endoglin/ALK1 leads to an enhancement of TGF-β1-mediated Smad1/Smad5 activation and signaling[72]. Recently, leucine-rich α_2-glycoprotein 1 (Lrg1) has been further shown to interact with the ECD of endoglin. This protein is a regulator of endothelial functions during angiogenesis. In addition to endoglin, it interacts with ALK5 and TβR Ⅱ directly and facilitates recruitment of ALK1 into the receptor complex thereby promoting Smad1/Smad5-signaling[73].

In contrast to the signaling type Ⅰ and type Ⅱ receptors, the type Ⅲ receptors betaglycan and endoglin do not possess a kinase activity in their short intracellular domains[3,53,41]. Nevertheless, respective domains have important functional implications for the interaction with the signaling receptors as described above. Although the C-termini of betaglycan and endoglin are very homologous to each other (Figure 2), several residues used as substrates by the signaling receptors are unique to endoglin[70].

Phosphorylation by a respective receptor serves as a switch to regulate the interaction with a certain receptor. Besides receptor interactions, other regulatory proteins have been identified which specifically bind to the C-terminal domain of FL-Eng. Using the two hybrid method, zyxin and zyxin-related protein-1 (ZRP-1) were found to specifically and exclusively, with respect to type Ⅲ receptors, interact with FL-Eng[63,64]. Association with FL-Eng redirects these proteins from focal adhesions to actin stress fibers and leads to endoglin dependent inhibition of cell migration[63,64]. Another protein identified in the yeast system is the dynein light chain member Tctex2β. In addition to FL-Eng, Tctex2β also interacts with TβR Ⅱ and betaglycan and it inhibits TGF-β signaling[65].

However, it has to be mentioned here that all these interaction screens have been solely performed using protein baits of the endoglin intracellular domain which have not been posttranslationally modified, *e.g.*, phosphorylated. The interaction of at least zyxin with endoglin is stronger with the so called -ΔSMA deletion mutant that lacks the 3'-carboxyl-terminal protein part harbouring the PDZ-domain[64]. In line, removal of this domain causes an increase in endoglin phosphorylation[70] implying that this modification (phosphorylation) most likely modulates/regulates protein-protein interaction with the carboxyterminal domain (CD) of endoglin. Therefore, it is most likely that the group of proteins able to interact with endoglin is currently somewhat underestimated.

Based upon the high homology of the CD of endoglin and betaglycan, it is not surprising that both β-arrestin2 and GIPC were found to associate with both proteins[66,67,74,75]. The interaction of β-arrestin2 and endoglin is lost in the absence of threonine 650 and increases when co-expressed with TβR Ⅱ and ALK1[66].

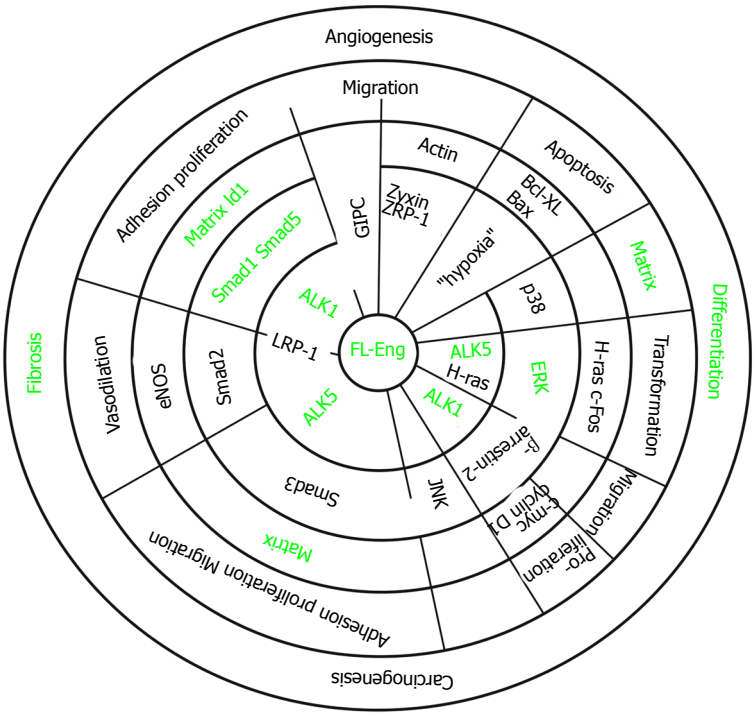

Figure 5 Association of endoglin with different signaling cascades. The concentrical circles display the hierarchy of signaling. Signaling starts at the membrane with receptors and adaptors (inner two circles). The next circle represents activated intermediates and adaptors. Thereafter, target genes are indicated. These are involved in shaping a cellular response which is part of a complex process (last circle). Partially open radial lines indicate that the corresponding molecules interact or interaction of molecules is mediated by the protein displayed on the radial line (LRP-1). The green font indicates items addressed in liver cells which are modulated by endoglin.

Whether the latter receptor regulates this interaction *via* phosphorylation is unclear since Thr650 is not a prominent ALK1 substrate[70]. On a functional level, β-arrestin2 causes endocytosis of the receptor complex, including endoglin, TβR Ⅱ and ALK1, and impacts MAPK-signaling in an endoglin-dependent manner[66]. In contrast to β-arrestin-2, the C terminus of the G alpha interacting protein (GAIP)-interacting protein (GIPC) binds to the C terminus of endoglin in a manner that is restricted to the endoglin class I PDZ-motif. This leads to a stabilization of endoglin at the plasma membrane and changes in Smad1/Smad5 activation and endothelial cell migration (see below)[67]. Moreover, GIPC mediates the interaction of endoglin and phosphatidylinositol 3-kinase in a TGF-β1 dependent manner to regulate endothelial cell sprouting and capillary tube stability[76].

ENDOGLIN FUNCTION AND IMPACT ON TGF-β SIGNAL TRANSDUCTION

Endoglin is an accessory receptor for TGF-β impacting various aspects of its signaling and biological functions. Special features for the full length, soluble and short forms of endoglin have been reported. In the following, we provide a brief overview about TGF-β signaling and the impact of the different endoglin protein variants. Functional aspects of FL-endoglin are summarized in Figure 5.

Brief overview of TGF-β signaling

Signaling by ligands of the TGF-β superfamily is initiated by binding of the ligand to a heterooligomeric membrane receptor complex. Binding of TGF-β1 is mediated by a homodimer of the TGF-β type Ⅱ receptor which in turn recruits and phosphorylates a type I receptor (ALK5 or ALK1) homodimer into the complex. After ligand binding, the receptor complexes are internalized in general *via* two different pathways. Endocytosis mediated by clathrin-coated vesicles, enriched for Smad anchor for receptor activation (SARA), leads to active signaling. Depending on the type I receptor involved, the signal is propagated to two different Smad protein subfamilies, with the specificities of ALK5 phosphorylating Smad2/Smad3 or ALK1 in triggering phosphorylation of Smad1/Smad5. Phosphorylated Smads bind to the common Smad4, translocate into the nucleus and regulate transcription of target genes. Of these, the I-Smads, *i.e.*, Smad6 and Smad7, are important regulators since they are direct target genes and shut off the signaling cascade at diverse points in a negative feedback loop. If internalization occurs *via* the lipid-rafts-caveolae-1, the receptors are bound to I-Smad/Smurf complexes targeting the receptor for ubiquitination and degradation[77].

Since this simple "core" of TGF-β signaling is involved in the regulation of a wide array of different target genes and control of diverse cellular responses, cells are endowed with a plethora of switches to adjust this cascade for their needs. Such cell type specific regulators for example are the type Ⅲ receptors, *i.e.*, betaglycan and endoglin, which are engaged in TGF-β receptor-complex formation and modulation of downstream signaling.

In the liver and especially in HSC, it has been assumed that the key operating TGF-β1 pathway is the ALK5/Smad3 branche that regulates proliferation, activation and profibrogenic responses of these cells. However, it has been anticipated that other signaling modalities like the ALK1/ALK5/Smad1/Smad5/Id1 axis is also engaged by TGF-β1 in regulating HSC physiology under normal and pathological conditions[40,78,79].

Impact of full length endoglin on TGF-β1-signaling

Analysis regarding the role of endoglin in signaling was primarily based on TGF-β-signaling and Smad-activation in monocytes and myoblasts[51,80]. Since it is known that endoglin is the candidate gene affected in HHT-1, detailed experimental work has been done using different endothelial cells[7,81]. So far the functional data regarding the involvement of endoglin in HSC are rather sparse. Endoglin is expressed in quiescent HSC and transdifferentiated myofibroblasts (MFB) and is transiently upregulated during cellular activation[40,41]. Upregulation of endoglin during activation/differentiation of cells is also seen in endothelial cells and monocytes[82,83]. Similar to other cell types, endoglin is not only affecting TGF-β1-signaling but is itself regulated by this ligand on the transcriptional level, most likely involving the Sp1 transcription factor[40,84-86]. As a mutual prerequisite, endoglin is membrane localized and interacts with and is phosphorylated by TβR Ⅱ in HSC[41,40]. Overexpression of endoglin causes an increased phosphorylation of Smad1/Smad5 in HSC of rat and mouse origin[40,79]. In line with the HSC data, it was previously found that endoglin enhances ALK1/Smad1/5 signaling in endothelial cells and other cell types[87], leading to increased proliferation and migration (characteristics of the activation phase of angiogenesis), responses which are negatively affected upon endoglin reduction[72,88,89]. However, other laboratories claimed that endoglin causes reduced activation of ALK1/Smad1/5 as well as reduced migration and proliferation[90] or even having no impact on Smad-signaling at all[66]. These differences might be explained in part by the experimental set up (method used to modulate the endoglin expression, *i.e.*, siRNA *vs.* knockout, concentration of the ligand, time scale of stimulation, cell type analyzed) and by the expression level of the two corresponding type I receptors, *i.e.*, ALK1 and ALK5, both of which are expressed in HSC[78]. On the other hand, ALK5/Smad3 signaling that inhibits proliferation and migration (characteristics of the resolution phase of angiogenesis) is blocked by endoglin[67,88,91]. Interestingly, in contrast to ALK5/Smad3 which is downregulated, the signaling *via* ALK5/Smad2 leading to increased eNOS expression/activity is promoted in endothelial cells[56,92]. This effect is in part due to a stabilization of the Smad2 protein[92].

Although collagen type I expression is reduced, the overexpression of endoglin has no significant impact on ALK5/Smad3/Smad2 activation in mouse and rat HSC cell lines[40,79]. An inhibitory role of endoglin in collagen type I expression has been well documented in diverse kinds of cells, including mesangial cells, fibroblast of different origins and myoblasts[87,93-95] and was attributed to a reduced Smad3 activation[87,94]. A contribution of MAPK in the endoglin dependent modulation of collagen expression and Smad3 phosphorylation was postulated for JNK1 and ERK1/2[94,96].

In HSC, endoglin causes an increase in TGF-β1 dependent ERK1/2 activation[79]. A positive effect of endoglin on ERK1/2 activation was also observed in human T cells upon crosslinking of endoglin[97]. In line with an enhancement of ERK1/2 phosphorylation, TGF-β1 mediated expression of the connective tissue growth factor (CTGF) is promoted by endoglin in HSC[79]. There are several other reports showing an ERK1/2 dependent expression of CTGF, once more underscoring these results[98,99]. Nevertheless, the activation of ERK1/2 and increased expression of CTGF by endoglin is most likely cell type specific. In endothelial cells and epidermal cells it was shown that endoglin, in association with β-arrestin2, leads to suppression of ERK1/2 activation and a change in the cellular distribution[66,100]. On the contrary, in myoblasts in which TGF-β1 and endoglin have only a minor effect on ERK1/2 activation, CTGF is reduced in the presence of endoglin[87,95]. A negative impact of endoglin on CTGF expression was also found in scleroderma fibroblasts by some groups[39,101]. However, in a subset of scleroderma fibroblasts it was shown that the TGF-β1/ALK1/Smad1 pathway mediates fibrogenic responses, *e.g.,* collagen I and CTGF expression, and that endoglin promotes this ALK1 pathway[102,103]. Finally, it was shown that ERK1/2 and Smad1 activation are functionally linked[102]. If endoglin-dependent up-regulation of ERK1/2 phosphorylation in HSC is directly linked to Smad1 activation and CTGF expression, and if ALK1 is involved in these responses is currently under investigation. Moreover, if the co-expressed betaglycan is involved in the up-regulation of CTGF is actually only speculative[101]. In addition, the basis of the forced expression of α-smooth muscle actin (α-SMA) in endoglin overexpressing cells needs to be analyzed[40,79]. One comprehensible option is a direct promoting effect on TGF-β1 signaling mediating α-SMA expression, which was shown to rely not exclusively on Smad3[104], or alternatively endoglin may cause a general shift in the transdifferentiation process leading finally to up-regulation of α-SMA.

Role of short (S-) endoglin on TGF-β1-signaling

Similar to FL-Eng, the S-Eng splice variant, although missing a large part of the C-terminal tail, binds to TGF-β1[3] and interacts with the signaling type Ⅱ receptor[40] and both type I receptors (ALK5 and ALK1)[44]. FL-Eng was shown to be phosphorylated at serine residues by TβR Ⅱ receptor[70] that fortuitously can be detected by a phospho-specific NF-κB antibody[105]. TβR Ⅱ-mediated phosphorylation of both isoforms of rat endoglin could be detected in HSC using this antibody[40], implying a functional association of endoglin and the TGF-β-signaling receptors in HSC. Both splice variants are co-expressed in endothelial cells and HSC and can form heteromeric L-/S-Endoglin dimers[40,43]. Nevertheless, S-Eng is unable to substitute for FL-Eng since animals that carry an S-Eng transgene on an Eng null background are not viable, implying that S-Eng alone is inappropriate to rescue the lethal phenotype[43]. Using the afore mentioned S-Eng overexpressing animals in a model for tumor angiogenesis and metastatic infiltration by injecting Lewis lung carcinoma (3LL) cells, it was

found that tumor growth is retarded when compared to control mice[43]. Even more, in a model of chemically induced skin tumors, overexpression of S-Eng in the vascular endothelium reduces benign tumor formation[43]. Nevertheless, functional data obtained in the rat system for the specific S-Eng variant yielded similar results when compared to FL-Eng[40]. Whether these results can be transferred to the mouse or human system is questionable due to the completely different C-termini.

Soluble endoglin: more than just a disease marker

As described above, endoglin can be shedded by MT1-MMP (MMP-14) from the cell surface to generate a soluble extracellular domain (sol-Eng) which reduces spontaneous and VEGF-induced endothelial sprouting[59]. In addition, the occurrence of sol-Eng has been observed in the serum/plasma of patients suffering from diverse tumors[106]. In pre-eclamptic women, the elevation sol-Eng precedes the onset of the disease, correlates with the severity of the disease and therefore its detection is of prognostic value[107]. Increased serum levels of sol-Eng have been found in cystic fibrosis associated liver disease (CFLD) patients, with the highest levels in patients suffering from HCV coupled with cirrhosis[28]. Significantly elevated sol-Eng levels are also observed in patients with hepatocellular carcinoma [Hepatocellular carcinoma (HCC)] combined with cirrhosis[108]. However, the role of sol-Eng in TGF-β1 signaling is presently controversial. Initially it was shown that the soluble domain is able to reduce TGF-β1-mediated reporter-gene activity and eNOS activation in endothelial cells[14]. In line with a ligand sequestering function, complexes of sol-Eng and TGF-β1 have been detected in serum of breast cancer patients using ELISA and co-immunoprecipitation[58]. Nevertheless, although part of the TGF-β1 ligand binding complex, a direct binding of TGF-β1 to endoglin is questionable[109,110]. If the signaling receptor type I and type Ⅱ are present/co-expressed, endoglin can be precipitated together with labelled ligand. If endoglin on the other hand is overexpressed in cells lacking type I and type Ⅱ receptor, there is no binding of TGF-β1 to endoglin[110]. The increase of the sol-Eng concentration in pre-eclamptic women and a few studies with a focus on sol-Eng function, using overexpression systems and luciferase assays, suggest that sol-Eng indeed has a functional role in TGF-β1 signaling[14,59]. In addition, we could show by co-immunoprecipitation that heterologous expressed sol-Eng is able to bind to TGF-β1 directly (SKM unpublished data) but experimental data suggest that it is unlikely for soluble endoglin to simply interfere with TGF-β1 signaling by competing with membrane bound type Ⅱ receptor for TGF-β1. Using a BIACore facility, the measured dissociation constants are 5 pM for TβR Ⅱ/TGF-β1[111] and in the micromolar range for sol-Eng/TGF-β1[112], underscoring the higher affinity of TβR Ⅱ for TGF-β1 compared to the soluble endoglin counterpart. On the other hand, Van Le *et al* found that CHO-overexpressed and purified soluble endoglin increased TGF-β1 mediated p3TP-lux activity in U937 monocytic cells[55] in which L-endoglin was shown to antagonize several TGF-β1-responses[80]. Nevertheless, direct ligand binding and functional mechanisms used by sol-Eng to affect cellular responses have to be analyzed in more detail in the future. There are currently no data focussing on functional aspects of sol-Eng, especially in the liver.

ENDOGLIN IN DISEASE

As outlined above, mutations that affect human endoglin function are inherited as autosomal dominant disorders and may cause AVM in different organs, including brain, lung and liver (Figure 6). In the following paragraphs we will highlight the pathogenesis of several of these disorders and associated diseases and give an overview about the important role of endoglin dysfunction in the pathology of liver fibrosis.

Hereditary hemorrhagic telangiectasia

Hereditary hemorrhagic telangiectasia (HHT, Osler-Weber-Rendu syndrome) is an autosomal dominant inherited vascular disorder with a variety of clinical manifestations. Common symptoms of this disease occur due to the forming of AVM in small and large blood vessels. This leads to epistaxis, gastrointestinal bleeding and microcytic anemia due to iron deficiency, along with characteristic mucocutaneous telangiectasia[113]. AVM are found in pulmonary, hepatic and cerebral vascular tissue (Figure 6). The diagnosis of HHT is based on these clinical features, which are summarized in consensus criteria known as the "Curaçao criteria"[114]. Rupture of AVM contributes to significant morbidity.

Mutations in at least five genes result in manifestation of hereditary hemorrhagic telangiectasia. However, about 85% of the cases develop due to mutations of the *ENG* gene (coding for endoglin) and *ACVRL1* (activin A receptor type Ⅱ-like 1 kinase 1, ALK1)[115]. This disease is usually autosomal dominantly inherited, varying in penetrance and expression. Juvenile Polyposis/Hereditary Hemorrhagic Telangiectasia (JPHT) is a rare juvenile form of HHT which is associated with polyposis and occurs due to mutations in the MADH4 gene coding for Smad4[116]. In gene linkage analyses, two other loci have been shown to be in a disequilibrium with HHT symptoms; one on chromosome 5, defining HHT-3[35], the other on chromosome 7[13], defining HHT-4. However specific genes on these chromosomes involved in disease formation remain to be identified. Mice deficient for endoglin or ALK1 expression show clinical features of HHT[117]. Eng knockout (null) mice are embryonically lethal, dying at day 10.5 p.c. due to impaired extraembryonic vascular development and several cardiac defects (see below). Heterozygous animals show clinical symptoms of HHT-1 with variable penetrance. Human patients with HHT-1 exhibit less endoglin expression in peripheral blood monocytes and newborn umbilical vein endothelial cells[118].

To prevent fatal clinical events like stroke, high-output heart failure, pulmonary hypertension and hemorrhage, the embolization of visceral AVM is a valuable course

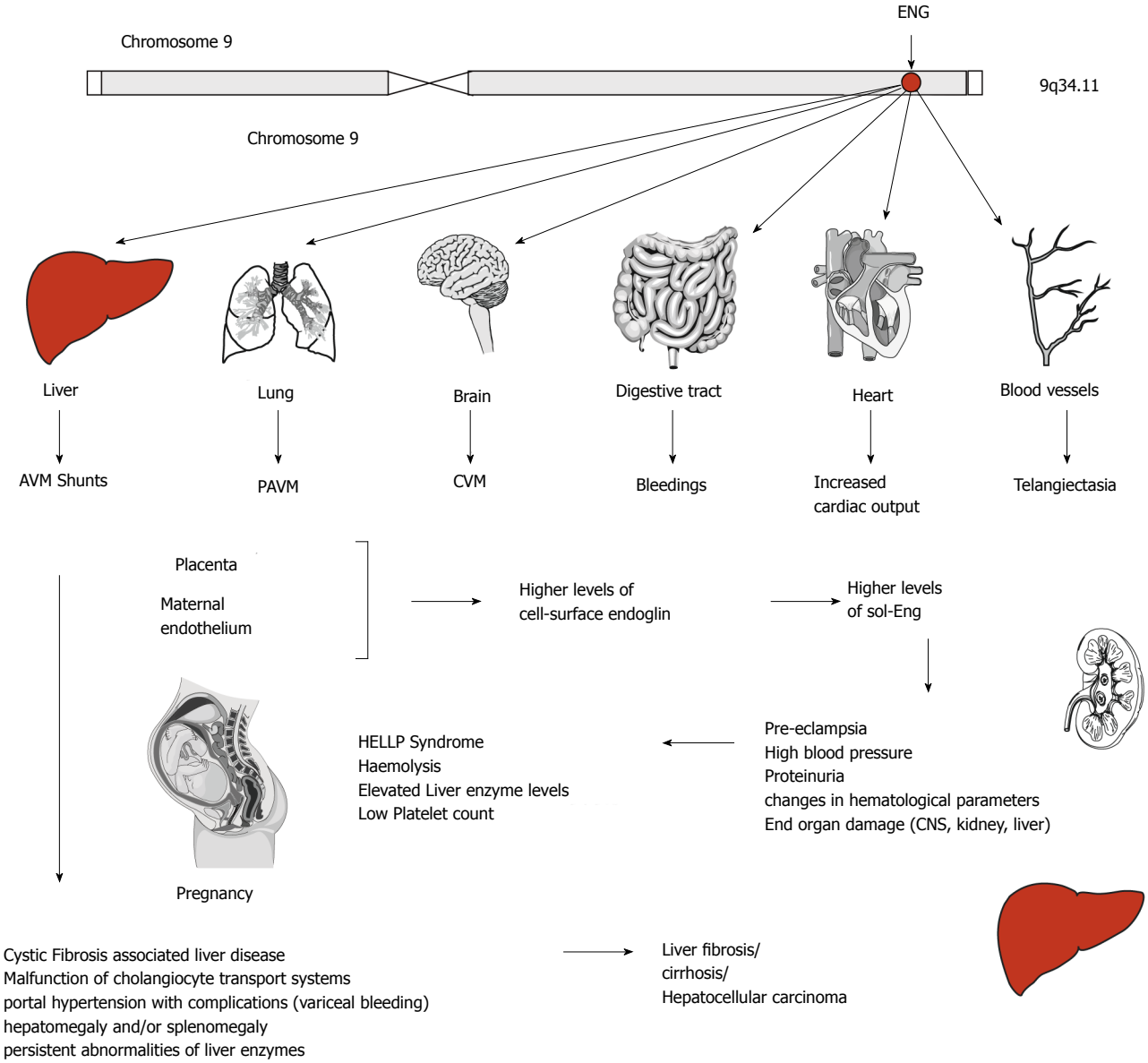

Figure 6 Endoglin and disease. The human endoglin gene (ENG) is located on the long arm of human chromosome 9. Mutations are inherited in an autosomal dominant manner and affect several organs. In liver, abnormal connection formed between blood vessels, arteriovenous malformations (AVM), malfunction of the cholangiocyte transport system gives rise to liver damage indicated by portal hypertension, persistent abnormalities of liver enzymes, hepatomegaly and/or splenomegaly, fibrosis, cirrhosis or even hepatocellular carcinoma. Intrahepatic connection between arteries and veins results in a large amount of blood bypasses for which the heart compensates by increasing the cardiac output resulting on long term in heart insufficiency. Similar arteriovenous (pulmonary AVM, cerebral AVM) are found in lung and brain. In the digestive tract bleedings occur and telangiectasias of blood vessels are found on the skin of the hands, face and mouth. During pregnancy, the placenta and the maternal endothelium produce higher levels of cell-surface endoglin that is shedded and leads to higher systemic concentration of soluble endoglin (sol-Eng) that leads to an imbalance of the antiangiogenic factors resulting in life-threatening obstetric complication (*e.g.*, pre-eclampsia, HELLP syndrome).

of treatment. Furthermore, symptomatical treatment approaches with antiangiogenic or antihormonal agents have been investigated. In some patients, the use of antiangiogenic therapies known from cancer therapy, such as thalidomide[119], lenalidomide[120] and bevacizumab[121], reduces the incidence of nasal and gastrointestinal bleeding. The β-receptor blocker propanolol, usually used for prophylaxis of esophageal variceal bleeding in patients with liver cirrhosis or the treatment in infantile haemangiomas, was able to decrease cellular migration and tube formation, concomitantly with reduced RNA and protein levels of ENG and ALK1 in cell culture[122]. Other studies showed that tamoxifen, an estrogen receptor antagonist, and the selective estrogen receptor modulator, raloxifene, can reduce episodes of epistaxis and transfusion requirements in patients suffering from nasal vascular malformations[123,124]. However, limited controlled studies, severe side effects of those drugs and the need for life long treatment limits the applicability for most patients.

Pre-eclampsia

Pre-eclampsia is a disease of high incidence (about 3%) in pregnant women with an onset after 20 wk of gestation. It complicates pregnancy and can lead to death of

mother and baby. The disease is characterized by new-onset hypertension (140 mmHg or diastolic blood pressure 90 mmHg) and proteinuria (excess of protein in the urine of at least 0.3 g of protein/d)[125]. Eclampsia is characterized by additionally occurring grand mal seizures[126]. Typical complications for the pregnant woman are the involvement of the central nervous system, acute renal or liver failure, and changes in hematological parameters. Women with pre-eclampsia are prone to higher lifetime cardiovascular morbidity, including hypertension and ischemic heart disease. Effects on the fetus can be severe and include prematurity, fetal growth restriction, oligohydramnios and placental abruption. A family history of pre-eclampsia, advanced maternal age, obesity or pregestational diabetes increases the mothers risk to develop this condition[127].

The pathophysiology of pre-eclampsia is still poorly understood. Prior to the development of clinical symptoms, cells migrating to the placenta lack the expression of endothelial surface adhesion markers. This leads to incomplete invasion of maternal arteries by the developing trophoblast, resulting in placental ischemia and the release of antiangiogenic factors, including sol-Eng and soluble fms-like tyrosine kinase (sFlt1)[128]. Vascular endothelial growth factor (VEGF) and placental growth factor are antagonized by soluble fms-like tyrosine kinase-1 (sFlt1 or sVEGFR-1) and sol-Eng antagonizes TGF-β1 and TGF-β3 activity[129]. These effects on vascular homeostasis promote changes in placental circulation. Numerous studies show the effect of VEGF and TGF-β signaling pathways on circulation and angiogenesis. These pathways directly influence the development of pre-eclampsia. By regulating endothelial cell proliferation, migration, vascular permeability and secretion, VEGF-A is an important ligand for angiogenesis. It binds to two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1). The soluble receptor VEGFR-1 (sFlt1) acts as an endogenous VEGF inhibitor. In patients with pre-eclampsia, sFlt1 is overexpressed in the maternal circulation[130], which corresponds to a decrease of VEGF and placental growth factor expression in the placenta of pre-eclampsia patients[131]. This leads to the development of major symptoms of the disease due to abnormal trapping of VEGFs. The role of sFlt1 is underlined by studies in which pregnant rats were treated with exogenous sFlt1, inducing severe pre-eclampsia. Immunoprecipitation of sFlt1 in cells derived from placental villous explants normalized their angiogenic responses[129].

In addition, the VEGF-signaling changes in the TGF-β signal transduction pathway promotes the development of pre-eclampsia. Placentas of pre-eclamptic women show increased levels of membrane-bound Eng and sol-Eng[14]. Hypoxia and oxidative stress seem to be important triggers for the release of sol-Eng, as shown in a study where oxysterol activation promoted MMP-14-mediated cleavage of sol-Eng in cells of trophoblast origin[132]. sol-Eng antagonizes TGF-β1 induced vasodilatation, leading to vascular hypertension[133-135]. The increase of systemic sol-Eng in pregnant women is a factor that prequels the onset of pre-eclampsia[106,136,137]. Modulating the TGF-β pathway, endoglin can, alone or together with sFlt1, induce pre-eclamsia symptoms in pregnant rats[14].

The pathogenesis of pre-eclampsia is defined by the imbalance of the anti-angiogenic factors, sFlt1 and sol-Eng, and the proangiogenic factors, placental growth factor, TGF-β and VEGF[138]. Current treatment concepts therefore include the use of antibodies and small molecules to sequester or limit synthesis of anti-angiogenic molecules. Improvement in blood pressure and renal function could be achieved after administration of exogenous VEGF in a preclinical model of pre-eclampsia, modulating the balance of angio- and anti-angiogenic factors[139]. Recently, a study using a dextran sulfate column to remove sFlt1 from the maternal circulation by extracorporeal apheresis showed a potential therapeutic approach for the treatment of pre-eclampsia[140]. Other studies using induction of hemoxygenase-1 with cobalt protoporphyrin in pre-eclamptic rats[141] and prevention of the release of sol-Eng by direct inhibition of MMP-14 showed promising results[142]. As mentioned before, any therapeutic approach must be safe for mother and fetus and should be evaluated by controlled studies. Currently these problems still limit any effective therapy.

HELLP Syndrome

The HELLP syndrome is a complex of maternal symptoms in pregnancy, including hemolysis, elevated liver enzymes and low platelet count. HELLP syndrome occurs in 0.2%-0.8% of pregnancies and is a serious threat for mother and child. 70%-80% of women expressing HELLP symptoms also suffer from pre-eclampsia[143]. As in pre-eclampsia, a previous HELLP pregnancy increases the risk of HELLP as well as pre-eclampsia in subsequent pregnancies, suggesting related pathogenetics. Anti-angiogenic factors play an important role in both symptom complexes. In comparison to pre-eclampsia, maternal blood levels of anti-angiogenic sFlt1 are similar, but HELLP shows higher sol-Eng levels[144]. The pathogenesis of symptoms defining HELLP is driven by those angiopathogenic mechanisms. Activated vascular endothelium leads to an inflammatory response, including coagulation and complement activation, increased white blood count and elevated levels of inflammatory cytokines such as TNF-α and von Willebrand factor, leading to clinical symptoms of disseminated coagulation in microvessels[144,145]. Activation of these inflammatory signaling cascades leads to hemolysis in response to microangiopathy, reduced liver blood flow with elevated liver enzymes and low platelet counts due to consumption of platelets by microvessel thrombosis (= HELLP).

Cystic fibrosis associated liver disease

Cystic fibrosis (CF, mucoviscidosis) is an autosomal recessive genetic disorder affecting lungs, pancreas, liver and intestine. A mutation in the gene for the protein

cystic fibrosis transmembrane conductance regulator (CFTR) causes an abnormal transport of chloride and sodium across an epithelium, resulting in viscous secretions[146]. The most severe symptoms affect the lungs, often causing lung transplantation or death in those patients. Gastrointestinal symptoms due to thick mucus are common[147] and cystic fibrosis associated liver disease (CFLD) is often (30%) diagnosed, accounting for 2.5% of overall mortality, representing the third most common cause of death in these patients[148].

Rath *et al*[28] showed in a recent study that patients suffering of CFLD show elevated serum levels of TIMP-4 and endoglin. Expression levels correlate with hepatic staging, therefore allowing, together with transient elastography, to increase the sensitivity for the non-invasive diagnosis of CFLD in patients suffering from CF. High endoglin levels showed a significant association with the severity of liver injury, suggesting an active role for endoglin in the pathology of liver fibrosis.

Endoglin in liver fibrosis and HCC

Liver fibrosis and cirrhosis is the outcome of most types of chronic liver injury. The excessive accumulation of extracellular matrix (ECM) proteins promotes hepatic scarring and eventually leads to organ failure[149]. In the pathogenesis of liver fibrosis, TGF-β is the most potent fibrogenic cytokine. It induces fibrosis through multiple mechanisms, including direct activation of HSC, stimulation of ECM production, as well as prompting the synthesis of tissue inhibitors of matrixmetalloproteases (TIMPs) and thereby inhibiting ECM degradation[150]. Knock-out mice with deletions in components of the TGF-β signaling cascade (TGF-β1, SMAD3 and MMP13) develop less severe fibrosis[151]. TGF-β ligands and receptors form a complex signaling network, which can be modulated by endoglin and betaglycan (TGF-β type Ⅲ receptor). By inhibiting ALK5-Smad2/3 and promoting ALK1-Smad1/5 signaling, endoglin can shift TGF-β downstream signals to pro-fibrogenic effects[40]. Presently, there is not much knowledge how the expression of the different endoglin isoforms and sol-Eng is regulated in diverse liver cell subpopulations but it was reported that the concentration of sol-Eng increases during hepatic fibrogenesis (see below). In previous studies, we could show that endoglin expression is increased in activated HSC *in vitro* and in murine models of liver injury (carbon tetrachloride application and bile duct ligation) *in vivo*[41]. HSC are the major source for ECM production in liver fibrosis. Endoglin overexpression leads to enhanced TGF-β-driven Smad1/5 phosphorylation and α-smooth muscle actin expression without affecting Smad2/3 signaling in these cells. By shifting TGF-β signaling from ALK5-Smad2/3 to ALK1-Smad1/5 pathway, endoglin exceeds a central role in TGF-β signal modulation and the development of liver fibrosis.

HCC develops most often (80%) in cirrhotic livers. Angiogenesis and irregular capillary distribution are a key feature for malignant lesions[152]. Blood vessels are needed to supply nutrients and oxygen to the growing tumors. Most malignant tumors as well as HCCs have developed efficient strategies to promote fast vessel growth. Angiogenesis is a highly regulated, complex process modulated by many intersecting pathways, including vascular endothelial growth factor (VEGF), TGF-β and endoglin[26], angiopoietins[153], Notch[154] and integrins[155]. Usually, pro-angiogenic and anti-angiogenic factors are tightly balanced. In contrast to physiological angiogenesis (*i.e.,* in wound healing), tumor angiogenesis is not controlled by normal physiological inhibition, resulting in an imbalance of pro-angiogenic and anti-angiogenic factors. By modulating TGF-β signaling, endoglin plays a crucial role in angiogenesis and tumor growth and could be linked to HCC[108], as well as esophageal cancer[156], breast carcinoma[157], colorectal cancer[158] and tumor angiogenesis[44].

EXPRESSION OF ENDOGLIN IN ISOLATED LIVER CELLS AND LIVER TISSUE

Endoglin expression has been studied in many different tissues and diseases. It is highly expressed on proliferating vascular endothelial cells[159,160]. However, Meurer *et al*[40,41] showed that endoglin is expressed on HSC and activated MFB as well. By molecular cloning of endoglin cDNA, surface labeling, immunoprecipitation and immunocytochemistry experiments, it could be shown that endoglin plays a significant role in liver injury and fibrosis development[40,41]. Endoglin expression is differentially regulated at the plasma membrane of HSCs and in activated myofibroblasts (MFB)[40,41]. Endoglin expression is increased in transdifferentiating HSC and in two models of liver fibrosis but not in hepatocytes. Furthermore, endoglin is expressed in cultured portal fibroblasts, representing another important fibrogenic cell type in biliary types of liver disease. Transient overexpression of endoglin leads to significantly increased TGF-β1-driven Smad1/5 phosphorylation and α-smooth muscle actin expression, while Smad2 phosphorylation is not changed[40]. These results are in line with a study by Lebrin *et al*[88] which showed endoglin promoting TGF-β1/ALK1-Smad1/5 signaling in endothelial cells.

To further investigate the influence of endoglin on TGF-β signal transduction, we recently established and characterized a new mouse HSC line expressing collagen 1(I) promoter/enhancer driven green fluorescent protein (GFP). These cells, originating from quiescent HSC, show an activated MFB phenotype in culture and express low endogenous endoglin concentrations. By selective overexpression of endoglin in these cells, stimulation with TGF-β and PDGF, and specific inhibition of endoglin/ALK signaling with antagonists, the differential effect of endoglin on downstream Smad-signaling could be shown[77].

Because of the complexity of endoglin and TGF-β signaling pathways, it is important to investigate the modulation of TGF-β signal transduction in cells of different origin. For example, Velasco *et al*[87] showed the differ-

ential effects of endoglin isoforms in L6E9 myoblasts[85]. Because these cells have no endogenous endoglin expression, this cell line is an ideal tool to selectively express specific isoforms of endoglin and show a different and sometimes opposing effect of L- and S-Eng isoforms on downstream regulation of TGF-β-induced responses. While endoglin expression is well investigated in vascular endothelial cells, HHT and tumor angiogenesis, the role of endoglin in liver disease is poorly understood. Liver cell lines overexpressing endoglin or single members of the TGF-β pathway, as well as cells with low endogenous endoglin expression and specifically induced endoglin expression are needed to further dissect the functional roles of endoglin in liver injury and fibrosis.

ANIMAL MODELS IN UNDERSTANDING ENDOGLIN FUNCTION

Endoglin deficiency in humans has a strong phenotype and is responsible for many diseases, such as HHT, preeclampsia liver fibrosis and cancer. To study its impact on the pathogenesis of those diseases, murine endoglin knockout models were needed. Because a complete homozygous endoglin knockout is embryonically lethal, several alternative strategies were established. Endoglin plays an important role in angiogenesis; a complete endoglin deficiency has fatal consequences in the development of heart and major vessels. To study the role of endoglin *in vivo* and its impact on HHT-1, Arthur *et al*[161] established a mouse carrying a targeted nonsense mutation (deletion of exons 9-11) in the endoglin gene. These mice already showed that endoglin expression is critical for early vascular development. Embryos with two mutated endoglin genes die at day 10 - 10.5 post coitum (dpc) due to cardiac malformations and a failure to form mature blood vessels in the yolk sac. Homozygous endoglin knockout embryos generated by a deletion of 609 bp including exon 1 show a similar phenotype as mice lacking TGF-β1 and the TGF-β receptor Ⅱ, suggesting that endoglin plays a crucial role in TGF-β signaling in early vascular development[162,163]. Li and co-workers reported that mice lacking functionally active endoglin by replacing the first two exons die from defective vascular development but do not show defective vasculogenesis, which is observed in mice lacking TGF-β1[163]. Loss of endoglin caused poor vascular smooth muscle cell (vSMC) development and arrested endothelial remodelling. Therefore, endoglin is required for the differential growth and sprouting of endothelial tubes and recruitment and differentiation of mesenchymal cells into vSMC and pericytes[164]. Both studies show slight differences in vascular embryonic development. Eng deficient mice generated by Li *et al*[163] die at day 11.5 dpc. While Arthur *et al*[161] used embryonic stem cells of 129/Ola origin, Li *et al*[163] generated endoglin knockout mice by targeting embryonic stem cells from 129/SVJ background. Those different approaches already suggest a strong impact of genetic background on murine models of Eng deficiency.

To overcome the problems of embryonic lethality and to study the effect of endoglin in disease, several groups have used alternative approaches to generate endoglin deficient mice. Allinson *et al*[164] for example generated a mouse in which the endoglin gene is flanked by loxP sites at exons 5 and 6. These mice show a normal phenotype comparable to wild type littermates. Using the *Cre*-loxP genetic recombination system and an appropriate *Cre* expressing mouse line, specific endoglin knockout mice can be created. To generate a null allele of the endoglin gene, the floxed construct was designed to allow a conditional deletion of exons 5 and 6, which would also lead to frameshift mutation in exon 7 before reaching a stop codon, resulting in a functional inactive endoglin[164].

Using this approach, two mouse models were generated expressing Cre in smooth muscle (SM22αcre) and endothelial cells (Tie2cre) to evaluate the role of endoglin in vascular smooth muscle and endothelial cells during angiogenesis[165]. In this study, endoglin null embryos show ectopic arterial expression of the venous specific marker COUPTF Ⅱ (chicken ovalbumin upstream promoter transcription factor Ⅱ). Normal expression of COUPTF Ⅱ was restored after endoglin re-expression in endothelial cells. COUPTF Ⅱ plays an important role in vascular development, including heart, blood vessels and smooth muscle cell differentiation. Endoglin induces changes in COUPTF Ⅱ expression patterns and therefore can influence vSMC recruitment and differentiation in angiogenesis.

Other groups used heterozygous endoglin knockout mice to investigate the function of endoglin and avoid embryonic lethality. Bourdeau *et al*[166] developed a mouse model with a single copy of the endoglin gene and another mouse line with a homozygous deletion of the endoglin gene. As already observed by Arthur *et al*[161], mice lacking any functional endoglin die at day 10.0-10.5 dpc due to defects in vessel and heart development. Embryos show a normal angiogenesis and vessel formation until hemorrhage occurs in the yolk sac around 9.0-10.5 dpc. Heart development stopped at day 9.0 and the atrioventricular canal endocardium did not undergo mesenchymal transformation and cushion-tissue formation. Similar to the study published by Arthur *et al*[161], Bourdeau *et al*[166] used 129/Ola origin on C57BL/6 background. The heterozygous mouse displays a multiorgan vascular phenotype similar to the human HHT, which is often caused by endoglin haploinsufficiency. To evaluate the impact of the genetic background on endoglin deficiency, different Eng/null mouse strains were generated. The 129/Ola strain developed HHT symptoms at an earlier age and with greater severity than C57BL/6 mice. The F2 strain intercrosses between both strains showed an intermediate phenotype. As in humans, Eng deficiency shows variable penetrance. Of 171 mice observed in this study over a 12 mo period, 50 developed clinical signs of HHT. Disease prevalence was high in the 129/Ola strain (72%), intermediate in the intercrosses (36%), and low in C57BL/6 backcrosses (7%)[166].

Using the heterozygous Eng null mouse generated by Bordeau *et al*[166], another study showed that endoglin is

required for paracrine TGF-β signaling between endothelial cells and adjacent smooth muscle cells to promote smooth muscle cell differentiation[167].

In primary cultures of endothelial cells generated from mice carrying only one functional Eng allele, a significantly reduced migration and proliferation along with increased collagen production, vascular endothelial growth factor (VEGF) secretion and decreased NO synthase expression was observed[168]. This again highlights the important role of endoglin in vascular pathology.

As outlined above, endoglin modulates both the ALK1 and ALK5 pathways. Park *et al*[169] generated an ALK1 conditional knockout mouse line. The specific deletion of ALK1 in vascular endothelial cells by an endothelial specific Cre was lethal through massive hemorrhage in the lungs. ALK1 deficient mice showed heavy pulmonary vascular malformations mimicking all pathological features of HHT-2, such as dilation of vessel lumen, thinning of vascular walls, loss of capillaries, development of excessive tortuous vessels, and AVM[169].

Dolinsec *et al*[170] used another approach to investigate endoglin deficiency in murine models without affecting embryonical vascular development[170]. By applying siRNA against endoglin to human and murine endothelial cells (HMEC-1, 2H11) *in vitro* and in TS/A mammary adenocarcinoma growing in BALB/c mice, they evaluated the therapeutic potential of siRNA in cancer treatment. *In vitro*, the transfection resulted in reduced levels of endoglin mRNA and protein, leading to a 60% decrease of endothelial cell proliferation. *In vivo* silencing of endoglin expression showed lower endoglin mRNA levels and a decreased number of tumor blood vessels resulting in significantly reduced TS/A tumor growth. The study demonstrated that siRNA molecules against endoglin have a good anti-angiogenic therapeutic potential[171].

The endoglin gene gives rise to two different isoforms resulting from differential splicing, *i.e.,* S- and L-Eng (for details see above). Pérez-Gómez *et al*[43] investigated the role of S-Eng *in vivo* using a mouse with ICAM-2 driven overexpression of human S-Eng on the vascular endothelium. Interestingly, breeding these mice to endoglin deficient mice did not rescue the embryogenic lethal phenotype. Furthermore, this study investigates the impact of S-Eng on carcinogenesis. Therefore, Lewis lung carcinoma cells were transplanted into mice expressing S-Eng. Carcinoma cells in these mice showed reduced tumor growth and less neovascularization. Additionally, benign papilloma formation was reduced significantly in respective S-Eng positive mice. These results show that S-Eng has anti-angiogenic properties in cancer development, showing new potential approaches for tumor therapy[43].

DIAGNOSTIC VALUE OF ENDOGLIN IN LIVER-ASSOCIATED DISEASES

Genetic testing

HHT is phenotypically heterogeneous both between affected families and amongst members of the same family in regard to penetrance and age of disease onset. There are hundreds of different mutations in the human *ENG* gene known that affect proper gene function. Although HHT is most common in Caucasians, disease causing mutations with ethnic-related differences also occur in Asians, Africans and Middle Eastern[171]. The overall incidence of HHT in North America is more frequent than initially estimated and ranges between 1:5000 and 1:10000[172], while the frequency in Europe varies between 1:2500 to 1:40000[173-175]. In a cohort of the northern part of Japan, the prevalence of HHT in the population was estimated to be 1:8000[176], demonstrating that HHT is more common among Asians than often assumed.

HHT is a dominantly inherited autosomal disorder and genetic testing of individuals with a known family history is generally performed for disease confirmation (Figure 7). In addition, pre-symptomatic screening of relatives of patients with a positive molecular diagnosis and in patients with suggestive (but not confirmatory) clinical features of HHT is well established[177].

At the molecular level, there is a large spectrum of different gene mutations that influence the expression, integrity and stability of the endoglin protein. Missense (nonsynonymous) mutations introducing different aa, nonsense mutations introducing premature stop codons, splice-site mutations that affect consensus splice donor sites and provoke exon skipping, frame shift and in frame deletions resulting in proteins with markedly different sizes, and several intronic mutations are rather common and show an ethnic and regional distribution[7-10,178-180]. However, the penetrance of the different mutations and gene variations are rather different and subtle genotype-phenotype correlations in HHT-1 have been reported, revealing that truncating mutations in *ENG* are associated with more affected organs and more severe hemorrhage than *ENG* missense mutations[13]. Pulse-chase experimentation and overexpression studies have further shown that several endoglin gene mutations form proteins that are only barely detectable, do not form heterodimers with normal endoglin, and are further unable to interfere with endoglin trafficking to the cell surface and remain intracellular as a precursor form[12,181]. On the contrary, another study that investigated six different missense and two truncation mutations have shown that not all mutants are unable to dimerize with normal endoglin, suggesting that haploinsufficiency and dominant-negative protein interactions both can cause HHT-1[12,182]. No homozygotes that carry two abnormal copies of the *ENG* gene have been reported so far, suggesting that this constellation is not compatible with life[183]. Likewise, mice lacking both copies of the *ENG* gene die at gestational day 10.0-10.5 due to defects in vessel and heart development[161].

However, there are four other genetic types of HHT identified that are not associated with alterations in the *ENG* gene. It is essential to know that there are likely to be differences in the normal requirements for the individual disease-causing genes in different vascular beds and cell types that, when affected by mutation, result in somewhat diverse clinical features and symptoms[183]. The onset of epistaxis for example was found to have

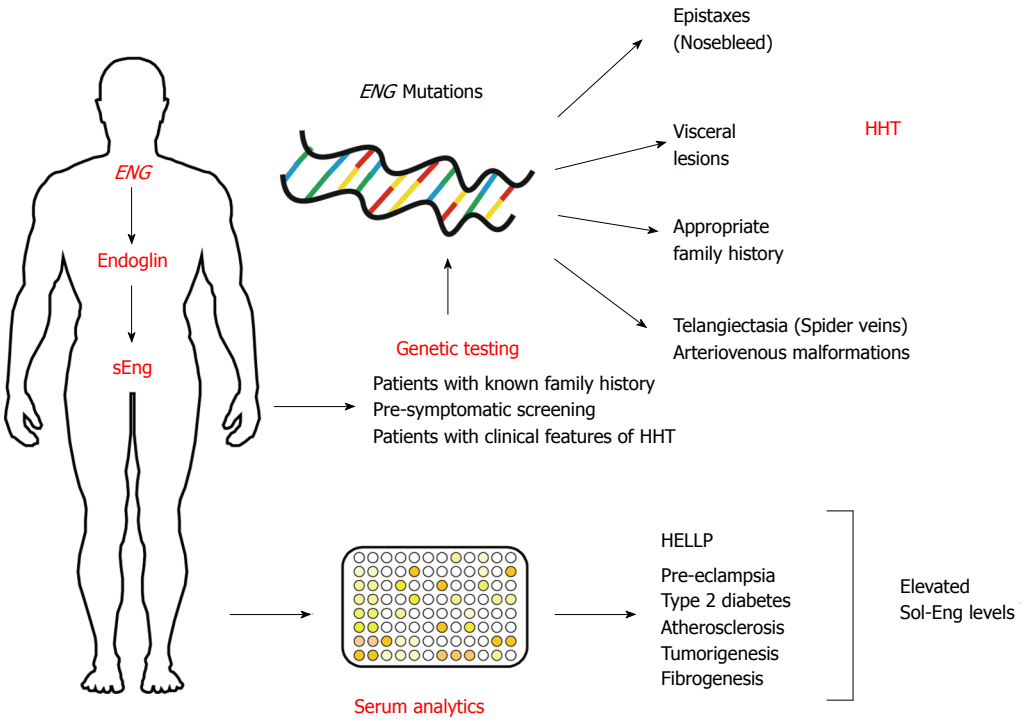

Figure 7 Endoglin in diagnostics. Several distinct mutations in the endoglin gene (ENG) give rise to hereditary hemorrhagic telangiectasia (HHT) that is mainly characterized by epistaxes (nosebleed), various visceral lesions, telangiectasia (spider veins) and arteriovenous malformations. Patients often show an appropriate family history. The clinical diagnosis "HHT" is made if three of the four classical signs (*i.e.*, epistaxes, visceral lesions, telangiectasia and family history) occur. Elevated levels of soluble endoglin have been reported in patients suffering from hemolysis, elevated liver enzymes and low platelets syndrome (HELLP), pre-eclampsia, type 2 diabetes, atherosclerosis, tumorigenesis in several organs, and fibrogenesis.

an earlier onset in patients with HHT-1 than those with HHT-2 and AVM of the brain and lungs were more common in respective patients, while hepatic and spinal AVM were noticed at a lower frequency in patients with HHT-2[13,178,184,185]. Based on all these findings, several guidelines were proposed in which the *ENG* gene should be first targeted for mutational screening when large visceral AVM in the lungs in patients younger than 45 years occur[185]. However, based on the fact that all 15 exons and their non-coding introns can be easily sequenced, it is self-evident that these molecular diagnostic tests have refined and supplemented the criteria that were first proposed for clinical diagnosis of HHT[114].

Serum measurements

Based on the finding that the serum or plasma concentration of sol-Eng is increased dramatically in several disease conditions, its predictive value for the outcome of various diseases is presently intensively discussed and a large variety of commercially available ELISA test systems that allow reliable and accurate detection of endoglin in biological fluids have been established by many companies. It was shown that serum sol-Eng that plays a major role as an anti-angiogenic factor increases two- and three-fold in preterm and term pregnancy compared to non-pregnant controls and further dramatically increases two to three months before the onset of pre-eclampsia and in patients with HELLP syndrome, suggesting that sol-Eng alone or in combination with other variables is usable as a biomarker with a high predictive value in pregnancy complications[14,106,186,187]. Other studies demonstrated that plasma sol-Eng levels are significant higher in patients with diabetes than in healthy control subjects and that the duration of diabetes is an independent predictor of plasma sol-Eng increase[17]. The measurement of sol-Eng also has predictive value for the progression of the atherosclerotic process and correlates well with the expression of eNOS in endothelium, repair of the vessel wall, plaque neoangiogenesis, production of collagen and stabilization of atherosclerotic lesions[19]. As an indicator of endothelial dysfunction, the measurement of sol-Eng was proposed to monitor the therapy efficacy during extracorporeal LDL-cholesterol elimination therapy for familial hypercholesterolemia[18]. Since endoglin expression was shown to be extremely relevant for cancer formation[159], it is not surprising that sol-Eng is a potential angiogenic marker to indicate and predict diseases associated with metastases[32,188-190]. Patients suffering from Alzheimer's disease were also found to have elevated levels of sol-Eng combined with decreased levels of TGF-β, possibly indicating impairment of cerebral circulation that is associated with this neurodegenerative process[24]. Of course, the wide expression pattern of endoglin that encompasses endothelial cells, subsets of bone marrow cells, activated macrophages, fibroblasts, chondrocytes, smooth muscle cells and pro-fibrogenic cells (*e.g.*, HSC) as well as its linkage with the TGF-β signaling pathways has further offered several new avenues in which sol-Eng

measurements might be beneficial. In regards to liver, it is well established that intrahepatic and circulating levels of endoglin are elevated in patients suffering from chronic hepatitis C infection, liver cirrhosis and carcinoma. In addition, there is a correlation of histological and serum markers of hepatic fibrosis and endoglin is abundantly expressed in hepatic sinusoidal endothelium of non-tumor tissues with cirrhosis[108,191,192]. Increased endoglin expression was recently also documented by proteomic profiling in patients suffering from cystic fibrosis associated liver disease[28]. Likewise, high circulating endoglin concentrations are correlated with a poor outcome for biliary atresia that represents a chronic progressive disorder of the extrahepatic and intrahepatic biliary system[27]. Therefore, there is no doubt that these measurements enrich the panel of available diagnostic options to identify proliferative disorders, including organ diseases that are associated with fibrogenesis.

CONCLUSION

Endoglin is found on many cell surfaces and plays a crucial role in TGF-β signaling. It forms homodimers and consists of a large extracellular domain, a hydrophobic transmembrane domain and a short cytoplasmic tail. This receptor binds to a large variety of extra- and intracellular binding partners and modulates numerous cellular properties, including morphology, migration, endocytic vesicular transport, microtubular structures and functionality of focal adhesion proteins. Several hundred independent *ENG* gene mutations result in HHT that is associated with various vascular lesions, mainly on the face, lips, hands and gastrointestinal mucosa. Recent work has demonstrated that endoglin expression is also altered during ongoing hepatic fibrogenesis. The unravelling of the underlying pathways that are associated with alterations in endoglin expression will be of fundamental interest, not only for establishment of potential new therapeutic options for HHT treatment, but might allow re-establishing the activities of Smad2/3 and Smad1/5/8 that are both part of TGF-β homeostasis and pathologically altered in ongoing and established organ fibrosis.

REFERENCES

1 **Gougos A**, Letarte M. Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line. *J Immunol* 1988; **141**: 1925-1933 [PMID: 3262644]

2 **Gougos A**, Letarte M. Primary structure of endoglin, an RGD-containing glycoprotein of human endothelial cells. *J Biol Chem* 1990; **265**: 8361-8364 [PMID: 1692830]

3 **Bellón T**, Corbí A, Lastres P, Calés C, Cebrián M, Vera S, Cheifetz S, Massague J, Letarte M, Bernabéu C. Identification and expression of two forms of the human transforming growth factor-beta-binding protein endoglin with distinct cytoplasmic regions. *Eur J Immunol* 1993; **23**: 2340-2345 [PMID: 8370410 DOI: 10.1002/eji.1830230943]

4 **Fernández-Ruiz E**, St-Jacques S, Bellón T, Letarte M, Bernabéu C. Assignment of the human endoglin gene (END) to 9q34--& gt; qter. *Cytogenet Cell Genet* 1993; **64**: 204-207 [PMID: 8404038 DOI: 0.1159/000133576]

5 **Pilz A**, Woodward K, Povey S, Abbott C. Comparative mapping of 50 human chromosome 9 loci in the laboratory mouse. *Genomics* 1995; **25**: 139-149 [PMID: 7774911]

6 **Qureshi ST**, Gros P, Letarte M, Malo D. The murine endoglin gene (Eng) maps to chromosome 2. *Genomics* 1995; **26**: 165-166 [PMID: 7782079 DOI: 10.1016/0888-7543(95)80099-8]

7 **McAllister KA**, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, Jackson CE, Helmbold EA, Markel DS, McKinnon WC, Murrell J. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet* 1994; **8**: 345-351 [PMID: 7894484 DOI: 10.1038/ng1294-345]

8 **Lesca G**, Plauchu H, Coulet F, Lefebvre S, Plessis G, Odent S, Rivière S, Leheup B, Goizet C, Carette MF, Cordier JF, Pinson S, Soubrier F, Calender A, Giraud S. Molecular screening of ALK1/ACVRL1 and ENG genes in hereditary hemorrhagic telangiectasia in France. *Hum Mutat* 2004; **23**: 289-299 [PMID: 15024723 DOI: 10.1007/s004390050008]

9 **Gallione CJ**, Scheessele EA, Reinhardt D, Duits AJ, Berg JN, Westermann CJ, Marchuk DA. Two common endoglin mutations in families with hereditary hemorrhagic telangiectasia in the Netherlands Antilles: evidence for a founder effect. Hum. *Genet* 2000; 107:40-44 [PMID: 10982033]

10 **Cymerman U**, Vera S, Karabegovic A, Abdalla S, Letarte M. Characterization of 17 novel endoglin mutations associated with hereditary hemorrhagic telangiectasia. *Hum Mutat* 2003; **21**: 482-492 [PMID: 12673790 DOI: 10.1002/humu.10203]

11 **Pece N**, Vera S, Cymerman U, White RI, Wrana JL, Letarte M. Mutant endoglin in hereditary hemorrhagic telangiectasia type 1 is transiently expressed intracellularly and is not a dominant negative. *J Clin Invest* 1997; **100**: 2568-2579 [PMID: 9366572 DOI: 10.1172/JCI119800]

12 **Pece-Barbara N**, Cymerman U, Vera S, Marchuk DA, Letarte M. Expression analysis of four endoglin missense mutations suggests that haploinsufficiency is the predominant mechanism for hereditary hemorrhagic telangiectasia type 1. *Hum Mol Genet* 1999; **8**: 2171-2181 [PMID: 10545596 DOI: 10.1093/hmg/8.12.2171]

13 **Bayrak-Toydemir P**, McDonald J, Markewitz B, Lewin S, Miller F, Chou LS, Gedge F, Tang W, Coon H, Mao R. Genotype-phenotype correlation in hereditary hemorrhagic telangiectasia: mutations and manifestations. *Am J Med Genet A* 2006; **140**: 463-470 [PMID: 16470787 DOI: 10.1002/ajmg.a.31101]

14 **Venkatesha S**, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA, Stillman IE, Roberts D, D'Amore PA, Epstein FH, Sellke FW, Romero R, Sukhatme VP, Letarte M, Karumanchi SA. Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* 2006; **12**: 642-649 [PMID: 16751767 DOI: 10.1038/nm1429]

15 **Luft FC**. Soluble endoglin (sEng) joins the soluble fms-like tyrosine kinase (sFlt) receptor as a pre-eclampsia molecule. *Nephrol Dial Transplant* 2006; **21**: 3052-3054 [PMID: 16870672 DOI: 10.1093/ndt/gfl439]

16 **Chen Y**, Hao Q, Kim H, Su H, Letarte M, Karumanchi SA, Lawton MT, Barbaro NM, Yang GY, Young WL. Soluble endoglin modulates aberrant cerebral vascular remodeling. *Ann Neurol* 2009; **66**: 19-27 [PMID: 19670444 DOI: 10.1002/ana.21710]

17 **Motawi TK**, Rizk SM, Ibrahim IA, El-Emady YF. Alterations in circulating angiogenic and anti-angiogenic factors in type 2 diabetic patients with neuropathy. *Cell Biochem Funct* 2013; **32**: 155-163 [PMID: 23913471 DOI: 10.1002/cbf.2987]

18 **Blaha M**, Cermanova M, Blaha V, Jarolim P, Andrys C, Blazek M, Maly J, Smolej L, Zajic J, Masin V, Zimova R, Rehacek V. Elevated serum soluble endoglin (sCD105) decreased during extracorporeal elimination therapy for familial hypercholesterolemia. *Atherosclerosis* 2008; **197**: 264-270

[PMID: 17540382 DOI: 10.1016/j.atherosclerosis.2012.03.001]
19 **Nachtigal P**, Zemankova Vecerova L, Rathouska J, Strasky Z. The role of endoglin in atherosclerosis. *Atherosclerosis* 2012; **224**: 4-11 [PMID: 22460049]
20 **Bassyouni IH**, El-Shazly R, Azkalany GS, Zakaria A, Bassyouni RH. Clinical significance of soluble-endoglin levels in systemic lupus erythematosus: possible association with anti-phospholipid syndrome. *Lupus* 2012; **21**: 1565-1570 [PMID: 22941564 DOI: 10.1177/0961203312460115]
21 **Kopczyńska E**, Dancewicz M, Kowalewski J, Makarewicz R, Kardymowicz H, Kaczmarczyk A, Tyrakowski T. Influence of surgical resection on plasma endoglin (CD105) level in non-small cell lung cancer patients. *Exp Oncol* 2012; **34**: 53-56 [PMID: 22453150]
22 **Blázquez-Medela AM**, García-Ortiz L, Gómez-Marcos MA, Recio-Rodríguez JI, Sánchez-Rodríguez A, López-Novoa JM, Martínez-Salgado C. Increased plasma soluble endoglin levels as an indicator of cardiovascular alterations in hypertensive and diabetic patients. *BMC Med* 2010; **8**: 86 [PMID: 21171985 DOI: 10.1186/1741-7015-8-86]
23 **Wipff J**, Avouac J, Borderie D, Zerkak D, Lemarechal H, Kahan A, Boileau C, Allanore Y. Disturbed angiogenesis in systemic sclerosis: high levels of soluble endoglin. *Rheumatology* (Oxford) 2008; **47**: 972-975 [PMID: 18477643 DOI: 10.1093/rheumatology/ken100]
24 **Juraskova B**, Andrys C, Holmerova I, Solichova D, Hrnciarikova D, Vankova H, Vasatko T, Krejsek J. Transforming growth factor beta and soluble endoglin in the healthy senior and in Alzheimer's disease patients. *J Nutr Health Aging* 2010; **14**: 758-761 [PMID: 21085906 DOI: 10.1007/s12603-010-0325-1]
25 **Davidson B**, Stavnes HT, Førsund M, Berner A, Staff AC. CD105 (Endoglin) expression in breast carcinoma effusions is a marker of poor survival. *Breast* 2010; **19**: 493-498 [PMID: 21078485 DOI: 10.1016/j.breast.2010.05.013]
26 **Bellone G**, Gramigni C, Vizio B, Mauri FA, Prati A, Solerio D, Dughera L, Ruffini E, Gasparri G, Camandona M. Abnormal expression of Endoglin and its receptor complex (TGF-β1 and TGF-β receptor II) as early angiogenic switch indicator in premalignant lesions of the colon mucosa. *Int J Oncol* 2010; **37**: 1153-1165 [PMID: 20878063 DOI: 10.3892/ijo_00000767]
27 **Preativatanyou K**, Honsawek S, Chongsrisawat V, Vejchapipat P, Theamboonlers A, Poovorawan Y. Correlation of circulating endoglin with clinical outcome in biliary atresia. *Eur J Pediatr Surg* 2010; **20**: 237-241 [PMID: 20383820 DOI: 10.1055/s-0030-1249695]
28 **Rath T**, Hage L, Kügler M, Menendez Menendez K, Zachoval R, Naehrlich L, Schulz R, Roderfeld M, Roeb E. Serum proteome profiling identifies novel and powerful markers of cystic fibrosis liver disease. *PLoS One* 2013; **8**: e58955 [PMID: 23516586 DOI: 10.1371/journal.pone.0058955]
29 **Chaiworapongsa T**, Romero R, Kusanovic JP, Savasan ZA, Kim SK, Mazaki-Tovi S, Vaisbuch E, Ogge G, Madan I, Dong Z, Yeo L, Mittal P, Hassan SS. Unexplained fetal death is associated with increased concentrations of anti-angiogenic factors in amniotic fluid. *J Matern Fetal Neonatal Med* 2010; **23**: 794-805 [PMID: 20199197 DOI: 10.3109/14767050903443467]
30 **Dietmann A**, Helbok R, Lackner P, Fischer M, Reindl M, Lell B, Issifou S, Kremsner PG, Schmutzhard E. Endoglin in African children with Plasmodium falciparum malaria: a novel player in severe malaria pathogenesis? *J Infect Dis* 2009; **200**: 1842-1848 [PMID: 19919302 DOI: 10.1086/648476]
31 **Fujita K**, Ewing CM, Chan DY, Mangold LA, Partin AW, Isaacs WB, Pavlovich CP. Endoglin (CD105) as a urinary and serum marker of prostate cancer. *Int J Cancer* 2009; **124**: 664-669 [PMID: 19004009 DOI: 10.1002/ijc.24007]
32 **Kuiper P**, Hawinkels LJ, de Jonge-Muller ES, Biemond I, Lamers CB, Verspaget HW. Angiogenic markers endoglin and vascular endothelial growth factor in gastroenteropancreatic neuroendocrine tumors. *World J Gastroenterol* 2011; **17**: 219-225 [PMID: 21245995 DOI: 10.3748/wjg.v17.i2.219]
33 **Johnson DW**, Berg JN, Baldwin MA, Gallione CJ, Marondel I, Yoon SJ, Stenzel TT, Speer M, Pericak-Vance MA, Diamond A, Guttmacher AE, Jackson CE, Attisano L, Kucherlapati R, Porteous ME, Marchuk DA. Mutations in the activin receptor-like kinase 1 gene in hereditary haemorrhagic telangiectasia type 2. *Nat Genet* 1996; **13**: 189-195 [PMID: 8640225 DOI: 10.1038/ng0696-189]
34 **Gallione CJ**, Repetto GM, Legius E, Rustgi AK, Schelley SL, Tejpar S, Mitchell G, Drouin E, Westermann CJ, Marchuk DA. A combined syndrome of juvenile polyposis and hereditary haemorrhagic telangiectasia associated with mutations in MADH4 (SMAD4). *Lancet* 2004; **363**: 852-859 [PMID: 15031030 DOI: 10.1016/S0140-6736(04)15732-2]
35 **Cole SG**, Begbie ME, Wallace GM, Shovlin CL. A new locus for hereditary haemorrhagic telangiectasia (HHT3) maps to chromosome 5. *J Med Genet* 2005; **42**: 577-582 [PMID: 15994879 DOI: 10.1136/jmg.2004.028712]
36 **Bayrak-Toydemir P**, McDonald J, Akarsu N, Toydemir RM, Calderon F, Tuncali T, Tang W, Miller F, Mao R. A fourth locus for hereditary hemorrhagic telangiectasia maps to chromosome 7. *Am J Med Genet A* 2006; **140**: 2155-2162 [PMID: 16969873 DOI: 10.1002/ajmg.a.31450]
37 **St-Jacques S**, Cymerman U, Pece N, Letarte M. Molecular characterization and in situ localization of murine endoglin reveal that it is a transforming growth factor-beta binding protein of endothelial and stromal cells. *Endocrinology* 1994; **134**: 2645-2657 [PMID: 8194490 DOI: 10.1210/en.134.6.2645]
38 **Roy-Chaudhury P**, Simpson JG, Power DA. Endoglin, a transforming growth factor-beta-binding protein, is upregulated in chronic progressive renal disease. *Exp Nephrol* 1997; **5**: 55-60 [PMID: 9052849]
39 **Leask A**, Abraham DJ, Finlay DR, Holmes A, Pennington D, Shi-Wen X, Chen Y, Venstrom K, Dou X, Ponticos M, Black C, Bernabeu C, Jackman JK, Findell PR, Connolly MK. Dysregulation of transforming growth factor beta signaling in scleroderma: overexpression of endoglin in cutaneous scleroderma fibroblasts. *Arthritis Rheum* 2002; **46**: 1857-1865 [PMID: 12124870 DOI: 10.1002/art.10333]
40 **Meurer SK**, Tihaa L, Borkham-Kamphorst E, Weiskirchen R. Expression and functional analysis of endoglin in isolated liver cells and its involvement in fibrogenic Smad signalling. *Cell Signal* 2011; **23**: 683-699 [PMID: 21146604 DOI: 10.1016/j.cellsig.2010.12.002]
41 **Meurer SK**, Tihaa L, Lahme B, Gressner AM, Weiskirchen R. Identification of endoglin in rat hepatic stellate cells: new insights into transforming growth factor β receptor signaling. *J Biol Chem* 2005; **280**: 3078-3087 [PMID: 15537649]
42 **Scherner O**, Meurer SK, Tihaa L, Gressner AM, Weiskirchen R. Endoglin differentially modulates antagonistic transforming growth factor-beta1 and BMP-7 signaling. *J Biol Chem* 2007; **282**: 13934-13943 [PMID: 17376778 DOI: 10.1074/jbc.M611062200]
43 **Pérez-Gómez E**, Eleno N, López-Novoa JM, Ramirez JR, Velasco B, Letarte M, Bernabéu C, Quintanilla M. Characterization of murine S-endoglin isoform and its effects on tumor development. *Oncogene* 2005; **24**: 4450-4461 [PMID: 15806144 DOI: 10.1038/sj.onc.1208644]
44 **Blanco FJ**, Bernabeu C. Alternative splicing factor or splicing factor-2 plays a key role in intron retention of the endoglin gene during endothelial senescence. *Aging Cell* 2011; **10**: 896-907 [PMID: 21668763 DOI: 10.1111/j.1474-9726.2011.00727.x]
45 **Blanco FJ**, Grande MT, Langa C, Oujo B, Velasco S, Rodriguez-Barbero A, Perez-Gomez E, Quintanilla M, López-Novoa JM, Bernabeu C. S-endoglin expression is induced in senescent endothelial cells and contributes to vascular pathology. *Circ Res* 2008; **103**: 1383-1392 [PMID: 18974388 DOI: 10.1161/CIRCRESAHA.108.176552]
46 **ten Dijke P**, Goumans MJ, Pardali E. Endoglin in angiogen-

esis and vascular diseases. *Angiogenesis* 2008; **11**: 79-89 [PMID: 18283546 DOI: 10.1007/s10456-008-9101-9]
47 **Llorca O**, Trujillo A, Blanco FJ, Bernabeu C. Structural model of human endoglin, a transmembrane receptor responsible for hereditary hemorrhagic telangiectasia. *J Mol Biol* 2007; **365**: 694-705 [PMID: 17081563 DOI: 10.1016/j.jmb.2006.10.015]
48 **Gougos A**, Letarte M. Biochemical characterization of the 44G4 antigen from the HOON pre-B leukemic cell line. *J Immunol* 1988; **141**: 1934-1940 [PMID: 3262645]
49 **Bork P**, Sander C. A large domain common to sperm receptors (Zp2 and Zp3) and TGF-beta type III receptor. *FEBS Lett* 1992; **300**: 237-240 [PMID: 1313375 DOI: 10.1016/0014-5793(92)80853-9]
50 **Jovine L**, Darie CC, Litscher ES, Wassarman PM. Zona pellucida domain proteins. *Annu Rev Biochem* 2005; **74**: 83-114 [PMID: 15952882 DOI: 10.1146/annurev.biochem.74.082803.133039]
51 **Letamendía A**, Lastres P, Botella LM, Raab U, Langa C, Velasco B, Attisano L, Bernabeu C. Role of endoglin in cellular responses to transforming growth factor-beta. A comparative study with betaglycan. *J Biol Chem* 1998; **273**: 33011-33019 [PMID: 9830054 DOI: 10.1074/jbc.273.49.33011]
52 **Bernabeu C**, Lopez-Novoa JM, Quintanilla M. The emerging role of TGF-beta superfamily coreceptors in cancer. *Biochim Biophys Acta* 2009; **1792**: 954-973 [PMID: 19607914 DOI: 10.1016/j.bbadis.2009.07.003]
53 **Ge AZ**, Butcher EC. Cloning and expression of a cDNA encoding mouse endoglin, an endothelial cell TGF-beta ligand. *Gene* 1994; **138**: 201-206 [PMID: 8125301 DOI: 10.1016/0378-1119(94)90808-7]
54 **Gregory A**. Structural and Functional Characteristics of a Soluble Form of Endoglin in the Context of Preeclampsia. A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Immunology, University of Toronto, Toronto, Canada, 2011. Available from: URL: https: //tspace.library.utoronto.ca/bitstream/1807/30615/1/Gregory_Allison_L_201111_MSc_thesis.pdf
55 **Van Le B**, Franke D, Svergun DI, Han T, Hwang HY, Kim KK. Structural and functional characterization of soluble endoglin receptor. *Biochem Biophys Res Commun* 2009; **383**: 386-391 [PMID: 19268655 DOI: 10.1016/j.bbrc.2009.02.162]
56 **Guerrero-Esteo M**, Sanchez-Elsner T, Letamendia A, Bernabeu C. Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-beta receptors I and II. *J Biol Chem* 2002; **277**: 29197-29209 [PMID: 12015308 DOI: 10.1074/jbc.M111991200]
57 **Raab U**, Velasco B, Lastres P, Letamendía A, Calés C, Langa C, Tapia E, López-Bote JP, Páez E, Bernabéu C. Expression of normal and truncated forms of human endoglin. *Biochem J* 1999; **339** (Pt 3): 579-588 [PMID: 10215596 DOI: 10.1042/0264-6021: 3390579]
58 **Li CG**, Wilson PB, Bernabeu C, Raab U, Wang JM, Kumar S. Immunodetection and characterisation of soluble CD105-TGFbeta complexes. *J Immunol Methods* 1998; **218**: 85-93 [PMID: 9819125 DOI: 10.1016/S0022-1759(98)00118-5]
59 **Hawinkels LJ**, Kuiper P, Wiercinska E, Verspaget HW, Liu Z, Pardali E, Sier CF, ten Dijke P. Matrix metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumor angiogenesis. *Cancer Res* 2010; **70**: 4141-4150 [PMID: 20424116 DOI: 10.1158/0008-5472.CAN-09-4466]
60 **Kumar S**, Pan CC, Bloodworth JC, Nixon A, Theuer C, Hoyt DG, Lee NY. Antibody-directed coupling of endoglin and MMP-14 is a key mechanism for endoglin shedding and deregulation of TGF-β signaling. *Oncogene* 2013 Sep 30; Epub ahead of print [PMID: 24077288 DOI: 10.1038/onc.2013.386]
61 **Velasco-Loyden G**, Arribas J, López-Casillas F. The shedding of betaglycan is regulated by pervanadate and mediated by membrane type matrix metalloprotease-1. *J Biol Chem* 2004; **279**: 7721-7733 [PMID: 14672946 DOI: 10.1074/jbc.M306499200]
62 **Yamashita H**, Ichijo H, Grimsby S, Morén A, ten Dijke P, Miyazono K. Endoglin forms a heteromeric complex with the signaling receptors for transforming growth factor-beta. *J Biol Chem* 1994; **269**: 1995-2001 [PMID: 8294451]
63 **Sanz-Rodriguez F**, Guerrero-Esteo M, Botella LM, Banville D, Vary CP, Bernabéu C. Endoglin regulates cytoskeletal organization through binding to ZRP-1, a member of the LIM family of proteins. *J Biol Chem* 2004; **279**: 32858-32868 [PMID: 151483178 DOI: 10.1074/jbc.M400843200]
64 **Conley BA**, Koleva R, Smith JD, Kacer D, Zhang D, Bernabéu C, Vary CP. Endoglin controls cell migration and composition of focal adhesions: function of the cytosolic domain. *J Biol Chem* 2004; **279**: 27440-27449 [PMID: 15084601 DOI: 10.1074/jbc.M312561200]
65 **Meng Q**, Lux A, Holloschi A, Li J, Hughes JM, Foerg T, McCarthy JE, Heagerty AM, Kioschis P, Hafner M, Garland JM. Identification of Tctex2beta, a novel dynein light chain family member that interacts with different transforming growth factor-beta receptors. *J Biol Chem* 2006; **281**: 37069-37080 [PMID: 16982625 DOI: 10.1074/jbc.M608614200]
66 **Lee NY**, Blobe GC. The interaction of endoglin with beta-arrestin2 regulates transforming growth factor-beta-mediated ERK activation and migration in endothelial cells. *J Biol Chem* 2007; **282**: 21507-21517 [PMID: 17540773 DOI: 10.1074/jbc.M700176200]
67 **Lee NY**, Ray B, How T, Blobe GC. Endoglin promotes transforming growth factor beta-mediated Smad 1/5/8 signaling and inhibits endothelial cell migration through its association with GIPC. *J Biol Chem* 2008; **283**: 32527-32533 [PMID: 18775991 DOI: 10.1074/jbc.M803059200]
68 **Blanco FJ**, Santibanez JF, Guerrero-Esteo M, Langa C, Vary CP, Bernabeu C. Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor-beta receptor complex. *J Cell Physiol* 2005; **204**: 574-584 [PMID: 15702480 DOI: 10.1002/jcp.20311]
69 **Lastres P**, Martín-Perez J, Langa C, Bernabéu C. Phosphorylation of the human-transforming-growth-factor-beta-binding protein endoglin. *Biochem J* 1994; **301** (Pt 3): 765-768 [PMID: 8053900]
70 **Koleva RI**, Conley BA, Romero D, Riley KS, Marto JA, Lux A, Vary CP. Endoglin structure and function: Determinants of endoglin phosphorylation by transforming growth factor-beta receptors. *J Biol Chem* 2006; **281**: 25110-25123 [PMID: 16785228 DOI: 10.1074/jbc.M601288200]
71 **Ray BN**, Lee NY, How T, Blobe GC. ALK5 phosphorylation of the endoglin cytoplasmic domain regulates Smad1/5/8 signaling and endothelial cell migration. *Carcinogenesis* 2010; **31**: 435-441 [PMID: 20042635 DOI: 10.1093/carcin/bgp327]
72 **Tian H**, Mythreye K, Golzio C, Katsanis N, Blobe GC. Endoglin mediates fibronectin/α5β1 integrin and TGF-β pathway crosstalk in endothelial cells. *EMBO J* 2012; **31**: 3885-3900 [PMID: 22940691 DOI: 10.1038/emboj.2012.246]
73 **Wang X**, Abraham S, McKenzie JA, Jeffs N, Swire M, Tripathi VB, Luhmann UF, Lange CA, Zhai Z, Arthur HM, Bainbridge JW, Moss SE, Greenwood J. LRG1 promotes angiogenesis by modulating endothelial TGF-β signalling. *Nature* 2013; **499**: 306-311 [PMID: 23868260 DOI: 10.1038/nature12345]
74 **Blobe GC**, Liu X, Fang SJ, How T, Lodish HF. A novel mechanism for regulating transforming growth factor beta (TGF-beta) signaling. Functional modulation of type III TGF-beta receptor expression through interaction with the PDZ domain protein, GIPC. *J Biol Chem* 2001; **276**: 39608-39617 [PMID: 11546783 DOI: 10.1074/jbc.M106831200]
75 **Chen W**, Kirkbride KC, How T, Nelson CD, Mo J, Frederick JP, Wang XF, Lefkowitz RJ, Blobe GC. Beta-arrestin 2 mediates endocytosis of type III TGF-beta receptor and down-

regulation of its signaling. *Science* 2003; **301**: 1394-1397 [PMID: 12958365 DOI: 10.1126/science.1083195]
76 **Lee NY**, Golzio C, Gatza CE, Sharma A, Katsanis N, Blobe GC. Endoglin regulates PI3-kinase/Akt trafficking and signaling to alter endothelial capillary stability during angiogenesis. *Mol Biol Cell* 2012; **23**: 2412-2423 [PMID: 22593212 DOI: 10.1091/mbc.E11-12-0993]
77 **Moustakas A**, Heldin CH. The regulation of TGFbeta signal transduction. *Development* 2009; **136**: 3699-3714 [PMID: 19855013 DOI: 10.1242/dev.030338]
78 **Wiercinska E**, Wickert L, Denecke B, Said HM, Hamzavi J, Gressner AM, Thorikay M, ten Dijke P, Mertens PR, Breitkopf K, Dooley S. Id1 is a critical mediator in TGF-beta-induced transdifferentiation of rat hepatic stellate cells. *Hepatology* 2006; **43**: 1032-1041 [PMID: 16628634 DOI: 10.1002/hep.21135]
79 **Meurer SK**, Alsamman M, Sahin H, Wasmuth HE, Kisseleva T, Brenner DA, Trautwein C, Weiskirchen R, Scholten D. Overexpression of endoglin modulates TGF-β1-signalling pathways in a novel immortalized mouse hepatic stellate cell line. *PLoS One* 2013; **8**: e56116 [PMID: 23437087 DOI: 10.1371/journal.pone.0056116]
80 **Lastres P**, Letamendía A, Zhang H, Rius C, Almendro N, Raab U, López LA, Langa C, Fabra A, Letarte M, Bernabéu C. Endoglin modulates cellular responses to TGF-beta 1. *J Cell Biol* 1996; **133**: 1109-1121 [PMID: 8655583 DOI: 10.1083/jcb.133.5.1109]
81 **van Meeteren LA**, ten Dijke P. Regulation of endothelial cell plasticity by TGF-β. *Cell Tissue Res* 2012; **347**: 177-186 [PMID: 21866313 DOI: 10.1007/s00441-011-1222-6]
82 **Lastres P**, Bellon T, Cabañas C, Sanchez-Madrid F, Acevedo A, Gougos A, Letarte M, Bernabeu C. Regulated expression on human macrophages of endoglin, an Arg-Gly-Asp-containing surface antigen. *Eur J Immunol* 1992; **22**: 393-397 [PMID: 1537377 DOI: 10.1002/eji.1830220216]
83 **Ríus C**, Smith JD, Almendro N, Langa C, Botella LM, Marchuk DA, Vary CP, Bernabéu C. Cloning of the promoter region of human endoglin, the target gene for hereditary hemorrhagic telangiectasia type 1. *Blood* 1998; **92**: 4677-4690 [PMID: 9845534]
84 **Botella LM**, Sánchez-Elsner T, Rius C, Corbí A, Bernabéu C. Identification of a critical Sp1 site within the endoglin promoter and its involvement in the transforming growth factor-beta stimulation. *J Biol Chem* 2001; **276**: 34486-34494 [PMID: 11432852 DOI: 10.1074/jbc.M011611200]
85 **Botella LM**, Sánchez-Elsner T, Sanz-Rodriguez F, Kojima S, Shimada J, Guerrero-Esteo M, Cooreman MP, Ratziu V, Langa C, Vary CP, Ramirez JR, Friedman S, Bernabéu C. Transcriptional activation of endoglin and transforming growth factor-beta signaling components by cooperative interaction between Sp1 and KLF6: their potential role in the response to vascular injury. *Blood* 2002; **100**: 4001-4010 [PMID: 12433697 DOI: 10.1182/blood.V100.12.4001]
86 **Sánchez-Elsner T**, Botella LM, Velasco B, Langa C, Bernabéu C. Endoglin expression is regulated by transcriptional cooperation between the hypoxia and transforming growth factor-beta pathways. *J Biol Chem* 2002; **277**: 43799-43808 [PMID: 12228247 DOI: 10.1074/jbc.M207160200]
87 **Velasco S**, Alvarez-Muñoz P, Pericacho M, Dijke PT, Bernabéu C, López-Novoa JM, Rodríguez-Barbero A. L- and S-endoglin differentially modulate TGFbeta1 signaling mediated by ALK1 and ALK5 in L6E9 myoblasts. *J Cell Sci* 2008; **121**: 913-919 [PMID: 18303046 DOI: 10.1242/jcs.023283]
88 **Lebrin F**, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, Mummery C, Arthur HM, ten Dijke P. Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction. *EMBO J* 2004; **23**: 4018-4028 [PMID: 15385967 DOI: 10.1038/sj.emboj.7600386]
89 **Jerkic M**, Rivas-Elena JV, Santibanez JF, Prieto M, Rodríguez-Barbero A, Perez-Barriocanal F, Pericacho M, Arévalo M, Vary CP, Letarte M, Bernabeu C, López-Novoa JM. Endoglin regulates cyclooxygenase-2 expression and activity. *Circ Res* 2006; **99**: 248-256 [PMID: 16840721 DOI: 10.1161/01.RES.0000236755.98627.69]
90 **Pece-Barbara N**, Vera S, Kathirkamathamby K, Liebner S, Di Guglielmo GM, Dejana E, Wrana JL, Letarte M. Endoglin null endothelial cells proliferate faster and are more responsive to transforming growth factor beta1 with higher affinity receptors and an activated Alk1 pathway. *J Biol Chem* 2005; **280**: 27800-27808 [PMID: 15923183 DOI: 10.1074/jbc.M503471200]
91 **Goumans MJ**, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J* 2002; **21**: 1743-1753 [PMID: 11927558 DOI: 10.1093/emboj/21.7.1743]
92 **Santibanez JF**, Letamendia A, Perez-Barriocanal F, Silvestri C, Saura M, Vary CP, Lopez-Novoa JM, Attisano L, Bernabeu C. Endoglin increases eNOS expression by modulating Smad2 protein levels and Smad2-dependent TGF-beta signaling. *J Cell Physiol* 2007; **210**: 456-468 [PMID: 17058229 DOI: 10.1002/jcp.20878]
93 **Diez-Marques L**, Ortega-Velazquez R, Langa C, Rodriguez-Barbero A, Lopez-Novoa JM, Lamas S, Bernabeu C. Expression of endoglin in human mesangial cells: modulation of extracellular matrix synthesis. *Biochim Biophys Acta* 2002; **1587**: 36-44 [PMID: 12009422 DOI: 10.1016/S0925-4439(02)00051-0]
94 **Guo B**, Slevin M, Li C, Parameshwar S, Liu D, Kumar P, Bernabeu C, Kumar S. CD105 inhibits transforming growth factor-beta-Smad3 signalling. *Anticancer Res* 2004; **24**: 1337-1345 [PMID: 15274293]
95 **Obreo J**, Díez-Marques L, Lamas S, Düwell A, Eleno N, Bernabéu C, Pandiella A, López-Novoa JM, Rodríguez-Barbero A. Endoglin expression regulates basal and TGF-beta1-induced extracellular matrix synthesis in cultured L6E9 myoblasts. *Cell Physiol Biochem* 2004; **14**: 301-310 [PMID: 15319534 DOI: 10.1159/000080340]
96 **Rodríguez-Barbero A**, Obreo J, Alvarez-Munoz P, Pandiella A, Bernabéu C, López-Novoa JM. Endoglin modulation of TGF-beta1-induced collagen synthesis is dependent on ERK1/2 MAPK activation. *Cell Physiol Biochem* 2006; **18**: 135-142 [PMID: 16914898 DOI: 10.1159/000095181]
97 **Schmidt-Weber CB**, Letarte M, Kunzmann S, Rückert B, Bernabéu C, Blaser K. TGF-{beta} signaling of human T cells is modulated by the ancillary TGF-{beta} receptor endoglin. *Int Immunol* 2005; **17**: 921-930 [PMID: 15967783 DOI: 10.1093/intimm/dxh272]
98 **Pickles M**, Leask A. Analysis of CCN2 promoter activity in PANC-1 cells: regulation by ras/MEK/ERK. *J Cell Commun Signal* 2007; **1**: 85-90 [PMID: 18481199 DOI: 10.1007/s12079-007-0008-9]
99 **Zhang X**, Arnott JA, Rehman S, Delong WG, Sanjay A, Safadi FF, Popoff SN. Src is a major signaling component for CTGF induction by TGF-beta1 in osteoblasts. *J Cell Physiol* 2010; **224**: 691-701 [PMID: 20432467 DOI: 10.1002/jcp.22173]
100 **Santibanez JF**, Pérez-Gómez E, Fernandez-L A, Garrido-Martin EM, Carnero A, Malumbres M, Vary CP, Quintanilla M, Bernabéu C. The TGF-beta co-receptor endoglin modulates the expression and transforming potential of H-Ras. *Carcinogenesis* 2010; **31**: 2145-2154 [PMID: 20884686 DOI: 10.1093/carcin/bgq199]
101 **Holmes AM**, Ponticos M, Shi-Wen X, Denton CP, Abraham DJ. Elevated CCN2 expression in scleroderma: a putative role for the TGFβ accessory receptors TGFβRIII and endoglin. *J Cell Commun Signal* 2011; **5**: 173-177 [PMID: 21769684 DOI: 10.1007/s12079-011-0140-4]
102 **Pannu J**, Nakerakanti S, Smith E, ten Dijke P, Trojanowska M. Transforming growth factor-beta receptor type I-dependent fibrogenic gene program is mediated via activation of Smad1 and ERK1/2 pathways. *J Biol Chem* 2007; **282**: 10405-10413

[PMID: 17317656 DOI: 10.1074/jbc.M611742200]
103 **Morris E**, Chrobak I, Bujor A, Hant F, Mummery C, Ten Dijke P, Trojanowska M. Endoglin promotes TGF-β/Smad1 signaling in scleroderma fibroblasts. *J Cell Physiol* 2011; **226**: 3340-3348 [PMID: 21344387 DOI: 10.1002/jcp.22690]
104 **Schnabl B**, Kweon YO, Frederick JP, Wang XF, Rippe RA, Brenner DA. The role of Smad3 in mediating mouse hepatic stellate cell activation. *Hepatology* 2001; **34**: 89-100 [PMID: 11431738 DOI: 10.1053/jhep.2001.25349]
105 **Tang H**, Low B, Rutherford SA, Hao Q. Thrombin induces endocytosis of endoglin and type-II TGF-beta receptor and down-regulation of TGF-beta signaling in endothelial cells. *Blood* 2005; **105**: 1977-1985 [PMID: 15522964 DOI: 10.1182/blood-2004-08-3308]
106 **Pérez-Gómez E**, Del Castillo G, Juan Francisco S, López-Novoa JM, Bernabéu C, Quintanilla M. The role of the TGF-β coreceptor endoglin in cancer. *ScientificWorldJournal* 2010; **10**: 2367-2384 [PMID: 21170488 DOI: 10.1100/tsw.2010.230]
107 **Levine RJ**, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R, Karumanchi SA. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med* 2006; **355**: 992-1005 [PMID: 16957146 DOI: 10.1056/NEJMoa055352]
108 **Yagmur E**, Rizk M, Stanzel S, Hellerbrand C, Lammert F, Trautwein C, Wasmuth HE, Gressner AM. Elevation of endoglin (CD105) concentrations in serum of patients with liver cirrhosis and carcinoma. *Eur J Gastroenterol Hepatol* 2007; **19**: 755-761 [PMID: 17700260 DOI: 10.1097/MEG.0b013e3282202bea]
109 **Cheifetz S**, Bellón T, Calés C, Vera S, Bernabeu C, Massagué J, Letarte M. Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. *J Biol Chem* 1992; **267**: 19027-19030 [PMID: 1326540]
110 **Barbara NP**, Wrana JL, Letarte M. Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-beta superfamily. *J Biol Chem* 1999; **274**: 584-594 [PMID: 9872992 DOI: 10.1074/jbc.274.2.584]
111 **De Crescenzo G**, Pham PL, Durocher Y, O'Connor-McCourt MD. Transforming growth factor-beta (TGF-beta) binding to the extracellular domain of the type II TGF-beta receptor: receptor capture on a biosensor surface using a new coiled-coil capture system demonstrates that avidity contributes significantly to high affinity binding. *J Mol Biol* 2003; **328**: 1173-1183 [PMID: 12729750 DOI: 10.1016/S0022-2836(03)00360-7]
112 **Castonguay R**, Werner ED, Matthews RG, Presman E, Mulivor AW, Solban N, Sako D, Pearsall RS, Underwood KW, Seehra J, Kumar R, Grinberg AV. Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. *J Biol Chem* 2011; **286**: 30034-30046 [PMID: 21737454 DOI: 10.1074/jbc.M111.260133]
113 **McDonald J**, Bayrak-Toydemir P, Pyeritz RE. Hereditary hemorrhagic telangiectasia: an overview of diagnosis, management, and pathogenesis. *Genet Med* 2011; **13**: 607-616 [PMID: 21546842 DOI: 10.1097/GIM.0b013e3182136d32]
114 **Shovlin CL**, Guttmacher AE, Buscarini E, Faughnan ME, Hyland RH, Westermann CJ, Kjeldsen AD, Plauchu H. Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). *Am J Med Genet* 2000; **91**: 66-67 [PMID: 10751092 DOI: 10.1002/(SICI)1096-8628(20000306)91: 1<66: : AID-AJMG12>3.0.CO; 2-P]
115 **Sadick H**, Hage J, Goessler U, Stern-Straeter J, Riedel F, Hoermann K, Bugert P. Mutation analysis of "Endoglin" and "Activin receptor-like kinase" genes in German patients with hereditary hemorrhagic telangiectasia and the value of rapid genotyping using an allele-specific PCR-technique. *BMC Med Genet* 2009; **10**: 53 [PMID: 19508727 DOI: 10.1186/1471-2350-10-53]
116 **Abdalla SA**, Letarte M. Hereditary haemorrhagic telangiectasia: current views on genetics and mechanisms of disease. *J Med Genet* 2006; **43**: 97-110 [PMID: 15879500 DOI: 10.1136/jmg.2005.030833]
117 **Srinivasan S**, Hanes MA, Dickens T, Porteous ME, Oh SP, Hale LP, Marchuk DA. A mouse model for hereditary hemorrhagic telangiectasia (HHT) type 2. *Hum Mol Genet* 2003; **12**: 473-482 [PMID: 12588795 DOI: 10.1093/hmg/ddg050]
118 **Bourdeau A**, Cymerman U, Paquet ME, Meschino W, McKinnon WC, Guttmacher AE, Becker L, Letarte M. Endoglin expression is reduced in normal vessels but still detectable in arteriovenous malformations of patients with hereditary hemorrhagic telangiectasia type 1. *Am J Pathol* 2000; **156**: 911-923 [PMID: 10702408 DOI: 10.1016/S0002-9440(10)64960-7]
119 **Bauditz J**, Lochs H. Angiogenesis and vascular malformations: antiangiogenic drugs for treatment of gastrointestinal bleeding. *World J Gastroenterol* 2007; **13**: 5979-5984 [PMID: 18023086 DOI: 10.3748/wjg.13.5979]
120 **Bowcock SJ**, Patrick HE. Lenalidomide to control gastrointestinal bleeding in hereditary haemorrhagic telangiectasia: potential implications for angiodysplasias? *Br J Haematol* 2009; **146**: 220-222 [PMID: 19438503 DOI: 10.1111/j.1365-2141.2009.07730.x]
121 **Dupuis-Girod S**, Ginon I, Saurin JC, Marion D, Guillot E, Decullier E, Roux A, Carette MF, Gilbert-Dussardier B, Hatron PY, Lacombe P, Lorcerie B, Rivière S, Corre R, Giraud S, Bailly S, Paintaud G, Ternant D, Valette PJ, Plauchu H, Faure F. Bevacizumab in patients with hereditary hemorrhagic telangiectasia and severe hepatic vascular malformations and high cardiac output. *JAMA* 2012; **307**: 948-955 [PMID: 22396517 DOI: 10.1001/jama.2012.250]
122 **Albiñana V**, Recio-Poveda L, Zarrabeitia R, Bernabéu C, Botella LM. Propranolol as antiangiogenic candidate for the therapy of hereditary haemorrhagic telangiectasia. *Thromb Haemost* 2012; **108**: 41-53 [PMID: 22552254 DOI: 10.1160/TH11-11-0809]
123 **Yaniv E**, Preis M, Shevro J, Nageris B, Hadar T. Anti-estrogen therapy for hereditary hemorrhagic telangiectasia - a long-term clinical trial. *Rhinology* 2011; **49**: 214-216 [PMID: 21743879]
124 **Albiñana V**, Bernabeu-Herrero ME, Zarrabeitia R, Bernabéu C, Botella LM. Estrogen therapy for hereditary haemorrhagic telangiectasia (HHT): Effects of raloxifene, on Endoglin and ALK1 expression in endothelial cells. *Thromb Haemost* 2010; **103**: 525-534 [PMID: 20135064 DOI: 10.1160/TH09-07-0425]
125 **Naljayan MV**, Karumanchi SA. New developments in the pathogenesis of preeclampsia. *Adv Chronic Kidney Dis* 2013; **20**: 265-270 [PMID: 23928392 DOI: 10.1053/j.ackd.2013.02.003]
126 **Karnad DR**, Guntupalli KK. Neurologic disorders in pregnancy. *Crit Care Med* 2005; **33**: S362-S371 [PMID: 16215360 DOI: 10.1097/01.CCM.0000182790.35728.F7]
127 **Brown MC**, Best KE, Pearce MS, Waugh J, Robson SC, Bell R. Cardiovascular disease risk in women with pre-eclampsia: systematic review and meta-analysis. *Eur J Epidemiol* 2013; **28**: 1-19 [PMID: 23397514 DOI: 10.1007/s10654-013-9762-6]
128 **Zhou Y**, Damsky CH, Fisher SJ. Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *J Clin Invest* 1997; **99**: 2152-2164 [PMID: 9151787 DOI: 10.1172/JCI119388]
129 **Ahmad S**, Ahmed A. Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. *Circ Res* 2004; **95**: 884-891 [PMID: 15472115 DOI: 10.1161/01.RES.0000147365.86159.f5]
130 **Staff AC**, Braekke K, Harsem NK, Lyberg T, Holthe MR. Circulating concentrations of sFlt1 (soluble fms-like tyrosine kinase 1) in fetal and maternal serum during pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol* 2005; **122**: 33-39 [PMID: 15935542 DOI: 10.1016/j.ejogrb.2004.11.015]

131 **Maynard SE**, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP, Karumanchi SA. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003; **111**: 649-658 [PMID: 12618519 DOI: 10.1172/JCI17189]

132 **Valbuena-Diez AC**, Blanco FJ, Oujo B, Langa C, Gonzalez-Nuñez M, Llano E, Pendas AM, Díaz M, Castrillo A, Lopez-Novoa JM, Bernabeu C. Oxysterol-induced soluble endoglin release and its involvement in hypertension. *Circulation* 2012; **126**: 2612-2624 [PMID: 23110859 DOI: 10.1161/CIRCULATIONAHA.112.101261]

133 **Jerkic M**, Rivas-Elena JV, Prieto M, Carrón R, Sanz-Rodríguez F, Pérez-Barriocanal F, Rodríguez-Barbero A, Bernabéu C, López-Novoa JM. Endoglin regulates nitric oxide-dependent vasodilatation. *FASEB J* 2004; **18**: 609-611 [PMID: 14734648]

134 **López-Novoa JM**, Bernabeu C. The physiological role of endoglin in the cardiovascular system. *Am J Physiol Heart Circ Physiol* 2010; **299**: H959-H974 [PMID: 20656886 DOI: 10.1152/ajpheart.01251.2009]

135 **Toporsian M**, Gros R, Kabir MG, Vera S, Govindaraju K, Eidelman DH, Husain M, Letarte M. A role for endoglin in coupling eNOS activity and regulating vascular tone revealed in hereditary hemorrhagic telangiectasia. *Circ Res* 2005; **96**: 684-692 [PMID: 15718503 DOI: 10.1161/01.RES.0000159936.38601.22]

136 **Gu Y**, Lewis DF, Wang Y. Placental productions and expressions of soluble endoglin, soluble fms-like tyrosine kinase receptor-1, and placental growth factor in normal and preeclamptic pregnancies. *J Clin Endocrinol Metab* 2008; **93**: 260-266 [PMID: 17956952 DOI: 10.1210/jc.2007-1550]

137 **Gilbert JS**, Gilbert SA, Arany M, Granger JP. Hypertension produced by placental ischemia in pregnant rats is associated with increased soluble endoglin expression. *Hypertension* 2009; **53**: 399-403 [PMID: 19075097 DOI: 10.1161/HYPERTENSIONAHA.108.123513]

138 **Powers RW**, Jeyabalan A, Clifton RG, Van Dorsten P, Hauth JC, Klebanoff MA, Lindheimer MD, Sibai B, Landon M, Miodovnik M. Soluble fms-Like tyrosine kinase 1 (sFlt1), endoglin and placental growth factor (PlGF) in preeclampsia among high risk pregnancies. *PLoS One* 2010; **5**: e13263 [PMID: 20948996 DOI: 10.1371/journal.pone.0013263]

139 **Li Z**, Zhang Y, Ying Ma J, Kapoun AM, Shao Q, Kerr I, Lam A, O'Young G, Sannajust F, Stathis P, Schreiner G, Karumanchi SA, Protter AA, Pollitt NS. Recombinant vascular endothelial growth factor 121 attenuates hypertension and improves kidney damage in a rat model of preeclampsia. *Hypertension* 2007; **50**: 686-692 [PMID: 17724276 DOI: 10.1161/HYPERTENSIONAHA.107.092098]

140 **Thadhani R**, Kisner T, Hagmann H, Bossung V, Noack S, Schaarschmidt W, Jank A, Kribs A, Cornely OA, Kreyssig C, Hemphill L, Rigby AC, Khedkar S, Lindner TH, Mallmann P, Stepan H, Karumanchi SA, Benzing T. Pilot study of extracorporeal removal of soluble fms-like tyrosine kinase 1 in preeclampsia. *Circulation* 2011; **124**: 940-950 [PMID: 21810665 DOI: 10.1161/CIRCULATIONAHA.111.034793]

141 **Cudmore M**, Ahmad S, Al-Ani B, Fujisawa T, Coxall H, Chudasama K, Devey LR, Wigmore SJ, Abbas A, Hewett PW, Ahmed A. Negative regulation of soluble Flt-1 and soluble endoglin release by heme oxygenase-1. *Circulation* 2007; **115**: 1789-1797 [PMID: 17389265 DOI: 10.1161/CIRCULATIONAHA.106.660134]

142 **Devy L**, Huang L, Naa L, Yanamandra N, Pieters H, Frans N, Chang E, Tao Q, Vanhove M, Lejeune A, van Gool R, Sexton DJ, Kuang G, Rank D, Hogan S, Pazmany C, Ma YL, Schoonbroodt S, Nixon AE, Ladner RC, Hoet R, Henderikx P, Tenhoor C, Rabbani SA, Valentino ML, Wood CR, Dransfield DT. Selective inhibition of matrix metalloproteinase-14 blocks tumor growth, invasion, and angiogenesis. *Cancer Res* 2009; **69**: 1517-1526 [PMID: 19208838 DOI: 10.1158/0008-5472.CAN-08-3255]

143 **Abildgaard U**, Heimdal K. Pathogenesis of the syndrome of hemolysis, elevated liver enzymes, and low platelet count (HELLP): a review. *Eur J Obstet Gynecol Reprod Biol* 2013; **166**: 117-123 [PMID: 23107053 DOI: 10.1016/j.ejogrb.2012.09.026]

144 **Reimer T**, Rohrmann H, Stubert J, Pecks U, Glocker MO, Richter DU, Gerber B. Angiogenic factors and acute-phase proteins in serum samples of preeclampsia and HELLP patients: a matched-pair analysis. *J Matern Fetal Neonatal Med* 2013; **26**: 263-269 [PMID: 23020582 DOI: 10.3109/14767058.2012.733747]

145 **Indraccolo U**, Gentile G, Manfreda VM, Pomili G. The development of disseminated intravascular coagulation in hemolysis, elevated liver enzymes, and low platelet count syndrome (HELLP) at very early gestational age. *Minerva Ginecol* 2008; **60**: 445-450 [PMID: 18854811]

146 **Lubamba B**, Dhooghe B, Noel S, Leal T. Cystic fibrosis: insight into CFTR pathophysiology and pharmacotherapy. *Clin Biochem* 2012; **45**: 1132-1144 [PMID: 22698459 DOI: 10.1016/j.clinbiochem.2012.05.034]

147 **Gelfond D**, Borowitz D. Gastrointestinal complications of cystic fibrosis. *Clin Gastroenterol Hepatol* 2013; **11**: 333-342; quiz e30-e31 [PMID: 23142604 DOI: 10.1016/j.cgh.2012.11.006]

148 **Leeuwen L**, Fitzgerald DA, Gaskin KJ. Liver disease in cystic fibrosis. *Paediatr Respir Rev* 2013 Jun 13; Epub ahead of print [PMID: 23769887 DOI: 10.1016/j.prrv.2013.05.001]

149 **Bataller R**, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; **115**: 209-218 [PMID: 15690074]

150 **Brenner DA**. Molecular pathogenesis of liver fibrosis. *Trans Am Clin Climatol Assoc* 2009; **120**: 361-368 [PMID: 19768189]

151 **Kanzler S**, Lohse AW, Keil A, Henninger J, Dienes HP, Schirmacher P, Rose-John S, zum Büschenfelde KH, Blessing M. TGF-beta1 in liver fibrosis: an inducible transgenic mouse model to study liver fibrogenesis. *Am J Physiol* 1999; **276**: G1059-G1068 [PMID: 10198351]

152 **Fakih M**. The evolving role of VEGF-targeted therapies in the treatment of metastatic colorectal cancer. *Expert Rev Anticancer Ther* 2013; **13**: 427-438 [PMID: 23432698 DOI: 10.1586/era.13.20]

153 **Fagiani E**, Christofori G. Angiopoietins in angiogenesis. *Cancer Lett* 2013; **328**: 18-26 [PMID: 22922303 DOI: 10.1016/j.canlet.2012.08.018]

154 **Benedito R**, Hellström M. Notch as a hub for signaling in angiogenesis. *Exp Cell Res* 2013; **319**: 1281-1288 [PMID: 23328307 DOI: 10.1016/j.yexcr.2013.01.010]

155 **Eklund L**, Saharinen P. Angiopoietin signaling in the vasculature. *Exp Cell Res* 2013; **319**: 1271-1280 [PMID: 23500414 DOI: 10.1016/j.yexcr.2013.03.011]

156 **Bellone G**, Solerio D, Chiusa L, Brondino G, Carbone A, Prati A, Scirelli T, Camandona M, Palestro G, Dei Poli M. Transforming growth factor-beta binding receptor endoglin (CD105) expression in esophageal cancer and in adjacent nontumorous esophagus as prognostic predictor of recurrence. *Ann Surg Oncol* 2007; **14**: 3232-3242 [PMID: 17682823 DOI: 10.1245/s10434-007-9528-z]

157 **Kumar S**, Ghellal A, Li C, Byrne G, Haboubi N, Wang JM, Bundred N. Breast carcinoma: vascular density determined using CD105 antibody correlates with tumor prognosis. *Cancer Res* 1999; **59**: 856-861 [PMID: 10029075]

158 **Yu JX**, Zhang XT, Liao YQ, Zhang QY, Chen H, Lin M, Kumar S. Relationship between expression of CD105 and growth factors in malignant tumors of gastrointestinal tract and its significance. *World J Gastroenterol* 2003; **9**: 2866-2869 [PMID: 14669355]

159 **Duff SE**, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. *FASEB J* 2003; **17**: 984-992 [PMID: 12773481 DOI: 10.1096/fj.02-0634rev]

160 **Wikström P**, Lissbrant IF, Stattin P, Egevad L, Bergh A. Endoglin (CD105) is expressed on immature blood vessels and is a marker for survival in prostate cancer. *Prostate* 2002; **51**: 268-275 [PMID: 11987155 DOI: 10.1002/pros.10083]

161 **Arthur HM**, Ure J, Smith AJ, Renforth G, Wilson DI, Torsney E, Charlton R, Parums DV, Jowett T, Marchuk DA, Burn J, Diamond AG. Endoglin, an ancillary TGFbeta receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev Biol* 2000; **217**: 42-53 [PMID: 10625534 DOI: 10.1006/dbio.1999.9534]

162 **Bourdeau A**, Dumont DJ, Letarte M. A murine model of hereditary hemorrhagic telangiectasia. *J Clin Invest* 1999; **104**: 1343-1351 [PMID: 10562296 DOI: 10.1172/JCI8088]

163 **Li DY**, Sorensen LK, Brooke BS, Urness LD, Davis EC, Taylor DG, Boak BB, Wendel DP. Defective angiogenesis in mice lacking endoglin. *Science* 1999; **284**: 1534-1537 [PMID: 10348742 DOI: 10.1126/science.284.5419.1534]

164 **Allinson KR**, Carvalho RL, van den Brink S, Mummery CL, Arthur HM. Generation of a floxed allele of the mouse Endoglin gene. *Genesis* 2007; **45**: 391-395 [PMID: 17506087 DOI: 10.1002/dvg.20284]

165 **Mancini ML**, Terzic A, Conley BA, Oxburgh LH, Nicola T, Vary CP. Endoglin plays distinct roles in vascular smooth muscle cell recruitment and regulation of arteriovenous identity during angiogenesis. *Dev Dyn* 2009; **238**: 2479-2493 [PMID: 19705428 DOI: 10.1002/dvdy.22066]

166 **Bourdeau A**, Faughnan ME, McDonald ML, Paterson AD, Wanless IR, Letarte M. Potential role of modifier genes influencing transforming growth factor-beta1 levels in the development of vascular defects in endoglin heterozygous mice with hereditary hemorrhagic telangiectasia. *Am J Pathol* 2001; **158**: 2011-2020 [PMID: 11395379 DOI: 10.1016/S0002-9440(10)64673-1]

167 **Carvalho RL**, Jonker L, Goumans MJ, Larsson J, Bouwman P, Karlsson S, Dijke PT, Arthur HM, Mummery CL. Defective paracrine signalling by TGFbeta in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. *Development* 2004; **131**: 6237-6247 [PMID: 15548578 DOI: 10.1242/dev.01529]

168 **Jerkic M**, Rodríguez-Barbero A, Prieto M, Toporsian M, Pericacho M, Rivas-Elena JV, Obreo J, Wang A, Pérez-Barriocanal F, Arévalo M, Bernabéu C, Letarte M, López-Novoa JM. Reduced angiogenic responses in adult Endoglin heterozygous mice. *Cardiovasc Res* 2006; **69**: 845-854 [PMID: 16405930 DOI: 10.1016/j.cardiores.2005.11.020]

169 **Park SO**, Lee YJ, Nguyen HL, Han C, Choi EJ, Oh SP. ALK1 signaling plays a pivotal role in regulation of genes involved in angiogenesis and vascular tone: implication on the pathogenetic mechanism for hereditary hemorrhagic telangiectasia 2 (HHT2). *FASEB J* 2008; **22**: 318

170 **Dolinsek T**, Markelc B, Sersa G, Coer A, Stimac M, Lavrencak J, Brozic A, Kranjc S, Cemazar M. Multiple delivery of siRNA against endoglin into murine mammary adenocarcinoma prevents angiogenesis and delays tumor growth. *PLoS One* 2013; **8**: e58723 [PMID: 23593103 DOI: 10.1371/journal.pone.0058723]

171 **Krex D**, Ziegler A, Schackert HK, Schackert G. Lack of association between endoglin intron 7 insertion polymorphism and intracranial aneurysms in a white population: evidence of racial/ethnic differences. *Stroke* 2001; **32**: 2689-2694 [PMID: 11692035 DOI: 10.1161/hs1101.098660]

172 **Daina E**, D'Ovidio F, Sabbà C. Introduction: hereditary hemorrhagic telangiectasia as a rare disease. *Curr Pharm Des* 2006; **12**: 1171-1172 [PMID: 16611098 DOI: 0.2174/138161206776361264]

173 **Plauchu H**, de Chadarévian JP, Bideau A, Robert JM. Age-related clinical profile of hereditary hemorrhagic telangiectasia in an epidemiologically recruited population. *Am J Med Genet* 1989; **32**: 291-297 [PMID: 2729347]

174 **Porteous ME**, Burn J, Proctor SJ. Hereditary haemorrhagic telangiectasia: a clinical analysis. *J Med Genet* 1992; **29**: 527-530 [PMID: 1518020 DOI: 10.1136/jmg.29.8.527]

175 **Schulte C**, Geisthoff U, Lux A, Kupka S, Zenner HP, Blin N, Pfister M. High frequency of ENG and ALK1/ACVRL1 mutations in German HHT patients. *Hum Mutat* 2005; **25**: 595 [PMID: 15880681 DOI: 10.1002/humu.9345]

176 **Dakeishi M**, Shioya T, Wada Y, Shindo T, Otaka K, Manabe M, Nozaki J, Inoue S, Koizumi A. Genetic epidemiology of hereditary hemorrhagic telangiectasia in a local community in the northern part of Japan. *Hum Mutat* 2002; **19**: 140-148 [PMID: 11793473 DOI: 10.1002/humu.10026]

177 **Bayrak-Toydemir P**, Mao R, Lewin S, McDonald J. Hereditary hemorrhagic telangiectasia: an overview of diagnosis and management in the molecular era for clinicians. *Genet Med* 2004; **6**: 175-191 [PMID: 15266205 DOI: 10.1097/01.GIM.0000132689.25644.7C]

178 **Shovlin CL**, Hughes JM, Scott J, Seidman CE, Seidman JG. Characterization of endoglin and identification of novel mutations in hereditary hemorrhagic telangiectasia. *Am J Hum Genet* 1997; **61**: 68-79 [PMID: 9245986 DOI: 10.1086/513906]

179 **Brusgaard K**, Kjeldsen AD, Poulsen L, Moss H, Vase P, Rasmussen K, Kruse TA, Hørder M. Mutations in endoglin and in activin receptor-like kinase 1 among Danish patients with hereditary haemorrhagic telangiectasia. *Clin Genet* 2004; **66**: 556-561 [PMID: 15521985 DOI: 10.1111/j.1399-0004.2004.00341.x]

180 **Wehner LE**, Folz BJ, Argyriou L, Twelkemeyer S, Teske U, Geisthoff UW, Werner JA, Engel W, Nayernia K. Mutation analysis in hereditary haemorrhagic telangiectasia in Germany reveals 11 novel ENG and 12 novel ACVRL1/ALK1 mutations. *Clin Genet* 2006; **69**: 239-245 [PMID: 16542389 DOI: 10.1111/j.1399-0004.2006.00574.x]

181 **Paquet ME**, Pece-Barbara N, Vera S, Cymerman U, Karabegovic A, Shovlin C, Letarte M. Analysis of several endoglin mutants reveals no endogenous mature or secreted protein capable of interfering with normal endoglin function. *Hum Mol Genet* 2001; **10**: 1347-1357 [PMID: 11440987 DOI: 10.1093/hmg/10.13.1347]

182 **Lux A**, Gallione CJ, Marchuk DA. Expression analysis of endoglin missense and truncation mutations: insights into protein structure and disease mechanisms. *Hum Mol Genet* 2000; **9**: 745-755 [PMID: 10749981 DOI: 10.1093/hmg/9.5.745]

183 **Govani FS**, Shovlin CL. Hereditary haemorrhagic telangiectasia: a clinical and scientific review. *Eur J Hum Genet* 2009; **17**: 860-871 [PMID: 19337313 DOI: 10.1038/ejhg.2009.35.]

184 **Berg J**, Porteous M, Reinhardt D, Gallione C, Holloway S, Umasunthar T, Lux A, McKinnon W, Marchuk D, Guttmacher A. Hereditary haemorrhagic telangiectasia: a questionnaire based study to delineate the different phenotypes caused by endoglin and ALK1 mutations. *J Med Genet* 2003; **40**: 585-590 [PMID: 12920067 DOI: 10.1136/jmg.40.8.585]

185 **Sabbà C**, Pasculli G, Lenato GM, Suppressa P, Lastella P, Memeo M, Dicuonzo F, Guant G. Hereditary hemorrhagic telangiectasia: clinical features in ENG and ALK1 mutation carriers. *J Thromb Haemost* 2007; **5**: 1149-1157 [PMID: 17388964 DOI: 10.1111/j.1538-7836.2007.02531.x]

186 **Stepan H**, Geipel A, Schwarz F, Krämer T, Wessel N, Faber R. Circulatory soluble endoglin and its predictive value for preeclampsia in second-trimester pregnancies with abnormal uterine perfusion. *Am J Obstet Gynecol* 2008; **198**: 175.e1-175.e6 [PMID: 18226617 DOI: 10.1016/j.ajog.2007.08.052.]

187 **Abdelaziz A**, Maher MA, Sayyed TM, Bazeed MF, Mohamed NS. Early pregnancy screening for hypertensive disorders in women without a-priori high risk. *Ultrasound Obstet Gynecol* 2012; **40**: 398-405 [PMID: 22689569 DOI: 10.1002/uog.11205.]

188 **Li C**, Guo B, Wilson PB, Stewart A, Byrne G, Bundred N, Kumar S. Plasma levels of soluble CD105 correlate with metastasis in patients with breast cancer. *Int J Cancer* 2000; **89**: 122-126 [PMID: 10754488]

189 **Takahashi N**, Kawanishi-Tabata R, Haba A, Tabata M, Haruta Y, Tsai H, Seon BK. Association of serum endoglin with metastasis in patients with colorectal, breast, and other solid tumors, and suppressive effect of chemotherapy on the serum endoglin. *Clin Cancer Res* 2001; **7**: 524-532 [PMID: 11297243]
190 **Li C**, Gardy R, Seon BK, Duff SE, Abdalla S, Renehan A, O'Dwyer ST, Haboubi N, Kumar S. Both high intratumoral microvessel density determined using CD105 antibody and elevated plasma levels of CD105 in colorectal cancer patients correlate with poor prognosis. *Br J Cancer* 2003; **88**: 1424-1431 [PMID: 12778073 DOI: 10.1038/sj.bjc.6600874]
191 **Clemente M**, Núñez O, Lorente R, Rincón D, Matilla A, Salcedo M, Catalina MV, Ripoll C, Iacono OL, Bañares R, Clemente G, García-Monzón C. Increased intrahepatic and circulating levels of endoglin, a TGF-beta1 co-receptor, in patients with chronic hepatitis C virus infection: relationship to histological and serum markers of hepatic fibrosis. *J Viral Hepat* 2006; **13**: 625-632 [PMID: 16907850 DOI: 10.1111/j.1365-2893.2006.00733.x]
192 **Yu D**, Zhuang L, Sun X, Chen J, Yao Y, Meng K, Ding Y. Particular distribution and expression pattern of endoglin (CD105) in the liver of patients with hepatocellular carcinoma. *BMC Cancer* 2007; **7**: 122 [PMID: 17608955 DOI: 10.1186/1471-2407-7-122]

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REVIEW

Ceruloplasmin-ferroportin system of iron traffic in vertebrates

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Core tip: The ceruloplasmin-ferroportin system represents the main pathway for cellular iron egress in vertebrates and it is responsible for physiological regulation of cellular iron levels. This review focuses on the structural and functional features of the two proteins, with special emphasis on their coordinate regulation at the transcriptional and post-transcriptional levels.

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Abstract

Safe trafficking of iron across the cell membrane is a delicate process that requires specific protein carriers. While many proteins involved in iron uptake by cells are known, only one cellular iron export protein has been identified in mammals: ferroportin (SLC40A1). Ceruloplasmin is a multicopper enzyme endowed with ferroxidase activity that is found as a soluble isoform in plasma or as a membrane-associated isoform in specific cell types. According to the currently accepted view, ferrous iron transported out of the cell by ferroportin would be safely oxidized by ceruloplasmin to facilitate loading on transferrin. Therefore, the ceruloplasmin-ferroportin system represents the main pathway for cellular iron egress and it is responsible for physiological regulation of cellular iron levels. The most recent findings regarding the structural and functional features of ceruloplasmin and ferroportin and their relationship will be described in this review.

INTRODUCTION

The importance of iron for all eukaryotes, and particularly for humans, is well established. Iron is fundamental for the transport, storage and activation of oxygen, for electron transport and for many other important metabolic processes. It is therefore not surprising that any genetic defect leading to iron imbalance can have severe consequences on our health. The loss of regulation of iron metabolism can lead to development of iron overload as seen in hereditary hemochromatosis, a common inherited disorder which may lead to progressive organ dysfunction. Conversely, iron deficiency is typical of many pathological states, such as the anemia of chronic disease or anemia associated with inflammation. In the last fifteen years, several new genes and proteins involved in iron disorders in animal models and in humans have been identified, which has greatly improved our understanding of the molecular mechanisms of iron absorption, the regulation of iron transport and general iron homeostasis in mammals[1-3].

Table 1 List of the most relevant papers

Topic	Ref.
Fpn identification and structure	[4,6,8,9,12,14,16]
Cp structure and function	[29,34,35,39,43,45,46,49,50,52,82]
Cp/Fpn connection	[40,42,52,53]
Transcriptional regulation of Cp/Fpn	[56-58,61,62,64,68,71]
Post-transcriptional regulation of Fpn	[21,24,73]
Aceruloplasminemia	[75-78,81,85]
Fpn disease	[10,88,89,91,92,94]

Cp: Ceruloplasmin; Fpn: Ferroportin.

Serum transferrin and the almost ubiquitously expressed transferrin receptor-1 (TfR1) represent the most important system for distribution and delivery of iron to the different organs of the body. Iron delivery to the bloodstream for transferrin-dependent transport is mediated by enterocytes, which release iron absorbed from the diet, and mostly by macrophages, which recycle iron from damaged and senescent erythrocytes. These specialized cells export iron through the recently identified protein ferroportin (SLC40A1, initially also named Ireg-1 or MTP-1), the only known mammalian iron exporter[4-6]. A group of enzymes that convert Fe^{2+} to Fe^{3+} collaborates with ferroportin, facilitating iron loading onto transferrin, which binds only Fe^{3+}. These enzymes belong to the family of the blue multicopper oxidases and possess ferroxidase activity; members of this family include ceruloplasmin, hephaestin and zyklopen in mammals.

In this review the most recent findings regarding the structural and functional features of ceruloplasmin and ferroportin and their relationships will be described. A list of the most relevant papers in the field is presented in Table 1.

FERROPORTIN, STRUCTURE AND FUNCTION

Human ferroportin (Fpn) is constituted by 571 amino acids, the corresponding SLC40A1 gene is located on chromosome 2 (2q32), it spans about 20 kb and has 8 exons. Fpn has been identified in many organisms and its amino acid sequences can be easily retrieved from annotated genome projects. The protein is well conserved, with over 60% identity between distantly related proteins such as human and zebrafish Fpn, indicating a wide distribution and a critical role for Fpn. This assumption is supported by the finding that inactivation of the Fpn gene in mice is embryonically lethal[7].

Fpn is a polytopic membrane protein with a predicted 9-12 transmembrane topology. A model proposed by Liu *et al*[8] suggested that Fpn has 12 transmembrane domains. A number of studies have indicated that the N-terminus of Fpn is cytosolic[8-11]. On the other hand, the location of the C-terminus is unclear, with studies based on epitope-tagged proteins supporting the hypothesis of a cytosolic localization[8,12] and other studies claiming that the C-terminus is extracellular. In particular, Yeh *et al*[13] suggested that the presence of the epitope might affect the topology of Fpn. It should be noted, however, that epitope-tagged Fpn is fully functional with respect to transport activity and regulation.

Putative structure of human ferroportin

Most questions regarding the structure and mechanism of action of Fpn could be answered by an experimentally determined three-dimensional structure of the protein. Unfortunately, such a structure will probably not be available in the near future due to the difficulties of obtaining crystals of membrane proteins. Therefore, functional studies of Fpn mostly rely on theoretical modeling to provide a framework for analysis of Fpn wild type and mutants.

Recently, two molecular models of human Fpn based on different approaches have been reported[9,14]. Both models predict that Fpn belongs to the major facilitator superfamily (MFS) of membrane transporters. Wallace and coworkers based their model on the topology proposed by Liu *et al*[8], and confirmed the intracellular localization of both N- and C-termini. They used the structure of the glycerol-3-phosphate transporter from *E. coli* as template for building a three-dimensional model of Fpn. Using the model, they showed that all reported loss-of-function Fpn mutations localize at the membrane/cytoplasm interface, while gain-of-function mutations are largely associated with the inner channel running down the axis of Fpn (see below for details on Fpn mutations and "ferroportin disease"). They concluded that the phenotypic variability of "ferroportin disease" likely arises from the different functional consequences of the various mutations.

On the other hand, using sensitive profile-profile alignment methods, Le Gac *et al*[14] provided an alignment of Fpn with MFS proteins. Along with the crystal structure of the *E. coli* EmrD antiporter, this alignment served as a basis for the homology modeling of the three-dimensional structure of Fpn. The authors focused their attention on key functional amino acids and disease-causing mutations, and showed that their model of Fpn could be used to identify critical amino acids. In particular, they proved the involvement of a specific tryptophan residue in both the iron export function and the mechanism of inhibition by hepcidin.

Neither model gives any clue about the localization of iron binding site(s) inside Fpn. We are currently building a different structural model of human Fpn using two MFS *E. coli* proteins (manuscript in preparation). A preliminary analysis shows that the model allows to postulate the presence of a potential iron binding site in the central cavity of the protein, whose relevance can be tested through measurement of the iron export ability of wild type and mutated Fpn. A depiction of our preliminary Fpn model and of the iron binding site is shown in Figure 1.

Oligomeric state of ferroportin

The multimeric structure of Fpn is still the subject of

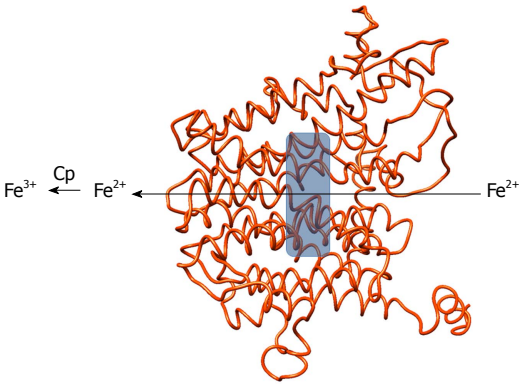

Figure 1 Structural model of human ferroportin viewed along the membrane plane. The gray box indicates the location of a putative iron-binding site, ferrous iron flows through the protein from the cell interior and is then oxidized by ceruloplasmin at the extracellular side. The figure was produced with Chimera[96].

much debate, with reports demonstrating that the protein is dimeric[10,12,15] while other studies have suggested that it is a monomer[11,16-19]. Most of the studies addressing the oligomeric state of Fpn have relied on the use of recombinant Fpn tagged with different epitopes. The techniques employed are mainly (but not only) co-immunoprecipitation, gel-filtration chromatography and cross-linking. Evaluation of the effect of co-transfection of wild type and mutant Fpn on iron export function and subcellular localization has also been taken into consideration[10,15,16,18,19]. Conflicting results on the multimeric structure of Fpn obtained by the methods outlined above can have many explanations: the efficiency of co-immunoprecipitation can depend on the tags (and antibodies) or the experimental conditions imposed on the cell lysates. For instance, different groups have reported that it is possible to co-immunoprecipitate Fpn-GFP and Fpn-flag while co-precipitation of Fpn-flag and Fpn-myc was less reproducible. Also, high expression levels of recombinant Fpn could be in part responsible for reported discrepancies. Some negative results obtained with different cross-linkers might be explained by the chemical features of the reagent (*i.e.*, group reactivity and spacer arm length), which can be suboptimal. Similarly, negative results obtained by fusion of Fpn to fluorescent/luminescent protein tags to exploit FRET or BRET do not necessarily imply the lack of Fpn dimers because these techniques are highly dependent on close spatial proximity of the probes. The most convincing evidence that Fpn is dimeric comes from cross-linking of endogenous Fpn in rat glioma C6 cells and bone marrow-derived macrophages, which resulted in doubling of the molecular mass of the protein[12]. This experimental set-up circumvents the possibility of artifacts due to the presence of the tags and/or overexpression of Fpn. In any case, the strength of the interaction between monomers appears to be quite low because differently tagged Fpn expressed separately and mixed after detergent-extraction from the lipid bilayer do not co-immunoprecipitate[10,12]. Multimerization of Fpn is particularly attractive to explain the dominant inheritance of "ferroportin disease" (see below).

Ferroportin and hepcidin

Fpn is the receptor for hepcidin, a peptide of 25 amino acids forming a bent β-hairpin stabilized by four disulfide bonds. Inflammatory states and/or increased iron stores trigger the hepatic synthesis of the peptide[20]. Binding of hepcidin to Fpn leads to the internalization and degradation of Fpn, resulting in impaired iron export[21].

Conflicting reports have been published on the molecular mechanism of hepcidin-induced Fpn degradation. In particular, there is no agreement on the possible phosphorylation by JAK2 kinase of two tyrosine residues on Fpn in hepcidin-triggered internalization of the protein[22,23]. On the other hand, Fpn is certainly ubiquitinated on lysine residues before degradation[23,24]. The hepcidin binding site has been identified on the extracellular loop of Fpn containing cysteine in position 326[25]. Cells expressing the C326S mutant Fpn export iron normally but do not bind the peptide and export iron even in the presence of hepcidin[26]. Modeling of the hepcidin-Fpn interaction suggested that Cys326 is involved in a thiol-dependent interaction with hepcidin, perhaps involving the disulfide framework of hepcidin, while Phe324 and Tyr333 may form crucial contacts with two phenylalanine residues on the hepcidin moiety[27].

CERULOPLASMIN, STRUCTURE AND FUNCTION

Structure of ceruloplasmin and of its copper binding sites

Ceruloplasmin (Cp) is an enzyme, ubiquitous among vertebrates, that belongs to the family of the multicopper oxidases. Members of this family posse multiple copper sites that can be classified, on the basis of their spectroscopic properties, in type 1, type 2 and type 3 sites[28]. Human Cp is constituted by 1046 amino acids; the *Cp* gene maps on chromosome 3 (3q23-q24), it spans about 65 kb and it is organized in 20 exons. Determination of the three-dimensional structure of Cp[29,30] has shown that this enzyme is made up of six domains arranged in a ternary symmetry. Domains 1 and 2, 3 and 4, and 5 and 6 interact with each other through extensive, highly packed hydrophobic interfaces, while polar interactions and loosely packed interfaces are observed between domains 2 and 3 and 4 and 5. Three of the six domains (domains 2, 4 and 6) bind a type 1 blue copper coordinated by nitrogen and sulphur ligands, supplied by histidine and cysteine residues arranged in tetrahedral geometry with an axial methionine ligand, which is absent in the type 1 site of domain 2.

Three more copper ions are coordinated by eight histidine ligands at the interface between domain 1 and 6. The latter copper ions represent the trinuclear cluster formed by two antiferromagnetically coupled type 3 and one type 2 copper ions. The oxidation of substrates is coupled to the reduction of oxygen to water in a mecha-

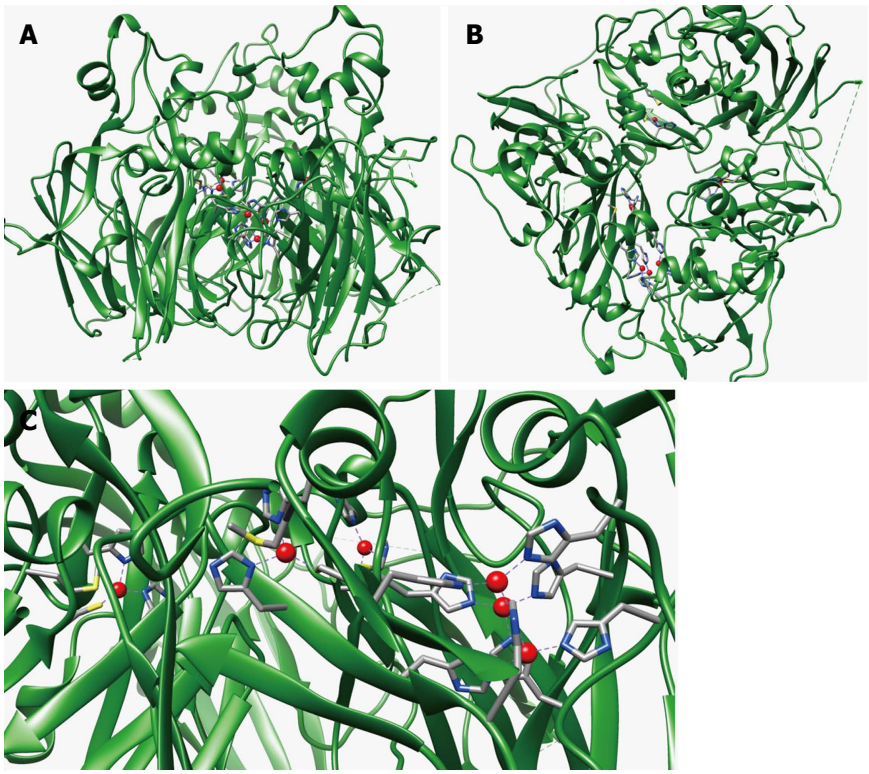

Figure 2 Structure of human ceruloplasmin. Overall structure of the protein (PDB 1KCW) in two orientations (A: Side view and B: Bottom view), the copper atoms are shown as red spheres, the side chains of the copper ligands are represented as sticks; C: Close-up view of the type 1 and trinuclear cluster catalytic copper binding sites. The figure was produced with Chimera[96].

nism involving electron transfer from the type 1 copper sites, the primary sites of substrate oxidation, to the trinuclear cluster, where oxygen binds and is reduced in a controlled way, *i.e.,* without release of potentially toxic intermediates (O_2^-, H_2O_2). While electron entrance at type 1 copper sites in domains 4 and 6 is established, the role of the blue copper ion in domain 2 is less clear. In fact, there is no experimental evidence from crystallographic data that reducing substrates can bind in domain 2. Moreover, site-directed mutagenesis at this copper site failed to modify either the spectroscopic or catalytic properties of the protein[31]. Thus, the blue copper ion in domain 2 could be an "evolutionary relic" or, alternatively, it could serve for still unknown other functions. Figure 2 reports the structure of human Cp and the localization of its copper sites.

Beside copper, other metals have been proposed to bind to Cp. In particular, refined crystallographic data showed an extra metal-binding site in domain 1, likely filled with a calcium ion. The finding of a calcium binding site is consistent with a previous study from our laboratory showing that human and sheep Cp bind divalent ions, and that this could be exploited in a one-step purification protocol based on the affinity of the protein for calcium ions[32].

Physiological role of ceruloplasmin

Cp is mainly synthesized by hepatocytes, where the P-type ATPase ATP7B incorporates copper into apo-Cp during transit through the trans-Golgi network[33], and secreted into the plasma where it is found at micromolar concentration. The molecular mechanism of copper loading of Cp by ATP7B is still unknown. Inspection of the structure of Cp shows that large solvent exposed loops connect the six domains of Cp. Despite a low degree of sequence homology, all these loops start with a C-X-R/K motif, with the cysteine residue stabilizing the loop by forming a disulfide bridge. Our recent work indicates that the basic residues of the five loops connecting the six domains of Cp, and the disulfide bridges that stabilize the loops, are required for proper copper loading by ATP7B[34].

A GPI-anchored form of Cp was initially identified on the plasma membrane of astrocytes[35] and leptomeningeal cells[36] in the CNS, in Sertoli cells[37] and in the retina[38]. Synthesis of this isoform is *via* alternative splicing of exons 19 and 20 where the last 5 amino acids are replaced by 30 alternative residues leading to addition of the GPI anchor[39]. More recently Cp-GPI has been detected also in macrophages[40], immune cells and hepatocytes[41] and in many other tissues[42], indicating a wider than anticipated distribution of this isoform.

Despite the knowledge of the details of the three-dimensional structure, the true biological function of Cp has been the subject of much debate mainly because Cp is a rather promiscuous enzyme, as regards the multitude of substrates it can act on and the possibility that copper bound to sites other than the active site can give rise to accessory activities. In fact, several functions have been attributed to Cp, ranging from copper transport to ferrous iron and biological amines oxidation, as well as antioxidant activity *via* prevention of the formation of free radicals in serum[43]. Conversely, pro-oxidant activity leading to LDL oxidation has also been attributed to Cp due to the presence of a seventh copper atom that is bound to a site unrelated to the active site[44]. However, among

various substrates, the enzyme displays the highest affinity for ferrous ions and a role for Cp in iron metabolism had been proposed as early as in 1966[45]. The study of the ferroxidase activity of Cp evidenced two K_m values which differ by approximately two orders of magnitude (K_{m1} 0.6 μmol/L and K_{m2} 50 μmol/L) and binding of Fe^{2+} in the vicinity of type 1 copper sites has been demonstrated by X-ray diffraction studies, soaking crystals of Cp with Fe^{2+}[46]. Cp is thought to promote iron release from cells, facilitating loading of the metal onto transferrin, which only binds Fe^{3+}. An important point regarding the ferroxidase activity of Cp is that Fe^{2+} readily oxidizes, at physiological pH, even in the absence of a protein catalyst. However, spontaneous oxidation of Fe^{2+} is potentially dangerous as it triggers the formation of oxygen radicals *via* Fenton chemistry. Thus ferroxidation by Cp would prevent iron-induced oxidative stress.

An increasing body of evidence supports earlier work[47,48] and points to an essential role for Cp in iron metabolism (and specifically in iron efflux from cells) *via* its ferroxidase activity. Stimulation of iron release from macrophages by Cp in the presence of apotransferrin and hypoxia has been demonstrated[49]. Targeted Cp gene disruption in mouse evidenced a striking impairment in the movement of iron out of reticuloendothelial cells and hepatocytes[50]. Moreover, increased deposition of iron in several regions of the CNS was noted in $Cp^{-/-}$ mice[51], and Cp-GPI was found to be required for iron efflux from astrocytes[52]. In addition, individuals carrying a defective gene coding for Cp, thus suffering from aceruloplasminemia, show normal copper homeostasis but present a severely impaired iron metabolism.

CERULOPLASMIN-FERROPORTIN CONNECTION

Ceruloplasmin is essential for ferroportin stability

The essential role of the ferroxidase activity of Cp in iron release from cells was attributed to facilitation of loading of the metal onto transferrin, which only binds Fe^{3+}. However, a new molecular connection between Cp and Fpn has been established by the finding that ferroxidase activity is required to stabilize Fpn at the cell surface in cells expressing Cp-GPI[40]. Thus, Cp can be considered as a second determinant of Fpn stability after hepcidin (Figure 3). As described in detail below, ferroxidase active Cp stabilizes Fpn at the plasma membrane supporting iron export (Figure 3A); on the other hand, absence of Cp or presence of an inactive Cp lead to degradation of Fpn in specific cell types (Figures 3B, C); hepcidin induces internalization and degradation of Fpn also if Cp is present (Figure 3D), unless hepcidin levels are very low. It is worth noting that removal of Fpn from the plasma membrane appears to be the only means to 'turn off' iron export from the cell because no inhibitor of Fpn is known.

The starting point was the observation that loss of Cp-GPI either by gene silencing or by incubation of rat C6 glioma cells and bone marrow macrophages with the copper chelator BCS led to disappearance of Fpn from the cell surface. Fpn was rapidly internalized and degraded in the absence of Cp-GPI. Addition of exogenous Cp or of the yeast ferroxidase Fet3p or of an iron chelator such as BPS or DFO, restored Fpn at the cell surface in cells silenced for Cp-GPI. The activity of the ferroxidase or the presence of the iron chelator were essential to lower the concentration of extracellular Fe^{2+} establishing an iron gradient and promoting removal of the metal from Fpn. In the absence of Cp-GPI, radioactive ^{59}Fe remained associated with Fpn and the protein was found to be ubiquitinated on Lys253. It can be hypothesized that a conformational state of Fpn with bound iron is recognized by a specific ubiquitin ligase, triggering degradation of the transporter. The requirement for a ferroxidase to maintain iron transport appears specific to cells that express Cp-GPI, because transfected Fpn is stable in many cell lines that do not express this isoform of Cp. In this respect, this new function of Cp is particularly relevant for brain iron metabolism because any factor affecting the ferroxidase activity of Cp-GPI cannot be compensated by circulating plasma Cp, which is unable to cross the blood-brain barrier. Iron uptake by endothelial cells of the blood-brain barrier takes place through the Tf-TfR1 system, how the metal is then moved out of these cells and taken up by CNS cells is still unclear. Recent data indicate that iron efflux from brain microvasculature endothelial cells is mediated by Fpn and requires the action of a ferroxidase, which can be either endogenous hephaestin or extracellular Cp[53]. These findings highlight once again the importance of ferroxidases for correct cellular iron management. Astrocytes are in close contact with the abluminal surface of capillary endothelial cells and therefore are ideally positioned to control the transport of metabolites between the blood and the neuropil. Since astrocytes are able to take up and release iron, they have been proposed to be largely responsible for distributing iron in the brain[54]. Therefore, Fpn and Cp-GPI would represent the central system for release of iron from astrocytes to meet the requirements of neurons and other brain cells.

A physical interaction between Cp and Fpn has not been evidenced despite many efforts; however, it has been reported that Cp is able to partially prevent hepcidin-induced internalization of Fpn when cells are treated with 0.15 μmol/L hepcidin[42]. This finding could be taken as an indication that Cp can compete with hepcidin for binding to Fpn, suggesting that probably such interaction exists but it is transient and/or too weak to be detected. A direct consequence of this hypothesis is that the Cp-binding site on Fpn would partially overlap with the hepcidin-binding site. An alternative explanation would be that Cp interacts with hepcidin, making the peptide unavailable for binding to Fpn.

Transcriptional regulation of the ceruloplasmin-ferroportin system

The Cp-Fpn functional connection is strengthened also

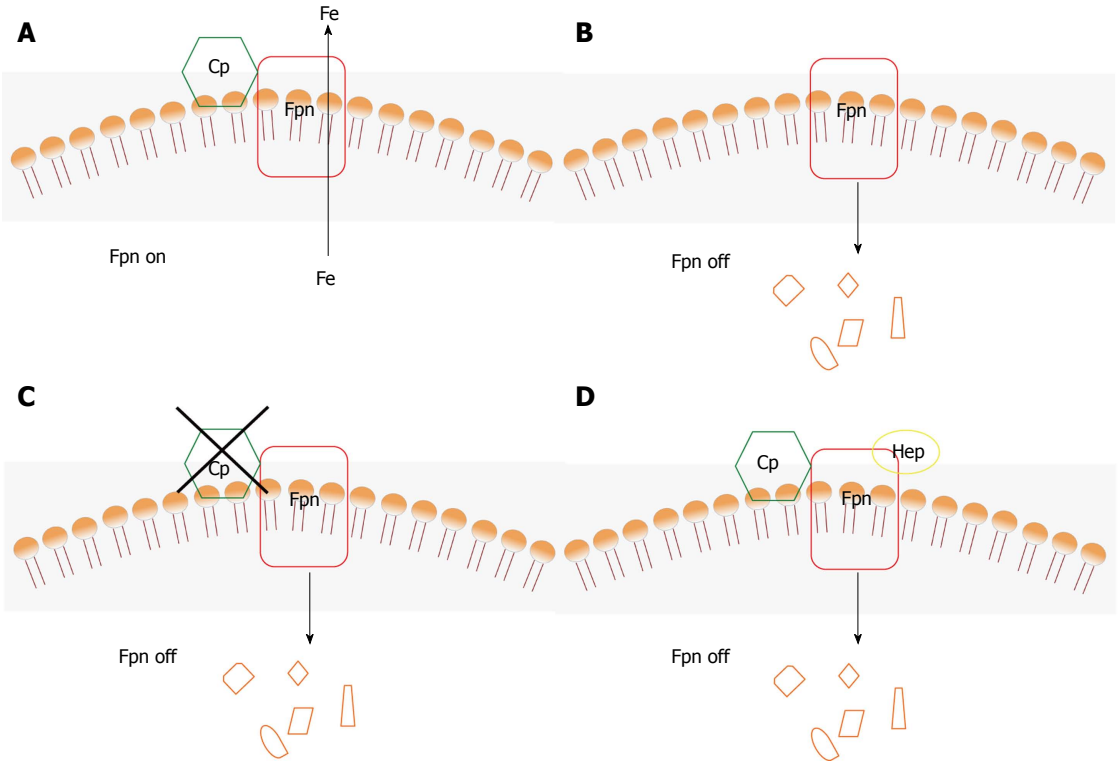

Figure 3 Scheme of the role of ceruloplasmin and hepcidin in the degradation of ferroportin. A: In the presence of Cp, Fpn is stable and exports iron; B: In the absence of Cp, Fpn is degraded; C: In the presence of inactive Cp, Fpn is degraded; D: In the presence of Cp and hepcidin, Fpn is degraded. Cp: Ceruloplasmin; Fpn: Ferroportin.

by the finding that expression of the two proteins can be coordinately regulated in specific cell types.

Cp was recognized to be an acute phase protein many years ago, and it is known to be induced in response to pro-inflammatory stimuli, such as IL-1β[55-57], INF-γ[58] and IL-6[59]. Recent data demonstrate that IL-6 mediates induction of Cp *via* the transcription factor FOXO1[60]. Metal-dependent regulation of Cp has not been conclusively assessed, although indirect effects of iron deficiency mediated by hypoxia-inducible factor-1 (HIF-1) have been reported[61].

Expression of Fpn is regulated by different stimuli: iron and transition metals, heme, hypoxia and inflammation among others. Many studies have highlighted a tissue-specific regulation of expression of Fpn and point to Fpn regulation by systemic rather than local signals of iron status. Actually, two layers of regulation are active to control Fpn: one at the level of mRNA (transcriptional and post-transcriptional) and one at the level of the protein (hepcidin-dependent and hepcidin-independent internalization and degradation). Moreover, any factor affecting hepcidin synthesis in turn will affect Fpn protein levels.

The Fpn promoter contains different response elements sensitive to hypoxia, heme/oxidative stress and metals. The presence of HIF-Responsive-Elements was evidenced using Fpn reporter constructs and HIF2α was demonstrated to be a direct activator of Fpn transcription[62]. It is worth noting that HIF2α expression has recently been shown to depend on IRP1[63], strengthening the link between iron and hypoxia. Metal-Responsive-Element induction of Fpn mediated by the transcription factor MTF-1 in response to zinc was recently demonstrated[64]. Antioxidant-Responsive-Elements enable up-regulation of Fpn transcription in response to heme *via* activation of the redox-sensitive transcription factor Nrf2 in mouse and human macrophages[65,66]. Other studies indicated that heme-induced Fpn transcription required the release of iron from heme[67]. Ultimately, these results link transcriptional control of Fpn synthesis directly and indirectly to iron levels: *i.e.*, iron is crucial for HIF2α stability and IRP1-mediated expression, iron mediates oxidative stress and activation of Nrf2.

Fpn is down-regulated by pro-inflammatory cytokines in reticuloendothelial cells, as demonstrated by the finding that treatment with IFN-γ and LPS reduced Fpn mRNA and iron release from monocytes[68,69]. Fpn mRNA and protein levels were also found to decrease significantly in astrocytes treated with LPS but not with IL-6 or TNF-α[70]. Interestingly, we have found that in rat C6 glioma cells Cp and Fpn are up-regulated by IL-1β, suggesting that the response of Fpn to cytokines might be tissue-specific[57]. The expression of Cp and Fpn in response to IL-1β requires the activation of MAP kinase pathways as a consequence of IL-1β receptor stimulation. Moreover, we have observed that IL-1β regulates the expression of Cp and Fpn genes through (1) p38 MAPK-mediated activation of C/EBP transcription factor; (2) ERK1/2-, JNK1- and partially p38 MAPK-dependent activation of AP-1; and (3) activation of NF-κB partially mediated by p38 MAPK[71]. A similar pathway was found

to activate Fpn expression in response to the isoflavone genistein[72]. In this case, p38 MAPK activation was found to be triggered by activation of the estrogen receptor β.

Post-transcriptional regulation of ferroportin

At the post-transcriptional level, Fpn expression is regulated by iron-responsive sequences both at the 5' UTR and at the 3' UTR. Repression of Fpn mRNA translation in conditions of iron deficiency was shown to be mediated by the well-characterized IRE/IRP system, due to the presence of an IRE sequence at the 5' UTR. Also the 3' UTR of Fpn plays a role in post-transcriptional regulation of expression through a recently discovered miRNA-dependent mechanism. microRNAs are small non-coding RNAs that bind the 3' UTR of target mRNAs driving translational repression or mRNA degradation. In particular, it has been demonstrated that miR-485-3p is induced during iron deficiency and it targets the 3' UTR of Fpn to reduce iron export in several cell lines and primary macrophages[73]. In duodenal and erythroid precursor cells alternative splicing produces an isoform of Fpn lacking the 5' IRE indicating that these cells can evade IRE/IRP-dependent translational repression[74] becoming sensitive to systemic rather than local (intracellular) cues. It would be interesting to evaluate whether miR-485-3p is expressed in these cell types and this isoform of Fpn is subject to miRNA-mediated control.

CERULOPLASMIN-FERROPORTIN SYSTEM AND PATHOLOGY

The importance of the ceruloplasmin-ferroportin system is highlighted by the fact that mutations in the Cp and Fpn genes lead to severe consequences. Impairment of the Cp-Fpn system is common to aceruloplasminemia and "ferroportin disease", two genetic diseases that share a common phenotype of iron overload.

Aceruloplasminemia

Aceruloplasminemia is a rare autosomal disease caused by mutations in the *Cp* gene[75,76]. Approximately forty mutations of the *Cp* gene have been so far described, including frameshift, nonsense and missense mutations[77,78]. Heterozygous individuals have partial Cp deficiency with normal iron metabolism and no clinical symptoms, with some exceptions. Homozygotes present iron overload mainly in the brain, but also in liver, pancreas and retina. Patients develop retinal degeneration, diabetes mellitus and neurological symptoms, which include ataxia, involuntary movements and dementia. Onset of clinical manifestations usually occurs in adulthood. Laboratory findings include absence of serum Cp ferroxidase activity (although low levels of Cp protein were reported in some cases), low transferrin saturation, high serum ferritin and moderate anemia; magnetic resonance imaging of the brain shows iron deposits in the basal ganglia, striatum, thalamus and dentate nucleus. These features place aceruloplasminemia in the group of disorders known as NBIA (neurodegeneration with brain iron accumulation), clearly distinguishing it from hereditary hemochromatosis (serum iron is high and the brain is usually not affected) and from disorders of copper metabolism, Menkes and Wilson disease, that are also characterized by low/absent serum Cp ferroxidase activity because of impaired functioning of copper ATPases ATP7A and ATP7B, respectively[33].

Iron-mediated oxidative stress has been shown to contribute to tissue injury and neuronal cell death in aceruloplasminemia. In particular, it has been suggested that astrocytes, which are the most affected cell type, accumulate iron and die from iron toxicity, while neuronal loss would be secondary to loss of metabolic support provided by astrocytes[79,80].

The ferroxidase activity of Cp-GPI plays a critical role in the targeting of Fpn to the plasma membrane in astrocytes and bone marrow-derived macrophages[40]. Thus, brain iron overload and low serum iron levels observed in aceruloplasminemia patients can be explained by impaired iron export from these cell types due to lack of active Cp. On the other hand, the origin of iron overload in liver and pancreas, which is observed in aceruloplasminemia patients has still to be clarified.

Actually, the situation is even more complicated. In fact, while it is obvious that frameshift and nonsense mutations produce a truncated non-functional Cp, *in vitro* characterization of missense mutants yielded some unexpected findings. The first mutants to be studied invariably lacked ferroxidase activity either due to retention in the endoplasmic reticulum (P177R) or to production as apo-Cp lacking copper (D58H, G631R Q692K and G969S), due to structural or folding defects[81-84]. Indeed, residue Pro177 is found in a hydrophobic pocket, while residues Gly631, Gln692 and Gly969 are close to type 1 copper sites, suggesting that substitutions in these positions can affect folding and copper binding. Residue Asp58 is located on the protein surface and it has been suggested that substitution with histidine could cause aberrant incorporation of copper. However, another set of mutants (I9F, Q146E, F198S, W264S, A331D, G606E, G876A) that we characterized based on their ability to stabilize Fpn on the plasma membrane of rat C6 glioma cells silenced for endogenous Cp-GPI, revealed that they were partly or fully functional[85]. Also other studies showed that some mutants (Y356H, G876A) appeared to partly retain ferroxidase activity, but were less efficient than wild type Cp in protecting Fpn from hepcidin[42]. In these cases, inspection of the structure of Cp suggests that the position of the mutations is such that the protein can retain ferroxidase activity.

A quite different scenario was apparent for mutant R701W, which has been found in a very young heterozygous patient with severe extrapyramidal movement coordination deficit[86]. Both isoforms of Cp R701W (secreted and GPI-anchored) were inactive due to lack of copper, and dominant over wild type Cp in glioma cells. Moreover, they induced dispersal of the Golgi apparatus and

"functional silencing" of ATP7B[85]. Of note, Cp R701W could load copper in appropriate conditions, in particular when Ccc2p, the yeast homologue of ATP7B, was co-expressed. The resulting holo-Cp R701W was fully functional with respect to stabilization of Fpn[85]. It was reported that Cp R701W expressed in HeLa cells retained some oxidase activity but it was unable to stabilize Fpn at the cell surface[42], raising the possibility that a threshold level of activity might be required to observe this stabilizing effect. Further investigations have demonstrated that Cp R701W caused massive production of reactive oxygen (ROS) species in the cell. Scavenging ROS production with different antioxidants, such as N-acetyl-cysteine, glutathione and zinc, restored Golgi morphology and rescued Fpn on the cell membrane[87]. Whether ROS are produced directly by Cp R701W or by other cellular systems such as NOX, remains to be established. Residue Arg701 is found in the surface-exposed loop connecting domains 4 and 5 of Cp and it is difficult to understand why replacement with tryptophan should cause such a dramatic phenotype.

Ferroportin disease

Hemochromatosis is the most common genetic iron overload disease, it is inherited recessively and it is caused by defects of genes (*HFE, TfR2, HJV, HAMP*) that ultimately lead to inefficient synthesis of hepcidin. Fpn missense mutations are responsible for a different form of hemochromatosis which exhibits autosomal dominant inheritance with rather heterogeneous phenotypes, the so-called "ferroportin disease"[88]. Decreased function of Fpn appears to be limiting for macrophage iron export but not for intestinal iron export, due to the very different amounts of the metal mobilized by enterocytes (1-2 mg/d) compared to reticuloendothelial cells (20-30 mg/d). Fpn missense mutants can give rise to two different phenotypes: iron overload in macrophages and low serum transferrin saturation due to mutants that are transport incompetent or are not correctly targeted to the plasma membrane (loss-of-function mutants); hepatocyte iron overload and high serum transferrin saturation due to mutants that are unable to respond to hepcidin (gain-of-function mutants)[89,90]. Most of the mutations identified so far appear to lead to loss-of-function of Fpn, affecting plasma membrane localization of the protein and (less commonly) iron export function.

Many studies on the molecular features of the Fpn mutants have attempted to correlate mutation with phenotype. However, such analyses are complicated by difficulties in establishing a satisfactory experimental model. In most cases, recombinant Fpn mutants have been overexpressed in HEK293T or polarized MDCK cells. Subcellular localization is determined by employing Fpn-GFP fusions, Fpn function is investigated by analyzing hepcidin-induced internalization and by assessing intracellular iron levels. Conflicting results have been reported for some Fpn mutants, possibly due to the different experimental systems and conditions employed. For example, expression of Fpn in polarized MDCK cells resulted primarily in plasma membrane localization for all 16 mutants examined[11], compared to nonpolarized HeLa or HEK293T cells where some intracellular staining was apparent but could be eliminated by treatment with cycloheximide. Discrepancies in hepcidin resistance can probably be attributed to differences in hepcidin concentration and time of incubation, such that partial resistance at low (0.4-0.7 μmol/L) hepcidin concentration[9,10,91,92] can become sensitivity at high (2 μmol/L) hepcidin concentration[11]. Also, if a mutant is found to be predominantly intracellular, impaired iron export or hepcidin-resistance would simply reflect unavailability of Fpn at the plasma membrane and not a true property of the mutant protein.

Resistance to hepcidin can derive from different mechanisms: mutation of residues belonging to the hepcidin-binding site (C326Y/S and S338R) or impairment of the mechanism of internalization of Fpn (Y64N, N144H/D/T)[26]. Mutation of other residues (G204S, Y501C, H507R) has been reported to result in hepcidin resistance[93-95], suggesting that the hepcidin-binding site is probably formed by residues belonging to more that one extracellular loop of Fpn.

Other mutations impact the iron transport function of Fpn for as yet unidentified reasons (I152F). In summary, it is evident that the difficulties of working *in vitro* with Fpn make it tricky to unequivocally link patient phenotype to molecular defects of Fpn. This is further complicated by phenotypic heterogeneity among patients carrying the same Fpn mutation[93], suggesting that modifier genes might influence the penetrance of the disease.

CONCLUSION

Less than fifteen years have passed from the initial discovery of Fpn and a huge amount of information has been gained on this elusive protein. However, many questions still require an answer regarding our understanding of the structure and function of Fpn and the full implications of the connection between Fpn and Cp. Fpn is predicted to belong to the MFS transporters that function with an alternate "inward open-outward open" mechanism, involving extensive conformational changes to translocate their substrate across the membrane. The molecular details of how Fpn works are still a mystery, it is also unknown if transport of iron is coupled to other ions (either as symport or antiport). Why does Cp stabilize Fpn only in specific cell types is not clear.

Future studies should be aimed at addressing these and many other questions, in order to gain a better understanding of how Fpn and Cp collaborate for correct iron handling by cells.

REFERENCES

1 **Hentze MW**, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell* 2010; **142**: 24-38 [PMID: 20603012 DOI: 10.1016/

j.cell.2010.06.028]
2 **Pantopoulos K**, Porwal SK, Tartakoff A, Devireddy L. Mechanisms of mammalian iron homeostasis. *Biochemistry* 2012; **51**: 5705-5724 [PMID: 22703180 DOI: 10.1021/bi300752r]
3 **Ganz T**. Systemic iron homeostasis. *Physiol Rev* 2013; **93**: 1721-1741 [PMID: 24137020 DOI: 10.1152/physrev.00008.2013]
4 **Abboud S**, Haile DJ. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J Biol Chem* 2000; **275**: 19906-19912 [PMID: 10747949 DOI: 10.1074/jbc.M000713200]
5 **Donovan A**, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A, Law TC, Brugnara C, Lux SE, Pinkus GS, Pinkus JL, Kingsley PD, Palis J, Fleming MD, Andrews NC, Zon LI. Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* 2000; **403**: 776-781 [PMID: 10693807 DOI: 10.1038/35001596]
6 **McKie AT**, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F, Hediger MA, Hentze MW, Simpson RJ. A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Mol Cell* 2000; **5**: 299-309 [PMID: 10882071 DOI: 10.1016/S1097-2765(00)80425-6]
7 **Donovan A**, Lima CA, Pinkus JL, Pinkus GS, Zon LI, Robine S, Andrews NC. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metab* 2005; **1**: 191-200 [PMID: 16054062 DOI: 10.1016/j.cmet.2005]
8 **Liu XB**, Yang F, Haile DJ. Functional consequences of ferroportin 1 mutations. *Blood Cells Mol Dis* 2005; **35**: 33-46 [PMID: 15935710 DOI: 10.1016/j.bcmd.2005.04.005]
9 **Wallace DF**, Harris JM, Subramaniam VN. Functional analysis and theoretical modeling of ferroportin reveals clustering of mutations according to phenotype. *Am J Physiol Cell Physiol* 2010; **298**: C75-C84 [PMID: 19846751 DOI: 10.1152/ajpcell.00621.2008]
10 **De Domenico I**, Ward DM, Nemeth E, Vaughn MB, Musci G, Ganz T, Kaplan J. The molecular basis of ferroportin-linked hemochromatosis. *Proc Natl Acad Sci USA* 2005; **102**: 8955-8960 [PMID: 15956209 DOI: 10.1073/pnas.0503804102]
11 **Rice AE**, Mendez MJ, Hokanson CA, Rees DC, Björkman PJ. Investigation of the biophysical and cell biological properties of ferroportin, a multipass integral membrane protein iron exporter. *J Mol Biol* 2009; **386**: 717-732 [PMID: 19150361 DOI: 10.1016/j.jmb.2008.12.063]
12 **De Domenico I**, Ward DM, Musci G, Kaplan J. Evidence for the multimeric structure of ferroportin. *Blood* 2007; **109**: 2205-2209 [PMID: 17077321 DOI: 10.1182/blood-2006-06-032516]
13 **Yeh KY**, Yeh M, Glass J. Interactions between ferroportin and hephaestin in rat enterocytes are reduced after iron ingestion. *Gastroenterology* 2011; **141**: 292-299, 299.e1 [PMID: 21473866 DOI: 10.1053/j.gastro.2011.03.059]
14 **Le Gac G**, Ka C, Joubrel R, Gourlaouen I, Lehn P, Mornon JP, Férec C, Callebaut I. Structure-function analysis of the human ferroportin iron exporter (SLC40A1): effect of hemochromatosis type 4 disease mutations and identification of critical residues. *Hum Mutat* 2013; **34**: 1371-1380 [PMID: 23784628 DOI: 10.1002.humu.22369]
15 **McGregor JA**, Shayeghi M, Vulpe CD, Anderson GJ, Pietrangelo A, Simpson RJ, McKie AT. Impaired iron transport activity of ferroportin 1 in hereditary iron overload. *J Membr Biol* 2005; **206**: 3-7 [PMID: 16440176 DOI: 10.1007/s00232-005-0768-1]
16 **Gonçalves AS**, Muzeau F, Blaybel R, Hetet G, Driss F, Delaby C, Canonne-Hergaux F, Beaumont C. Wild-type and mutant ferroportins do not form oligomers in transfected cells. *Biochem J* 2006; **396**: 265-275 [PMID: 16457665 DOI: 10.1042/BJ20051682]
17 **Pignatti E**, Mascheroni L, Sabelli M, Barelli S, Biffo S, Pietrangelo A. Ferroportin is a monomer in vivo in mice. *Blood Cells Mol Dis* 2006; **36**: 26-32 [PMID: 16380275 DOI: 10.1016/j.bcmd.2005.11.001]
18 **Schimanski LM**, Drakesmith H, Talbott C, Horne K, James JR, Davis SJ, Sweetland E, Bastin J, Cowley D, Townsend AR. Ferroportin: lack of evidence for multimers. *Blood Cells Mol Dis* 2008; **40**: 360-369 [PMID: 17977032 DOI: 10.1016/j.bcmd.2007.09.007]
19 **Song G**, Jiang Q, Xu T, Liu YL, Xu ZG, Guo ZY. A convenient luminescence assay of ferroportin internalization to study its interaction with hepcidin. *FEBS J* 2013; **280**: 1773-1781 [PMID: 23413836 DOI: 10.1111/febs.12192]
20 **Ganz T**, Nemeth E. Iron imports. IV. Hepcidin and regulation of body iron metabolism. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G199-G203 [PMID: 16407589 DOI: 10.1152/ajpgi.00412.2005]
21 **Nemeth E**, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, Ganz T, Kaplan J. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 2004; **306**: 2090-2093 [PMID: 15514116 DOI: 10.1126/science.1104742]
22 **De Domenico I**, Lo E, Ward DM, Kaplan J. Hepcidin-induced internalization of ferroportin requires binding and cooperative interaction with Jak2. *Proc Natl Acad Sci USA* 2009; **106**: 3800-3805 [PMID: 19234114 DOI: 10.1073/pnas.0900453106]
23 **Qiao B**, Sugianto P, Fung E, Del-Castillo-Rueda A, Moran-Jimenez MJ, Ganz T, Nemeth E. Hepcidin-induced endocytosis of ferroportin is dependent on ferroportin ubiquitination. *Cell Metab* 2012; **15**: 918-924 [PMID: 22682227 DOI: 10.1016/j.cmet.2012.03.018]
24 **De Domenico I**, Ward DM, Langelier C, Vaughn MB, Nemeth E, Sundquist WI, Ganz T, Musci G, Kaplan J. The molecular mechanism of hepcidin-mediated ferroportin down-regulation. *Mol Biol Cell* 2007; **18**: 2569-2578 [PMID: 17475779 DOI: 10.1091/mbc.E07-01-0060]
25 **De Domenico I**, Nemeth E, Nelson JM, Phillips JD, Ajioka RS, Kay MS, Kushner JP, Ganz T, Ward DM, Kaplan J. The hepcidin-binding site on ferroportin is evolutionarily conserved. *Cell Metab* 2008; **8**: 146-156 [PMID: 18680715 DOI: 10.1016/j.cmet.2008.07.002]
26 **Fernandes A**, Preza GC, Phung Y, De Domenico I, Kaplan J, Ganz T, Nemeth E. The molecular basis of hepcidin-resistant hereditary hemochromatosis. *Blood* 2009; **114**: 437-443 [PMID: 19383972 DOI: 10.1182/blood-2008-03-146134]
27 **Preza GC**, Ruchala P, Pinon R, Ramos E, Qiao B, Peralta MA, Sharma S, Waring A, Ganz T, Nemeth E. Minihepcidins are rationally designed small peptides that mimic hepcidin activity in mice and may be useful for the treatment of iron overload. *J Clin Invest* 2011; **121**: 4880-4888 [PMID: 22045566 DOI: 10.1172/JCI57693]
28 **Solomon EI**, Sundaram UM, Machonkin TE. Multicopper Oxidases and Oxygenases. *Chem Rev* 1996; **96**: 2563-2606 [PMID: 11848837 DOI: 10.1021/cr950046o]
29 **Zaitseva I**, Zaitsev V, Card G, Moshkov K, Bax B, Ralph A, Lindley P. The X-ray structure of human serum ceruloplasmin at 3.1Å: nature of the copper centers. *J Biol Inorg Chem* 1996; **1**: 15-23 [DOI: 10.1007/s007750050018]
30 **Bento I**, Peixoto C, Zaitsev VN, Lindley PF. Ceruloplasmin revisited: structural and functional roles of various metal cation-binding sites. *Acta Crystallogr D Biol Crystallogr* 2007; **63**: 240-248 [PMID: 17242517 DOI: 10.1107/S090744490604947X]
31 **Bielli P**, Bellenchi GC, Calabrese L. Site-directed mutagenesis of human ceruloplasmin: . production of a proteolytically stable protein and structure-activity relationships of type 1 sites. *J Biol Chem* 2001; **276**: 2678-2685 [PMID: 11042176 DOI: 10.1074/jbc.M007176200]
32 **Musci G**, Bonaccorsi di Patti MC, Petruzzelli R, Giartosio A, Calabrese L. Divalent cation binding to ceruloplasmin. *Biometals* 1996; **9**: 66-72 [PMID: 8574094]
33 **Lutsenko S**, Barnes NL, Bartee MY, Dmitriev OY. Function

and regulation of human copper-transporting ATPases. *Physiol Rev* 2007; **87**: 1011-1046 [PMID: 17615395 DOI: 10.1152/physrev.00004.2006]

34 **Maio N**, Polticelli F, De Francesco G, Rizzo G, Bonaccorsi di Patti MC, Musci G. Role of external loops of human ceruloplasmin in copper loading by ATP7B and Ccc2p. *J Biol Chem* 2010; **285**: 20507-20513 [PMID: 20430895 DOI: 10.1074/jbc.M109.090027]

35 **Patel BN**, David S. A novel glycosylphosphatidylinositol-anchored form of ceruloplasmin is expressed by mammalian astrocytes. *J Biol Chem* 1997; **272**: 20185-20190 [PMID: 9242695 DOI: 10.1074/jbc.272.32.20185]

36 **Mittal B**, Doroudchi MM, Jeong SY, Patel BN, David S. Expression of a membrane-bound form of the ferroxidase ceruloplasmin by leptomeningeal cells. *Glia* 2003; **41**: 337-346 [PMID: 12555201 DOI: 10.1002/glia.10158]

37 **Fortna RR**, Watson HA, Nyquist SE. Glycosyl phosphatidylinositol-anchored ceruloplasmin is expressed by rat Sertoli cells and is concentrated in detergent-insoluble membrane fractions. *Biol Reprod* 1999; **61**: 1042-1049 [PMID: 10491642 DOI: 10.1095/biolreprod61.4.1042]

38 **Chen L**, Dentchev T, Wong R, Hahn P, Wen R, Bennett J, Dunaief JL. Increased expression of ceruloplasmin in the retina following photic injury. *Mol Vis* 2003; **9**: 151-158 [PMID: 12724641]

39 **Patel BN**, Dunn RJ, David S. Alternative RNA splicing generates a glycosylphosphatidylinositol-anchored form of ceruloplasmin in mammalian brain. *J Biol Chem* 2000; **275**: 4305-4310 [PMID: 10660599 DOI: 10.1074/jbc.275.6.4305]

40 **De Domenico I**, Ward DM, di Patti MC, Jeong SY, David S, Musci G, Kaplan J. Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin. *EMBO J* 2007; **26**: 2823-2831 [PMID: 17541408 DOI: 10.1038/sj.emboj.7601735]

41 **Marques L**, Auriac A, Willemetz A, Banha J, Silva B, Canonne-Hergaux F, Costa L. Immune cells and hepatocytes express glycosylphosphatidylinositol-anchored ceruloplasmin at their cell surface. *Blood Cells Mol Dis* 2012; **48**: 110-120 [PMID: 22178061 DOI: 10.1016/j.bcmd.2011.11.005]

42 **Kono S**, Yoshida K, Tomosugi N, Terada T, Hamaya Y, Kanaoka S, Miyajima H. Biological effects of mutant ceruloplasmin on hepcidin-mediated internalization of ferroportin. *Biochim Biophys Acta* 2010; **1802**: 968-975 [PMID: 20655381 DOI: 10.1016/j.bbadis.2010.07.011]

43 **Floris G**, Medda R, Padiglia A, Musci G. The physiopathological significance of ceruloplasmin. A possible therapeutic approach. *Biochem Pharmacol* 2000; **60**: 1735-1741 [PMID: 11108788 DOI: 10.1016/S0006-2952(00)00399-3]

44 **Mukhopadhyay CK**, Mazumder B, Lindley PF, Fox PL. Identification of the prooxidant site of human ceruloplasmin: a model for oxidative damage by copper bound to protein surfaces. *Proc Natl Acad Sci USA* 1997; **94**: 11546-11551 [PMID: 9326646]

45 **Osaki S**, Johnson DA, Frieden E. The possible significance of the ferrous oxidase activity of ceruloplasmin in normal human serum. *J Biol Chem* 1966; **241**: 2746-2751 [PMID: 5912351]

46 **Lindley PF,** Card G, Zaitseva I, Zaitsev V, Reinhammar B, Selin-Lindgren E, Yoshida K. An X-ray structural study of human ceruloplasmin in relation to ferroxidase activity. *J Biol Inorg Chem* 1997; **2**: 454-463 [DOI: 10.1007/s007750050156]

47 **Ragan HA**, Nacht S, Lee GR, Bishop CR, Cartwright GE. Effect of ceruloplasmin on plasma iron in copper-deficient swine. *Am J Physiol* 1969; **217**: 1320-1323 [PMID: 5346295]

48 **Roeser HP**, Lee GR, Nacht S, Cartwright GE. The role of ceruloplasmin in iron metabolism. *J Clin Invest* 1970; **49**: 2408-2417 [PMID: 5480864 DOI: 10.1172/JCI106460]

49 **Sarkar J**, Seshadri V, Tripoulas NA, Ketterer ME, Fox PL. Role of ceruloplasmin in macrophage iron efflux during hypoxia. *J Biol Chem* 2003; **278**: 44018-44024 [PMID: 12952974 DOI: 10.1074/jbc.M304926200]

50 **Harris ZL**, Durley AP, Man TK, Gitlin JD. Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. *Proc Natl Acad Sci USA* 1999; **96**: 10812-10817 [PMID: 10485908 DOI: 10.1073/pnas.96.19.10812]

51 **Patel BN**, Dunn RJ, Jeong SY, Zhu Q, Julien JP, David S. Ceruloplasmin regulates iron levels in the CNS and prevents free radical injury. *J Neurosci* 2002; **22**: 6578-6586 [PMID: 12151537]

52 **Jeong SY**, David S. Glycosylphosphatidylinositol-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. *J Biol Chem* 2003; **278**: 27144-27148 [PMID: 12743117 DOI: 10.1074/jbc.M301988200]

53 **McCarthy RC**, Kosman DJ. Ferroportin and exocytoplasmic ferroxidase activity are required for brain microvascular endothelial cell iron efflux. *J Biol Chem* 2013; **288**: 17932-17940 [PMID: 23640881 DOI: 10.1074/jbc.M113.455428]

54 **Dringen R**, Bishop GM, Koeppe M, Dang TN, Robinson SR. The pivotal role of astrocytes in the metabolism of iron in the brain. *Neurochem Res* 2007; **32**: 1884-1890 [PMID: 17551833 DOI: 10.1007/s11064-007-9375-0]

55 **Barber EF**, Cousins RJ. Interleukin-1--stimulated induction of ceruloplasmin synthesis in normal and copper-deficient rats. *J Nutr* 1988; **118**: 375-381 [PMID: 3258371]

56 **Kuhlow CJ**, Krady JK, Basu A, Levison SW. Astrocytic ceruloplasmin expression, which is induced by IL-1beta and by traumatic brain injury, increases in the absence of the IL-1 type 1 receptor. *Glia* 2003; **44**: 76-84 [PMID: 12951659 DOI: 10.1002/glia.10273]

57 **di Patti MC**, Persichini T, Mazzone V, Polticelli F, Colasanti M, Musci G. Interleukin-1beta up-regulates iron efflux in rat C6 glioma cells through modulation of ceruloplasmin and ferroportin-1 synthesis. *Neurosci Lett* 2004; **363**: 182-186 [PMID: 15172111 DOI: 10.1016/j.neulet.2004.04.005]

58 **Mazumder B**, Mukhopadhyay CK, Prok A, Cathcart MK, Fox PL. Induction of ceruloplasmin synthesis by IFN-gamma in human monocytic cells. *J Immunol* 1997; **159**: 1938-1944 [PMID: 9257859]

59 **Conley L**, Geurs TL, Levin LA. Transcriptional regulation of ceruloplasmin by an IL-6 response element pathway. *Brain Res Mol Brain Res* 2005; **139**: 235-241 [PMID: 15979198 DOI: 10.1016/j.molbrainres.2005.05.027]

60 **Sidhu A**, Miller PJ, Hollenbach AD. FOXO1 stimulates ceruloplasmin promoter activity in human hepatoma cells treated with IL-6. *Biochem Biophys Res Commun* 2011; **404**: 963-967 [PMID: 21185807 DOI: 10.1016/j.bbrc.2010.12.089]

61 **Mukhopadhyay CK**, Mazumder B, Fox PL. Role of hypoxia-inducible factor-1 in transcriptional activation of ceruloplasmin by iron deficiency. *J Biol Chem* 2000; **275**: 21048-21054 [PMID: 10777486 DOI: 10.1074/jbc.M000636200]

62 **Taylor M**, Qu A, Anderson ER, Matsubara T, Martin A, Gonzalez FJ, Shah YM. Hypoxia-inducible factor-2α mediates the adaptive increase of intestinal ferroportin during iron deficiency in mice. *Gastroenterology* 2011; **140**: 2044-2055 [PMID: 21419768 DOI: 10.1053/j.gastro.2011.03.007]

63 **Wilkinson N**, Pantopoulos K. IRP1 regulates erythropoiesis and systemic iron homeostasis by controlling HIF2α mRNA translation. *Blood* 2013; **122**: 1658-1668 [PMID: 23777768 DOI: 10.1182/blood-2013-03-492454]

64 **Troadec MB**, Ward DM, Lo E, Kaplan J, De Domenico I. Induction of FPN1 transcription by MTF-1 reveals a role for ferroportin in transition metal efflux. *Blood* 2010; **116**: 4657-4664 [PMID: 20688958 DOI: 10.1182/blood-2010-04-278614]

65 **Marro S**, Chiabrando D, Messana E, Stolte J, Turco E, Tolosano E, Muckenthaler MU. Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position -7007 of the FPN1 promoter. *Haematologica* 2010; **95**: 1261-1268 [PMID: 20179090 DOI:

10.3324/haematol.2009.020123]
66 **Harada N**, Kanayama M, Maruyama A, Yoshida A, Tazumi K, Hosoya T, Mimura J, Toki T, Maher JM, Yamamoto M, Itoh K. Nrf2 regulates ferroportin 1-mediated iron efflux and counteracts lipopolysaccharide-induced ferroportin 1 mRNA suppression in macrophages. *Arch Biochem Biophys* 2011; **508**: 101-109 [PMID: 21303654 DOI: 10.1016/j.abb.2011.02.001]
67 **Delaby C**, Pilard N, Puy H, Canonne-Hergaux F. Sequential regulation of ferroportin expression after erythrophagocytosis in murine macrophages: early mRNA induction by haem, followed by iron-dependent protein expression. *Biochem J* 2008; **411**: 123-131 [PMID: 18072938 DOI: 10.1042/BJ20071474]
68 **Yang F**, Liu XB, Quinones M, Melby PC, Ghio A, Haile DJ. Regulation of reticuloendothelial iron transporter MTP1 (Slc11a3) by inflammation. *J Biol Chem* 2002; **277**: 39786-39791 [PMID: 12161425 DOI: 10.1074/jbc.M201485200]
69 **Ludwiczek S**, Aigner E, Theurl I, Weiss G. Cytokine-mediated regulation of iron transport in human monocytic cells. *Blood* 2003; **101**: 4148-4154 [PMID: 12522003 DOI: 10.1182/blood-2002-08-2459]
70 **Urrutia P**, Aguirre P, Esparza A, Tapia V, Mena NP, Arredondo M, González-Billault C, Núñez MT. Inflammation alters the expression of DMT1, FPN1 and hepcidin, and it causes iron accumulation in central nervous system cells. *J Neurochem* 2013; **126**: 541-549 [PMID: 23506423 DOI: 10.1111/jnc.12244]
71 **Persichini T**, Maio N, di Patti MC, Rizzo G, Toscano S, Colasanti M, Musci G. Interleukin-1β induces ceruloplasmin and ferroportin-1 gene expression via MAP kinases and C/EBPβ, AP-1, and NF-κB activation. *Neurosci Lett* 2010; **484**: 133-138 [PMID: 20727382 DOI: 10.1016/j.neulet.2010.08.034]
72 **Persichini T**, Maio N, di Patti MC, Rizzo G, Colasanti M, Musci G. Genistein up-regulates the iron efflux system in glial cells. *Neurosci Lett* 2010; **470**: 145-149 [PMID: 20056127 DOI: 10.1016/j.neulet.2009.12.074]
73 **Sangokoya C**, Doss JF, Chi JT. Iron-responsive miR-485-3p regulates cellular iron homeostasis by targeting ferroportin. *PLoS Genet* 2013; **9**: e1003408 [PMID: 23593016 DOI: 10.1371/journal.pgen.1003408]
74 **Zhang DL**, Hughes RM, Ollivierre-Wilson H, Ghosh MC, Rouault TA. A ferroportin transcript that lacks an iron-responsive element enables duodenal and erythroid precursor cells to evade translational repression. *Cell Metab* 2009; **9**: 461-473 [PMID: 19416716 DOI: 10.1016/j.cmet.2009.03.006]
75 **Harris ZL**, Takahashi Y, Miyajima H, Serizawa M, MacGillivray RT, Gitlin JD. Aceruloplasminemia: molecular characterization of this disorder of iron metabolism. *Proc Natl Acad Sci USA* 1995; **92**: 2539-2543 [PMID: 7708681 DOI: 10.1073/pnas.92.7.2539]
76 **Yoshida K**, Furihata K, Takeda S, Nakamura A, Yamamoto K, Morita H, Hiyamuta S, Ikeda S, Shimizu N, Yanagisawa N. A mutation in the ceruloplasmin gene is associated with systemic hemosiderosis in humans. *Nat Genet* 1995; **9**: 267-272 [PMID: 7539672 DOI: 10.1038/ng0395-267]
77 **McNeill A**, Pandolfo M, Kuhn J, Shang H, Miyajima H. The neurological presentation of ceruloplasmin gene mutations. *Eur Neurol* 2008; **60**: 200-205 [PMID: 18667828 DOI: 10.1159/000148691]
78 **Kono S**. Aceruloplasminemia. *Curr Drug Targets* 2012; **13**: 1190-1199 [PMID: 22515740 DOI: 10.2174/138945012802002320]
79 **Jeong SY**, David S. Age-related changes in iron homeostasis and cell death in the cerebellum of ceruloplasmin-deficient mice. *J Neurosci* 2006; **26**: 9810-9819 [PMID: 16988052 DOI: 10.1523/JNEUROSCI.2922-06.2006]
80 **Oshiro S**, Kawamura K, Zhang C, Sone T, Morioka MS, Kobayashi S, Nakajima K. Microglia and astroglia prevent oxidative stress-induced neuronal cell death: implications for aceruloplasminemia. *Biochim Biophys Acta* 2008; **1782**: 109-117 [PMID: 18187051 DOI: 10.1016/j.bbadis.2007.12.002]
81 **Hellman NE**, Kono S, Miyajima H, Gitlin JD. Biochemical analysis of a missense mutation in aceruloplasminemia. *J Biol Chem* 2002; **277**: 1375-1380 [PMID: 11689569 DOI: 10.1074/jbc.M109123200]
82 **Hellman NE**, Kono S, Mancini GM, Hoogeboom AJ, De Jong GJ, Gitlin JD. Mechanisms of copper incorporation into human ceruloplasmin. *J Biol Chem* 2002; **277**: 46632-46638 [PMID: 12351628 DOI: 10.1074/jbc.M206246200]
83 **Kono S**, Suzuki H, Takahashi K, Takahashi Y, Shirakawa K, Murakawa Y, Yamaguchi S, Miyajima H. Hepatic iron overload associated with a decreased serum ceruloplasmin level in a novel clinical type of aceruloplasminemia. *Gastroenterology* 2006; **131**: 240-245 [PMID: 16831606 DOI: 10.1053/j.gastro.2006.04.017]
84 **Hofmann WP**, Welsch C, Takahashi Y, Miyajima H, Mihm U, Krick C, Zeuzem S, Sarrazin C. Identification and in silico characterization of a novel compound heterozygosity associated with hereditary aceruloplasminemia. *Scand J Gastroenterol* 2007; **42**: 1088-1094 [PMID: 17710675 DOI: 10.1080/00365520701278810]
85 **di Patti MC**, Maio N, Rizzo G, De Francesco G, Persichini T, Colasanti M, Polticelli F, Musci G. Dominant mutants of ceruloplasmin impair the copper loading machinery in aceruloplasminemia. *J Biol Chem* 2009; **284**: 4545-4554 [PMID: 19095659 DOI: 10.1074/jbc.M805688200]
86 **Kuhn J**, Miyajima H, Takahashi Y, Kunath B, Hartmann-Klosterkoetter U, Cooper-Mahkorn D, Schaefer M, Bewermeyer H. Extrapyramidal and cerebellar movement disorder in association with heterozygous ceruloplasmin gene mutation. *J Neurol* 2005; **252**: 111-113 [PMID: 15654567 DOI: 10.1007/s00415-005-0608-3]
87 **Persichini T**, De Francesco G, Capone C, Cutone A, di Patti MC, Colasanti M, Musci G. Reactive oxygen species are involved in ferroportin degradation induced by ceruloplasmin mutant Arg701Trp. *Neurochem Int* 2012; **60**: 360-364 [PMID: 22281056 DOI: 10.1016/j.neuint.2012.01.010]
88 **Pietrangelo A**. The ferroportin disease. *Blood Cells Mol Dis* 2004; **32**: 131-138 [PMID: 14757427 DOI: 10.1016/j.bcmd.2003.08.003]
89 **Mayr R**, Janecke AR, Schranz M, Griffiths WJ, Vogel W, Pietrangelo A, Zoller H. Ferroportin disease: a systematic meta-analysis of clinical and molecular findings. *J Hepatol* 2010; **53**: 941-949 [PMID: 20691492 DOI: 10.1016/j.jhep.2010.05.016]
90 **Kasvosve I**. Effect of ferroportin polymorphism on iron homeostasis and infection. *Clin Chim Acta* 2013; **416**: 20-25 [PMID: 23178444 DOI: 10.1016/j.cca.2012.11.013]
91 **De Domenico I**, McVey Ward D, Nemeth E, Ganz T, Corradini E, Ferrara F, Musci G, Pietrangelo A, Kaplan J. Molecular and clinical correlates in iron overload associated with mutations in ferroportin. *Haematologica* 2006; **91**: 1092-1095 [PMID: 16885049]
92 **Drakesmith H**, Schimanski LM, Ormerod E, Merryweather-Clarke AT, Viprakasit V, Edwards JP, Sweetland E, Bastin JM, Cowley D, Chinthammitr Y, Robson KJ, Townsend AR. Resistance to hepcidin is conferred by hemochromatosis-associated mutations of ferroportin. *Blood* 2005; **106**: 1092-1097 [PMID: 15831700 DOI: 10.1182/blood-2005-02-0561]
93 **Détivaud L**, Island ML, Jouanolle AM, Ropert M, Bardou-Jacquet E, Le Lan C, Mosser A, Leroyer P, Deugnier Y, David V, Brissot P, Loréal O. Ferroportin diseases: functional studies, a link between genetic and clinical phenotype. *Hum Mutat* 2013; **34**: 1529-1536 [PMID: 23943237 DOI: 10.1002/humu.22396]
94 **Létocart E**, Le Gac G, Majore S, Ka C, Radio FC, Gourlaouen I, De Bernardo C, Férec C, Grammatico P. A novel missense mutation in SLC40A1 results in resistance to hepcidin and confirms the existence of two ferroportin-associated iron overload diseases. *Br J Haematol* 2009; **147**: 379-385 [PMID: 19709084 DOI: 10.1111/j.1365-2141.2009.07834.x]

95 **Mayr R**, Griffiths WJ, Hermann M, McFarlane I, Halsall DJ, Finkenstedt A, Douds A, Davies SE, Janecke AR, Vogel W, Cox TM, Zoller H. Identification of mutations in SLC40A1 that affect ferroportin function and phenotype of human ferroportin iron overload. *Gastroenterology* 2011; **140**: 2056-263, 2063.e1 [PMID: 21396368 DOI: 10.1053/j.gastro.2011.02.064]

96 **Pettersen EF**, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* 2004; **25**: 1605-1612 [PMID: 15264254 DOI: 10.1002/jcc.20084]

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REVIEW

FBW7-mediated ubiquitination and degradation of KLF5

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Abstract

Krüppel-like factor (KLF) family proteins are transcription factors that regulate numerous cellular functions, such as cell proliferation, differentiation, and cell death. Posttranslational modification of KLF proteins is important for their transcriptional activities and biological functions. One KLF family member with important roles in cell proliferation and tumorigenesis is KLF5. The function of KLF5 is tightly controlled by post-translational modifications, including SUMOylation, phosphorylation, and ubiquitination. Recent studies from our lab and others' have demonstrated that the tumor suppressor FBW7 is an essential E3 ubiquitin ligase that targets KLF5 for ubiquitination and degradation. KLF5 contains functional Cdc4 phospho-degrons (CPDs), which are required for its interaction with FBW7. Mutation of CPDs in KLF5 blocks the ubiquitination and degradation of KLF5 by FBW7. The protein kinase Glycogen synthase kinase 3β is involved in the phosphorylation of KLF5 CPDs. In both cancer cell lines and mouse models, it has been shown that FBW7 regulates the expression of KLF5 target genes through the modulation of KLF5 stability. In this review, we summarize the current progress on delineating FBW7-mediated KLF5 ubiquitination and degradation.

Key words: Krüppel-like factor 5; FBW7; Ubiquitin proteasome system; Degradation; Krüppel-like factor family

Core tip: The protein levels of Krüppel-like factor (KLF)5 are tightly controlled in cell. Ubiquitination and destruction of KLF5 *via* FBW7, a famous tumor suppressor, has proved to have important roles in multiple cellular progresses by different studies. Here, we summarize these studies and show the physiological and pathological significance of FBW7-mediated degradation of KLF5.

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INTRODUCTION

Krüppel-like factor (KLF) family proteins are important transcription factors that regulate numerous cellular processes[1]. KLF5 is a member of the KLF family that has been well-studied and shown to play a key role in mediating multiple cellular activities, such as proliferation and differentiation, in both normal and tumor cells[2]. Post-translational modifications of KLF5, including ubiquitination, SUMOylation, acetylation, and phosphorylation, can impact both the stability and activity of KLF5, thus affecting its downstream cellular functions[3-8].

FBW7 is the mammalian homolog of CDC4 in *Saccharomyces cerevisiae* and SEL10 in C. elegans. It is a component of the SCF (SKP1-CUL1-F-box protein) ubiquitin

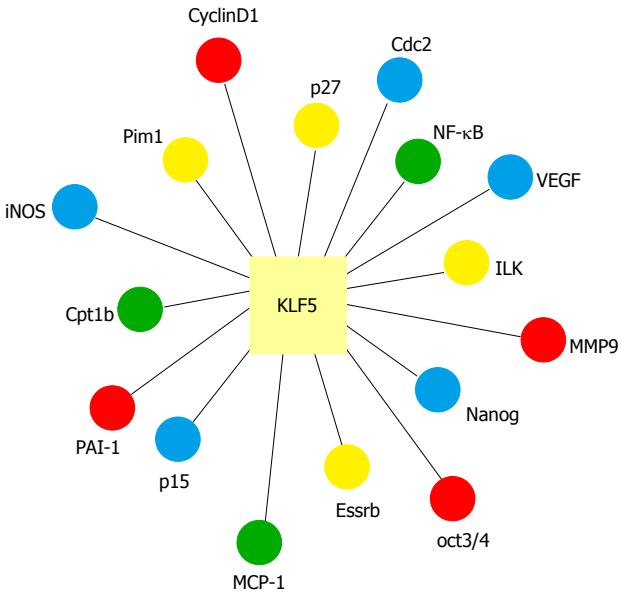

Figure 1 Regulation of gene expression by Krüppel-like factor 5. VEGF: Vascular endothelial growth factor; MCP-1: Monocyte chemoattractant protein-1; NK-κB: Nuclear factor κB; MMP-9: Matrix metalloproteinase-9; PAI-1: Plasminogen activator inhibitor-1; iNOS: Inducible nitric oxide synthase.

ligase complex. FBW7 is thought to have an important role in tumor biology by serving as a critical regulator of several oncoproteins, and mutations of FBW7 are found in a rapidly expanding number of human neoplasms[9].

In this review, we summarize the progress of research on FBW7-mediated KLF5 degradation and ubiquitination and show the physiological and pathological significance of KLF5 regulation by FBW7.

KRÜPPEL-LIKE FACTOR FAMILY AND KLF5

KLFs are a family of transcription factors with homologies to the Krüppel protein and the transcription factor Sp1 in Drosophila melanogaster and mammals, respectively[1]. To date, 17 mammalian KLFs have been identified, all of which contain three zinc finger motifs at the carboxyl-terminals, which are responsible for binding to GC-rich DNA sequences[10,11]. The KLFs have been demonstrated to play essential roles in development, immunity and cancer[1,10-15].

KLF5, also known as BTEB2 and IKLF, is an important KLF factor. KLF5 is widely expressed in various tissues, including lung, colon, intestine, and pancreas[2,16-19]. KLF5 is located at chromosomal position 13q22.1 in the human genome. It is involved in the regulation of diverse cellular functions, including cell cycle, proliferation, apoptosis, differentiation and stem cell self-renewal, by regulating the expression of numerous genes (Figure 1)[2,20-23]. Previous studies have shown that KLF5 plays a pivotal role in regulating cardiovascular remodeling[24-26]. Heterozygous KLF5-knockout mice showed reduced responses to cardiac injury, angiogenesis, hypertrophy and fibrosis[24,25]. In addition, KLF5 activity is regulated by other transcriptional regulators and nuclear receptors that are also involved in cardiovascular remodeling and injury response[24,25]. In tumor biology, KLF5 also has context-dependent proliferative or anti-proliferative activities in cancer cells and may function as either a tumor suppressor or an oncoprotein[27-29].

The functions of KLF5 are tightly controlled by post-translational modifications, including ubiquitination, SUMOylation, acetylation and phosphorylation[3-8,21,30,31]. For example, the SUMOylation of Lys151 and Lys202 regulates KLF5 nuclear localization[3]. Phosphorylation of KLF5 by PKC may enhance the transcriptional activities of KLF5 by promoting its interaction with CREB-binding protein[21]. In addition, KLF5 activity is also regulated by its acetylation status[4]. Moreover, KLF5 is a short-lived protein in cells and its protein level is tightly controlled by the ubiquitin-proteasome system[5-8,31,32]. Several E3 ubiquitin ligases, such as Smurf2, WWP1 and EFP, have been shown to degrade KLF5[7,31,32]. In 2010, Dr. Chen C's group and our laboratory both reported that KLF5 is targeted for ubiquitination and degradation by the E3 ubiquitin ligase FBW7[6,8]. In the past three years, several studies from different groups have also provided evidence strongly supporting KLF5 as an essential FBW7 substrate under both physiological and pathological conditions[6-8,31-34].

UBIQUITIN-PROTEASOME SYSTEM AND FBW7

Cellular protein levels are tightly controlled by protein degradation. The ubiquitin-proteasome system (UPS) is the major pathway for the degradation of approximately 90% of all proteins in cells[35-37]. The UPS acts by promoting protein ubiquitination and delivering the ubiquitinated proteins to the 26S proteasome for degradation[36]. The UPS is an enzymatic cascade containing three enzymes: enzyme-1 (E1), the ubiquitin-activating enzyme; E2, the ubiquitin carrier protein (ubiquitin-conjugating enzyme); and E3, the ubiquitin-protein ligase. E3 determines the specificity of protein degradation[35]. To date, more than 600 E3s have been identified in mammals and categorized into either the RING or HECT family of E3 ubiquitin ligases[38-40].

FBW7 (F-box and WD repeat domain-containing 7, also named CDC4, SEL10, or AGO) is the substrate recognition subunit of the E3 ubiquitin ligase complex SCF^{FBW7} (Skp1-Cullin-FBW7), which can target various proteins that are involved in cell proliferation for degradation[9]. Many substrates of FBW7 have been identified, including c-Myc, Cyclin E, Notch, TGIF, c-Jun, Mcl-1, p100 and so on (Table 1)[41-56]. There are three known isoforms of FBW7 with different subcellular localizations, including FBW7α, FBW7β and FBW7γ[9,57]. FBW7α is mainly localized to the nucleoplasm. FBW7β contains a transmembrane domain and is localized to the cytosol. FBW7γ is localized to the nucleolus *via* a nucleolar localization signal at its N terminus[9]. Each FBW7 isoform

Table 1 Sequences of Cdc4 phospho-degrons in FBW7 substrates

Substrate	Cdc4 phospho-degron	Phospho-site
CyclinE	LLTPPQSG	T380 S384
Myc	LPTPPLSP	T58 S62
JUN	GETPPLSP	T239 S243
NOTCH1	FLTPSPE	T2512
TGIF	FNTPPPTP	T235 T239
SRC3	VHSPMASS	S505 S509
mTOR	LLTPSIHL	T631
MCL1	DGSLPSTP	S159 T163 S121
KLF5	LNTPDLDM/PPSPPSSE/ NLTPPPSY	T244 S303 T324
KLF2	PDTPPLSPD/LLTPPSSP	T171 S175 T243 S247
SREBP	TLTPPPSDAGSP	T426 S430 S434
SV40 large T antigen	PPTPPPEP	T701
MED13/MED13L	SSVTLTPPTS	T326
NF-κB2	LPSPPTSDSDSD	S707 S711
C/EBPα	HPTPPPTP	T222 T226
C/EBPσ	QPTPPQSP	T157 S161
HIF1a	DQTPSPSDGSTRQSS	T497 S451
AuroraA	LSYCHSK/NSSKPSN	S245 S387
C-Myb	LMTPVSED	T572 S556 S528
NRF1	LFSPEVE	S350
PGC1	PLTPESPN/GLTPPTTP	T263 T295

NK-κB: Nuclear factor κB; KLF: Krüppel-like factor.

contains a F-box domain and WD40 repeats. The F-box domain contains approximately 40 amino acids that are involved in recruiting the SCF complex through direct interaction with SKP1. WD40 repeats are thought to form multiple contacts with various substrates[57-62].

FBW7 recognizes its substrates through a conserved phospho-epitope known as the Cdc4 phospho-degron (CPD), in which a central phospho-threonine/serine is embedded within hydrophobic residues in a I/L-I/L/P-pT-P-<K/R>4 (where K and R are unfavorable residues at positions 2 to 5) motif[9]. Most of the FBW7 substrates contain at least one conserved CPD, and the phosphorylation of the central Ser/Thr is usually mediated by the protein kinase Glycogen synthase kinase 3 (GSK-3)β[61,63,64].

Numerous studies have demonstrated that FBW7 functions as a tumor suppressor in various cancers. Mutant FBW7 is frequently found in human tumors. For example, amino acid substitutions such as Q264R, H460R, and R465C have been found in breast cancer, cholangiocarcinoma and colon cancer, respectively[52,65-67].

FBW7 INTERACTS WITH KLF5 *IN VIVO* AND *IN VITRO*

KLF5 contains several potential CPDs[6]. Data from Dr. Chen's group and our laboratory have indicated that all three isoforms of FBW7 can bind to KLF5 *in vivo*[6,8]. Mass spectrometry data have also shown that endogenous KLF5 can be co-purified with FBW7 in different cell types[46]. The interaction of KLF5 with FBW7 is dependent on the KLF5 CPD(s). Mutations within the KLF5 CPDs were shown to abolish the interaction. In addition, FBW7 binds to KLF5 *via* the WD40 repeats on FBW7. This interaction is also dependent on the phosphorylation of KLF5 CPDs by GSK3β, and inhibition of GSK3β activity can reduce FBW7 binding to KLF5. GSK3β activity is regulated by various extracellular stimuli such as Wnt and growth factors[68,69], but it is still unclear whether the interaction between KLF5 and FBW7 is also regulated by extracellular signals.

FBW7 TARGETS KLF5 FOR UBIQUITINATION AND DEGRADATION

As a component of the SCF E3 ubiquitin ligase complex, co-expression of FBW7α or FBW7γ was shown to markedly promote the degradation of co-expressed KLF5, which could be blocked by the proteasome inhibitor MG132. In contrast, other F-box-containing proteins such as β-TrCP1, FBXW2, FBXW5 and FBXW8 had little effect on KLF5 stability. FBW7 with its F-box domain deleted or the WD40 domain of FBW7 alone failed to mediate KLF5 degradation, suggesting that FBW7-mediated KLF5 degradation requires the recruitment of other components of SCF E3 ligase. R338 residue in FBW7 is considered as a key residue in regulating the interaction of FBW7 with its substrates. Mutation of R338 to lysine blocks FBW7 mediated KLF5 degradation (Figure 2). Depletion of endogenous FBW7 significantly increased the amount of endogenous KLF5 protein without affecting the KLF5 mRNA level. KLF5 protein level was also upregulated in FBW7-deficient DLD1 cells and the half-life of endogenous KLF5 was dramatically extended in these cells compared with the WT DLD1 cells.

Moreover, FBW7 also promotes KLF5 ubiquitination *in vitro* and *in vivo*. The ubiquitination of KLF5 by FBW7 is dependent on the phosphorylation of KLF5 CPDs. Mutation of KLF5 CPDs dramatically blocked FBW7-induced KLF5 ubiquitination.

In addition to FBW7, WWP1, EFP and Smurf2 were also identified as E3 ligases that can target KLF5 for degradation[7,31,32]. Both WWP1 and Smurf2 belong to the HECT E3 ubiquitin ligase family[70,71]. Unlike FBW7, WWP1 and Smurf2 degrade KLF5 in a phosphorylation-independent manner. Interestingly, FBW7 and WWP1 appear to degrade KLF5 in a compensatory manner because knockdown of WWP1 was shown to cause an increase in FBW7 expression, and vice versa[8]. Degradation of KLF5 by multiple E3 ubiquitin ligases signifies the importance of the regulation of KLF5 protein stability under various physiological and pathological conditions[5-8,31-34].

KLF5 CONTAINS CPDS THAT ARE REQUIRED FOR ITS DEGRADATION THROUGH FBW7

FBW7 targets a substrate for degradation through the CPD consensus sites on the substrate[63]. KLF5 contains three potential CPDs: 242-LNTPDLDM, 301-PPSPPSSE and 322-NLTPPPSY (Table 1). Mutations of individual

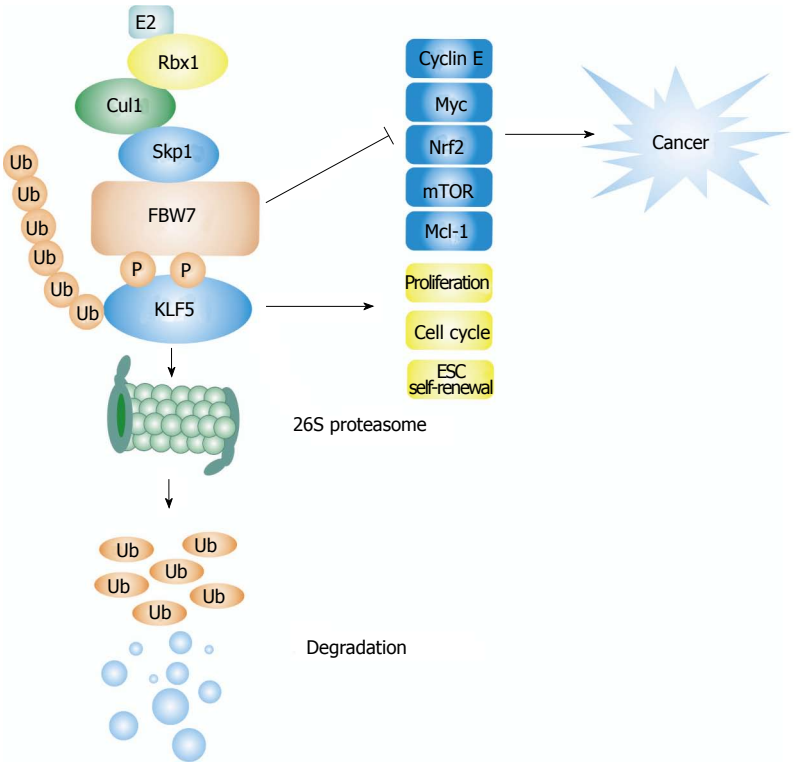

Figure 2 A model for FBW7 mediated Krüppel-like factor 5 degradation. SCFFBW7 recognizes KLF5 *via* conserved Cdc4 phospho-degron (CPD) in KLF5, GSK3 phosphorylates the threonine of the CPD, which facilitates the degradation of KLF5. FBW7 plays an important role in tumor suppression *via* targeting numerous oncoproteins for degradation, such as Myc, cyclin E, mammalian target of rapamycin (Mtor), Mcl-1, and so on. KLF5 has an important role in regulating cellular functions, including promoting cell proliferation, cell cycle, and embryonic stem cell (ESC) self-renewal. FBW7 promotes KLF5 ubiquitination and degradation through 26S proteasome. KLF: Krüppel-like factor.

CPDs in mouse KLF5 were shown to have a minor effect on FBW7-mediated degradation. However, simultaneous mutations of two CPDs markedly blocked KLF5 interaction with FBW7 and KLF5 degradation. Mutations of all three CPDs completely abolished FBW7-induced KLF5 ubiquitination and degradation. Although KLF5 contains three CPDs, both Dr. Chen's group and ours have found that phosphorylation of Ser303 in 301-PPSPPSSE is especially essential for FBW7-mediated degradation. In addition, Dr. Vincent W Yang's group also found that P301 in KLF5 CPD is important for interaction between FBW7 and KLF5 and FBW7-mediated degradation of KLF5. P301S KLF5, a somatic mutation in KLF5 found in human colorectal cancer tissues, has a higher transcriptional activity than WT KLF5 and is resistant to FBW7α-mediated degradation, suggesting that P301S KLF5 mutant play an oncogenic role in colorectal cancer[72].

GSK3α IS A KEY PROTEIN KINASE FOR KLF5 PHOSPHORYLATION AND DEGRADATION

GSK-3 is a serine/threonine protein kinase[73] that phosphorylates the central serine/threonine residues in the CPDs of numerous FBW7 substrates[9], including KLF5. Co-expression of KLF5 with GSK3β was shown to promote KLF5 phosphorylation and KLF5 interaction with FBW7. Data from *in vitro* phosphorylation assays indicated that phosphorylation of wild-type KLF5 by GSK3β was much greater than that of a CPD-deficient KLF peptide, indicating that the KLF5 CPDs are phosphorylation targets of GSK3β. Inhibition of GSK3β by LiCl was shown to block FBW7-mediated KLF5 degradation. Conversely, KLF5 degradation was enhanced in the presence of the constitutively active GSK3β-S9A. Dr. Chen's group reported similar results, and together these data indicate that GSK3β is required for FBW7-mediated degradation of KLF5.

Protein phosphorylation by GSK3β requires the phosphorylation of the priming phosphate group on a Ser/Thr residue that is located at the +4 position of a target residue[63]. For example, phosphorylation of c-Myc at T58 by GSK3β requires prior mitogen-activated protein kinase-dependent phosphorylation at serine S62[74-77]. Two of the KLF5 CPDs, 301-PPSPPSSE and 322-NLTPPPSY, contain a Ser at the +4 position. The protein kinase(s) that is involved in the phosphorylation of priming sites on KLF5 CPDs is still unknown.

REGULATION OF CANCER CELL PROLIFERATION BY FBW7-MEDIATED KLF5 DEGRADATION

We have previously shown that FBW7 negatively regulates the biological activity of KLF5[6]. An earlier study has also shown that KLF5 promotes the growth and proliferation of colorectal cancer cells[78]. Co-expression of FBW7 with KLF5 significantly inhibited the wild-type KLF5-mediated cell proliferation but had little effect on the proliferation of cells containing a CPD-mutant KLF5[6]. FBW7 can also inhibit the expression of KLF5 target genes, such as survivin, which regulates mitosis and caspase activity[79]. A high level of KLF5 has also been correlated with low survival in breast cancer patients[28].

Dr. Chen and his colleagues have determined the expression of FBW7 and KLF5 in multiple cancer cell lines, including HeLa, MCF10A, and 184B5 cells. Interestingly, they found that degradation of KLF5 by FBW7 is dependent on both the cell type and the FBW7 isoform[8]. For example, in 184B5 mammary gland cells, knockdown of FBW7α but not of the FBW7β and FBW7γ isoforms, upregulated the expression of KLF5 and its downstream target FGF-BP, which is a known promoter of breast cancer cell proliferation[8,80], suggesting that the different isoforms of FBW7 specifically regulate KLF5 stability and activity in breast cells.

REGULATION OF KLF5 BY FBW7 IN MOUSE MODELS

Recently, several lines of evidence from mouse models indicate that KLF5 stability can be regulated by FBW7 *in vivo*[33,34,81]. As mentioned above, mutations of FBW7 occur frequently in multiple cancers, including those of the lung, colorectum, stomach, blood, pancreas, and endometrium. FBW7 R482Q is one of the loss-of-function mutants that have been identified in various cancers. A mouse model harboring the R482Q mutation was generated in Dr. Ian Tomlinson's laboratory. Interestingly, the protein levels of KLF5 and TGIF1 were upregulated in the lungs of the heterozygous mutant mice, but the mRNA levels of these two genes remained the same between the mutant and the wild type mice[33,34]. Further investigation revealed that the levels of KLF5 and TGIF1 were also upregulated in normal intestine and adenomas of FBW7-deficient or FBW7-mutant mice. These data serve as strong *in vivo* evidences for KLF5 regulation by FBW7.

Regulation of KLF5 target gene expression by FBW7 has also been demonstrated in a mouse model[81]. Kumadaki *et al*[81] showed that *in vivo* knockdown of FBW7 significantly increased the hepatic expression of PPARγ2 as well as its targeted genes. More importantly, the degradation of KLF5 by FBW7 was associated with the inhibition of PPARγ2 expression. Thus, these findings suggested that degradation of KLF5 by FBW7 contributes to hepatic lipid metabolism.

CONCLUSION

In summary, FBW7 is an E3 ubiquitin ligase for KLF5. KLF5 contains functional CPDs that are phosphorylated by GSK3β, thus promoting the interaction between KLF5 and the WD40 domain of FBW7. This interaction subsequently leads to KLF5 ubiquitination and degradation by the ubiquitin-proteasome system. Mutation or deletion of FBW7 in cancer cells results in increased level of the KLF5 protein due to impaired degradation of KLF5, which in turn causes increased expression of KLF5 target genes, many of which can promote cell proliferation. Moreover, the KLF5 protein level is tightly controlled by FBW7 under normal physiological conditions, thus affecting many developmental and metabolic processes. In summary, the FBW7-KLF5 axis is important for both normal cellular activities, such as lipid metabolism, and cancer cell proliferation. This pathway may therefore serve as a novel target for cancer therapy

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REFERENCES

1 **McConnell BB**, Yang VW. Mammalian Krüppel-like factors in health and diseases. *Physiol Rev* 2010; **90**: 1337-1381 [PMID: 20959618 DOI: 10.1152/physrev.00058.2009]
2 **Dong JT**, Chen C. Essential role of KLF5 transcription factor in cell proliferation and differentiation and its implications for human diseases. *Cell Mol Life Sci* 2009; **66**: 2691-2706 [PMID: 19448973 DOI: 10.1007/s00018-009-0045-z]
3 **Du JX**, Bialkowska AB, McConnell BB, Yang VW. SUMOylation regulates nuclear localization of Krüppel-like factor 5. *J Biol Chem* 2008; **283**: 31991-32002 [PMID: 18782761 DOI: 10.1074/jbc.M803612200]
4 **Matsumura T**, Suzuki T, Aizawa K, Munemasa Y, Muto S, Horikoshi M, Nagai R. The deacetylase HDAC1 negatively regulates the cardiovascular transcription factor Krüppel-like factor 5 through direct interaction. *J Biol Chem* 2005; **280**: 12123-12129 [PMID: 15668237 DOI: 10.1074/jbc.M410578200]
5 **Chen C**, Sun X, Ran Q, Wilkinson KD, Murphy TJ, Simons JW, Dong JT. Ubiquitin-proteasome degradation of KLF5 transcription factor in cancer and untransformed epithelial cells. *Oncogene* 2005; **24**: 3319-3327 [PMID: 15735697 DOI: 10.1038/sj.onc.1208497]
6 **Liu N**, Li H, Li S, Shen M, Xiao N, Chen Y, Wang Y, Wang W, Wang R, Wang Q, Sun J, Wang P. The Fbw7/human CDC4 tumor suppressor targets proproliferative factor KLF5 for ubiquitination and degradation through multiple phosphodegron motifs. *J Biol Chem* 2010; **285**: 18858-18867 [PMID: 20388706 DOI: 10.1074/jbc.M109.099440]
7 **Du JX**, Hagos EG, Nandan MO, Bialkowska AB, Yu B, Yang VW. The E3 ubiquitin ligase SMAD ubiquitination regulatory factor 2 negatively regulates Krüppel-like factor 5 protein. *J Biol Chem* 2011; **286**: 40354-40364 [PMID: 21953463 DOI: 10.1074/jbc.M111.258707]
8 **Zhao D**, Zheng HQ, Zhou Z, Chen C. The Fbw7 tumor suppressor targets KLF5 for ubiquitin-mediated degradation and suppresses breast cell proliferation. *Cancer Res* 2010; **70**: 4728-4738 [PMID: 20484041 DOI: 10.1158/0008-5472.CAN-10-0040]
9 **Welcker M**, Clurman BE. FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation. *Nat Rev Cancer* 2008; **8**: 83-93 [PMID: 18094723 DOI: 10.1038/nrc2290]
10 **Dang DT**, Pevsner J, Yang VW. The biology of the mammalian Krüppel-like family of transcription factors. *Int J Biochem Cell Biol* 2000; **32**: 1103-1121 [PMID: 11137451]
11 **Turner J**, Crossley M. Mammalian Krüppel-like transcription factors: more than just a pretty finger. *Trends Biochem Sci* 1999; **24**: 236-240 [PMID: 10366853]
12 **Lania L**, Majello B, De Luca P. Transcriptional regulation by the Sp family proteins. *Int J Biochem Cell Biol* 1997; **29**: 1313-1323 [PMID: 9570130]
13 **Bieker JJ**. Krüppel-like factors: three fingers in many pies. *J Biol Chem* 2001; **276**: 34355-34358 [PMID: 11443140 DOI: 10.1074/jbc.R100043200]
14 **Kaczynski J**, Cook T, Urrutia R. Sp1- and Krüppel-like transcription factors. *Genome Biol* 2003; **4**: 206 [PMID: 12620113]

15 **Black AR**, Black JD, Azizkhan-Clifford J. Sp1 and krüppel-like factor family of transcription factors in cell growth regulation and cancer. *J Cell Physiol* 2001; **188**: 143-160 [PMID: 11424081 DOI: 10.1002/jcp.1111]

16 **Sogawa K**, Imataka H, Yamasaki Y, Kusume H, Abe H, Fujii-Kuriyama Y. cDNA cloning and transcriptional properties of a novel GC box-binding protein, BTEB2. *Nucleic Acids Res* 1993; **21**: 1527-1532 [PMID: 8479902]

17 **Conkright MD**, Wani MA, Anderson KP, Lingrel JB. A gene encoding an intestinal-enriched member of the Krüppel-like factor family expressed in intestinal epithelial cells. *Nucleic Acids Res* 1999; **27**: 1263-1270 [PMID: 9973612]

18 **Shi H**, Zhang Z, Wang X, Liu S, Teng CT. Isolation and characterization of a gene encoding human Kruppel-like factor 5 (IKLF): binding to the CAAT/GT box of the mouse lactoferrin gene promoter. *Nucleic Acids Res* 1999; **27**: 4807-4815 [PMID: 10572182]

19 **Ohnishi S**, Ohnami S, Laub F, Aoki K, Suzuki K, Kanai Y, Haga K, Asaka M, Ramirez F, Yoshida T. Downregulation and growth inhibitory effect of epithelial-type Krüppel-like transcription factor KLF4, but not KLF5, in bladder cancer. *Biochem Biophys Res Commun* 2003; **308**: 251-256 [PMID: 12901861]

20 **Chen C**, Benjamin MS, Sun X, Otto KB, Guo P, Dong XY, Bao Y, Zhou Z, Cheng X, Simons JW, Dong JT. KLF5 promotes cell proliferation and tumorigenesis through gene regulation and the TSU-Pr1 human bladder cancer cell line. *Int J Cancer* 2006; **118**: 1346-1355 [PMID: 16184550 DOI: 10.1002/ijc.21533]

21 **Zhang Z**, Teng CT. Phosphorylation of Kruppel-like factor 5 (KLF5/IKLF) at the CBP interaction region enhances its transactivation function. *Nucleic Acids Res* 2003; **31**: 2196-2208 [PMID: 12682370]

22 **Jiang J**, Chan YS, Loh YH, Cai J, Tong GQ, Lim CA, Robson P, Zhong S, Ng HH. A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat Cell Biol* 2008; **10**: 353-360 [PMID: 18264089 DOI: 10.1038/ncb1698]

23 **Parisi S**, Passaro F, Aloia L, Manabe I, Nagai R, Pastore L, Russo T. Klf5 is involved in self-renewal of mouse embryonic stem cells. *J Cell Sci* 2008; **121**: 2629-2634 [PMID: 18653541 DOI: 10.1242/jcs.027599]

24 **Nagai R**, Suzuki T, Aizawa K, Shindo T, Manabe I. Significance of the transcription factor KLF5 in cardiovascular remodeling. *J Thromb Haemost* 2005; **3**: 1569-1576 [PMID: 16102021 DOI: 10.1111/j.1538-7836.2005.01366.x]

25 **Shindo T**, Manabe I, Fukushima Y, Tobe K, Aizawa K, Miyamoto S, Kawai-Kowase K, Moriyama N, Imai Y, Kawakami H, Nishimatsu H, Ishikawa T, Suzuki T, Morita H, Maemura K, Sata M, Hirata Y, Komukai M, Kagechika H, Kadowaki T, Kurabayashi M, Nagai R. Krüppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. *Nat Med* 2002; **8**: 856-863 [PMID: 12101409 DOI: 10.1038/nm738]

26 **Santulli G**, Basilicata MF, De Simone M, Del Giudice C, Anastasio A, Sorriento D, Saviano M, Del Gatto A, Trimarco B, Pedone C, Zaccaro L, Iaccarino G. Evaluation of the anti-angiogenic properties of the new selective αVβ3 integrin antagonist RGDechiHCit. *J Transl Med* 2011; **9**: 7 [PMID: 21232121 DOI: 10.1186/1479-5876-9-7]

27 **McConnell BB**, Klapproth JM, Sasaki M, Nandan MO, Yang VW. Krüppel-like factor 5 mediates transmissible murine colonic hyperplasia caused by Citrobacter rodentium infection. *Gastroenterology* 2008; **134**: 1007-1016 [PMID: 18395082 DOI: 10.1053/j.gastro.2008.01.013]

28 **Tong D**, Czerwenka K, Heinze G, Ryffel M, Schuster E, Witt A, Leodolter S, Zeillinger R. Expression of KLF5 is a prognostic factor for disease-free survival and overall survival in patients with breast cancer. *Clin Cancer Res* 2006; **12**: 2442-2448 [PMID: 16638850 DOI: 10.1158/1078-0432.CCR-05-0964]

29 **Yagi N**, Manabe I, Tottori T, Ishihara A, Ogata F, Kim JH, Nishimura S, Fujiu K, Oishi Y, Itaka K, Kato Y, Yamauchi M, Nagai R. A nanoparticle system specifically designed to deliver short interfering RNA inhibits tumor growth in vivo. *Cancer Res* 2009; **69**: 6531-6538 [PMID: 19654315 DOI: 10.1158/0008-5472.CAN-08-3945]

30 **Miyamoto S**, Suzuki T, Muto S, Aizawa K, Kimura A, Mizuno Y, Nagino T, Imai Y, Adachi N, Horikoshi M, Nagai R. Positive and negative regulation of the cardiovascular transcription factor KLF5 by p300 and the oncogenic regulator SET through interaction and acetylation on the DNA-binding domain. *Mol Cell Biol* 2003; **23**: 8528-8541 [PMID: 14612398]

31 **Chen C**, Sun X, Guo P, Dong XY, Sethi P, Cheng X, Zhou J, Ling J, Simons JW, Lingrel JB, Dong JT. Human Kruppel-like factor 5 is a target of the E3 ubiquitin ligase WWP1 for proteolysis in epithelial cells. *J Biol Chem* 2005; **280**: 41553-41561 [PMID: 16223724 DOI: 10.1074/jbc.M506183200]

32 **Zhao KW**, Sikriwal D, Dong X, Guo P, Sun X, Dong JT. Oestrogen causes degradation of KLF5 by inducing the E3 ubiquitin ligase EFP in ER-positive breast cancer cells. *Biochem J* 2011; **437**: 323-333 [PMID: 21542805 DOI: 10.1042/BJ20101388]

33 **Davis H**, Lewis A, Behrens A, Tomlinson I. Investigation of the atypical FBXW7 mutation spectrum in human tumours by conditional expression of a heterozygous propellor tip missense allele in the mouse intestines. *Gut* 2014; **63**: 792-799 [PMID: 23676439 DOI: 10.1136/gutjnl-2013-304719]

34 **Davis H**, Lewis A, Spencer-Dene B, Tateossian H, Stamp G, Behrens A, Tomlinson I. FBXW7 mutations typically found in human cancers are distinct from null alleles and disrupt lung development. *J Pathol* 2011; **224**: 180-189 [PMID: 21503901 DOI: 10.1002/path.2874]

35 **Hershko A**. Ubiquitin: roles in protein modification and breakdown. *Cell* 1983; **34**: 11-12 [PMID: 6309404]

36 **Ciechanover A**. The ubiquitin-proteasome proteolytic pathway. *Cell* 1994; **79**: 13-21 [PMID: 7923371]

37 **Ravid T**, Hochstrasser M. Diversity of degradation signals in the ubiquitin-proteasome system. *Nat Rev Mol Cell Biol* 2008; **9**: 679-690 [PMID: 18698327 DOI: 10.1038/nrm2468]

38 **Nakayama KI**, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* 2006; **6**: 369-381 [PMID: 16633365 DOI: 10.1038/nrc1881]

39 **Petroski MD**, Deshaies RJ. Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* 2005; **6**: 9-20 [PMID: 15688063 DOI: 10.1038/nrm1547]

40 **Cardozo T**, Pagano M. The SCF ubiquitin ligase: insights into a molecular machine. *Nat Rev Mol Cell Biol* 2004; **5**: 739-751 [PMID: 15340381 DOI: 10.1038/nrm1471]

41 **Bengoechea-Alonso MT**, Ericsson J. A phosphorylation cascade controls the degradation of active SREBP1. *J Biol Chem* 2009; **284**: 5885-5895 [PMID: 19126544 DOI: 10.1074/jbc.M807906200]

42 **Kwon YW**, Kim IJ, Wu D, Lu J, Stock WA, Liu Y, Huang Y, Kang HC, DelRosario R, Jen KY, Perez-Losada J, Wei G, Balmain A, Mao JH. Pten regulates Aurora-A and cooperates with Fbxw7 in modulating radiation-induced tumor development. *Mol Cancer Res* 2012; **10**: 834-844 [PMID: 22513362 DOI: 10.1158/1541-7786.MCR-12-0025]

43 **Lee EC**, Frolov A, Li R, Ayala G, Greenberg NM. Targeting Aurora kinases for the treatment of prostate cancer. *Cancer Res* 2006; **66**: 4996-5002 [PMID: 16707419 DOI: 10.1158/0008-5472.CAN-05-2796]

44 **Yumimoto K**, Matsumoto M, Onoyama I, Imaizumi K, Nakayama KI. F-box and WD repeat domain-containing-7 (Fbxw7) protein targets endoplasmic reticulum-anchored osteogenic and chondrogenic transcriptional factors for degradation. *J Biol Chem* 2013; **288**: 28488-28502 [PMID: 23955342 DOI: 10.1074/jbc.M113.465179]

45 **Kanei-Ishii C**, Nomura T, Takagi T, Watanabe N, Nakayama KI, Ishii S. Fbxw7 acts as an E3 ubiquitin ligase that targets

c-Myb for nemo-like kinase (NLK)-induced degradation. *J Biol Chem* 2008; **283**: 30540-30548 [PMID: 18765672 DOI: 10.1074/jbc.M804340200]

46 **Davis MA**, Larimore EA, Fissel BM, Swanger J, Taatjes DJ, Clurman BE. The SCF-Fbw7 ubiquitin ligase degrades MED13 and MED13L and regulates CDK8 module association with Mediator. *Genes Dev* 2013; **27**: 151-156 [PMID: 23322298 DOI: 10.1101/gad.207720.112]

47 **Tan M**, Zhao Y, Kim SJ, Liu M, Jia L, Saunders TL, Zhu Y, Sun Y. SAG/RBX2/ROC2 E3 ubiquitin ligase is essential for vascular and neural development by targeting NF1 for degradation. *Dev Cell* 2011; **21**: 1062-1076 [PMID: 22118770 DOI: 10.1016/j.devcel.2011.09.014]

48 **Biswas M**, Phan D, Watanabe M, Chan JY. The Fbw7 tumor suppressor regulates nuclear factor E2-related factor 1 transcription factor turnover through proteasome-mediated proteolysis. *J Biol Chem* 2011; **286**: 39282-39289 [PMID: 21953459 DOI: 10.1074/jbc.M111.253807]

49 **Fukushima H**, Matsumoto A, Inuzuka H, Zhai B, Lau AW, Wan L, Gao D, Shaik S, Yuan M, Gygi SP, Jimi E, Asara JM, Nakayama K, Nakayama KI, Wei W. SCF(Fbw7) modulates the NFkB signaling pathway by targeting NFkB2 for ubiquitination and destruction. *Cell Rep* 2012; **1**: 434-443 [PMID: 22708077 DOI: 10.1016/j.celrep.2012.04.002]

50 **Busino L**, Millman SE, Scotto L, Kyratsous CA, Basrur V, O'Connor O, Hoffmann A, Elenitoba-Johnson KS, Pagano M. Fbxw7α- and GSK3-mediated degradation of p100 is a pro-survival mechanism in multiple myeloma. *Nat Cell Biol* 2012; **14**: 375-385 [PMID: 22388891 DOI: 10.1038/ncb2463]

51 **Olson BL**, Hock MB, Ekholm-Reed S, Wohlschlegel JA, Dev KK, Kralli A, Reed SI. SCFCdc4 acts antagonistically to the PGC-1alpha transcriptional coactivator by targeting it for ubiquitin-mediated proteolysis. *Genes Dev* 2008; **22**: 252-264 [PMID: 18198341 DOI: 10.1101/gad.1624208]

52 **Akhoondi S**, Sun D, von der Lehr N, Apostolidou S, Klotz K, Maljukova A, Cepeda D, Fiegl H, Dafou D, Marth C, Mueller-Holzner E, Corcoran M, Dagnell M, Nejad SZ, Nayer BN, Zali MR, Hansson J, Egyhazi S, Petersson F, Sangfelt P, Nordgren H, Grander D, Reed SI, Widschwendter M, Sangfelt O, Spruck C. FBXW7/hCDC4 is a general tumor suppressor in human cancer. *Cancer Res* 2007; **67**: 9006-9012 [PMID: 17909001 DOI: 10.1158/0008-5472.CAN-07-1320]

53 **Flügel D**, Görlach A, Kietzmann T. GSK-3β regulates cell growth, migration, and angiogenesis via Fbw7 and USP28-dependent degradation of HIF-1α. *Blood* 2012; **119**: 1292-1301 [PMID: 22144179 DOI: 10.1182/blood-2011-08-375014]

54 **Kim DS**, Zhang W, Millman SE, Hwang BJ, Kwon SJ, Clayberger C, Pagano M, Krensky AM. Fbw7γ-mediated degradation of KLF13 prevents RANTES expression in resting human but not murine T lymphocytes. *Blood* 2012; **120**: 1658-1667 [PMID: 22797700 DOI: 10.1182/blood-2012-03-415968]

55 **Galli F**, Rossi M, D'Alessandra Y, De Simone M, Lopardo T, Haupt Y, Alsheich-Bartok O, Anzi S, Shaulian E, Calabrò V, La Mantia G, Guerrini L. MDM2 and Fbw7 cooperate to induce p63 protein degradation following DNA damage and cell differentiation. *J Cell Sci* 2010; **123**: 2423-2433 [PMID: 20571051 DOI: 10.1242/jcs.061010]

56 **Welcker M**, Clurman BE. The SV40 large T antigen contains a decoy phosphodegron that mediates its interactions with Fbw7/hCdc4. *J Biol Chem* 2005; **280**: 7654-7658 [PMID: 15611062 DOI: 10.1074/jbc.M413377200]

57 **Spruck CH**, Strohmaier H, Sangfelt O, Müller HM, Hubalek M, Müller-Holzner E, Marth C, Widschwendter M, Reed SI. hCDC4 gene mutations in endometrial cancer. *Cancer Res* 2002; **62**: 4535-4539 [PMID: 12183400]

58 **Matsumoto A**, Onoyama I, Nakayama KI. Expression of mouse Fbxw7 isoforms is regulated in a cell cycle- or p53-dependent manner. *Biochem Biophys Res Commun* 2006; **350**: 114-119 [PMID: 16989775 DOI: 10.1016/j.bbrc.2006.09.003]

59 **Kimura T**, Gotoh M, Nakamura Y, Arakawa H. hCDC4b, a regulator of cyclin E, as a direct transcriptional target of p53. *Cancer Sci* 2003; **94**: 431-436 [PMID: 12824889]

60 **Bai C**, Sen P, Hofmann K, Ma L, Goebl M, Harper JW, Elledge SJ. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell* 1996; **86**: 263-274 [PMID: 8706131]

61 **Orlicky S**, Tang X, Willems A, Tyers M, Sicheri F. Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. *Cell* 2003; **112**: 243-256 [PMID: 12553912]

62 **Hao B**, Oehlmann S, Sowa ME, Harper JW, Pavletich NP. Structure of a Fbw7-Skp1-cyclin E complex: multisite-phosphorylated substrate recognition by SCF ubiquitin ligases. *Mol Cell* 2007; **26**: 131-143 [PMID: 17434132 DOI: 10.1016/j.molcel.2007.02.022]

63 **Nash P**, Tang X, Orlicky S, Chen Q, Gertler FB, Mendenhall MD, Sicheri F, Pawson T, Tyers M. Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. *Nature* 2001; **414**: 514-521 [PMID: 11734846 DOI: 10.1038/35107009]

64 **Verma R**, Annan RS, Huddleston MJ, Carr SA, Reynard G, Deshaies RJ. Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science* 1997; **278**: 455-460 [PMID: 9334303]

65 **Wei G,** Wang Y, Zhang P, Lu J, Mao JH. Evaluating the prognostic significance of FBXW7 expression level in human breast cancer by a meta-analysis of transcriptional profiles. *J Cancer Sci Ther* 2012; **4**: 299-305 [PMID: 23105958 DOI: 10.4172/1948-5956.1000158]

66 **Ibusuki M**, Yamamoto Y, Shinriki S, Ando Y, Iwase H. Reduced expression of ubiquitin ligase FBXW7 mRNA is associated with poor prognosis in breast cancer patients. *Cancer Sci* 2011; **102**: 439-445 [PMID: 21134077 DOI: 10.1111/j.1349-7006.2010.01801.x]

67 **Grim JE**. Fbxw7 hotspot mutations and human colon cancer: mechanistic insights from new mouse models. *Gut* 2014; **63**: 707-709 [PMID: 24000292 DOI: 10.1136/gutjnl-2013-305144]

68 **Cohen P**, Frame S. The renaissance of GSK3. *Nat Rev Mol Cell Biol* 2001; **2**: 769-776 [PMID: 11584304 DOI: 10.1038/35096075]

69 **Jope RS**, Yuskaitis CJ, Beurel E. Glycogen synthase kinase-3 (GSK3): inflammation, diseases, and therapeutics. *Neurochem Res* 2007; **32**: 577-595 [PMID: 16944320 DOI: 10.1007/s11064-006-9128-5]

70 **Chen C**, Zhou Z, Ross JS, Zhou W, Dong JT. The amplified WWP1 gene is a potential molecular target in breast cancer. *Int J Cancer* 2007; **121**: 80-87 [PMID: 17330240 DOI: 10.1002/ijc.22653]

71 **David D**, Nair SA, Pillai MR. Smurf E3 ubiquitin ligases at the cross roads of oncogenesis and tumor suppression. *Biochim Biophys Acta* 2013; **1835**: 119-128 [PMID: 23164545 DOI: 10.1016/j.bbcan.2012.11.003]

72 **Bialkowska AB**, Liu Y, Nandan MO, Yang VW. A colon cancer-derived mutant of Krüppel-like factor 5 (KLF5) is resistant to degradation by glycogen synthase kinase 3β (GSK3β) and the E3 ubiquitin ligase F-box and WD repeat domain-containing 7α (FBW7α). *J Biol Chem* 2014; **289**: 5997-6005 [PMID: 24398687 DOI: 10.1074/jbc.M113.508549]

73 **Cohen P**, Goedert M. GSK3 inhibitors: development and therapeutic potential. *Nat Rev Drug Discov* 2004; **3**: 479-487 [PMID: 15173837 DOI: 10.1038/nrd1415]

74 **Sears R**, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes Dev* 2000; **14**: 2501-2514 [PMID: 11018017]

75 **Yeh E**, Cunningham M, Arnold H, Chasse D, Monteith T, Ivaldi G, Hahn WC, Stukenberg PT, Shenolikar S, Uchida T, Counter CM, Nevins JR, Means AR, Sears R. A signalling pathway controlling c-Myc degradation that impacts onco-

genic transformation of human cells. *Nat Cell Biol* 2004; **6**: 308-318 [PMID: 15048125 DOI: 10.1038/ncb1110]

76 **Yada M**, Hatakeyama S, Kamura T, Nishiyama M, Tsunematsu R, Imaki H, Ishida N, Okumura F, Nakayama K, Nakayama KI. Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. *EMBO J* 2004; **23**: 2116-2125 [PMID: 15103331 DOI: 10.1038/sj.emboj.7600217]

77 **Welcker M**, Orian A, Jin J, Grim JE, Harper JW, Eisenman RN, Clurman BE. The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. *Proc Natl Acad Sci USA* 2004; **101**: 9085-9090 [PMID: 15150404 DOI: 10.1073/pnas.0402770101]

78 **Yang Y**, Goldstein BG, Chao HH, Katz JP. KLF4 and KLF5 regulate proliferation, apoptosis and invasion in esophageal cancer cells. *Cancer Biol Ther* 2005; **4**: 1216-1221 [PMID: 16357509]

79 **Cheung CH**, Huang CC, Tsai FY, Lee JY, Cheng SM, Chang YC, Huang YC, Chen SH, Chang JY. Survivin - biology and potential as a therapeutic target in oncology. *Onco Targets Ther* 2013; **6**: 1453-1462 [PMID: 24204160 DOI: 10.2147/OTT.S33374]

80 **Zheng HQ**, Zhou Z, Huang J, Chaudhury L, Dong JT, Chen C. Krüppel-like factor 5 promotes breast cell proliferation partially through upregulating the transcription of fibroblast growth factor binding protein 1. *Oncogene* 2009; **28**: 3702-3713 [PMID: 19668233 DOI: 10.1038/onc.2009.235]

81 **Kumadaki S**, Karasawa T, Matsuzaka T, Ema M, Nakagawa Y, Nakakuki M, Saito R, Yahagi N, Iwasaki H, Sone H, Takekoshi K, Yatoh S, Kobayashi K, Takahashi A, Suzuki H, Takahashi S, Yamada N, Shimano H. Inhibition of ubiquitin ligase F-box and WD repeat domain-containing 7α (Fbw7α) causes hepatosteatosis through Krüppel-like factor 5 (KLF5)/peroxisome proliferator-activated receptor γ2 (PPARγ2) pathway but not SREBP-1c protein in mice. *J Biol Chem* 2011; **286**: 40835-40846 [PMID: 21911492 DOI: 10.1074/jbc.M111.235283]

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Extracellular *O*-linked β-*N*-acetylglucosamine: Its biology and relationship to human disease

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Abstract

The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc)ylation of cytoplasmic and nuclear proteins regulates basic cellular functions and is involved in the etiology of neurodegeneration and diabetes. Intracellular *O*-GlcNAcylation is catalyzed by a single *O*-GlcNAc transferase, *O*-GlcNAc transferase (OGT). Recently, an atypical *O*-GlcNAc transferase, extracellular *O*-linked β-*N*-acetylglucosamine (EOGT), which is responsible for the modification of extracellular *O*-GlcNAc, was identified. Although both OGT and EOGT are regulated through the common hexosamine biosynthesis pathway, EOGT localizes to the lumen of the endoplasmic reticulum and transfers GlcNAc to epidermal growth factor-like domains in an OGT-independent manner. In *Drosophila*, loss of *Eogt* gives phenotypes similar to those caused by defects in the apical extracellular matrix. Dumpy, a membrane-anchored apical extracellular matrix protein, was identified as a major *O*-GlcNAcylated protein, and EOGT mediates Dumpy-dependent cell adhesion. In mammals, extracellular *O*-GlcNAc was detected on extracellular proteins including heparan sulfate proteoglycan 2, Nell1, laminin subunit alpha-5, Pamr1, and transmembrane proteins, including Notch receptors. Although the physiological function of *O*-GlcNAc in mammals has not yet been elucidated, exome sequencing identified homozygous *EOGT* mutations in patients with Adams-Oliver syndrome, a rare congenital disorder characterized by aplasia cutis congenita and terminal transverse limb defects. This review summarizes the current knowledge of extracellular *O*-GlcNAc and its implications in the pathological processes in Adams-Oliver syndrome.

Key words: Extracellular *O*-linked β-*N*-acetylglucosamine; Notch; Adams-Oliver syndrome

Core tip: The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) on extracellular protein domains is the most recently identified *O*-glycosylation of epidermal growth factor repeat-containing proteins such as Notch receptors. This *O*-GlcNAc modification occurs in the secretory pathway by an endoplasmic reticulum-resident *O*-GlcNAc transferase, extracellular *O*-linked β-*N*-acetylglucosamine (*EOGT*). In *Drosophila*, Dumpy, a membrane-tethered cuticle protein, was identified as a major *O*-GlcNAcylated protein that mediates the interaction between epithelial cells and the extracellular matrix. In mammals, extracellular *O*-GlcNAc was detected on Hspg2, Nell1, Lama5, Pamr1, and Notch receptors, although the physiological function of *O*-GlcNAc in mammals has not yet been elucidated. However, the recent finding that *EOGT* is a causative gene for Adams-Oliver syndrome provided important insights into the significance of extracellular *O*-GlcNAc in mammals. This review summarizes the current knowledge of extracellular *O*-GlcNAc and its implications in the pathological processes in Adams-Oliver syndrome.

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INTRODUCTION

O-linked β-*N*-acetylglucosamine (*O*-GlcNAc) was first identified in 1984 as a cell-surface saccharide moiety on intact lymphocytes[1]. Later studies, however, revealed that *O*-GlcNAc is present on nuclear, cytosolic, and mitochondrial proteins. This modification is prevalent in multicellular organisms, where more than 1000 *O*-GlcNAcylated proteins have been identified[2]. Intracellular *O*-GlcNAcylation is reversible, and its cycling is dynamically regulated by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase[3-5]. A large number of studies have indicated that *O*-GlcNAcylation is involved in various cellular functions, including transcription, epigenesis, cellular signaling, cell differentiation, and glucose sensing[6-9]. It had long been believed that *O*-GlcNAc is a unique intracellular modification and that OGT is the sole enzyme catalyzing the *O*-GlcNAc transfer reaction. However, extracellular *O*-GlcNAc was recently discovered on the extracellular domains of Notch receptors (Figure 1A). In this minireview, we will focus on extracellular *O*-GlcNAc and its relevance to human disease.

EXTRACELLULAR *O*-GLCNAC ON EGF DOMAINS

The first example of the *O*-GlcNAc modification of extracellular protein domains was the 20th EGF domain (EGF20) of *Drosophila* Notch expressed in S2 cells. Biochemical analyses revealed that *O*-GlcNAcylation occurs on the threonine located between the fifth and sixth cysteine[10]. Moreover, *in vivo* studies revealed that *O*-GlcNAc is abundantly expressed in the *Drosophila* cuticle[11]. Among cuticle proteins, Dumpy, a giant 2.5-MDa membrane-anchored cuticle protein containing a very large number of EGF-like domains (308 EGF-like repeats), was identified as a major *O*-GlcNAcylated protein[11]. In addition to Notch and Dumpy, Delta and Serrate, ligands for Notch receptors, have been shown to be *O*-GlcNAcylated by extracellular *O*-linked β-*N*-acetylglucosamine (EOGT)[10,12] (Figure 1B) in *Drosophila* S2 cells.

Similar to intracellular *O*-GlcNAc, extracellular *O*-GlcNAc is conserved in mammals but can be subjected to subsequent modification. The co-expression of Notch1 with EOGT in HEK293T cells suggests that the *O*-GlcNAc moiety is further modified with galactose to form *O*-linked *N*-acetyl-lactosamine (*O*-LacNAc)[13]. Recently, five extracellular *O*-GlcNAcylated proteins [Hspg2(Perlecan), Nell1, Lama5, Pamr1, and Notch2] were identified by a modified chemical/enzymatic photochemical cleavage approach for enriching *O*-GlcNAcylated peptides from mouse cerebrocortical brain tissue[14]. Another carbohydrate analysis revealed that *O*-GlcNAcylation occurs in the native thrombospondin-1 (TSP1) purified from platelets as well as in the recombinant TSP1 fragments expressed in insect High Five cells[15] (Figure 1B). The sequence alignment of *O*-GlcNAcylated proteins suggests that the predictive consensus sequence for the modification is C^5XXGX(T/S)GXXC^6, where C^5 and C^6 are the fifth and sixth conserved cysteines of the EGF domain, respectively. It should be noted, however, that no experimental data are available to indicate whether the C^5XXGX(T/S)GXXC^6 sequence is necessary or sufficient for the modification[10].

EOGT IS RESPONSIBLE FOR EXTRACELLULAR *O*-GLCNAC

In contrast to the OGT-catalyzed intracellular modification, the addition of *O*-GlcNAc onto extracellular proteins is mediated by a distinct *O*-GlcNAc transferase, the EGF-domain specific *O*-GlcNAc transferase (EOGT)[11,13]. *Eogt* is evolutionarily conserved from *Caenorhabditis elegans* to humans. EOGT contains a hydrophobic region corresponding to a signal peptide and a KDEL-like ER-retrieval sequence at the carboxyl terminus (Figure 2A)[11]. EOGT exhibits no similarity to OGT, but it is phylogenetically related to plant xylosyltransferases. EOGT possesses a putative UDP-GlcNAc-binding DXD motif[12]. EOGT specifically utilizes uridine diphosphate (UDP)-GlcNAc as a sugar donor, and its *in vitro* enzyme activity is enhanced in the presence of divalent cations, especially Mn^{2+}[11,13].

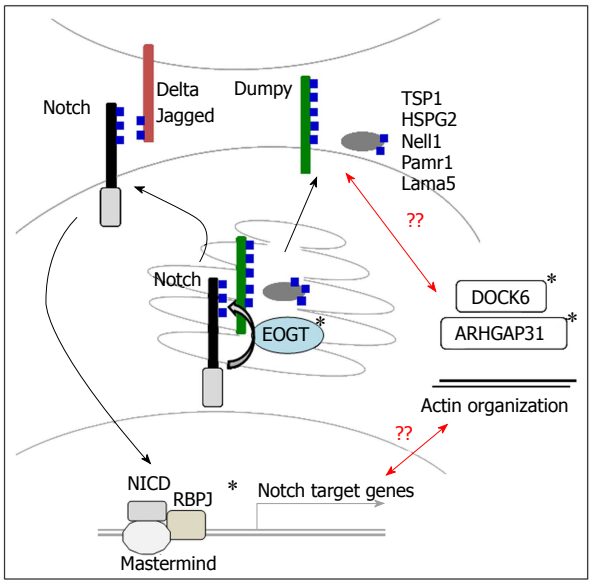
Because the levels of *O*-GlcNAcylation on Notch are increased by treatment with glucosamine or GlcNAc[8], it is suggested that the hexosamine biosynthesis pathway (HBP) is upstream of extracellular *O*-GlcNAc modification. The end product of the HBP is UDP-GlcNAc, which is utilized by EOGT as a donor substrate to modify proteins with *O*-GlcNAc in the ER. The transport of UDP-GlcNAc across the ER or Golgi membrane is mediated by nucleotide-sugar transporters[16-19]. However, it remains unclear which UDP-GlcNAc transporters are required for *O*-GlcNAcylation by EOGT.

Although *EOGT* expression has been detected in all adult mouse tissues, its expression is highest in the lung and lowest in the skeletal muscles[13]. During mouse development, high expression was detected in the growing edge of the limb buds; the expression was localized to the digits of the four limbs at later stages[20].

BIOLOGICAL FUNCTION OF EXTRACELLULAR *O*-GLCNAC IN *DROSOPHILA*

The biological function of extracellular *O*-GlcNAc was first suggested by the phenotype of the *Eogt* mutant in

A



B

***O*-GlcNAcylated proteins**	***O*-GlcNAc site**	**Protein function**	**Species**	**Ref.**
Notch1	LNAFSCQCMPGYTGQKC	Receptor for Notch signaling	*Drosophila*	[10]
Dumpy	Unspecified	Apical extracellular matrix protein	*Drosophila*	[11,12]
Hspg2 (Perlecan)	ACAPGYTGRRCES	heparan sulfate proteoglycan/extracellular matrix component	Mouse	[14]
Nell1	VCPSGFTGSHCEK	Glycoprotein involved in bone physiology	Mouse	[14]
Lama5	TCPPGLSGERCDT	One of the vertebrate laminin alpha chains	Mouse	[14]
Pamr1	ACLAGYTGQRCEN	Regeneration-associated Muscle protease homolog	Mouse	[14]
Notch2	VCSPGFTGQRCNI (?)	Receptor for Notch signaling	Mouse	[14]
thrombospondin-1	CGACPPGYSGNGIQCTLELVPR	Adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions	Human	[15]
Delta	Unspecified	Ligand for Notch signaling	Drosophila	[11,12]
Serrate	Unspecified	Ligand for Notch signaling	Drosophila	[12]

Figure 1 Extracellular *O*-linked β-*N*-acetylglucosamine. A: The *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc)ylation of extracellular protein domains is a newly identified translational modification of epidermal growth factor (EGF) domains, including Notch, HSPG2, Pamr1, and Lama5. Extracellular *O*-GlcNAc is mediated by EOGT in the endoplasmic reticulum (ER). Mutations in EOGT were recently identified in patients with Adams-Oliver syndrome (AOS). The role of EOGT in the pathogenesis of AOS is currently unknown. Given that RBPJ, a transcriptional factor for Notch signaling, is a causative gene for AOS, *O*-GlcNAcylation of Notch receptors by EOGT might regulate Notch receptor trafficking or Notch-ligand interactions. ARHGAP31 or DOCK6, another causative gene for AOS, affects the actin cytoskeleton by regulating Cdc42 and Rac1 activity. Thus, another possibility is that the *O*-GlcNAcylation of unidentified cell adhesion molecules by EOGT affects actin dynamics. It should be noted, however, that Dumpy homologues are not present in mammals. The *O*-GlcNAcylation of Notch ligands was reported in Drosophila. The causative genes for AOS are shown by asterisks; B: Summary of proteins with extracellular *O*-GlcNAc identified to date.

Drosophila[11]. Although the *Eogt* mutant does not exhibit the classical Notch phenotype, it shows defects in the wings, notum, and cuticle (*i.e.*, wing blistering, vortex, and cuticle detachment), similar to the *dumpy* mutant[11,12]. As mentioned above, Dumpy is a membrane-tethered protein that represents a major *O*-GlcNAcylated protein in the cuticle[11]. Moreover, the genetic interaction and phenotypic similarity between *Eogt* and *dumpy* suggests that EOGT is required for Dumpy-dependent epithelial cell-matrix interactions.

Previous studies using *Eogt* mutant embryos suggested that *O*-GlcNAc is required for the correct targeting of Dumpy into the chitinous matrix, possibly by mediating interactions with other components in the extracellular matrix (ECM)[11]. Currently, the molecular mechanisms by which Dumpy mediates cell adhesion are unknown, and thus the precise mechanism by which *O*-GlcNAc mediates cell adhesion must await the functional characterization of Dumpy. However, it is intriguing to speculate that multiple *O*-GlcNAc moieties arranged regularly along the EGF repeats of Dumpy have the ability to associate with unidentified chitin (a polymer of GlcNAc)-binding lectins in the ECM, thereby enabling the cuticle assembly/maintenance required for epidermis adhesion.

Interestingly, comprehensive genetic interaction studies revealed an interaction between *Eogt* and pyrimidine metabolism in the wing blister phenotype[12]. Thus, an alternative possibility is that loss of *Eogt* directs the increased UDP-GlcNAc pool in the cytoplasm. This will lead to elevated pyrimidine synthesis, such as uracil, that is likely to promote wing blistering[12]. If this is the case, EOGT might regulate pyrimidine metabolism by *O*-GlcNAcylating Dumpy. The contribution of pyrimidine metabolism to the *Eogt* phenotype was also suggested by the genetic interaction between *Eogt* and the Notch signaling genes,

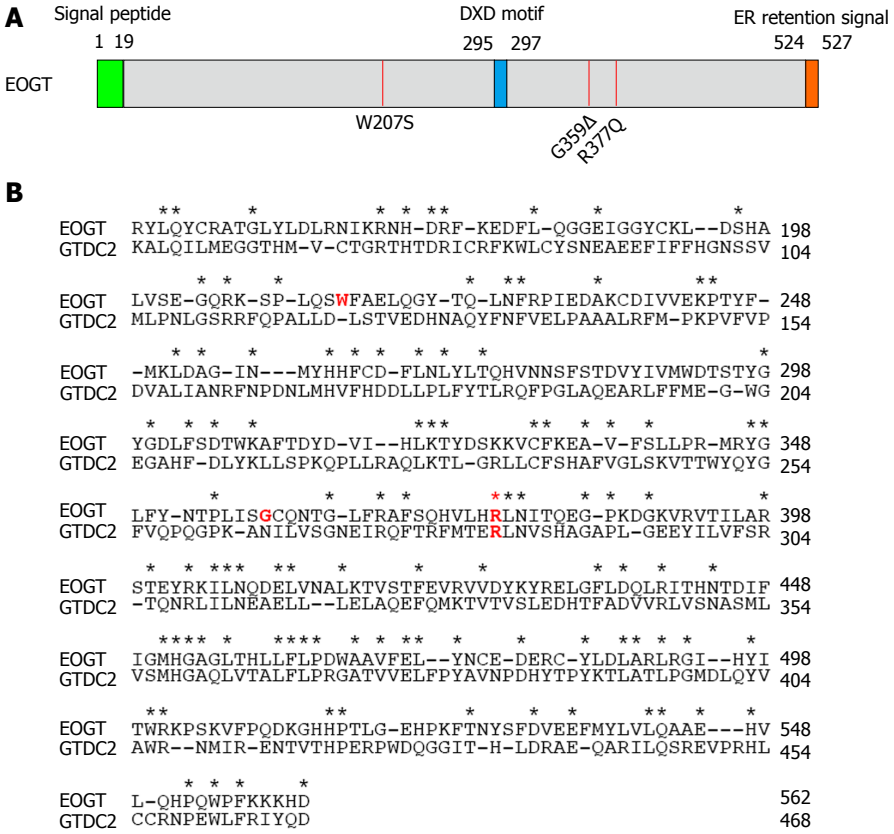


Figure 2 Extracellular *O*-linked β-*N*-acetylglucosamine mutations found in Adams-Oliver syndrome. A: A schematic representation of the primary structure of EOGT. The amino-terminal signal peptide is shown in yellow and the carboxyl-terminal Lys-Asp-Glu-Leu-like endoplasmic reticulum (ER) retrieval signal is in orange. The putative DXD motif involved in binding the nucleotide sugar is shown in blue. The position of each mutation is indicated by a red line; B: The amino acid sequence alignment of mouse EOGT (NP_780522, 149-562 aa) and mouse GTDC2/EOGT-L (Q8BW41, 55-468 aa). Identical amino acid residues are indicated by asterisks. Amino acid residues corresponding to the mutations in patients with Adams-Oliver syndrome are highlighted by red letters. EOGT: Extracellular *O*-linked β-*N*-acetylglucosamine.

which are involved in pyrimidine synthesis regulation[12].

EXTRACELLULAR *O*-GLCNAC AND ITS RELATIONSHIP TO ADAMS-OLIVER SYNDROME

The significance of the *O*-GlcNAcylated proteins was only tested in the context of Dumpy function, and the physiological roles of *O*-GlcNAc in mammals have not been investigated. However, exome sequencing in Adams-Oliver syndrome (AOS) patients provided important insights into the significance of extracellular *O*-GlcNAc in mammals. AOS is a rare congenital disorder characterized by vertex scalp defects [aplasia cutis congenital (ACC)] and terminal transverse limb defects (TTLDs)[21]. Recently, homozygous mutations in *EOGT* were identified in some patients with AOS[20,22]. These mutations include missense mutations (W207S and R377Q) and a frame shift mutation that creates a premature stop codon (G359Dfs*28) (Figure 2A). Currently, the blood levels of extracellular *O*-GlcNAc, the sugar moiety and its metabolites in the patients have not yet been investigated. However, the frame shift mutation in *EOGT* likely abolishes the enzyme activity because the truncated form of EOGT lacks the putative catalytic region containing the sequences conserved between EOGT and GTDC2, another ER-resident GlcNAc transferase modifying α-dystroglycan[23-25] (Figure 2B). The biochemical properties of the W207S and R377Q mutations have not yet been addressed. However, the R377 residue of EOGT is conserved in GTDC2. Thus, it is likely that the R377 residue may be important for GlcNAc transferase activity in EOGT and GTDC2 and that the R377Q mutation impairs the *O*-GlcNAc transferase activity of EOGT.

AOS is genetically heterogeneous, and its molecular pathology appears complex. In addition to *EOGT*, homozygous mutations of *DOCK6,* gain-of-function mutations of *ARHGAP31*, and heterozygous mutations for *RBPJ* were reported in AOS[26-28] (Figure 1A). *ARHGAP31* and *DOCK6* encode proteins that regulate the activity of key regulators of the actin cytoskeleton, RAC1 and CDC42. Accordingly, patient fibroblasts harboring disease-causing *ARHGAP31* or *DOCK6* mutations exhibited disorganized cytoskeletons and morphologies[27,28]. By contrast, *EOGT* mutant fibroblasts showed a typical spindle appearance comparable to that of control fibroblasts[22]. Therefore, it appears that EOGT does not directly affect the actin cytoskeleton, although the pos-

sibility remains that EOGT affects actin dynamism in restricted cell-types other than fibroblasts.

EXTRACELLULAR *O*-GLCNAC AND NOTCH SIGNALING

Another intriguing possibility for the role of EOGT in the pathogenesis of AOS involves Notch regulation because *RBPJ* encodes the transcriptional factor for Notch signaling. It has been reported that disease-causing *RBPJ* mutations decrease binding to the Notch target promoter, *HES1*[26]. Therefore, if EOGT and RBPJ act through a common signaling pathway in AOS, EOGT might positively regulate Notch signaling by the *O*-GlcNAcylation of Notch receptors. It should be noted, however, that no experimental data are available to support this hypothesis.

In *Drosophila*, *O*-GlcNAcylated EGF domains could be simultaneously modified with other *O*-glycosylations, namely *O*-fucose and *O*-glucose. *O*-fucosylation and *O*-glucosylation are catalyzed by ER-resident glycosyltransferases, POFUT1/Ofut1[29] and POGLUT1/Rumi[30]. These enzymes play indispensable roles for Notch signaling by affecting the trafficking, processing, and ligand-binding ability of Notch receptors[30-37]. In contrast, *O*-GlcNAc is dispensable for the majority of Notch receptor functions because *Eogt* mutants failed to exhibit apparent defects in most Notch-dependent biological processes, including embryonic neurogenesis, wing margin formation, and wing vein specification[11]. Given that the mutation of *Ofut1* or *rumi* does not produce Dumpy-like phenotypes, *O*-GlcNAcylation and *O*-fucosylation/*O*-glucosylation appears to be significant for the separate protein functions and distinct developmental processes in *Drosophila*. Nonetheless, there remains the possibility that these *O*-glycosylations may have partially redundant roles for Notch function, which would be revealed by genetic interaction studies between *Eogt* and *rumi*/*Poglut1* or *Eogt* and *Ofut1*/*Pofut1*.

Currently, no animal models for AOS have been established, and no AOS-related phenotypes were reported in *RBPJ* heterozygous mice[38]. In this regard, it would be interesting to investigate whether *EOGT* mutant mice would serve as a disease model for AOS.

CONCLUSION

The *O*-GlcNAc on extracellular protein domains is the most recently identified *O*-glycosylation of EGF repeat-containing proteins such as Notch receptors. This *O*-GlcNAc modification occurs in the secretory pathway by EOGT in the ER. In *Drosophila*, Dumpy was identified as a major *O*-GlcNAcylated protein that contributes to the interaction between epithelial cells and cuticles. Recent reports revealed that the mutations in *EOGT* cause AOS. However, the significance of the *O*-GlcNAcylated proteins was only tested in the context of Dumpy function in *Drosophila*, and the roles of *O*-GlcNAc in mammals have not been elucidated. In mammals, extracellular *O*-GlcNAc was detected on the TSP1, Hspg2, Nell1, Lama5, Pamr1, and Notch receptors[14,15]. Considering that a number of extracellular and transmembrane proteins are potentially *O*-GlcNAcylated by EOGT, additional studies will be required to address the roles of extracellular *O*-GlcNAc in Notch-dependent and independent biological processes in mammals as well as the molecular pathogenesis of human disease.

REFERENCES

1 **Torres CR**, Hart GW. Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. *J Biol Chem* 1984; **259**: 3308-3317 [PMID: 6421821]

2 **Hart GW**, Slawson C, Ramirez-Correa G, Lagerlof O. Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. *Annu Rev Biochem* 2011; **80**: 825-858 [PMID: 21391816 DOI: 10.1146/annurev-biochem-060608-102511]

3 **Yang X**, Ongusaha PP, Miles PD, Havstad JC, Zhang F, So WV, Kudlow JE, Michell RH, Olefsky JM, Field SJ, Evans RM. Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance. *Nature* 2008; **451**: 964-969 [PMID: 18288188 DOI: 10.1038/nature06668]

4 **Zachara NE**, Hart GW. O-GlcNAc a sensor of cellular state: the role of nucleocytoplasmic glycosylation in modulating cellular function in response to nutrition and stress. *Biochim Biophys Acta* 2004; **1673**: 13-28 [PMID: 15238246 DOI: 10.1016/j.bbagen.2004.03.016]

5 **Capotosti F**, Guernier S, Lammers F, Waridel P, Cai Y, Jin J, Conaway JW, Conaway RC, Herr W. O-GlcNAc transferase catalyzes site-specific proteolysis of HCF-1. *Cell* 2011; **144**: 376-388 [PMID: 21295698 DOI: 10.1016/j.cell.2010.12.030]

6 **Love DC**, Krause MW, Hanover JA. O-GlcNAc cycling: emerging roles in development and epigenetics. *Semin Cell Dev Biol* 2010; **21**: 646-654 [PMID: 20488252 DOI: 10.1016/j.semcdb.2010.05.001]

7 **O'Donnell N**, Zachara NE, Hart GW, Marth JD. Ogt-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability. *Mol Cell Biol* 2004; **24**: 1680-1690 [PMID: 14749383 DOI: 10.1128/Mcb.24.4.1680-1690.2004]

8 **Ogawa M**, Mizofuchi H, Kobayashi Y, Tsuzuki G, Yamamoto M, Wada S, Kamemura K. Terminal differentiation program of skeletal myogenesis is negatively regulated by O-GlcNAc glycosylation. *Biochim Biophys Acta* 2012; **1820**: 24-32 [PMID: 22056510 DOI: 10.1016/j.bbagen.2011.10.011]

9 **Ogawa M**, Sakakibara Y, Kamemura K. Requirement of decreased O-GlcNAc glycosylation of Mef2D for its recruitment to the myogenin promoter. *Biochem Biophys Res Commun* 2013; **433**: 558-562 [PMID: 23523791 DOI: 10.1016/j.bbrc.2013.03.033]

10 **Matsuura A**, Ito M, Sakaidani Y, Kondo T, Murakami K, Furukawa K, Nadano D, Matsuda T, Okajima T. O-linked N-acetylglucosamine is present on the extracellular domain of notch receptors. *J Biol Chem* 2008; **283**: 35486-35495 [PMID: 18948267 DOI: 10.1074/Jbc.M806202200]

11 **Sakaidani Y**, Nomura T, Matsuura A, Ito M, Suzuki E, Murakami K, Nadano D, Matsuda T, Furukawa K, Okajima T. O-linked-N-acetylglucosamine on extracellular protein domains mediates epithelial cell-matrix interactions. *Nat Commun* 2011; **2**: 583 [PMID: 22158438 DOI: 10.1038/ncomms1591]

12 **Müller R**, Jenny A, Stanley P. The EGF repeat-specific

O-GlcNAc-transferase Eogt interacts with notch signaling and pyrimidine metabolism pathways in Drosophila. *PLoS One* 2013; **8**: e62835 [PMID: 23671640 DOI: 10.1371/journal.pone.0062835]

13 **Sakaidani Y**, Ichiyanagi N, Saito C, Nomura T, Ito M, Nishio Y, Nadano D, Matsuda T, Furukawa K, Okajima T. O-linked-N-acetylglucosamine modification of mammalian Notch receptors by an atypical O-GlcNAc transferase Eogt1. *Biochem Biophys Res Commun* 2012; **419**: 14-19 [PMID: 22310717 DOI: 10.1016/j.bbrc.2012.01.098]

14 **Alfaro JF**, Gong CX, Monroe ME, Aldrich JT, Clauss TR, Purvine SO, Wang Z, Camp DG, Shabanowitz J, Stanley P, Hart GW, Hunt DF, Yang F, Smith RD. Tandem mass spectrometry identifies many mouse brain O-GlcNAcylated proteins including EGF domain-specific O-GlcNAc transferase targets. *Proc Natl Acad Sci USA* 2012; **109**: 7280-7285 [PMID: 22517741 DOI: 10.1073/pnas.1200425109]

15 **Hoffmann BR**, Liu Y, Mosher DF. Modification of EGF-like module 1 of thrombospondin-1, an animal extracellular protein, by O-linked N-acetylglucosamine. *PLoS One* 2012; **7**: e32762 [PMID: 22403705 DOI: 10.1371/journal.pone.0032762]

16 **Ishida N**, Kawakita M. Molecular physiology and pathology of the nucleotide sugar transporter family (SLC35). *Pflugers Arch* 2004; **447**: 768-775 [PMID: 12759756 DOI: 10.1007/s00424-003-1093-0]

17 **Ashikov A**, Routier F, Fuhlrott J, Helmus Y, Wild M, Gerardy-Schahn R, Bakker H. The human solute carrier gene SLC35B4 encodes a bifunctional nucleotide sugar transporter with specificity for UDP-xylose and UDP-N-acetylglucosamine. *J Biol Chem* 2005; **280**: 27230-27235 [PMID: 15911612 DOI: 10.1074/jbc.M504783200]

18 **Maszczak-Seneczko D**, Sosicka P, Olczak T, Jakimowicz P, Majkowski M, Olczak M. UDP-N-acetylglucosamine transporter (SLC35A3) regulates biosynthesis of highly branched N-glycans and keratan sulfate. *J Biol Chem* 2013; **288**: 21850-21860 [PMID: 23766508 DOI: 10.1074/jbc.M113.460543]

19 **Sesma JI**, Esther CR, Kreda SM, Jones L, O'Neal W, Nishihara S, Nicholas RA, Lazarowski ER. Endoplasmic reticulum/golgi nucleotide sugar transporters contribute to the cellular release of UDP-sugar signaling molecules. *J Biol Chem* 2009; **284**: 12572-12583 [PMID: 19276090 DOI: 10.1074/jbc.M806759200]

20 **Shaheen R**, Aglan M, Keppler-Noreuil K, Faqeih E, Ansari S, Horton K, Ashour A, Zaki MS, Al-Zahrani F, Cueto-González AM, Abdel-Salam G, Temtamy S, Alkuraya FS. Mutations in EOGT confirm the genetic heterogeneity of autosomal-recessive Adams-Oliver syndrome. *Am J Hum Genet* 2013; **92**: 598-604 [PMID: 23522784 DOI: 10.1016/j.ajhg.2013.02.012]

21 **Algaze C**, Esplin ED, Lowenthal A, Hudgins L, Tacy TA, Selamet Tierney ES. Expanding the phenotype of cardiovascular malformations in Adams-Oliver syndrome. *Am J Med Genet A* 2013; **161A**: 1386-1389 [PMID: 23613382 DOI: 10.1002/ajmg.a.35864]

22 **Cohen I**, Silberstein E, Perez Y, Landau D, Elbedour K, Langer Y, Kadir R, Volodarsky M, Sivan S, Narkis G, Birk OS. Autosomal recessive Adams-Oliver syndrome caused by homozygous mutation in EOGT, encoding an EGF domain-specific O-GlcNAc transferase. *Eur J Hum Genet* 2014; **22**: 374-378 [PMID: 23860037 DOI: 10.1038/ejhg.2013.159]

23 **Yoshida-Moriguchi T**, Willer T, Anderson ME, Venzke D, Whyte T, Muntoni F, Lee H, Nelson SF, Yu L, Campbell KP. SGK196 is a glycosylation-specific O-mannose kinase required for dystroglycan function. *Science* 2013; **341**: 896-899 [PMID: 23929950 DOI: 10.1126/science.1239951]

24 **Ogawa M**, Nakamura N, Nakayama Y, Kurosaka A, Manya H, Kanagawa M, Endo T, Furukawa K, Okajima T. GTDC2 modifies O-mannosylated α-dystroglycan in the endoplasmic reticulum to generate N-acetyl glucosamine epitopes reactive with CTD110.6 antibody. *Biochem Biophys Res Commun* 2013; **440**: 88-93 [PMID: 24041696 DOI: 10.1016/j.bbrc.2013.09.022]

25 **Yagi H**, Nakagawa N, Saito T, Kiyonari H, Abe T, Toda T, Wu SW, Khoo KH, Oka S, Kato K. AGO61-dependent GlcNAc modification primes the formation of functional glycans on α-dystroglycan. *Sci Rep* 2013; **3**: 3288 [PMID: 24256719 DOI: 10.1038/srep03288]

26 **Hassed SJ**, Wiley GB, Wang S, Lee JY, Li S, Xu W, Zhao ZJ, Mulvihill JJ, Robertson J, Warner J, Gaffney PM. RBPJ mutations identified in two families affected by Adams-Oliver syndrome. *Am J Hum Genet* 2012; **91**: 391-395 [PMID: 22883147 DOI: 10.1016/j.ajhg.2012.07.005]

27 **Shaheen R**, Faqeih E, Sunker A, Morsy H, Al-Sheddi T, Shamseldin HE, Adly N, Hashem M, Alkuraya FS. Recessive mutations in DOCK6, encoding the guanidine nucleotide exchange factor DOCK6, lead to abnormal actin cytoskeleton organization and Adams-Oliver syndrome. *Am J Hum Genet* 2011; **89**: 328-333 [PMID: 21820096 DOI: 10.1016/j.ajhg.2011.07.009]

28 **Southgate L**, Machado RD, Snape KM, Primeau M, Dafou D, Ruddy DM, Branney PA, Fisher M, Lee GJ, Simpson MA, He Y, Bradshaw TY, Blaumeiser B, Winship WS, Reardon W, Maher ER, FitzPatrick DR, Wuyts W, Zenker M, Lamarche-Vane N, Trembath RC. Gain-of-function mutations of ARHGAP31, a Cdc42/Rac1 GTPase regulator, cause syndromic cutis aplasia and limb anomalies. *Am J Hum Genet* 2011; **88**: 574-585 [PMID: 21565291 DOI: 10.1016/j.ajhg.2011.04.013]

29 **Wang Y**, Shao L, Shi S, Harris RJ, Spellman MW, Stanley P, Haltiwanger RS. Modification of epidermal growth factor-like repeats with O-fucose. Molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. *J Biol Chem* 2001; **276**: 40338-40345 [PMID: 11524432 DOI: 10.1074/jbc.M107849200]

30 **Acar M**, Jafar-Nejad H, Takeuchi H, Rajan A, Ibrani D, Rana NA, Pan H, Haltiwanger RS, Bellen HJ. Rumi is a CAP10 domain glycosyltransferase that modifies Notch and is required for Notch signaling. *Cell* 2008; **132**: 247-258 [PMID: 18243100 DOI: 10.1016/j.cell.2007.12.016]

31 **Okajima T**, Irvine KD. Regulation of notch signaling by o-linked fucose. *Cell* 2002; **111**: 893-904 [PMID: 12526814]

32 **Sasamura T**, Sasaki N, Miyashita F, Nakao S, Ishikawa HO, Ito M, Kitagawa M, Harigaya K, Spana E, Bilder D, Perrimon N, Matsuno K. neurotic, a novel maternal neurogenic gene, encodes an O-fucosyltransferase that is essential for Notch-Delta interactions. *Development* 2003; **130**: 4785-4795 [PMID: 12917292 DOI: 10.1242/dev.00679]

33 **Sasamura T**, Ishikawa HO, Sasaki N, Higashi S, Kanai M, Nakao S, Ayukawa T, Aigaki T, Noda K, Miyoshi E, Taniguchi N, Matsuno K. The O-fucosyltransferase O-fut1 is an extracellular component that is essential for the constitutive endocytic trafficking of Notch in Drosophila. *Development* 2007; **134**: 1347-1356 [PMID: 17329366 DOI: 10.1242/dev.02811]

34 **Shi S**, Stanley P. Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. *Proc Natl Acad Sci USA* 2003; **100**: 5234-5239 [PMID: 12697902 DOI: 10.1073/pnas.0831126100]

35 **Fernandez-Valdivia R**, Takeuchi H, Samarghandi A, Lopez M, Leonardi J, Haltiwanger RS, Jafar-Nejad H. Regulation of mammalian Notch signaling and embryonic development by the protein O-glucosyltransferase Rumi. *Development* 2011; **138**: 1925-1934 [PMID: 21490058 DOI: 10.1242/dev.060020]

36 **Brückner K**, Perez L, Clausen H, Cohen S. Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature* 2000; **406**: 411-415 [PMID: 10935637 DOI: 10.1038/35019075]

37 **Moloney DJ**, Panin VM, Johnston SH, Chen J, Shao L, Wilson R, Wang Y, Stanley P, Irvine KD, Haltiwanger RS, Vogt TF. Fringe is a glycosyltransferase that modifies Notch. *Nature* 2000; **406**: 369-375 [PMID: 10935626 DOI:

10.1038/35019000]
38 **Han H,** Tanigaki K, Yamamoto N, Kuroda K, Yoshimoto M, Nakahata T, Ikuta K, Honjo T. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol* 2002; **14**: 637-645 [PMID: 12039915]

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MINIREVIEWS

Regulation and function of signal transducer and activator of transcription 3

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Core tip: The differential subcellular localization of signal transducer and activator of transcription 3 makes it play distinct functions in transcriptional regulation, cell proliferation and cellular respiration, thus contributing to development, reproduction and tumorigenesis in physiological and pathological conditions.

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Abstract

Signal transducer and activator of transcription 3 (STAT3), a member of the STAT family, is a key regulator of many physiological and pathological processes. Significant progress has been made in understanding the transcriptional control, posttranslational modification, cellular localization and functional regulation of STAT3. STAT3 can translocate into the nucleus and bind to specific promoter sequences, thereby exerting transcriptional regulation. Recent studies have shown that STAT3 can also translocate into mitochondria, participating in aerobic respiration and apoptosis. In addition, STAT3 plays an important role in inflammation and tumorigenesis by regulating cell proliferation, differentiation and metabolism. Conditional knockout mouse models make it possible to study the physiological function of STAT3 in specific tissues and organs. This review summarizes the latest advances in the understanding of the expression, regulation and function of STAT3 in physiological and tumorigenic processes.

INTRODUCTION

Signal transducer and activator of transcription factors (STATs) are a family of transcription factors that regulate cell growth, survival, differentiation, and motility. Structural studies identified that STAT proteins consist of an N-terminal domain, a coiled-coil domain, a DNA-binding domain, a Src homology 2 (SH2) domain and a transactivation domain, of which the DNA-binding domain is required for the recognition of specific binding sequences. Until now, seven members of the STAT family have been identified and characterized, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. Despite the difference from canonical oncogenes, STAT3 has been recognized as a critical regulator in tumor cells since its identification[1]. STAT3 is over-expressed or activated by various carcinogenic agents, and can induce cell proliferation, differentiation and anti-apoptosis by activating the target genes, including STAT3, c-Myc and p53[2]. STAT3 exists in two main isoforms, full-length STAT3α

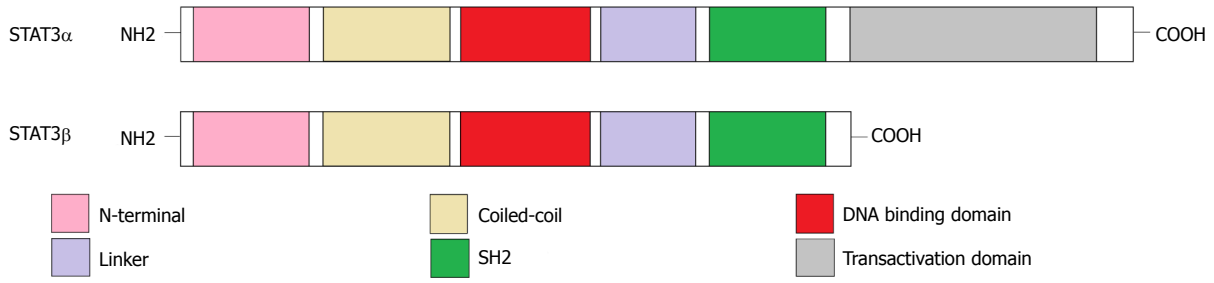

Figure 1 Domain structure of signal transducer and activator of transcription (3α and 3β). The signal transducer and activator of transcription 3α (STAT3α) protein is composed of N-terminal, coiled-coil, DNA binding, linker, SH2, and transactivation domains. However, the transactivation domain is absent in the alternative splicing variant, STAT3β.

and truncated STAT3β generated by alternative splicing. Under normal circumstances, STAT3α is the main isoform expressed in cells. STAT3β can competitively bind to the promoter of STAT3α target genes and inhibit the transactivation function of STAT3α. Additionally, STAT3β has its own specific target genes that differ from those of STAT3α[3].

STAT3 protein exists in a latent or inactive form in the cytoplasm. STAT3 can be activated by receptor-associated kinases and phosphorylated at various phosphorylation sites, particularly at Tyr-705 and Ser-727. Previous studies suggested that only phosphorylated STAT3 (p-STAT3) can translocate into the nucleus. However, recent data indicated that the nuclear translocation and transcriptional activity are partially independent of phosphorylation pathways[4]. Furthermore, STAT3 may translocate into mitochondria to control cell metabolism independent of its transcriptional regulatory activity[5]. Here we review the emerging biochemical and biological data on STAT3 and discuss its comprehensive roles in animal development and etiopathology of various diseases.

TRANSCRIPTIONAL REGULATION OF STAT3

STAT3 protein is expressed at a basal level in cells but rapidly increases once activated by specific cytokines. STAT3 is a critical factor in interleukin-6 (IL-6) induced gene regulation. STAT3 can be phosphorylated by IL-6 signal pathway, whereas IL-6 can also activate STAT3 at the transcriptional level. The level of STAT3 mRNA increases 1 h after IL-6 treatment and reaches to the maximum value at 3 h. There is an IL-6 response element (IL-6RE) in the promoter of STAT3 which contains a low affinity STAT3-binding element and a cAMP-responsive element (CRE). STAT3 executes its regulation in cooperation with this CRE-binding protein through self-activation[6].

In diabetic mice, estrogen administration can increase the level of STAT3 mRNA. There is a binding site of estrogen receptor α (ERα) in STAT3 promoter. Estrogen treatment induces the accumulation of ERα on STAT3 promoter and regulates the expression of STAT3[7]. STAT3 overexpression in tumor cells is related to the cytoplasmic/nuclear accumulation of β-catenin and the activation of β-catenin/T-cell factors (TCF) pathway. β-catenin is a key mediator in cell adhesion and signal transduction. Overexpression of β-catenin enhances both STAT3 mRNA and protein levels. There is a functional TCF binding element in STAT3 promoter, indicating that β-catenin/TCF may participate in the regulation of STAT3 expression[8].

The suppressors of cytokine signaling (SOCS) family consists of eight members, including SOCS1 to SOCS7 and cytokine-inducible SH2 domain proteins (CIS)[9]. SOCS proteins exist at low levels in resting cells and dramatically increase after STAT activation. SOCS proteins serve as classic negative regulatory factors of STAT activation[10]. Among them, SOCS3, a target gene of STAT3, contributes to negative feedback regulation of the JAK/STAT3 signal pathway, and inhibits the self-activation of STAT3[11]. Bone marrow SOCS3 deficient mice exhibit overexpression of STAT3 and continuous activation of the JAK/STAT3 signal pathway, suggesting that STAT3 expression is negatively regulated by SOCS3[12].

POST-TRANSCRIPTIONAL REGULATION OF STAT3 EXPRESSION

Human *STAT3* gene is located on the long (q) arm of chromosome 17 at position 21.31. The encoding product of the STAT3 gene is an 89 kDa protein[13]. Further study identified a cDNA clone encoding a variant of STAT3 (named STAT3β), which is different from classic STAT3 (named STAT3α). Compared to STAT3α, STAT3β is the truncated form and lacks the internal domain of 50 base pairs located near the C-terminus (Figure 1). The encoding product of STAT3β is an 80 kDa protein. Under normal conditions, STAT3β exists in various cells, such as monocytes, lymphocytes and neutrophil granulocytes. In COS cells, STAT3β is phosphorylated at tyrosine sites by IL-5R treatment and binds to the palindromic IL-6/interferon-γ response element (pIRE) located in the promoter of intercellular adhesion molecule-1 (ICAM-1). However, this phosphorylated STAT3β exhibits a negative transcriptional regulation through inhibiting the transactivation potential of STAT3α, suggesting that STAT3β may be a dominant-negative regulator of transcription and

promotes apoptosis[14].

Depending on context, truncated STAT3β can be phosphorylated at tyrosine 705 and bind to DNA sequence that is equal to that bound by STAT3α with negative transcriptional regulation. Overexpression of STAT3β can induce apoptosis and inhibit tumor growth[15,16]. However, alternative splicing regulation by antisense oligodeoxynucleotides targeting STAT3 can specifically shift the expression from STAT3α to STAT3β. High expression of endogenous STAT3β promotes cell apoptosis and leads to cell cycle arrest. This apoptosis-promoting effect of STAT3β is independent on the inhibition of STAT3α target genes. Several genes that differ from classic STAT3α target genes are specifically decreased by STAT3β knockdown, including lens epithelium-derived growth factor, p300/CBP-associated factor, Cyclin C, peroxisomal biogenesis factor 1 and STAT1β[3], indicating that STAT3β may promote cell apoptosis through regulating its own specific target genes in addition to negative transcriptional regulation of STAT3α.

POST-TRANSLATIONAL MODIFICATION OF STAT3

STAT3 phosphorylation

STAT3 protein exists in the cytoplasm as an inactive form until phosphorylation by receptor-associated kinases. Activated JAK kinases phosphorylate STAT3 through binding of the SH2 domain to a phosphorylated tyrosine residue, by which the C-terminus of p-STAT3 triggers its release from receptor, and form a homo- or hetero-dimerization of p-STAT3. Dimerized STAT3 translocates to the nucleus and binds to the promoters bearing cognate DNA-binding sequences[17]. STAT3 can be also phosphorylated by other tyrosine kinases, such as the Src family. However, such Src-induced STAT3 phosphorylation does not always result in STAT3 activation[18]. Tyrosine phosphorylation is necessary for STAT3 activity. In addition, serine phosphorylation at residue 727 of STAT3 also leads to the up-regulation of the transcriptional activity. STAT3 phosphorylation at Ser-727 is mediated by MAPK, P38 and c-Jun N-terminal kinase (JNK) pathways, and involved in transcriptional regulation of the target genes of STAT3[19]. Ser-727 mutant STAT3 knock-in mice display impaired development and survival process[20]. Recently, several articles reported that un-phosphorylated STAT3 can interact with nuclear factor-κB (NF-κB). Un-phosphorylated STAT3 (U-STAT3)/NF-κB complex translocates into the nucleus and activates the expression of NF-κB target genes[21].

STAT3 acetylation

Protein acetylation is a crucial post-translational modification of gene expression and involved in extensive physiological and pathological processes[22]. Investigation on protein acetylation is focused on the alteration of chromatin structure and activation of transcription factors. The inhibition of histone deacetylases (HDACs) can induce the acetylation of STAT3 at Lys-685, and acetylated STAT3 (Ac-STAT3) regulates the function of dendritic cells through activating the transcription of indoleamine 2,3-dioxygenase[23].

The significant increase in STAT3 acetylation at Lys-685 is detected in tumor tissues. CD44, a transmembrane glycoprotein, has been recognized as a marker for tumor cells. Activated CD44 can bind STAT3 and p300 in the nucleus and acetylate STAT3 at Lys-685. CD44/Ac-STAT3 complex activates cyclinD1 expression by binding to its promoter, leading to cell proliferation[24]. Additionally, Ac-STAT3 may be the major determinant for promoter methylation of tumor suppressor genes. DNA methyltransferase 1 (DNMT1) is primarily involved in the maintenance of methylation. Ac-STAT3/DNMT1 complex can induce gene silencing through binding to target genes, leading to increased CpG island methylation. STAT3 mutant at Lys685 exhibits impaired STAT3 acetylation and tumor growth. Acetylation inhibitors and HDAC activators can inhibit STAT3 acetylation with demethylation and reactivation of several tumor-suppressor genes, including cyclin-dependent kinase inhibitor 2A (CDKN2A), deleted in lung and esophageal cancer 1 (DLEC1) and STAT1. In triple-negative breast cancer cells and melanoma, Ac-STAT3 is related to the methylation of the ERα gene. Therefore, inhibition of Ac-STAT3 is favorable for hormone therapy through reactivating ERα expression[25].

Other post-translational modification of STAT3

Except for phosphorylation and acetylation, STAT1 and STAT3 are also subjected to SUMOylation through binding to small ubiquitin-like modifier (SUMO). STAT3 SUMOylation suppresses the transcriptional activity of STAT3 by affecting STAT3 phosphorylation and dimerization[26].

STAT3 LOCALIZATION AND FUNCTION

Nucleo-cytoplasmic shuttling of p-STAT3

Since protein synthesis and modification are processed in the cytoplasm, most transcription factors need to pass through the nuclear pore complex and enter into the nucleus to exert their transcriptional activity. In general, proteins that have a molecular weight greater than 50 kDa require specific structural domain named nuclear localization sequence (NLS) and nuclear export sequence (NES). Both NLS- and NES-containing proteins can recognize and combine with specific soluble carriers to mediate the nucleo-cytoplasmic trafficking[27]. Most NLS can recognize importin α and co-regulate the shuttling of proteins through interacting with importin β1[28].

The transcriptional regulatory activity of STAT3 is dependent on nuclear translocation. The distinction between STAT3 and other STAT members is that activated STAT3 can shuttle between the cytoplasm and nucleus, and accumulate in the nucleus to play the role in tran-

scriptional activation. In the canonical nuclear translocation, p-STAT3 is released from the receptor, forms a homo- or hetero-dimer, and translocates into the nucleus. Importin α3 can specifically recognize the coiled-coil domain and mediate the nucleo-cytoplasmic shuttling of STAT3 protein[29].

Nucleo-cytoplasmic shuttling of U-STAT3

Previous studies showed that STAT3 protein acquires its DNA binding activity only in a phosphorylated form. However, recent studies indicated that the transcriptional activation of STAT3 in the nucleus is also independent of phosphorylation[21]. Both phosphorylated and unphosphorylated STAT3 proteins exist in the nucleus and regulate different target genes. Data from fluorescently-labeled STAT3 mutants in STAT3 deficient cells show that U-STAT3 can shuttle constitutively between the cytoplasm and nucleus under the condition of NLS and NES mutation, indicating that the nuclear accumulation of U-STAT3 is independent of the binding of NLS or NES and importins. Both native gel electrophoresis and dual-focus fluorescence correlation spectroscopy identify that the N-terminal domain is essential for dimer formation and nuclear accumulation of U-STAT3. The monomeric N-terminal deletion mutant can be phosphorylated and dimerized in response to IL-6 treatment without nuclear accumulation. Therefore, the N-terminal domain has an important role in nucleo-cytoplasmic trafficking of U-STAT3[30].

STAT3 in mitochondria

Except for the classic transcriptional regulation during cell proliferation and differentiation through nuclear translocation, STAT3 translocation in different organelles may regulate cell metabolism and be involved in a broad range of biological functions independent of transcriptional activity. For instance, phosphorylated STAT3 at Serine 727 (P-Ser-STAT3) is localized to the mitochondria of hepatocytes and myocardial cells. STAT3 deficient cells exhibit a low activity of complex I and Ⅱ[31], suggesting that STAT3 regulates mitochondrial respiration *via* electron transport chain. Data from co-immunoprecipitation indicate that the translocation of STAT3 to mitochondria is mediated by the presequence receptor Tom20[32]. However, the mechanism that STAT3 alters mitochondrial respiration is controversial. There is an unfavorable ratio of complexes Ⅰ / Ⅱ and STAT3 in cardiac tissue, which implied the existence of an additional mechanism of STAT3 regulation of ATP production *in vivo*[33]. The sirtuin 1 (SIRT1), a NAD-dependent deacetylase, is located in the nucleus and known as a key factor regulating and controlling the mitochondrial bioenergetics by means of activating gene expression through deacetylating some important signal molecules, such as STAT3. In Sirt1-null cells, there is a significantly higher serine-phosphorylated STAT3 level in mitochondria with an increase in the mitochondrial bioenergetics and ATP formation[34].

In eukaryotes, the primary function of mitochondria is aerobic respiration and energy production, in which the reactive oxygen species (ROS) is the inevitable by-products. During the process of ischemia-reperfusion injury in the myocardium, the opening of mitochondrial permeability transition pore (MPTP) is a major response to cardiomyocyte death, while the ROS from respiratory chain is the primary endogenous reason for MPTP opening. Mitochondria play a major role in cardio-protection, most likely by preventing MPTP opening, while mitochondrial STAT3 has an impact on inhibiting MPTP opening and cardio-protection. In calcium-induced MPTP opening model, STAT3-KO mitochondria tolerate less induction of MPTP opening. The function of STAT3 in MPTP stability may be carried out through binding to cyclophilin D[32]. Another study found that GRIM-19-induced mitochondrial STAT3 location may involve in TNF-mediated necroptosis[35].

It is identified that cancer cells have the feature of metabolic turnover in aerobic glycolysis - the Warburg effect[36], in which STAT3 acts as a central mediator of cell metabolism through both HIF-1α-dependent and -independent mechanisms. Oncogenic signals activate STAT3 phosphorylation and induce STAT3 translocation into the nucleus where it regulates HIF-1α expression. Mitochondrial STAT3 displays Serine 727 phosphorylation, while tyrosine phosphorylation or DNA binding activity is not detected, unlike canonical transcriptional activation. p-Ser-STAT3 located in mitochondria shows many metabolic functions and induces malignant transformation mediated by oncogenic Ras[37]. Fibroblast growth factor receptor 4-R388 (FGFR4-R388), a known single nucleotide polymorphism which promotes breast cancer cell motility and invasiveness, can promote mitochondrial cytochrome c activity and induce pituitary tumor cell growth through STAT3 serine phosphorylation. Therefore, serine phosphorylation of STAT3 and mitochondrial translocation may contribute to tumor cell transformation and tumorigenesis[38].

FUNCTION OF STAT3 IN PATHOPHYSIOLOGY AND DEVELOPMENT

STAT3 in stem cells

Mouse embryonic stem cells (ES cells) are pluripotent cells derived from the inner cell mass of blastocysts. The self-renewal and pluripotency of ES cells depend on leukemia inhibitory factor (LIF) and bone morphogenetic protein 2 (BMP2) during *in vitro* culture[39]. Based on chromatin immunoprecipitation-deep sequencing (ChIP-seq), 13 specific transcriptional factors (Nanog, Oct4, STAT3, Smad1, Sox2, Zfx, c-Myc, n-Myc, Klf4, Esrrb, Tcfcp2l1, E2f1, and CTCF) and 2 transcription regulators (p300 and Suz12) are identified in the regulatory network of ES cells, and these factors are involved in LIF and BMP signaling pathways, and play important roles in self-

renewal, reprogramming and pluripotency of ES cells[40].

LIF activates STAT3 through the Janus kinase (JAK) signal pathway. p-STAT3 is functionally associated with the transcriptional regulation of target genes for the self-renewal of ES cells, including Kruppel-like factors (Klf4 and Klf5)[41]. Furthermore, persistently activated STAT3 can maintain the self-renewal process without LIF[42]. Transcriptional factors Nanog and STAT3 are the molecular markers of ES cells. Nanog and STAT3 co-regulate the transcriptional activation of STAT3 target genes through binding to their promoters, such as α2M and Nanog promoters. This activation is abrogated by eliminating LIF, indicating that the function of Nanog and STAT3 is dependent on the LIF signal pathway[43]. Overexpression of STAT3 target genes, such as Klf4 and Klf5[41], has been shown to promote self-renewal of ES cells, while knockdown of these genes has no impact on the self-renewal in the presence of LIF or STAT3[44]. Gastrulation brain homeobox 2 (Gbx2), a LIF/STAT3 target gene, can facilitate the pluripotency of ES cells when over-expressed without LIF and STAT3[45]. These results illustrated that LIF/STAT3 may act upstream to trigger the maintenance of ES cells through activating a range of downstream target genes.

STAT3 in proliferation and apoptosis

P-STAT3 can activate proliferation-related genes to promote cell proliferation. Moreover, U-STAT3 can bind to the promoters of pro-apoptotic genes and inhibit their expression in tumor cells, but not in normal cells. Inhibitors of STAT3 phosphorylation or dominant-negative STAT3 mutants facilitate the expression of pro-apoptosis factors, suggesting that STAT3 plays a dominant role in regulating cell proliferation and anti-apoptosis[46]. STAT3 knockout mice exhibit complete embryonic lethality. STAT3 deficient embryos show a rapid degeneration on day 7 of pregnancy, highlighting the important role of STAT3 in embryo development[47]. Conditional ablation of STAT3 in myocardial cells leads to higher susceptibility to drug-induced heart failure[48]. In addition, ischemic preconditioning can induce the phosphorylation of STAT3 at Tyr-705 and Ser-727 in myocardial cells. However, the expression of cardio-protective factor (COX-2 and HO-1) and anti-apoptotic proteins [Mcl-1, Bcl-x (L) and c-FLIP (S)] is elevated in normal cells 24 h later, but not in STAT3 deficient cells[49]. These results illustrated the function of STAT3 in anti-inflammation and anti-apoptosis.

Mammary gland involution initiates at the ending of lactation, involving extensive apoptosis of the secretory alveolar epithelium and inflammatory response. Although STAT3 is expressed in the mammary gland throughout the whole reproductive cycle, it is only activated by LIF on the day of delivery and at 6-12 h after weaning[50]. STAT3 has an important role in mammary gland involution. Conditional ablation of STAT3 in mammary cells causes delayed involution of the mammary gland[51]. STAT3 is involved in the apoptotic process of mammary epithelial cells and tissue remodeling through inducing the expression of pro-apoptotic factors and regulating the balance of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP)[52]. Mammary STAT3 deficient mice have impaired accumulation of inflammatory factors, macrophages and mastocytes in the mammary gland[53]. In addition, p-STAT3 in mammary epithelial cells is also involved in lysosomal-mediated cell death pathway through up-regulating the expression of lysosomal proteases cathepsin B and L[54]. Therefore, STAT3 expression in the mammary gland may participate in apoptosis under physiological conditions.

STAT3 in tumorigenesis and cancer-related inflammation

As a key transcriptional factor, p-STAT3 can translocate into the nucleus and bind to specific DNA sequences to activate the expression of target genes, including c-Myc and FGFR2, consequently regulating the proliferation, differentiation and anti-apoptosis of tumor cells[55,56]. Furthermore, acetylated STAT3 can induce the down-regulation of tumor suppressor genes through promoter methylation and facilitate tumorigenesis. MicroRNAs are short non-coding RNAs (ncRNAs) mediating post-transcriptional down-regulation of target genes and functioning in cell proliferation and apoptosis. MicroRNA-21 (miR-21) is an oncogene that contributes to anti-apoptosis in most tumor cells. There are two strictly conserved STAT3 binding sites in the enhancer of miR-21. MiR-21 induction by IL-6 is STAT3-dependent. ChIP results also confirm the accumulation of STAT3 in the upstream enhancer of miR-21[57], indicating that IL-6/STAT3 pathway contributes to miR-21 induction.

Chronic infection and inflammation contribute to about 15% of human cancers. The inflammatory response can induce necrotic cell death accompanied with activation of numerous cytokines, growth factors and chemokines which facilitate cell proliferation and survival[58]. The STAT3 signal pathway is the major intrinsic pathway for inflammation in tumor cells. STAT3 activates many inflammatory-related genes including BCL-XL, intercellular adhesion molecule 1 and vascular endothelial growth factor, and is involved in the maintenance of inflammatory environment[59]. NF-κB has the ability to induce the expression of inflammatory mediators, and is the major pathway functioning in inflammation-induced carcinogenesis and anti-tumor immunity. The signaling pathways of STATs, especially STAT3, are closely related with NF-κB signaling[60]. The inflammatory factor IL-6, the target gene of NF-κB, is the important STAT3 activator. In tumor cells, STAT3 directly interacts with NF-κB, translocates into the nucleus and contributes to the constitutive NF-κB activation in cancer. In addition, STAT3 binding to NF-κB also regulates numerous oncogenic and inflammatory genes[61].

Targeting the STAT3 pathway should be a promising and novel form of treatment for human cancers. Blocking STAT3 by siRNAs, antisense oligonucleotides, dominant-negative mutants, and specific inhibitors of

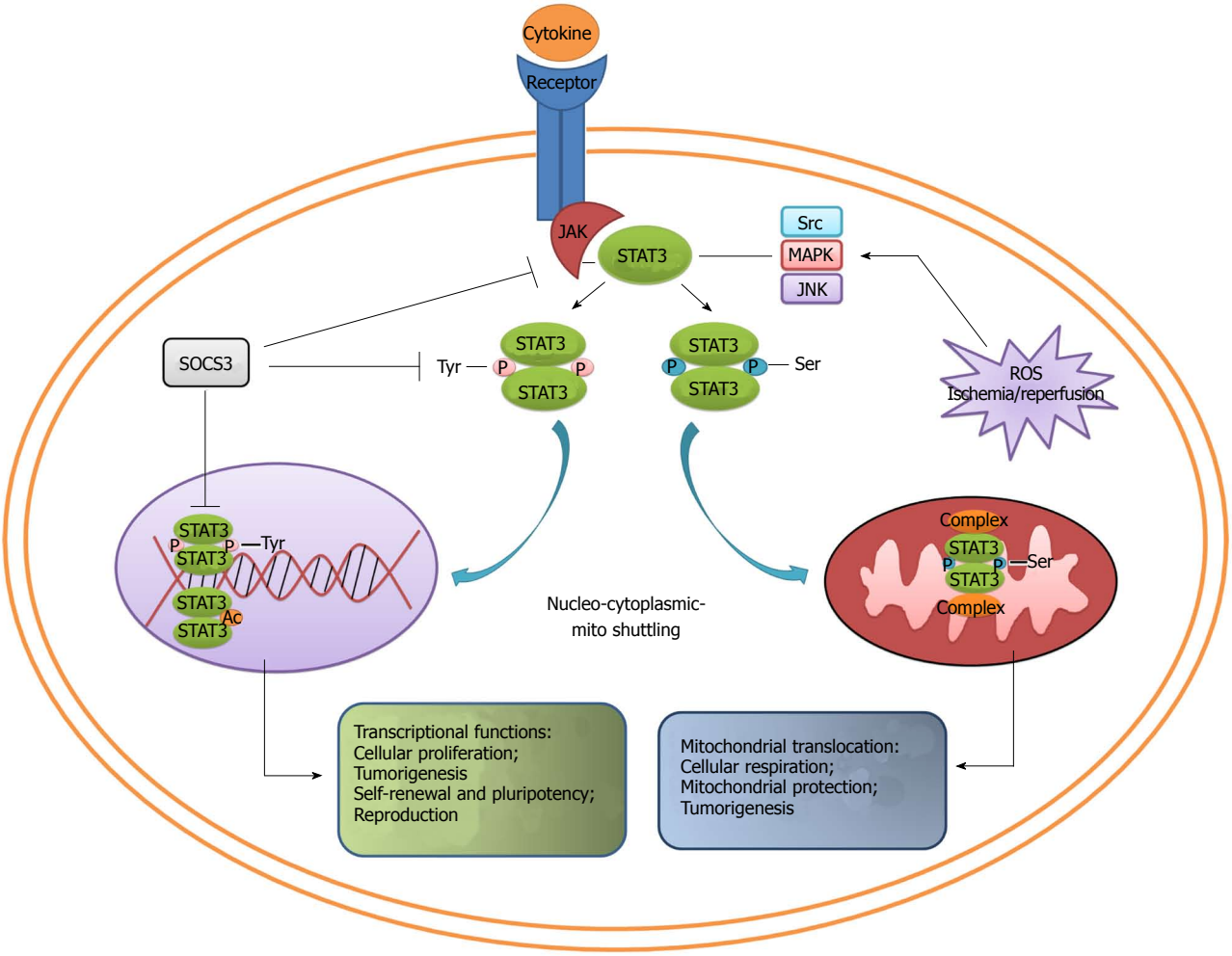

Figure 2 Converging roles of signal transducer and activator of transcription 3. Different signals can selectively trigger signal transducer and activator of transcription 3 phosphorylation. Tyr-phosphorylated STAT3 translocates into the nucleus and regulates gene expression, thus playing an important role in cell proliferation, tumorigenesis, self-renewal and pluripotency. On the other hand, Ser-phosphorylated STAT3 translocates into mitochondria, binds with the complexes in respiratory chain, and ultimately maintains the cellular respiration and mitochondrial protection. MAPK: Mitogen-activated protein kinase; ROS: Reactive oxygen species; JNK: c-Jun N-terminal kinase; JAK: Janus kinase.

STAT3 in combination with chemotherapeutics can synergistically inhibit the growth, invasion and metastasis of carcinoma cells[62-64]. Therefore, inhibiting STAT3 signals are a promising therapeutic target for most types of human cancers with constitutively activated STAT3.

STAT3 in reproduction

In mammals, the uterus is receptive to blastocyst during a restricted time termed as "implantation window". LIF is expressed at a high level during implantation window in humans and mice. LIF deficient mice display embryo implantation failure[65]. In mouse uterus, STAT3 protein is expressed and phosphorylated in the luminal epithelium on day 4 of pregnancy. LIF treatment induces the STAT3 phosphorylation in mouse uterine luminal epithelium isolated from day 4 of pregnancy but not for days 3 and 5[66]. LIF antagonist (LA, truncated LIF protein) injection led to the failure of mouse embryo implantation through inhibiting STAT3 phosphorylation[67]. In humans, LIF and STAT3 are expressed in decidual tissues during early pregnancy. LIF can activate STAT3 phosphorylation in both non-decidualized and decidualized human endometrial stromal cells *in vitro*[68], indicating that LIF/STAT3 signaling is involved in human embryo implantation and decidualization.

To investigate the function of STAT3 during embryo implantation, a cell-permeable STAT3 peptide inhibitor is injected into mouse uterine lumen before implantation, which significantly reduces embryo implantation by 70%. STAT3 phosphorylation in uterine luminal epithelium activated by LIF and some LIF targeted genes, such as Irg1, is significantly inhibited by STAT3 inhibitors both *in vivo* and *in vitro*[69]. Meanwhile, the injection of STAT3 decoy into uterine lumen during implantation also causes implantation failure[70]. Co-immunoprecipitation assay showed that STAT3 can bind to progesterone receptor A (PR-A) and co-regulate the embryo implantation and decidualization in mice. Conditional ablation of STAT3 only in PR-positive cells ($PR^{cre/+}Stat3^{f/f}$; $Stat3^{d/d}$) is used to investigate the role of STAT3 in reproduction. Conditional ablation of STAT3 in the uterus ($Stat3^{d/d}$) results in embryo implantation failure. Furthermore, $Stat3^{d/d}$ mice

are also defective in hormonally induced decidual reaction[71], suggesting that the interaction between STAT3 and PR is essential for successful implantation.

CONCLUSION

STAT3 is a key transcription factor and regulates a multitude of genes important for proliferation, differentiation, apoptosis, inflammation and tumorigenesis. STAT3 expression and activity are regulated through alternative splicing, post-translational modification and subcellular localization. STAT3β, the new isoform of STAT3, participates in apoptosis and plays a role distinct from STAT3α. Despite the different mechanism, STAT3 activation through phosphorylation or acetylation can facilitate tumorigenesis synergistically. STAT3 shuttles among the cytoplasm, nucleus, mitochondria and some other possible organelles, and exerts its diverse functions in transcriptional regulation, cellular respiration, proliferation and apoptosis. A variety of animal models reveal that STAT3 is essential for embryo development, pluripotency maintenance of stem cells, embryo implantation and decidualization. Increasing evidence confirms that STAT3 is a key modulator of cancer and inflammation (Figure 2). Hence, further clarification of the biological function of STAT3 will validate its promising application prospect for gene therapy in multi-directions.

REFERENCES

1 **Bromberg J**, Wang TC. Inflammation and cancer: IL-6 and STAT3 complete the link. *Cancer Cell* 2009; **15**: 79-80 [PMID: 19185839 DOI: 10.1016/j.ccr.2009.01.009]

2 **Kathiria AS**, Neumann WL, Rhees J, Hotchkiss E, Cheng Y, Genta RM, Meltzer SJ, Souza RF, Theiss AL. Prohibitin attenuates colitis-associated tumorigenesis in mice by modulating p53 and STAT3 apoptotic responses. *Cancer Res* 2012; **72**: 5778-5789 [PMID: 22869582 DOI: 10.1158/0008-5472.CAN-12-0603]

3 **Zammarchi F**, de Stanchina E, Bournazou E, Supakorndej T, Martires K, Riedel E, Corben AD, Bromberg JF, Cartegni L. Antitumorigenic potential of STAT3 alternative splicing modulation. *Proc Natl Acad Sci USA* 2011; **108**: 17779-17784 [PMID: 22006329 DOI: 10.1073/pnas.1108482108]

4 **Timofeeva OA**, Chasovskikh S, Lonskaya I, Tarasova NI, Khavrutskii L, Tarasov SG, Zhang X, Korostyshevskiy VR, Cheema A, Zhang L, Dakshanamurthy S, Brown ML, Dritschilo A. Mechanisms of unphosphorylated STAT3 transcription factor binding to DNA. *J Biol Chem* 2012; **287**: 14192-14200 [PMID: 22378781 DOI: 10.1074/jbc.M111.323899]

5 **Shaw PE**. Could STAT3 provide a link between respiration and cell cycle progression? *Cell Cycle* 2010; **9**: 4294-4296 [PMID: 20962592 DOI: 10.4161/cc.9.21.13677]

6 **Ichiba M**, Nakajima K, Yamanaka Y, Kiuchi N, Hirano T. Autoregulation of the Stat3 gene through cooperation with a cAMP-responsive element-binding protein. *J Biol Chem* 1998; **273**: 6132-6138 [PMID: 9497331 DOI: 10.1074/jbc.273.11.6132]

7 **Gao H**, Bryzgalova G, Hedman E, Khan A, Efendic S, Gustafsson JA, Dahlman-Wright K. Long-term administration of estradiol decreases expression of hepatic lipogenic genes and improves insulin sensitivity in ob/ob mice: a possible mechanism is through direct regulation of signal transducer and activator of transcription 3. *Mol Endocrinol* 2006; **20**: 1287-1299 [PMID: 16627594 DOI: 10.1210/me.2006-0012]

8 **Yan S**, Zhou C, Zhang W, Zhang G, Zhao X, Yang S, Wang Y, Lu N, Zhu H, Xu N. beta-Catenin/TCF pathway upregulates STAT3 expression in human esophageal squamous cell carcinoma. *Cancer Lett* 2008; **271**: 85-97 [PMID: 18602747 DOI: 10.1016/j.canlet.2008.05.035]

9 **Haan S**, Wüller S, Kaczor J, Rolvering C, Nöcker T, Behrmann I, Haan C. SOCS-mediated downregulation of mutant Jak2 (V617F, T875N and K539L) counteracts cytokine-independent signaling. *Oncogene* 2009; **28**: 3069-3080 [PMID: 19543316 DOI: 10.1038/onc.2009.155]

10 **Leroith D**, Nissley P. Knock your SOCS off! *J Clin Invest* 2005; **115**: 233-236 [PMID: 15690080]

11 **Ji Y**, Wang Z, Li Z, Li K, Le X, Zhang T. Angiotensin II induces angiogenic factors production partly via AT1/JAK2/STAT3/SOCS3 signaling pathway in MHCC97H cells. *Cell Physiol Biochem* 2012; **29**: 863-874 [PMID: 22613986 DOI: 10.1159/000171034]

12 **Qin H**, Yeh WI, De Sarno P, Holdbrooks AT, Liu Y, Muldowney MT, Reynolds SL, Yanagisawa LL, Fox TH, Park K, Harrington LE, Raman C, Benveniste EN. Signal transducer and activator of transcription-3/suppressor of cytokine signaling-3 (STAT3/SOCS3) axis in myeloid cells regulates neuroinflammation. *Proc Natl Acad Sci USA* 2012; **109**: 5004-5009 [PMID: 22411837 DOI: 10.1073/pnas.1117218109]

13 **Akira S**, Nishio Y, Inoue M, Wang XJ, Wei S, Matsusaka T, Yoshida K, Sudo T, Naruto M, Kishimoto T. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell* 1994; **77**: 63-71 [PMID: 7512451 DOI: 10.1016/0092-8674(94)90235-6]

14 **Caldenhoven E**, van Dijk TB, Solari R, Armstrong J, Raaijmakers JA, Lammers JW, Koenderman L, de Groot RP. STAT3beta, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription. *J Biol Chem* 1996; **271**: 13221-13227 [PMID: 8675499]

15 **Huang Y**, Qiu J, Dong S, Redell MS, Poli V, Mancini MA, Tweardy DJ. Stat3 isoforms, alpha and beta, demonstrate distinct intracellular dynamics with prolonged nuclear retention of Stat3beta mapping to its unique C-terminal end. *J Biol Chem* 2007; **282**: 34958-34967 [PMID: 17855361 DOI: 10.1074/jbc.M704548200]

16 **Ng IH**, Ng DC, Jans DA, Bogoyevitch MA. Selective STAT3-α or -β expression reveals spliceform-specific phosphorylation kinetics, nuclear retention and distinct gene expression outcomes. *Biochem J* 2012; **447**: 125-136 [PMID: 22799634 DOI: 10.1042/BJ20120941]

17 **Liu L**, Nam S, Tian Y, Yang F, Wu J, Wang Y, Scuto A, Polychronopoulos P, Magiatis P, Skaltsounis L, Jove R. 6-Bromoindirubin-3′-oxime inhibits JAK/STAT3 signaling and induces apoptosis of human melanoma cells. *Cancer Res* 2011; **71**: 3972-3979 [PMID: 21610112 DOI: 10.1158/0008-5472.CAN-10-3852]

18 **Michels S**, Trautmann M, Sievers E, Kindler D, Huss S, Renner M, Friedrichs N, Kirfel J, Steiner S, Endl E, Wurst P, Heukamp L, Penzel R, Larsson O, Kawai A, Tanaka S, Sonobe H, Schirmacher P, Mechtersheimer G, Wardelmann E, Büttner R, Hartmann W. SRC signaling is crucial in the growth of synovial sarcoma cells. *Cancer Res* 2013; **73**: 2518-2528 [PMID: 23580575 DOI: 10.1158/0008-5472.CAN-12-3023]

19 **Tworkoski K**, Singhal G, Szpakowski S, Zito CI, Bacchiocchi A, Muthusamy V, Bosenberg M, Krauthammer M, Halaban R, Stern DF. Phosphoproteomic screen identifies potential therapeutic targets in melanoma. *Mol Cancer Res* 2011; **9**: 801-812 [PMID: 21521745 DOI: 10.1158/1541-7786.MCR-10-0512]

20 **Shen Y**, Schlessinger K, Zhu X, Meffre E, Quimby F, Levy DE, Darnell JE. Essential role of STAT3 in postnatal survival and growth revealed by mice lacking STAT3 serine 727 phosphorylation. *Mol Cell Biol* 2004; **24**: 407-419 [PMID:

14673173 DOI: 10.1128/MCB.24.1.407-419.2004]
21 **Yang J**, Stark GR. Roles of unphosphorylated STATs in signaling. *Cell Res* 2008; **18**: 443-451 [PMID: 18364677 DOI: 10.1038/cr.2008.41]
22 **Hohl M**, Wagner M, Reil JC, Müller SA, Tauchnitz M, Zimmer AM, Lehmann LH, Thiel G, Böhm M, Backs J, Maack C. HDAC4 controls histone methylation in response to elevated cardiac load. *J Clin Invest* 2013; **123**: 1359-1370 [PMID: 23434587 DOI: 10.1172/JCI61084]
23 **Sun Y**, Chin YE, Weisiger E, Malter C, Tawara I, Toubai T, Gatza E, Mascagni P, Dinarello CA, Reddy P. Cutting edge: Negative regulation of dendritic cells through acetylation of the nonhistone protein STAT-3. *J Immunol* 2009; **182**: 5899-5903 [PMID: 19414739 DOI: 10.4049/jimmunol.0804388]
24 **Lee JL**, Wang MJ, Chen JY. Acetylation and activation of STAT3 mediated by nuclear translocation of CD44. *J Cell Biol* 2009; **185**: 949-957 [PMID: 19506034 DOI: 10.1083/jcb.200812060]
25 **Lee H**, Zhang P, Herrmann A, Yang C, Xin H, Wang Z, Hoon DS, Forman SJ, Jove R, Riggs AD, Yu H. Acetylated STAT3 is crucial for methylation of tumor-suppressor gene promoters and inhibition by resveratrol results in demethylation. *Proc Natl Acad Sci USA* 2012; **109**: 7765-7769 [PMID: 22547799 DOI: 10.1073/pnas.1205132109]
26 **Droescher M**, Begitt A, Marg A, Zacharias M, Vinkemeier U. Cytokine-induced paracrystals prolong the activity of signal transducers and activators of transcription (STAT) and provide a model for the regulation of protein solubility by small ubiquitin-like modifier (SUMO). *J Biol Chem* 2011; **286**: 18731-18746 [PMID: 21460228 DOI: 10.1074/jbc.M111.235978]
27 **Lu Q**, Lu Z, Liu Q, Guo L, Ren H, Fu J, Jiang Q, Clarke PR, Zhang C. Chromatin-bound NLS proteins recruit membrane vesicles and nucleoporins for nuclear envelope assembly via importin-α/β. *Cell Res* 2012; **22**: 1562-1575 [PMID: 22847741 DOI: 10.1038/cr.2012.113]
28 **Hayashi K**, Morita T. Differences in the nuclear export mechanism between myocardin and myocardin-related transcription factor A. *J Biol Chem* 2013; **288**: 5743-5755 [PMID: 23283978 DOI: 10.1074/jbc.M112.408120]
29 **Liu L**, McBride KM, Reich NC. STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-alpha3. *Proc Natl Acad Sci USA* 2005; **102**: 8150-8155 [PMID: 15919823 DOI: 10.1073/pnas.0501643102]
30 **Vogt M**, Domoszlai T, Kleshchanok D, Lehmann S, Schmitt A, Poli V, Richtering W, Müller-Newen G. The role of the N-terminal domain in dimerization and nucleocytoplasmic shuttling of latent STAT3. *J Cell Sci* 2011; **124**: 900-909 [PMID: 21325026 DOI: 10.1242/jcs.072520]
31 **Wegrzyn J**, Potla R, Chwae YJ, Sepuri NB, Zhang Q, Koeck T, Derecka M, Szczepanek K, Szelag M, Gornicka A, Moh A, Moghaddas S, Chen Q, Bobbili S, Cichy J, Dulak J, Baker DP, Wolfman A, Stuehr D, Hassan MO, Fu XY, Avadhani N, Drake JI, Fawcett P, Lesnefsky EJ, Larner AC. Function of mitochondrial Stat3 in cellular respiration. *Science* 2009; **323**: 793-797 [PMID: 19131594 DOI: 10.1126/science.1164551]
32 **Boengler K**, Hilfiker-Kleiner D, Heusch G, Schulz R. Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion. *Basic Res Cardiol* 2010; **105**: 771-785 [PMID: 20960209 DOI: 10.1007/s00395-010-0124-1]
33 **Phillips D**, Reilley MJ, Aponte AM, Wang G, Boja E, Gucek M, Balaban RS. Stoichiometry of STAT3 and mitochondrial proteins: Implications for the regulation of oxidative phosphorylation by protein-protein interactions. *J Biol Chem* 2010; **285**: 23532-23536 [PMID: 20558729 DOI: 10.1074/jbc.C110.152652]
34 **Bernier M**, Paul RK, Martin-Montalvo A, Scheibye-Knudsen M, Song S, He HJ, Armour SM, Hubbard BP, Bohr VA, Wang L, Zong Y, Sinclair DA, de Cabo R. Negative regulation of STAT3 protein-mediated cellular respiration by SIRT1 protein. *J Biol Chem* 2011; **286**: 19270-19279 [PMID: 21467030 DOI: 10.1074/jbc.M110.200311]
35 **Shulga N**, Pastorino JG. GRIM-19-mediated translocation of STAT3 to mitochondria is necessary for TNF-induced necroptosis. *J Cell Sci* 2012; **125**: 2995-3003 [PMID: 22393233 DOI: 10.1242/jcs.103093]
36 **Haigis MC**, Deng CX, Finley LW, Kim HS, Gius D. SIRT3 is a mitochondrial tumor suppressor: a scientific tale that connects aberrant cellular ROS, the Warburg effect, and carcinogenesis. *Cancer Res* 2012; **72**: 2468-2472 [PMID: 22589271 DOI: 10.1158/0008-5472.CAN-11-3633]
37 **Gough DJ**, Corlett A, Schlessinger K, Wegrzyn J, Larner AC, Levy DE. Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science* 2009; **324**: 1713-1716 [PMID: 19556508 DOI: 10.1126/science.1171721]
38 **Tateno T**, Asa SL, Zheng L, Mayr T, Ullrich A, Ezzat S. The FGFR4-G388R polymorphism promotes mitochondrial STAT3 serine phosphorylation to facilitate pituitary growth hormone cell tumorigenesis. *PLoS Genet* 2011; **7**: e1002400 [PMID: 22174695 DOI: 10.1371/journal.pgen.1002400]
39 **Niwa H**, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev* 1998; **12**: 2048-2060 [PMID: 9649508 DOI: 10.1101/gad.12.13.2048]
40 **Chen X**, Xu H, Yuan P, Fang F, Huss M, Vega VB, Wong E, Orlov YL, Zhang W, Jiang J, Loh YH, Yeo HC, Yeo ZX, Narang V, Govindarajan KR, Leong B, Shahab A, Ruan Y, Bourque G, Sung WK, Clarke ND, Wei CL, Ng HH. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell* 2008; **133**: 1106-1117 [PMID: 18555785 DOI: 10.1016/j.cell.2008.04.043]
41 **Bourillot PY**, Aksoy I, Schreiber V, Wianny F, Schulz H, Hummel O, Hubner N, Savatier P. Novel STAT3 target genes exert distinct roles in the inhibition of mesoderm and endoderm differentiation in cooperation with Nanog. *Stem Cells* 2009; **27**: 1760-1771 [PMID: 19544440 DOI: 10.1002/stem.110]
42 **Matsuda T**, Nakamura T, Nakao K, Arai T, Katsuki M, Heike T, Yokota T. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J* 1999; **18**: 4261-4269 [PMID: 10428964 DOI: 10.1093/emboj/18.15.4261]
43 **Torres J**, Watt FM. Nanog maintains pluripotency of mouse embryonic stem cells by inhibiting NFkappaB and cooperating with Stat3. *Nat Cell Biol* 2008; **10**: 194-201 [PMID: 18223644 DOI: 10.1038/ncb1680]
44 **Jiang J**, Chan YS, Loh YH, Cai J, Tong GQ, Lim CA, Robson P, Zhong S, Ng HH. A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat Cell Biol* 2008; **10**: 353-360 [PMID: 18264089 DOI: 10.1038/ncb1698]
45 **Tai CI**, Ying QL. Gbx2, a LIF/Stat3 target, promotes reprogramming to and retention of the pluripotent ground state. *J Cell Sci* 2013; **126**: 1093-1098 [PMID: 23345404 DOI: 10.1242/jcs.118273]
46 **Timofeeva OA**, Tarasova NI, Zhang X, Chasovskikh S, Cheema AK, Wang H, Brown ML, Dritschilo A. STAT3 suppresses transcription of proapoptotic genes in cancer cells with the involvement of its N-terminal domain. *Proc Natl Acad Sci USA* 2013; **110**: 1267-1272 [PMID: 23288901 DOI: 10.1073/pnas.1211805110]
47 **Schindler CW**. Series introduction. JAK-STAT signaling in human disease. *J Clin Invest* 2002; **109**: 1133-1137 [PMID: 11994400]
48 **Jacoby JJ**, Kalinowski A, Liu MG, Zhang SS, Gao Q, Chai GX, Ji L, Iwamoto Y, Li E, Schneider M, Russell KS, Fu XY. Cardiomyocyte-restricted knockout of STAT3 results in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age. *Proc Natl Acad Sci USA* 2003; **100**: 12929-12934 [PMID: 14566054 DOI: 10.1073/pnas.2134694100]

49 **Bolli R**, Stein AB, Guo Y, Wang OL, Rokosh G, Dawn B, Molkentin JD, Sanganalmath SK, Zhu Y, Xuan YT. A murine model of inducible, cardiac-specific deletion of STAT3: its use to determine the role of STAT3 in the upregulation of cardioprotective proteins by ischemic preconditioning. *J Mol Cell Cardiol* 2011; **50**: 589-597 [PMID: 21223971 DOI: 10.1016/j.yjmcc.2011.01.002]

50 **Kritikou EA**, Sharkey A, Abell K, Came PJ, Anderson E, Clarkson RW, Watson CJ. A dual, non-redundant, role for LIF as a regulator of development and STAT3-mediated cell death in mammary gland. *Development* 2003; **130**: 3459-3468 [PMID: 12810593 DOI: 10.1242/dev.00578]

51 **Humphreys RC**, Bierie B, Zhao L, Raz R, Levy D, Hennighausen L. Deletion of Stat3 blocks mammary gland involution and extends functional competence of the secretory epithelium in the absence of lactogenic stimuli. *Endocrinology* 2002; **143**: 3641-3650 [PMID: 12193580 DOI: 10.1210/en.2002-220224]

52 **Baxter FO**, Neoh K, Tevendale MC. The beginning of the end: death signaling in early involution. *J Mammary Gland Biol Neoplasia* 2007; **12**: 3-13 [PMID: 17340185 DOI: 10.1007/s10911-007-9033-9]

53 **Hughes K**, Wickenden JA, Allen JE, Watson CJ. Conditional deletion of Stat3 in mammary epithelium impairs the acute phase response and modulates immune cell numbers during post-lactational regression. *J Pathol* 2012; **227**: 106-117 [PMID: 22081431 DOI: 10.1002/path.3961]

54 **Kreuzaler PA**, Staniszewska AD, Li W, Omidvar N, Kedjouar B, Turkson J, Poli V, Flavell RA, Clarkson RW, Watson CJ. Stat3 controls lysosomal-mediated cell death in vivo. *Nat Cell Biol* 2011; **13**: 303-309 [PMID: 21336304 DOI: 10.1038/ncb2171]

55 **Ochi A**, Graffeo CS, Zambirinis CP, Rehman A, Hackman M, Fallon N, Barilla RM, Henning JR, Jamal M, Rao R, Greco S, Deutsch M, Medina-Zea MV, Bin Saeed U, Ego-Osuala MO, Hajdu C, Miller G. Toll-like receptor 7 regulates pancreatic carcinogenesis in mice and humans. *J Clin Invest* 2012; **122**: 4118-4129 [PMID: 23023703 DOI: 10.1172/JCI63606]

56 **Wei W**, Liu W, Cassol CA, Zheng W, Asa SL, Ezzat S. The breast cancer susceptibility gene product fibroblast growth factor receptor 2 serves as a scaffold for regulation of NF-κB signaling. *Mol Cell Biol* 2012; **32**: 4662-4673 [PMID: 22988296 DOI: 10.1128/MCB.00935-12]

57 **Löffler D**, Brocke-Heidrich K, Pfeifer G, Stocsits C, Hackermüller J, Kretzschmar AK, Burger R, Gramatzki M, Blumert C, Bauer K, Cvijic H, Ullmann AK, Stadler PF, Horn F. Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood* 2007; **110**: 1330-1333 [PMID: 17496199 DOI: 10.1182/blood-2007-03-081133]

58 **Coussens LM**, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860-867 [PMID: 12490959 DOI: 10.1038/nature01322]

59 **Yu H**, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer* 2009; **9**: 798-809 [PMID: 19851315 DOI: 10.1038/nrc2734]

60 **Lee H**, Deng J, Xin H, Liu Y, Pardoll D, Yu H. A requirement of STAT3 DNA binding precludes Th-1 immunostimulatory gene expression by NF-κB in tumors. *Cancer Res* 2011; **71**: 3772-3780 [PMID: 21502401 DOI: 10.1158/0008-5472.CAN-10-3304]

61 **He G**, Karin M. NF-κB and STAT3 - key players in liver inflammation and cancer. *Cell Res* 2011; **21**: 159-168 [PMID: 21187858 DOI: 10.1038/cr.2010.183]

62 **Ma J**, Wang S, Zhao M, Deng XS, Lee CK, Yu XD, Liu B. Therapeutic potential of cladribine in combination with STAT3 inhibitor against multiple myeloma. *BMC Cancer* 2011; **11**: 255 [PMID: 21679466 DOI: 10.1186/1471-2407-11-255]

63 **Souissi I**, Najjar I, Ah-Koon L, Schischmanoff PO, Lesage D, Le Coquil S, Roger C, Dusanter-Fourt I, Varin-Blank N, Cao A, Metelev V, Baran-Marszak F, Fagard R. A STAT3-decoy oligonucleotide induces cell death in a human colorectal carcinoma cell line by blocking nuclear transfer of STAT3 and STAT3-bound NF-κB. *BMC Cell Biol* 2011; **12**: 14 [PMID: 21486470 DOI: 10.1186/1471-2121-12-14]

64 **Kortylewski M**, Swiderski P, Herrmann A, Wang L, Kowolik C, Kujawski M, Lee H, Scuto A, Liu Y, Yang C, Deng J, Soifer HS, Raubitschek A, Forman S, Rossi JJ, Pardoll DM, Jove R, Yu H. In vivo delivery of siRNA to immune cells by conjugation to a TLR9 agonist enhances antitumor immune responses. *Nat Biotechnol* 2009; **27**: 925-932 [PMID: 19749770 DOI: 10.1038/nbt.1564]

65 **Zhang S**, Lin H, Kong S, Wang S, Wang H, Wang H, Armant DR. Physiological and molecular determinants of embryo implantation. *Mol Aspects Med* 2013; **34**: 939-980 [PMID: 23290997 DOI: 10.1016/j.mam.2012.12.011]

66 **Cheng JG**, Chen JR, Hernandez L, Alvord WG, Stewart CL. Dual control of LIF expression and LIF receptor function regulate Stat3 activation at the onset of uterine receptivity and embryo implantation. *Proc Natl Acad Sci USA* 2001; **98**: 8680-8685 [PMID: 11438698 DOI: 10.1073/pnas.151180898]

67 **White CA**, Zhang JG, Salamonsen LA, Baca M, Fairlie WD, Metcalf D, Nicola NA, Robb L, Dimitriadis E. Blocking LIF action in the uterus by using a PEGylated antagonist prevents implantation: a nonhormonal contraceptive strategy. *Proc Natl Acad Sci USA* 2007; **104**: 19357-19362 [PMID: 18042698 DOI: 10.1073/pnas.0710110104]

68 **Shuya LL**, Menkhorst EM, Yap J, Li P, Lane N, Dimitriadis E. Leukemia inhibitory factor enhances endometrial stromal cell decidualization in humans and mice. *PLoS One* 2011; **6**: e25288 [PMID: 21966484 DOI: 10.1371/journal.pone.0025288]

69 **Catalano RD**, Johnson MH, Campbell EA, Charnock-Jones DS, Smith SK, Sharkey AM. Inhibition of Stat3 activation in the endometrium prevents implantation: a nonsteroidal approach to contraception. *Proc Natl Acad Sci USA* 2005; **102**: 8585-8590 [PMID: 15937114 DOI: 10.1073/pnas.0502343102]

70 **Nakamura H**, Kimura T, Koyama S, Ogita K, Tsutsui T, Shimoya K, Taniguchi T, Koyama M, Kaneda Y, Murata Y. Mouse model of human infertility: transient and local inhibition of endometrial STAT-3 activation results in implantation failure. *FEBS Lett* 2006; **580**: 2717-2722 [PMID: 16647058 DOI: 10.1016/j.febslet.2006.04.029]

71 **Lee JH**, Kim TH, Oh SJ, Yoo JY, Akira S, Ku BJ, Lydon JP, Jeong JW. Signal transducer and activator of transcription-3 (Stat3) plays a critical role in implantation via progesterone receptor in uterus. *FASEB J* 2013; **27**: 2553-2563 [PMID: 23531596 DOI: 10.1096/fj.12-225664]

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ORIGINAL ARTICLE

Functional analysis of human Na^+/K^+-ATPase familial or sporadic hemiplegic migraine mutations expressed in *Xenopus* oocytes

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Abstract

AIM: Functional characterization of ATP1A2 mutations that are related to familial or sporadic hemiplegic migraine (FHM2, SHM).

METHODS: cRNA of human Na^+/K^+-ATPase α_2- and β_1-subunits were injected in *Xenopus laevis* oocytes. FHM2 or SHM mutations of residues located in putative α/β interaction sites or in the α_2-subunit's C-terminal region were investigated. Mutants were analyzed by the two-electrode voltage-clamp (TEVC) technique on *Xenopus* oocytes. Stationary K^+-induced Na^+/K^+ pump currents were measured, and the voltage dependence of apparent K^+ affinity was investigated. Transient currents were recorded as ouabain-sensitive currents in Na^+ buffers to analyze kinetics and voltage-dependent pre-steady state charge translocations. The expression of constructs was verified by preparation of plasma membrane and total membrane fractions of cRNA-injected oocytes.

RESULTS: Compared to the wild-type enzyme, the mutants G900R and E902K showed no significant differences in the voltage dependence of K^+-induced currents, and analysis of the transient currents indicated that the extracellular Na^+ affinity was not affected. Mutant G855R showed no pump activity detectable by TEVC. Also for L994del and Y1009X, pump currents could not be recorded. Analysis of the plasma and total membrane fractions showed that the expressed proteins were not or only minimally targeted to the plasma membrane. Whereas the mutation K1003E had no impact on K^+ interaction, D999H affected the voltage dependence of K^+-induced currents. Furthermore, kinetics of the transient currents was altered compared to the wild-type enzyme, and the apparent affinity for extracellular Na^+ was reduced.

CONCLUSION: The investigated FHM2/SHM mutations influence protein function differently depending on the structural impact of the mutated residue.

Key words: Na^+/K^+-ATPase; Electrophysiology; Voltage dependence; Familial hemiplegic migraine; C-terminus; β-subunit

Core tip: Mutations of the human *ATP1A2* gene, which encodes the Na^+/K^+-ATPase α_2-subunit, are associated with familial hemiplegic migraine (FHM2) that is inherited in an autosomal dominant fashion. We studied seven ATP1A2 mutations related to FHM2 or sporadic hemiplegic migraine by electrophysiological and biochemical methods to characterize functional impairments. The mutations G855R, G900R, E902K, L994del, D999H, K1003E and Y1009X were selected according to their structural importance: in putative interaction sites between α- and β-subunit and in the α-subunit's C-terminal region. Some of these mutations showed a severe loss of function, and we discuss the functional and physiological consequences in order to better un-

derstand the molecular basis for neurological impairments.

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INTRODUCTION

Migraine is a common neurological disease, and the different forms are defined by the International Headache Society criteria[1]. Familial hemiplegic migraine (FHM) and sporadic hemiplegic migraine (SHM) are rare autosomal-dominant subforms of migraine with aura. These syndromes are associated with some degree of motor weakness (hemiparesis) and other neurological symptoms during the aura phase. FHM is inherited in an autosomal dominant fashion and genetically heterogeneous. There are a number of mutations related to FHM in three different genes: the *CACNA1A* gene (FHM1) coding for the neuronal $Ca_V2.1$ calcium channel[2,3], the *ATP1A2* gene (FHM2) encoding the α_2-subunit of the Na^+/K^+-ATPase[4], and the *SCN1A* gene (FHM3) encoding the neuronal $Na_V1.1$ sodium channel[5]. The clinical symptoms of SHM are identical to those of FHM but without affected family members.

The Na^+/K^+-ATPase is a transmembrane protein which transports two K^+ ions in and three Na^+ ions out of the cell upon hydrolysis of ATP (Figure 1A). This electrogenic P-type ATPase assumes two principal conformational changes during its reaction cycle. Upon binding of three intracellular Na^+ ions in the ATP-bound E_1 conformation, the phosphorylated intermediate with occluded Na^+, $E_1P(3Na^+)$, is formed, followed by a change to the phosphorylated $E_2P(3Na^+)$ conformation, from which Na^+ ions are released to the extracellular medium. Because of the increased affinity for K^+ in this configuration, two K^+ ions bind subsequently, which triggers the dephosphorylation, and binding of intracellular ATP accelerates the conformational change from E_2 to E_1. At last, the K^+ ions dissociate to the cytoplasm. The sequential translocation of Na^+ and K^+ ions requires strict cation specificity of the phosphorylation and dephosphorylation reactions. According to the $3Na^+/2K^+$ stoichiometry of transport, electrogenic turnover activity of the Na^+/K^+-ATPase corresponds to outward movement of one positive charge per reaction cycle, and the major electrogenic event has been shown to take place during extracellular release or reverse binding of Na^+[6-8]. This has been suggested to arise from passage of Na^+ ions through a narrow, high-field access channel or 'ion well'[9,10].

The Na^+/K^+-ATPase consists of at least two mandatory subunits (Figure 1B). The large catalytic α-subunit is composed of ten transmembrane domains (M1-M10), which are linked by five extracellular and four intracellular loops. The smaller regulatory β-subunit is a single-span transmembrane protein (βM) with an ectodomain exhibiting several glycosylation sites. Several isoforms of both subunits are expressed in human cells in a tissue-specific manner. In human brain, the α_2-subunit is mainly expressed in glial cells (astrocytes), and loss-of-function of the Na^+/K^+-ATPase can result in neuronal hyperexcitability, which is commonly explained as follows. The Na^+/K^+-ATPase maintains the gradients for K^+ and Na^+ ions, which are essential for the accurate function of secondary active transporters or ion channels, whose activities depend on these gradients. On one hand, changes of the Na^+ gradient influence, first, the activity of the Na^+/Ca^{2+} exchanger (NCX) which is crucial for, *e.g.*, Ca^{2+} signaling. Second, the ability of the glial Na^+/glutamate symporter to remove the neurotransmitter glutamate from the synaptic cleft is affected. On the other hand, an altered K^+ gradient impairs the repolarizing activity of neuronal K^+ channels, which is critical for setting the threshold of action potential generation. Hyperkalemia is known to trigger the phenomenon of cortical spreading depression (CSD), the putatively causal mechanism of the aura phase during a migraine attack[11].

Up to now, far more than 50 mutations of the *ATP1A2* gene, which are associated with SHM or FHM2, have been described in literature[12,13]. Yet, most of these mutations have not been studied by electrophysiological techniques, which is a prerequisite for a better understanding of the functional consequences on enzyme activity.

In continuation of previous works[14,15], we studied seven FHM2 or SHM mutations, which are located in regions that are putatively critical for transport properties of the human Na^+/K^+-ATPase α_2-subunit, (Figure 1B), with the two-electrode voltage-clamp technique (TEVC) and biochemical methods to analyze protein expression. Since mutations in the α_2-subunit's C-terminal region were shown to have complex effects on enzyme activity, cation affinities and voltage dependence[16-19], we analyzed four mutations in the transmembrane segment αM10 and in the C-terminus (L994del, K1003E[13], D999H[20] and Y1009X[21]) to further understand structure-function relationships in the C-terminal region. Furthermore, interactions between the α- and β-subunit are not satisfactorily clarified so far. Especially, the highly conserved SYGQ motif in the αM7/M8-loop is believed to interact with the β-ectodomain[22,23]. The FHM2 mutations G900R[24] and E902K[25] are located within this motif and were functionally analyzed in this work. In addition, Gly852 (αM7) has previously been shown to interact with two tyrosines of the βM[26]. In this work, we show that the FHM2 mutation G855R[27] which is located near this interaction site, has severe consequences on the mutant protein's plasma membrane expression.

MATERIALS AND METHODS

Mutagenesis

As described before[14,19], human Na^+/K^+-ATPase α_2- and β_1-subunit cDNAs were subcloned into a modified pCD-

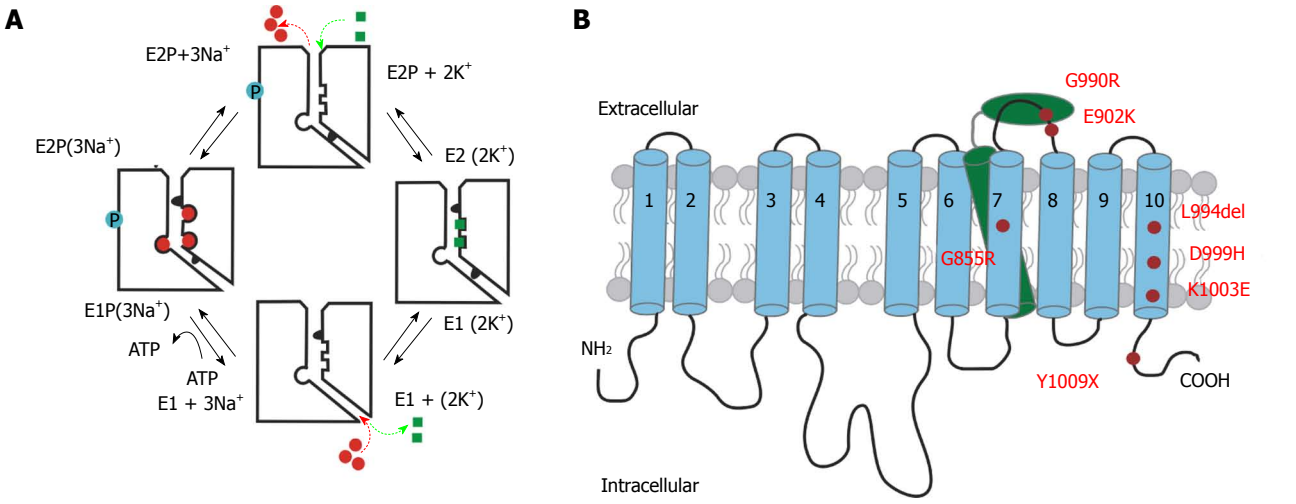

Figure 1 Reaction mechanism and structural detail of the Na^+/K^+-ATPase. A: Schematic reaction cycle of one Na^+/K^+-ATPase pump molecule. The cytosolic side is shown at the bottom of each molecule depicted with an ion pathway to the right, whereas the extracellular side is set at the top. Na^+ ions are shown as red circles, and K^+ ion are shown as green squares. Blue circles depict the phosphorylated state; B: Simplified structure of the Na^+/K^+-ATPase indicating FHM2/SHM mutation positions studied in this work. The α-subunit is composed of ten transmembrane domains (blue). The N- and C-terminus are located intracellularly. The β-subunit comprises only one transmembrane domain (green) and a large ectodomain with several glycosylation sites. FHM2/SHM mutations are marked in red.

NA3.1 vector. To distinguish the activity of the heterologously expressed constructs from the endogenous *Xenopus* Na^+/K^+-ATPase, the mutations Q116R and N127D were introduced in the human α_2-subunit to reduce the ouabain sensitivity (IC_{50} in a mmol/L range)[28]. This construct is herein referred to as "RD-WT". Mutants were designed by introducing mutations into the RD-WT α_2-construct by site-directed mutagenesis (Quikchange® kit, Stratagene). All PCR-derived fragments were verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Two-electrode voltage-clamp

cRNA synthesis was carried out with the T7 mMessage mMachine kit (Ambion, Austin, TX). 25 ng of α_2- and 2.5 ng of β_1-subunit cRNAs were coinjected into oocytes of *Xenopus laevis*. After three days incubation in ORI buffer (contents in mmol/L: 110 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, pH 7.4, and 50 mg/L gentamycin) at 18 ℃, oocytes were subjected to a Na^+ loading procedure preceding experiments to elevate $[Na^+]_{in}$. For this purpose, oocytes were incubated for 45 min in Na^+ loading solution (contents in mmol/L: 110 NaCl, 2.5 sodium citrate, 5 MOPS, 5 TRIS, pH 7.4) and stored subsequently in Na^+ buffer (in mmol/L: 100 NaCl, 1 $CaCl_2$, 5 $BaCl_2$, 2 $MgCl_2$ and 2.5 MOPS, 2.5 TRIS, pH 7.4) for at least 30 min.

Currents were recorded at room temperature (21 ℃-23 ℃) using a Turbotec 10CX amplifier (NPI instruments, Tamm, Germany) and pClamp 10 software (Axon Inst., Union City, CA). Solutions used for measurements were: Na^+ buffer (in mmol/L: 100 NaCl, 1 $CaCl_2$, 5 $BaCl_2$, 2 $MgCl_2$, 2.5 MOPS, 2.5 TRIS, 0.01 ouabain, pH 7.4), and K^+ buffers with distinct K^+ concentrations, which were prepared by adding appropriate amounts of KCl to Na^+ buffer.

Stationary currents

K^+-induced currents were determined as the difference of currents measured in a distinct K^+ buffer and currents measured in Na^+ buffer. Oocytes were subjected to the following voltage pulse protocol: from -30 mV holding potential, cells were clamped to potentials between +60 mV and -140 mV (in -20 mV decrements) for 200 ms, followed by a pulse back to -30 mV. All currents within one experiment were normalized to the pump current amplitude at 10 mmol/L K^+ and 0 mV. To determine the apparent affinity for extracellular K^+, voltage-dependent $K_{0.5}(K^+_{ex})$ values were determined using fits of a Hill equation $I = \frac{I_{max}}{1+\left(\frac{K_{0.5}}{[K^+]}\right)^{n_H}}$ to the normalized K^+-induced currents at a given membrane potential ($K_{0.5}$ is the concentration at half-maximal current, and n_H is the Hill coefficient). n_H values from the fits were between 1 and 1.5.

Analysis of transient currents

To obtain kinetic information about extracellular Na^+ binding/release and the voltage-dependent distribution of pump molecules between E_1P and E_2P states, pre-steady state currents under Na^+/Na^+ exchange conditions were recorded. These ouabain-sensitive transient currents were calculated as the difference between currents measured in Na^+ buffer with 10 μmol/L ouabain (blocking only the endogenous Na^+ pump) and in the presence of 10 mmol/L ouabain (to inhibit the RD-mutated enzyme). Data were fitted by using a monoexponential function, excluding the first 3-5 ms to eliminate capacitive artifacts, yielding time constants τ and amplitudes A. The translocated charge Q was determined from the product $A \times \tau$. The resulting Q(V) curves were approximated by a

Boltzmann function:

$$Q(V) = Q_{min} + \frac{Q_{max}-Q_{min}}{1 + \exp\left(\frac{z_q \times F(V-V_{0.5})}{RT}\right)}$$

where Q_{max} and Q_{min} are the saturation values of Q(V), $V_{0.5}$ is the half-maximal voltage at which equal distribution of E_1P and E_2P states is achieved, z_q the fractional charge, F the Faraday constant, R the molar gas constant, T the temperature, and V the membrane potential. After fitting, the translocated charge values were normalized to saturating values (Q_{max} - Q_{min}) after subtracting Q_{min}.

Isolation of membrane fractions from oocytes

To assess impairments in plasma membrane targeting or expression of mutant proteins that showed no pump current activity in TEVC experiments, plasma membrane (PM) and total membrane (TM) fractions were isolated from oocytes injected with cRNA of the constructs as described before[14,29]. All obtained samples were dissolved in SDS-PAGE sample buffer, and the amount of protein corresponding to the equivalent of two oocytes was separated by 10%SDS-PAGE and blotted on nitrocellulose membranes. Since oocytes are homogenous in size, the procedure of loading the equivalent of a certain number of cells provides an internal loading standard, as shown previously[15]. The α_2-subunits of Na^+/K^+-ATPase were detected with the specific polyclonal antibody AB9094 (Chemicon, Temecula, CA). Afterwards, blots were incubated with a HRP-conjugated secondary antibody (Dako, Glostrup, Denmark). Proteins were visualized by an enhanced chemiluminescence reaction (Roche, Mannheim, Germany).

Structural examinations and figures

Structural inspections of the Na^+/K^+-ATPase (PDB structure entry 3B8E) were carried out with Swiss PDB viewer 3.7. Figures were prepared with PyMOL 1.0r1 (http://www.pymol.org). Data analysis and figure presentation were carried out with Origin 7.0 (OriginLab Corp., Northampton, MA).

Statistical analysis

Statistical analyses were carried out based on the Student's *t*-test for independent samples. The significance level $P < 0.05$ is indicated in the figures by an "a" above the data points reaching this significance level.

RESULTS

Stationary K^+-induced pump currents and apparent K^+_{ex} affinity

From the investigated ATP1A2 mutants, only G900R, E902K, D999H and K1003E showed K^+-induced currents with amplitudes that were sufficiently large for electrophysiological analysis (> 10 nA, Figure 2), whereas no measurable pump activity could be detected for the mutants G855R, L994del and Y1009X. For G900R, E902K and K1003E, the bell-shaped I(V) curves at different $[K^+]_{ex}$ did not differ significantly from those of the RD-WT enzyme. This voltage dependence of currents is due to the extracellular competition between K^+ and Na^+ ions for the two "shared" cation binding sites. With proceeding hyperpolarization of the membrane, reverse binding of extracellular Na^+ is stimulated and K^+ pump activity inhibited[30,31].

For D999H, in contrast, the voltage dependence of K^+-induced currents apparently deviated from RD-WT behavior (Figure 2C). In general, at negative potentials, the current amplitudes of the mutant were small compared to RD-WT amplitudes (data not shown), but at +60 mV, they were in the same range as RD-WT amplitudes (100-200 nA). We suppose that the activity of the D999H construct was similar to the RD-WT enzyme at positive potentials. In contrast to the RD-WT enzyme, the I(V) curves of D999H at high K^+ concentrations (2, 5, 10 mmol/L) were nearly constant at potentials between -100 to -40 mV and even increased at hyperpolarization below -100 mV, indicating that the inhibition of K^+ pump activity by reverse binding of extracellular Na^+ is not as efficient as in the RD-WT enzyme. At potentials more positive than -20 mV, the K^+-induced currents started to rise steeply, which shows that positive membrane potentials had a stronger effect on enzyme activity of the D999H mutant compared to the RD-WT enzyme in this voltage range.

As for the apparent K^+ affinity in Na^+ containing buffers, $K_{0.5}(K^+)$ values were determined from K^+-induced currents at different $[K^+]_{ex}$ and plotted as a function of the membrane potential (Figure 3). For G900R, E902K, K1003E and RD-WT, the voltage dependence of $K_{0.5}(K^+)$ values can be approximated by a parabolic function. The minimal $K_{0.5}(K^+)$ values were similar, with values between 1.09-1.25 mmol/L (Table 1). For the RD-WT enzyme, the apparent K^+ affinity decreases at negative potentials because the reverse binding of extracellular Na^+ is stimulated. In contrast, the $K_{0.5}(K^+)$ values determined for mutant D999H did not increase at hyperpolarization, but were nearly voltage-independent between -140 mV and -40 mV (Figure 3C). The minimal $K_{0.5}(K^+)$ value was 0.67 mmol/L and shifted to negative potentials. Apparently for D999H, extracellular Na^+ does not compete as efficiently with K^+ as for the RD-WT enzyme, which indicates a reduced affinity of the mutant for extracellular Na^+ (or destabilization of the Na^+-bound E_2 state). To further investigate this question, the electrogenic Na^+/Na^+ exchange mode was examined.

Electrogenic Na^+/Na^+ exchange

To investigate changes in apparent Na^+_{ex} affinity, we measured transient currents under Na^+/Na^+ exchange conditions (ouabain-sensitive currents, 0 mmol/L K^+). Representative transient currents of the RD-WT enzyme are shown as inset in Figure 4E, and the reciprocal time constants of the charge translocation are shown in Figure 5. Basically, the voltage dependence of the reciprocal time constants determined for mutants G900R, E902K and K1003E conformed to that of the RD-WT protein.

Table 1 Minimal $K_{0.5}$ values from $[K^+]_{ex}$ dependence of pump currents and parameters of Boltzmann fits to Q(V) curves derived from transient currents (means ± SE)

	Minimal $K_{0.5}$ (K^+)/mmol/L	Membrane potential at minimum/mV	$V_{0.5}$/mV	zq
RD-WT	1.12 ± 0.01	-6.2 ± 1.5	0.9 ± 1.3	0.77 ± 0.02
G900R	1.09 ± 0.04	0.2 ± 4.7	0.3 ± 3.1	0.76 ± 0.02
E902K	1.25 ± 0.03	-15.2 ± 2.3	-2.1 ± 2.1	0.81 ± 0.02
D999H	0.67 ± 0.08	-97.6 ± 4.4	-67 ± 14	0.33 ± 0.11
K1003E	1.10 ± 0.03	6.6 ± 3.3	-11.3 ± 4.3	0.75 ± 0.06

However, kinetics of charge translocation was slightly faster for these mutants compared to the RD-WT enzyme. Especially for G900R and E902K, the rise of the reciprocal time constants (τ^{-1}) at hyperpolarizing potentials was enhanced.

The voltage dependence of charge translocation is shown in Figure 4 and provides information about the distribution of pump molecules between E_1P and E_2P states[32]. For the mutants G900R and E902K, the Q(V) curves are similar to that of the RD-WT protein, and the $V_{0.5}$ values in particular did not differ (Table 1). The $V_{0.5}$ value of mutant K1003E was shifted by -5 to -15 mV. This hints at a slightly reduced apparent Na^+_{ex} affinity of this mutant[10,33], which, however, does not seem to impair function in terms of the voltage dependence and the amplitudes of K^+-induced currents (Figure 2D).

The D999H mutation had more severe consequences on Na^+/Na^+ exchange. In general, the transient current signals were fast and small compared to the RD-WT enzyme (data not shown). In addition, the Q(V) curve of translocated charge was linearly dependent on membrane potential, and saturating values were not clearly detectable within the investigated voltage range (Figure 4C). Hence, the approximation with a Boltzmann function and determination of $V_{0.5}$ proved to be difficult. For fitting, the z_q value (Table 1) was reduced until the fitted function superposed the Q values. For this reason, the determined z_q can only be regarded as an upper limit, and with a value of 0.33, z_q was very small compared to the RD-WT enzyme (0.77). Since $V_{0.5}$ also directly depends on the quality of the fit, it is likely that the shift of $V_{0.5}$ by about -70 mV is only a rough estimate for the lower limit of the actual shift. Nonetheless, this strong negative shift shows that D999H has a considerably reduced affinity for extracellular Na^+ since very strong hyperpolarization is required to force Na^+ ions into the binding sites and to enable the subsequent conformational change to E_1P[10,33]. This is in good agreement with the simultaneously reduced $K_{0.5}(K^+)$ values at negative potentials. Furthermore, kinetics of the D999H transient currents was less voltage-dependent than for the RD-WT protein (Figure 5C). τ^{-1} values varied between 200 and 300 s^{-1} at potentials below 0 mV and increased up to 400 s^{-1} at depolarization. These results show that the apparent affinities for Na^+ and K^+ (or stabilization of the cation-occluded state) as well as charge translocation and kinetics of the Na^+/Na^+ exchange reaction were significantly affected by this mutation.

Plasma membrane protein expression

Since the constructs G855R, L994del and Y1009X did not yield measurable Na^+/K^+ pump currents in TEVC experiments, it was necessary to examine whether or not these proteins were expressed in oocytes and properly targeted to the plasma membrane. For this purpose, plasma membrane (PM) and total intracellular membrane (TM) fractions were prepared using oocytes that had been injected with cRNA of these constructs. Representative Western blots with TM and PM fractions of G855R, L994del (Figure 6C) and Y1009X (Figure 6B) are shown in Figure 6. Densitometric analysis of four Western blots prepared from independent cell batches indicated a disturbed expression pattern of these mutants (Figure 6C). By trend, larger amounts of mutant proteins could be detected in the TM fraction than for the RD-WT protein, which in turn was highly concentrated in the PM fraction. However, analysis of the PM fractions showed that the mutants were not or only minimally expressed in the plasma membrane. The band intensities of PM fractions were only 10%-20% of RD-WT values. Thus, G855R, L994del and Y1009X accumulate in cytoplasmic membranes, and targeting to the plasma membrane was disturbed by these mutations.

DISCUSSION

α/β-interactions

Several studies have shown that the C-terminal ectodomain of the β-subunit is important for modulation of cation transport by the Na^+/K^+-ATPase[34-36]. A motif of eight amino acids (Asp897-Tyr905, amino acid sequence DSYGQEWTY) in the αM7/M8-loop seems to be of special importance. Interactions of the β-subunit with this sequence element that encompasses a highly conserved SYGQ motif were identified as crucial for correct folding of newly synthesized α-subunits in the endoplasmic reticulum, and furthermore, it is suspected that an hypothetical sequence motif for proteolytic degradation is masked by these interactions[22,37,38]. Four FHM2/SHM-associated mutations have been identified in the extracellular αM7/M8-loop so far: W887R, G900R, E902K and R908Q[4,24,25,39]. W887R and R908Q, which are not directly located in the SYGQ motif, have already been analyzed[26,40].

The W887R construct was found to be correctly targeted to the plasma membrane of *Xenopus* oocytes[40],

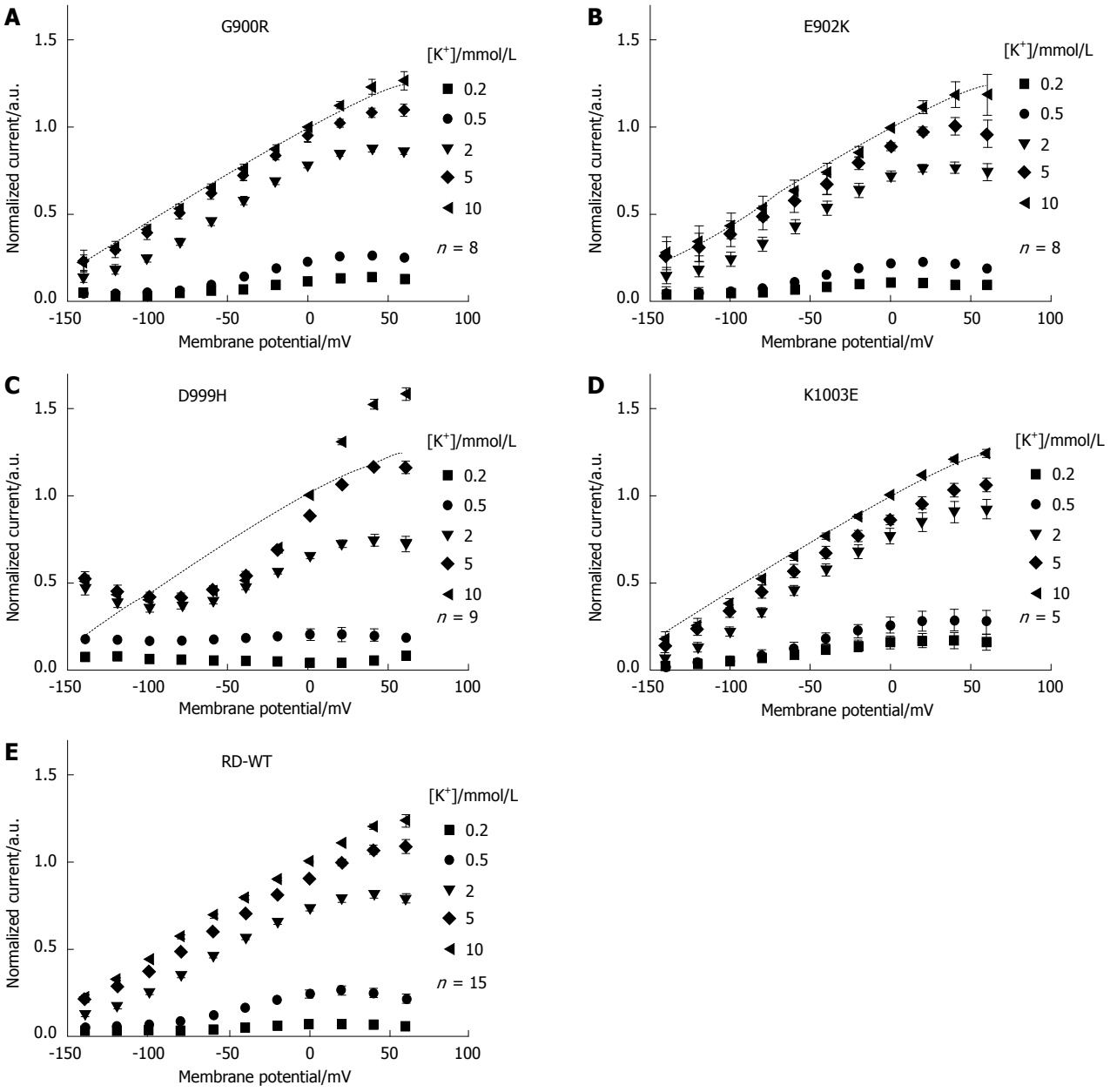

Figure 2 Voltage and $[K^+]_{ex}$ dependence of stationary currents for ATP1A2 RD-WT, G900R, K902E, D999H and K1003E. A-E: Dependence of K^+-induced stationary currents of the RD-WT enzyme (E) and the mutants G900R (A), E902K (B), D999H (C) and K1003E (D) on the extracellular K^+ concentration and on membrane potential. $[K^+]_{ex}$-dependent currents were calculated as the difference between currents induced by voltage steps first in presence of different $[K^+]_{ex}$ and then at $[K^+]_{ex}$ = 0. The amplitudes at $[K^+]_{ex}$ = 10 mmol/L and 0 mV were used for normalization. Different $[K^+]_{ex}$ are indicated by symbols. The RD-WT curve at 10 mmol/L K^+ is superimposed as dotted line for comparison. Data are means ± SE obtained from 5-15 cells of at least three batches.

but this mutation caused a complete loss-of-function and a strongly reduced ouabain affinity. Koenderink *et al*[29] argued that Trp887 might rather have an influence on Arg880, which is critical for ouabain sensitivity, than on targeting-relevant interactions between α- and β-subunits. However, the loss of catalytic function might be due to disturbed α/β-interactions during ion transport. The R908Q mutation, which is very close to the SYGQ motif, indeed affected targeting, since plasma membrane expression in *Xenopus* oocytes was reduced compared to the RD-WT protein, which easily explains the diminished pump currents[26]. The highly conserved residues Gly900 and Glu902 are located directly in the SYGQ motif and are presumably important for interactions with the β-ectodomain. It was expected that the mutations G900R, which substitutes the small unpolar glycine with a large positively charged arginine, and E902K, where the negatively charged glutamic acid is replaced by a positively charged lysine, would have a strong effect on function. However, both constructs showed no differences compared to the RD-WT enzyme, neither regarding pump activity (Figure 2A, B) nor the apparent affinities for extracellular K^+ ($K_{0.5}(K^+)$ values in Figure 3A, B) and for extracellular Na^+ (Q(V) curves and $V_{0.5}$ values in Figure 4A, B). Presumably, either these amino acids are not directly interacting with the β-subunit, or the positively

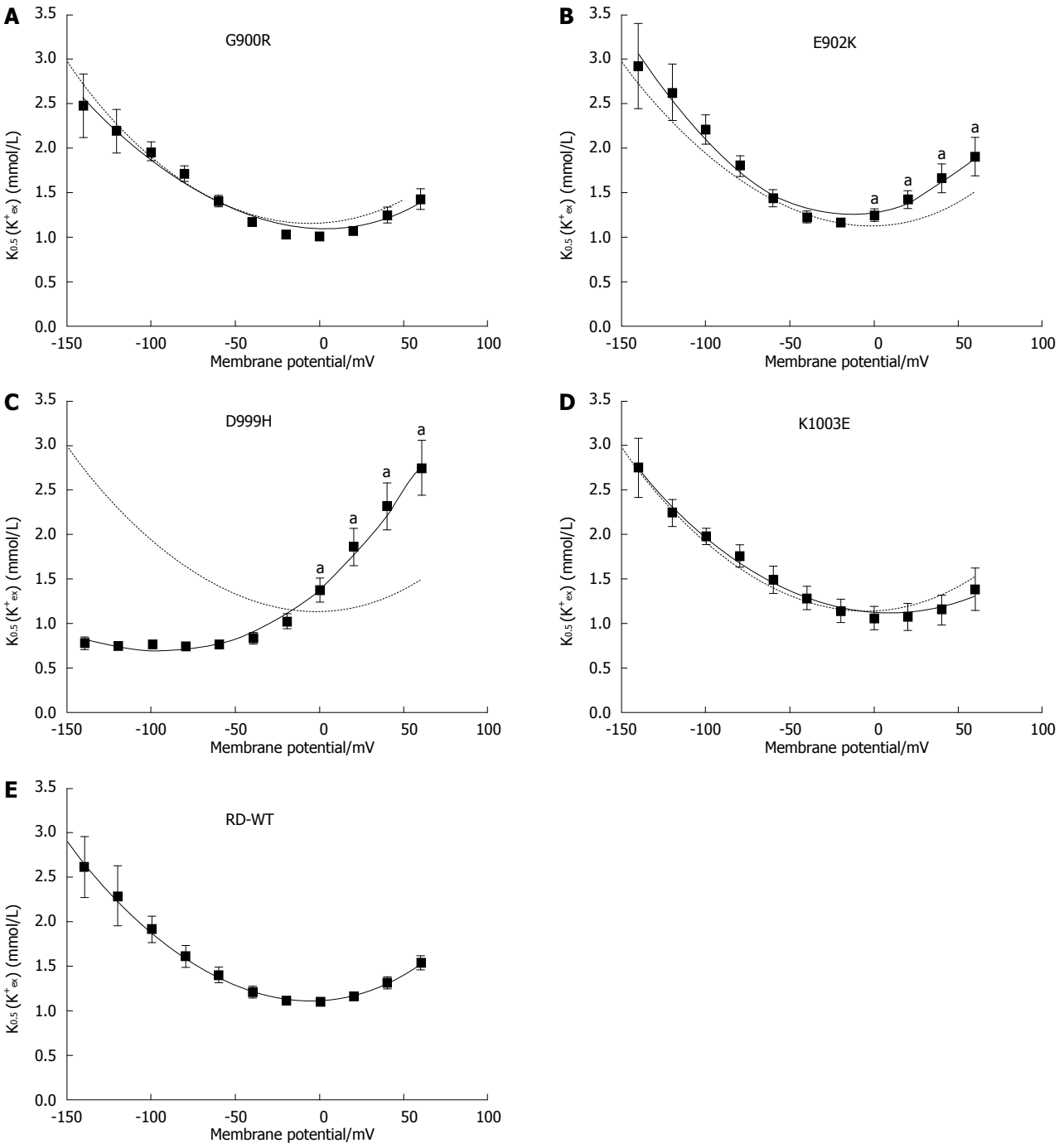

Figure 3 Apparent K^+ affinity. A-E: $K_{0.5}$ values for the $[K^+]_{ex}$ dependence of stationary currents at different membrane potentials for the RD-WT enzyme (E) and the mutants G900R (A), E902K (B), D999H (C) and K1003E (D), as calculated from fits of a Hill function to the data in Figure 2, respectively. Data were approximated with polynomial functions of second or third (D999H) grade to determine the minimum. The curve derived from RD-WT data is superimposed as dotted line for comparison. An "a" indicates that the data point was significantly different from the RD-WT data ($^aP < 0.05$ *vs* RD-WT, Student's *t*-test). Data are means ± SE obtained from 5-15 cells of at least three batches.

charged side chains of arginine and lysine do not interfere with α/β-interactions, at least under the conditions of our study.

According to the crystal structure of the Na^+/K^+-ATPase[16,23], Tyr39 and Tyr43 of βM can directly interact with residues at positions 848-856 in αM7 (Figure 7A). Especially, interactions between Gly852 (M7) and both aforementioned tyrosines of the β-subunit seem to stabilize the E_2 conformation, and, as confirmed by mutagenesis studies[26,41], not only are hydrogen bonds involved, but also the aromatic ring system of the tyrosines. The β-subunit stabilizes the orientation of αM7 and, consequently, also the position of αM5 because Tyr851 (αM7) can interact with Asn780 in αM5. These interactions are relevant for conformational stabilization during K^+ transport[26].

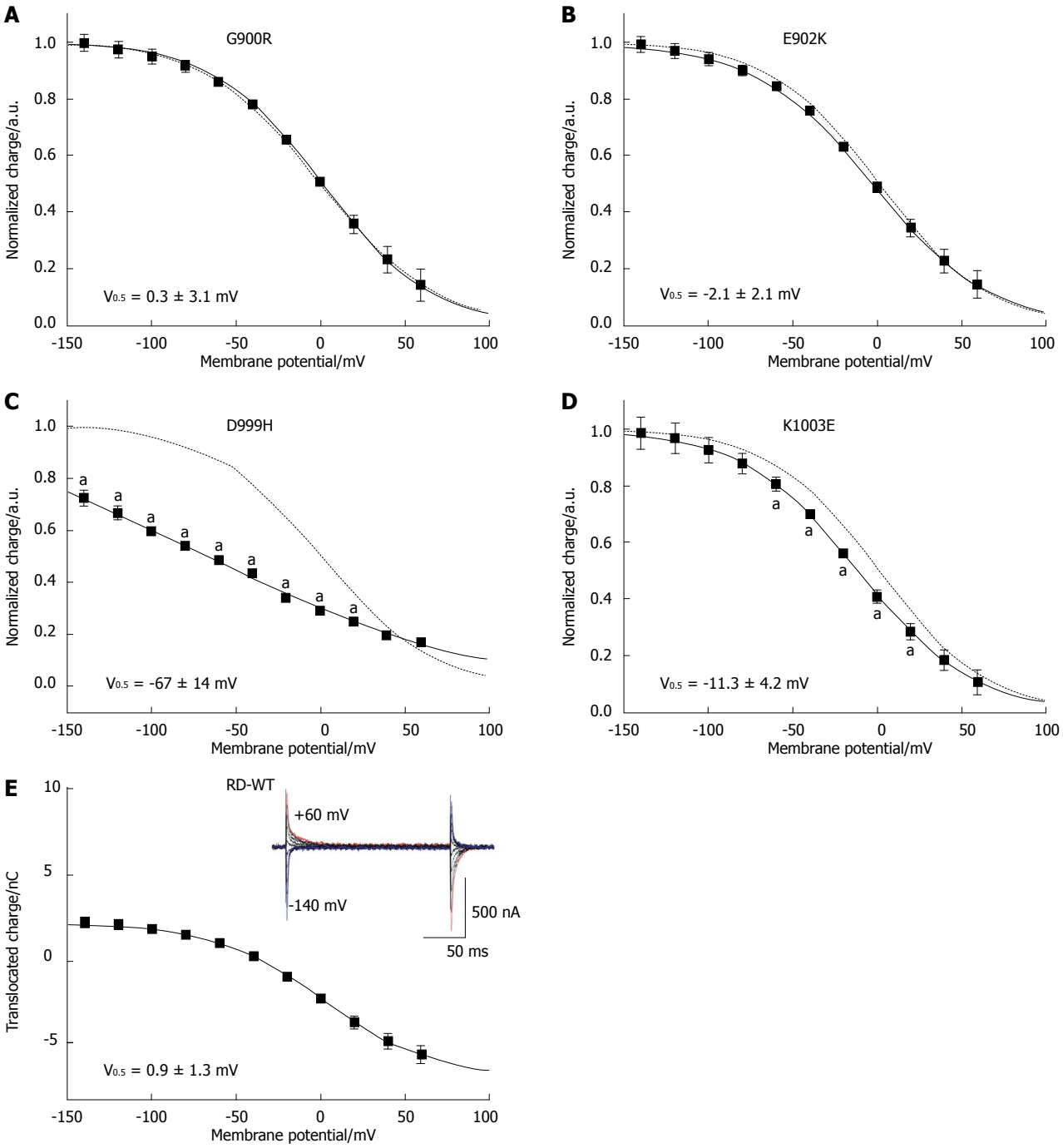

Figure 4 Voltage dependence of translocated transient charge. A-E: Normalized Q(V) curves from ouabain-sensitive transient currents for the RD-WT enzyme (E), and for the mutants G900R (A), E902K (B), D999H (C) and K1003E (D). Fits of a Boltzmann function to the data are superimposed. Q_{min} and Q_{max} determined by the fit were used for normalization. The Boltzmann curve of the RD-WT enzyme is shown as a dotted line for comparison. Transient current signals are shown in a box for the RD-WT enzyme in panel (E). An "a" indicates that the data point was significantly different from the RD-WT data ($^aP < 0.05$ *vs* RD-WT, Student's *t*-test).

Gly855 is separated by three positions from Gly852, but due to the α-helical structure, it is oriented towards αM5 rather than to βM (Figure 7A). Two mutations at this position have been identified in patients with hemiplegic migraine forms: G855R (FHM2)[27] and G855V (SHM)[13], with G855R presumably having a stronger effect on Na^+/K^+-ATPase function. Our study indeed shows that the G855R mutant protein is not correctly targeted to the plasma membrane of *Xenopus* oocytes (Figure 6A, C) although it could well be detected in the total intracellular membrane fraction. However, disruption of α/β-interactions would cause degradation of the protein already in the ER. It is conceivable that the long side chain of the introduced arginine might disturb the structure in a way that transmembrane domains (especially αM7 and αM5) are not correctly positioned. Here, we cannot clarify if the integration in the plasma membrane of G855R is affected because of deficient α/β-interactions or because

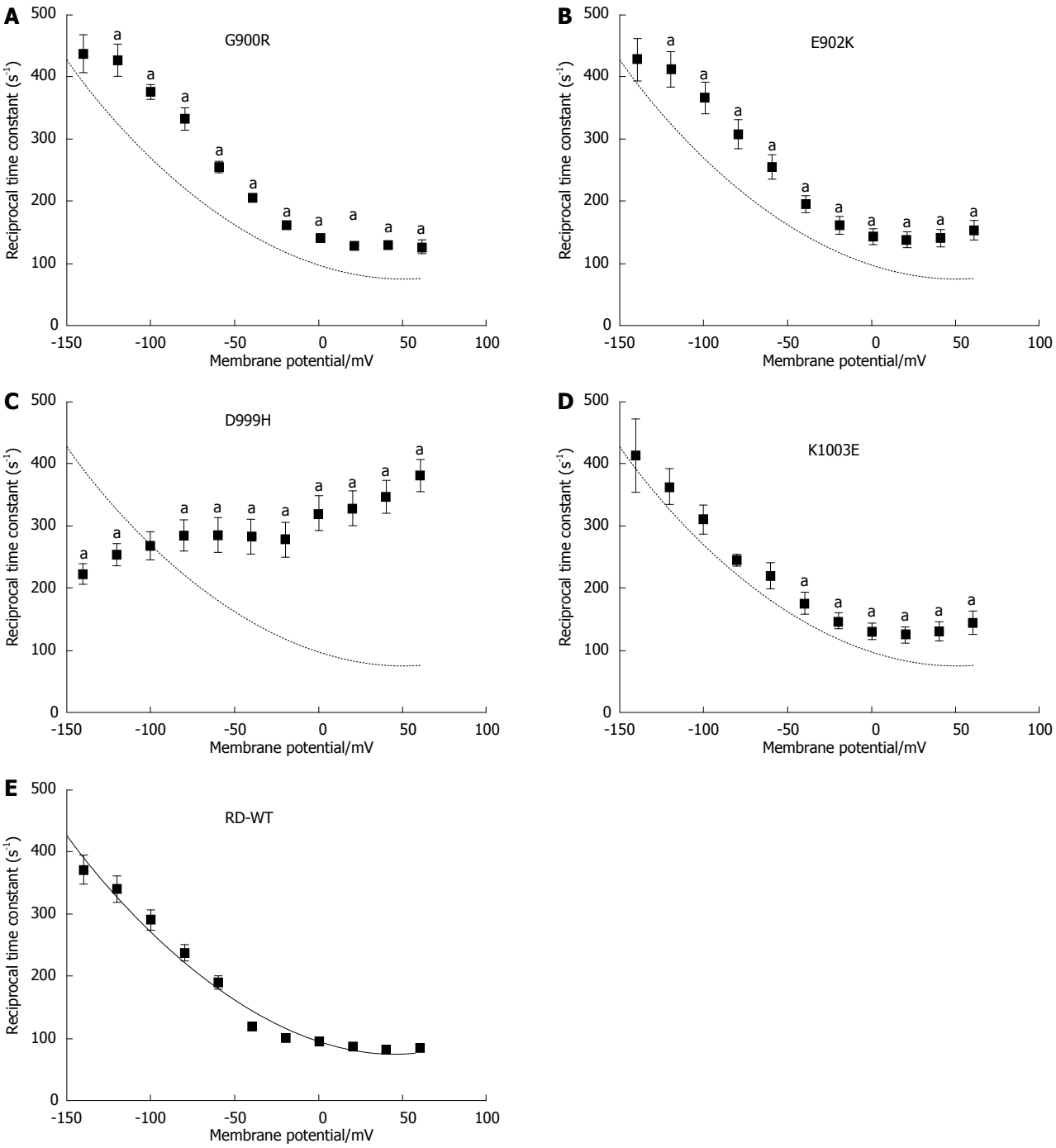

Figure 5 Reciprocal time constants of transient currents. A-E: Voltage dependence of reciprocal time constants τ-1 from ouabain-sensitive transient currents of RD-WT enzyme (E) and the mutants G900R (A), E902K (B), D999H (C) and K1003E (D) under K^+-free Na^+/Na^+ exchange conditions. The fit of a polynomial function to RD-WT values is superimposed as a dotted line. An "a" indicates that the data point was significantly different from the RD-WT data ($^aP < 0.05$ *vs* RD-WT, Student's *t*-test). Data are means ± SE from 5-21 oocytes of at least three batches.

of misfolding, but Gly855 seems to be a critical position.

In this context, the effect of Y1009X and L994del, which are not targeted to the plasma membrane either but are present in the TM fraction (Figure 6), might be of interest. As shown in Figure 7A, the flexible C-terminus (orange) of the α-subunit is oriented towards a region between βM and αM7, in interaction distance to Lys770 in αM5 (Figure 7B). It was suggested that Tyr998 in αM10 directly interacts with βM[23]. The Y1009X mutant protein lacks the 11 C-terminal residues, and in L994del, the 25 C-terminal amino acid residues are shifted N-terminally by one position. These modifications in the C-terminus might affect the orientation of αM7 and αM5 and thereby, correct protein folding. To what extent α/β-interactions are influenced cannot be clarified in this study.

C-terminal region

A number of functional studies imply that the C-terminus

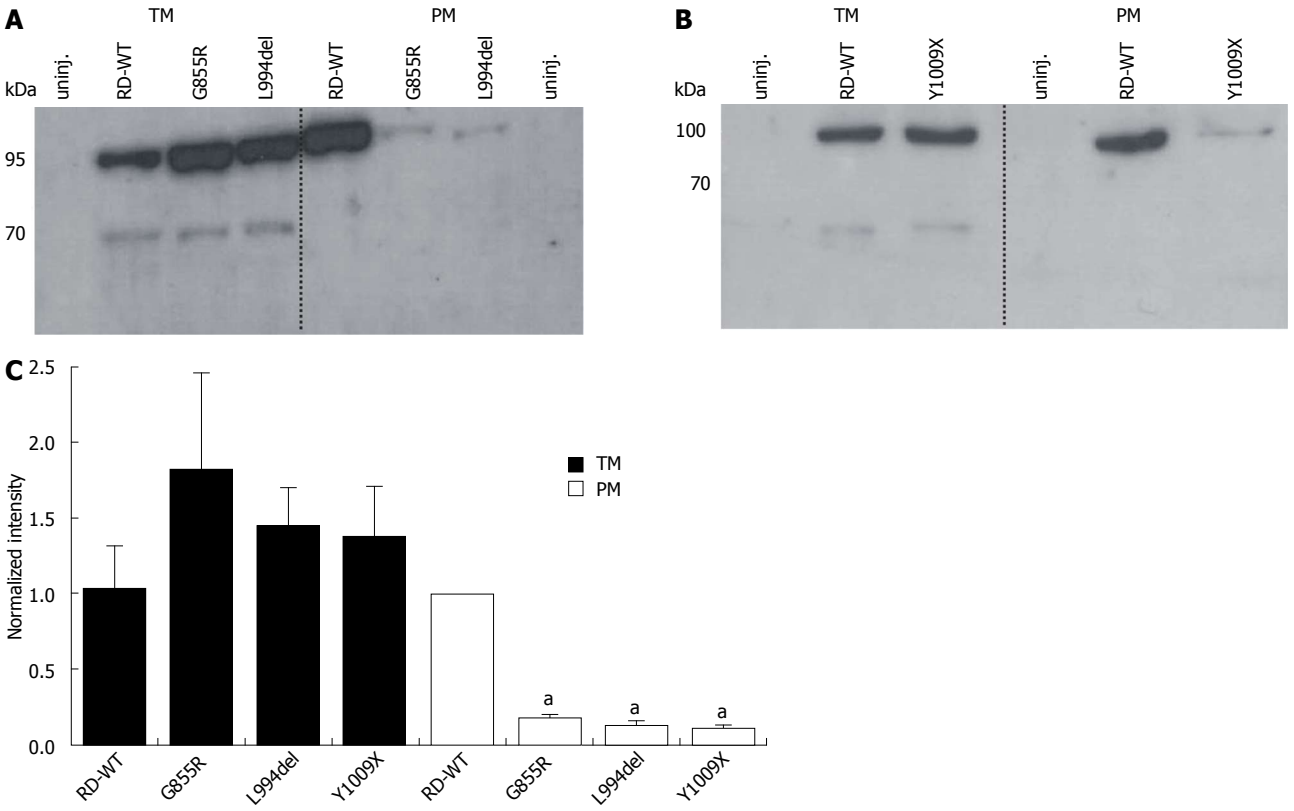

Figure 6 Protein expression of the constructs G855R, L994del and Y1009X. A, B: Representative Western blots for the constructs G855R, L994del (A) and Y1009X (B) compared to the RD-WT enzyme and non-injected cells. Samples of total intracellular membrane (TM, left) and plasma membrane (PM, right) fractions corresponding to the protein amount of two oocytes were loaded in each lane (the number of cell equivalents serves as internal loading standard); C: Densitometric analysis of band intensities from at least four Western blots of G855R, L994del, Y1009X and RD-WT. The program ImageJ 1.44o (Wayne Rasband, United States) was used for analysis. In each experiment, signals were normalized to the intensity of the RD-WT signal from PM samples. An "a" indicates that the data point was significantly different from the RD-WT data ($^aP < 0.05$ *vs* RD-WT, Student's *t*-test). Data are means ± SD.

is intimately involved in the stabilization of the third Na^+ binding site[16,19,42,43], including analyses of mutations which are suspected to trigger neurological diseases. Elongation of the C-terminus provoked different functional abnormalities. Investigations on a mutation found in a patient with rapid-onset dystonia parkinsonism, where the α_3-subunit's C-terminus is extended by one tyrosine, implied a direct participation of the C-terminus in Na^+ binding[43]. Another C-terminal mutation X1021R (mutation of the stop codon resulting in an elongation of the C-terminus by 28 amino acids) was analyzed electrophysiologically in *Xenopus* oocytes[14]. Interestingly, this mutation affected the apparent Na^+_{ex} affinity of the enzyme in a similar way as the D999H mutation. The Q(V) curve of transient currents of X1021R was comparably shallow, as for D999H (Figure 4C), and linear in the tested potential range. The z_q value was reduced to 0.3 for both mutations, which implies that Na^+ release and rebinding is less voltage-dependent. Furthermore, the reciprocal time constants of transient currents showed inverse voltage dependence compared to the RD-WT enzyme (kinetics accelerated with increasing potentials, Figure 5C). Similar curves were also detected for other C-terminally mutated enzymes like ΔYY or ΔKE(S/T)YY (deletion of the last two or five amino acids, depending on species isoform)[17-19]. The transient currents correlate with the movement of the third Na^+ ion through a substantial fraction of the membrane dielectric, which reaches its bindings site through a high-field access channel[10,33]. The $\tau^{-1}(V)$ curve measured for D999H (Figure 5C) or ΔYY[19] corresponds to a voltage dependence that is predicted by Vasilyev *et al*[19,44] for a reaction cycle in which the intra- and extracellular access for Na^+ to its binding sites is facilitated. In conclusion, the C-terminus stabilizes the Na^+-occluded state. This argumentation is also shared by Vedovato and Gadsby, who argued that the C-terminally deleted mutations increase the free energy for $E_1P(3Na^+)$[18]. This destabilization manifests in a faster conformational change or in a faster access/release of intracellular Na^+ ions, which means that the function of the $E_1P(3Na^+)$ state is impaired and correct closure of an intracellular occlusion gate for Na^+ ions is not assured.

Not only are the two terminal tyrosines involved in this stabilization, but also the residues Arg937 (αM8/M9-loop), Asp999 (M10) and Arg1002 (M10) are part of a network of interactions with these tyrosines (Figure 7B). The FHM2/SHM mutations R937P, R1002Q[42] and D999H, as well as the ΔYY or ΔKE(S/T)YY sequence variants have similar effects on transient currents (kinetics and Q(V) distribution). The functional studies all show that the C-terminus not only regulates the apparent Na^+_{ex} affinity in the E_2P conformation, but also the Na^+_{in} affin-

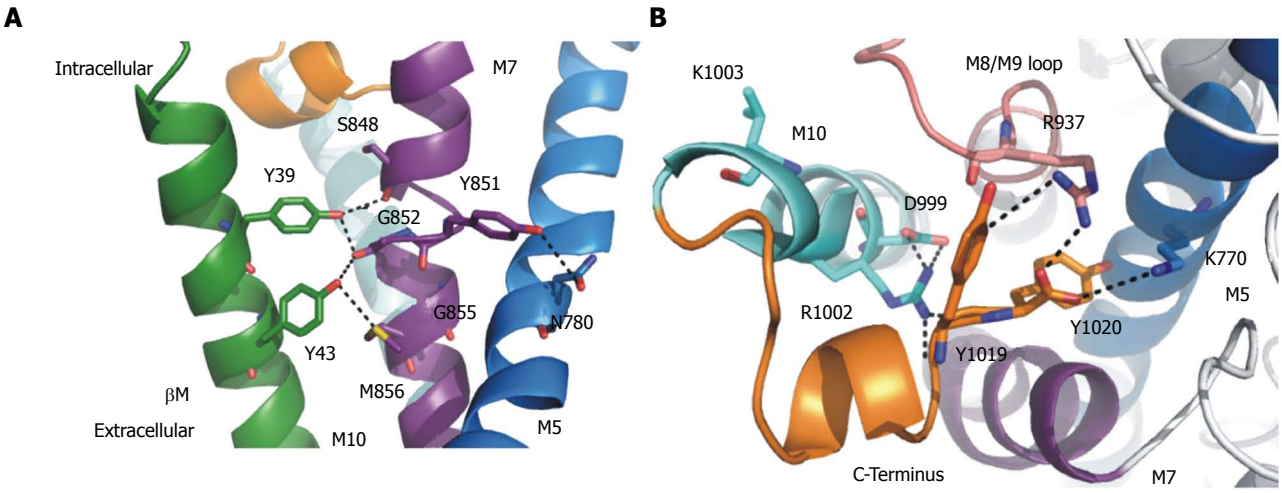

Figure 7 Structural details of the C-terminal region and α/β-interactions. A: Structural details (PDB structure entry 3B8E) of putative interactions between the α- and β-subunit. Interacting residues are shown as sticks. Tyr39 and Tyr43 in the β-transmembrane domain (green) can interact with αM7 (purple). The α-helix is unwound at residue Gly952 (αM7). Tyr851 can form hydrogen bonds to Asn780 in αM5 (marine), which is part of the K^+ binding site Ⅰ and Ⅱ. Also shown is αM10 (light blue) with the C-terminus (orange) of the α-subunit; B: Structural details of the C-terminal region viewed from the intracellular side. Possibly interacting residues are shown in sticks. The C-terminal Tyr1019 and Tyr1020 (orange) can interact with Arg1002 in αM10 (light blue), Arg937 (αM8/M9-loop in purple) and Lys770 in αM5 (marine). Asp999 (αM10) can form hydrogen bonds to Arg1002. Lys1003 (αM10) is not involved in the C-terminal network.

ity in the E_1 conformation[17,19,43]. Based on molecular dynamics simulations of the wild-type enzyme and C-terminally mutated α_2-subunits, it was proposed that the amino acids Arg937, Asp999, Arg1002 and Tyr1019/1020 form an intracellular ion pathway with Asp930 at its end, which controls the access to the third Na^+ binding site depending on the protonation state of Asp930[42]. Our study confirms that Asp999 is at least indirectly involved in the stabilization of Na^+ binding because its substitution by a histidine affected electrogenicity and kinetics of Na^+ charge translocation in a similar fashion. In contrast, the overall electrophysiological data of K1003E did not show severe functional abnormalities, and with regard to the crystal structure of the Na^+/K^+-ATPase, we conclude that Lys1003 (αM10) is not directly involved in the C-terminal network (Figure 7B).

Functional consequences

Dysfunction of the Na^+/K^+-ATPase affects excitatory processes in the CNS, especially in patients suffering from hemiplegic migraine. How do the mutations studied in this work affect the physiological processes in neuronal signaling cascades, since the α_2-isoform in human brain is mainly expressed in astrocytes and not in neurons? The CSD phenomenon is discussed as pathophysiological mechanism of the migraine aura. It is promoted by hyperexcitability caused by insufficient removal of K^+ and neurotransmitters such as glutamate from the synaptic cleft, which is the primary function of astrocytes. The glial Na^+/K^+-ATPase is directly involved in K^+ transport, and it indirectly influences glutamate and Ca^{2+} transport by regulating the Na^+ gradient, which is the energy source of the glutamate transporter (EAAT) and the Na^+/Ca^{2+}-exchanger (NCX).

G900R, E902K and K1003E did not show significant functional abnormalities compared to the RD-WT enzyme, at least under the conditions tested here. It is possible that these mutations impair the enzymatic function in human cells *e.g.* due to different temperature conditions (37 ℃ as opposed to oocytes, which need to be kept at room temperature), as shown previously for another FHM2 mutation P979L[15]. Furthermore, the constructs G855R, L994del and Y1009X exhibited strongly reduced expression in the plasma membrane (Figure 6). This hints at an incomplete or improper folding of the protein so that these mutants could not be correctly targeted to the plasma membrane. In patients with such mutations, the pump enzyme is seriously damaged, and cannot contribute to the maintenance of ion gradients or to the removal of K^+. As a consequence, hyperexcitability is probable.

Compared to all other mutants in this study, which gave rise to measurable Na^+/K^+ pump currents, the D999H mutation had the largest impact on pump function. The voltage dependence of Na^+/K^+ pump activity was shifted to positive potentials compared to the RD-WT enzyme (Figure 2C). We suppose that K^+ transport of this construct is only effective at around zero or positive membrane potentials. Since the α_2-isoform is dominant in astrocytes with resting potentials at -85 to -90 mV, this mutant exhibits a severe loss-of-function. K^+ cannot be removed efficiently from the synaptic cleft at negative potentials, which lowers the excitation threshold and may trigger CSD. Furthermore, regarding the negative shift of the Q(V) curve (Na^+/Na^+ exchange conditions, Figure 4C) and the low $K_{0.5}(K^+)$ values at hyperpolarization (Figure 3C), we conclude that the apparent affinity for extracellular Na^+ is reduced in the D999H mutant. As explained above, Asp999 is part of the C-terminal interaction network which plays a role in Na^+ binding (especially concerning stabilization of the third Na^+ binding site, Figure 7B). Mutations at positions Arg937 and Tyr1019/Tyr1020, which are also part of this network, affected the

affinity for both, intra- and extracellular Na^+[17,19,43]. The ATP1A2 α_2-isoform (expressed in non-excitable cells of the CNS) has a slightly increased Na^+_{in} affinity compared to the α_3-isoform[45,46], which is expressed in neurons. This is advantageous because enzyme activity in astrocytes presumably depends mainly on the increase of the intracellular Na^+ concentration. In other words, $[Na^+]_{in}$ is the important factor determining the sensitivity of the Na^+/K^+-ATPase towards increasing extracellular K^+[47]. For instance, the intracellular Na^+ concentration increases upon glutamate uptake by EAAT, and this stimulates pump activity and K^+ transport. Accordingly, a reduced Na^+_{in} affinity would constrain forward pumping with serious consequences for the recovery of the neuronal resting potential.

In effect, K^+ and glutamate removal from the synaptic cleft not only depends on Na^+/K^+-ATPase activity, but other transporting enzymes are also involved. Furthermore, the penetrance of ATP1A2 mutations can be low or heterogenous because of a large diversity of phenotypic expression depending on genetic and environmental conditions[48-50]. In consequence, physiological impacts of α_2-mutations vary and provoke clinical symptoms of different severity.

In conclusion, this study shows that the investigated FHM2/SHM mutations influence protein function differently depending on the structural impacts of the mutated residue, and thereby, the spectrum of molecular phenotypes of ATP1A2 mutations is widened. We have identified at least two positions that are critical for correct protein function, with Asp999 being involved in Na^+-binding and with Gly855 being essential for plasma membrane targeting. The functional analysis of FHM2/SHM mutations are mandatory to elucidate structure-function relationships of the Na^+/K^+-ATPase and, furthermore, to identify biochemical linkage between impairments of protein function and neurological diseases. Our results may help to understand molecular mechanisms in order to develop a basic approach for future therapeutic strategies.

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COMMENTS

Background

The Na^+/K^+-ATPase is a very important transmembrane protein in the signaling cascade and it has been investigated for over 50 years. There are still open questions concerning details of the reaction mechanism and structure-function relationships. In patients suffering from a genetically inherited subform of migraine with aura (familial hemiplegic migraine), mutations of the *ATP1A2* gene, which codes for the α_2-subunit of the Na^+/K^+-ATPase, have been identified.

Research frontiers

To clarify structure-function relationships of the Na^+/K^+-ATPase, different methods have to be applied like molecular dynamics simulations, crystallography, mutagenesis studies together with biochemical assays or electrophysiology. Especially, interactions between the two mandatory enzyme subunits, the role of the α-subunit's C-terminus and the detailed mechanism of Na^+ binding remain unclear. This study analyzed ATP1A2 mutants functionally by electrophysiological and biochemical methods to clarify some of these questions.

Innovations and breakthroughs

More than 50 mutations of the ATP1A2 gene associated with familial hemiplegic migraine have been identified, but many of them have not been functionally analyzed. This study identifies critical structure elements of the Na^+/K^+-ATPase and discusses their impact on correct protein function. After publication of the first crystal structure, many efforts were made to clarify the role of the α-subunit's C-terminus and its structural interaction. The authors show in this study that Asp999 is indeed part of the C-terminal network and is critical for Na^+ binding. Furthermore, the authors have identified Gly855 to be a very critical position for correct protein function.

Applications

This study helps to elucidate structure-function relationships of the Na^+/K^+-ATPase and its correlation with neurological diseases. It is mandatory to understand the molecular basis of genotype-phenotype relations and to develop therapeutic approaches and future therapeutic strategies.

Terminology

The Na^+/K^+-ATPase is an ion pump. This transmembrane protein maintains the electrochemical gradients for sodium and potassium ions, which are necessary for the transmission of stimuli in neurons or muscle cells. The Na^+/K^+-ATPase can be inhibited by ouabain, a cardiac glycoside which was used for the treatment of heart diseases. *Xenopus* oocytes are the eggs of the African Clawed Frog. They are used for the expression of proteins like ion channels or ion pumps to study ion transport by electrophysiological methods. The two-electrode voltage-clamp technique is used to measure changes in conductivity and ion currents over the cell membrane. With this method, it is possible to control the membrane potential of the cell and to analyze current-voltage relationships of ion-transporting membrane proteins.

Peer review

This paper represent a very good piece of scientific information, it provides information on the consequences of mutations in the Na,K-ATPase alpha subunit, measured by voltage clamp.

REFERENCES

1 **Headache Classification Subcommittee of the International Headache Society.** The International Classification of Headache Disorders: 2nd edition. *Cephalalgia* 2004; **24** Suppl 1: 9-160 [PMID: 14979299 DOI: 10.1111/j.1468-2982.2003.00824.x]

2 **Ophoff RA**, Terwindt GM, Vergouwe MN, van Eijk R, Oefner PJ, Hoffman SM, Lamerdin JE, Mohrenweiser HW, Bulman DE, Ferrari M, Haan J, Lindhout D, van Ommen GJ, Hofker MH, Ferrari MD, Frants RR. Familial hemiplegic migraine and episodic ataxia type-2 are caused by mutations in the Ca2+ channel gene CACNL1A4. *Cell* 1996; **87**: 543-552 [PMID: 8898206 DOI: 10.1016/S0092-8674(00)81373-2]

3 **Ducros A**, Denier C, Joutel A, Cecillon M, Lescoat C, Vahedi K, Darcel F, Vicaut E, Bousser MG, Tournier-Lasserve E. The clinical spectrum of familial hemiplegic migraine associated with mutations in a neuronal calcium channel. *N Engl J Med* 2001; **345**: 17-24 [PMID: 11439943 DOI: 10.1056/NEJM200107053450103]

4 **De Fusco M**, Marconi R, Silvestri L, Atorino L, Rampoldi L, Morgante L, Ballabio A, Aridon P, Casari G. Haploinsufficiency of ATP1A2 encoding the Na+/K+ pump alpha2 subunit associated with familial hemiplegic migraine type 2. *Nat Genet* 2003; **33**: 192-196 [PMID: 12539047 DOI: 10.1038/ng1081]

5 **Dichgans M**, Freilinger T, Eckstein G, Babini E, Lorenz-Depiereux B, Biskup S, Ferrari MD, Herzog J, van den Maagdenberg AM, Pusch M, Strom TM. Mutation in the neuronal voltage-gated sodium channel SCN1A in familial hemiplegic migraine. *Lancet* 2005; **366**: 371-377 [PMID: 16054936 DOI: 10.1016/S0140-6736(05)66786-4]

6 **Fendler K**, Grell E, Haubs M, Bamberg E. Pump currents generated by the purified Na+K+-ATPase from kidney on black lipid membranes. *EMBO J* 1985; **4**: 3079-3085 [PMID: 3004932]

7 **Nakao M**, Gadsby DC. Voltage dependence of Na translocation by the Na/K pump. *Nature* 1986; **323**: 628-630 [PMID: 2430183 DOI: 10.1038/323628a0]

8 **Wuddel I**, Apell HJ. Electrogenicity of the sodium transport pathway in the Na,K-ATPase probed by charge-pulse experiments. *Biophys J* 1995; **69**: 909-921 [PMID: 8519991 DOI: 10.1016/S0006-3495(95)79965-9]

9 **Sagar A**, Rakowski RF. Access channel model for the voltage dependence of the forward-running Na+/K+ pump. *J Gen Physiol* 1994; **103**: 869-893 [PMID: 8035166 DOI: 10.1085/jgp.103.5.869]

10 **Holmgren M**, Wagg J, Bezanilla F, Rakowski RF, De Weer P, Gadsby DC. Three distinct and sequential steps in the release of sodium ions by the Na+/K+-ATPase. *Nature* 2000; **403**: 898-901 [PMID: 10706288 DOI: 10.1038/35002599]

11 **Eikermann-Haerter K**, Negro A, Ayata C. Spreading depression and the clinical correlates of migraine. *Rev Neurosci* 2013; **24**: 353-363 [PMID: 23907418 DOI: 10.1515/revneuro-2013-0005]

12 **Morth JP**, Poulsen H, Toustrup-Jensen MS, Schack VR, Egebjerg J, Andersen JP, Vilsen B, Nissen P. The structure of the Na+,K+-ATPase and mapping of isoform differences and disease-related mutations. *Philos Trans R Soc Lond B Biol Sci* 2009; **364**: 217-227 [PMID: 18957371 DOI: 10.1098/rstb.2008.0201]

13 **Riant F**, Ducros A, Ploton C, Barbance C, Depienne C, Tournier-Lasserve E. De novo mutations in ATP1A2 and CACNA1A are frequent in early-onset sporadic hemiplegic migraine. *Neurology* 2010; **75**: 967-972 [PMID: 20837964 DOI: 10.1212/WNL.0b013e3181f25e8f]

14 **Tavraz NN**, Friedrich T, Dürr KL, Koenderink JB, Bamberg E, Freilinger T, Dichgans M. Diverse functional consequences of mutations in the Na+/K+-ATPase alpha2-subunit causing familial hemiplegic migraine type 2. *J Biol Chem* 2008; **283**: 31097-31106 [PMID: 18728015 DOI: 10.1074/jbc.M802771200]

15 **Tavraz NN**, Dürr KL, Koenderink JB, Freilinger T, Bamberg E, Dichgans M, Friedrich T. Impaired plasma membrane targeting or protein stability by certain ATP1A2 mutations identified in sporadic or familial hemiplegic migraine. *Channels* (Austin) 2009; **3**: 82-87 [PMID: 19372756 DOI: 10.4161/chan.3.2.8085]

16 **Morth JP**, Pedersen BP, Toustrup-Jensen MS, Sørensen TL, Petersen J, Andersen JP, Vilsen B, Nissen P. Crystal structure of the sodium-potassium pump. *Nature* 2007; **450**: 1043-1049 [PMID: 18075585 DOI: 10.1038/nature06419]

17 **Yaragatupalli S**, Olivera JF, Gatto C, Artigas P. Altered Na+ transport after an intracellular alpha-subunit deletion reveals strict external sequential release of Na+ from the Na/K pump. *Proc Natl Acad Sci USA* 2009; **106**: 15507-15512 [PMID: 19706387 DOI: 10.1073/pnas.0903752106]

18 **Vedovato N**, Gadsby DC. The two C-terminal tyrosines stabilize occluded Na/K pump conformations containing Na or K ions. *J Gen Physiol* 2010; **136**: 63-82 [PMID: 20548052 DOI: 10.1085/jgp.201010407]

19 **Meier S**, Tavraz NN, Dürr KL, Friedrich T. Hyperpolarization-activated inward leakage currents caused by deletion or mutation of carboxy-terminal tyrosines of the Na+/K+-ATPase {alpha} subunit. *J Gen Physiol* 2010; **135**: 115-134 [PMID: 20100892 DOI: 10.1085/jgp.200910301]

20 **Fernandez DM**, Hand CK, Sweeney BJ, Parfrey NA. A novel ATP1A2 gene mutation in an Irish familial hemiplegic migraine kindred. *Headache* 2008; **48**: 101-108 [PMID: 18184292 DOI: 10.1111/j.1526-4610.2007.00848.x]

21 **Gallanti A**, Tonelli A, Cardin V, Bussone G, Bresolin N, Bassi MT. A novel de novo nonsense mutation in ATP1A2 associated with sporadic hemiplegic migraine and epileptic seizures. *J Neurol Sci* 2008; **273**: 123-126 [PMID: 18644608 DOI: 10.1016/j.jns.2008.06.006]

22 **Béguin P**, Hasler U, Staub O, Geering K. Endoplasmic reticulum quality control of oligomeric membrane proteins: topogenic determinants involved in the degradation of the unassembled Na,K-ATPase alpha subunit and in its stabilization by beta subunit assembly. *Mol Biol Cell* 2000; **11**: 1657-1672 [PMID: 10793142 DOI: 10.1091/mbc.11.5.1657]

23 **Shinoda T**, Ogawa H, Cornelius F, Toyoshima C. Crystal structure of the sodium-potassium pump at 2.4 A resolution. *Nature* 2009; **459**: 446-450 [PMID: 19458722 DOI: 10.1038/nature07939]

24 **Deprez L**, Peeters K, Van Paesschen W, Claeys KG, Claes LR, Suls A, Audenaert D, Van Dyck T, Goossens D, Del-Favero J, De Jonghe P. Familial occipitotemporal lobe epilepsy and migraine with visual aura: linkage to chromosome 9q. *Neurology* 2007; **68**: 1995-2002 [PMID: 17460155 DOI: 10.1212/01.wnl.0000307659.43996.ca]

25 **Jurkat-Rott K**, Freilinger T, Dreier JP, Herzog J, Göbel H, Petzold GC, Montagna P, Gasser T, Lehmann-Horn F, Dichgans M. Variability of familial hemiplegic migraine with novel A1A2 Na+/K+-ATPase variants. *Neurology* 2004; **62**: 1857-1861 [PMID: 15159495 DOI: 10.1212/01.WNL.0000127310.11526.FD]

26 **Dürr KL**, Tavraz NN, Dempski RE, Bamberg E, Friedrich T. Functional significance of E2 state stabilization by specific alpha/beta-subunit interactions of Na,K- and H,K-ATPase. *J Biol Chem* 2009; **284**: 3842-3854 [PMID: 19064992 DOI: 10.1074/jbc.M808101200]

27 **de Vries B**, Stam AH, Kirkpatrick M, Vanmolkot KR, Koenderink JB, van den Heuvel JJ, Stunnenberg B, Goudie D, Shetty J, Jain V, van Vark J, Terwindt GM, Frants RR, Haan J, van den Maagdenberg AM, Ferrari MD. Familial hemiplegic migraine is associated with febrile seizures in an FHM2 family with a novel de novo ATP1A2 mutation. *Epilepsia* 2009; **50**: 2503-2504 [PMID: 19874388 DOI: 10.1111/j.1528-1167.2009.02186.x]

28 **Price EM**, Lingrel JB. Structure-function relationships in the Na,K-ATPase alpha subunit: site-directed mutagenesis of glutamine-111 to arginine and asparagine-122 to aspartic acid generates a ouabain-resistant enzyme. *Biochemistry* 1988; **27**: 8400-8408 [PMID: 2853965 DOI: 10.1021/bi00422a016]

29 **Koenderink JB**, Geibel S, Grabsch E, De Pont JJ, Bamberg E, Friedrich T. Electrophysiological analysis of the mutated Na,K-ATPase cation binding pocket. *J Biol Chem* 2003; **278**: 51213-51222 [PMID: 14532287 DOI: 10.1074/jbc.M306384200]

30 **Nakao M**, Gadsby DC. [Na] and [K] dependence of the Na/K pump current-voltage relationship in guinea pig ventricular myocytes. *J Gen Physiol* 1989; **94**: 539-565 [PMID: 2607334 DOI: 10.1085/jgp.94.3.539]

31 **Rakowski RF**, Gadsby DC, De Weer P. Stoichiometry and voltage dependence of the sodium pump in voltage-clamped, internally dialyzed squid giant axon. *J Gen Physiol* 1989; **93**: 903-941 [PMID: 2544655 DOI: 10.1085/jgp.93.5.903]

32 **Rakowski RF**. Charge movement by the Na/K pump in Xenopus oocytes. *J Gen Physiol* 1993; **101**: 117-144 [PMID: 8382257 DOI: 10.1085/jgp.101.1.117]

33 **Holmgren M**, Rakowski RF. Charge translocation by the Na+/K+ pump under Na+/Na+ exchange conditions: intracellular Na+ dependence. *Biophys J* 2006; **90**: 1607-1616 [PMID: 16326910 DOI: 10.1529/biophysj.105.072942]

34 **Jaunin P**, Jaisser F, Beggah AT, Takeyasu K, Mangeat P, Rossier BC, Horisberger JD, Geering K. Role of the transmembrane and extracytoplasmic domain of beta subunits in subunit assembly, intracellular transport, and functional expression of Na,K-pumps. *J Cell Biol* 1993; **123**: 1751-1759 [PMID: 8276895 DOI: 10.1083/jcb.123.6.1751]

35 **Hasler U**, Wang X, Crambert G, Béguin P, Jaisser F, Horisberger JD, Geering K. Role of beta-subunit domains in the assembly, stable expression, intracellular routing, and functional properties of Na,K-ATPase. *J Biol Chem* 1998; **273**: 30826-30835 [PMID: 9804861 DOI: 10.1074/jbc.273.46.30826]

36 **Eakle KA**, Kabalin MA, Wang SG, Farley RA. The influence of beta subunit structure on the stability of Na+/K(+)-ATPase complexes and interaction with K+. *J Biol Chem* 1994; **269**: 6550-6557 [PMID: 8120007]

37 **Colonna TE**, Huynh L, Fambrough DM. Subunit interactions in the Na,K-ATPase explored with the yeast two-hybrid system. *J Biol Chem* 1997; **272**: 12366-12372 [PMID: 9139681 DOI: 10.1074/jbc.272.19.12366]

38 **Geering K**. The functional role of beta subunits in oligomeric P-type ATPases. *J Bioenerg Biomembr* 2001; **33**: 425-438 [PMID: 11762918]

39 **de Vries B**, Freilinger T, Vanmolkot KR, Koenderink JB, Stam AH, Terwindt GM, Babini E, van den Boogerd EH, van den Heuvel JJ, Frants RR, Haan J, Pusch M, van den Maagdenberg AM, Ferrari MD, Dichgans M. Systematic analysis of three FHM genes in 39 sporadic patients with hemiplegic migraine. *Neurology* 2007; **69**: 2170-2176 [PMID: 18056581 DOI: 10.1212/01.wnl.0000295670.01629.5a]

40 **Koenderink JB**, Zifarelli G, Qiu LY, Schwarz W, De Pont JJ, Bamberg E, Friedrich T. Na,K-ATPase mutations in familial hemiplegic migraine lead to functional inactivation. *Biochim Biophys Acta* 2005; **1669**: 61-68 [PMID: 15843000 DOI: 10.1016/j.bbamem.2005.01.003]

41 **Hasler U**, Crambert G, Horisberger JD, Geering K. Structural and functional features of the transmembrane domain of the Na,K-ATPase beta subunit revealed by tryptophan scanning. *J Biol Chem* 2001; **276**: 16356-16364 [PMID: 11278434 DOI: 10.1074/jbc.M008778200]

42 **Poulsen H**, Khandelia H, Morth JP, Bublitz M, Mouritsen OG, Egebjerg J, Nissen P. Neurological disease mutations compromise a C-terminal ion pathway in the Na(+)/K(+)-ATPase. *Nature* 2010; **467**: 99-102 [PMID: 20720542 DOI: 10.1038/nature09309]

43 **Blanco-Arias P**, Einholm AP, Mamsa H, Concheiro C, Gutiérrez-de-Terán H, Romero J, Toustrup-Jensen MS, Carracedo A, Jen JC, Vilsen B, Sobrido MJ. A C-terminal mutation of ATP1A3 underscores the crucial role of sodium affinity in the pathophysiology of rapid-onset dystonia-parkinsonism. *Hum Mol Genet* 2009; **18**: 2370-2377 [PMID: 19351654 DOI: 10.1093/hmg/ddp170]

44 **Vasilyev A**, Khater K, Rakowski RF. Effect of extracellular pH on presteady-state and steady-state current mediated by the Na+/K+ pump. *J Membr Biol* 2004; **198**: 65-76 [PMID: 15138746 DOI: 10.1007/s00232-004-0660-4]

45 **Blanco G**, Mercer RW. Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol* 1998; **275**: F633-F650 [PMID: 9815123]

46 **Crambert G**, Hasler U, Beggah AT, Yu C, Modyanov NN, Horisberger JD, Lelièvre L, Geering K. Transport and pharmacological properties of nine different human Na, K-ATPase isozymes. *J Biol Chem* 2000; **275**: 1976-1986 [PMID: 10636900 DOI: 10.1074/jbc.275.3.1976]

47 **Rose CR**, Ransom BR. Gap junctions equalize intracellular Na+ concentration in astrocytes. *Glia* 1997; **20**: 299-307 [PMID: 9262234 DOI: 10.1002/(SICI)1098-1136(199708)20:]

48 **Ducros A**, Joutel A, Vahedi K, Cecillon M, Ferreira A, Bernard E, Verier A, Echenne B, Lopez de Munain A, Bousser MG, Tournier-Lasserve E. Mapping of a second locus for familial hemiplegic migraine to 1q21-q23 and evidence of further heterogeneity. *Ann Neurol* 1997; **42**: 885-890 [PMID: 9403481 DOI: 10.1002/ana.410420610]

49 **Haan J**, Terwindt GM, van den Maagdenberg AM, Stam AH, Ferrari MD. A review of the genetic relation between migraine and epilepsy. *Cephalalgia* 2008; **28**: 105-113 [PMID: 18197881 DOI: 10.1111/j.1468-2982.2007.01460.x]

50 **Shyti R**, de Vries B, van den Maagdenberg A. Migraine genes and the relation to gender. *Headache* 2011; **51**: 880-890 [PMID: 21631474 DOI: 10.1111/j.1526-4610.2011.01913.x]

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ORIGINAL ARTICLE

Binding of rhodopsin and rhodopsin analogues to transducin, rhodopsin kinase and arrestin-1

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Abstract

AIM: To investigate the interaction of reconstituted rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin with transducin, rhodopsin kinase and arrestin-1.

METHODS: Rod outer segments (ROS) were isolated from bovine retinas. Following bleaching of ROS membranes with hydroxylamine, rhodopsin and rhodopsin analogues were generated with the different retinal isomers and the concentration of the reconstituted pigments was calculated from their UV/visible absorption spectra. Transducin and arrestin-1 were purified to homogeneity by column chromatography, and an enriched-fraction of rhodopsin kinase was obtained by extracting freshly prepared ROS in the dark. The guanine nucleotide binding activity of transducin was determined by Millipore filtration using β,γ-imido-(^{3}H)-guanosine 5′-triphosphate. Recognition of the reconstituted pigments by rhodopsin kinase was determined by autoradiography following incubation of ROS membranes containing the various regenerated pigments with partially purified rhodopsin kinase in the presence of (γ-^{32}P) ATP. Binding of arrestin-1 to the various pigments in ROS membranes was determined by a sedimentation assay analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

RESULTS: Reconstituted rhodopsin and rhodopsin analogues containing 9-*cis*-retinal and 13-*cis*-retinal rendered an absorption spectrum showing a maximum peak at 498 nm, 486 nm and about 467 nm, respectively, in the dark; which was shifted to 380 nm, 404 nm and about 425 nm, respectively, after illumination. The percentage of reconstitution of rhodopsin and the rhodopsin analogues containing 9-*cis*-retinal and 13-*cis*-retinal was estimated to be 88%, 81% and 24%, respectively. Although only residual activation of transducin was observed in the dark when reconstituted rhodopsin and 9-*cis*-retinal-rhodopsin was used, the rhodopsin analogue containing the 13-*cis* isomer of retinal was capable of activating transducin independently of light. Moreover, only a basal amount of the reconstituted rhodopsin and 9-*cis*-retinal-rhodopsin was phosphorylated by rhodopsin kinase in the dark, whereas the pigment containing the 13-*cis*-retinal was highly phosphorylated by rhodopsin kinase even in the dark. In addition, arrestin-1 was incubated with rhodopsin, 9-*cis*-retinal-rhodopsin or 13-*cis*-retinal-rhodopsin. Experiments were performed using both phosphorylated and non-phosphorylated regenerated pigments. Basal amounts of arrestin-1 interacted with rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin under dark and light conditions. Residual arrestin-1 was also recognized by the phosphorylated rhodopsin and phosphorylated 9-*cis*-retinal-rhodopsin in the dark. However, arrestin-1 was recognized by phosphorylated 13-*cis*-

retinal-rhodopsin in the dark. As expected, all reformed pigments were capable of activating transducin and being phosphorylated by rhodopsin kinase in a light-dependent manner. Additionally, all reconstituted photolyzed and phosphorylated pigments were capable of interacting with arrestin-1.

CONCLUSION: In the dark, the rhodopsin analogue containing the 13-*cis* isomer of retinal appears to fold in a pseudo-active conformation that mimics the active photointermediate of rhodopsin.

Key words: Rhodopsin; Rhodopsin analogues; 9-*cis*-Retinal; 11-*cis*-Retinal; 13-*cis*-Retinal; Photointermediates; Transducin; Rhodopsin kinase; Arrestin-1; Visual process

Core tip: Rhodopsin is a specialized G protein-coupled receptors composed of a single polypeptide chain, opsin, and a covalently linked 11-*cis*-retinal. It is well known that rhodopsin uses the 11-*cis* form of retinal exclusively as the chromophore. Retinal analogues have long been used to probe the chromophore binding pocket and to study ligand-protein relationships to better understand the photochemical cis-trans isomerization of rhodopsin. However, little is known about the interactions of rhodopsin analogues with other proteins in the visual cascade. Here, we were able to reconstitute a rhodopsin analogue containing 13-*cis*-retinal. We compared the binding of reconstituted rhodopsin, 9-cis-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin to transducin, rhodopsin kinase and arrestin-1, both in the dark and under illumination. Interestingly, we found that in the dark the rhodopsin analogue containing the 13-*cis* isomer of retinal appears to fold in a pseudo-active conformation that mimics the active photointermediate of rhodopsin.

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INTRODUCTION

G protein-coupled receptors (GPCRs) activate signaling paths in response to a diverse number of stimuli such as photons, Ca^{2+}, organic odorants, amines, hormones, nucleotides, nucleosides, peptides, lipids and even large proteins[1]. All GPCRs share a conserved seven-transmembrane-helix structural bundle connected by six loops of varying lengths. Binding of specific ligands to the transmembrane or extracellular domains of members of the GPCR superfamily causes conformational changes that act as a switch to relay the signal to heterotrimeric G proteins that in turn evoke further intracellular responses[2].

The dim-light photoreceptor rhodopsin is a highly specialized GPCR composed of a single polypeptide chain of 348 amino acids that conforms the apoprotein opsin, and a covalently linked 11-*cis*-retinal chromophore that is tightly packed within the bundle of helices[3,4]. The chromophore is bound to the ε-amino group of Lys296, located in the seventh helix (TM7) *via* a protonated Schiff base linkage. In the ground state this charge is stabilized by the counter-ion Glu113 that is located in the third helix (TM3)[5]. Another important structural feature of the 11-*cis*-retinal chromophore in rhodopsin is its extended polyene structure, which accounts for its visible absorption properties and allows for resonance structures[6].

In rhodopsin, 11-*cis*-retinal serves both as the chromophore and as an inverse agonist that holds the visual pigment protein in an inactive conformation. Absorption of a photon by the 11-*cis*-retinal of rhodopsin causes its photoisomerization to the all-*trans* form[7], converting the ligand into an agonist, and leading to a conformational change of the protein moiety that triggers the signal transduction cascade *via* reactions of the G protein transducin. Following *cis-trans* isomerization of the chromophore, rhodopsin relaxes through a series of photoproducts, which have been identified by their characteristic absorption spectra. One of the photointermediates, metarhodopsin Ⅱ (meta Ⅱ), is the active conformation of rhodopsin responsible of binding transducin and initiating the signaling process. Transducin, which is arranged as two units, the α subunit and the βγ-complex, transmits the visual stimuli by activating a potent cGMP phosphodiesterase known as PDE6. The resulting decrease in the cytosolic concentration of cGMP causes the closure of cation-specific cGMP-gated channels located in the plasma membrane, leading to the hyperpolarization of the rod cell. Additional protein molecules participate in modulating the duration of the signal and the achievement of the appropriate response[8]. Particularly, the phosphorylation of photoactivated rhodopsin by rhodopsin kinase, also known as GPCR kinase 1 or GRK1, and its interaction with arrestin-1, are both involved in signal desensitization since the transducin activation phase is terminated by the interaction of meta Ⅱ with rhodopsin kinase and arrestin-1[9,10]. Subsequently, the retinal Schiff base is hydrolysed and the photolysed all-*trans*-retinal is released from its binding site. Regeneration of the light sensitive rhodopsin ground state requires the supply of new 11-*cis*-retinal through the so-called retinoid cycle[11,12].

It is well known that the rod visual pigment rhodopsin uses the 11-*cis* form of retinal exclusively as the chromophore, and the strict selection of this isomer appears to have occurred early in the evolution of visual function. Under certain pathological conditions, however, also the 9-*cis* configuration of retinal is observed, which generates a pigment known as isorhodopsin[13]. Retinal analogues have long been used to probe the chromophore binding pocket and to study ligand-protein relationships to better understand the photochemical *cis-trans* isomerization of rhodopsin[14] and cone opsins[15]. Yet, little is known about the interac-

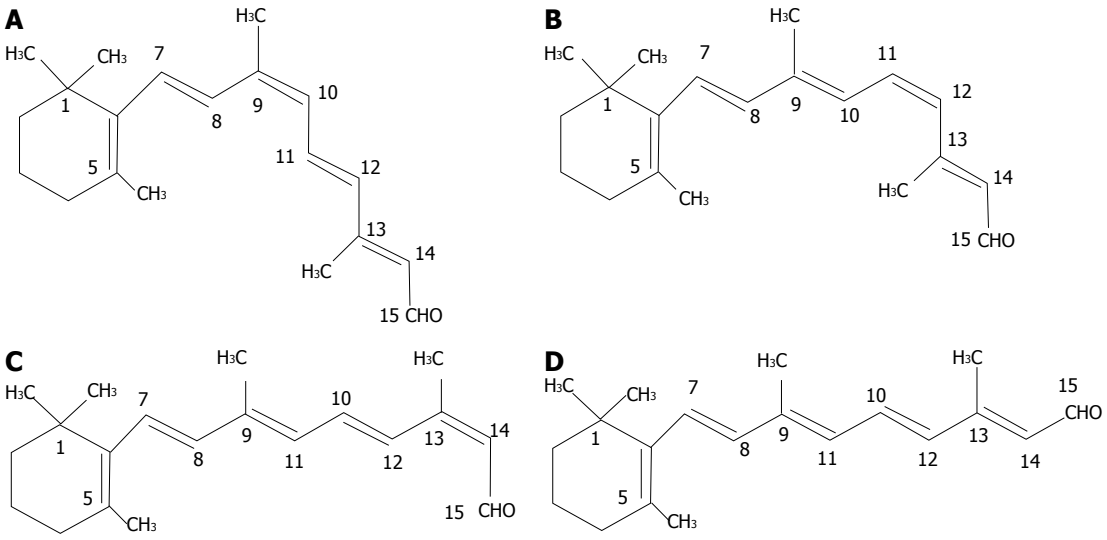

Figure 1 Structures of retinal analogues. A: 9-*cis*-retinal; B: 11-*cis*-retinal; C: 13-*cis*-retinal; D: all-*trans*-retinal.

tions of rhodopsin analogues with other proteins in the visual cascade. Although, it has been reported that the retinal binding site in the inactive state of rhodopsin can accommodate the 7-*cis*, 9-*cis* and 11-*cis* isomers of retinal, but not the longer all-*trans* or 13-*cis* isomers[16], in the present work we were able to reconstitute a rhodopsin analogue containing 13-*cis*-retinal, in addition to photoreceptor proteins containing 9-*cis*-retinal and 11-*cis*-retinal. We compared the binding of reconstituted rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin to transducin, rhodopsin kinase and arrestin-1, both in the dark and under illumination. The chemical structures of the geometrical retinal isomers used here and of all-*trans*-retinal are shown in Figure 1.

MATERIALS AND METHODS

Materials

Bovine eyes were obtained from the nearest abattoir (Beneficiadora Diagon, CA, Matadero Caracas, Venezuela). Retinae were extracted in the dark, under red light, and were maintained frozen at -80 ℃. Reagents were purchased from the following sources: β,γ-imido-(^{3}H)-guanosine 5'-triphosphate [(8-^{3}H) GMPpNp] (17.9 Ci/mmol) and (γ-^{32}P) ATP (3000 Ci/mmol), Amersham; 9-*cis*-retinal, 13-*cis*-retinal, bovine serum albumine (BSA), hydroxylamine, phytic acid or inositol hexakisphosphate (IP_6), n-dodecyl β-D-maltoside, and DEAE-cellulose, Sigma-Aldrich; ATP, heparine-sepharose and concanavalin A-Sepharose 4B, Pharmacia; molecular weight pre-stained protein markers, and Bradford reagent, Bio-Rad; anti-rabbit IgG antibodies conjugated to alkaline phsophatase, KPL; bromocloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT), and molecular weight protein standards, Promega; X-ray films, Kodak. The 11-*cis*-retinal was donated by Dr. Debra Thompson, University of Michigan, United States.

Preparation of rod outer segments and washed membranes

Rod outer segments (ROS) were isolated from frozen bovine retinas as described previously[17]. Dark depleted ROS membranes were prepared by washing ROS with 5 mmol/L Tris-HCl (pH = 7.4), 2 mmol/L EDTA, and 5 mmol/L β-mercaptoethanol until no significant amount of peripheral proteins was released with the wash buffer. ROS and dark-depleted ROS membranes were stored in the dark at -80 ℃. Rhodopsin concentration was calculated from its UV/visible absorption spectra, using its molar extinction coefficient (40700 $M^{-1}cm^{-1}$, at 500 nm)[18]. In addition, rhodopsin was identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using anti-bovine rhodopsin polyclonal antibodies raised in mice.

Purification of transducin

Transducin was obtained from ROS prepared under room light, at 4 ℃, following the affinity procedure described by Kühn[19]. GTP (100 μmol/L) was employed to elute transducin from the washed illuminated ROS, and transducin was further purified to homogeneity by anion exchange chromatography on a DEAE-cellulose column as described elsewhere[20]. Fractions containing transducin were identified by SDS-PAGE and Western blot using anti-bovine transducin polyclonal antibodies raised in mice.

Preparation of an Enriched fraction of Rhodopsin Kinase

Freshly prepared ROS were washed three times with an isotonic buffer containing 70 mmol/L potassium phosphate (pH = 6.8), 5 mmol/L magnesium acetate, 5 mmol/L β-mercaptoethanol, and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Following centrifugation, the washed ROS pellet was hypotonically extracted with 5 mmol/L Tris-HCl (pH = 7.4), 5 mmol/L magnesium acetate, 5 mmol/L β-mercaptoethanol, and 0.1 mmol/L PMSF[21]. Under these conditions, soluble proteins and proteins weakly associated with the membrane, including transducin, cGMP phosphodiesterase PDE6, arrestin-1, and rhodopsin kinase, appear in the supernatant gener-

ated after centrifugation. This supernatant was considered as the enriched fraction of rhodopsin kinase. The whole procedure was carried out at 4 ℃, in the dark under red light.

Purification of arrestin-1

Arrestin-1 was purified following the procedure described by Buczylko *et al*[22]. Frozen bovine retinas were homogenized with 10 mmol/L Hepes (pH = 7.5), 0.1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol, under dim red light, at 4 ℃. Following centrifugation at 70000 *g*, for 25 min, the supernatant containing the soluble proteins was chromatographed on a DEAE-cellulose column, previously equilibrated in the same buffer. The column was washed with 10 mmol/L Hepes (pH = 7.5), 15 mmol/L NaCl, 0.1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol (Buffer A) until the absorbance at 280 nm dropped below 0.1. Adsorbed proteins were eluted with a 0 to 150 mmol/L linear gradient of NaCl in Buffer A, and the fractions containing arrestin-1 were identified by SDS-PAGE and Western blot using anti-bovine arrestin-1 polyclonal antibodies prepared in rabbits. These fractions were pooled and applied to a heparin-sepharose column, which was previously equilibrated with 10 mmol/L Hepes (pH = 7.5), 100 mmol/L NaCl, 0.1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol (Buffer B). Arrestin-1 was eluted using a gradient of 0 to 8 mmol/L phytic acid in Buffer B. The peak of arrestin-1 was pooled, dialyzed against Buffer A, applied to a second heparin-sepharose column, and eluted with 10 mmol/L Hepes (pH = 7.5), 400 mmol/L NaCl, 0,1 mmol/L PMSF, 1 mmol/L β-mercaptoethanol.

Bleaching of rhodopsin in washed ROS membranes

Washed ROS membranes were incubated with 50 mmol/L hydroxylamine in 10 mmol/L Tris-HCl (pH = 7.4), at 4 ℃, for 15 min, under illumination with a tungsten 100 W lamp. Then, the mixture was centrifuged at 50000 *g* for 20 min, at 4 ℃. The supernatant was discarded and the pellet was washed twice with 5 mmol/L Tris-HCl (pH = 7.4), 5 mmol/L magnesium acetate, 5 mmol/L β-mercaptoethanol.

Regeneration of rhodopsin and rhodopsin analogues

Samples of bleached washed ROS membranes containing about 25 μmol/L of opsin were resuspended in 10 mmol/L Tris-HCl (pH = 7.4). Then, appropriate aliquots of stock solutions of 9-*cis*-retinal, 11-*cis*-retinal, and 13-*cis*-retinal prepared in ethanol were added in the dark. A molar ratio of 3:1 retinal to opsin was used for the reconstitution of the pigment with the 9-*cis*-retinal and 11-*cis*-retinal isomers, whereas a ratio of 15:1 retinal to opsin was employed for the regeneration of the rhodopsin analogue containing the 13-*cis*-retinal isomer. Following an overnight incubation, at room temperature, all samples were centrifuged at 50000 *g*, for 20 min, at 4 ℃. The regeneration of the pigments was followed by UV-Vis spectroscopy using the extinction coefficient of rhodopsin[18]. The excess of 9-*cis*-retinal, 11-*cis*-retinal, and 13-*cis*-retinal was eliminated by washing the membranes containing the reconstituted pigments with 2% BSA in 10 mmol/L Tris-HCl (pH = 7.4). BSA was then removed by successive washes with 5 mmol/L Tris-HCl (pH 7.4), 5 mmol/L magnesium acetate, 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol. ROS membranes containing the reconstituted pigments were resuspended in 5 mmol/L Tris-HCl (pH = 7.4), 100 mmol/L NaCl, 1 mmol/L magnesium acetate, 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol.

Binding of (8-^{3}H) GMPpNp to transducin

Guanine nucleotide binding was measured by Millipore filtration using (8-^{3}H) GMPpNp, a radioactive non-hydrolyzable analogue of GTP, as previously described[23].

Phosphorylation of reconstituted rhodopsin and rhodopsin analogues

ROS membranes containing the reconstituted pigments were incubated with a 50-μL aliquot of an enriched fraction of rhodopsin kinase, in the presence of 50 mmol/L Tris-HCl (pH = 7.5), 12 mmol/L $MgCl_2$, 20 mmol/L KF, 40 μmol/L [γ-^{32}P] ATP (specific activity about 4500 cpm/pmol), 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol. Following incubation for 1 h, at room temperature, under illumination with a 100 W tungsten lamp, the phosphorylated membranes were centrifuged at 100000 *g*, for 20 min, at 4 ℃. Identical control experiments were carried out in the dark. Samples were separated by SDS-PAGE and the phosphorylated bands were identified by autoradiography following staining and drying of the gels.

Regeneration of phosphorylated rhodopsin and phosphorylated rhodopsin analogues

ROS containing 1.9 mg of rhodopsin were sedimented by centrifugation at 100000 *g* for 20 min, and resuspended in 50 mmol/L Tris-HCl (pH = 7.5), 12 mmol/L $MgCl_2$, 20 mmol/L KF, 40 μmol/L ATP, 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol, in the presence of a 50-μL aliquot of an enriched fraction of rhodopsin kinase. Following illumination for 1 h with a tungsten 100 W lamp, the mixture was centrifuged and the resulting pellet containing the phosphorylated protein was resuspended in 10 mmol/L Tris-HCl (pH = 7.4). Phosphorylated rhodopsin was bleached with 50 mmol/L hydroxylamine to obtain phosphorylated opsin. Samples of phosphorylated opsin were reconstituted with 9-*cis*-retinal, 11-*cis*-retinal and 13-*cis*-retinal as described above.

Interaction of reconstituted rhodopsin and rhodopsin analogues with arrestin-1

The binding of arrestin-1 to the pigments reconstituted in washed ROS membranes was determined according to Gurevich *et al*[24], with slight modifications. Briefly, samples of arrestin-1 (14 μg) were incubated with 12 μg of the regenerated pigments, for 1 h, at room temperature. Experiments were performed in 100 μL of 5 mmol/L Tris-HCl (pH = 7.4), 5 mmol/L magnesium acetate, 0.1

mmol/L PMSF, 5 mmol/L β-mercaptoethanol, both in the dark and under illumination, using phosphorylated and non-phosphorylated pigments (about molar ratio of 1:1 arrestin-1 to pigment). The original mixture, and the resulting supernatant and pellet after centrifugation at 100000 *g*, for 20 min, were separated by SDS-PAGE. The gels were colored by silver staining and the bands of arrestin-1 and rhodopsin or rhodopsin analogues were evaluated by densitometry.

Other procedures

Protein concentration was determined as reported by Bradford[25] using BSA as protein standard. SDS-PAGE was carried out on 1.5-mm thick slab gels containing 12% polyacrylamide as described by Laemmli[26]. Coomassie blue R-250 or silver staining was used for protein visualization. For Western blot analyses, the proteins were electrophoretically transferred from the gels to nitrocellulose sheets (0.45 μm pore size) as reported by Towbin *et al*[27]. Rhodopsin was purified to homogeneity by batchwise affinity chromatography on concanavalin A-Sepharose[28], using n-dodecyl β-D-maltoside instead of n-octyl β-D-glucopyranoside as the detergent. Polyclonal antibodies against rhodopsin and transducin were prepared in mice as described[29]. Purified arrestin-1 was used to raise polyclonal antibodies in rabbit serum following the procedure described by Harlow *et al*[30].

Statistical analysis

For statistical analysis, mean value comparisons were performed by using the Student *t*-test or Anova and Krunskal-Wallis test. *P*-values below 0.05 were considered significant. Data in all histograms are graphed as mean ± SD.

RESULTS

Analysis by SDS-PAGE showed that isolated ROS membranes contained all the proteins involved in the photoexcitation process (Figure 2A), including rhodopsin, transducin, cGMP phosphodiesterase PDE6, arrestin-1 and rhodopsin kinase[19]. As revealed by Western blot using anti-rhodopsin polyclonal antibodies, the major polypeptide band with an apparent molecular mass of approximately 35 kDa corresponded to rhodopsin (Figure 2B). Since rhodopsin has a tendency to oligomerize, higher order oligomers of rhodopsin, such as dimers, trimers, *etc*., were also detected by immunoblotting (Figure 2B). Rhodopsin polypeptide bands were observed in the original ROS sample and remained in the pellet following the washing procedure (Figure 2A and B). The presence of rhodopsin was also demonstrated by measuring the UV/visible absorption spectra of the samples and estimating the ratio of the absorbance at 280 nm to the absorbance at 500 nm[28,31]. Crude ROS showed a spectral ratio $A_{280\ nm}/A_{500\ nm}$ of 2.68, which decreased to 2.05 in dark-depleted ROS membranes after removal of the peripheral proteins (Figure 2C).

Rhodopsin was bleached by exposing washed ROS membranes to light in the presence of hydroxylamine. This treatment caused the complete detachment of the retinal chromophore. Rhodopsin and rhodopsin analogues containing the 9-*cis* and 13-*cis* isomers of retinal were reformed by incubating opsin with an excess of each retinal in the dark. The regeneration of rhodopsin is shown in Figure 2D as an example. As illustrated in Figure 2D, the 11-*cis*-retinal molecule possessed a broad absorption band at about 370 nm that overlapped with the absorption peak of the reconstituted rhodopsin pigment. Washes in the presence of BSA completely removed the residual retinal (Figure 2D).

As can be seen in Figure 3, pigments were reconstituted after the addition of the three retinal isomers to opsin in the dark. Reconstituted rhodopsin rendered the characteristic absorption spectrum of rhodopsin in the dark (Figure 3B), showing a maximum peak at 498 nm (about 500 nm). Following illumination, this band was shifted to 380 nm that corresponded to the meta Ⅱ photointermediate. In the dark, the reconstituted pigment analogue containing 9-*cis*-retinal (isorhodopsin) showed an absorbance peak at 486 nm (Figure 3A), which was slightly blue shifted in comparison to rhodopsin. Once photolyzed, the maximum of illuminated isorhodopsin was obtained at 404 nm which was slightly red shifted in comparison to meta Ⅱ. The absorption spectra of the rhodopsin analogue containing 13-*cis*-retinal showed broader bands than rhodopsin and isorhodopsin, under both, dark and light conditions (Figure 3C). In the dark, the absorption peak of 13-*cis*-retinal-rhodopsin was blue shifted showing its maximum at about 467 nm. After photolysis, the highest absorption peak of the illuminated 13-*cis*-retinal-rhodopsin was acquired at about 425 nm, more red shifted than meta Ⅱ and illuminated isorhodopsin. The percentage of reconstitution of the three pigments was estimated by comparing the absorption values at their maximum wavelength, using the extinction coefficient of rhodopsin as an approximate value[18], and the amount of total protein determined for each sample by the method of Bradford[25]. Our results showed that rhodopsin and isorhodopsin were reconstituted with a yield of 88% and 81%, respectively, whereas the rhodopsin analogue containing the 13-*cis* isomer of retinal was reformed with a yield of only 24%.

A partially purified transducin sample was initially obtained by GTP elution from illuminated ROS membranes. Then, transducin was purified to homogeneity by chromatography on a DEAE-cellulose column (Figure 4A). The elution of transducin was evaluated by measuring the rhodopsin- and light-dependent guanine nucleotide binding by a filtration assay using (8-^{3}H) GMPpNp. SDS-PAGE revealed that the same fractions comprising the GMPpNp binding activity also contained the polypeptide bands corresponding to the α-, β-, and γ-subunits of transducin (Figure 4A, Inset, top). In addition, anti-transducin polyclonal antibodies that preferentially detect

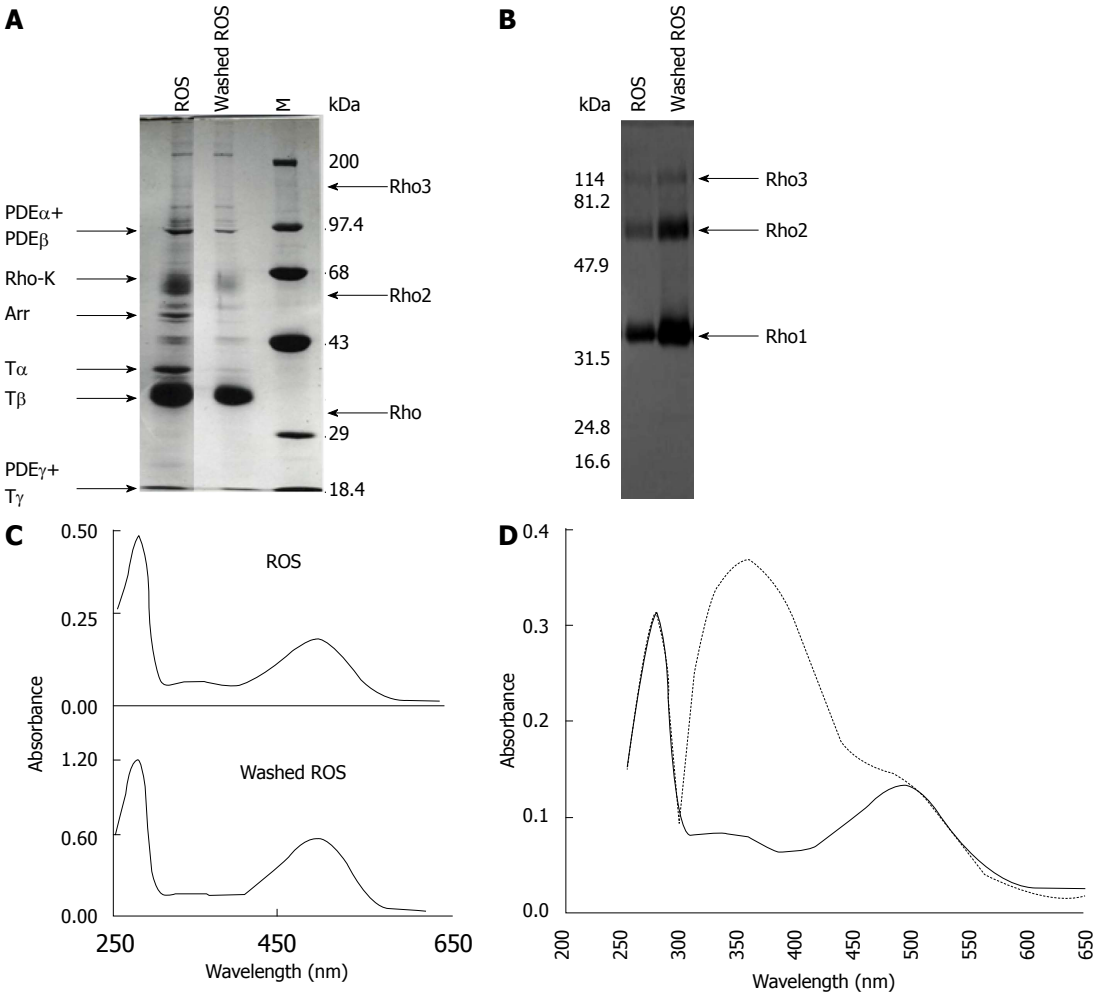

Figure 2 Isolation of rod outer segments, preparation of washed rod outer segments membranes, and reconstitution of rhodopsin. A: ROS were isolated from frozen bovine retinas and were hypotonically washed in the dark until no peripheral proteins were released. Arrows indicate the migration of rhodopsin (Rho), rhodopsin oligomers (Rho2 and Rho3), α-, β- and γ-subunits of the cGMP phosphodiesterase PDE6 (PDEα, PDEβ and PDEγ), α-, β- and γ-subunits of transducin (Tα, Tβ and Tγ), rhodopsin kinase (Rho-K), and arrestin-1 (Arr); B: ROS and dark-depleted ROS membranes were separated by SDS-PAGE, electrotransferred to a nitrocellulose filter and analyzed using polyclonal anti-rhodopsin antibodies. Arrows point out the migration of rhodopsin (Rho), rhodopsin dimers (Rho2), and rhodopsin trimers (Rho3). C: Absorption spectra of solubilized ROS and washed-ROS membranes in the dark; D: Regeneration of rhodopsin. A sample of depleted ROS membranes was bleached with hydroxylamine and incubated with an excess of 11-cis-retinal. Shown is the UV/visible spectra of rhodopsin in the dark, before (dashed line) and after (continuous line) removing the excess of 11-*cis*-retinal by washing with BSA. M: Molecular weight markers; ROS: Rod outer segments.

the α-subunit of transducin also recognized the α-subunit in the fractions containing the protein peak (Figure 4A, Inset, bottom).

Transducin binding to reconstituted rhodopsin and rhodopsin analogues was evaluated by measuring their capacity to induce the exchange of guanine nucleotides on transducin. The amount of reconstituted pigment, instead of the total amount of protein, was employed to normalize the reported values. As shown in Figure 5, all reformed pigments were capable of catalyzing the GMPpNp binding activity of transducin in a light-dependent manner. As expected, little activation of transducin (about 10%-15%) was observed in the dark when reconstituted rhodopsin and isorhodopsin were employed (Figure 5). Moreover, the apoprotein opsin was unable of inducing the exchange of GMPpNp on transducin (data not shown). In contrast and surprisingly, the rhodopsin analogue containing the 13-*cis* isomer of retinal was capable of activating transducin independently of light (about 40%) (Figure 5), suggesting that this pigment possesses a conformation in the dark that is similar to that of meta Ⅱ.

Figure 4B (left) shows the polypeptide composition of an aliquot of the enriched fraction of rhodopsin kinase, compared with samples of ROS and washed ROS membranes. This partially purified fraction of rhodopsin kinase contained polypeptide bands that corresponded to reported ROS peripheral proteins (transducin, cGMP phosphodiesterase PDE6, arrestin-1, rhodopsin kinase, *etc*.). As shown in Figure 4B (right) by autoradiography, intact ROS included active rhodopsin kinase given that rhodopsin was specifically phosphorylated in a light-dependent manner. Phosphorylated rhodopsin oligomers were also obtained in the crude ROS sample (Figure 4B, right). The enriched fraction of rhodopsin kinase was also capable of phosphorylating rhodopsin in washed-ROS membranes and under illumination (Figure 4B,

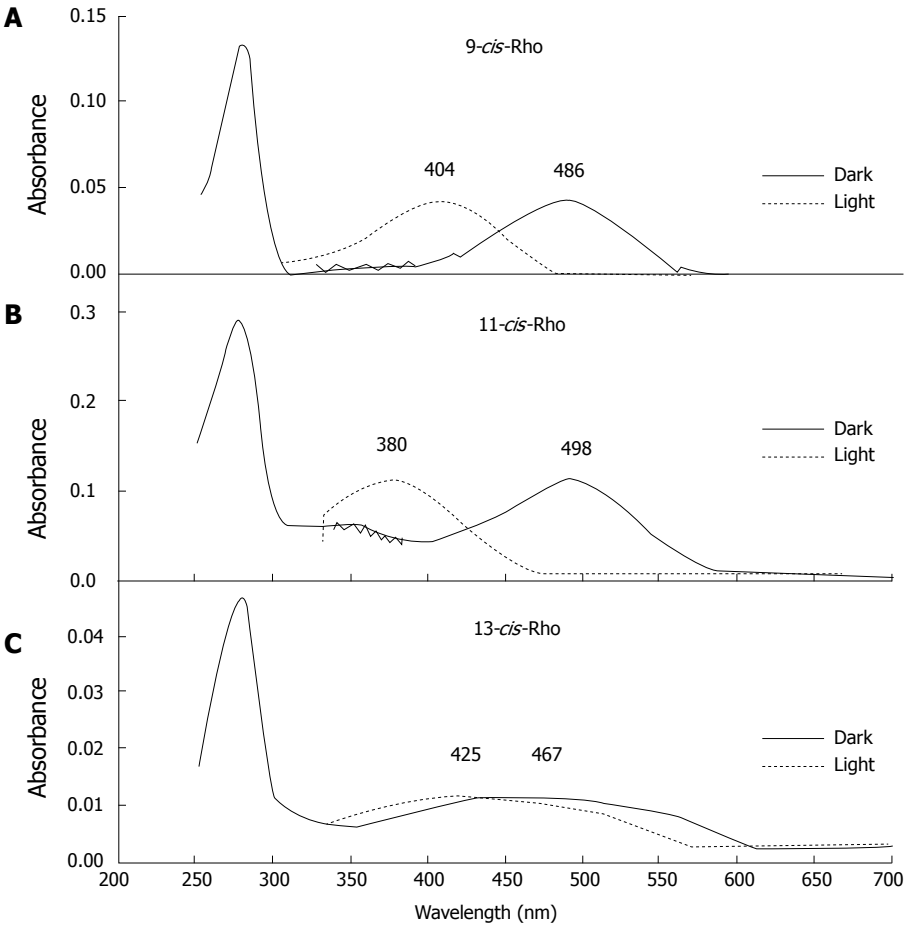

Figure 3 Absorption spectra of rhodopsin and rhodopsin analogues. Absorption spectrum of 9-*cis*-retinal-rhodopsin (9-*cis*-Rho) (A), rhodopsin (11-*cis*-Rho) (B) and 13-*cis*-retinal-rhodopsin (13-*cis*-Rho) (C) in the dark (continuous line) and under illumination (dashed line). Shown are the maximum wavelengths for each pigment.

right).

The ability of the reconstituted rhodopsin and rhodopsin analogues to serve as substrates for rhodopsin kinase was then measured by incubating each sample with an aliquot of the enriched fraction of rhodopsin kinase. As shown in Figure 6A by Coomassie blue staining, the same amount of each reconstituted protein was loaded in the gel lanes. Figure 6B illustrates by autoradiography that an enriched fraction of rhodopsin kinase was capable of phosphorylating all the reformed pigments in a light-dependent manner. Only basal amounts of the reconstituted rhodopsin and isorhodopsin samples were phosphorylated by rhodopsin kinase in the dark (Figure 6B). Opsin behaved similar to inactive rhodopsin given that the apoprotein was not phosphorylated by rhodopsin kinase (data not shown). However, the pigment containing the 13-*cis*-retinal was highly phosphorylated by rhodopsin kinase even in the dark (Figure 6B). Autoradiograms were quantified by densitometry in Figure 6C, corroborating the results qualitatively obtained in Figure 6B. The amount of regenerated pigment, instead of the total amount of protein, was used to normalize the reported values. These results suggest that 13-*cis*-retinal-rhodopsin, in its dark state, folds in a conformation that appears to be comparable to that of meta Ⅱ, given that it can be recognized by rhodopsin kinase even in the absence of light.

Arrestin-1 was purified to homogeneity by using three consecutive chromatography steps: (1) a DEAE-cellulose column; (2) a heparin-sepharose column that was eluted with a gradient of phytic acid; and (3) a second heparin-sepharose column that was eluted with 400 mmol/L NaCl[21]. Figure 4C shows the protein profile obtained after the last chromatography step. The elution of arrestin-1 was evaluated by SDS-PAGE analysis, which showed a polypeptide band with an apparent molecular mass of approximately 50 kDa (Figure 4C, Inset, top). This band was specifically recognized by anti-arrestin-1 polyclonal antibodies (Figure 4C, Inset, bottom). The ability of arrestin-1 to interact with the reconstituted rhodopsin and rhodopsin analogues was then evaluated by an affinity binding procedure. Arrestin-1 was incubated with rhodopsin, isorhodopsin or the 13-*cis*-retinal-rhodopsin, which were reconstituted using washed ROS membranes. Experiments were performed both in the dark and under illumination, and using phosphorylated and non-phosphorylated pigments. An experiment using opsin was also included as a control. After centrifugation, the resulting supernatants and pellets of all the samples were separated by SDS-PAGE. The interaction between arrestin-1 and the three pigments was determined qualitatively by

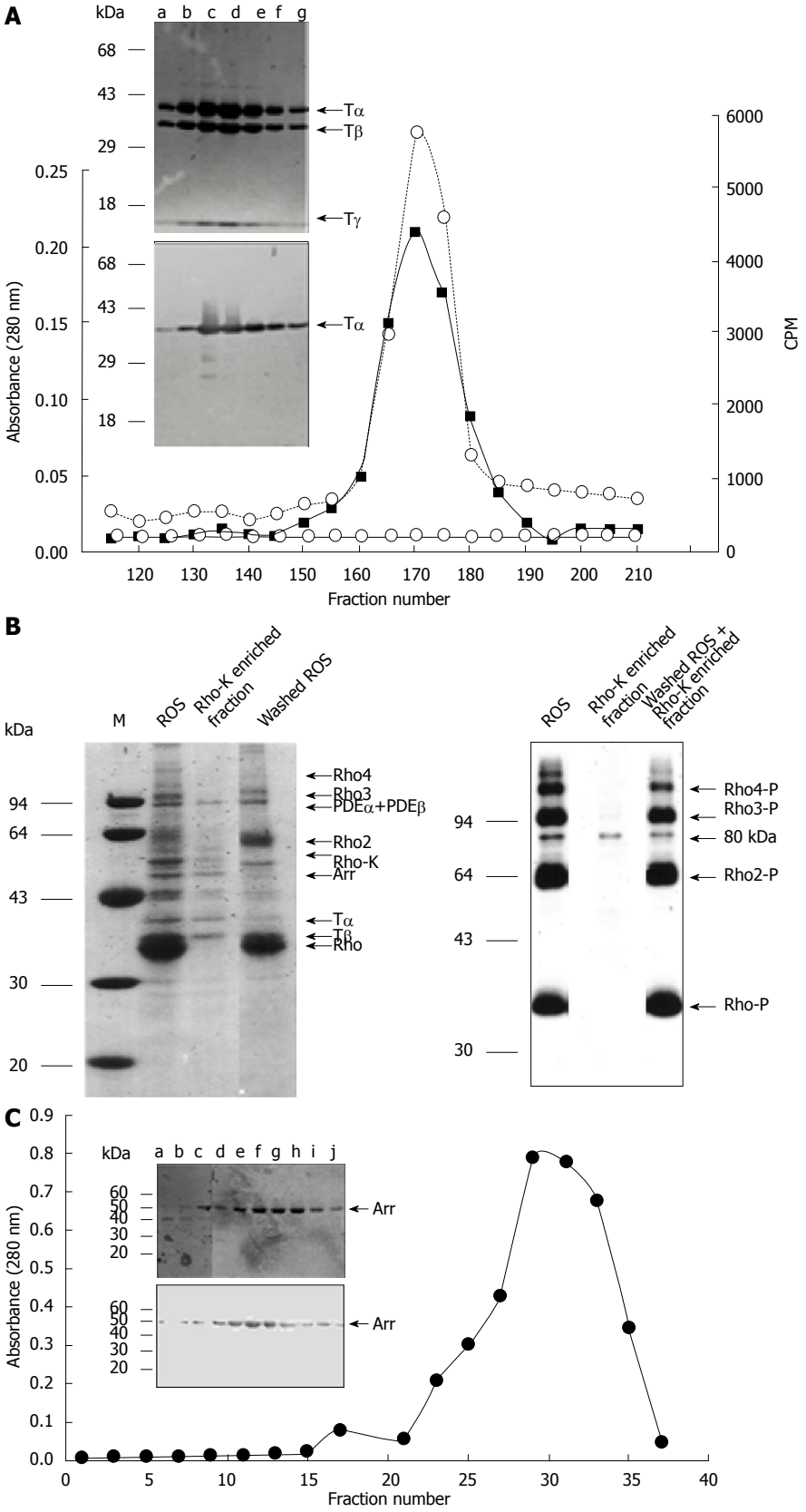

Figure 4 Purification of transducin and arrestin-1, and preparation of an enriched fraction of rhodopsin kinase. A: Transducin was purified to homogeneity on a DEAE-cellulose column. The elution profile was monitored at 280 nm (■). Fractions were analyzed for [8-^{3}H] GMPpNp binding activity (CPM) in the absence (○, continuous line) or presence (○, dashed line) of light-activated rhodopsin (as dark-depleted ROS membranes). Fractions were also examined by SDS-PAGE (Inset, top) and Western blot using anti-transducin polyclonal antibodies (Inset, bottom). Lanes a, b, c, d, e, f, and g correspond to column fractions Nº 155, 160, 165, 170, 175, 180 and 185, respectively. Arrows indicate the migration of α-, β- and γ-subunits of transducin (Tα, Tβ and Tγ); B: Autoradiography showing the light-induced *in vitro* phosphorylation of rhodopsin by rhodopsin kinase (Rho-K). Left, Coomassie blue staining; Right, Autoradiography. Samples of intact ROS membranes, a partially purified fraction of Rho-K, or a mixture of dark-depleted ROS membranes together with the enriched fraction of Rho-K were incubated with [γ-^{32}P] ATP under light conditions as described in Materials and Methods. Arrows indicate the migration of phosphorylated rhodopsin (Rho), rhodopsin dimers (Rho2), rhodopsin trimers (Rho3) and rhodopsin tetramers (Rho4). A polypeptide band of 80 kDa was phosphorylated in the Rho-K enriched fraction. M: Molecular weight markers; C: Arrestin-1 was purified to homogeneity after three consecutive chromatography steps, a DEAE-cellulose column, a heparin-sepharose column eluted with a gradient of phytic acid, and a second heparin-sepharose column eluted by increasing the salt concentration in the buffer. Shown is the elution profile of the last heparin-sepharose column, which was monitored at 280 nm (•). Fractions were inspected by SDS-PAGE (Inset, top) and Western blot using anti-arrestin-1 polyclonal antibodies (Inset, bottom). Lanes a, b, c, d, e, f, g, h, i, and j correspond to column fractions Nº 17, 21, 23, 25, 27, 29, 31, 33, 35, and 36. Arrows indicate the migration of arrestin-1 (Arr). ROS: Rod outer segments.

measuring the amount of arrestin-1 that was translocated from the initial mixture to the pellet. No arrestin-1 was bound to non-phosphorylated apoprotein opsin in the dark or light (Figure 7A and B, lane P). Moreover, as seen in the same figure (Figure 7A and B, lane P), no arrestin-1 interacted with phosphorylated opsin in the dark or light. Basal amounts of arrestin-1 interacted with rhodopsin, isorhodopsin and the 13-*cis*-retinal-rhodopsin complex, both in the dark and under illumination (Figure 7A and B, lane P), and as expected, all reformed photolyzed and

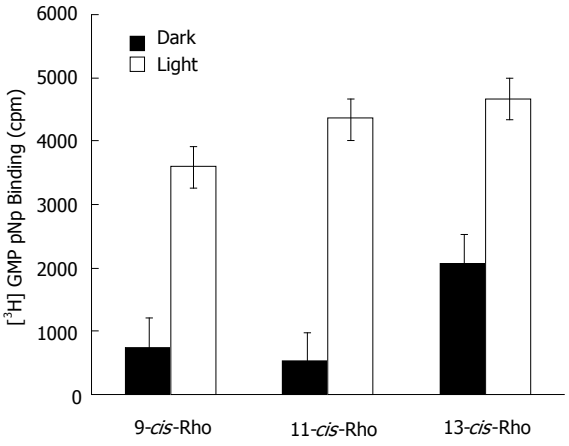

Figure 5 Activation of transducin by reconstituted rhodopsin and rhodopsin analogues. Binding of guanine nucleotides to transducin was evaluated by Millipore filtration using [8-^{3}H] GMPpNp. Dark and white bars correspond to experiments performed under dark and light conditions, respectively. Duplicate assays of three independent experiments were carried out. Mean ± SD are reported. Differences with *P*-values < 0.05 were considered significant.

phosphorylated pigments were capable of recognizing and binding arrestin-1 (Figure 7B, lane P). Although only residual arrestin-1 was bound to phosphorylated rhodopsin and phosphorylated isorhodopsin in the dark (Figure 7A, lane P), arrestin-1 was efficiently recognized by phosphorylated 13-*cis*-retinal-rhodopsin in the dark (Figure 7A, lane P). The silver stained gels shown in Figure 7A and B were quantified by densitometry (Figure 7C) and confirmed the results described above. The amount of reconstituted pigment, instead of the total amount of protein, was used to normalize the reported values shown in the histograms. The interaction of arrestin-1 with phosphorylated 13-*cis*-retinal-rhodopsin in the dark is consistent with our findings using transducin and rhodopsin kinase, that suggest that the rhodopsin analogue containing the 13-*cis* isomer of retinal exists as a pseudo-active state even without illumination.

DISCUSSION

To study ligand binding pockets in proteins, specific analogues with systematically altered chemical property in their structural moieties have usually been employed to establish structure-activity relationships with regard to their functional groups. Retinal has four C = C double bonds that give rise to the four mono-*cis* isomers, the 7-*cis*, 9-*cis*, 11-*cis* and 13-*cis* forms. These isomers undergo *cis*–*trans* isomerization upon photoexcitation. The chromophore of rhodopsin is 11-*cis*-retinal and, thus, in its absence, opsin is not photosensitive and no visual function exists. Here, the 9-*cis* and 13-*cis* retinal isomers have been used to probe the rhodopsin chromophore binding pocket and to study ligand-protein relationships to better understand the photochemical *cis-trans* isomerization of rhodopsin.

The production of 11-*cis*-retinal occurs in the retinal pigment epithelium. One of the more abundant proteins in this tissue is RPE65, which has been shown to be essential for the conversion of all-*trans*-retinyl ester to 11-*cis*-retinol[32]. Leber's congenital amaurosis, a childhood blinding disorder, results from disruption of a number of genes, but in many cases, the gene for RPE65 is defective[33-36]. When RPE65 is mutated or lacking, as in the RPE65 knockout mouse and Leber's congenital amaurosis, visual function is impaired[32]. However, in the RPE65 knockout mouse, where synthesis of 11-*cis*-retinal does not occur, a minimal visual response from rod photoreceptors is obtained, which is mediated by isorhodopsin, the rod pigment formed with 9-*cis*-retinal, rather

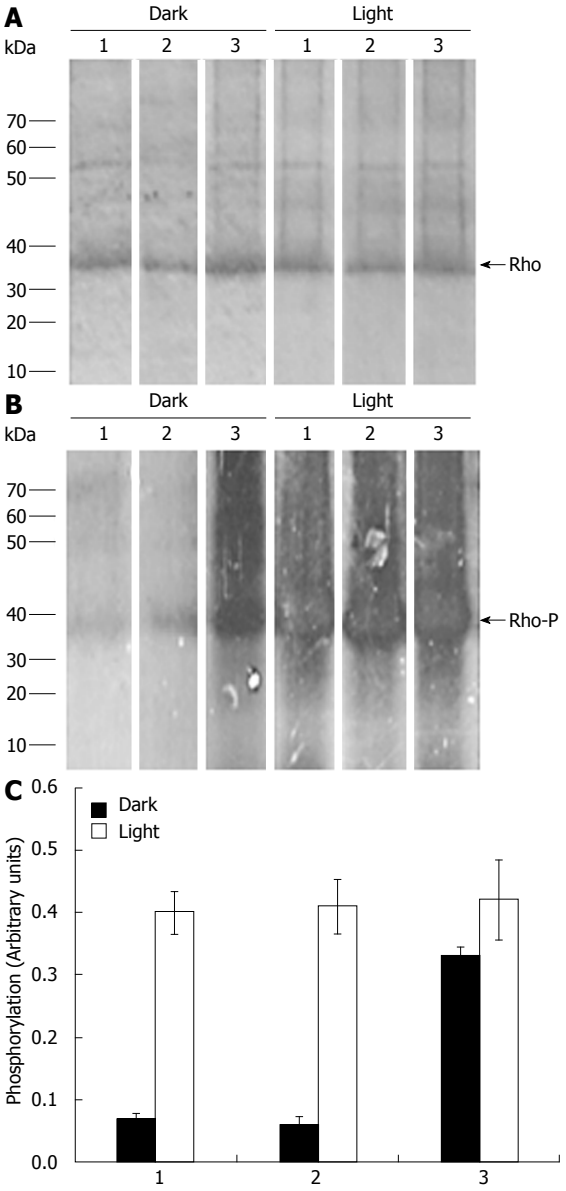

Figure 6 Ability of the reconstituted rhodopsin and rhodopsin analogues to serve as substrates for rhodopsin kinase. A: Coomassie blue staining; B: Autoradiography. Arrows indicate the migration of rhodopsin (Rho) and phosphorylated Rho (Rho-P); C: Densitometry of the autoradiograms shown in B. Dark and white bars correspond to experiments performed under dark and light conditions, respectively. Mean ± SD of three independent experiments are reported. Differences with P-values below 0.05 were considered significant.1: 9-*cis*-Rho; 2: 11-*cis*-Rho; 3: 13-*cis*-Rho.

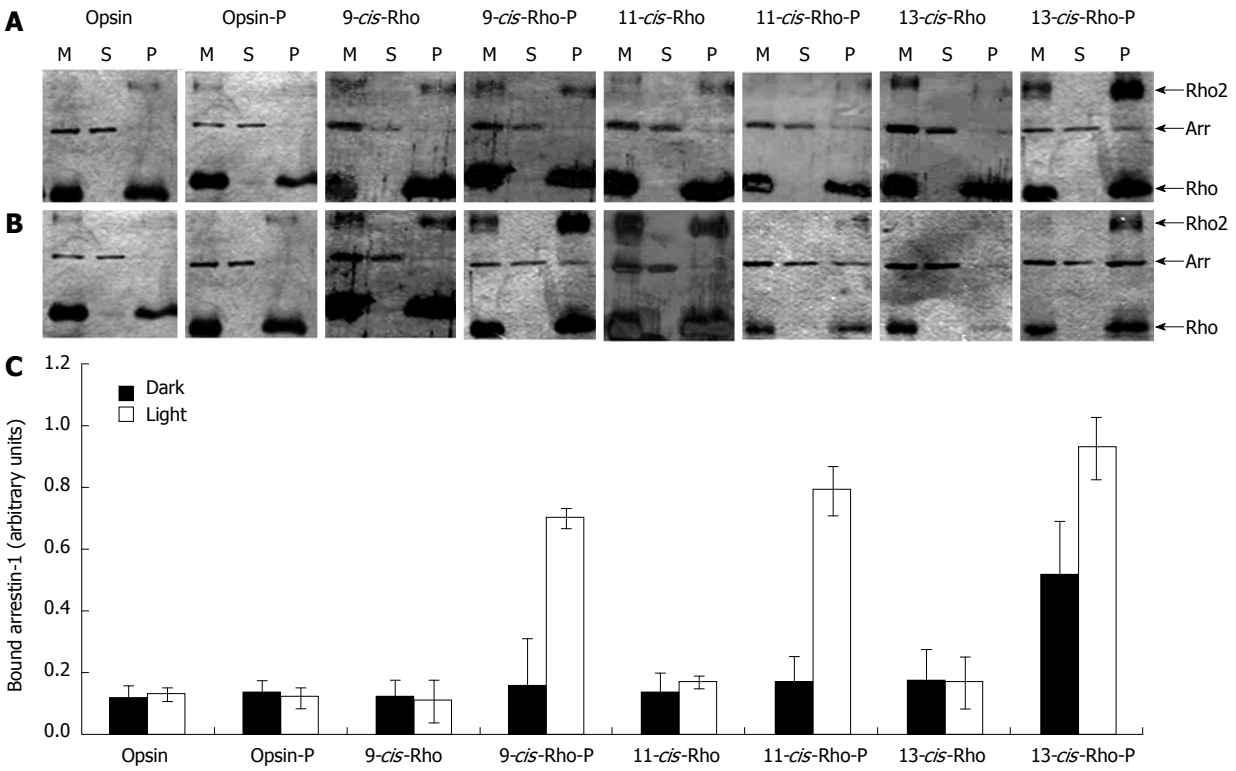

Figure 7 Ability of arrestin-1 to interact with the reconstituted rhodopsin and rhodopsin analogues. Arrestin-1 was combined with membranes containing opsin, phosphorylated opsin (opsin-P), isorhodopsin (9-*cis*-Rho), phosphorylated isorhodopsin (9-*cis*-Rho-P), rhodopsin (11-*cis*-Rho), phosphorylated rhodopsin (11-*cis*-Rho-P), 13-*cis*-retinal-rhodopsin (13-*cis*-Rho), and phosphorylated 13-*cis*-retinal-rhodopsin (13-*cis*-Rho-P), under dark (Panel A) and light (Panel B) conditions. The mixtures (M) were centrifuged and aliquots of each mixture and of the resulting supernatants (S) and pellets (P) were separated by SDS-PAGE. Gels were colored by silver staining. In Panel C, the amount of arrestin-1 that interacted with the phosphorylated pigments in the pellet fraction was quantified by densitometry. Mean ± SD of three independent experiments are reported. Differences with *P*-values < 0.05 were considered significant Arrows indicate the migration of rhodopsin (Rho), arrestin-1 (Arr), and rhodopsin dimers (Rho2).

than rhodopsin[13]. Isorhodopsin, is photosensitive and appears to be very similar to rhodopsin, as determined in numerous *in vitro* studies and experiments using intact retinae and isolated photoreceptors[37,38]. Then, although endogenous 9-*cis*-retinal has not been reported in the retina, the high expression of 9-*cis*-retinol dehydrogenase (RDH4/RDH5) in the retinal pigment epithelium[39,40] suggests that 9-*cis*-retinal could be generated in that tissue. Actually, 9-cis-retinoids do exist in many tissues, with highest concentrations in liver and kidney, and are essential for gene regulation, growth and development[41,42]. In contrast, the 13-*cis* configuration of retinal has never been observed in vision and as such is not physiologically relevant in the visual process. Nevertheless, all-*trans*-retinal is an essential component of type I , or microbial, opsins such as bacteriorhodopsins, channelrhodopsins, sensory rhodopsins and halorhodopsin. Type I opsin genes are found in prokaryotes, algae, and fungi, where they control diverse functions such as phototaxis, energy storage, development, and retinal biosynthesis[43]. Using microbial opsin genes, prokaryotes can transduce light to shift proton gradients, modulate chloride balance, or switch flagellar motor direction, whereas motile algae transduce light to change flagellar beating to direct locomotion toward environments optimally illuminated for their photosynthetic requirements. In these seven-trans-membrane-segment receptor proteins, light causes the all-*trans*-retinal to become 13-*cis*-retinal, which then cycles back to all-*trans*-retinal in the dark state. Unlike the situation with rhodopsin, in which the retinal-protein linkage is hydrolyzed after photoisomerization[44], the activated retinal molecule in type I opsins, 13-*cis*-retinal, does not dissociate from its opsin protein, but thermally reverts to the all-*trans* state while maintaining a covalent bond to its protein partner[45]. Accordingly, 13-*cis*-retinal is physiologically crucial in those organisms that possess type I opsins.

The regular instability of 11-*cis*-retinal limits its commercial availability. The standard procedure used to prepare 11-*cis*-retinal consist of an isomerization reaction of all-*trans*-retinal by irradiation under 436 nm[46-48], which generates a mixture of 9-*cis*-retinal, 11-*cis*-retinal and 13-*cis*-retinal that requires to be separated by chromatography techniques, such as alumina column chromatography, thin-layer chromatography, high-performance liquid chromatography (HPLC), or flash countercurrent chromatography (FCCC). Photochemical and enzymatic processing of retinoids in the eye is essential for perception of the light signal and for sustaining vision by regeneration of visual pigments[12]. Specifically, the photoisomerized all-*trans*-retinal is converted back to the 11-*cis*-retinal chromophore by an enzymatic pathway of chemical

reactions termed the retinoid cycle[11,12]. Why the 9-*cis*- and 13-*cis*-isomers of retinal are not formed in the eye in addition to 11-*cis*-retinal? The retinal G protein-coupled receptor (RGR) is a protein that structurally resembles visual pigments and other G protein-coupled receptors. RGR appears to play a role as a photoisomerase in the production of 11-*cis*-retinal. The proposed function of RGR, in a complex with 11-*cis*-retinol dehydrogenase (RDH5), is to regenerate 11-*cis*-retinal under light conditions[49]. Maeda *et al*[50] evaluated the role of RGR using RGR single knockout mice, and RGR and RDH5 double knockout mice, under various conditions. The most striking phenotype of RGR knockout mice after illumination included light-dependent formation of 9-*cis*- and 13-*cis*-retinoid isomers. These isomers were not formed in wild-type mice because either all-*trans*-retinal is bound to RGR and protected from isomerization to 9-*cis*- or 13-*cis*-retinal or because RGR is able to eliminate these isomers directly or indirectly. These results suggest that RGR and RDH5 are likely to function in the retinoid cycle.

In the present manuscript, we focused on comparing the interactions of rhodopsin and rhodopsin analogues containing 9-*cis*-retinal and 13-*cis*-retinal with other proteins of the visual cascade, such as transducin, rhodopsin kinase and arrestin-1. Under dark conditions, 13-*cis*-retinal-rhodopsin was capable of catalyzing transducin GDP/GTP exchange and was highly phosphorylated by rhodopsin kinase. Since 13-*cis*-retinal-rhodopsin behaves like active rhodopsin independently of light, and given that both transducin activation and phosphorylation by rhodopsin kinase require the generation by photolysis of the meta II intermediate of rhodopsin, we propose that the structure of dark 13-*cis*-retinal-rhodopsin adopts a tridimensional conformation that mimics the active photoproduct of rhodopsin. Moreover, arrestin-1 was also efficiently recognized by phosphorylated 13-*cis*-retinal-rhodopsin in the dark. As shown by Gurevich *et al*[24], arrestin-1 binds phosphorylated light-activated rhodopsin with remarkable selectivity. However, arrestin-1 binding to an equal amount of dark (inactive) phosphorylated rhodopsin or active unphosphorylated rhodopsin (light-activated rhodopsin) is 10-20 times lower, whereas its binding to inactive unphosphorylated rhodopsin is barely detectable[24]. Thus, rhodopsin activation or phosphorylation alone promotes relatively weak arrestin-1 interaction. In addition, arrestin-1 binding to phosphorylated light-activated rhodopsin is many times greater than the sum of dark phosphorylated rhodopsin and light-activated rhodopsin levels, suggesting that the binding mechanism is more sophisticated than a simple cooperative two-site interaction. Gurevich *et al*[24] proposed a model positing that arrestin-1 has two sensor sites, an activation sensor that binds receptor elements that change conformation upon activation, and a phosphate sensor that binds receptor attached phosphates. When the receptor is phosphorylated and active at the same time, both sensors bind. Simultaneous engagement of the two sensor sites allows arrestin-1 transition into the active high affinity receptor-binding state. Since the conformation of dark 13-*cis*-retinal-rhodopsin appears to mimic the structure of the meta II photointermediate, phosphorylated 13-*cis*-retinal-rhodopsin seems to be sufficient to be recognized by arrestin-1 even in the absence of light.

Since the 9-*cis*, 11-*cis*, and 13-*cis* isomers of retinal are not planar, changes at the *cis* configuration in the polyene structure may cause important non-planar distortions in the retinal molecule that in turn may affect its longitudinal size. Employing the molecular orbital program MOPAC (version 1.11), we determined the structures of minimal energy for the various retinal isomers used here. The distances from carbon C-2 to carbon C-15 were found to be 10.84 Å, 10.96 Å, and 11.54 Å for 11-*cis*-retinal, 9-*cis*-retinal, and 13-*cis*-retinal, respectively. The retinal molecule reaches its longest longitude in its all-*trans* configuration (13.02 Å). A clear relationship between the size of each isomer and its accessibility to the chromophore binding pocket in the apoprotein opsin can be established when these theoretical distances were taken in consideration and contrasted with the percentage of pigment that was regenerated with each retinal isomer. 11-*cis*-Retinal and 13-*cis*-retinal, which corresponded to the shortest and longest isomers, showed the highest and lowest percentage of pigment reconstitution, respectively. Thus, it is evident that some size restrictions exist within the prosthetic group binding site. In addition, structural differences may occur when the various retinal isomers are incorporated and accommodated into the apoprotein to reform the distinct pigments.

It is known that the spectral properties of 9-*cis*-retinal, 11-*cis*-retinal, and 13-*cis*-retinal are very similar; all three compounds show absorption maxima at 365-370 nm. Interaction between 11-*cis*-retinal and opsin generates the ground state of rhodopsin with its characteristic peak at about 500 nm. The red shift of 11-*cis*-retinal in rhodopsin is a result of the protonated Schiff base linkage between the aldehyde and the ε-amino group of Lys296, which is stabilized by the Glu113 counter-ion. Moreover, the positive charge is delocalized through the polyene moiety of retinal. Rhodopsin is constrained in an inactive conformation because binding of 11-*cis*-retinal to Lys296 *via* the protonated Schiff base induces changes in rhodopsin' s helical transmembrane domain and cytoplasmic surface that prevent interaction with native transducin, rhodopsin kinase and arrestin-1. Upon photoisomerization of 11-*cis*-retinal to all-*trans*-retinal, the receptor undergoes major structural rearrangements that include displacement of the positively charged Schiff base from its interaction with negatively charged Glu113. Based on this mechanism of action, a bulky ligand might affect and modify the regular distance between the Glu113 counter-ion and the retinal attachment site in the protein, affecting in turn the spectroscopic properties of the regenerated pigment. Blue shifts in isorhodopsin (λ_{max} = 486 nm) and 13-*cis*-retinal-rhodopsin (λ_{max} - 467 nm) correlate well with the increase in longitudinal size of 9-*cis*-retinal and 13-*cis*-retinal compared to 11-*cis*-retinal. During the rho-

dopsin photocycle, the protein relaxes through a series of distinct photointermediates, each with characteristic UV/visible absorption maxima. Most of these intermediates can only be trapped by using ultra freezing temperatures. The metarhodopsin I photointermediate (meta I), which is the inactive precursor of meta II, possesses a characteristic peak at 478 nm. Interestingly, the spectroscopic properties of 13-*cis*-retinal-rhodopsin in the dark were comparable to those of meta I. The resemblance of meta I and the 13-*cis*-retinal-rhodopsin pigment might cause the pseudo-activation state seen for the latter even without illumination. Since meta I can only be generated following freeze-trapping at -40 ℃, and at temperatures below the phase transition temperature of the surrounding lipids, it was not viable for us to carry out a direct comparison between the properties of meta I and the 13-*cis*-retinal-rhodopsin analogue.

Rhodopsin pigment regeneration studies using available retinal isomers showed that stable isomeric pigments can be formed using a diversity of isomers such as 11-*cis*, 9-*cis*, 7-*cis*, 9,13-*dicis*, 7,13-*dicis*, 9,11-*dicis*, 7,11-*dicis*, 7,9-*dicis*, 7,9,11-*tricis*, 7,9,13-*tricis*, *etc*., with varying rates of pigment formation[51]. With the exception of 9-*cis*-retinal, all isomers required much longer times to give isomeric pigments at reduced yields[51]. By using the crystal structures of rhodopsin, Liu *et al*[52] reproduced the binding cavity of rhodopsin containing the 11-*cis*-retinal, and examined whether other isomers were capable of being accommodated within the pocket. When the 9-*cis* and 7-*cis* isomers of retinal were tested it was clear that all atoms of the two isomeric pigment analogs fitted well within the binding cavity. However, when the pigment was replaced with atoms of the 13-*cis* protonated Schiff base, it was clear that the 13-methyl group and partly C13 and C14 of the 13-*cis* chromophore was projected far beyond the binding pocket overlapping with atoms in the β-sheet of the loop that connects the TM4 and TM5 helices[52]. These results confirm that steric restrictions exist in the binding cavity and explain previous reports showing nonbinding of the 13-*cis* or all-*trans* isomers to the inactive state of the protein[16], as well as our results that showed a low percentage of regeneration of the 13-*cis*-retinal-rhodopsin analog. The much reduced rate for pigment formation for the 13-*cis* isomer and other retinal isomers is likely due to the altered ring conformations, the relocated 9-methyl groups, and shifts of the polyene chain.

The C-9 and C-13 methyl groups of the 11-*cis*-retinal appear to be pivotal elements in ligand-receptor communication. For instance, 9- and 13-demethylretinals yielded analogue pigments, but with an increase in constitutive activity and/or much reduced physiological activity[53,54]. Ebrey *et al*[55] observed that 13-demethyl-rhodopsin, which is opsin regenerated with 11-*cis*-13-demethyl-retinal, activated transducin as measured by cGMP-phosphodiesterase PDE6 activity in the dark. This finding was surprising, since 13-demethyl-retinal lacks only the methyl group in position 13. However, the 9-*cis* isomer of 13-demethylretinal like all the other activating pigments required light[55]. When, 11-*cis*-13-demethyl-retinal was preincubated with opsin in the dark, significant phosphorylation was observed[56]. The activity was increased when the all-*trans* isomer was used, but decreased with 9-*cis*-13-demethyl-retinal. The results obtained by Buczyłko *et al*[56] were consistent with the observations of Ebrey *et al*[55]. Deletion of methyl groups to form 9-demethyl and 13-demethyl analogues, as well as addition of a methyl group at C10 or C12, shifted the meta I/meta II equilibrium toward meta I, such that the retinal analogues behaved like partial agonists[54]. To examine the steric limits of the 9-methyl and 13-methyl binding pocket of opsin, deGrip *et al*[57] prepared cyclopropyl and isopropyl derivatives of 11-*cis*- and 9-*cis*-retinal, at C-9 and C-13, and of α-retinal at C-9. Most isopropyl analogues showed very poor binding, whereas most cyclopropyl derivatives exhibit intermediate binding activity. The data of deGrip *et al*[57] were in line with the growing body of evidence showing that the interplay between a receptor and its ligand is very finely tuned. Small modification of a ligand can already alter this interplay and thereby redirect the conformational space of a receptor, leading to a different activity profile. Here we have shown that 13-*cis*-retinal-rhodopsin behaves as a pseudo-active pigment in the dark. Similar to 11-*cis*-13-demethyl-retinal-rhodopsin, the structure of 13-*cis*-retinal-rhodopsin probably embraces a tridimensional conformational fold that mimics to some extent the active meta II photointermediate of rhodopsin. Consequently, 13-*cis*-retinal-rhodopsin is capable of interacting with transducin, rhodopsin kinase and arrestin-1 even without illumination. Palczewski *et al*[58] have also shown that active pseudo-photoproducts, which stimulate transducin activation and opsin phosphorylation by rhodopsin kinase, are formed with opsin and retinal analogues lacking the 13 methyl or the terminal two carbons of the polyene chain as well as with opsin and all-*trans*-retinal. Other reports have also shown that an activated receptor may be generated without illumination by addition of all-*trans*-retinal or its analogues to opsin[56,59-61]. Cohen *et al*[59] found that transducin activation by the all-*trans*-retinal-opsin complex was strongly pH-dependent with the most efficient catalysis at pH = 5-6. Hofmann *et al*[60] demonstrated that free all-*trans*-retinal can react with the apoprotein to form pseudo-photoproducts that are spectrally identical to the photoinduced metarhodopsin species (meta I/II/III). By measuring the increased phosphorylation of opsin by rhodopsin kinase, Buczyłko *et al*[56] showed that the potency of stimulation depended on the chemical and isomeric nature of the analogues and the length of the polyene chain. For example all-*trans*-C17 aldehyde was the most effective in stimulation of opsin phosphorylation, while longer (all-*trans*-retinal) and shorter analogues (all-*trans*-C15 aldehyde) were less potent. All-*trans*-C22 aldehyde was not effective suggesting that the length of this retinoid excluded it from the binding to opsin, while the shortest aldehyde, all-*trans*-C12 aldehyde, was only modestly effective. This specificity suggested a unique inter-

action of opsin with retinoids, rather than a nonspecific lipid-like effect or interaction with peripheral amines[56]. Ligand-free opsin is also capable of activating transducin, although at a much reduced level than light-activated rhodopsin[61,62], but this activity was enhanced by a factor of about 10 by the presence of all-*trans*-retinal. Interestingly, when the sizes of the various isomers of retinal used in the present work were compared, 13-*cis*-retinal was more alike to all-*trans*-retinal than 9-*cis*-retinal or 11-*cis*-retinal.

Various tridimensional conformations of the photoreceptor protein have been solved. Park *et al*[63] reported the X-ray crystal structure of ligand-free native opsin from bovine retinal rod cells. Compared to rhodopsin[64], opsin shows prominent structural changes in the conserved E(D)RY and NPxxY(x)$_{5,6}$F motifs and in the transmembrane fifth to transmembrane seventh regions (TM5-TM7). These structural changes reorganize the empty retinal-binding pocket to disclose two openings that may serve for the entry and exit of retinal. The lack of the interacting prosthetic group causes distinct structural alterations in the retinal-binding pocket. For example, part of the space occupied by the β-ionone ring of retinal is filled in opsin with the side chains of some aromatic residues[63]. In rhodopsin, retinal is held along the polyene chain by amino acids located in TM3, TM6 and loop E2[64]. In opsin, the extracellular part of TM3 and loop E2 are slightly moved away from helices TM5-TM7. Thereby, the retinal-binding pocket becomes wider towards the retinal attachment site in Lys296, and the ε-amino group of Lys296 does not seem to be involved in a salt bridge with Glu113, which corresponds to the retinal Schiff base counter-ion in the rhodopsin dark state, or with Glu181, which was proposed to be part of a complex counter-ion which forms in meta I [65]. Moreover, it has been shown that opsin can readily adopt inactive and active conformations *in vitro*, and low pH and a synthetic peptide derived from the C terminus of the α-subunit of transducin stabilized this active conformation of opsin[66]. Scheerer *et al*[67] reported the crystal structure of the complex between active opsin and the carboxy terminus peptide of the α-subunit of transducin, and clear conformational differences can be detected when the structures of inactive and active opsin are compared. More recently, Choe *et al*[68] used the low pH induced-active conformation of opsin to obtain crystals of meta Ⅱ, by soaking crystals of active opsin with all-*trans*-retinal. They presented the crystal structures of meta Ⅱ alone or in complex with a C-terminal fragment derived from the α-subunit of transducin. The binding site for all-*trans*-retinal appears to be preformed in the active conformation of opsin because the presence of retinal in the meta Ⅱ structures causes only a small adjustment of some amino acid side chains[68], while the Lys296 side chain, which is more flexible in ligand-free opsin[63], becomes ordered due to its linkage with retinal. From the crystal structures of rhodopsin, opsin, activated opsin and meta Ⅱ, it is clear that changes in the prosthetic group binding pocket occur in each of the different conformations of the protein, and receptor can make use of the conformational flexibility of the ligand and the variability of its interaction with the binding site.

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COMMENTS

Background

G protein-coupled receptors activate signaling paths in response to a diverse number of stimuli such as photons, Ca^{2+}, organic odorants, amines, hormones, nucleotides, nucleosides, peptides, lipids and even large proteins. The dim-light photoreceptor rhodopsin is a highly specialized G protein-coupled receptor composed of the apoprotein opsin, and a covalently linked 11-*cis*-retinal chromophore.

Research frontiers

Little is known about the interactions of rhodopsin analogues with other proteins in the visual cascade. Although, it has been reported that the retinal binding site in the inactive state of rhodopsin can accommodate the 7-*cis*, 9-*cis* and 11-*cis* isomers of retinal, but not the longer all-*trans* or 13-*cis* isomers[16], in the present work we were able to reconstitute a rhodopsin analogue containing 13-*cis*-retinal, in addition to photoreceptor proteins containing 9-*cis*-retinal and 11-*cis*-retinal.

Innovations and breakthroughs

This study compared the binding of reconstituted rhodopsin, 9-*cis*-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin to transducin, rhodopsin kinase and arrestin-1, both in the dark and under illumination.

Applications

The rhodopsin analogue containing the 13-*cis* isomer of retinal was capable of activating transducin and was highly phosphorylated by rhodopsin kinase independently of light. Arrestin-1 was also efficiently recognized by phosphorylated 13-*cis*-retinal-rhodopsin in the dark.

Peer review

This manuscript by Araujo *et al* is aimed to study if reconstituted rhodopsin, 9-cis-retinal-rhodopsin and 13-*cis*-retinal-rhodopsin interact with transducin, rhodopsin kinase and arrestin-1. The authors isolated rod outer segments (ROS) from bovine retinas, generated rhodopsin and rhodopsin analogues with the different retinal isomers, purified transducin and arrestin-1 to homogeneity, and obtained an enriched-fraction of rhodopsin kinase by extracting freshly prepared ROS. The authors characterized the reconstituted rhodopsin and rhodopsin analogues through three sets of experiments: activation of transducin, ability to serve as substrates for rhodopsin kinase, and binding to arrestin-1. Different approaches including column chromatography, guanine nucleotide binding assay, *in vitro* phosphorylation, *etc.* were used. They found that rhodopsin analogue harboring the 13-*cis* isomer of retinal is capable of activating transducin in a light-independent way. They concluded that the rhodopsin analogue containing the 13-*cis* isomer of retinal seems to fold in a pseudo-active conformation that mimics the active photointermediate of rhodopsin.

REFERENCES

1 **Bockaert J**, Pin JP. Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J* 1999; **18**: 1723-1729 [PMID: 10202136 DOI: 10.1093/emboj/18.7.1723]
2 **Iiri T**, Farfel Z, Bourne HR. G-protein diseases furnish a model for the turn-on switch. *Nature* 1998; **394**: 35-38 [PMID: 9665125 DOI: 10.1038/27831]
3 **Ovchinnikov YuA.** Rhodopsin and bacteriorhodopsin: structure-function relationships. *FEBS Lett* 1982; **148**: 179-191 [PMID: 6759163 DOI: 10.1016/0014-5793(82)80805-3]
4 **Hargrave PA**, McDowell JH, Curtis DR, Wang JK, Juszczak E, Fong SL, Rao JK, Argos P. The structure of bovine rho-

dopsin. *Biophys Struct Mech* 1983; **9**: 235-244 [PMID: 6342691 DOI: 10.1007/BF00535659]
5 **Sakmar TP**. Rhodopsin: a prototypical G protein-coupled receptor. *Prog Nucleic Acid Res Mol Biol* 1998; **59**: 1-34 [PMID: 9427838 DOI: 10.1016/S0079-6603(08)61027-2]
6 **Rando RR**. Polyenes and vision. *Chem Biol* 1996; **3**: 255-262 [PMID: 8807853 DOI: 10.1016/S1074-5521(96)90105-2]
7 **Yoshizawa T**, Wald G. Pre-lumirhodopsin and the bleaching of visual pigments. *Nature* 1963; **197**: 1279-1286 [PMID: 14002749 DOI: 10.1038/1971279a0]
8 **Pugh EN**, Nikonov S, Lamb TD. Molecular mechanisms of vertebrate photoreceptor light adaptation. *Curr Opin Neurobiol* 1999; **9**: 410-418 [PMID: 10448166 DOI: 10.1016/S0959-4388(99)80062-2]
9 **Krupnick JG**, Gurevich VV, Benovic JL. Mechanism of quenching of phototransduction. Binding competition between arrestin and transducin for phosphorhodopsin. *J Biol Chem* 1997; **272**: 18125-18131 [PMID: 9218446 DOI: 10.1074/jbc.272.29.18125]
10 **Palczewski K**. G protein-coupled receptor rhodopsin. *Annu Rev Biochem* 2006; **75**: 743-767 [PMID: 16756510 DOI: 10.1146/annurev.biochem.75.103004.142743]
11 **Lamb TD**, Pugh EN. Dark adaptation and the retinoid cycle of vision. *Prog Retin Eye Res* 2004; **23**: 307-380 [PMID: 15177205 DOI: 10.1016/j.preteyeres.2004.03.001]
12 **McBee JK**, Palczewski K, Baehr W, Pepperberg DR. Confronting complexity: the interlink of phototransduction and retinoid metabolism in the vertebrate retina. *Prog Retin Eye Res* 2001; **20**: 469-529 [PMID: 11390257 DOI: 10.1016/S1350-9462(01)00002-7]
13 **Fan J**, Rohrer B, Moiseyev G, Ma JX, Crouch RK. Isorhodopsin rather than rhodopsin mediates rod function in RPE65 knock-out mice. *Proc Natl Acad Sci USA* 2003; **100**: 13662-13667 [PMID: 14578454 DOI: 10.1073/pnas.2234461100]
14 **Lou J**, Tan Q, Karnaukhova E, Berova N, Nakanishi K, Crouch RK. Synthetic retinals: convenient probes of rhodopsin and visual transduction process. *Methods Enzymol* 2000; **315**: 219-237 [PMID: 10736705 DOI: 10.1016/S0076-6879(00)15846-X]
15 **Kono M**, Crouch RK. Probing human red cone opsin activity with retinal analogues. *J Nat Prod* 2011; **74**: 391-394 [PMID: 21314100 DOI: 10.1021/np100749j]
16 **Matsumoto H**, Yoshizawa T. Recognition of opsin to the longitudinal length of retinal isomers in the formation of rhodopsin. *Vision Res* 1978; **18**: 607-609 [PMID: 664347 DOI: 10.1016/0042-6989(78)90212-2]
17 **Bubis J**. Effect of detergents and lipids on transducin photoactivation by rhodopsin. *Biol Res* 1998; **31**: 59-71 [PMID: 10347747]
18 **Wald G**, Brown PK. The molar extinction of rhodopsin. *J Gen Physiol* 1953; **37**: 189-200 [PMID: 13109155 DOI: 10.1085/jgp.37.2.189]
19 **Kühn H**. Light- and GTP-regulated interaction of GTPase and other proteins with bovine photoreceptor membranes. *Nature* 1980; **283**: 587-589 [PMID: 6101903 DOI: 10.1038/283587a0]
20 **Bubis J**. Improved purification of transducin subunits from bovine retinal rod outer segments. *Biol Res* 1995; **28**: 291-299 [PMID: 9251760]
21 **Medina R**, Perdomo D, Bubis J. The hydrodynamic properties of dark- and light-activated states of n-dodecyl beta-D-maltoside-solubilized bovine rhodopsin support the dimeric structure of both conformations. *J Biol Chem* 2004; **279**: 39565-39573 [PMID: 15258159 DOI: 10.1074/jbc.M402446200]
22 **Buczyłko J**, Palczewski K. Purification of arrestin from bovine retinas. In: Hargrave PA: photoreceptor cells. San Diego: Academic Press, 1993: 226-236 [DOI: 10.1016/B978-0-12-185279-5.50021-2]
23 **Bubis J**, Ortiz JO, Möller C. Chemical modification of transducin with iodoacetic acid: transducin-alpha carboxymethylated at Cys(347) allows transducin binding to Light-activated rhodopsin but prevents its release in the presence of GTP. *Arch Biochem Biophys* 2001; **395**: 146-157 [PMID: 11697851 DOI: 10.1006/abbi.2001.2550]
24 **Gurevich VV**, Benovic JL. Visual arrestin interaction with rhodopsin. Sequential multisite binding ensures strict selectivity toward light-activated phosphorylated rhodopsin. *J Biol Chem* 1993; **268**: 11628-11638 [PMID: 8505295]
25 **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254 [PMID: 942051 DOI: 10.1016/0003-2697(76)90527-3]
26 **Laemmli UK**. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680-685 [PMID: 5432063 DOI: 10.1038/227680a0]
27 **Towbin H**, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; **76**: 4350-4354 [PMID: 388439 DOI: 10.1073/pnas.76.9.4350]
28 **Litman BJ**. Purification of rhodopsin by concanavalin A affinity chromatography. *Methods Enzymol* 1982; **81**: 150-153 [PMID: 7098858 DOI: 10.1016/S0076-6879(82)81025-2]
29 **Bubis J**, Millan EJ, Martinez R. Identification of guanine nucleotide binding proteins from Trypanosoma cruzi. *Biol Res* 1993; **26**: 177-188 [PMID: 7670530]
30 **Harlow E**, Lane D. Immunizations. In: Antibodies. A Laboratory Manual. New York: Cold Spring Harbor, 1988: 53-137
31 **Hong K**, Knudsen PJ, Hubbell WL. Purification of rhodopsin on hydroxyapatite columns, detergent exchange, and recombination with phospholipids. *Methods Enzymol* 1982; **81**: 144-150 [PMID: 6285125 DOI: 10.1016/S0076-6879(82)81024-0]
32 **Redmond TM**, Yu S, Lee E, Bok D, Hamasaki D, Chen N, Goletz P, Ma JX, Crouch RK, Pfeifer K. Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nat Genet* 1998; **20**: 344-351 [PMID: 9843205 DOI: 10.1038/3813]
33 **Gu SM**, Thompson DA, Srikumari CR, Lorenz B, Finckh U, Nicoletti A, Murthy KR, Rathmann M, Kumaramanickavel G, Denton MJ, Gal A. Mutations in RPE65 cause autosomal recessive childhood-onset severe retinal dystrophy. *Nat Genet* 1997; **17**: 194-197 [PMID: 9326941 DOI: 10.1038/ng1097-194]
34 **Lorenz B**, Gyürüs P, Preising M, Bremser D, Gu S, Andrassi M, Gerth C, Gal A. Early-onset severe rod-cone dystrophy in young children with RPE65 mutations. *Invest Ophthalmol Vis Sci* 2000; **41**: 2735-2742 [PMID: 10937591]
35 **Marlhens F**, Bareil C, Griffoin JM, Zrenner E, Amalric P, Eliaou C, Liu SY, Harris E, Redmond TM, Arnaud B, Claustres M, Hamel CP. Mutations in RPE65 cause Leber's congenital amaurosis. *Nat Genet* 1997; **17**: 139-141 [PMID: 9326927 DOI: 10.1038/ng1097-139]
36 **Thompson DA**, Gyürüs P, Fleischer LL, Bingham EL, McHenry CL, Apfelstedt-Sylla E, Zrenner E, Lorenz B, Richards JE, Jacobson SG, Sieving PA, Gal A. Genetics and phenotypes of RPE65 mutations in inherited retinal degeneration. *Invest Ophthalmol Vis Sci* 2000; **41**: 4293-4299 [PMID: 11095629]
37 **Hubbard R**, Wald G. Cis-trans isomers of vitamin A and retinene in the rhodopsin system. *J Gen Physiol* 1952; **36**: 269-315 [PMID: 13011282 DOI: 10.1085/jgp.36.2.269]
38 **Pepperberg DR**, Brown PK, Lurie M, Dowling JE. Visual pigment and photoreceptor sensitivity in the isolated skate retina. *J Gen Physiol* 1978; **71**: 369-396 [PMID: 660156 DOI: 10.1085/jgp.71.4.369]
39 **Romert A**, Tuvendal P, Simon A, Dencker L, Eriksson U. The identification of a 9-cis retinol dehydrogenase in the mouse embryo reveals a pathway for synthesis of 9-cis retinoic acid. *Proc Natl Acad Sci USA* 1998; **95**: 4404-4409 [PMID: 9539749 DOI: 10.1073/pnas.95.8.4404]
40 **Romert A**, Tuvendal P, Tryggvason K, Dencker L, Eriksson U. Gene structure, expression analysis, and membrane

topology of RDH4. *Exp Cell Res* 2000; **256**: 338-345 [PMID: 10739682 DOI: 10.1006/excr.2000.4817]
41 **Heyman RA**, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C. 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* 1992; **68**: 397-406 [PMID: 1310260 DOI: 10.1016/0092-8674(92)90479-V]
42 **Paik J**, Vogel S, Piantedosi R, Sykes A, Blaner WS, Swisshelm K. 9-cis-retinoids: biosynthesis of 9-cis-retinoic acid. *Biochemistry* 2000; **39**: 8073-8084 [PMID: 10891090 DOI: 10.1021/bi992152g]
43 **Spudich JL**. The multitalented microbial sensory rhodopsins. *Trends Microbiol* 2006; **14**: 480-487 [PMID: 17005405 DOI: 10.1016/j.tim.2006.09.005]
44 **Hofmann KP**, Scheerer P, Hildebrand PW, Choe HW, Park JH, Heck M, Ernst OP. A G protein-coupled receptor at work: the rhodopsin model. *Trends Biochem Sci* 2009; **34**: 540-552 [PMID: 19836958 DOI: 10.1016/j.tibs.2009.07.005]
45 **Haupts U**, Tittor J, Bamberg E, Oesterhelt D. General concept for ion translocation by halobacterial retinal proteins: the isomerization/switch/transfer (IST) model. *Biochemistry* 1997; **36**: 2-7 [PMID: 8993311 DOI: 10.1021/bi962014g]
46 **Shichida Y**, Imai H, Imamoto Y, Fukada Y, Yoshizawa T. Is chicken green-sensitive cone visual pigment a rhodopsin-like pigment? A comparative study of the molecular properties between chicken green and rhodopsin. *Biochemistry* 1994; **33**: 9040-9044 [PMID: 8049204 DOI: 10.1021/bi00197a002]
47 **Matsumoto H**, Nakamura Y, Tachibanaki S, Kawamura S, Hirayama M. Stimulatory effect of cyanidin 3-glycosides on the regeneration of rhodopsin. *J Agric Food Chem* 2003; **51**: 3560-3563 [PMID: 12769524 DOI: 10.1021/jf034132y]
48 **He M**, Du W, Du Q, Zhang Y, Li B, Ke C, Ye Y, Du Q. Isolation of the retinal isomers from the isomerization of all-trans-retinal by flash countercurrent chromatography. *J Chromatogr A* 2013; **1271**: 67-70 [PMID: 23219476 DOI: 10.1016/j.chroma.2012.11.022]
49 **Chen P**, Lee TD, Fong HK. Interaction of 11-cis-retinol dehydrogenase with the chromophore of retinal g protein-coupled receptor opsin. *J Biol Chem* 2001; **276**: 21098-21104 [PMID: 11274198 DOI: 10.1074/jbc.M010441200]
50 **Maeda T**, Van Hooser JP, Driessen CA, Filipek S, Janssen JJ, Palczewski K. Evaluation of the role of the retinal G protein-coupled receptor (RGR) in the vertebrate retina in vivo. *J Neurochem* 2003; **85**: 944-956 [PMID: 12716426 DOI: 10.1046/j.1471-4159.2003.01741.x]
51 **Liu RS,** Matsumoto H, Kini A, Asato AE, Denny M, Kropf A, DeGrip WJ. Seven new hindered isomeric rhodopsins: A reexamination of the stereospecificity of the binding site of bovine opsin. *Tetrahedron* 1984; **40**: 473-482 [DOI: 10.1016/0040-4020(84)85052-8]
52 **Liu RS**, Hammond GS, Mirzadegan T. Possible role of the 11-cis-retinyl conformation in controlling the dual decay processes of excited rhodopsin. *Proc Natl Acad Sci USA* 2005; **102**: 10783-10787 [PMID: 16043701 DOI: 10.1073/pnas.0501665102]
53 **Meyer CK**, Bohme M, Ockenfels A, Gartner W, Hofmann KP, Ernst OP. Signaling states of rhodopsin. Retinal provides a scaffold for activating proton transfer switches. *J Biol Chem* 2000; **275**: 19713-19718 [PMID: 10770924 DOI: 10.1074/jbc.M000603200]
54 **Vogel R**, Lüdeke S, Siebert F, Sakmar TP, Hirshfeld A, Sheves M. Agonists and partial agonists of rhodopsin: retinal polyene methylation affects receptor activation. *Biochemistry* 2006; **45**: 1640-1652 [PMID: 16460011 DOI: 10.1021/bi052196r]
55 **Ebrey T**, Tsuda M, Sassenrath G, West JL, Waddell WH. Light activation of bovine rod phosphodiesterase by non-physiological visual pigments. *FEBS Lett* 1980; **116**: 217-219 [PMID: 6250883 DOI: 10.1016/0014-5793(80)80647-8]
56 **Buczyłko J**, Saari JC, Crouch RK, Palczewski K. Mechanisms of opsin activation. *J Biol Chem* 1996; **271**: 20621-20630 [PMID: 8702809 DOI: 10.1074/jbc.271.34.20621]
57 **deGrip WJ**, Bovee-Geurts PH, Wang Y, Verhoeven MA, Lugtenburg J. Cyclopropyl and isopropyl derivatives of 11-cis and 9-cis retinals at C-9 and C-13: subtle steric differences with major effects on ligand efficacy in rhodopsin. *J Nat Prod* 2011; **74**: 383-390 [PMID: 21309593 DOI: 10.1021/np100744v]
58 **Palczewski K**, Jäger S, Buczyłko J, Crouch RK, Bredberg DL, Hofmann KP, Asson-Batres MA, Saari JC. Rod outer segment retinol dehydrogenase: substrate specificity and role in phototransduction. *Biochemistry* 1994; **33**: 13741-13750 [PMID: 7947785 DOI: 10.1021/bi00250a027]
59 **Cohen GB**, Oprian DD, Robinson PR. Mechanism of activation and inactivation of opsin: role of Glu113 and Lys296. *Biochemistry* 1992; **31**: 12592-12601 [PMID: 1472495 DOI: 10.1021/bi00165a008]
60 **Hofmann KP**, Pulvermüller A, Buczyłko J, Van Hooser P, Palczewski K. The role of arrestin and retinoids in the regeneration pathway of rhodopsin. *J Biol Chem* 1992; **267**: 15701-15706 [PMID: 1386362]
61 **Jäger S**, Palczewski K, Hofmann KP. Opsin/all-trans-retinal complex activates transducin by different mechanisms than photolyzed rhodopsin. *Biochemistry* 1996; **35**: 2901-2908 [PMID: 8608127 DOI: 10.1021/bi9524068]
62 **Surya A**, Foster KW, Knox BE. Transducin activation by the bovine opsin apoprotein. *J Biol Chem* 1995; **270**: 5024-5031 [PMID: 7890610]
63 **Park JH**, Scheerer P, Hofmann KP, Choe HW, Ernst OP. Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature* 2008; **454**: 183-187 [PMID: 18563085 DOI: 10.1038/nature07063]
64 **Palczewski K**, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 2000; **289**: 739-745 [PMID: 10926528 DOI: 10.1126/science.289.5480.739]
65 **Lüdeke S**, Beck M, Yan EC, Sakmar TP, Siebert F, Vogel R. The role of Glu181 in the photoactivation of rhodopsin. *J Mol Biol* 2005; **353**: 345-356 [PMID: 16169009 DOI: 10.1016/j.jmb.2005.08.039]
66 **Vogel R**, Siebert F. Conformations of the active and inactive states of opsin. *J Biol Chem* 2001; **276**: 38487-38493 [PMID: 11502747 DOI: 10.1074/jbc.M105423200]
67 **Scheerer P**, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP, Ernst OP. Crystal structure of opsin in its G-protein-interacting conformation. *Nature* 2008; **455**: 497-502 [PMID: 18818650 DOI: 10.1038/nature07330]
68 **Choe HW**, Kim YJ, Park JH, Morizumi T, Pai EF, Krauss N, Hofmann KP, Scheerer P, Ernst OP. Crystal structure of metarhodopsin II. *Nature* 2011; **471**: 651-655 [PMID: 21389988 DOI: 10.1038/nature09789]

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Contents

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EDITORIAL

Can short-term fasting protect against doxorubicin-induced cardiotoxicity?

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Abstract

Doxorubicin (Dox) is one of the most effective chemotherapeutic agents used in the treatment of several types of cancer. However the use is limited by cardiotoxicity. Despite extensive investigation into the mechanisms of toxicity and preventative strategies, Dox-induced cardiotoxicity still remains a major cause of morbidity and mortality in cancer survivors. Thus, continued research into preventative strategies is vital. Short-term fasting has proven to be cardioprotective against a variety of insults. Despite the potential, only a few studies have been conducted investigating its ability to prevent Dox-induced cardiotoxicity. However, all show proof-of-principle that short-term fasting is cardioprotective against Dox. Fasting affects a plethora of cellular processes making it difficult to discern the mechanism(s) translating fasting to cardioprotection, but may involve suppression of insulin and insulin-like growth factor-1 signaling with stimulated autophagy. It is likely that additional mechanisms also contribute. Importantly, the literature suggests that fasting may enhance the antitumor activity of Dox. Thus, fasting is a regimen that warrants further investigation as a potential strategy to prevent Dox-induced cardiotoxicity. Future research should aim to determine the optimal regimen of fasting, confirmation that this regimen does not interfere with the antitumor properties of Dox, as well as the underlying mechanisms exerting the cardioprotective effects.

Key words: Fasting; Doxorubicin; Cardiotoxicity; Cardioprotection

Core tip: Doxorubicin (Dox)-induced cardiotoxicity remains a significant cause of morbidity and mortality in cancer survivors, despite the intensive investigation of potential protective strategies. Studies have shown that short-term fasting induces cardioprotective effects against Dox-induced injury. Importantly, evidence suggests that fasting may enhance the antitumor effects of Dox. Thus, short-term fasting may be a feasible practice that can easily be incorporated into the treatment plans of cancer patients.

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INTRODUCTION

Doxorubicin (Dox) is one of the most effective chemotherapeutic agents currently used in the treatment of haematological malignancies and solid tumors such as breast cancer. It is a quinone-containing anthracycline antibiotic. Its mechanism of antitumor activity has been shown to

involve binding to topoisomerase-Ⅱ α, thereby resulting in DNA strand breaks and apoptosis[1]. Despite its effectiveness, its use is limited due to cardiotoxicity. In animal models, Dox has been shown to be hepatotoxic as well, but in humans it is the cardiotoxicity which primarily limits its use[2,3]. High cumulative doses of Dox are the most powerful predictor of Dox-induced congestive heart failure[4]. One early study reported that 4% of patients receiving a dose of 500-550 mg/m^2 and 18% and 36% of patients receiving 551 mg/m^2 or higher, respectively, experienced heart failure, which is often refractory to conventional therapy[4,5]. The clinical outcome for these patients is poor[4]. A variety of approaches to prevent cardiotoxicity have been tested, however, their efficacy has been limited[4]. Thus, continued investigation of viable strategies to protect the heart from Dox-induced toxicity is of vital importance.

MECHANISMS OF DOX-INDUCED CARDIOTOXICITY

Mechanisms of Dox-induced cardiotoxicity have not been clearly elucidated, but have been shown to involve oxidative stress, mitochondrial dysfunction, and apoptosis. For example, Dox treatment has been shown to increase mitochondrial depolarization, fission, and ROS production while decreasing the rate of ATP synthesis and content[6-10]. Lipid peroxidation, reduced aconitase activity (a marker of oxidative stress), and alterations in the expression and activity of antioxidant enzymes, such as superoxide dismutase (SOD), are also evident[11,12]. Oxidative stress and mitochondrial dysfunction can induce apoptosis which leads to loss of post-mitotic myocytes and altered cardiac function[8,13,14]. It has long been known that Dox can induce oxidative stress *via* semiquinone redox cycling, however it is unclear if this is the specific mechanism of cardiotoxicity since ROS scavengers failed to prevent cardiac toxicity in several studies[15,16]. Recently, topoisomerase-Ⅱ β has been shown to be a molecular target of Dox in cardiomyocytes[17]. Cardiac myocytes do not express topoisomerase-Ⅱ α, the molecular target in tumor cells[18]. Zhang *et al*[17] demonstrated that cardiomyocyte-specific deletion of topoisomerase-Ⅱ β prevented Dox-induced cardiotoxicity. Furthermore, the deletion prevented Dox-induced DNA damage and transcriptional changes that are responsible for impaired mitochondrial biogenesis, ROS formation, and apoptosis. Thus, the mechanism of Dox-induced cardiotoxicity may involve molecular targeting of topoisomerase-Ⅱ β as well as the potential contribution of semiquinone redox cycling.

PROTECTIVE STRATEGIES AGAINST DOX-INDUCED CARDIOTOXICITY

Currently, available therapies to effectively prevent cardiotoxicity in patients treated with Dox are limited. Thus, the first line of defense is to limit the cumulative dose of Dox. However, lowering cumulative dose may translate to reduced treatment efficacy[19]. Another strategy to protect against cardiotoxicity has been to alter the mode of delivery of Dox, such as encapsulation in liposomes, which aims to target the delivery to the tumor, thereby, reducing plasma concentrations of Dox. The United States Food and Drug Administration has approved one liposomal doxorubicin, Doxil[19]. Shorter-term clinical trials have shown that liposomal doxorubicin can reduce early cardiotoxicity while having the same antineoplastic efficacy as conventional doxorubicin[19]. Although, liposomal doxorubicin has shown promise in reducing cardiotoxicity, currently, it is still mainstream to use conventional Dox. Utilizing antioxidants or iron chelators to reduce Dox-induced oxidative stress has been another tested strategy, but with limited success[19]. Dexrazoxane, an iron chelating agent, has shown the most promise in reducing oxidative stress and cardiotoxicity, however, with some limitations. Most studies have shown that Dexrazoxane is safe, however, some have shown that dexrazoxane may cause myelosuppression and also increase the risk of second malignancies[20,21]. Furthermore, it has been shown that the efficacy of dexrazoxane may vary between sexes, with less benefit in males[19,22]. Despite extensive investigation and numerous tested strategies to prevent cardiotoxicity, success has been limited. Dox-induced cardiotoxicity still remains a major cause of morbidity and mortality in cancer survivors[19]. Thus, exploration of additional strategies to prevent Dox-induced cardiotoxicity is paramount.

A cardioprotective strategy that warrants further exploration is fasting. Fasting and/or caloric restriction (CR) has been shown to protect the heart from a variety of conditions and insults. For example, intermittent fasting protects the heart from ischemic damage and attenuates post-MI cardiac remodeling[23]. Furthermore, calorie restriction has proven protective against coronary artery disease, the process of aging on the cardiovascular system, as well as drug toxicities, including doxorubicin-induced cardiotoxicity[24-27]. Mitra *et al*[26] demonstrated that 40+ days of a 35% calorie restricted diet lead to 100% protection against Dox-induced cardiotoxicity and death while all of the rodents administered with Dox in the *ad libitum* fed group died. However, long term CR regimens, such as this, are not feasible in cancer patients since they typically suffer from malnutrition and other complications. Therefore, short-term fasting may be an alternative approach. Indeed, Raffaghello *et al*[28] reported that 48-60 h of complete fasting prevented organ toxicity induced by chemotherapy in various species of female mice, however, etoposide rather than Dox was used in the study. Kawaguchi *et al*[29] demonstrated that 48 h of complete fasting prior to Dox administration mitigated the Dox-induced impairment in cardiac function in adult GFP-LC-3 transgenic mice, as determined by left ventricular ejection fraction (LVEF), systolic pressure (LVSP), end diastolic pressure (LVEDP), and +dP/dt. Microscopy revealed attenuation

Table 1 Summary of unpublished data

Dependent variable	Control (mean ± SEM)	Dox (mean ± SEM)	*P* value
Aconitase activity (nmol/min per milligram protein)	14.46 ± 3.68	23.74 ± 3.25	0.08
SOD activity (units/mg protein)	0.026 ± 0.003	0.026 ± 0.002	0.904
SOD1 content	1025 ± 110.2	949 ± 91.6	0.603
SOD2 content	275.6 ± 23.25	288.1 ± 23.71	0.715
Procaspase-12 content	36.90 ± 6.14	24.28 ± 4.19	0.1
Procaspase-9 content	28.59 ± 1.57	25.33 ± 3.61	0.5
Procaspase-8 content	68.10 ± 11.90	82.90 ± 0.93	0.34
Caspase-3 activity (arbitrary OD/mg protein)	0.951 ± 0.676	0.490 ± 0.295	0.524
Caspase-9 activity (arbitrary OD/mg protein)	1.084 ± 0.809	0.462 ± 0.255	0.451
FIS1 content	563.6 ± 76.6	474.3 ± 68.8	0.4
DRP1 content	1294.9 ± 109.8	1187.5 ± 73.5	0.421
MFN1 content	5443.5 ± 786.8	4607.8 ± 627.0	0.417
MFN2 content	2001.5 ± 456.8	2053.6 ± 330.2	0.926
OPA1 content	6019.5 ± 739.3	6143.6 ± 601.0	0.897
PINK1 content	3343.0 ± 206.9	3422.0 ± 263.4	0.824
Parkin content	4192.0 ± 1009.0	4157.0 ± 1629.0	0.986
p62 content	1895.7 ± 272.7	1896.7 ± 252.2	0.998

Protein content determined by Western blot (units are "normalized OD"). SOD: Superoxide dismutase.

of LV dilatation, myocardial atrophy, and fibrosis[29]. *In vitro*, a caloric restriction mimetic, 2-deoxyglucose (2-DG), was shown to exhibit cardioprotective properties against Dox using neonatal rat cardiomyocytes isolated from 0-2 d old Harlan Sprague-Dawley rat neonates[30]. Thus, the literature supports that fasting may be an effective regimen to protect against Dox-induced cardiotoxicity.

Unpublished data from our laboratory (Table 1) may also suggest that short-term fasting may provide cardioprotection against Dox. Six-week old male F344 rats were treated with a single injection of Dox (20 mg/kg) or saline. Tissues were harvested for analysis 24-h post injection with the aim of determining the effects of Dox on the mitochondrial dynamics and mitophagy machinery. In order to remove the external variable of Dox-induced anorexia, we fasted both groups of animals upon treatment. Studies have shown that animals treated with Dox reduce their food and water intake by up to 70% for several days[31]. Using this experimental design, the results were unexpected. Dox did not affect any markers of oxidative stress or apoptosis that were assessed in the heart. Dox did not affect aconitase activity, superoxide dismutase (SOD) activity, nor the protein content of cytosolic SOD1 and mitochondrial SOD2. Expression and activation of caspase-12, caspase-9, and caspase-8 were assessed *via* Western analysis, as well as caspase-3 and -9 enzyme activities, and were not affected by Dox. As previously mentioned, the original aim of the study was to investigate the effects of Dox on the mitochondrial dynamics and mitophagy machinery with the hypothesis that Dox treatment would increase the protein content of FIS1 and DRP1 (fission regulators) and decrease the content of MFN1, MFN2 and OPA1 (fusion regulators) thus favoring mitochondrial fission, which is most often associated with oxidative stress, mitochondrial dysfunction and apoptosis[32-34]. Under the current fasting conditions, Dox did not affect the content of any of these primary regulators. Regulators of mitophagy were also assessed. Dox did not affect the content of PINK1, Parkin, or p62 (a marker of mitophagy) under these fasting conditions. We do know that Dox exerted a biological effect in these animals since many of these variables were altered in the liver. Furthermore, the treatment significantly affected the proteome lysine acetylation status in the heart, inducing deacetylation (Figure 1), although the significance of this observation is currently unknown. Because previously published studies have reported that acute Dox treatment does affect many of these variables and processes[8-11,35-37], we believe that complete fasting of the animals in our study may have exerted an unintended cardioprotective effect against the Dox-induced insult. However, further investigation is required to confirm our interpretation of the data. Although this work was done using an acute model of Dox cardiotoxicity, since short-term fasting may be able to protect against the high dose used in the acute model, it is likely that it may also be protective against lower doses used in chronic models of Dox cardiotoxicity which mimics more closely the clinical use of Dox in patients. In summary, short-term fasting may extend similar benefits as longer term CR in regards to cardioprotection against Dox-induced injury.

MECHANISM OF FASTING-INDUCED CARDIOPROTECTION AGAINST DOX TOXICITY

Fasting and caloric deprivation affect a plethora of cellular processes such as mitochondrial dynamics and biogenesis, energy metabolism, oxidative stress, autophagy, and survival signaling pathways, thus making it difficult to discern the mechanism(s) responsible for the cardioprotection[38-42]. Kawaguchi *et al*[29] concluded that the protection against Dox-induced injury extended by 48-h of fasting prior to treatment was due to restoration of autophagy. Autophagy is a conserved process among eukaryotic cells

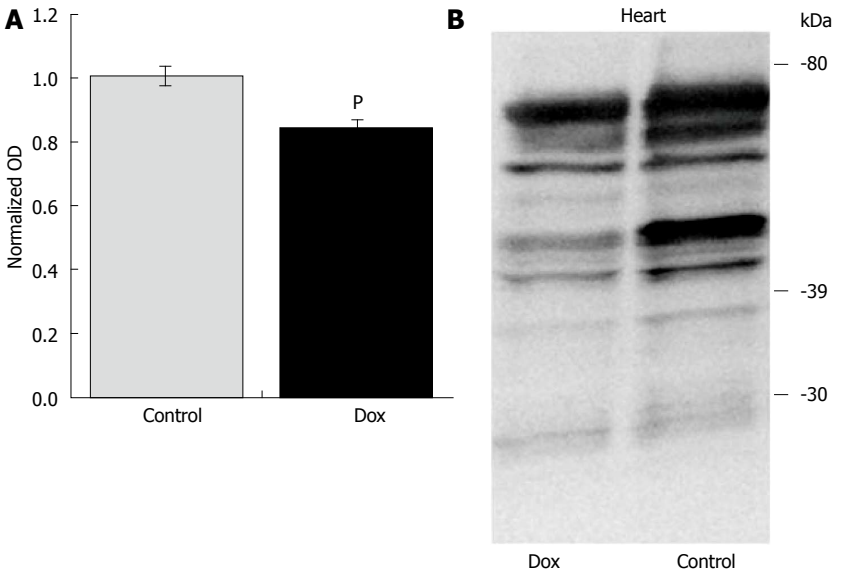

Figure 1 Acute doxorubicin treatment induces proteome lysine deacetylation in the hearts of fasted animals. A: Graphical representation of results; B: Representative Western blot. $P = 0.0016$.

that sequesters cellular material *via* formation of a multi-membrane-bound vacuole, an autophagosome, followed by degradation of the material *via* fusion of the autophagosome with a lysosome[43]. Autophagy can enhance cellular function and survival by degrading damaged or unwanted proteins and organelles such as mitochondria, as well as by modulating apoptosis[44]. Indeed, stimulation of autophagy has been shown to be cardioprotective from a variety of damaging stimuli[44]. Kawaguchi *et al*[29] reported that the inhibition of autophagy by Dox was due to inhibition of AMP-activated protein kinase (AMPK). Prior fasting prevented the Dox-induced inhibition of AMPK. Although fasting was able to reverse the effects of Dox on autophagy, no experimental methods were employed to identify restoration of autophagy as the underlying factor for cardioprotection. Furthermore, no other processes known to be affected by fasting were assessed in the study. Moreover, several studies have shown that stimulation of autophagy contributes to Dox-induced cardiotoxicity and protection is provided *via* inhibition of autophagy[43]. Thus the role of autophagy in Dox-induced cardiotoxicity, whether protective or pathological, is still under question. Therefore, the underlying mechanism(s) of fasting-induced cardioprotection against Dox remains to be determined and is likely due to a combination of mechanisms[30].

EFFECTS OF FASTING ON TUMOR CELL KILLING

It is critical that a potential cardioprotective agent or regimen does not interfere with the goal of cancer treatment. CR has long been shown to have antineoplastic effects. CR can slow the intrinsic rate of aging and prevent the onset of age-related pathologies, including cancer[45,46]. Furthermore, CR mimetics, such as 2-DG, have been shown to inhibit tumor growth[47]. Moreover, 2-DG has been shown to enhance the antitumor efficacy of Dox both *in vitro* and *in vivo*[48,49]. Short-term (48-60 h) fasting was shown to enhance death of cancer cells, prevent organ toxicity, and increase survival in chemotherapy treated mice, however the chemotherapy tested was etoposide, not Dox[28]. Interestingly, Raffaghello *et al*[28] noted that fasting longer than 60 h worsened outcomes. Thus, there may be a window of optimal duration of fasting to maximize beneficial effects. Many of the benefits of fasting and caloric restriction have been shown to be, at least in part, due to decreased circulating levels of insulin and reduced insulin-like growth factor-1 receptor (IGF-1R) signaling[50,51]. Seventy-two hours of fasting reduced circulating IGF-1 by 70% and increased the level of the IFG-1 binding protein (IGFBP) by 11x[52]. Survival time, after Dox treatment, was extended by delaying metastasis of highly aggressive melanoma and prevented Dox-induced toxicity in liver-specific IGF-1-deficient (LID) mice compared to non-LID mice[52]. Ninety days after inoculation with the melanoma cancer cells, all non-LID mice that were treated with Dox had died from either cancer metastases or Dox toxicity. 60% of LID mice treated with Dox were cancer-free with no signs of toxicity[52]. Thus, the evidence supports that fasting may be a safe regimen to use in conjunction with Dox in order to prevent cardiotoxicity.

CONCLUSION

In conclusion, Dox-induced cardiotoxicity remains a significant cause of morbidity and mortality in cancer survivors despite the intensive investigation of potential protective strategies. Studies have shown that short-term fasting induces cardioprotective effects against Dox-induced injury. Importantly, evidence suggests that fasting may enhance the antitumor effects of Dox. It seems that short-term fasting would be a feasible practice that can easily be incorporated into the treatment plans of cancer patients. Thus, short-term fasting is a strategy warranting further exploration. Further studies, both preclinical and clinical, should reveal the optimal regimen of fasting,

confirmation that this regimen does not interfere with the antitumor properties of Dox, as well as the underlying mechanisms exerting the cardioprotective effects.

REFERENCES

1 **Tewey KM**, Rowe TC, Yang L, Halligan BD, Liu LF. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* 1984; **226**: 466-468 [PMID: 6093249 DOI: 10.1126/science.6093249]

2 **Kalender Y**, Yel M, Kalender S. Doxorubicin hepatotoxicity and hepatic free radical metabolism in rats. The effects of vitamin E and catechin. *Toxicology* 2005; **209**: 39-45 [PMID: 15725512 DOI: 10.1016/j.tox.2004.12.003]

3 **Patel N**, Joseph C, Corcoran GB, Ray SD. Silymarin modulates doxorubicin-induced oxidative stress, Bcl-xL and p53 expression while preventing apoptotic and necrotic cell death in the liver. *Toxicol Appl Pharmacol* 2010; **245**: 143-152 [PMID: 20144634 DOI: 10.1016/j.taap.2010.02.002]

4 **Takemura G**, Fujiwara H. Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. *Prog Cardiovasc Dis* 2007; **49**: 330-352 [PMID: 17329180 DOI: 10.1016/j.pcad.2006.10.002]

5 **Lefrak EA**, Pitha J, Rosenheim S, Gottlieb JA. A clinicopathologic analysis of adriamycin cardiotoxicity. *Cancer* 1973; **32**: 302-314 [PMID: 4353012]

6 **Bugger H**, Guzman C, Zechner C, Palmeri M, Russell KS, Russell RR. Uncoupling protein downregulation in doxorubicin-induced heart failure improves mitochondrial coupling but increases reactive oxygen species generation. *Cancer Chemother Pharmacol* 2011; **67**: 1381-1388 [PMID: 20809120 DOI: 10.1007/s00280-010-1441-7]

7 **Danz ED**, Skramsted J, Henry N, Bennett JA, Keller RS. Resveratrol prevents doxorubicin cardiotoxicity through mitochondrial stabilization and the Sirt1 pathway. *Free Radic Biol Med* 2009; **46**: 1589-1597 [PMID: 19303434 DOI: 10.1016/j.freeradbiomed.2009.03.011]

8 **Pointon AV**, Walker TM, Phillips KM, Luo J, Riley J, Zhang SD, Parry JD, Lyon JJ, Marczylo EL, Gant TW. Doxorubicin in vivo rapidly alters expression and translation of myocardial electron transport chain genes, leads to ATP loss and caspase 3 activation. *PLoS One* 2010; **5**: e12733 [PMID: 20856801 DOI: 10.1371/journal.pone.0012733]

9 **Parra V**, Eisner V, Chiong M, Criollo A, Moraga F, Garcia A, Härtel S, Jaimovich E, Zorzano A, Hidalgo C, Lavandero S. Changes in mitochondrial dynamics during ceramide-induced cardiomyocyte early apoptosis. *Cardiovasc Res* 2008; **77**: 387-397 [PMID: 18006463 DOI: 10.1093/cvr/cvm029]

10 **Marechal X**, Montaigne D, Marciniak C, Marchetti P, Hassoun SM, Beauvillain JC, Lancel S, Neviere R. Doxorubicin-induced cardiac dysfunction is attenuated by ciclosporin treatment in mice through improvements in mitochondrial bioenergetics. *Clin Sci* (Lond) 2011; **121**: 405-413 [PMID: 21605084 DOI: 10.1042/CS20110069]

11 **Ascensão A**, Magalhães J, Soares JM, Ferreira R, Neuparth MJ, Marques F, Oliveira PJ, Duarte JA. Moderate endurance training prevents doxorubicin-induced in vivo mitochondriopathy and reduces the development of cardiac apoptosis. *Am J Physiol Heart Circ Physiol* 2005; **289**: H722-H731 [PMID: 15792986 DOI: 10.1152/ajpheart.01249.2004]

12 **Minotti G**, Ronchi R, Salvatorelli E, Menna P, Cairo G. Doxorubicin irreversibly inactivates iron regulatory proteins 1 and 2 in cardiomyocytes: evidence for distinct metabolic pathways and implications for iron-mediated cardiotoxicity of antitumor therapy. *Cancer Res* 2001; **61**: 8422-8428 [PMID: 11731422]

13 **Childs AC**, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C. Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2: Bax ratio. *Cancer Res* 2002; **62**: 4592-4598 [PMID: 12183413]

14 **Ueno M**, Kakinuma Y, Yuhki K, Murakoshi N, Iemitsu M, Miyauchi T, Yamaguchi I. Doxorubicin induces apoptosis by activation of caspase-3 in cultured cardiomyocytes in vitro and rat cardiac ventricles in vivo. *J Pharmacol Sci* 2006; **101**: 151-158 [PMID: 16766856 DOI: 10.1254/jphs.FP0050980]

15 **Myers C**, Bonow R, Palmeri S, Jenkins J, Corden B, Locker G, Doroshow J, Epstein S. A randomized controlled trial assessing the prevention of doxorubicin cardiomyopathy by N-acetylcysteine. *Semin Oncol* 1983; **10**: 53-55 [PMID: 6340204]

16 **Martin E**, Thougaard AV, Grauslund M, Jensen PB, Bjorkling F, Hasinoff BB, Tjørnelund J, Sehested M, Jensen LH. Evaluation of the topoisomerase II-inactive bisdioxopiperazine ICRF-161 as a protectant against doxorubicin-induced cardiomyopathy. *Toxicology* 2009; **255**: 72-79 [PMID: 19010377 DOI: 10.1016/j.tox.2008.10.011]

17 **Zhang S**, Liu X, Bawa-Khalfe T, Lu LS, Lyu YL, Liu LF, Yeh ET. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat Med* 2012; **18**: 1639-1642 [PMID: 23104132 DOI: 10.1038/nm.2919]

18 **Capranico G**, Tinelli S, Austin CA, Fisher ML, Zunino F. Different patterns of gene expression of topoisomerase II isoforms in differentiated tissues during murine development. *Biochim Biophys Acta* 1992; **1132**: 43-48 [PMID: 1380833 DOI: 10.1016/0167-4781(92)90050-A]

19 **Lipshultz SE**, Adams MJ, Colan SD, Constine LS, Herman EH, Hsu DT, Hudson MM, Kremer LC, Landy DC, Miller TL, Oeffinger KC, Rosenthal DN, Sable CA, Sallan SE, Singh GK, Steinberger J, Cochran TR, Wilkinson JD. Long-term cardiovascular toxicity in children, adolescents, and young adults who receive cancer therapy: pathophysiology, course, monitoring, management, prevention, and research directions: a scientific statement from the American Heart Association. *Circulation* 2013; **128**: 1927-1995 [PMID: 24081971 DOI: 10.1161/CIR.0b013e3182a88099]

20 **Tebbi CK**, London WB, Friedman D, Villaluna D, De Alarcon PA, Constine LS, Mendenhall NP, Sposto R, Chauvenet A, Schwartz CL. Dexrazoxane-associated risk for acute myeloid leukemia/myelodysplastic syndrome and other secondary malignancies in pediatric Hodgkin's disease. *J Clin Oncol* 2007; **25**: 493-500 [PMID: 17290056 DOI: 10.1200/JCO.2005.02.3879]

21 **Curran CF**, Narang PK, Reynolds RD. Toxicity profile of dexrazoxane (Zinecard, ICRF-187, ADR-529, NSC-169780), a modulator of doxorubicin cardiotoxicity. *Cancer Treat Rev* 1991; **18**: 241-252 [PMID: 1842575 DOI: 10.1016/0305-7372(91)90015-R]

22 **Lipshultz SE**, Scully RE, Lipsitz SR, Sallan SE, Silverman LB, Miller TL, Barry EV, Asselin BL, Athale U, Clavell LA, Larsen E, Moghrabi A, Samson Y, Michon B, Schorin MA, Cohen HJ, Neuberg DS, Orav EJ, Colan SD. Assessment of dexrazoxane as a cardioprotectant in doxorubicin-treated children with high-risk acute lymphoblastic leukaemia: long-term follow-up of a prospective, randomised, multicentre trial. *Lancet Oncol* 2010; **11**: 950-961 [PMID: 20850381 DOI: 10.1016/S1470-2045(10)70204-7]

23 **Ahmet I**, Wan R, Mattson MP, Lakatta EG, Talan M. Cardioprotection by intermittent fasting in rats. *Circulation* 2005; **112**: 3115-3121 [PMID: 16275865 DOI: 10.1161/CIRCULATIONAHA.105.563817]

24 **Varady KA**, Bhutani S, Church EC, Klempel MC. Short-term modified alternate-day fasting: a novel dietary strategy for weight loss and cardioprotection in obese adults. *Am J Clin Nutr* 2009; **90**: 1138-1143 [PMID: 19793855 DOI: 10.3945/ajcn.2009.28380]

25 **Ahmet I**, Tae HJ, de Cabo R, Lakatta EG, Talan MI. Effects of calorie restriction on cardioprotection and cardiovascular

health. *J Mol Cell Cardiol* 2011; **51**: 263-271 [PMID: 21586294 DOI: 10.1016/j.yjmcc.2011.04.015]
26 **Mitra MS**, Donthamsetty S, White B, Latendresse JR, Mehendale HM. Mechanism of protection of moderately diet restricted rats against doxorubicin-induced acute cardiotoxicity. *Toxicol Appl Pharmacol* 2007; **225**: 90-101 [PMID: 17904602 DOI: 10.1016/j.taap.2007.07.018]
27 **Vucevic D**, Mladenovic D, Ninkovic M, Aleksic V, Stankovic MN, Stankovic M, Jorgacevic B, Vukicevic RJ, Radosavljevic T. The effects of caloric restriction against ethanol-induced oxidative and nitrosative cardiotoxicity and plasma lipids in rats. *Exp Biol Med* (Maywood) 2013; **238**: 1396-1405 [PMID: 24157589 DOI: 10.1177/1535370213506806]
28 **Raffaghello L**, Lee C, Safdie FM, Wei M, Madia F, Bianchi G, Longo VD. Starvation-dependent differential stress resistance protects normal but not cancer cells against high-dose chemotherapy. *Proc Natl Acad Sci USA* 2008; **105**: 8215-8220 [PMID: 18378900 DOI: 10.1073/pnas.0708100105]
29 **Kawaguchi T**, Takemura G, Kanamori H, Takeyama T, Watanabe T, Morishita K, Ogino A, Tsujimoto A, Goto K, Maruyama R, Kawasaki M, Mikami A, Fujiwara T, Fujiwara H, Minatoguchi S. Prior starvation mitigates acute doxorubicin cardiotoxicity through restoration of autophagy in affected cardiomyocytes. *Cardiovasc Res* 2012; **96**: 456-465 [PMID: 22952253 DOI: 10.1093/cvr/cvs282]
30 **Chen K**, Xu X, Kobayashi S, Timm D, Jepperson T, Liang Q. Caloric restriction mimetic 2-deoxyglucose antagonizes doxorubicin-induced cardiomyocyte death by multiple mechanisms. *J Biol Chem* 2011; **286**: 21993-22006 [PMID: 21521688 DOI: 10.1074/jbc.M111.225805]
31 **Gilliam LA**, Ferreira LF, Bruton JD, Moylan JS, Westerblad H, St Clair DK, Reid MB. Doxorubicin acts through tumor necrosis factor receptor subtype 1 to cause dysfunction of murine skeletal muscle. *J Appl Physiol* (1985) 2009; **107**: 1935-1942 [PMID: 19779154 DOI: 10.1152/japplphysiol.00776.2009]
32 **Palmer CS**, Osellame LD, Stojanovski D, Ryan MT. The regulation of mitochondrial morphology: intricate mechanisms and dynamic machinery. *Cell Signal* 2011; **23**: 1534-1545 [PMID: 21683788 DOI: 10.1016/j.cellsig.2011.05.021]
33 **Lee YJ**, Jeong SY, Karbowski M, Smith CL, Youle RJ. Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. *Mol Biol Cell* 2004; **15**: 5001-5011 [PMID: 15356267 DOI: 10.1091/mbc.E04-04-0294]
34 **Chen H**, Chomyn A, Chan DC. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *J Biol Chem* 2005; **280**: 26185-26192 [PMID: 15899901 DOI: 10.1074/jbc.M503062200]
35 **Kavazis AN**, Smuder AJ, Min K, Tümer N, Powers SK. Short-term exercise training protects against doxorubicin-induced cardiac mitochondrial damage independent of HSP72. *Am J Physiol Heart Circ Physiol* 2010; **299**: H1515-H1524 [PMID: 20833957 DOI: 10.1152/ajpheart.00585.2010]
36 **Smuder AJ**, Kavazis AN, Min K, Powers SK. Doxorubicin-induced markers of myocardial autophagic signaling in sedentary and exercise trained animals. *J Appl Physiol* (1985) 2013; **115**: 176-185 [PMID: 23703114 DOI: 10.1152/japplphysiol.00924.2012]
37 **Abd El-Aziz MA**, Othman AI, Amer M, El-Missiry MA. Potential protective role of angiotensin-converting enzyme inhibitors captopril and enalapril against adriamycin-induced acute cardiac and hepatic toxicity in rats. *J Appl Toxicol* 2001; **21**: 469-473 [PMID: 11746193 DOI: 10.1002/jat.782]
38 **Sorensen M**, Sanz A, Gómez J, Pamplona R, Portero-Otín M, Gredilla R, Barja G. Effects of fasting on oxidative stress in rat liver mitochondria. *Free Radic Res* 2006; **40**: 339-347 [PMID: 16517498 DOI: 10.1080/10715760500250182]
39 **Rambold AS**, Kostelecky B, Elia N, Lippincott-Schwartz J. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. *Proc Natl Acad Sci USA* 2011; **108**: 10190-10195 [PMID: 21646527 DOI: 10.1073/pnas.1107402108]
40 **Houtkooper RH**, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. *Nat Rev Mol Cell Biol* 2012; **13**: 225-238 [PMID: 22395773 DOI: 10.1038/nrm3293]
41 **Di Simplicio P**, Rossi R, Falcinelli S, Ceserani R, Formento ML. Antioxidant status in various tissues of the mouse after fasting and swimming stress. *Eur J Appl Physiol Occup Physiol* 1997; **76**: 302-307 [PMID: 9349643 DOI: 10.1007/s004210050252]
42 **Hayashida S**, Arimoto A, Kuramoto Y, Kozako T, Honda S, Shimeno H, Soeda S. Fasting promotes the expression of SIRT1, an NAD+ -dependent protein deacetylase, via activation of PPARalpha in mice. *Mol Cell Biochem* 2010; **339**: 285-292 [PMID: 20148352 DOI: 10.1007/s11010-010-0391-z]
43 **Dirks-Naylor AJ**. The role of autophagy in doxorubicin-induced cardiotoxicity. *Life Sci* 2013; **93**: 913-916 [PMID: 24404586 DOI: 10.1016/j.lfs.2013.10.013]
44 **Gottlieb RA**, Finley KD, Mentzer RM. Cardioprotection requires taking out the trash. *Basic Res Cardiol* 2009; **104**: 169-180 [PMID: 19242643 DOI: 10.1007/s00395-009-0011-9]
45 **Dirks AJ**, Leeuwenburgh C. Caloric restriction in humans: potential pitfalls and health concerns. *Mech Ageing Dev* 2006; **127**: 1-7 [PMID: 16226298 DOI: 10.1016/j.mad.2005.09.001]
46 **Longo VD**, Fontana L. Calorie restriction and cancer prevention: metabolic and molecular mechanisms. *Trends Pharmacol Sci* 2010; **31**: 89-98 [PMID: 20097433 DOI: 10.1016/j.tips.2009.11.004]
47 **Zhang XD**, Deslandes E, Villedieu M, Poulain L, Duval M, Gauduchon P, Schwartz L, Icard P. Effect of 2-deoxy-D-glucose on various malignant cell lines in vitro. *Anticancer Res* 2006; **26**: 3561-3566 [PMID: 17094483]
48 **Zhang F**, Aft RL. Chemosensitizing and cytotoxic effects of 2-deoxy-D-glucose on breast cancer cells. *J Cancer Res Ther* 2009; **5** Suppl 1: S41-S43 [PMID: 20009293 DOI: 10.4103/0973-1482.55140]
49 **Maschek G**, Savaraj N, Priebe W, Braunschweiger P, Hamilton K, Tidmarsh GF, De Young LR, Lampidis TJ. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. *Cancer Res* 2004; **64**: 31-34 [PMID: 14729604 DOI: 10.1158/0008-5472.CAN-03-3294]
50 **Katic M**, Kahn CR. The role of insulin and IGF-1 signaling in longevity. *Cell Mol Life Sci* 2005; **62**: 320-343 [PMID: 15723168 DOI: 10.1007/s00018-004-4297-y]
51 **Sell Ch**. Caloric restriction and insulin-like growth factors in aging and cancer. *Horm Metab Res* 2003; **35**: 705-711 [PMID: 14710349 DOI: 10.1055/s-2004-814156]
52 **Lee C**, Safdie FM, Raffaghello L, Wei M, Madia F, Parrella E, Hwang D, Cohen P, Bianchi G, Longo VD. Reduced levels of IGF-I mediate differential protection of normal and cancer cells in response to fasting and improve chemotherapeutic index. *Cancer Res* 2010; **70**: 1564-1572 [PMID: 20145127 DOI: 10.1158/0008-5472.CAN-09-3228]

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EDITORIAL

Life is more than a computer running DNA software

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Abstract

In his recent interview for the Guardian Craig Venter is elaborating about a household appliance for the future, Digital Biological Converter (DBC). Current prototype, which can produce DNA, is a box attached to the computer which receives DNA sequences over the internet to synthesize DNA; later in future also viruses, proteins, and living cells. This would help the household members to produce, *e.g.*, insulin, virus vaccines or phages that fight antibiotic resistant bacteria. In more distant future, Craig Venter's hope is that the DBC will generate living cells *via* so-called "Universal Recipient Cell". This platform will allow digitally transformed genomes, downloaded from the internet, to form new cells fitted for the particular needs such as therapeutics, food, fuel or cleaning water. In contrast to this, the authors propose that DNA sequences of genomes do not represent 1:1 depictions of unequivocal coding structures such as genes. In light of the variety of epigenetic markings, DNA can store a multitude of further meanings hidden under the superficial grammar of nucleic acid sequences.

Key words: DNA; Genome; Information; Life; Non-coding RNA; Synthetic biology; Virus

Core tip: Craig Venter is elaborating a box attached to a computer that receives DNA sequences over the internet to synthesize DNA. As a leading expert in the field of synthetic biology, he is convinced that "life is a DNA software system", and all living things are reducible to DNA sequences. In contrast to this, the authors propose that DNA sequences of genomes do not represent 1:1 depictions of unequivocal coding structures such as genes. In light of the variety of epigenetic markings, DNA can store a multitude of further meanings hidden under the superficial grammar of nucleic acid sequences.

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INTRODUCTION

In his recent interview for the Guardian[1], Craig Venter is elaborating about a household appliance for the future, the Digital Biological Converter (DBC). The current prototype, which can produce DNA, is a box attached to a computer that receives DNA sequences over the internet to synthesize DNA; in future, it will be able to do the same for viruses, proteins and living cells. This would help the household members to produce, for example, insulin, virus vaccines or phages that fight antibiotic-resistant bacteria. Additionally, it could help future Martian colonists, giving them vaccines, antibiotics or personalized drugs that they would need on Mars. If there should be DNA-based life, a digital version could be transmitted to earth without danger of contaminating the home-planet's biosphere[1]. In the more distant future, Craig Venter's hope is that the DBC will generate living cells *via* the so-called "Universal Recipient Cell". This platform will allow digitally transformed genomes, downloaded from

the internet, to form new cells for particular needs (therapeutics, food, fuel or cleaning water)[1]. The final aim is to establish a "Digital Life Sending Unit" allowing biological teleportation. This unit will sample unknown organisms, perhaps on Mars, then analyse their sequences and generate digital DNA files that will be used by the receiving DBC to re-generate these organisms at new places, such as on Earth.

NOTHING ELSE THAN A SOFTWARE SYSTEM?

Some readers might be reminded of Goethe's Dr Faustus' pact with Mephisto and its goal to create a "homunculus" or similar dreams of living robots that would invade space and time, covered by an abundance of science fiction poetries. But Craig Venter is a leading expert in the field of synthetic biology, in which scientists design new biological systems, *i.e.*, synthetic life. He insists that his insertion of a synthetic bacterial (*Mycoplasma genitalium*) genome into a living recipient bacterium (*Mycoplasma capricolum*) represents the "world's first synthetic life" because the synthetic cell replicated itself into a colony of bacteria, containing only the synthetic DNA.

We do not want to enter here the debate of whether his indisputable achievements represent true creation of new life or represent just some kind of a copy-paste approach. We can also be pretty sure that he and his company will achieve several further technological breakthroughs in the near future. However, we would like to make very clear that his conclusions about the nature of Life are not justified. For example, Craig Venter is convinced that it would be possible, in principle, to synthetically create most complex organisms: "I can't explain consciousness yet, but like anything else it will be explainable at the molecular level, the cellular level and therefore the DNA coding level"[1]. In his view, the question of Erwin Schrödinger What is life? has been answered. "Life is a DNA software system", and all living things are reducible to DNA sequences[1]. The DNA-based software creates as well as directs the more visible "hardware" of life, such as proteins and cells[1-3].

This DNA-centric concept looks clear and straightforward. However, it can work only if the theoretical background on which Craig Venter makes his conclusions is correct. In his view[1-3], organisms are mechanistic apparatus-like molecular structures that work as computing machines according the algorithm-based programs encoded in the DNA storage medium. The syntax structure of DNA follows Francis Crick's central dogma of molecular biology "DNA-RNA-anything else". But is this view coherent with recent empirical knowledge? Are cellular organisms only robot-like computing machines that function strictly according to their algorithm-based programming? Or, rather, are they coordinated complex entities that share bio-communication properties that may vary according to different context-specific needs? Is DNA the unequivocal syntax for sequences out of which one can construct living cells, viruses and phages for a household appliance? Or is the superficial molecular syntax of DNA solely the result of evolution's long inserts and deletions of an abundance of various genetic parasites that shape host genomes? The most crucial questions are: do DNA sequences contain a hidden deep grammar structure that varies according to the meaning and context of environmental insults; do DNA sequences match with high fidelity environmental circumstances that led to epigenetic markings and memory? If yes, this would then mean that the identical DNA sequence may have various-even contradictory-meanings. In fact, this scenario is emerging as true[4-8].

EPIGENETICS: HIDDEN DEEP GRAMMAR

Interestingly, in complex genomes like humans, the coding genes are about 1.5% of the total genome whereas the abundance of non-coding RNAs are about 98.5%. This means Craig Venter's household appliance box could focus only on the 1.5% coding sequences. The DNA sequences of genomes do not represent 1:1 depictions of unequivocal coding structures such as genes, but in light of the variety of epigenetic markings-with its executives RNA editing and alternative splicing-can store a multitude of further meanings[4-8].

This means epigenetic marking saves energy costs like in human language. A limited repertoire of signs, and a limited number of rules to combine these signs correctly, enables signs using agents to generate an unlimited number of sentences with a superficial grammar in the visible text and an abundance of connotations by marking through gestures and other conscious and unconscious bodily expressions such as the movements of three hundred different eye muscles[9].

Are organisms computing machines that fulfil what the DNA program determines? The machine metaphor in molecular biology is an old-fashioned narrative[9] that would like to reduce life to physics and chemistry. Manfred Eigen and Sydney Brenner expanded the concept by adding also information: "Life = physics + chemistry + information"[10,11]. But they defined information according to the mathematical theory of language as used by John von Neumann and Alan Turing in their concept of self-reproducing automata, a chimera that has remained for the last 80 years at a conceptual stage without any functional realization[9,12].

Similar to the algorithm-based computing machines of Turing and von Neumann, Venter's concept of DNA as a software system relies on these computation models. However, these models cannot explain: (1) *de novo* generation of new functional nucleic acid sequences; (2) their context-dependent recombination; and (3) the abundance of mobile regulatory elements being active in all essential processes of life such as replication, transcription, translation, repair and immune defence, all of which are organized by an abundance of small and large RNAs[4-8].

Today, we know that these RNAs predated the emer-

gence of DNA and many of these RNA-world descendants-even RNA viruses-remain as defective parts of genetic parasites in host cellular genomes as exapted and endogenized tools to regulate gene functions[13-17].

RNA-WORLD AGENT ACTIVITIES

Endogenous viruses, transposons, retrotransposons, long terminal repeats, non-long terminal repeats, long interspersed nuclear elements, short interspersed nuclear elements, group I introns, group II introns, phages and plasmids are currently investigated examples that use genomic DNA as their preferred live habitat. This means that DNA is not solely a genetic storage medium that serves as a read and write medium as an evolutionary protocol, but it is also a (quasi-)species-specific ecological niche[4,17]. A great variety of such mobile genetic elements infect, insert, delete, cut and paste, copy and paste and spread within the genome. They change host genetic identities either by insertion, recombination or the epigenetic regulation of genetic content, and co-evolve with the host and interact in a module-like manner. In this respect, they play vital roles in evolutionary and developmental processes. In contrast to accidental point mutations, integration at various preferred sites is not a randomly occurring process but is coherent with the genetic content of the host; otherwise, important protein-coding regions would be damaged, causing disease or even lethal consequences for the host organism[17].

Therefore, DNA organized in chromatin is far more complex than the human-made "software system", except that we are confusing the algorithm-based simulation of real-life storage with the real life, the computer machines with the living cells and organisms, and the self-reproducing automatons with the real-life organisms that can replicate since the origins of life[5,9,14].

Although various complex attempts to simulate early evolution and emergence of life have been accomplished, no complete living cell with all of its components (cell membranes, organelles, microtubules, chromosomes, *etc.*) has yet been engineered. Although hundreds of announcements have been made within the last 60 years, not one of them has been successfully completed.

BIOCOMMUNICATION AND NATURAL GENOME EDITING

The logical alternatives to the concepts of synthetic biology are not "guilty of a kind of modern day vitalism" as suggested by Craig Venter[1,2]. The alternative is the full range of nucleic acid sequence-based life and the agents that are competent to arrange and rearrange DNA information according to their real-life needs. Communication between cells, tissues, organs and organisms cannot be predicted or simulated by computing machines, because biocommunication does not function mechanistically and is not algorithm dependent[18]. The genome itself, *via* natural genome editing[19], generates large amounts of coherent new sequences and inserts these into DNA genomes without damaging essential protein-coding regions. This is not possible for any human-made software. Therefore, despite the bold visions of Craig Venter, it will not be possible to create digital life in the future. The 20th century DNA-based models and concept cannot integrate current empirical data into a coherent picture of how the real life functions. We need new concepts that will be able to integrate all the currently available empirical data on viruses, mobile genetic elements and the abundance of non-coding RNAs most relevant for genome shaping, regulation and evolution[20-26].

CONCLUSION

Despite the theoretical concepts of Turing and von Neumann, and the abundance of announcements of self-reproducing machines, the vision of digital life files that can be used as modules for generating life units will remain on the theoretical stage. The main reason is that the 20th century DNA-based models cannot integrate current empirical data into a coherent picture of how the real life functions: nucleic acid sequences do not represent unequivocal meanings that can be expressed in protein bodies, but depend on context, *i.e.*, epigenetic markings, RNA-editing and alternative splicing that vary according to environmental circumstances, even though the DNA remains identical.

REFERENCES

1 **Corby Z.** Craig Venter: 'This isn't a fantasy look at the future. We are doing the future'. The Guardian/The Observer 13 October, 2013. Available from: URL: http: //www.theguardian.com/science/2013/oct/13/craig-ventner-mars
2 **Venter C.** Life at the Speed of Light. From the Double Helix to the Dawn of Digital Life. New York: Viking, 2013
3 **Roosth S.** The Godfather, Part II. *Science* 2013; **342:** 312-313
4 **Villarreal LP.** Viruses and the Evolution of Life. Washington: ASM Press, 2005
5 **Baluška F**, Witzany G. At the dawn of a new revolution in life sciences. *World J Biol Chem* 2013; **4**: 13-15 [PMID: 23710294 DOI: 10.4331/wjbc.v4.i2.13]
6 **Mattick JS**, Taft RJ, Faulkner GJ. A global view of genomic information--moving beyond the gene and the master regulator. *Trends Genet* 2010; **26**: 21-28 [PMID: 19944475 DOI: 10.1016/j.tig.2009.11.002.Epub]
7 **Mercer TR**, Mattick JS. Understanding the regulatory and transcriptional complexity of the genome through structure. *Genome Res* 2013; **23**: 1081-1088 [PMID: 23817049 DOI: 10.1101/gr.156612.113]
8 **Qureshi IA**, Mehler MF. Emerging roles of non-coding RNAs in brain evolution, development, plasticity and disease. *Nat Rev Neurosci* 2012; **13**: 528-541 [PMID: 22814587 DOI: 10.1038/nrn3234]
9 **Witzany G**, Baluška F. Life's code script does not code itself. The machine metaphor for living organisms is outdated. *EMBO Rep* 2012; **13**: 1054-1056 [PMID: 23146891 DOI: 10.1038/embor.2012.166]
10 **Eigen M.** From Strange Simplicity to Complex Familiarity A Treatise on Matter, Information, Life, and Thought. Oxford: Oxford University Press, 2013
11 **Brenner S**. Turing centenary: Life's code script. *Nature* 2012; **482**: 461 [PMID: 22358811 DOI: 10.1038/482461a]

12 **Witzany G**. Can mathematics explain the evolution of human language? *Commun Integr Biol* 2011; **4**: 516-520 [PMID: 22046452 DOI: 10.4161/cib.4.5.16426.Epub]
13 **Zuckerkandl E**, Cavalli G. Combinatorial epigenetics, "junk DNA", and the evolution of complex organisms. *Gene* 2011; **390:** 232-242 [PMID: 17223284]
14 **Lozada-Chávez I**, Stadler PF, Prohaska SJ. "Hypothesis for the modern RNA world": a pervasive non-coding RNA-based genetic regulation is a prerequisite for the emergence of multicellular complexity. *Orig Life Evol Biosph* 2011; **41**: 587-607 [PMID: 22322874 DOI: 10.1007/s11084-011-9262-1]
15 **Koonin EV**. On the origin of cells and viruses: primordial virus world scenario. *Ann N Y Acad Sci* 2009; **1178**: 47-64 [PMID: 19845627 DOI: 10.1111/j.1749-6632.2009.04992.x]
16 **Baluska F**. Cell-cell channels, viruses, and evolution: via infection, parasitism, and symbiosis toward higher levels of biological complexity. *Ann N Y Acad Sci* 2009; **1178**: 106-119 [PMID: 19845631 DOI: 10.1111/j.1749-6632.2009.04995.x]
17 **Shapiro JA.** Evolution: A view from the 21st century. Washington: FT Press
18 **Witzany G.** Biocommunication and Natural Genome Editing. Dortrecht: Springer, 2010
19 **Witzany G, editor.** Natural Genetic Engineering and Natural Genome Editing. Cambridge: Wiley & Sons, 2010
20 **Brosius J**. The contribution of RNAs and retroposition to evolutionary novelties. *Genetica* 2003; **118**: 99-116 [PMID: 12868601]
21 **Venner S**, Feschotte C, Biémont C. Dynamics of transposable elements: towards a community ecology of the genome. *Trends Genet* 2009; **25**: 317-323 [PMID: 19540613 DOI: 10.1016/j.tig.2009.05.003.Epub]
22 **Witzany G.** From Molecular Entities to Competent Agents: Viral Infection-derived Consortia act as Natural Genetic Engineers. In: Witzany G, editor. Viruses: Essential Agents of Life. Dortrecht: Springer, 2012: 407-419
23 **Slotkin RK**, Martienssen R. Transposable elements and the epigenetic regulation of the genome. *Nat Rev Genet* 2007; **8**: 272-285 [PMID: 17363976 DOI: 10.1038/nrg2072]
24 **Villarreal LP**. Viral ancestors of antiviral systems. *Viruses* 2011; **3**: 1933-1958 [PMID: 22069523 DOI: 10.3390/v3101933.Epub]
25 **Baluška F.** Evolution in revolution. Paradigm shift in our understanding of life and biological evolution. *Commun Integr Biol* 2011; **4:** 521-523 [DOI: 10.4161/cib.4.5.17702]
26 **Witzany G**. The agents of natural genome editing. *J Mol Cell Biol* 2011; **3**: 181-189 [PMID: 21459884 DOI: 10.1093/jmcb/mjr005.Epub]

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FIELD OF VISION

Pragmatic turn in biology: From biological molecules to genetic content operators

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Abstract

Erwin Schrödinger's question "What is life?" received the answer for decades of "physics + chemistry". The concepts of Alain Turing and John von Neumann introduced a third term: "information". This led to the understanding of nucleic acid sequences as a natural code. Manfred Eigen adapted the concept of Hammings "sequence space". Similar to Hilbert space, in which every ontological entity could be defined by an unequivocal point in a mathematical axiomatic system, in the abstract "sequence space" concept each point represents a unique syntactic structure and the value of their separation represents their dissimilarity. In this concept molecular features of the genetic code evolve by means of self-organisation of matter. Biological selection determines the fittest types among varieties of replication errors of quasi-species. The quasi-species concept dominated evolution theory for many decades. In contrast to this, recent empirical data on the evolution of DNA and its forerunners, the RNA-world and viruses indicate cooperative agent-based interactions. Group behaviour of quasi-species consortia constitute de novo and arrange available genetic content for adaptational purposes within real-life contexts that determine epigenetic markings. This review focuses on some fundamental changes in biology, discarding its traditional status as a subdiscipline of physics and chemistry.

Key words: Life; Information; Quasi-species; Incompleteness theorem; Genetic content operators

Core tip: Meaning in natural languages/codes and communication is context dependent. In contrast, artificial formalizable (algorithm based) languages employ a "universal" syntax in order to determine meaning independent of the contextual circumstances. It is empirically evident that no natural language speaks itself as no natural code codes itself. It always requires living agents that share a competence to generate and interpret these natural codes. Therefore I suppose that changes in the genetic code, which are of evolutionary relevance, are rather the result of fine-tuned processes by a large network of mobile genetic elements, persistent viruses, its defectives and other genetic parasites that alter DNA sequences. In this respect DNA remains as ecosphere habitat for social interacting RNA inhabitants. This represents a pragmatic turn in biology from syntax centered molecular biology to pragmatics centered agents interactions.

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INTRODUCTION

Sydney Brenner recently described the radical revolution in life sciences in the early 1950s: the occupation of biology by quantum mechanics examining the fundamental questions of matter and energy followed by the rise of genetics that showed that chromosomes were the carriers of genes. The discovery of the double helix resulted in

the new paradigm that information is physically embodied in DNA sequences of four different types[1]. In contrast to the years before 1953, the question of "information" now became central: the components of DNA are simple chemicals, but the biological complexity that can be generated by the information of different sequences is revolutionary. The fundamental concept that integrated this new biological "information" with matter and energy was enshrined in the universal Turing machine and von Neumann's self-reproducing machines[2-4]. Consequently it follows that biology is physics with computation[5]. This was the core paradigm of molecular biology for almost the next half-century. The crucial step in the serious discussion of "information" as an essential part of definitions of "life" was taken by Manfred Eigen.

MANFRED EIGEN COMBINES PHYSICS, CHEMISTRY, MATHEMATICS AND INFORMATION THEORY

In a series of articles and books Manfred Eigen developed a model of how the essential features of life and its inherent complexity can be explained by physical properties of matter[6,7]. If certain chemical properties exist on a planet and certain physical conditions obtain, life will start by self-reproducing macromolecular cycles which act in a complementary way. On the one hand there are "information"-carrying nucleic acids which build a reproductive cycle. On the other there are functional amino acids which build the protein bodies. Both code-systems together can build a catalytic "hypercycle" which is the basis of the self-reproductivity of life.

Both parts can be reconstructed physically. Nucleic acids (information) and proteins (function) represent a closed system, because there is no function without information, and information gets meaning from function. "Mutations" are replication errors with selective advantage, *i.e.*, instabilities in this system represent irreversible thermodynamic processes. A series of such mutations in nucleic acid sequences leads to quasi-species that are mutant distributions of primitive replicating entities. Such dynamic distributions of genomes that share genetic variation, competition and selection generate the fittest types ("master copies") and therefore avoid "error thresholds", *i.e.*, excessively high mutation rates, in that information cannot further reproduce. The resulting evolution of life is an optimising process in that Darwinian selection evaluates the fittest results of mutations[8,9].

Manfred Eigen adapted the concept of Hammings "sequence space" to explain hypercycle concept by physical properties of matter. Similar to Hilbert space, in which every ontological entity could be defined by an unequivocal point in a mathematical axiomatic system, in the abstract , information space" concept each point represents a unique syntactic structure and the value of their separation represents their dissimilarity. In this concept molecular features of the genetic code evolve by means of self-organisation of matter. Each point in the sequence space can be occupied by one of four different nucleotides. But each point can also be represented by digital computation (1 and 0)[7].

BIOLINGUISTICS, BIOINFORMATICS, SYSTEMS BIOLOGY AND SYNTHETIC BIOLOGY

A series of varieties of mathematical theories of language emerged such as Biolinguistics, Bioinformatics, and systems biology. They all interpret and investigate genetic structures in the light of linguistic categories as quantifiable sets of signs[10-12] and use statistical methods and algorithms to identify genetic sequence orders.

An emerging hybrid of information-theoretical aspects of nucleic acid language is synthetic biology. Its theoretical assumptions clearly derive from systems biology and information theory and generally from a mathematical theory of language. Proponents of synthetic biology want to deconstruct complex biological systems into its parts and artificially reconstruct and even evolve biological systems[13]. This kind of artificial molecular design could serve as an appropriate tool in genetic engineering for, *e.g.*, new vaccines, immune functions, *etc.* This rather mechanistic concept depends on syntax structure identification that represents meaning/function. The context-dependent epigenetic imprinting which represents a deep grammar hidden in the superficial grammar of nucleic acid sequences is not the focus of synthetic biology approaches. In contrast to predominant genetic engineering synthetic biology tries to construct complex biological systems which are then subject to selection processes. They are expected to be mutation-resistant in a certain sense.

UNEXPECTED EARTHQUAKE IN THE FOUNDATIONS OF MATHEMATICS

The original mainstream assumptions regarding the several mathematical theories of language are still present in concepts, curricula and even the definition of life and animated nature[14,15]. The conviction of an exact science based on exact definitions of scientific sentences in contrast to non-scientific ones is at the basis of scientific communities and their self-understanding[16].

The history of science or even sociology of knowledge evidences the interesting fact that it is still largely ignored that 50 years ago the basis of this world view was shaken to the core. The belief that mathematics was the best tool for depicting the physical reality of matter and natural laws marginalised world views other than mathematical ones[16-19].

In his Unvollständigkeitssatz (incompleteness theorem) Manfred Gödel investigated a formal system converting a meta-theoretical statements into an arithmetical one[20]. He strove to convert the statements formulated in a meta-language into the object language S. This led

Gödel to two prominent and critical conclusions: (1) If system S is consistent, then it will contain at least one formally indeterminable sentence. This means that onev sentence is inevitably present that can be neither proved nor disproved within the system; and (2) If system S is consistent, then this consistency of S cannot be proved within S.

The consequence of the incompleteness theorem for the automaton theory of Turing and von Neumann was significant: a machine can principally calculate only those functions for which an algorithm can be provided. Sign-mediated interactions between living organisms in which the meaning of the signs depends on real life circumstances relay on non-formalizable sequence generation, for which no algorithm can be provided. Essential functions of every natural language, such as non-formalizable features are not object of algorithm based calculations. Living organisms are no machines[21].

PRAGMATIC TURN IN BIOLOGY: NATURAL GENETIC CONTENT OPERATORS EDIT GENOMES

Manfred Eigen's concept of natural languages/codes and the current concepts embraced by bioinformatics, biolinguistics, systems biology and synthetic biology are not coherent with current knowledge about key features of natural languages or codes, *i.e.*, the three levels of rules that govern natural code use by competent code-using groups: combinatorial rules (syntax), contextual rules (semantics) and context-dependent rules (pragmatics). In all mathematical theories of language the syntax determines semantics (function), but in natural codes pragmatics (context) determines semantics. Pragmatic rules do not exist in Eigen's concept. Natural code-inherent rules are absent in abiotic matter that is determined strictly by natural laws: no syntax, pragmatic or semantic rules are present if water freezes to ice. Therefore the explanation of the evolution of biological macromolecules in Eigen's concept as well as in other mathematical theories of language cannot explain the evolution of natural codes and its inherent rules[22-25].

RNAS THAT ORGANIZE GENETIC CONTENT COMPOSITION

The change from a read-only-memory genome with copying errors to a read-and-write genome with active change operators is fundamental. In contrast to the decades long assumption that the driving forces of evolution were chance mutation (statistical replication errors) and selection it is now recognised that although mutation is an empirical fact it does not contribute very much to genetic novelty. Key roles now act as non-random genetic change operators in the production of complex evolutionary inventions[26-28].

Now we can investigate several key players that organise the genetic content compositions of host organisms such as, *e.g.*, endogenous viruses and its defectives, transposons, retrotransposons, LTRs (long terminal repeats), non-LTRs (non-long terminal repeats), LINEs (long interspersed nuclear elements), SINEs (short interspersed nuclear elements), ALUs, group I introns, group II introns, phages, plasmids[29-31]. We now recognize that DNA is not solely a genetic storage medium but is also a kind of ecological habitat. Many of such mobile genetic elements have been found within the last 40 years as inhabitants of all genomes[32-35]. Some cut and paste, others copy and paste and both spread within the genome. They modify host genetic identities through insertion, recombination, or the epigenetic regulation of genetic content. They co-evolve with the host, interact in a modular manner and additionally generate highly adaptive immune systems for host organisms from the simplest prokaryotes (CRISPRs/Cas system) to the most complex eukaryotes (VJD-Systems). Such mobile genetic elements shape both genome architecture and regulation. Therefore they are agents of change not only over evolutionary time but also in real time as domesticated agents[36-38].

FROM MOLECULAR BIOLOGICAL ENTITIES TO SOCIAL GROUPS

The question arises how these RNA populations, it's closely related RNA viruses and their complex interactions can be explained and understood without mathematical theories of language. How should we investigate non-coding RNA interactions, competencies and even their role in epigenetic imprinting without formalisable tools? This world of life processes is dominated by RNA, whereas DNA remains a habitat, an ecosphere of interacting RNAs that behave like inhabitants and as genetic information storage[39-43].

All these these RNAs, share a secondary structure like a hairpin, or a stem-loop. In more complex ligated consortia of such stem loops we can look at tRNAs, or ribosomal subunits, RNA polymerases or a great variety of RNA viruses and its defectives as listed above. The RNA stem loops have two characteristic parts: stems that consist of base-paired nucleic acids and loops, bulges and junctions that consist of unpaired regions limited by stems. Most interesting from an evolutionary perspective are two recently found key features[44-46]: (1) Randomly associated RNAs that have no evolutionary history show the same structure-dependent compositional bias as ribosomal RNAs. This means that the differences do not depend on selection processes but on the overall composition of the RNA consortium; and (2) The singular RNA stem loop behaves like a random assembly of nucleotides without selective forces and underlies physico-chemical laws exclsusively Only if stem loops build groups, they share a culture of interactional patterns and a history of defined timescales, *i.e.*, they underlie biological selective forces.

This looks like the true split of life and non-life processes. To better understand behavioral motifs of RNA

stem-loop swarms and viruses, one should add group membership features that are absent in the inanimate world. The basic tool of such RNAs is their complementary composition of base-pairing stems and not base-pairing loops, the result of an inherent property of RNA chemicals, the foldback of polyRNAs. The variety of regulations on protein coding genes as well as the processing of these regulatory RNAs by phases of splicing and editing RNA transcripts makes its algorithm based predictability nearly impossible because of its complexity[39].

These populations of RNAs share properties with RNA viruses, which have defined capabilities. In contrast to DNA viruses RNA viruses have much smaller genomes on RNA bases without proofreading and repair. In contrast to the previous perspective (mutation, *i.e.,* replication error) the new perspective assembles the property of invention of new sequence contents, *de novo*, that have not existed before and for which no algorithms are available in principle. This is important for variation and innovation, as well as infection, immunity and identity, for both diversified viral and cellular populations[47-50].

RNA STEM LOOP STRUCTURES CONSTITUTE LIFE

This change in perspective from molecules to agent-based behaviour will look at interactions of RNA viruses, DNA viruses, RNA-DNA viruses, viral swarms, and sub-viral groups like any ligated RNA stem loop groups that cooperate and coordinate (regulate) within cellular genomes as replication-relevant co-players[51-53]. Or they interact as suppression-relevant silencers or as infection-derived modular tools of non-coding RNAs that have built consortia of complementary agents that function together such as retrovirus-derived remnants, such as LTRs, non-LTRs, group Ⅱ introns, rRNAs, tRNAs, spliceosomes, editosomes, and other counterbalancing modules[54-58]. Such populations determine regulations in many ways and may newly adapt different functions. The use of a natural language or code depends on consortia of living agents, because natural languages and codes function according to rules. In contrast to the inevitability of natural laws rule-following is a feature of social interaction and not solely one of physico-chemical necessity[58].

Investigating syntactic sequences without knowing something about the real-life context of code-using agents is senseless because syntactic structures do not represent unequivocally semantic meaning. Quantifiable analyses of signs, words and sequences cannot extract meaning. Only in a restricted (statistical) sense this is possible through sequence comparison.

EVOLUTIONARY GENETIC INVENTION IS NOT THE RESULT OF REPLICATION ERRORS

The virosphere in particular exemplifies how genetic innovation derives from novel nucleic acid sequences and their combination[59]. If cells are infected by more than one virus, the genomes of different viruses are copackaged into the viral progeny. During reverse transcription the reverse transcriptase switches between two or more templates, generating a new DNA sequence[60]. Similar sequence generations are known in various co-infection events such as the combination of external RNA viruses and persistent endogenous retrovirus, infectious RNA viruses with former viruses, retaining defective parts which can be combined into new sequence orders of still functioning viruses[52,53]. Interestingly, not only viruses generate de novo, or combine and recombine sequences. With this innovation competence quasispecies-consortia (qs-c) transfer this adaptive principle also to all forms of cellular life. The defective parts of infectious genetic parasites represent an abundance of appropriate tools for cellular needs, documented in the variety of non-coding RNAs which are essential actors in all stages of cellular life such as transcription, translation, repair, recombination and immune functions[60-65].

REMEMBER GÖDEL: NATURAL CODES ARE OPEN "SYSTEMS"

RNA group membership can be described by its various features. But this membership can never be completely specified, since it can always be further parasitised by unknown and even unpredictable parasites. This essential feature renders the ability to specify membership absolutely impossible. Additionally this means absolute immunity in this open "system" is impossible in principle. This "insecurity" provides the inherent capacity for novelty, that is, the precondition for greater complexity. It seems we are here at the core competence of variation the essential feature for biological selection.

How do agents emerge from ribozymes to form identity of replicators and then form groups that learn membership? The emergence of single RNA stem-loops solely depends on physico-chemical properties. As mentioned above, if stem-loop groups build complex consortia biological selection and social interactions emerge that are not present in a purely chemical world[44-46]. This looks coherent with the results of sociology and the evolution of natural languages. Natural languages and codes depend on competent agents that followsemiotic (syntactic, pragmatic, semantic) rules, and rule-following are social interactions. This means one agent alone cannot generate or follow a rule. Evolution of identity implies emergence of self/non-self differentiation competence. This is a crucial step from single RNA stem loops to RNA stem loop groups[28,36,45,46].

RNA GROUP BUILDING: CONTEXT DETERMINES MEANING

If we apply some interactional motifs of RNA agents

to form biotic structures that follow biological selection processes and not mere physico-chemical reaction patterns we must also look at the group-building of RNA stem-loop structures.

As previously mentioned it has been found that single stem loops react in a purely physico-chemical reaction mode without selective forces, regardless of whether they derived randomly or are constructed under in vitro conditions[39,46]. Conversely, if these single RNA stem loops build groups they overrule pure physico-chemical reaction patterns and emerge as biological selection forces: biological identities of self/non-self recognition and preclusion, immune functions, dynamically changing (adapting) membership roles. A single alteration in a base-pairing RNA stem that leads to a new bulge may dynamically alter not only a single stem loop but the whole group identity from which this stem loop containing the newly emerged bulge derives[39,46].

Simple self-ligating RNA stem loops can build much larger groups of RNA stem loops that serve to increase complexity[66]. This may lead to ribozymatic consortia, which later on build success stories, such as the merger of the two subunits of transfer RNAs or RNA-dependent RNA-polymerases for replication of RNA through RNA or the subunits of ribosomal RNAs, all of them being former groups that evolved and functioned for different reasons than those applicable to subsequent conserved modes[67-69].

If RNA fragments self-ligate into self-replicating ribozymes they constitute networks. For example, three-membered networks represent highly cooperative growth behavior. If such networks compete directly with selfish autocatalytic cycles, the former grow faster. This clearly indicates the ability of RNA populations to evolve into higher complexity through cooperation which clearly outruns selfishness[46].

Another intriguing example of the biological (selective) group-building competence of RNA stem loop consortia is the chemical interaction based on the molecular syntax in stem-loop "kissing", in that single-stranded regions of RNA stem loops bind according to Chargaff rules to other single-stranded stem loop structures to unite and build more complex group identities for several functions, such as dimerisation of genomic RNA in viruses, *e.g.*, HIV 1. Such complementary interactions are also important in RNA replication of the hepatitis C virus[70-72].

Complex three-dimensional structures can be built by consortia of single RNA sequence strings. One of the most interesting structures is the pseudoknot composed of two helical segments connected by single-stranded regions or loops. Bases in the single-stranded loop are base-pairings with bases outside the loop. This interaction pattern clearly depends on the rules of molecular syntax but is initiated for adaptational purposes by different ecosphere habitat dynamics. So the results of these interactions may lead to structurally diverse groups with important different biological roles such as the catalytic core of key players of the present RNA world, *i.e.*, ribozymes, self-splicing introns, telomerase and its context-dependent altering gene expression by inducing ribosomal frameshifting in several viruses[73-75].

Most interestingly, the base-pairing in pseudoknots is strictly context-sensitive and base-pairs overlap with one another in sequence positions. This leads to the limits of algorithm-based prediction models such as dynamic programming or stochastic context-free grammars. This indicates the natural language nature of nucleic acid code3aw which represents the possibility of coherent de novo generation and context-dependent alterations for a diversity of different meanings (functions) for the same syntax structures.

CONCLUSION

How long will biology remain a subdiscipline of physics and chemistry? As I have tried to demonstrate, the investigation methods of natural languages/codes such as the genetic code (in terms of both its superficial syntax and the deep grammar hidden as a result of epigenetic imprintings) in the light of mathematical theories of language and its derivatives such as biolinguistics, bioinformatics, systems biology and synthetic biology can lead to quantifiable, *i.e.*, statistical, results which can be compared, measured and computed. The question remains whether it is sensible, to measure, investigate and compare the wavelength and modulations of phonetic utterances of humans to extract a meaning? Can we extract semantics from investigations of certain features of syntax structure?

In natural languages/codes it is not the structure of syntax that determines the meaning of sequences. In nearly all cases it is the hidden deep grammar which determines meaning for the recipient of the message. The deep grammar depends on how the superficial syntax is marked: in the genetic code by epigenetic imprintings or in sign sequences of utterances by gestures and emphasis. In all cases the hidden deep grammar decides whether a competent recipient can understand the intended meaning of the sender or not.

The real-life world in which natural sign users are included decides the meaning of a natural language or code, not the in vitro experimental set ups, the universal grammar or similar algorithm-based components. In contrast with previous approaches the real action between interactors determines what signs of communication and coordination are used to express what should be transported, what is intended, and what is focused. The real actions are the driving force of content and represent the context which determines the meaning of thoughts and interpretations. Therefore pragmatics is of essential relevance to identify the meaning of natural languages/codes, not syntax or semantics.

This aspect is missing completely in Eigens concept of a sequence space in which each nucleotide sequence occupies a unique position that can be computed by digi-

tal units. Because each nucleotide sequence can have several meanings, depending on the contextual use, sequence space position can not explain the variety of its functions.

This means the mathematical concept of language and its derivatives is based upon a fundamental error. Natural languages/codes are not the core objects of natural sciences because the latter"s tools for appropriate investigations are rather limited and cannot lead to a full explanation or understanding. As a consequence we need a pragmatic turn in biology to liberate this discipline from its role as a subdiscipline of physics and chemistry.

REFERENCES

1 **Brenner S**. History of science. The revolution in the life sciences. *Science* 2012; **338**: 1427-1428 [PMID: 23239722 DOI: 10.1126/science.1232919]
2 **Turing A**. Computing machinery and intelligence. *Mind* 1950; **59**: 433-460
3 **Neumann J**. Theory of Self-Reproducing Automata. Urbana/London: University of Illinois Press, 1966
4 **Shannon CE**, Weaver W. The Mathematical Theory of Communication. Urbana: University of Illinois Press, 1949
5 **Brenner S**. Turing centenary: Life's code script. *Nature* 2012; **482**: 461 [PMID: 22358811 DOI: 10.1038/482461a]
6 **Eigen M**. Selforganization of matter and the evolution of biological macromolecules. *Naturwissenschaften* 1971; **58**: 465-523 [PMID: 4942363]
7 **Eigen M**. The origin of genetic information: viruses as models. *Gene* 1993; **135**: 37-47 [PMID: 8276276]
8 **Biebricher CK**, Eigen M. The error threshold. *Virus Res* 2005; **107**: 117-127 [PMID: 15649558]
9 **Eigen M**. From Strange Simplicity to Complex Familiarity A Treatise on Matter, Information, Life, and Thought. Oxford: Oxford University Press, 2013
10 **Dong S**, Searls DB. Gene structure prediction by linguistic methods. *Genomics* 1994; **23**: 540-551 [PMID: 7851881]
11 **Chomsky N**. Current Issues in Linguistic Theory. London: The Hague: Mouton, 1964
12 **Zhang HY**. The evolution of genomes and language. *EMBO Rep* 2006; **7**: 748-749 [PMID: 16880816 DOI: 10.1038/sj.embor.7400756]
13 **Venter C**. Life at the Speed of Light. From the Double Helix to the Dawn of Digital Life. New York: Viking, 2013
14 **Nowak MA**, Krakauer DC. The evolution of language. *Proc Natl Acad Sci USA* 1999; **96**: 8028-8033 [PMID: 10393942]
15 **Nowak MA**, Komarova NL, Niyogi P. Evolution of universal grammar. *Science* 2001; **291**: 114-118 [PMID: 11141560]
16 **Carnap R**. Foundations of Logic and Mathematics. Chicago: University of Chicago Press, 1939
17 **Hilbert D**, Bernays P, Grundlagen der Mathematik. Vol I. Berlin, New York: Springer, 1934
18 **Hilbert D,** Bernays P, Grundlagen der Mathematik. Vol II. Berlin, New York: Springer, 1939
19 **Whitehead AN**, Russell B. Principia Mathematica, 3 vols. Cambridge: Cambridge University Press, 1910, 1912, 1913
20 **Gödel K.** Über formal unentscheidbare Sätze der Principia Mathematica und verwandter Systeme. *Monatshefte für Mathematik und Physik* 1931; **38:** 173-198
21 **Witzany G**. Life: The Communicative Structure. Norderstedt: Libri BoD, 2000
22 **Witzany G**. Biocommunication and Natural Genome Editing. Dordrecht: Springer, 2010
23 **Witzany G**. Can mathematics explain the evolution of human language? *Commun Integr Biol* 2011; **4**: 516-520 [PMID: 22046452 DOI: 10.4161/cib.4.5.16426]
24 **Witzany G**, Baluška F. Turing: A formal clash of codes. *Nature* 2012; **483**: 541 [PMID: 22460888 DOI: 10.1038/483541c]
25 **Witzany G**, Baluška F. Life's code script does not code itself. The machine metaphor for living organisms is outdated. *EMBO Rep* 2012; **13**: 1054-1056 [PMID: 23146891 DOI: 10.1038/embor.2012.166]
26 **Ben-Jacob E**. Bacterial wisdom, Goedel"s theorem and creative genomic webs. *Physica A* 1998; **248**: 57-76
27 **Shapiro JA**. How life changes itself: the Read-Write (RW) genome. *Phys Life Rev* 2013; **10**: 287-323 [PMID: 23876611 DOI: 10.1016/j.plrev.2013.07.001]
28 **Villarreal LP**. Origin of Group Identity: Viruses, addiction and cooperation. New York: Springer, 2009
29 **Witzany G.** Natural Genetic Engineering and Natural Genome Editing. New York: Wiley & Blackwell, 2010
30 **Witzany G**. Noncoding RNAs: persistent viral agents as modular tools for cellular needs. *Ann N Y Acad Sci* 2009; **1178**: 244-267 [PMID: 19845641 DOI: 10.1111/j.1749-6632.2009.04989.x]
31 **Weiner AM**. SINEs and LINEs: troublemakers, saboteurs, benefactors, ancestors. In: Gesteland RF, Cech TR, Atkins JF, editors. The RNA World, 3rd ed. New York: Cold Spring Harbor Laboratory Press, 2006: 507-534
32 **Witzany G**. Viruses: Essential Agents of Life. Dortrecht: Springer, 2012
33 **Koonin EV**, Senkevich TG, Dolja VV. The ancient Virus World and evolution of cells. *Biol Direct* 2006; **1**: 29 [PMID: 16984643]
34 **Koonin EV**. On the origin of cells and viruses: primordial virus world scenario. *Ann N Y Acad Sci* 2009; **1178**: 47-64 [PMID: 19845627 DOI: 10.1111/j.1749-6632.2009.04992.x]
35 **Villarreal LP**, Witzany G. The DNA Habitat and its RNA Inhabitants. At the dawn of RNA sociology. *Genom Ins* 2013; **6**: 1-12[DOI: 10.4137/GEI.S11490]
36 **Villarreal L**. Viruses and host evolution: virus-mediated self identity. *Adv Exp Med Biol* 2012; **738**: 185-217 [PMID: 22399381 DOI: 10.1007/978-1-4614-1680-7_12]
37 **Villarreal LP**. The source of self: genetic parasites and the origin of adaptive immunity. *Ann N Y Acad Sci* 2009; **1178**: 194-232 [PMID: 19845639 DOI: 10.1111/j.1749-6632.2009.05020.x]
38 **Villarreal LP**. Viral ancestors of antiviral systems. *Viruses* 2011; **3**: 1933-1958 [PMID: 22069523 DOI: 10.3390/v3101933]
39 **Villarreal LP,** Witzany G. Rethinking quasispecies theory: From fittest type to cooperative consortia. *World J Biol Chem* 2013; **4**: 79-90 [PMID: 24340131 DOI: 10.4331/wjbc.v4.i4.79]
40 **Moelling K**. What contemporary viruses tell us about evolution: a personal view. *Arch Virol* 2013; **158**: 1833-1848 [PMID: 23568292]
41 **Forterre P**, Prangishvili D. The major role of viruses in cellular evolution: facts and hypotheses. *Curr Opin Virol* 2013; **3**: 558-565 [PMID: 23870799 DOI: 10.1016/j.coviro.2013.06.013]
42 **Witzany G**. From Molecular Entities to Competent Agents: Viral Infection-derived Consortia act as Natural Genetic Engineers. In: Witzany G, editor. Viruses: Essential Agents of Life. Dortrecht: Springer. 2012: 407-419
43 **Le Rouzic A**, Capy P. Population genetics models of competition between transposable element subfamilies. *Genetics* 2006; **174**: 785-793 [PMID: 16888345]
44 **Smit S**, Yarus M, Knight R. Natural selection is not required to explain universal compositional patterns in rRNA secondary structure categories. *RNA* 2006; **12**: 1-14 [PMID: 16373489]
45 **Gwiazda S**, Salomon K, Appel B, Müller S. RNA self-ligation: from oligonucleotides to full length ribozymes. *Biochimie* 2012; **94**: 1457-1463 [PMID: 22465106 DOI: 10.1016/j.biochi.2012.03.015]
46 **Vaidya N**, Manapat ML, Chen IA, Xulvi-Brunet R, Hayden EJ, Lehman N. Spontaneous network formation among cooperative RNA replicators. *Nature* 2012; **491**: 72-77 [PMID: 23075853 DOI: 10.1038/nature11549]
47 **Briones C**, Stich M, Manrubia SC. The dawn of the RNA

World: toward functional complexity through ligation of random RNA oligomers. *RNA* 2009; **15**: 743-749 [PMID: 19318464 DOI: 10.1261/rna.1488609]
48 **Villarreal LP**. The Addiction Module as a Social Force. In: Viruses: Essential Agents of Life. In: Witzany G, editor. Dordrecht: Springer, 2012: 107-145
49 **Venner S**, Feschotte C, Biémont C. Dynamics of transposable elements: towards a community ecology of the genome. *Trends Genet* 2009; **25**: 317-323 [PMID: 19540613 DOI: 10.1016/j.tig.2009.05.003]
50 **Stoddard B**, Belfort M. Social networking between mobile introns and their host genes. *Mol Microbiol* 2010; **78**: 1-4 [PMID: 20545861 DOI: 10.1111/j.1365-2958.2010.07217.x]
51 **Dinter-Gottlieb G**. Viroids and virusoids are related to group I introns. *Proc Natl Acad Sci USA* 1986; **83**: 6250-6254 [PMID: 3462692]
52 **Evans LH**, Alamgir AS, Owens N, Weber N, Virtaneva K, Barbian K, Babar A, Malik F, Rosenke K. Mobilization of endogenous retroviruses in mice after infection with an exogenous retrovirus. *J Virol* 2009; **83**: 2429-2435 [PMID: 19116259 DOI: 10.1128/JVI.01926-08]
53 **Diemer GS**, Stedman KM. A novel virus genome discovered in an extreme environment suggests recombination between unrelated groups of RNA and DNA viruses. *Biol Direct* 2012; **7**: 13 [PMID: 22515485 DOI: 10.1186/1745-6150-7-13]
54 **Witzany G**. The agents of natural genome editing. *J Mol Cell Biol* 2011; **3**: 181-189 [PMID: 21459884 DOI: 10.1093/jmcb/mjr005]
55 **Mruk I**, Kobayashi I. To be or not to be: regulation of restriction-modification systems and other toxin-antitoxin systems. *Nucleic Acids Res* 2014; **42**: 70-86 [PMID: 23945938 DOI: 10.1093/nar/gkt711]
56 **Kobayashi I**. Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution. *Nucleic Acids Res* 2001; **29**: 3742-3756 [PMID: 11557807]
57 **Garrett RA**, Vestergaard G, Shah SA. Archaeal CRISPR-based immune systems: exchangeable functional modules. *Trends Microbiol* 2011; **19**: 549-556 [PMID: 21945420 DOI: 10.1016/j.tim.2011.08.002]
58 **Villarreal LP**, Witzany G. Viruses are essential agents within the roots and stem of the tree of life. *J Theor Biol* 2010; **262**: 698-710 [PMID: 19833132 DOI: 10.1016/j.jtbi.2009.10.014]
59 **Witzany G**. Viruses: Essential Agents of Life. Springer, 2012
60 **Hu WS**, Temin HM. Retroviral recombination and reverse transcription. *Science* 1990; **250**: 1227-1233 [PMID: 1700865]
61 **Sciamanna I**, Vitullo P, Curatolo A, Spadafora C. Retrotransposons, reverse transcriptase and the genesis of new genetic information. *Gene* 2009; **448**: 180-186 [PMID: 19631262 DOI: 10.1016/j.gene.2009.07.011]
62 **Pathak KB**, Nagy PD. Defective Interfering RNAs: Foes of Viruses and Friends of Virologists. *Viruses* 2009; **1**: 895-919 [PMID: 21994575 DOI: 10.3390/v1030895]
63 **Cheng LK**, Unrau PJ. Closing the circle: replicating RNA with RNA. *Cold Spring Harb Perspect Biol* 2010; **2**: a002204 [PMID: 20554706 DOI: 10.1101/cshperspect.a002204]
64 **Li D**, Lott WB, Lowry K, Jones A, Thu HM, Aaskov J. Defective interfering viral particles in acute dengue infections. *PLoS One* 2011; **6**: e19447 [PMID: 21559384 DOI: 10.1371/journal.pone.0019447]
65 **Brosius J**. RNAs from all categories generate retrosequences that may be exapted as novel genes or regulatory elements. *Gene* 1999; **238**: 115-134 [PMID: 10570990]
66 **Müller S**, Appel B, Krellenberg T, Petkovic S. The many faces of the hairpin ribozyme: structural and functional variants of a small catalytic RNA. *IUBMB Life* 2012; **64**: 36-47 [PMID: 22131309 DOI: 10.1002/iub.575]
67 **Maizels N**, Weiner AM. Phylogeny from function: evidence from the molecular fossil record that tRNA originated in replication, not translation. *Proc Natl Acad Sci USA* 1994; **91**: 6729-6734 [PMID: 8041690]
68 **Dick TP**, Schamel WA. Molecular evolution of transfer RNA from two precursor hairpins: implications for the origin of protein synthesis. *J Mol Evol* 1995; **41**: 1-9 [PMID: 7608982]
69 **Sun FJ**, Caetano-Anollés G. Evolutionary patterns in the sequence and structure of transfer RNA: early origins of archaea and viruses. *PLoS Comput Biol* 2008; **4**: e1000018 [PMID: 18369418 DOI: 10.1371/journal.pcbi.1000018]
70 **Paillart JC**, Skripkin E, Ehresmann B, Ehresmann C, Marquet R. A loop-loop "kissing" complex is the essential part of the dimer linkage of genomic HIV-1 RNA. *Proc Natl Acad Sci USA* 1996; **93**: 5572-5577 [PMID: 8643617]
71 **Friebe P**, Boudet J, Simorre JP, Bartenschlager R. Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. *J Virol* 2005; **79**: 380-392 [PMID: 15596831]
72 **Lodmell JS**, Ehresmann C, Ehresmann B, Marquet R. Convergence of natural and artificial evolution on an RNA loop-loop interaction: the HIV-1 dimerization initiation site. *RNA* 2000; **6**: 1267-1276 [PMID: 10999604]
73 **Rivas E**, Eddy SR. A dynamic programming algorithm for RNA structure prediction including pseudoknots. *J Mol Biol* 1999; **285**: 2053-2068 [PMID: 9925784]
74 **Staple DW**, Butcher SE. Pseudoknots: RNA structures with diverse functions. *PLoS Biol* 2005; **3**: e213 [PMID: 15941360 DOI: 10.1371/journal.pbio.0030213]
75 **Gupta A**, Rahman R, Li K, Gribskov M. Identifying complete RNA structural ensembles including pseudoknots. *RNA Biol* 2012; **9**: 187-199 [PMID: 22418849 DOI: 10.4161/rna.18386]

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TOPIC HIGHLIGHT

WJBC 5th Anniversary Special Issues (2): Proteomics

In 2014, can we do better than CA125 in the early detection of ovarian cancer?

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Abstract

Ovarian cancer is a lethal gynecologic malignancy with greater than 70% of women presenting with advanced stage disease. Despite new treatments, long term outcomes have not significantly changed in the past 30 years with the five-year overall survival remaining between 20% and 40% for stage Ⅲ and Ⅳ disease. In contrast patients with stage Ⅰ disease have a greater than 90% five-year overall survival. Detection of ovarian cancer at an early stage would likely have significant impact on mortality rate. Screening biomarkers discovered at the bench have not translated to success in clinical trials. Existing screening modalities have not demonstrated survival benefit in completed prospective trials. Advances in high throughput screening are making it possible to evaluate the development of ovarian cancer in ways never before imagined. Data in the form of human "-omes" including the proteome, genome, metabolome, and transcriptome are now available in various packaged forms. With the correct pooling of resources including prospective collection of patient specimens, integration of high throughput screening, and use of molecular heterogeneity in biomarker discovery, we are poised to make progress in ovarian cancer screening. This review will summarize current biomarkers, imaging, and multimodality screening strategies in the context of emerging technologies.

Key words: Ovarian cancer; Screening; Biomarker; Detection; Diagnostic imaging; Proteomics; Adnexal mass

Core tip: Ovarian cancer is a lethal gynecologic malignancy with five-year survival of only 20% to 40% for advanced stage disease. Detection at an early stage would likely have significant impact on mortality rate. Advances in high throughput screening with the human "-omes" including the proteome, genome, metabolome, and transcriptome are now available in various packaged forms. To make progress in screening we need greater emphasis on prospective collection of patient specimens, integration of high throughput screening, and use of molecular heterogeneity in biomarker discovery.

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INTRODUCTION

Ovarian cancer is a lethal gynecologic malignancy with greater than 70% of women presenting with advanced stage disease[1]. Worldwide it is estimated there are 225500 new cases of ovarian cancer and 140200 deaths every

year including 14030 deaths in the United States alone[2,3]. Primary treatment for advanced stage disease involves both surgery and chemotherapy.

Despite new treatments, long term outcomes have not significantly changed in the past 30 years with the five-year overall survival remaining between 30 and 40%[3]. Greater than 60% of advanced stage patients will develop recurrent disease[4]. Patients with advanced stage disease have a five-year overall survival between 20% and 40%, in stark contrast to the greater than 90% five-year overall survival of patients identified and treated with stage I disease[5-7].

Given the poor prognosis for patients with advanced stage disease, effective screening modalities are needed to identify patients with early stage disease. The majority of women with early stage disease are asymptomatic, and unfortunately when they do present for diagnosis, three quarters are found to have regional or distant metastases[7]. Preliminary evaluations of screening with serum markers, pelvic ultrasounds, and multimodality strategies have demonstrated potential benefit in the earlier identification of ovarian cancer[8,9]. With these encouraging results, prospective screening trials have been undertaken as the scientific community continues to increase the number of potential biomarkers and imaging tests which might assist with identification of early stage ovarian cancer in asymptomatic women.

In the United States a woman's lifetime risk of developing ovarian cancer is 1 in 70 and the prevalence of ovarian cancer in postmenopausal women over the age of 50 is 1 in 2500[10]. To minimize harms while identifying women at risk, a positive predictive value (PPV) of 10% is needed, requiring a sensitivity of greater than 75% and specificity of 99.6% to identify one case of ovarian cancer for every ten operations[10]. It is unlikely that one biomarker test will meet this criteria given the high specificity needed[11].

The ideal biomarker or panel of biomarkers is obtained through noninvasive means such as a bodily fluid: blood, saliva, urine, and cervical mucous are possibilities[12]. Advances in high throughput screening have made it possible to evaluate the human genome with the hope of better understanding genetic and epigenetic changes associated with the development of ovarian cancer. Enormous amounts of data in the form of human "-omes" including the proteome, genome, metabolome, and transcriptome are now available in various packaged forms. The Cancer Genome Atlas (TCGA) recently completed a comprehensive genomic and epigenomic evaluation of over three hundred high-grade serous ovarian cancer samples with microarray analyses and massively parallel sequencing coupled with hybrid affinity capture[13].

Ovarian cancer represents a very diverse group of tumors. Scientific endeavors such as TCGA are now making it possible to better delineate characteristics of various subtypes. The continued quest for a strategy that meets the need to identify asymptomatic women in the general population may depend on the ability to parse out the origins of ovarian cancer. The epithelial category, which accounts for 90% of all ovarian cancers, consists of the following subtypes: (1) serous (50%); (2) endometrioid (10%-25%); (3) mucinous (5%-10%); (4) clear cell (4%-5%); (5) undifferentiated carcinomas (5%); and (6) transitional cells (rare)[14]. These ovarian tumors are likely distinct diseases with different cells of origin and driver mutations, united under one term due to their predilection for dissemination to the ovary and related pelvic organs[15]. A pitfall of the past may be failure to develop screening strategies based on differences among these tumors. It is not yet clear if one screening strategy or separate approaches will be needed to identify patients with these tumor types at an early stage of disease. This review will summarize current biomarkers, imaging, and multimodality screening strategies in the context of emerging technologies.

BIOMARKERS AND EXISTING ALGORITHMS

Initially described by Bast *et al*[16] in 1981, cancer antigen 125 (CA125) was recognized by the murine monoclonal antibody OC-125 as an antigenic determinant on a high molecular-weight glycoprotein. It is the most widely studied biomarker in ovarian cancer screening. Measurement of CA125 can be performed with different commercial assays resulting in a certain degree of variation. The majority of assays appear to be both clinically reliable and correlative, nonetheless, new quantitative methods including mass spectrometry are under investigation[17,18]. As part of its development, CA125 underwent molecular cloning and was found to have characteristics of mucin, receiving the name MUC16[19].

In adults, CA125 is expressed in tissues derived from coelomic epithelium (mesothelial cells of the peritoneum, pleura, and pericardium) and Mullerian (tubal, endometrial, and endocervical) epithelia, as well as epithelia of the pancreas, colon, gall bladder, lung, kidney, and stomach[20,21]. CA125 can be elevated in a number of conditions unrelated to ovarian cancer, resulting in decreased specificity and PPV. Diverticulitis, endometriosis, liver cirrhosis, uterine fibroids, menstruation, pregnancy, benign ovarian neoplasms, and other malignancies (pancreatic, bladder, breast, liver, lung) can all result in an elevated CA125[11].

When values below 35 U/L are designated as normal, CA125 is elevated in 80% of epithelial ovarian cancers[22]. CA125 is elevated in approximately 50%-60% of stage I epithelial ovarian cancers and 75%-90% of patients with advanced stage disease[21,23]. The sensitivity of CA125 to identify early stage disease is limited as a screening tool. With evaluation of 22000 volunteers and over 50000 serum CA125 samples with a median follow up of 8.6 years, Jacobs *et al*[24] demonstrated CA125 levels in women without ovarian cancer remained static or decreased over time while levels associated with malignancy tended to increase[8,24]. Based on these findings, the Risk

of Ovarian Cancer Algorithm (ROCA) was developed incorporating an individual's age specific incidence of ovarian cancer and CA125 profile to triage women into various risk categories[8]. ROCA increased the sensitivity of CA125 from 62% to 86% for detection of preclinical ovarian cancer while maintaining a specificity of 98%[25]. A randomized control trial to evaluate ROCA consisted of 13582 postmenopausal women over the age of 50 and demonstrated a specificity of 99.8% (CI: 99.7% to 99.9%) and positive predictive value of 19% (CI: 4.1% to 45.6%)[26]. This model has been incorporated into various multimodality screening strategies in an attempt to optimize sensitivity, specificity, and positive predictive value.

The quest for other biomarker candidates has continued because a single CA125 value at a given time point will not reach a specificity of 99.6%, and approximately 20% of ovarian cancers may not express this antigen. Human epididymis protein (HE4), found primarily in the epithelia of normal genital tissues and made up of two whey acidic protein (WAP) domains and a four disulfide core, is elevated in epithelial ovarian cancer[27,28]. HE4 is overexpressed in 50% of clear cell, 93% of serous, and 100% of endometrioid cancers but is not overexpressed in mucinous tumors[28]. Identified initially as an mRNA transcript specific to the distal epididymal tissue, genomic advances with microarray gene expression profiling demonstrated HE4 is highly-expressed in ovarian cancer[29,30]. HE4 has greater specificity in the premenopausal age group than CA125 given it does not appear to be expressed at high levels in the setting of benign conditions such as endometriomas[31-33]. HE4 represents a victory for genomic strategies in the search for potentially effective biomarkers with microarray gene expression[34].

In a systemic review of women with suspected gynecologic disease HE4 demonstrated a higher specificity (93% *vs* 78%) and similar sensitivity (79%) to CA125 when distinguishing benign disease from ovarian cancer[35]. Studies have demonstrated a potential benefit in combining HE4 and CA125 when quantifying risk potential malignancy in the evaluation of a pelvic mass[36,37]. Even with new technology, it is unlikely that an individual biomarker will reach a specificity of 99.6%, positive predictive value of 10%, and sensitivity greater than 75% when screening an asymptomatic general population.

In efforts to further triage women in the detection of ovarian cancer, progress has been made in the development of algorithms to delineate malignancy in the setting of an adnexal mass. Woman appropriately referred to a gynecologic oncologist have better outcomes including survival, demonstrating the potential importance of these triage tests[38,39]. The Risk of Malignancy Index (RMI), developed by Jacobs *et al*[40] in 1990, is a formula which incorporates a woman's CA125 level, ultrasound score, and menopausal status to determine her likelihood of malignancy in the setting of an adnexal mass. Since that time two other algorithms have been developed for assessment of malignancy risk in women with adnexal masses: the Risk of Malignancy Algorithm (ROMA) and the OVA1 test[41,42]. The ROMA algorithm is based on serum levels of HE4 and CA125 with menopausal status[41]. OVA1, with the exception of CA125, is made up of biomarkers discovered through mass spectrometry: β-2 microglobulin, transferrin, transthyretin, and apolipoprotein[42,43]. Various studies have been published evaluating the effectiveness of RMI, ROMA, and OVA1, as well as other strategies to help delineate the likelihood of malignancy in the setting of a pelvic mass. Table 1 provides a summary of various algorithms and assays used to predict likelihood of malignancy.

OVA1 and ROMA each have benefits and disadvantages. Prospective multi-institutional trials and cost-benefit analysis are needed before definitive conclusions can be drawn regarding these tests[34]. Table 2 lists sensitivities and specificities for various modalities in the setting of a pelvic mass. Based on available data, OVA1 and ROMA likely have similar sensitivities, but ROMA appears to have greater specificity (75% *vs* 43%) which may impact cost-effectiveness and referral patterns from general gynecologists reticent to lose patients with benign masses to gynecologic oncologists[53]. OVA1, based largely on mass spectrometry with proteomics, and ROMA, made possible by the incorporation of a microarray gene-expression based discovery in HE4, represent hopeful advancements in the ability to identify women with malignancy earlier than had been in the past. These are not screening tests for the general population, but represent potential tools to further triage of women to the appropriate providers once the decision for surgical intervention has been made.

Various other biomarkers and biomarker panels are currently under development for both the prediction of malignancy in the setting of a pelvic mass and in asymptomatic women. Table 3 lists various single biomarker and multi-biomarker panels with sensitivities and specificities for ovarian cancer detection. An important consideration with all of these tests is the ultimate need to demonstrate benefit for patients through reduction in morbidity and mortality while minimizing harm. The advancements of technology combined with our exponentially growing knowledge of the human "-omes" have outpaced our ability to reliably test these discoveries through clinical settings in a timely fashion.

AREAS OF GROWTH IN BIOMARKER DISCOVERY

High throughput technology in conjunction with TCGA has now made it possible to combine multiplex assays with data from the proteome, genome, metabolome, and transcriptome. Within proteomics, biomarker panels have been developed in an attempt to increase sensitivity for ovarian cancer detection due to the heterogeneous make up of subtypes (Table 3). Biomarker discovery in proteomics is usually based on two-dimensional gel electrophoresis, mass spectrometry (MS), and/or protein microarrays in combination with bioinformatics analysis[65].

Table 1 Screening algorithms and commercially available assays

Algorithm or assay *(Screening population)*	How it works
ROCA *(asymptomatic general population)*	1 Compares a woman's longitudinal CA-125 pattern to the change-point CA-125 profile seen in women with ovarian cancer and the flat CA-125 profiles seen in women without ovarian cancer[1] 2 Based on the ROCA result, women get triaged into one of three groups[1]: (1) Low Risk: continue annual CA-125 testing (2) Intermediate Risk: repeat CA-125 test 3 mo later (3) High Risk: receive TVS and referral to a gynecologic oncologist 3 After each additional CA-125 value, ROCA is recalculated and a new recommendation is made[1]
ROMA *(known pelvic mass)*	1 Uses both HE-4 and CA-125 test levels to evaluate patients as low or high risk for ovarian cancer[8] 2 A predictive index (PI) is calculated using different equations for pre-menopausal and post-menopausal women[8] 3 The PI is then inserted into the ROMA algorithm to predict the probability of ovarian cancer[8]
RMI *(known pelvic mass)*	Uses menopausal status, ultrasound findings, and serum CA-125 levels to determine malignancy risk[40]
OVA1 *(known pelvic mass)*	1 A multivariate index assay that incorporates CA-125, transferrin, transthyretin (prealbumin), apolipoprotein A1, and beta-2-microglobulin[41] 2 An algorithm is used to generate an ovarian malignancy risk score between 0 and 10[41] 3 OVA1 scores greater than or equal to 5.0 (premenopausal) or 4.4 (postmenopausal) result in high risk stratification and referral to a gynecologic oncologist[41]
LR-1 *(known pelvic mass)*	1 An ultrasound-based prediction model 2 Twelve variables are used to calculate a probability of malignancy[88]: (1) personal history of ovarian cancer (2) current hormonal therapy (3) age of the patient (4) maximum diameter of the lesion (5) pain during examination (6) ascites (7) blood flow within a solid papillary projection (8) a purely solid tumor (9) maximum diameter of the solid component (10) irregular internal cyst walls (11) acoustic shadows (12) color score
LR-2 *(known pelvic mass)*	1 An ultrasound-based prediction model 2 Uses six variables to calculate a probability of malignancy[90]: (1) patient's age (2) presence of ascites (3) presence of blood flow within a papillary projection (4) maximal diameter of solid components (5) irregular internal cyst walls (6) presence of acoustic shadows

ROCA: Risk of ovarian cancer algorithm; ROMA: Risk of ovarian malignancy algorithm; RMI: Risk of malignancy index; OVA1: Vermillion Inc. OVA1 ® blood test; LR-1: International ovarian tumor analysis logistic regression model 1;LR-2: International ovarian tumor analysis logistic regression model 2; ROCA: Risk of ovarian cancer algorithm; ROMA: Risk of Malignancy Algorithm.

MS with matrix assisted laser desorption and ionization time of flight (MALDI-TOF) and surface-enhanced laser desorption and ionization time of flight (SELDI-TOF) allow for the entire protein complement of a patient sample to be evaluated in rapid high throughput fashion[12,64]. Protein microarrays can be used to profile the proteome of cell populations using antigen-antibody interactions[66]. Protein microarrays are made up of two major classes: (1) forward-phase arrays (FPA) with antibodies arrayed and probed with cell lysates; and (2) reverse-phase arrays (RPA) with cell lysates arrayed and probed with antibodies[65].

Unfortunately, proteomics has not resulted in the major breakthroughs previously anticipated. An important consideration here is the biological samples used when identifying potential biomarkers. Various studies have demonstrated protein biomarkers perform very differently in the detection of ovarian cancer when analyzed in prospectively collected samples from asymptomatic patients[67,68]. Future proteomic discovery may best focus on samples from patients prospectively followed until diagnosis in larger population based trials. Incorporation of methods aimed at depletion of abundant serum proteins such as acute phase reactants, and the use of multiplex bead-based immunossays may allow for identification of low abundance or low concentration proteins not previously identified[12].

MS continues to serve as an important tool to explore the thousands of proteins relevant to ovarian cancer and has now been extended to use in glycomics, metabolomics, MALDI-MS imaging, and autoantibody signatures for biomarker discovery[69]. Glycosylation or the addition of carbohydrates to nascent proteins is a common post-translational modification that is potentially altered in a

Table 2 Specificity and sensitivity results of various screening strategies in the setting of a pelvic mass

Algorithm or assay	Ref.	Sensitivity (%)	Specificity (%)
ROMA	Karlsen *et al*[44]	94.4	76.5
	Moore *et al*[45]	94.3	75
	Sandri *et al*[46]	91.2	75
		89.3	81.7
	Van Gorp *et al*[89]	84.7	76.8
	Sandri *et al*[46]	84.4	90
	Chan *et al*[47]	89.2	87.3
	Kaijser *et al*[90]	84	80
RMI	Karlsen *et al*[44]	94.4	81.5
	Håkansson *et al*[48]	92	82
	Moore *et al*[45]	84.6	75
	Van den Akker[49]	81	85
	Van Gorp *et al*[89]	76	92.4
OVA1	Bristow *et al*[50]	92.4	53.5
	Longoria *et al*[52]	92.2	49.4
OVA1 +	Bristow *et al*[50]	95.7	50.7
Clinical assessment	Longoria *et al*[52]	95.3	44.2
LR-1	Kaijser *et al*[88]	93	77
LR-2	Nunes *et al*[51]	97	69
	Kaijser *et al*[88]	92	75
	Kaijser *et al*[90]	93.8	81.9
TVS	van Nagell *et al*[8]	86.4	98.8

ROMA: Risk of ovarian malignancy algorithm; RMI: Risk of malignancy index; OVA1: Vermillion Inc. OVA1® blood test; LR-1: International ovarian tumor analysis logistic regression model 1; LR-2: International ovarian tumor analysis logistic regression model 2; TVS: Transvaginal ultrasonography.

malignant state[69-71]. There is evidence to indicate various histologic subtypes of ovarian cancer exhibit different glycoproteins[72]. This is encouraging given the significant heterogeneity of ovarian cancer. The differences seen in glycomics may assist in screening algorithms which can be developed with this heterogeneity in mind.

Evaluation of the metabolome through MS has demonstrated differences in metabolites in patients with and without epithelial ovarian cancer[73,74]. Existing concerns with the study of metabolites include the significant variation in metabolic response and extensive biotransformation from the site of malignancy to fluids such as serum or plasma[75]. Study of the peptidome within the low-molecular weight proteome in ovarian cancer has been limited by the potential loss of peptides bound to carrier proteins during sample processing, although attempts have been made to mitigate this with isolation and enrichment of carrier proteins prior to MS evaluation[69,76]. Ovarian cancer diagnoses may also be aided with the uses of anti-tumor autoantibody signatures and MALDI-MS imaging; however, these areas of research are preliminary with MS[77,78].

Separate from the use of MS, there is a growing role for microRNAs in the development of ovarian cancer biomarkers[79]. MicroRNAs are a class of small noncoding RNAs which impact gene expression by targeting multiple messenger RNAs and triggering translation repression and/or RNA degradation[80]. Aberrant expression of microRNAs in ovarian cancer indicate they may act as a novel class of oncogenes or tumor-suppressor genes[79]. Five microRNAs (miR-200a, miR-100, miR-141, miR-200b, and miR-200c) have been found to be consistently differentially regulated in epithelial ovarian cancer and may assist in the development of biomarkers[81]. The future is promising with these techniques; however, validation strategies and appropriate patient samples are vital to improving success in clinical testing. No individual biomarker or biomarker panel has been developed which meets the sensitivity, specificity, and PPV criteria desired for screening in a general population.

IMAGING

There has been an immense effort placed in the evaluation of screening with radiologic technology. A systematic approach to the diagnosis of ovarian tumors with imaging is necessary given the majority of women have benign lesions, and unnecessary interventions should be avoided without placing patients at risks for advanced stage disease[82]. Available imaging modalities include ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), and 18F-fluorodeoxyglucose positron emission tomography (FDG-PET). Pelvic ultrasound has been the most studied imaging modality in ovarian cancer screening. Of 48053 postmenopausal women in the ultrasound group of the United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS), 4367 asymptomatic women (9.1%CI: 8.8%-9.3%) had abnormal adnexal morphology with an overall absolute risk of epithelial ovarian cancer of 1.08% (95%CI: 0.79%-1.43%) and a 1 in 22 risk of epithelial ovarian cancer if the abnormal findings included solid elements[83].

In a single arm prospective screening cohort, the University of Kentucky Ovarian Cancer Screening Trial,

Table 3 Results of serum marker panels for the detection of ovarian cancer

Serum marker(s)	Ref.	Sensitivity (%)	Specificity (%)
CA-125	[1]Karlsen *et al*[44]	91.7	75
	[1]Chan *et al*[47]	90.8	67.2
	[1]Leung *et al*[123]	89	90
	[1]Sandri *et al*[46]	84.4	80
	[1]Montagnana *et al*[54]	83	100
	[1]Sandri *et al*[46]	73.1	90
	Yang *et al*[55]	62.5	80
	Havrilesky *et al*[56]	45.9-58.5	98.2
	[1]Moore *et al*[37]	43.3	95
	Jacob *et al*[57]	12.5	90.1-93.9
HE-4	[1]Montagnana *et al*[54]	98	100
	Yang *et al*[55]	96.2	83.8
	[1]Karlsen *et al*[44]	91.3	75
	[1]Sandri *et al*[46]	83.1	90
	Havrilesky *et al*[56]	82.7-92.5	86.3
	[1]Moore *et al*[37]	72.9	95
	Jacob *et al*[57]	62.5	81.8-85.9
	[1]Chan *et al*[47]	56.9	96.9
CA-125, HE-4	[1]Moore *et al*[37]	76.4	95
	[1]Moore *et al*[41]	88.7	74.7
CA 125, leptin, PRL, OPN, IGFII, MIF	Visintin *et al*[58]	95.3	99.4
CA 125, CRP, SAA, IL-6, IL-8	Edgell *et al*[59]	94.1	91.3
CA-125, apoA-I, TTR, TF	Su *et al*[60]	89-97	91-99
CA 125, HE4, CEA, VCAM-1	Yurkovetsky *et al*[61]	86–93	98
CA 125, ApoA1, TTR	Kim *et al*[62]	93.9	95
	Zhang *et al*[42]	74	97
CA 125, CA 19-9, EGFR, CRP, myoglobin, ApoA1, ApoCIII, MIP-1a, IL-6, IL-18, tenascin C	[1]Amonkar *et al*[63]	91.3	88.5
CA-125, OVX1[r], LASA,CA15-3, CA72-4	Nossov *et al*[11]	90.6	93.2
CA 125, CA 72-4, CA 15-3, M-CSF	[1]Skates *et al*[64]	70	98
LPA	Nossov *et al*[11]	90-100	90
FOLR1	[1]Leung *et al*[123]	62	90
M-CSF	Nossov *et al*[11]	61-68	93
SMRP	[1]Moore *et al*[37]	53.7	95

[1]Study involved patients presenting with a pelvic mass; CA: Cancer antigen; HE-4: Human epididymis protein 4; PRL: Prolactin; OPN: Osteopontin; IGFII: Insulin-like growth factor II; MIF: Macrophage inhibitory factor; CRP: C-reactive protein; SAA: Serum amyloid A; IL: Interleukin; apoA-I: Apolipoprotein A-I; TTR: Transthyretin; TF: Transferrin; CEA: Carcinoembryonic antigen; VCAM-1: Vascular cell adhesion protein 1; EGFR: Epidermal growth factor receptor; ApoCIII: Apolipoprotein CIII; MIP-1a: Macrophage inflammatory protein-1alph; OVX1: Mouse antibody generated by immunizing mice with antigenic preparations from multiple OC cell lines[21]; LASA: Lipid-associated sialic acid; M-CSF: Macrophage colony-stimulating factor; LPA: Lipoprotein A; FOLR1: Folate receptor 1; SMRP: Soluble mesothelin-related peptide.

asymptomatic women 25 years or older with a documented family history of ovarian cancer and asymptomatic women 50 years or older were screened with annual transvaginal ultrasound[84]. Serial ultrasonography in this trial demonstrated many ovarian abnormalities resolve in follow up: 63.2% of women with an initially abnormal ultrasound were found to have resolution on subsequent imaging[85]. Observation with serial imaging may help improve positive predictive value and decrease false positive results in screening trials[85]. Of 37293 women who underwent annual screening, the five-year disease-free survival rate for women with ovarian cancer in the screening group, including those who developed ovarian cancer within one year of a normal ultrasound (false negative), was 74.8% ± 6.6%. In contrast, a group of unscreened women with ovarian cancer treated at the same institution with the same surgical and chemotherapeutic protocols had a five-year disease free survival of 53.7% ± 2.3%, *P*-value < 0.01[86]. Ultrasound screening does not impact disease-free survival by itself. Ultimately, the goal of ultrasound screening is to identify patients with early stage disease who can be treated before the malignancy becomes advanced. While the results from this study are encouraging, the mortality benefit may have been impacted by a healthy volunteer effect and lead time detection rather than impact on the natural history of ovarian cancer[87].

Although screening in an asymptomatic population ultimately provides the best opportunity to improve survival in women with ovarian cancer, there has been progress made in the development of imaging algorithms designed for those women with a known adnexal mass. The International Ovarian Tumor Analysis (IOTA) group has developed various approaches to characterize adnexal masses as malignant or benign with ultrasound guidelines. These approaches can be divided into two strategies: the first consisting of risk prediction with two logistic regression models (LR1 and LR2) based on demographic and ultrasound variables (Table 1), and the second based on simple ultrasound features that are descriptors of benign or malignant masses[88]. In women with a pelvic mass the sensitivity and specificity of ROMA and the RMI were

compared to subjective assessment by skilled ultrasonographers in a prospective cohort study of women[89]. The sensitivity of ROMA, RMI, and expert ultrasonographers were 84.7% (77.9% to 90.0%), 76.0% (68.4% to 82.6%), and 96.7% (92.4% to 98.9%) respectively, and the specificity was 76.8% (70.7% to 82.2%), 92.4% (88.1% to 95.5%), and 90.2% (85.5% to 93.7%) respectively[89]. Generalizability of these results may not be possible based on its location, the cohort, and the ultrasonographers used. The study took place at one single tertiary care center in Europe with experienced ultrasonographers and a high prevalence of malignant disease in the cohort[89].

In a different study, a cross-sectional cohort of 360 patients with adnexal masses undergoing surgery was retrospectively evaluated with ROMA and LR2[90]. This study demonstrated decreased sensitivity and specificity for ROMA *vs* LR2 in both premenopausal and postmenopausal patients, with overall sensitivity 84.0% *vs* 93.8%, and specificity 80% *vs* 81.9%, respectively[90]. While this result indicates LR2 may be a more effective screening test in the setting of adnexal mass, prospective randomized control trials are needed before conclusions can be made regarding the use of algorithms which include biomarkers such as HE4 and CA125 (ROMA) *vs* ultrasound-based prediction models such as LR2. Table 2 lists sensitivities and specificities for various modalities in the setting of a pelvic mass.

Currently no prospective randomized studies support the use of imaging as a single strategy in screening for ovarian cancer. At this time, given ultrasound is relatively inexpensive, available widely, and can provide tissue specific information with a presumptively risk-free technology; it is the method of choice for initial evaluation of an adnexal mass and estimating risk of malignancy[82]. In asymptomatic postmenopausal women, the ultrasound screening arm results of the UKCTOCS expected in 2015 will help elucidate the role of ultrasound in population-based screening strategies. At this time is it unlikely ultrasound will significantly reduce mortality in primary screening, but it may be extremely important in reducing false positive rates in multimodality screening[91]. Existing ultrasound-based strategies evaluating the likelihood of malignancy in the setting of a known adnexal mass are based on those who have already been scheduled for surgery. Comparative prospective studies are needed to determine efficacy and effect on survival in women who have surgery based on prediction models using proposed ultrasound-based strategies with and without biomarkers such as CA125 and HE4.

MULTIMODALITY SCREENING

The promising results of imaging in population-based screening for ovarian cancer have led to large scale multimodality strategies. Prior prospective studies demonstrating CA125 and ultrasound were feasible screening modalities have given way to prospective randomized multimodality screening trials involving ultrasound, serum biomarkers, and risk calculations using patient demographics[92,93]. The Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) is a multicenter randomized control trial of 78216 asymptomatic women aged 55 to 74 years who underwent multimodality screening or usual care between November 1993 and July 2001 with management of positive screens left to the discretion of the patient's physician[94]. Multimodality screening consisted of annual testing for three years with transvaginal ultrasound and serum CA125 with a cutoff of 35 U/mL followed by CA125 alone for an additional two years[94]. After four rounds of screening, the PPV and cancer yield per 10000 women screened in the multimodality screening arm remained similar across screening rounds at 1.0% to 1.3% and 4.7 to 6.2 cancers respectively with the overall ratio of surgeries to screen-detected cancers 19.5 to 1[95]. After a median follow up of 12.4 years (25th% to 75th%, 10.9 to 13.0), no mortality benefit was found with combination transvaginal ultrasound and CA125 using an absolute cutoff: 118 deaths due to ovarian cancer (3.1 per 10000 person-years) in the intervention group and 100 deaths (2.6 per 10000 person-years) in the usual care group (mortality rate ratio, 1.18; 95%CI: 0.82-1.71)[96].

The Japanese Shizuoka Cohort Study of Ovarian Cancer Screening is a randomized control trial of 82487 low risk postmenopausal women between 1985 and 1999 with the intervention arm consisting of annual ultrasound and CA125 with a cutoff value[97]. The strategy achieved a sensitivity of 77.1% and specificity of 99.9% with a nonsignificant difference in the proportion of stage I ovarian cancers identified, 63% in the screened group *vs* 38% in the control group, *P*-value = 0.2285[97]. Mortality results from this trial have not yet been published and, as such, conclusions cannot be drawn from this trial regarding the benefit of screening in an asymptomatic population.

The UKCTOCS is a randomized prospective multiarm ovarian cancer screening study in the United Kingdom. This trial, made up of 202638 post-menopausal women aged 50 to 74, randomizes women in a 2:1:1 format to three arms: (1) control; (2) annual screening with ultrasound; and (3) a multimodality strategy that takes advantage of ROCA to triage women to various substrategies[98]. These substrategies include transvaginal ultrasound and/or repeat CA125 at defined time points[98]. In the prevalence screen of the UKCTOCS, ultrasonography alone was compared to multimodality screening (ROCA as a primary test followed by transvaginal ultrasound as a secondary test or repeat CA125 if indicated). With regard to primary invasive epithelial and tubal cancers, the multimodality screening arm demonstrated a higher specificity compared to the ultrasonography arm (99.8% *vs* 98.2%), *P*-value < 0.001, while the difference in sensitivity was not statistically significant (89.4% *vs* 84.9%), *P*-value = 0.564[98].

A single-arm prospective cohort study of 4051 average-risk postmenopausal women in the United States was performed over 11 years using a two-stage ovarian cancer

screening strategy (CA125 interpreted through ROCA with subsequent repeat CA125 or transvaginal ultrasound as indicated) with a PPV of 40% for invasive ovarian cancer and specificity of 99.9% (95%CI: 99.7%-100%)[1]. The results from both the UKCTOCS and two-stage strategy in the United States indicate the use of ROCA to interpret CA125 may be effective in triaging women to subsequent follow-up categories that impact both screening outcomes.

When comparing the UKCTOCS prevalence screen results to the PLCO trial results, both the UKCTOCS multimodality arm (89.4% *vs* 51.7%) and the ultrasound arm (75.0% *vs* 67.4%) had higher sensitivities[99]. When CA125 values were retrospectively evaluated with ROCA within the PLCO data set no mortality benefit was seen; best-case and stage-shift scenarios resulted in 25 and 19 deaths prevented with ROCA for relative risks of 0.90 (95%CI: 0.69-1.17) and 0.95 (95%CI: 0.74-1.23), respectively[100]. In addition to the use of absolute cutoff value for CA125, other concerns have been raised regarding the PCLO trial design including leaving management of positive screens to the discretion of the treating physician and 40.6% of ovarian cancer diagnosis took place after the screening ended[101]. Use of an individualized algorithm that tracks a patient over time will likely provide the best combination of sensitivity, specificity, and PPV. For this reason, the UKCTOCS and its incorporation of ROCA in the multimodality screening arm, represents the best opportunity yet to identify a potential screening strategy. The results from the final mortality analysis in the UKCTOCS will be reported in 2015 and provide significant insight into whether population-based screening in asymptomatic women is possible with currently available imaging and biomarkers.

SYMPTOM-BASED SCREENING

Screening efforts in ovarian cancer have largely focused on asymptomatic women in the general population or women with known adnexal masses requiring further dichotomization for treatment purposes. Women with ovarian cancer do have physical symptoms such as abdominal pain, bloating, and bowel irregularity that may serve as a potential trigger for diagnosis. In a case-control study comparing woman with ovarian cancer to age and race matched controls, more than 90% of cases reported at least one symptom and symptoms were cited as the most common reason for the doctor visit leading to diagnosis (74%)[102]. Two feasibility studies have been performed demonstrating symptom-based screening in women is possible[103,104]. A symptom index was created with a sensitivity of 56.7% for early stage disease and 79.5% for advanced stage disease, and a specificity of 90% for women greater than 50 years of age and 86.7% for women less than 50 years of age[105]. Based on patient interviews performed with 812 women with ovarian cancer and 1313 population-based controls, the symptom index and symptoms established in consensus recommendations had a PPV of 0.6%-1.1% overall and less than 0.5% for early-stage disease[106]. The identification of specific symptoms associated with ovarian cancer has value, but recognition of symptoms alone will not significantly improve overall survival from ovarian cancer[107]. A cross sectional study of 160 women evaluated with use of this symptom index found that the addition of CA125, HE4, or the ROMA to a positive symptom index increased PPV when determining malignancy *vs* benign process in patients with a known adnexal mass[108]. At this time, given no effective screening tool has been proven in a prospective model, physicians should continue to discuss potential symptoms with their patients in an effort to increase self-awareness regarding warning signs for ovarian cancer.

SCREENING IN HIGH RISK PATIENTS

Familial genetic predisposition makes up approximately 10% of ovarian cancers with germline mutations in *BRCA1/BRCA2* and mismatch repair (*MMR*) genes in Lynch syndrome being the most common[109]. Women with *BRCA1* and *BRCA2* mutations have a cumulative lifetime risk of ovarian cancer of 40%-50% and 20%-30% respectively, while the DNA MMR genes, including those that predispose to Lynch syndrome, result in a cumulative lifetime risk of ovarian cancer ranging from 6.7% to 12%[110]. As seen with improved survival in BRCA-associated ovarian cancers, inherited ovarian cancers may have biological differences which allow treatment at time of screen detection to have significant benefit[111].

Currently, no prospective studies exist which demonstrate a mortality benefit by screening high risk asymptomatic patients. The United Kingdom Familial Ovarian Cancer Screening Study (FOCSS) has recently completed a phase 1 trial in which 3563 women at greater than a 10% risk of ovarian or fallopian tube cancer were screened with annual transvaginal ultrasound and CA125 for a mean of 3.2 years[109]. Sensitivity for detection of incident ovarian and fallopian tube cancers at one year after last annual screen was 81.3% (95%CI: 54.3%-96.0%) if occult cancers were classified as false negatives, and the PPV was 25.5% (95%CI: 14.3%-40.0%) with only four women undergoing surgery for each case of detected cancer[109]. As part of phase II of the FOCSS, screening frequency will increase to every four months, ROCA will be incorporated into the decision tree, and the threshold and work-up for repeat tests will be per protocol. The Gynecologic Oncology Group (GOG) and Cancer Genetics Network have recently completed GOG 199, a prospective study screening women at high risk of ovarian cancer with the use of ROCA and transvaginal ultrasound[112].

For women with Hereditary Breast/Ovarian Cancer Syndrome who have not undergone risk reducing bilateral salpingo-oophorectomy, the National Comprehensive Cancer Network recommends screening with transvaginal ultrasound and CA125 every 6 mo starting at age 30 or 5 to 10 years prior to the earliest age at diagnosis of ovarian can-

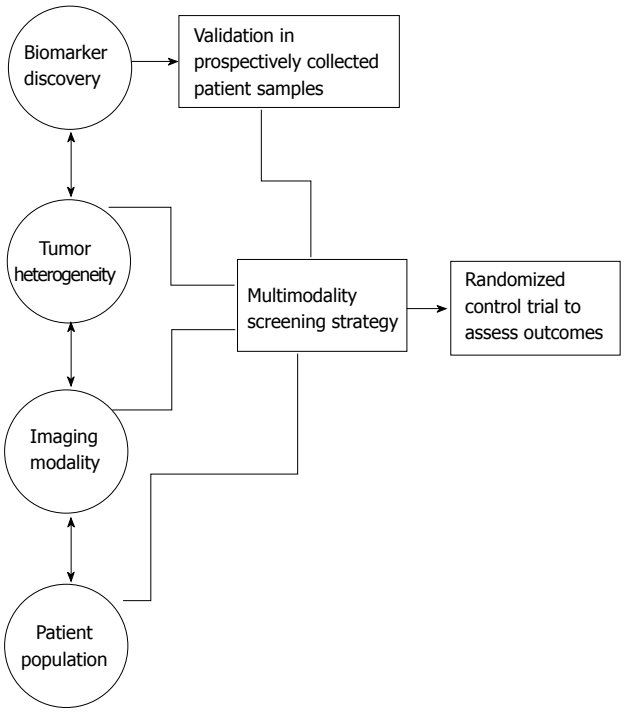

Figure 1 Biomarker candidates must be tested in patient samples collected prior to the onset of physical symptoms in ovarian cancer screening strategies.

cer in relatives[113]. Given the potential biologic differences associated with high risk patients, screening asymptomatic women within this population may have greater benefit than in the general population. The FOCSS phase Ⅱ results and GOG 199 will provide evidence regarding potential screening benefits and assist with strategy optimization.

FUTURE CONSIDERATIONS

Despite the technological advances which have been made, our current approach to screening strategies in ovarian cancer has inherent difficulties which need to be overcome. Directly impacting our ability to screen asymptomatic women for ovarian cancer is the evolving reclassification of this heterogeneous group of tumors. Results from the recently completed study of serous ovarian cancer through TCGA demonstrate significant genomic heterogeneity even within one subtype of epithelial ovarian cancer, high grade serous carcinoma[13].

As in colorectal cancer and cervical cancer, identification of a precursor lesion or lesions will improve our ability to screen for the disease. These precursor lesions are likely varied based on the subtype of ovarian cancer. With high grade serous carcinoma, a precursor lesion may develop in the fimbria of the fallopian tube (serous tubal intraepithelial carcinoma also known as a STIC) or in an ovarian cortical inclusion cyst during implantation of fimbrial epithelium on the denuded ovarian surface with ovulation[114]. Genetic evaluation links both clear cell and endometrioid carcinomas to precursor lesions within endometriosis[115]. A new model that considers both morphologic and molecular characteristics separates epithelial ovarian tumors into two categories: type Ⅰ tumors are low-grade serous, low-grade endometrioid, clear cell, and mucinous tumors which usually present as large cystic masses within one ovary, while type Ⅱ tumors are composed of high-grade serous, high-grade endometrioid, malignant mixed mesodermal (carcinosarcoma), and undifferentiated carcinomas which commonly present as advanced stage disease[116].

A focus on identification of the origins of these groups of tumors will lead to more effective screening strategies in an asymptomatic population. For example, evaluating blood samples of patients found to have STICs at the time of prophylactic bilateral salpingo-oophorectomy may prove useful in identifying biomarkers for preclinical serous carcinoma[12]. Type Ⅰ tumors tend to be genetically stable with mutations in various genes including *PTEN*, *BRAF*, β-*catenin* and *KRAS*, while type Ⅱ tumors have a high level of genetic instability and commonly have a *TP53* mutation[117]. Biomarker panels and multimodality screening may achieve better sensitivity and specificity with screening strategies based on differences in both cell origin and genetics among these varied tumors.

In addition to the varied origin and molecular heterogeneity, the time course of ovarian cancer development still eludes understanding. The time required for development of invasive disease or progression from stage Ⅰ to stage Ⅲ remains unknown[118]. This information is likely specific to the various ovarian tumors, and improved categorization through molecular advances will better elucidate the time course of disease. For example, type Ⅰ tumors appear to follow a developed path of transformation with stepwise progression from a benign lesion to a malignant tumor[119]. It has been proposed that ovarian cancer screening strategies should focus on type Ⅱ tumors with the goal to identify low volume disease rather than early stage, as high grade serous carcinomas represent 75% of all ovarian cancers and result in the majority of deaths[113,120]. Low volume advanced stage disease may be more easily resectable at the time of tumor debulking, but advanced stage patients still have a worse prognosis than those patients who are treated with early stage disease. Identification of early stage disease will have the greatest benefit on mortality, and will require a shift from current approaches to incorporate advances made in the understanding of tumor heterogeneity in this malignancy.

Five phases of biomarker development have been previously proposed: (1) the preclinical exploratory phase; (2) the clinical assay and validation stage; (3) the retrospective longitudinal study; (4) prospective screening evaluation; and (5) randomized control trials[121]. The preclinical exploratory phase must take advantage of developments in high-throughput screening technologies to more effectively identify potential biomarkers among the thousands of candidate molecules. For example, a biomarker discovery platform which incorporates proteome and transcriptome comparisons of serum, tissue, ascites,

cancer cell lines, and animal models through mass spectrometry and microarray technology makes it possible to take advantage of these immense data sets[122]. Folate receptor 1 protein, developed through use of proteomics, transcriptomics, and bioinformatics, demonstrates the incorporation of various technologic platforms that make it possible to identify new biomarkers[123].

Further efforts must be devoted to the collection of appropriate patient specimens in prospective trials. Within ovarian cancer, the majority of biomarkers are evaluated with patient samples taken at the time of diagnosis, usually advanced stage disease. It is not surprising that biomarkers discovered in an advanced disease setting do not perform with the same sensitivity or specificity in a prospective trial in which the goal is diagnosis of early stage disease. Prospectively collected samples in asymptomatic women provide a better understanding of the ability of candidate biomarkers to detect cancer prior to physical symptoms[124]. The prospective specimen collection retrospective blinded evaluation (PRoBE) study design mandates samples are collected prospectively, stored in a similar fashion, and once outcome status is defined, used to validate biomarkers in a blinded fashion with randomly selected cases and controls[125]. Given the low prevalence of ovarian cancer in the general population, pooling of resources is necessary to make advances in biomarker discovery. The National Cancer Institute's Early Detection Research Network assists with development of prospective patient samples under the PRoBE study design[126]. PLCO samples have been used in this fashion to test potential biomarkers[67,68]. Development of a large scale collection of samples prospectively in asymptomatic women on a national or international level would provide the ability to validate biomarkers and predict lead time in the discovery of ovarian cancer prior to physical symptoms (Figure 1).

The final phase of biomarker design is a randomized control trial, with the goal of ovarian cancer screening to demonstrate a mortality benefit in the studied population. This mortality benefit must be considered in the context of the number needed to treat to reach such a benefit. A systematic review and meta-analysis of available screening trials involving asymptomatic women found no reduction in ovarian cancer-specific or all-cause mortality [relative risk (RR), 1.08; 95%CI: 0.84-1.38; and 1.0; 95%CI: 0.84-1.38 respectively][127]. While this analysis does not include results from the UKCTOCS which will not be available until 2015, it does demonstrate that prospective trials within current paradigms have failed to meet major goals.

In the PLCO trial 1080 women underwent surgery in the setting of false positive results and 163 (15%) experienced a complication[96]. Based on review of available clinical trials, 6% of women with false positive screening results experienced a severe complication while undergoing surgery[127]. These patients underwent potential harm without benefit. A mortality benefit is necessary to justify the potential harm associated with false positives. If the UKCTOCS and/or the Japanese cohort fail to show a benefit in mortality, this may be explained by lead time bias in which slow growing tumors are detected more commonly by screening than fast growing lethal serous epithelial ovarian cancers[91]. Type Ⅰ tumors, which tend to be slow growing and more indolent than type Ⅱ tumors, were detected twice as often as type Ⅱ tumors in the ultrasound arm of the UKCTOCS (32 borderline or type Ⅰ tumors *vs* 15 type Ⅱ tumors) despite a higher prevalence of type Ⅱ tumors in epithelial ovarian cancer[83]. If this same pattern is seen through the UKCTOCS in 2015, it is unlikely there will be a mortality benefit given the better prognosis associated with the majority of borderline and type Ⅰ tumors compared to type Ⅱ tumors.

While further prospective screening trials will take place, ovarian cancer screening in the asymptomatic general population results in potential harms without proven benefit at this time. Guidelines from the American College of Obstetrics and Gynecology, the Society of Gynecologic Oncologists, the United States Preventive Services Task Force, and the American Cancer Society do not recommend screening for ovarian cancer in asymptomatic low-risk women in the general population[128-130].

CONCLUSION

Ovarian cancer is deadly at advanced stage, and as the quest for an optimal screening strategy continues, it is apparent there are risks associated with false positives and invasive tests. When surveyed, 80% of women without risk factors or symptoms for ovarian cancer in the University of Kentucky cohort felt that they would definitely want to participate in ovarian cancer screening starting at age 50[131]. Various avenues continue to be investigated in ovarian cancer screening including imaging, protein profiles, specific symptoms, and combinations of these, as well as other modalities. An expanded and shared biobank of patient specimens collected before development of symptoms and advanced disease is needed. It is with these precious samples that high throughput technology and human "-omes" will have the most positive impact on identification of screening modalities. Emerging technology will allow science to evaluate biological data in ways never imagined. With the correct pooling of resources, including prospective collection of patient specimens, integration of high throughput screening, and use of molecular heterogeneity in biomarker discovery, we are poised to make progress in ovarian cancer screening. If we are prudent in trial design and altruistic in the sharing of resources such as biological samples, identification of an effective screening modality for ovarian cancer is within our capabilities.

REFERENCES

1 **Lu KH**, Skates S, Hernandez MA, Bedi D, Bevers T, Leeds L, Moore R, Granai C, Harris S, Newland W, Adeyinka O, Gef-

fen J, Deavers MT, Sun CC, Horick N, Fritsche H, Bast RC. A 2-stage ovarian cancer screening strategy using the Risk of Ovarian Cancer Algorithm (ROCA) identifies early-stage incident cancers and demonstrates high positive predictive value. *Cancer* 2013; **119**: 3454-3461 [PMID: 23983047 DOI: 10.1002/cncr.28183]

2 **Ferlay J**, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893-2917 [PMID: 21351269 DOI: 10.1002/ijc.25516]

3 **Siegel R**, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013; **63**: 11-30 [PMID: 23335087 DOI: 10.3322/caac.21166]

4 **Salani R**, Santillan A, Zahurak ML, Giuntoli RL, Gardner GJ, Armstrong DK, Bristow RE. Secondary cytoreductive surgery for localized, recurrent epithelial ovarian cancer: analysis of prognostic factors and survival outcome. *Cancer* 2007; **109**: 685-691 [PMID: 17219441 DOI: 10.1002/cncr.22447]

5 **Cannistra SA**. Cancer of the ovary. *N Engl J Med* 2004; **351**: 2519-2529 [PMID: 15590954 DOI: 10.1056/NEJMra041842]

6 **Howlader N**, Noone AM, Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA. SEER Cancer Statistics Review, 1975-2010. SEER online, cited 2013-04. Available from: URL: http: //seer.cancer.gov/csr/1975_2010/

7 **Holschneider CH**, Berek JS. Ovarian cancer: epidemiology, biology, and prognostic factors. *Semin Surg Oncol* 2000; **19**: 3-10 [PMID: 10883018 DOI: 10.1002/1098-2388(200007/08)]

8 **Jacobs IJ**, Skates SJ, MacDonald N, Menon U, Rosenthal AN, Davies AP, Woolas R, Jeyarajah AR, Sibley K, Lowe DG, Oram DH. Screening for ovarian cancer: a pilot randomised controlled trial. *Lancet* 1999; **353**: 1207-1210 [PMID: 10217079 DOI: 10.1016/S0140-6736(98)10261-1]

9 **van Nagell JR**, DePriest PD, Reedy MB, Gallion HH, Ueland FR, Pavlik EJ, Kryscio RJ. The efficacy of transvaginal sonographic screening in asymptomatic women at risk for ovarian cancer. *Gynecol Oncol* 2000; **77**: 350-356 [PMID: 10831341 DOI: 10.1006/gyno.2000.5816]

10 **Bast RC**, Brewer M, Zou C, Hernandez MA, Daley M, Ozols R, Lu K, Lu Z, Badgwell D, Mills GB, Skates S, Zhang Z, Chan D, Lokshin A, Yu Y. Prevention and early detection of ovarian cancer: mission impossible? *Recent Results Cancer Res* 2007; **174**: 91-100 [PMID: 17302189 DOI: 10.1007/978-3-540-37696-5_9]

11 **Nossov V**, Amneus M, Su F, Lang J, Janco JM, Reddy ST, Farias-Eisner R. The early detection of ovarian cancer: from traditional methods to proteomics. Can we really do better than serum CA-125? *Am J Obstet Gynecol* 2008; **199**: 215-223 [PMID: 18468571 DOI: 10.1016/j.ajog.2008.04.009]

12 **Nolen BM**, Lokshin AE. Protein biomarkers of ovarian cancer: the forest and the trees. *Future Oncol* 2012; **8**: 55-71 [PMID: 22149035 DOI: 10.2217/fon.11.135]

13 **Cancer Genome Atlas Research Network.** Integrated genomic analyses of ovarian carcinoma. *Nature* 2011; **474**: 609-615 [PMID: 21720365]

14 **Vaughan S**, Coward JI, Bast RC, Berchuck A, Berek JS, Brenton JD, Coukos G, Crum CC, Drapkin R, Etemadmoghadam D, Friedlander M, Gabra H, Kaye SB, Lord CJ, Lengyel E, Levine DA, McNeish IA, Menon U, Mills GB, Nephew KP, Oza AM, Sood AK, Stronach EA, Walczak H, Bowtell DD, Balkwill FR. Rethinking ovarian cancer: recommendations for improving outcomes. *Nat Rev Cancer* 2011; **11**: 719-725 [PMID: 21941283 DOI: 10.1038/nrc3144]

15 **Chen VW**, Ruiz B, Killeen JL, Coté TR, Wu XC, Correa CN. Pathology and classification of ovarian tumors. *Cancer* 2003; **97**: 2631-2642 [PMID: 12733128 DOI: 10.1002/cncr.11345]

16 **Bast RC**, Feeney M, Lazarus H, Nadler LM, Colvin RB, Knapp RC. Reactivity of a monoclonal antibody with human ovarian carcinoma. *J Clin Invest* 1981; **68**: 1331-1337 [PMID: 7028788 DOI: 10.1172/JCI110380]

17 **Weiland F**, Martin K, Oehler MK, Hoffmann P. Deciphering the Molecular Nature of Ovarian Cancer Biomarker CA125. *Int J Mol Sci* 2012; **13**: 10568-10582 [PMID: 22949880 DOI: 10.3390/ijms130810568]

18 **Davelaar EM**, van Kamp GJ, Verstraeten RA, Kenemans P. Comparison of seven immunoassays for the quantification of CA 125 antigen in serum. *Clin Chem* 1998; **44**: 1417-1422 [PMID: 9665418]

19 **Yin BW**, Lloyd KO. Molecular cloning of the CA125 ovarian cancer antigen: identification as a new mucin, MUC16. *J Biol Chem* 2001; **276**: 27371-27375 [PMID: 11369781 DOI: 10.1074/jbc.M103554200]

20 **Kabawat SE**, Bast RC, Bhan AK, Welch WR, Knapp RC, Colvin RB. Tissue distribution of a coelomic-epithelium-related antigen recognized by the monoclonal antibody OC125. *Int J Gynecol Pathol* 1983; **2**: 275-285 [PMID: 6196309 DOI: 10.1097/00004347-198303000-00005]

21 **Jacobs I**, Bast RC. The CA 125 tumour-associated antigen: a review of the literature. *Hum Reprod* 1989; **4**: 1-12 [PMID: 2651469]

22 **Cramer DW**, O'Rourke DJ, Vitonis AF, Matulonis UA, Dijohnson DA, Sluss PM, Crum CP, Liu BC. CA125 immune complexes in ovarian cancer patients with low CA125 concentrations. *Clin Chem* 2010; **56**: 1889-1892 [PMID: 20943848 DOI: 10.1373/clinchem.2010.153122]

23 **Woolas RP**, Xu FJ, Jacobs IJ, Yu YJ, Daly L, Berchuck A, Soper JT, Clarke-Pearson DL, Oram DH, Bast RC Jr. Elevation of multiple serum markers in patients with stage I ovarian cancer. *J Natl Cancer Inst* 1993; **85**: 1748-1751 [PMID: 8411259 DOI: 10.1093/jnci/85.21.1748]

24 **Jacobs IJ**, Skates S, Davies AP, Woolas RP, Jeyerajah A, Weidemann P, Sibley K, Oram DH. Risk of diagnosis of ovarian cancer after raised serum CA 125 concentration: a prospective cohort study. *BMJ* 1996; **313**: 1355-1358 [PMID: 8956699 DOI: 10.1136/bmj.313.7069.1355]

25 **Gentry-Maharaj A**, Menon U. Screening for ovarian cancer in the general population. *Best Pract Res Clin Obstet Gynaecol* 2012; **26**: 243-256 [PMID: 22182415 DOI: 10.1016/j.bpobgyn.2011.11.006]

26 **Skates SJ**, Menon U, MacDonald N, Rosenthal AN, Oram DH, Knapp RC, Jacobs IJ. Calculation of the risk of ovarian cancer from serial CA-125 values for preclinical detection in postmenopausal women. *J Clin Oncol* 2003; **21**: 206s-210s [PMID: 12743136 DOI: 10.1200/JCO.2003.02.955]

27 **Menon U**, Skates SJ, Lewis S, Rosenthal AN, Rufford B, Sibley K, Macdonald N, Dawnay A, Jeyarajah A, Bast RC, Oram D, Jacobs IJ. Prospective study using the risk of ovarian cancer algorithm to screen for ovarian cancer. *J Clin Oncol* 2005; **23**: 7919-7926 [PMID: 16258091 DOI: 10.1200/JCO.2005.01.6642]

28 **Hellström I**, Raycraft J, Hayden-Ledbetter M, Ledbetter JA, Schummer M, McIntosh M, Drescher C, Urban N, Hellström KE. The HE4 (WFDC2) protein is a biomarker for ovarian carcinoma. *Cancer Res* 2003; **63**: 3695-3700 [PMID: 12839961]

29 **Drapkin R**, von Horsten HH, Lin Y, Mok SC, Crum CP, Welch WR, Hecht JL. Human epididymis protein 4 (HE4) is a secreted glycoprotein that is overexpressed by serous and endometrioid ovarian carcinomas. *Cancer Res* 2005; **65**: 2162-2169 [PMID: 15781627 DOI: 10.1158/0008-5472.CAN-04-3924]

30 **Kirchhoff C**, Habben I, Ivell R, Krull N. A major human epididymis-specific cDNA encodes a protein with sequence homology to extracellular proteinase inhibitors. *Biol Reprod* 1991; **45**: 350-357 [PMID: 1686187 DOI: 10.1095/biolreprod45.2.350]

31 **Moore RG**, MacLaughlan S, Bast RC. Current state of biomarker development for clinical application in epithelial ovarian cancer. *Gynecol Oncol* 2010; **116**: 240-245 [PMID:

19879639 DOI: 10.1016/j.ygyno.2009.09.041]
32 **Huhtinen K**, Suvitie P, Hiissa J, Junnila J, Huvila J, Kujari H, Setälä M, Härkki P, Jalkanen J, Fraser J, Mäkinen J, Auranen A, Poutanen M, Perheentupa A. Serum HE4 concentration differentiates malignant ovarian tumours from ovarian endometriotic cysts. *Br J Cancer* 2009; **100**: 1315-1319 [PMID: 19337252 DOI: 10.1038/sj.bjc.6605011]
33 **Moore RG**, Miller MC, Steinhoff MM, Skates SJ, Lu KH, Lambert-Messerlian G, Bast RC. Serum HE4 levels are less frequently elevated than CA125 in women with benign gynecologic disorders. *Am J Obstet Gynecol* 2012; **206**: 351.e1-351.e8 [PMID: 22284961 DOI: 10.1016/j.ajog.2011.12.029]
34 **Leung F**, Diamandis EP, Kulasingam V. From bench to bedside: discovery of ovarian cancer biomarkers using high-throughput technologies in the past decade. *Biomark Med* 2012; **6**: 613-625 [PMID: 23075239 DOI: 10.2217/bmm.12.70]
35 **Ferraro S**, Braga F, Lanzoni M, Boracchi P, Biganzoli EM, Panteghini M. Serum human epididymis protein 4 vs carbohydrate antigen 125 for ovarian cancer diagnosis: a systematic review. *J Clin Pathol* 2013; **66**: 273-281 [PMID: 23426716 DOI: 10.1136/jclinpath-2012-201031]
36 **Nolen B**, Velikokhatnaya L, Marrangoni A, De Geest K, Lomakin A, Bast RC, Lokshin A. Serum biomarker panels for the discrimination of benign from malignant cases in patients with an adnexal mass. *Gynecol Oncol* 2010; **117**: 440-445 [PMID: 20334903 DOI: 10.1016/j.ygyno.2010.02.005]
37 **Moore RG**, Brown AK, Miller MC, Skates S, Allard WJ, Verch T, Steinhoff M, Messerlian G, DiSilvestro P, Granai CO, Bast RC. The use of multiple novel tumor biomarkers for the detection of ovarian carcinoma in patients with a pelvic mass. *Gynecol Oncol* 2008; **108**: 402-408 [PMID: 18061248 DOI: 10.1016/j.ygyno.2007.10.017]
38 **Engelen MJ**, Kos HE, Willemse PH, Aalders JG, de Vries EG, Schaapveld M, Otter R, van der Zee AG. Surgery by consultant gynecologic oncologists improves survival in patients with ovarian carcinoma. *Cancer* 2006; **106**: 589-598 [PMID: 16369985 DOI: 10.1002/cncr.21616]
39 **Vernooij F**, Heintz P, Witteveen E, van der Graaf Y. The outcomes of ovarian cancer treatment are better when provided by gynecologic oncologists and in specialized hospitals: a systematic review. *Gynecol Oncol* 2007; **105**: 801-812 [PMID: 17433422 DOI: 10.1016/j.ygyno.2007.02.030]
40 **Jacobs I**, Oram D, Fairbanks J, Turner J, Frost C, Grudzinskas JG. A risk of malignancy index incorporating CA 125, ultrasound and menopausal status for the accurate preoperative diagnosis of ovarian cancer. *Br J Obstet Gynaecol* 1990; **97**: 922-929 [PMID: 2223684 DOI: 10.1111/j.1471-0528.1990.tb02448.x]
41 **Moore RG**, McMeekin DS, Brown AK, DiSilvestro P, Miller MC, Allard WJ, Gajewski W, Kurman R, Bast RC, Skates SJ. A novel multiple marker bioassay utilizing HE4 and CA125 for the prediction of ovarian cancer in patients with a pelvic mass. *Gynecol Oncol* 2009; **112**: 40-46 [PMID: 18851871 DOI: 10.1016/j.ygyno.2008.08.031]
42 **Zhang Z**, Bast RC, Yu Y, Li J, Sokoll LJ, Rai AJ, Rosenzweig JM, Cameron B, Wang YY, Meng XY, Berchuck A, Van Haaften-Day C, Hacker NF, de Bruijn HW, van der Zee AG, Jacobs IJ, Fung ET, Chan DW. Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 2004; **64**: 5882-5890 [PMID: 15313933 DOI: 10.1158/0008-5472.CAN-04-0746]
43 **Ueland FR**, Desimone CP, Seamon LG, Miller RA, Goodrich S, Podzielinski I, Sokoll L, Smith A, van Nagell JR, Zhang Z. Effectiveness of a multivariate index assay in the preoperative assessment of ovarian tumors. *Obstet Gynecol* 2011; **117**: 1289-1297 [PMID: 21606739 DOI: 10.1097/AOG.0b013e31821b5118]
44 **Karlsen MA**, Sandhu N, Høgdall C, Christensen IJ, Nedergaard L, Lundvall L, Engelholm SA, Pedersen AT, Hartwell D, Lydolph M, Laursen IA, Høgdall EV. Evaluation of HE4, CA125, risk of ovarian malignancy algorithm (ROMA) and risk of malignancy index (RMI) as diagnostic tools of epithelial ovarian cancer in patients with a pelvic mass. *Gynecol Oncol* 2012; **127**: 379-383 [PMID: 22835718 DOI: 10.1016/j.ygyno.2012.07.106]
45 **Moore RG**, Jabre-Raughley M, Brown AK, Robison KM, Miller MC, Allard WJ, Kurman RJ, Bast RC, Skates SJ. Comparison of a novel multiple marker assay *vs* the Risk of Malignancy Index for the prediction of epithelial ovarian cancer in patients with a pelvic mass. *Am J Obstet Gynecol* 2010; **203**: 228.e1-228.e6 [PMID: 20471625 DOI: 10.1016/j.ajog.2010.03.043]
46 **Sandri MT**, Bottari F, Franchi D, Boveri S, Candiani M, Ronzoni S, Peiretti M, Radice D, Passerini R, Sideri M. Comparison of HE4, CA125 and ROMA algorithm in women with a pelvic mass: correlation with pathological outcome. *Gynecol Oncol* 2013; **128**: 233-238 [PMID: 23200911 DOI: 10.1016/j.ygyno.2012.11.026]
47 **Chan KK**, Chen CA, Nam JH, Ochiai K, Wilailak S, Choon AT, Sabaratnam S, Hebbar S, Sickan J, Schodin BA, Sumpaico WW. The use of HE4 in the prediction of ovarian cancer in Asian women with a pelvic mass. *Gynecol Oncol* 2013; **128**: 239-244 [PMID: 23063998 DOI: 10.1016/j.ygyno.2012.09.034]
48 **Håkansson F**, Høgdall EV, Nedergaard L, Lundvall L, Engelholm SA, Pedersen AT, Hartwell D, Høgdall C. Risk of malignancy index used as a diagnostic tool in a tertiary centre for patients with a pelvic mass. *Acta Obstet Gynecol Scand* 2012; **91**: 496-502 [PMID: 22229703 DOI: 10.1111/j.1600-0412.2012.01359.x]
49 **van den Akker PA**, Aalders AL, Snijders MP, Kluivers KB, Samlal RA, Vollebergh JH, Massuger LF. Evaluation of the Risk of Malignancy Index in daily clinical management of adnexal masses. *Gynecol Oncol* 2010; **116**: 384-388 [PMID: 19959215 DOI: 10.1016/j.ygyno.2009.11.014]
50 **Bristow RE**, Smith A, Zhang Z, Chan DW, Crutcher G, Fung ET, Munroe DG. Ovarian malignancy risk stratification of the adnexal mass using a multivariate index assay. *Gynecol Oncol* 2013; **128**: 252-259 [PMID: 23178277 DOI: 10.1016/j.ygyno.2012.11.022]
51 **Nunes N**, Yazbek J, Ambler G, Hoo W, Naftalin J, Jurkovic D. Prospective evaluation of the IOTA logistic regression model LR2 for the diagnosis of ovarian cancer. *Ultrasound Obstet Gynecol* 2012; **40**: 355-359 [PMID: 22223587 DOI: 10.1002/uog.11088]
52 **Longoria TC**, Ueland FR, Zhang Z, Chan DW, Smith A, Fung ET, Munroe DG, Bristow RE. Clinical performance of a multivariate index assay for detecting early-stage ovarian cancer. *Am J Obstet Gynecol* 2014; **210**: 78.e1-78.e9 [PMID: 24055582 DOI: 10.1016/j.ajog.2013.09.017]
53 **Nolen BM**, Lokshin AE. Biomarker testing for ovarian cancer: clinical utility of multiplex assays. *Mol Diagn Ther* 2013; **17**: 139-146 [PMID: 23552992 DOI: 10.1007/s40291-013-0027-6]
54 **Montagnana M**, Lippi G, Ruzzenente O, Bresciani V, Danese E, Scevarolli S, Salvagno GL, Giudici S, Franchi M, Guidi GC. The utility of serum human epididymis protein 4 (HE4) in patients with a pelvic mass. *J Clin Lab Anal* 2009; **23**: 331-335 [PMID: 19774626 DOI: 10.1002/jcla.20340]
55 **Yang Z**, Luo Z, Zhao B, Zhang W, Zhang J, Li Z, Li L. Diagnosis and preoperative predictive value of serum HE4 concentrations for optimal debulking in epithelial ovarian cancer. *Oncol Lett* 2013; **6**: 28-34 [PMID: 23946773]
56 **Havrilesky LJ**, Whitehead CM, Rubatt JM, Cheek RL, Groelke J, He Q, Malinowski DP, Fischer TJ, Berchuck A. Evaluation of biomarker panels for early stage ovarian cancer detection and monitoring for disease recurrence. *Gynecol Oncol* 2008; **110**: 374-382 [PMID: 18584856 DOI: 10.1016/j.ygyno.2008.04.041]
57 **Jacob F**, Meier M, Caduff R, Goldstein D, Pochechueva T, Hacker N, Fink D, Heinzelmann-Schwarz V. No benefit

from combining HE4 and CA125 as ovarian tumor markers in a clinical setting. *Gynecol Oncol* 2011; **121**: 487-491 [PMID: 21420727 DOI: 10.1016/j.ygyno.2011.02.022]

58 **Visintin I**, Feng Z, Longton G, Ward DC, Alvero AB, Lai Y, Tenthorey J, Leiser A, Flores-Saaib R, Yu H, Azori M, Rutherford T, Schwartz PE, Mor G. Diagnostic markers for early detection of ovarian cancer. *Clin Cancer Res* 2008; **14**: 1065-1072 [PMID: 18258665 DOI: 10.1158/1078-0432.CCR-07-1569]

59 **Edgell T**, Martin-Roussety G, Barker G, Autelitano DJ, Allen D, Grant P, Rice GE. Phase II biomarker trial of a multimarker diagnostic for ovarian cancer. *J Cancer Res Clin Oncol* 2010; **136**: 1079-1088 [PMID: 20082099 DOI: 10.1007/s00432-009-0755-5]

60 **Su F**, Lang J, Kumar A, Ng C, Hsieh B, Suchard MA, Reddy ST, Farias-Eisner R. Validation of candidate serum ovarian cancer biomarkers for early detection. *Biomark Insights* 2007; **2**: 369-375 [PMID: 19662218]

61 **Yurkovetsky Z**, Skates S, Lomakin A, Nolen B, Pulsipher T, Modugno F, Marks J, Godwin A, Gorelik E, Jacobs I, Menon U, Lu K, Badgwell D, Bast RC, Lokshin AE. Development of a multimarker assay for early detection of ovarian cancer. *J Clin Oncol* 2010; **28**: 2159-2166 [PMID: 20368574 DOI: 10.1200/JCO.2008.19.2484]

62 **Kim YW**, Bae SM, Lim H, Kim YJ, Ahn WS. Development of multiplexed bead-based immunoassays for the detection of early stage ovarian cancer using a combination of serum biomarkers. *PLoS One* 2012; **7**: e44960 [PMID: 22970327 DOI: 10.1371/journal.pone.0044960]

63 **Amonkar SD**, Bertenshaw GP, Chen TH, Bergstrom KJ, Zhao J, Seshaiah P, Yip P, Mansfield BC. Development and preliminary evaluation of a multivariate index assay for ovarian cancer. *PLoS One* 2009; **4**: e4599 [PMID: 19240799 DOI: 10.1371/journal.pone.0004599]

64 **Skates SJ**, Horick N, Yu Y, Xu FJ, Berchuck A, Havrilesky LJ, de Bruijn HW, van der Zee AG, Woolas RP, Jacobs IJ, Zhang Z, Bast RC. Preoperative sensitivity and specificity for early-stage ovarian cancer when combining cancer antigen CA-125II, CA 15-3, CA 72-4, and macrophage colony-stimulating factor using mixtures of multivariate normal distributions. *J Clin Oncol* 2004; **22**: 4059-4066 [PMID: 15381683 DOI: 10.1200/JCO.2004.03.091]

65 **Zhang B**, Barekati Z, Kohler C, Radpour R, Asadollahi R, Holzgreve W, Zhong XY. Proteomics and biomarkers for ovarian cancer diagnosis. *Ann Clin Lab Sci* 2010; **40**: 218-225 [PMID: 20689132 DOI: 10.1007/s12010-012-9829-y]

66 **Moshkovskii SA**, Serebryakova MV, Kuteykin-Teplyakov KB, Tikhonova OV, Goufman EI, Zgoda VG, Taranets IN, Makarov OV, Archakov AI. Ovarian cancer marker of 11.7 kDa detected by proteomics is a serum amyloid A1. *Proteomics* 2005; **5**: 3790-3797 [PMID: 16121334 DOI: 10.1002/pmic.200401205]

67 **Zhu CS**, Pinsky PF, Cramer DW, Ransohoff DF, Hartge P, Pfeiffer RM, Urban N, Mor G, Bast RC, Moore LE, Lokshin AE, McIntosh MW, Skates SJ, Vitonis A, Zhang Z, Ward DC, Symanowski JT, Lomakin A, Fung ET, Sluss PM, Scholler N, Lu KH, Marrangoni AM, Patriotis C, Srivastava S, Buys SS, Berg CD. A framework for evaluating biomarkers for early detection: validation of biomarker panels for ovarian cancer. *Cancer Prev Res* (Phila) 2011; **4**: 375-383 [PMID: 21372037 DOI: 10.1158/1940-6207.CAPR-10-0193]

68 **Cramer DW**, Bast RC, Berg CD, Diamandis EP, Godwin AK, Hartge P, Lokshin AE, Lu KH, McIntosh MW, Mor G, Patriotis C, Pinsky PF, Thornquist MD, Scholler N, Skates SJ, Sluss PM, Srivastava S, Ward DC, Zhang Z, Zhu CS, Urban N. Ovarian cancer biomarker performance in prostate, lung, colorectal, and ovarian cancer screening trial specimens. *Cancer Prev Res* (Phila) 2011; **4**: 365-374 [PMID: 21372036 DOI: 10.1158/1940-6207.CAPR-10-0195]

69 **Leung F**, Musrap N, Diamandis E, Kulasingam V. Advances in mass spectrometry-based technologies to direct personalized medicine in ovarian cancer. *Translational Proteomics* 2013; **1**: 74-86 [DOI: 10.1016/j.trprot.2013.08.001]

70 **Adamczyk B**, Tharmalingam T, Rudd PM. Glycans as cancer biomarkers. *Biochim Biophys Acta* 2012; **1820**: 1347-1353 [PMID: 22178561 DOI: 10.1016/j.bbagen.2011.12.001]

71 **Tian Y**, Yao Z, Roden RB, Zhang H. Identification of glycoproteins associated with different histological subtypes of ovarian tumors using quantitative glycoproteomics. *Proteomics* 2011; **11**: 4677-4687 [PMID: 22113853 DOI: 10.1002/pmic.201000811]

72 **Hua S**, Williams CC, Dimapasoc LM, Ro GS, Ozcan S, Miyamoto S, Lebrilla CB, An HJ, Leiserowitz GS. Isomer-specific chromatographic profiling yields highly sensitive and specific potential N-glycan biomarkers for epithelial ovarian cancer. *J Chromatogr A* 2013; **1279**: 58-67 [PMID: 23380366 DOI: 10.1016/j.chroma.2012.12.079]

73 **Zhang T**, Wu X, Ke C, Yin M, Li Z, Fan L, Zhang W, Zhang H, Zhao F, Zhou X, Lou G, Li K. Identification of potential biomarkers for ovarian cancer by urinary metabolomic profiling. *J Proteome Res* 2013; **12**: 505-512 [PMID: 23163809 DOI: 10.1021/pr3009572]

74 **Fan L**, Zhang W, Yin M, Zhang T, Wu X, Zhang H, Sun M, Li Z, Hou Y, Zhou X, Lou G, Li K. Identification of metabolic biomarkers to diagnose epithelial ovarian cancer using a UPLC/QTOF/MS platform. *Acta Oncol* 2012; **51**: 473-479 [PMID: 22283470 DOI: 10.3109/0284186X.2011.648338]

75 **Schmidt CW**. Metabolomics: what's happening downstream of DNA. *Environ Health Perspect* 2004; **112**: A410-A415 [PMID: 15159214 DOI: 10.1289/ehp.112-a410]

76 **Lowenthal MS**, Mehta AI, Frogale K, Bandle RW, Araujo RP, Hood BL, Veenstra TD, Conrads TP, Goldsmith P, Fishman D, Petricoin EF, Liotta LA. Analysis of albumin-associated peptides and proteins from ovarian cancer patients. *Clin Chem* 2005; **51**: 1933-1945 [PMID: 16099937 DOI: 10.1373/clinchem.2005.052944]

77 **Philip R**, Murthy S, Krakover J, Sinnathamby G, Zerfass J, Keller L, Philip M. Shared immunoproteome for ovarian cancer diagnostics and immunotherapy: potential theranostic approach to cancer. *J Proteome Res* 2007; **6**: 2509-2517 [PMID: 17547437 DOI: 10.1021/pr0606777]

78 **El Ayed M**, Bonnel D, Longuespée R, Castelier C, Franck J, Vergara D, Desmons A, Tasiemski A, Kenani A, Vinatier D, Day R, Fournier I, Salzet M. MALDI imaging mass spectrometry in ovarian cancer for tracking, identifying, and validating biomarkers. *Med Sci Monit* 2010; **16**: BR233-BR245 [PMID: 20671603]

79 **Zhang B**, Cai FF, Zhong XY. An overview of biomarkers for the ovarian cancer diagnosis. *Eur J Obstet Gynecol Reprod Biol* 2011; **158**: 119-123 [PMID: 21632171 DOI: 10.1016/j.ejogrb.2011.04.023]

80 **Iorio MV**, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Ménard S, Croce CM. MicroRNA signatures in human ovarian cancer. *Cancer Res* 2007; **67**: 8699-8707 [PMID: 17875710 DOI: 10.1158/0008-5472.CAN-07-1936]

81 **Chen Y**, Zhang L, Hao Q. Candidate microRNA biomarkers in human epithelial ovarian cancer: systematic review profiling studies and experimental validation. *Cancer Cell Int* 2013; **13**: 86 [PMID: 23978303 DOI: 10.1186/1475-2867-13-86]

82 **Manegold-Brauer G**, Bellin AK, Tercanli S, Lapaire O, Heinzelmann-Schwarz V. The special role of ultrasound for screening, staging and surveillance of malignant ovarian tumors: distinction from other methods of diagnostic imaging. *Arch Gynecol Obstet* 2014; **289**: 491-498 [PMID: 24253338 DOI: 10.1007/s00404-013-3081-8]

83 **Sharma A**, Apostolidou S, Burnell M, Campbell S, Habib M, Gentry-Maharaj A, Amso N, Seif MW, Fletcher G, Singh N, Benjamin E, Brunell C, Turner G, Rangar R, Godfrey K, Oram D, Herod J, Williamson K, Jenkins H, Mould T,

Woolas R, Murdoch J, Dobbs S, Leeson S, Cruickshank D, Fourkala EO, Ryan A, Parmar M, Jacobs I, Menon U. Risk of epithelial ovarian cancer in asymptomatic women with ultrasound-detected ovarian masses: a prospective cohort study within the UK collaborative trial of ovarian cancer screening (UKCTOCS).*Ultrasound Obstet Gynecol* 2012; **40**: 338-344 [PMID: 22911637 DOI: 10.1002/uog.12270]

84 **van Nagell JR**, DePriest PD, Ueland FR, DeSimone CP, Cooper AL, McDonald JM, Pavlik EJ, Kryscio RJ. Ovarian cancer screening with annual transvaginal sonography: findings of 25,000 women screened. *Cancer* 2007; **109**: 1887-1896 [PMID: 17373668 DOI: 10.1002/cncr.22594]

85 **Pavlik EJ**, Ueland FR, Miller RW, Ubellacker JM, DeSimone CP, Elder J, Hoff J, Baldwin L, Kryscio RJ, van Nagell JR. Frequency and disposition of ovarian abnormalities followed with serial transvaginal ultrasonography. *Obstet Gynecol* 2013; **122**: 210-217 [PMID: 23969786 DOI: 10.1097/AOG.0b013e318298def5]

86 **van Nagell JR**, Miller RW, DeSimone CP, Ueland FR, Podzielinski I, Goodrich ST, Elder JW, Huang B, Kryscio RJ, Pavlik EJ. Long-term survival of women with epithelial ovarian cancer detected by ultrasonographic screening. *Obstet Gynecol* 2011; **118**: 1212-1221 [PMID: 22105249 DOI: 10.1097/AOG.0b013e318238d030]

87 **Jacobs I**, Menon U. Can ovarian cancer screening save lives? The question remains unanswered. *Obstet Gynecol* 2011; **118**: 1209-1211 [PMID: 22105248 DOI: 10.1097/AOG.0b013e31823b49b3]

88 **Kaijser J**, Bourne T, Valentin L, Sayasneh A, Van Holsbeke C, Vergote I, Testa AC, Franchi D, Van Calster B, Timmerman D. Improving strategies for diagnosing ovarian cancer: a summary of the International Ovarian Tumor Analysis (IOTA) studies. *Ultrasound Obstet Gynecol* 2013; **41**: 9-20 [PMID: 23065859 DOI: 10.1002/uog.12323]

89 **Van Gorp T**, Veldman J, Van Calster B, Cadron I, Leunen K, Amant F, Timmerman D, Vergote I. Subjective assessment by ultrasound is superior to the risk of malignancy index (RMI) or the risk of ovarian malignancy algorithm (ROMA) in discriminating benign from malignant adnexal masses. *Eur J Cancer* 2012; **48**: 1649-1656 [PMID: 22226481 DOI: 10.1016/j.ejca.2011.12.003]

90 **Kaijser J**, Van Gorp T, Van Hoorde K, Van Holsbeke C, Sayasneh A, Vergote I, Bourne T, Timmerman D, Van Calster B. A comparison between an ultrasound based prediction model (LR2) and the risk of ovarian malignancy algorithm (ROMA) to assess the risk of malignancy in women with an adnexal mass. *Gynecol Oncol* 2013; **129**: 377-383 [PMID: 23360924 DOI: 10.1016/j.ygyno.2013.01.018]

91 **Campbell S**. Ovarian cancer: role of ultrasound in preoperative diagnosis and population screening. *Ultrasound Obstet Gynecol* 2012; **40**: 245-254 [PMID: 22911634 DOI: 10.1002/uog.12281]

92 **Einhorn N**, Sjövall K, Knapp RC, Hall P, Scully RE, Bast RC, Zurawski VR. Prospective evaluation of serum CA 125 levels for early detection of ovarian cancer. *Obstet Gynecol* 1992; **80**: 14-18 [PMID: 1603484 DOI: 10.1016/0378-5122(93)90150-G]

93 **Campbell S**, Bhan V, Royston P, Whitehead MI, Collins WP. Transabdominal ultrasound screening for early ovarian cancer. *BMJ* 1989; **299**: 1363-1367 [PMID: 2513964 DOI: 10.1136/bmj.299.6712.1363]

94 **Buys SS**, Partridge E, Greene MH, Prorok PC, Reding D, Riley TL, Hartge P, Fagerstrom RM, Ragard LR, Chia D, Izmirlian G, Fouad M, Johnson CC, Gohagan JK. Ovarian cancer screening in the Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening trial: findings from the initial screen of a randomized trial. *Am J Obstet Gynecol* 2005; **193**: 1630-1639 [PMID: 16260202 DOI: 10.1016/j.ajog.2005.05.005]

95 **Partridge E**, Kreimer AR, Greenlee RT, Williams C, Xu JL, Church TR, Kessel B, Johnson CC, Weissfeld JL, Isaacs C, Andriole GL, Ogden S, Ragard LR, Buys SS. Results from four rounds of ovarian cancer screening in a randomized trial. *Obstet Gynecol* 2009; **113**: 775-782 [PMID: 19305319 DOI: 10.1097/AOG.0b013e31819cda77]

96 **Buys SS**, Partridge E, Black A, Johnson CC, Lamerato L, Isaacs C, Reding DJ, Greenlee RT, Yokochi LA, Kessel B, Crawford ED, Church TR, Andriole GL, Weissfeld JL, Fouad MN, Chia D, O'Brien B, Ragard LR, Clapp JD, Rathmell JM, Riley TL, Hartge P, Pinsky PF, Zhu CS, Izmirlian G, Kramer BS, Miller AB, Xu JL, Prorok PC, Gohagan JK, Berg CD. Effect of screening on ovarian cancer mortality: the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Randomized Controlled Trial. *JAMA* 2011; **305**: 2295-2303 [PMID: 21642681 DOI: 10.1001/jama.2011.766]

97 **Klompmaker J**, Jansen HW, Veth RP, de Groot JH, Nijenhuis AJ, Pennings AJ. Porous polymer implant for repair of meniscal lesions: a preliminary study in dogs. *Biomaterials* 1991; **12**: 810-816 [PMID: 1764550 DOI: 10.1111/j.1525-1438.2007.01035.x]

98 **Menon U**, Gentry-Maharaj A, Hallett R, Ryan A, Burnell M, Sharma A, Lewis S, Davies S, Philpott S, Lopes A, Godfrey K, Oram D, Herod J, Williamson K, Seif MW, Scott I, Mould T, Woolas R, Murdoch J, Dobbs S, Amso NN, Leeson S, Cruickshank D, McGuire A, Campbell S, Fallowfield L, Singh N, Dawnay A, Skates SJ, Parmar M, Jacobs I. Sensitivity and specificity of multimodal and ultrasound screening for ovarian cancer, and stage distribution of detected cancers: results of the prevalence screen of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS). *Lancet Oncol* 2009; **10**: 327-340 [PMID: 19282241 DOI: 10.1016/S1470-2045(09)70026-9]

99 **Menon U**, Kalsi J, Jacobs I. The UKCTOCS experience--reasons for hope? *Int J Gynecol Cancer* 2012; **22** Suppl 1: S18-S20 [PMID: 22543913 DOI: 10.1097/IGC.0b013e318251cb47]

100 **Pinsky PF**, Zhu C, Skates SJ, Black A, Partridge E, Buys SS, Berg CD. Potential effect of the risk of ovarian cancer algorithm (ROCA) on the mortality outcome of the Prostate, Lung, Colorectal and Ovarian (PLCO) trial. *Int J Cancer* 2013; **132**: 2127-2133 [PMID: 23065684 DOI: 10.1002/ijc.27909]

101 **Menon U**, Gentry-Maharaj A, Jacobs I. Ovarian cancer screening and mortality. *JAMA* 2011; **306**: 1544; author reply 1544-1545 [PMID: 21990294 DOI: 10.1001/jama.2011.1461]

102 **Vine MF**, Calingaert B, Berchuck A, Schildkraut JM. Characterization of prediagnostic symptoms among primary epithelial ovarian cancer cases and controls. *Gynecol Oncol* 2003; **90**: 75-82 [PMID: 12821345 DOI: 10.1016/S0090-8258(03)00175-6]

103 **Goff BA**, Lowe KA, Kane JC, Robertson MD, Gaul MA, Andersen MR. Symptom triggered screening for ovarian cancer: a pilot study of feasibility and acceptability. *Gynecol Oncol* 2012; **124**: 230-235 [PMID: 22063459 DOI: 10.1016/j.ygyno.2011.10.032]

104 **Rufford BD**, Jacobs IJ, Menon U. Feasibility of screening for ovarian cancer using symptoms as selection criteria. *BJOG* 2007; **114**: 59-64 [PMID: 17233861 DOI: 10.1111/j.1471-0528.2006.01153.x]

105 **Goff BA**, Mandel LS, Drescher CW, Urban N, Gough S, Schurman KM, Patras J, Mahony BS, Andersen MR. Development of an ovarian cancer symptom index: possibilities for earlier detection. *Cancer* 2007; **109**: 221-227 [PMID: 17154394 DOI: 10.1002/cncr.22371]

106 **Rossing MA**, Wicklund KG, Cushing-Haugen KL, Weiss NS. Predictive value of symptoms for early detection of ovarian cancer. *J Natl Cancer Inst* 2010; **102**: 222-229 [PMID: 20110551 DOI: 10.1093/jnci/djp500]

107 **Cass I**, Karlan BY. Ovarian cancer symptoms speak out--but what are they really saying? *J Natl Cancer Inst* 2010; **102**: 211-212 [PMID: 20110550 DOI: 10.1093/jnci/djp525]

108 **Pitta Dda R**, Sarian LO, Barreta A, Campos EA, Andrade LL, Fachini AM, Campbell LM, Derchain S. Symptoms,

CA125 and HE4 for the preoperative prediction of ovarian malignancy in Brazilian women with ovarian masses. *BMC Cancer* 2013; **13**: 423 [PMID: 24044637 DOI: 10.1186/1471-2407-13-423]

109 **Rosenthal AN**, Fraser L, Manchanda R, Badman P, Philpott S, Mozersky J, Hadwin R, Cafferty FH, Benjamin E, Singh N, Evans DG, Eccles DM, Skates SJ, Mackay J, Menon U, Jacobs IJ. Results of annual screening in phase I of the United Kingdom familial ovarian cancer screening study highlight the need for strict adherence to screening schedule. *J Clin Oncol* 2013; **31**: 49-57 [PMID: 23213100 DOI: 10.1200/JCO.2011.39.7638]

110 **Bolton KL**, Ganda C, Berchuck A, Pharaoh PD, Gayther SA. Role of common genetic variants in ovarian cancer susceptibility and outcome: progress to date from the Ovarian Cancer Association Consortium (OCAC). *J Intern Med* 2012; **271**: 366-378 [PMID: 22443200 DOI: 10.1111/j.1365-2796.2011.02509.x]

111 **Long KC**, Kauff ND. Screening for familial ovarian cancer: a ray of hope and a light to steer by. *J Clin Oncol* 2013; **31**: 8-10 [PMID: 23213103 DOI: 10.1200/JCO.2012.45.4678]

112 **Greene MH**, Piedmonte M, Alberts D, Gail M, Hensley M, Miner Z, Mai PL, Loud J, Rodriguez G, Basil J, Boggess J, Schwartz PE, Kelley JL, Wakeley KE, Minasian L, Skates S. A prospective study of risk-reducing salpingo-oophorectomy and longitudinal CA-125 screening among women at increased genetic risk of ovarian cancer: design and baseline characteristics: a Gynecologic Oncology Group study. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 594-604 [PMID: 18349277 DOI: 10.1158/1055-9965.EPI-07-2703]

113 **National Comprehensive Cancer Network.** NCCN Clinical Practice Guidelines in Oncology-Genetic/Familial High-Risk Assessment: Breast and Ovarian Cancer. Version 1, 2014

114 **Nik NN**, Vang R, Shih IeM, Kurman RJ. Origin and pathogenesis of pelvic (ovarian, tubal, and primary peritoneal) serous carcinoma. *Annu Rev Pathol* 2014; **9**: 27-45 [PMID: 23937438 DOI: 10.1146/annurev-pathol-020712-163949]

115 **Wiegand KC**, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, Senz J, McConechy MK, Anglesio MS, Kalloger SE, Yang W, Heravi-Moussavi A, Giuliany R, Chow C, Fee J, Zayed A, Prentice L, Melnyk N, Turashvili G, Delaney AD, Madore J, Yip S, McPherson AW, Ha G, Bell L, Fereday S, Tam A, Galletta L, Tonin PN, Provencher D, Miller D, Jones SJ, Moore RA, Morin GB, Oloumi A, Boyd N, Aparicio SA, Shih IeM, Mes-Masson AM, Bowtell DD, Hirst M, Gilks B, Marra MA, Huntsman DG. ARID1A mutations in endometriosis-associated ovarian carcinomas. *N Engl J Med* 2010; **363**: 1532-1543 [PMID: 20942669 DOI: 10.1056/NEJMoa1008433]

116 **Kurman RJ**, Shih IeM. Molecular pathogenesis and extra-ovarian origin of epithelial ovarian cancer--shifting the paradigm. *Hum Pathol* 2011; **42**: 918-931 [PMID: 21683865 DOI: 10.1016/j.humpath.2011.03.003]

117 **Kurman RJ**, Shih IeM. Pathogenesis of ovarian cancer: lessons from morphology and molecular biology and their clinical implications. *Int J Gynecol Pathol* 2008; **27**: 151-160 [PMID: 18317228 DOI: 10.1097/PGP.0b013e318161e4f5]

118 **Rauh-Hain JA**, Krivak TC, Del Carmen MG, Olawaiye AB. Ovarian cancer screening and early detection in the general population. *Rev Obstet Gynecol* 2011; **4**: 15-21 [PMID: 21629494]

119 **Lim D**, Oliva E. Precursors and pathogenesis of ovarian carcinoma. *Pathology* 2013; **45**: 229-242 [PMID: 23478230 DOI: 10.1097/PAT.0b013e32835f2264]

120 **Kurman RJ**, Visvanathan K, Roden R, Wu TC, Shih IeM. Early detection and treatment of ovarian cancer: shifting from early stage to minimal volume of disease based on a new model of carcinogenesis. *Am J Obstet Gynecol* 2008; **198**: 351-356 [PMID: 18395030 DOI: 10.1016/j.ajog.2008.01.005]

121 **Pepe MS**, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, Winget M, Yasui Y. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst* 2001; **93**: 1054-1061 [PMID: 11459866 DOI: 10.1093/jnci/93.14.1054]

122 **Kulasingam V**, Pavlou MP, Diamandis EP. Integrating high-throughput technologies in the quest for effective biomarkers for ovarian cancer. *Nat Rev Cancer* 2010; **10**: 371-378 [PMID: 20383179 DOI: 10.1038/nrc2831]

123 **Leung F**, Dimitromanolakis A, Kobayashi H, Diamandis EP, Kulasingam V. Folate-receptor 1 (FOLR1) protein is elevated in the serum of ovarian cancer patients. *Clin Biochem* 2013; **46**: 1462-1468 [PMID: 23528302 DOI: 10.1016/j.clinbiochem.2013.03.010]

124 **Anderson GL**, McIntosh M, Wu L, Barnett M, Goodman G, Thorpe JD, Bergan L, Thornquist MD, Scholler N, Kim N, O'Briant K, Drescher C, Urban N. Assessing lead time of selected ovarian cancer biomarkers: a nested case-control study. *J Natl Cancer Inst* 2010; **102**: 26-38 [PMID: 20042715 DOI: 10.1093/jnci/djp438]

125 **Pepe MS**, Feng Z. Improving biomarker identification with better designs and reporting. *Clin Chem* 2011; **57**: 1093-1095 [PMID: 21666069 DOI: 10.1373/clinchem.2011.164657]

126 **Feng Z**, Kagan J, Pepe M, Thornquist M, Ann Rinaudo J, Dahlgren J, Krueger K, Zheng Y, Patriotis C, Huang Y, Sorbara L, Thompson I, Srivastava S. The Early Detection Research Network's Specimen reference sets: paving the way for rapid evaluation of potential biomarkers. *Clin Chem* 2013; **59**: 68-74 [PMID: 23193062 DOI: 10.1373/clinchem.2012.185140]

127 **Reade CJ**, Riva JJ, Busse JW, Goldsmith CH, Elit L. Risks and benefits of screening asymptomatic women for ovarian cancer: a systematic review and meta-analysis. *Gynecol Oncol* 2013; **130**: 674-681 [PMID: 23822892 DOI: 10.1016/j.ygyno.2013.06.029]

128 American College of Obstetrics and Gynecologists Committee on Gynecologic Practice. Committee Opinion No. 477: the role of the obstetrician-gynecologist in the early detection of epithelial ovarian cancer. *Obstet Gynecol* 2011; **117**: 742-746 [PMID: 21343791 DOI: 10.1097/AOG.0b013e31821477db]

129 **Moyer VA**. Screening for ovarian cancer: U.S. Preventive Services Task Force reaffirmation recommendation statement. *Ann Intern Med* 2012; **157**: 900-904 [PMID: 22964825 DOI: 10.7326/0003-4819-157-11-201212040-00539]

130 **Smith RA**, Brooks D, Cokkinides V, Saslow D, Brawley OW. Cancer screening in the United States, 2013: a review of current American Cancer Society guidelines, current issues in cancer screening, and new guidance on cervical cancer screening and lung cancer screening. *CA Cancer J Clin* 2013; **63**: 88-105 [PMID: 23378235 DOI: 10.3322/caac.21174]

131 **Pavlik EJ**, van Nagell JR. Ovarian cancer screening--what women want. *Int J Gynecol Cancer* 2012; **22** Suppl 1: S21-S23 [PMID: 22543915 DOI: 10.1097/IGC.0b013e318251cbc2]

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TOPIC HIGHLIGHT

Lei Zheng, MD, PhD, Assistant Professor, *Series Editor*

MicroRNA regulation network in colorectal cancer metastasis

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Abstract

Colorectal cancer is the third most common cancer worldwide. Metastasis is a major cause of colorectal cancer-related death. Mechanisms of metastasis remain largely obscure. MicroRNA is one of the most important epigenetic regulators by targeting mRNAs post-transcriptionally. Accumulated evidence has supported its significant role in the metastasis of colorectal cancer, including epithelial-mesenchymal transition and angiogenesis. Dissecting microRNAome potentially identifies specific microRNAs as biomarkers of colorectal cancer metastasis. Better understanding of the complex network of microRNAs in colorectal cancer metastasis provide new insights in the biological process of metastasis and in the potential targets for colorectal cancer therapies and for diagnosis of recurrent and metastatic colorectal cancer.

Key words: MicroRNAs; Colorectal neoplasms; Neoplasm metastasis

Core tip: MicroRNA is one of the most important epigenetic regulators by targeting mRNAs post-transcriptionally. This article has reviewed the new evidence that has supported the significant role of microRNAs in the metastasis of colorectal cancer. Better understanding of the complex network of microRNAs in colorectal cancer metastasis provide new insights in the biological process of metastasis and in the potential targets for colorectal cancer therapies and for diagnosis of recurrent and metastatic colorectal cancer.

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INTRODUCTION

Colorectal cancer is the third most common cancer in both males and females worldwide. The major cause of death from colorectal cancer is the development of metastatic diseases. For those patients who have metastatic diseases at the time of diagnosis, five-year survival rate is only 12%[1,2]. However, the molecular mechanism of metastasis still remains obscure. Multiple cellular character-

istics of neoplastic cells including epithelial-mesenchymal transition (EMT), invasion and migration, and changes in the tumor microenvironment such as angiogenesis are believed to be important for the metastatic process. Genetic abnormalities accounting for metastasis have not been identified while accumulated evidence suggests that microRNAs may play a crucial role in the epigenetic regulation of the multi-step process of metastasis.

MicroRNAs are a family of small non-coding 18-22nt RNAs, which function in the post-transcriptional regulation of gene expression, by targeting mRNA for cleavage or translational repression[3]. In the nucleus, the DNA coding sequences of miRNA are firstly transcribed into pri-miRNA by RNA polymerase Ⅱ. After transcription, the enzyme named Drosha cuts pri-miRNA into pre-microRNA, which is then transported into cytoplasm and further cleaved into mature microRNA by Dicer. Only one strand of the mature microRNA will be incorporated into RNA-induced silencing complex (RISC), where microRNA play its silencing functions by interacting its target mRNA[4].

Accumulated evidence has suggested that microRNAs directly participate in the tumorigenesis process of colorectal cancer, especially through the posttranscriptional regulation. Some microRNAs play roles in colorectal tumorigenesis by regulating the functional pathways of tumor suppressor genes. For example, microRNAs including miR-135a/b and miR-122a were able to inactivate the adenomatous polyposis coli (APC) tumor suppressor gene and the APC-mediated pathways, providing an alternative, epigenetic mechanism for the inactivation of this tumor suppressor gene. In the APC-mutated mouse model, microRNAs including miR-31, miR-137 and miR-215 were found to be differentially regulated in association with colorectal adenoma formation, suggesting that these microRNAs may be regulated by the APC pathway and are involved in the early stage of colorectal cancer development[5-7]. Similarly, microRNAs including miR-34, miR-145 and miR-107 are regulated by p53 to mediate the function of p53 in cell survival, proliferation and angiogenesis, respectively[8,9]. Therefore, it has been well established that microRNA is involved in the initial stage of colorectal tumorigenesis through the APC pathway and in the advanced stage of colorectal tumorigenesis through the p53 pathway. More recently, a large body of evidence has also supported the role of microRNAs in colorectal cancer metastasis. Here we will review the function of microRNAs in the regulation of EMT and angiogenesis-two biological processes that are important for colorectal cancer metastasis.

DISSECT MICRORNAOME IN COLORECTAL CANCER AND METASTASIS

To discover aberrantly expressed microRNAs in colorectal cancer, microarray analysis was commonly used for dissecting microRNAome[10]. A microRNA microarray analysis in a discovery cohort of 84 colorectal cancer patients comparing microRNA expression profiles between colorectal tumors and paired non-tumorous tissues identified 37 microRNAs differentially expressed in colorectal cancer. Five microRNAs (miR-20a, miR-21, miR-106a, miR-181b, and miR-203) that were most differentially expressed between tumors and non-tumorous tissues and whose expression levels were also associated with patient survivals were selected for further validation. In the validation cohort, it was found that miR-21 was preferentially expressed in colorectal tumors at more advanced TNM stages. In both discovery and validation cohorts, higher miR-21 expression level was significantly associated with poorer survival[11]. Similarly, in another study, 49 microRNAs were identified to be significantly differentially expressed comparing between rectal cancer and adjacent non-tumorous mucosa. Among them, miR-135b was shown to be significantly correlated to disease-free and cancer-specific survival in the validation cohort[12].

Although these microRNAome studies included patients across different stages, there is still a lack of identification of microRNAs associated specifically with metastatic colorectal cancer. Shen *et al*[13] compared the miRNA expression profiles between colorectal cancers with liver metastasis and those without metastasis and found 28 differentially expressed microRNAs. Among them, four microRNAs (miR-150*, miR-125b-2*, miR-1179 and miR-139-3p) are up-regulated in colorectal cancer with metastasis. Although this study provided microRNAome data for colorectal cancer with metastasis, the sample size was small, including only 3 paired samples; and the clinical survival data was not available.

While the microRNAome specific for colorectal cancer metastasis is still highly desired, the research on microRNAome continues to strive ahead with microarray analysis on both tumoral and stromal tissues of colorectal cancer and with microarray analysis on colorectal tumors at different locations, of different subtypes, and with different mismatch repair status. Single nucleotide polymorphism (SNP) variations in microRNA coding sequences and 3'-UTR have been studied[14-18]. These new researches have shed lights on the future research direction of miroRNA in metastatic colorectal cancer.

MICRORNAS IN PATHWAYS OF COLORECTAL CANCER METASTASIS

Despite genetic mutation is still considered as one of the key characteristics in the primary occurrence of colorectal cancer, the metastasis of colorectal cancer appears to be closely associated with epigenetic regulation, such as DNA promoter methylation, histone modification and microRNAs[19,20]. microRNAs have been demonstrated to be involved in two major pathways of colorectal cancer metastasis, EMT and angiogenesis.

MicroRNAs in epithelial-mesenchymal transition

EMT is the biologic process that the polarized epithelial cells transit to a mesenchymal cell phenotype. During

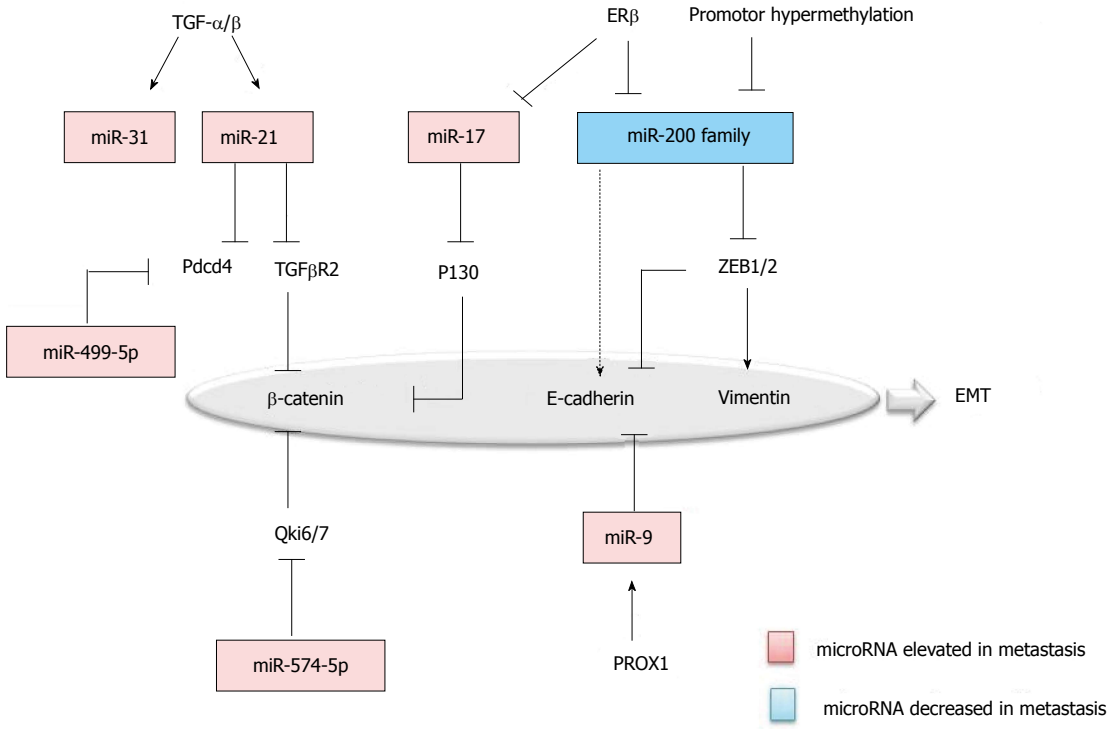

Figure 1 MicroRNAs regulation in epithelial–mesenchymal transition of colorectal cancer metastasis. EMT: Epithelial–mesenchymal transition; PROX1: Prospero Homeobox 1.

the course of metastasis, neoplastic cells undergo EMT, suggesting a mechanistic role of EMT in metastasis. In EMT, proteins such as Vimentin, β-Catenin, TCF8-ZEB1, E-Cadherin, Snail and Slug, are known to be specifically up or down-regulated and have been established as the markers of EMT[21]. Similarly, microRNAs associated with colorectal cancer, were also found to be specifically regulated in EMT (Figure 1).

First, TGF-β/Wnt signaling pathway is one of the prominent pathways in EMT. miR-21 and miR-31 were uncovered as the downstream effectors of TGF-β in colon carcinoma cells[22]. miR-21 is significantly elevated in the colorectal cancer with metastasis and negatively regulates the tumor suppressor programmed cell death 4 (Pdcd4) and TGF-β receptor 2, accompanied by the decrease of the downstreaming β-catenin[23,24]. Second, β-catenin was inversely correlated with Qki6/7 and P130, which are down-regulated by miR-574-5p and miR-17, respectively in colorectal carcinoma cells[25,26]. Third, the miR-200 Family was recognized as a master regulator of the epithelial phenotype, which is decreased in colorectal cancer with metastasis. The miR-200 family repressed the EMT by targeting the ZEB1/2, which down-regulate E-cadherin and up-regulate Vimentin. Furthermore, in the upstream of the miR-200 family, promoter methylation and ERβ can be the cause of decrease in colorectal metastasis[25,27,28]. Fourth, miR-9 has been suggested to be another regulator for E-cadherin. Prospero Homeobox 1 (PROX1) was shown to promote EMT by inhibiting E-cadherin *via* miR-9 in colon carcinoma cells[29,30]. Last, but not the least, microRNAs like miR-499-5p and miR-212 also function in regulating EMT, by targeting Pdcd4 and manganese superoxide dismutase (Mn-SOD)[31,32].

The mesenchymal to epithelial transition (MET) is a reverse biological process of EMT and has recently been suggested to be important for the metastatic cancer cells, by regaining epithelial properties, to establish their colonization in distant organs. MiR-147 was recently found to be able to induce MET in colon cancer cell lines by targeting the TGF-β signaling pathway[33]. Further exploring the roles of microRNAs in MET may provide a deep insight into how the MET is executed in the cancer metastasis.

Thus, many characteristic markers of EMT such as Vimentin, β-Catenin, TCF8-ZEB1, E-Cadherin, Snail and Slug, are targeted by microRNAs. Moreover, many microRNAs were found to have their targets in different types of cancer. For one example, miR-200, by targeting ZEB-1/2, is a tumor suppressor in both breast cancer and colorectal cancer. For another example, ectopic expression of miR-17 plays a role in regulating cancer cell invasion and migration of other malignancy types including colorectal cancer, breast cancer, head and neck cancer by targeting the TGF-β signaling pathway[34]. Therefore, understanding the roles of microRNAs in colorectal cancer metastasis may identify potential therapeutic targets for the treatment of many different types of malignancies.

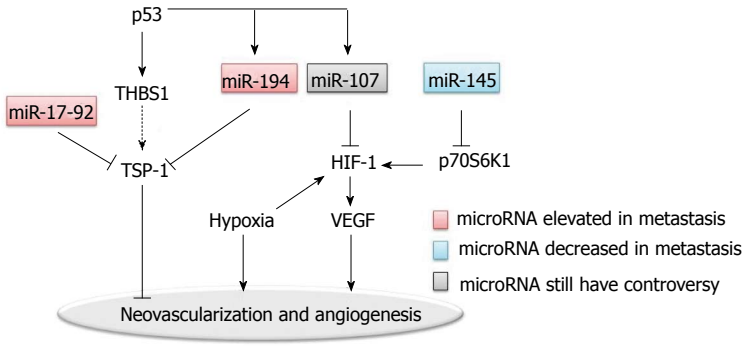

Figure 2 MicroRNAs regulation in the angiogenesis. HIF: Hypoxia inducible factor; VEGF: Vascular endothelial growth factors; THBS1: Thrombospondin; TSP-1: Thrombospondin-1.

MicroRNAs in angiogenesis

Angiogenesis is considered to be essential for the establishment of blood supply for metastatic lesions in the distant organs. Many pro-angiogenic and anti-angiogenic factors are involved (Figure 2). Tumor angiogenesis is associated with intravasation and extravasation of metastatic cancer cells, suggesting that many crucial molecules in angiogenesis and their corresponding receptors participate in the metastatic process[35]. One of the best studied molecular mechanisms involves the vascular endothelial growth factors (VEGF) and the VEGF receptors. In addition, intratumoral blood vessel is the main route of chemotherapy or targeted drug delivery; but the effectiveness of drug delivery is unsatisfactory due to the disorganized neovascularization in tumors. Clinically, VEGF targeted therapy is used for treating metastatic colorectal cancer, supporting the role of angiogenesis in colorectal cancer metastasis[36,37].

Hypoxia is one of the dominant characteristics of tumor microenvironment; and hypoxia inducible factor (HIF)-1 is one of the key regulators in hypoxia induced angiogenesis[38]. Hypoxic microenvironment drives tumor cells to undergo an angiogenic switch which leads to the production of pro-angiogenesis proteins such as VEGF. The VEGF pathway-mediated neovascularization is also driven partly by additional hypoxia responsive signals including basic FGF (bFGF) and placental growth factor (PlGF).

Epigenetic regulation *via* microRNAs may be one of the major regulatory mechanisms for the disorganization of neovascularization in tumor[39]. MiR-107 was shown to function as a suppresser of HIF-1 and VEGF expression[40]. In addition to miR-107, miR-145 was found to be a regulator of HIF-1 in colon cancer, by targeting p70S6K1 post-transcriptionally[42].

Another well characterized regulator for tumor angiogenesis is thrombospondin-1 (TSP-1), which belongs to the thrombospondins family[43]. TSP-1 acts as a barrier to neovascularization in tumors. MiR-17-92 and miR-194 were both found to repress TSP-1, thereby promoting the angiogenesis in colon cancers[40,44]. Interestingly, MiR-17-92 is a polycistronic microRNA cluster. The precursor transcript derived from this cluster gene produces six mature microRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1[45]. Overexpression of the miR-17-92 cluster was observed in multiple tumor types including colon cancer. As a polycistronic cluster, it can coordinate multiple functions in tumorigenesis including increasing angiogenesis, promoting proliferation and inhibiting differentiation[45,46]. The miR-17-92 microRNA cluster is known to be regulated by the Myc oncogene. The Myc-activated miR-17-92 can stimulate tumor angiogenesis by attenuating the TGF-β signaling pathway, which provides an alternative target for miR-17-92 in addition to TSP-1[47].

Both miR-17-92 and miR-194 are upregulated by the p53 tumor suppressor. miR-194 also negatively regulates the thrombospondin (THBS1) mRNA. On another hand, it is known that p53 induces the transcription of THBS1, which would have further induced the expression of TSP-1, since enhanced expression of THBS1 leads to the induction of TSP-1. Interestingly, p53 does not induce the expression of TSP-1. Thus, it is possible that TSP-1 is suppressed by miR-194 and miR-17-92, which both are regulated by p53[40]. Such a complex regulatory network, by providing multiple feedback mechanisms, allows a more precise regulation in angiogenesis.

MICRORNAS AS POTENTIAL DIAGNOSTIC MARKERS FOR METASTASIS IN COLORECTAL CANCER

MicroRNA is studied as a potential diagnostic marker, considering its commonly conserved existence and remarkable stability[48]. Although plasma CEA has been used as a diagnostic marker for colorectal cancer for decades, one study had shown that only 59% of 417 monitored patients with recurrence had a preceding elevation of CEA concentration[49]. Several recent studies thus have been conducted on the identification of plasma circulating microRNAs, attempting to develop more sensitive and specific detection methods[50-53]. Especially, methods for detecting the circulating microRNAs are attracting because of their noninvasive nature. Recently, a panel of 8 plasma microRNAs (miR-532-3p, miR-331, miR-195, miR-17, miR-142-3p, miR-15b, miR-532, and miR-652) was found to be able to distinguish colonic polyps from healthy controls. In addition, this study pointed out that a panel of 3 plasma miRNAs (miR-431,miR-15b, and miR-139-3p) distinguished Stage IV CRC from controls[54].

However, there is still a lack of circulating microR-

Table 1 Summary of microRNAs involved in the tumorigenesis and metastasis of colorectal cancer

	microRNA	Description	Ref.
microRNAs differentially expressed in colorectal cancers	miR-21	Associated to more advanced TNM stages and poorer survival	[11]
	miR-135b	Associated to disease-free and cancer-specific survival	[12]
	miR-150*, miR-125b-2*, miR-1179 and miR-139-3p	Up-regulated in colorectal cancer with metastasis.	[13]
microRNAs as diagnostic markers	Serum miR-29a	Up-regulated in patients with metastasis	[55]
	Circulating miR-221	Associated to poor survival	[53]
	Plasma miR-31	Up-regulated in stage Ⅲ and Ⅳ	[51]
microRNAs involved in epithelial-mesenchymal transition	miR-21	Pdcd4,TGFβ receptor 2	[23,24]
	miR-574-5p,miR-17	Qki6/7,P130	[25,26]
	miR-200	ZEB1/2	[27,28]
	miR-9	E-cadherin	[29,30]
	miR-499-5p	Pdcd4	[31]
	miR-212	MnSOD	[32]
microRNAs involved in Angiogenesis	miR-17-92	TSP-1	[44]
	miR-194	TSP-1,THBS1	[40]
	miR-107	HIF-1,VEGF,DAPK,KLF4	[41]
	miR-145	p70S6K1	[42]

MnSOD: Manganese superoxide dismutase; TSP-1: Thrombospondin-1; THBS1: Thrombospondin; HIF: Hypoxia inducible factor; VEGF: Vascular endothelial growth factors.

NAs as an ideal biomarker for recurrence and metastasis. Plasma miR-92 and miR-29 were significantly elevated in colorectal cancer patients, but demonstrated no significant difference between different stages of colorectal cancer[50,52]. Nevertheless, in a more recent study by Wang *et al*[55] serum level of the miR-29a was found to be significantly higher in colorectal cancer patients with metastasis comparing to those without metastasis, with a sensitivity of 75% and a specificity of 75%. Similarly, circulating miR-221 was demonstrated to be a significant prognostic factor associated with poor overall survival. Whether it can be used for early detection of recurrence is not studied[53]. Another two more recent studies found several promising microRNAs for the detection of recurrence. Kanaan *et al*[51] demonstrated that plasma miR-31, miR-135b, miR-1 and miR-133a have a 100% sensitivity and a 80% specificity in detecting colorectal cancer, and also found that miR-31 was significantly more upregulated in stage Ⅲ and Ⅳ than stage Ⅰ and Ⅱ. Hofsli *et al*[56] reported that in their "training" study conducted with with serum samples from 30 patients with stage Ⅳ colon cancer and from 10 healthy controls, 375 miRNAs were found to be more abundant in the sera from colon cancer patients than those from healthy controls, including miR-103, miR-107, and miR-221. These miRNAs were also found in sera from patients with stage Ⅰ-Ⅱ colon cancer; however, their roles in stage Ⅳ colon cancer remain interesting to be explored.

CONCLUSION

microRNAs play a substantial role in the epigenetic regulation of colorectal cancer metastasis. Differential expression of microRNAs is reported in the metastasis of colorectal cancer comparing to non-metastatic colorectal cancer (Table 1). More importantly, these microRNAs appear to form a network to coordinate the regulation of the metastatic process. Studies investigating the association of microRNAs with the metastasis process are highly desired. Plasma microRNA remains to be identified as a noninvasive biomarker for early diagnosis of colorectal cancer metastasis. Enlightened by the function of microRNAs in the EMT process and angiogenesis, mechanisms of colorectal cancer metastasis may be revealed by dissecting the regulatory network of microRNAs.

REFERENCES

1 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

2 **American Cancer Society**. Cancer Facts & Figures 2013. Atlanta: American Cancer Society, 2013

3 **Bartel DP**. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281-297 [PMID: 14744438]

4 **Lee YS**, Dutta A. MicroRNAs in cancer. *Annu Rev Pathol* 2009; **4**: 199-227 [PMID: 18817506 DOI: 10.1146/annurev.pathol.4.110807.092222]

5 **Nagel R**, le Sage C, Diosdado B, van der Waal M, Oude Vrielink JA, Bolijn A, Meijer GA, Agami R. Regulation of the adenomatous polyposis coli gene by the miR-135 family in colorectal cancer. *Cancer Res* 2008; **68**: 5795-5802 [PMID: 18632633 DOI: 10.1158/0008-5472.CAN-08-0951]

6 **Necela BM**, Carr JM, Asmann YW, Thompson EA. Differential expression of microRNAs in tumors from chronically inflamed or genetic (APC(Min/+)) models of colon cancer. *PLoS One* 2011; **6**: e18501 [PMID: 21532750 DOI: 10.1371/journal.pone.0018501]

7 **Wang X**, Lam EK, Zhang J, Jin H, Sung JJ. MicroRNA-122a functions as a novel tumor suppressor downstream of adenomatous polyposis coli in gastrointestinal cancers. *Biochem Biophys Res Commun* 2009; **387**: 376-380 [PMID: 19607815 DOI: 10.1016/j.bbrc.2009.07.034]

8 **Feng Z**, Zhang C, Wu R, Hu W. Tumor suppressor p53 meets microRNAs. *J Mol Cell Biol* 2011; **3**: 44-50 [PMID: 21278451 DOI: 10.1093/jmcb/mjq040]

9 **He L**, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, Xue W, Zender L, Magnus J, Ridzon D, Jackson AL, Linsley PS, Chen C, Lowe SW, Cleary MA, Hannon GJ. A microRNA component of the p53 tumour suppressor network. *Nature* 2007; **447**: 1130-1134 [PMID: 17554337 DOI: 10.1038/na-

ture05939]
10 **Cummins JM**, He Y, Leary RJ, Pagliarini R, Diaz LA, Sjoblom T, Barad O, Bentwich Z, Szafranska AE, Labourier E, Raymond CK, Roberts BS, Juhl H, Kinzler KW, Vogelstein B, Velculescu VE. The colorectal microRNAome. *Proc Natl Acad Sci USA* 2006; **103**: 3687-3692 [PMID: 16505370 DOI: 10.1073/pnas.0511155103]
11 **Schetter AJ**, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 2008; **299**: 425-436 [PMID: 18230780 DOI: 10.1001/jama.299.4.425]
12 **Gaedcke J**, Grade M, Camps J, Søkilde R, Kaczkowski B, Schetter AJ, Difilippantonio MJ, Harris CC, Ghadimi BM, Møller S, Beissbarth T, Ried T, Litman T. The rectal cancer microRNAome--microRNA expression in rectal cancer and matched normal mucosa. *Clin Cancer Res* 2012; **18**: 4919-4930 [PMID: 22850566 DOI: 10.1158/1078-0432.CCR-12-0016]
13 **Lin M**, Chen W, Huang J, Gao H, Ye Y, Song Z, Shen X. MicroRNA expression profiles in human colorectal cancers with liver metastases. *Oncol Rep* 2011; **25**: 739-747 [PMID: 21174058 DOI: 10.3892/or.2010.1112]
14 **Balaguer F**, Moreira L, Lozano JJ, Link A, Ramirez G, Shen Y, Cuatrecasas M, Arnold M, Meltzer SJ, Syngal S, Stoffel E, Jover R, Llor X, Castells A, Boland CR, Gironella M, Goel A. Colorectal cancers with microsatellite instability display unique miRNA profiles. *Clin Cancer Res* 2011; **17**: 6239-6249 [PMID: 21844009 DOI: 10.1158/1078-0432.CCR-11-1424]
15 **Landi D**, Gemignani F, Landi S. Role of variations within microRNA-binding sites in cancer. *Mutagenesis* 2012; **27**: 205-210 [PMID: 22294768 DOI: 10.1093/mutage/ger055]
16 **Nishida N**, Nagahara M, Sato T, Mimori K, Sudo T, Tanaka F, Shibata K, Ishii H, Sugihara K, Doki Y, Mori M. Microarray analysis of colorectal cancer stromal tissue reveals upregulation of two oncogenic miRNA clusters. *Clin Cancer Res* 2012; **18**: 3054-3070 [PMID: 22452939 DOI: 10.1158/1078-0432.CCR-11-1078]
17 **Sarver AL**, French AJ, Borralho PM, Thayanithy V, Oberg AL, Silverstein KA, Morlan BW, Riska SM, Boardman LA, Cunningham JM, Subramanian S, Wang L, Smyrk TC, Rodrigues CM, Thibodeau SN, Steer CJ. Human colon cancer profiles show differential microRNA expression depending on mismatch repair status and are characteristic of undifferentiated proliferative states. *BMC Cancer* 2009; **9**: 401 [PMID: 19922656 DOI: 10.1186/1471-2407-9-401]
18 **Slattery ML**, Wolff E, Hoffman MD, Pellatt DF, Milash B, Wolff RK. MicroRNAs and colon and rectal cancer: differential expression by tumor location and subtype. *Genes Chromosomes Cancer* 2011; **50**: 196-206 [PMID: 21213373 DOI: 10.1002/gcc.20844]
19 **Kerr D**. Clinical development of gene therapy for colorectal cancer. *Nat Rev Cancer* 2003; **3**: 615-622 [PMID: 12894249 DOI: 10.1038/nrc1147]
20 **Goel A**, Boland CR. Epigenetics of colorectal cancer. *Gastroenterology* 2012; **143**: 1442-1460.e1 [PMID: 23000599 DOI: 10.1053/j.gastro.2012.09.032]
21 **Kalluri R**, Weinberg RA. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009; **119**: 1420-1428 [PMID: 19487818 DOI: 10.1172/JCI39104]
22 **Cottonham CL**, Kaneko S, Xu L. miR-21 and miR-31 converge on TIAM1 to regulate migration and invasion of colon carcinoma cells. *J Biol Chem* 2010; **285**: 35293-35302 [PMID: 20826792 DOI: 10.1074/jbc.M110.160069]
23 **Asangani IA**, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, Allgayer H. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene* 2008; **27**: 2128-2136 [PMID: 17968323 DOI: 10.1038/sj.onc.1210856]
24 **Yu Y**, Kanwar SS, Patel BB, Oh PS, Nautiyal J, Sarkar FH, Majumdar AP. MicroRNA-21 induces stemness by down-regulating transforming growth factor beta receptor 2 (TGFβR2) in colon cancer cells. *Carcinogenesis* 2012; **33**: 68-76 [PMID: 22072622 DOI: 10.1093/carcin/bgr246]
25 **Edvardsson K**, Nguyen-Vu T, Kalasekar SM, Pontén F, Gustafsson JÅ, Williams C. Estrogen receptor β expression induces changes in the microRNA pool in human colon cancer cells. *Carcinogenesis* 2013; **34**: 1431-1441 [PMID: 23436804 DOI: 10.1093/carcin/bgt067]
26 **Ji S**, Ye G, Zhang J, Wang L, Wang T, Wang Z, Zhang T, Wang G, Guo Z, Luo Y, Cai J, Yang JY. miR-574-5p negatively regulates Qki6/7 to impact β-catenin/Wnt signalling and the development of colorectal cancer. *Gut* 2013; **62**: 716-726 [PMID: 22490519 DOI: 10.1136/gutjnl-2011-301083]
27 **Chen ML**, Liang LS, Wang XK. miR-200c inhibits invasion and migration in human colon cancer cells SW480/620 by targeting ZEB1. *Clin Exp Metastasis* 2012; **29**: 457-469 [PMID: 22407310 DOI: 10.1007/s10585-012-9463-7]
28 **Davalos V**, Moutinho C, Villanueva A, Boque R, Silva P, Carneiro F, Esteller M. Dynamic epigenetic regulation of the microRNA-200 family mediates epithelial and mesenchymal transitions in human tumorigenesis. *Oncogene* 2012; **31**: 2062-2074 [PMID: 21874049 DOI: 10.1038/onc.2011.383]
29 **Lu MH**, Huang CC, Pan MR, Chen HH, Hung WC. Prospero homeobox 1 promotes epithelial-mesenchymal transition in colon cancer cells by inhibiting E-cadherin via miR-9. *Clin Cancer Res* 2012; **18**: 6416-6425 [PMID: 23045246 DOI: 10.1158/1078-0432.CCR-12-0832]
30 **Zhu L**, Chen H, Zhou D, Li D, Bai R, Zheng S, Ge W. MicroRNA-9 up-regulation is involved in colorectal cancer metastasis via promoting cell motility. *Med Oncol* 2012; **29**: 1037-1043 [PMID: 21562850 DOI: 10.1007/s12032-011-9975-z]
31 **Liu X**, Zhang Z, Sun L, Chai N, Tang S, Jin J, Hu H, Nie Y, Wang X, Wu K, Jin H, Fan D. MicroRNA-499-5p promotes cellular invasion and tumor metastasis in colorectal cancer by targeting FOXO4 and PDCD4. *Carcinogenesis* 2011; **32**: 1798-1805 [PMID: 21934092 DOI: 10.1093/carcin/bgr213]
32 **Meng X**, Wu J, Pan C, Wang H, Ying X, Zhou Y, Yu H, Zuo Y, Pan Z, Liu RY, Huang W. Genetic and epigenetic down-regulation of microRNA-212 promotes colorectal tumor metastasis via dysregulation of MnSOD. *Gastroenterology* 2013; **145**: 426-436.e1-6 [PMID: 23583431 DOI: 10.1053/j.gastro.2013.04.004]
33 **Lee CG**, McCarthy S, Gruidl M, Timme C, J. YT. MicroRNA-147 Induces a Mesenchymal-To-Epithelial Transition (MET) and Reverses EGFR Inhibitor Resistance. *PloS One* 2014; **9:** e84597 [DOI: 10.1371/journal.pone.0084597.g001]
34 **Mogilyansky E**, Rigoutsos I. The miR-17/92 cluster: a comprehensive update on its genomics, genetics, functions and increasingly important and numerous roles in health and disease. *Cell Death Differ* 2013; **20**: 1603-1614 [PMID: 24212931 DOI: 10.1038/cdd.2013.125]
35 **Weis S**, Cui J, Barnes L, Cheresh D. Endothelial barrier disruption by VEGF-mediated Src activity potentiates tumor cell extravasation and metastasis. *J Cell Biol* 2004; **167**: 223-229 [PMID: 15504909 DOI: 10.1083/jcb.200408130]
36 **Rmali KA**, Puntis MC, Jiang WG. Tumour-associated angiogenesis in human colorectal cancer. *Colorectal Dis* 2007; **9**: 3-14 [PMID: 17181841 DOI: 10.1111/j.1463-1318.2006.01089.x]
37 **Sun W**. Angiogenesis in metastatic colorectal cancer and the benefits of targeted therapy. *J Hematol Oncol* 2012; **5**: 63 [PMID: 23057939 DOI: 10.1186/1756-8722-5-63]
38 **Liao D**, Johnson RS. Hypoxia: a key regulator of angiogenesis in cancer. *Cancer Metastasis Rev* 2007; **26**: 281-290 [PMID: 17603752 DOI: 10.1007/s10555-007-9066-y]
39 **Heusschen R**, van Gink M, Griffioen AW, Thijssen VL. MicroRNAs in the tumor endothelium: novel controls on the angioregulatory switchboard. *Biochim Biophys Acta* 2010; **1805**: 87-96 [PMID: 19782719 DOI: 10.1016/j.bbcan.2009.09.005]

40 **Sundaram P**, Hultine S, Smith LM, Dews M, Fox JL, Biyashev D, Schelter JM, Huang Q, Cleary MA, Volpert OV, Thomas-Tikhonenko A. p53-responsive miR-194 inhibits thrombospondin-1 and promotes angiogenesis in colon cancers. *Cancer Res* 2011; **71**: 7490-7501 [PMID: 22028325 DOI: 10.1158/0008-5472.CAN-11-1124]

41 **Chen HY**, Lin YM, Chung HC, Lang YD, Lin CJ, Huang J, Wang WC, Lin FM, Chen Z, Huang HD, Shyy JY, Liang JT, Chen RH. miR-103/107 promote metastasis of colorectal cancer by targeting the metastasis suppressors DAPK and KLF4. *Cancer Res* 2012; **72**: 3631-3641 [PMID: 22593189 DOI: 10.1158/0008-5472.CAN-12-0667]

42 **Xu Q**, Liu LZ, Qian X, Chen Q, Jiang Y, Li D, Lai L, Jiang BH. MiR-145 directly targets p70S6K1 in cancer cells to inhibit tumor growth and angiogenesis. *Nucleic Acids Res* 2012; **40**: 761-774 [PMID: 21917858 DOI: 10.1093/nar/gkr730]

43 **Lawler J**. Thrombospondin-1 as an endogenous inhibitor of angiogenesis and tumor growth. *J Cell Mol Med* 2002; **6**: 1-12 [PMID: 12003665]

44 **Dews M**, Homayouni A, Yu D, Murphy D, Sevignani C, Wentzel E, Furth EE, Lee WM, Enders GH, Mendell JT, Thomas-Tikhonenko A. Augmentation of tumor angiogenesis by a Myc-activated microRNA cluster. *Nat Genet* 2006; **38**: 1060-1065 [PMID: 16878133 DOI: 10.1038/ng1855]

45 **Olive V**, Jiang I, He L. mir-17-92, a cluster of miRNAs in the midst of the cancer network. *Int J Biochem Cell Biol* 2010; **42**: 1348-1354 [PMID: 20227518 DOI: 10.1016/j.biocel.2010.03.004]

46 **Volinia S**, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA* 2006; **103**: 2257-2261 [PMID: 16461460 DOI: 10.1073/pnas.0510565103]

47 **Dews M**, Fox JL, Hultine S, Sundaram P, Wang W, Liu YY, Furth E, Enders GH, El-Deiry W, Schelter JM, Cleary MA, Thomas-Tikhonenko A. The myc-miR-17~92 axis blunts TGF{beta} signaling and production of multiple TGF{beta}-dependent antiangiogenic factors. *Cancer Res* 2010; **70**: 8233-8246 [PMID: 20940405 DOI: 10.1158/0008-5472.CAN-10-2412]

48 **Ichikawa D**, Komatsu S, Konishi H, Otsuji E. Circulating microRNA in digestive tract cancers. *Gastroenterology* 2012; **142**: 1074-1078.e1 [PMID: 22433392 DOI: 10.1053/j.gastro.2012.03.008]

49 **Moertel CG**, Fleming TR, Macdonald JS, Haller DG, Laurie JA, Tangen C. An evaluation of the carcinoembryonic antigen (CEA) test for monitoring patients with resected colon cancer. *JAMA* 1993; **270**: 943-947 [PMID: 8141873 DOI: 10.1001/jama.1993.03510080047030]

50 **Huang Z**, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. *Int J Cancer* 2010; **127**: 118-126 [PMID: 19876917 DOI: 10.1002/ijc.25007]

51 **Kanaan Z**, Rai SN, Eichenberger MR, Roberts H, Keskey B, Pan J, Galandiuk S. Plasma miR-21: a potential diagnostic marker of colorectal cancer. *Ann Surg* 2012; **256**: 544-551 [PMID: 22868372 DOI: 10.1097/SLA.0b013e318265bd6f]

52 **Ng EK**, Chong WW, Jin H, Lam EK, Shin VY, Yu J, Poon TC, Ng SS, Sung JJ. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. *Gut* 2009; **58**: 1375-1381 [PMID: 19201770 DOI: 10.1136/gut.2008.167817]

53 **Pu XX**, Huang GL, Guo HQ, Guo CC, Li H, Ye S, Ling S, Jiang L, Tian Y, Lin TY. Circulating miR-221 directly amplified from plasma is a potential diagnostic and prognostic marker of colorectal cancer and is correlated with p53 expression. *J Gastroenterol Hepatol* 2010; **25**: 1674-1680 [PMID: 20880178 DOI: 10.1111/j.1440-1746.2010.06417.x]

54 **Kanaan Z**, Roberts H, Eichenberger MR, Billeter A, Ocheretner G, Pan J, Rai SN, Jorden J, Williford A, Galandiuk S. A plasma microRNA panel for detection of colorectal adenomas: a step toward more precise screening for colorectal cancer. *Ann Surg* 2013; **258**: 400-408 [PMID: 24022433 DOI: 10.1097/SLA.0b013e3182a15bcc]

55 **Wang LG**, Gu J. Serum microRNA-29a is a promising novel marker for early detection of colorectal liver metastasis. *Cancer Epidemiol* 2012; **36**: e61-e67 [PMID: 22018950 DOI: 10.1016/j.canep.2011.05.002]

56 **Hofsli E**, Sjursen W, Prestvik WS, Johansen J, Rye M, Tranø G, Wasmuth HH, Hatlevoll I, Thommesen L. Identification of serum microRNA profiles in colon cancer. *Br J Cancer* 2013; **108**: 1712-1719 [PMID: 23558896 DOI: 10.1038/bjc.2013.121]

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REVIEW

KAPtain in charge of multiple missions: Emerging roles of KAP1

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Abstract

KAP1/TRIM28/TIF1β was identified nearly twenty years ago as a universal transcriptional co-repressor because it interacts with a large KRAB-containing zinc finger protein (KRAB-ZFP) transcription factor family. Many studies demonstrate that KAP1 affects gene expression by regulating the transcription of KRAB-ZFP-specific loci, trans-repressing as a transcriptional co-repressor or epigenetically modulating chromatin structure. Emerging evidence suggests that KAP1 also functions independent of gene regulation by serving as a SUMO/ubiquitin E3 ligase or signaling scaffold protein to mediate signal transduction. KAP1 is subjected to multiple post-translational modifications (PTMs), including serine/tyrosine phosphorylation, SUMOylation, and acetylation, which coordinately regulate KAP1 function and its protein abundance. KAP1 is involved in multiple aspects of cellular activities, including DNA damage response, virus replication, cytokine production and stem cell pluripotency. Moreover, knockout of KAP1 results in embryonic lethality, indicating that KAP1 is crucial for embryonic development and possibly impacts a wide-range of (patho)physiological manifestations. Indeed, studies from conditional knockout mouse models reveal that KAP1-deficiency significantly impairs vital physiological processes, such as immune maturation, stress vulnerability, hepatic metabolism, gamete development and erythropoiesis. In this review, we summarize and evaluate current literatures involving the biochemical and physiological functions of KAP1. In addition, increasing studies on the clinical relevance of KAP1 in cancer will also be discussed.

Key words: KRAB domain-associated protein 1; Transcriptional co-repressor; Post-translational modification; Chromatin remodeling; KRAB-containing zinc finger protein

Core tip: This review article primarily summarizes the current findings of KAP1/TRIM28/TIF1β, with focuses on its biochemical and physiological functions. Both the canonical transcriptional co-repressor function and the transcriptional-independent roles of KAP1 are discussed in detail. We highlight the post-translational modifications and the compartmentalized localization of KAP1 and suggest that the function of KAP1 could be spatial and temporal regulated in multiple physiological circumstances. Finally, we summarize the clinical relevance of KAP1 in cancer and discuss the possibility to translate the mechanistic studies of KAP1 to human pathophysiology in the future.

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INTRODUCTION

KRAB domain-associated protein 1 (KAP1), also known as

tripartite motif-containing 28 (TRIM28) or transcriptional intermediary factor 1 beta (TIF1β) was identified as an interacting protein for Krüppel-associated box zinc finger proteins (KRAB-ZFPs) in 1996[1-4]. Since then, KAP1 has been reported in regulating multiple aspects of physiology, for examples, cell differentiation, DNA damage response (DDR), virus replication, immune response and tumorigenesis (Figure 1). As KAP1 binds to the conserved KRAB repression domain, which is present in many transcription factors, KAP1 is considered as a critical transcriptional co-repressor[2-4]. For instance, many proteins involved in chromatin remodeling or histone modification, such as heterochromatin-associated protein 1 (HP1), nuclear co-repressor (N-CoR), histone deacetylase (HDAC), chromodomain helicase DNA binding protein 3/nucleosome remodeling deacetylase (CHD3/NuRD), histone methyltransferases (HMTs), have been identified in KAP1-containing complexes[5-9] (Table 1). Consequently, KAP1 epigenetically regulates gene expression through multiple transcriptional co-repressor complexes. In addition to regulating KRAB-ZFPs, the activity of transcription factors lacking KRAB domain, such as c-Myc and E2F1, can also be regulated by KAP1[10-14]. KAP1 is subjected to multiple post-translational modifications (PTMs), including phosphorylation and SUMOylation (Figure 2). We and others have demonstrated that these PTMs coordinately regulate the gene repressive function of KAP1[15-18].

Deletion of *Kap1* in mouse embryo leads to embryonic lethality[19], suggesting that KAP1 is critical during embryonic development and should be involved in a wide-range of biological/physiological processes. Although most of the KAP1 studies have been focusing on its transcriptional functions, emerging evidence suggests that KAP1 also exerts transcription-independent function. Given that KAP1 functions as a scaffold protein to constitute KAP1-containing complexes that regulate chromatin structure, it also plays an important role in maintaining genome stability by facilitating DNA repair in response to DNA damage through chromatin remodeling[20-22]. Moreover, the RING and the plant homeodomain (PHD) domains of KAP1 possess intrinsic enzymatic activity to potentially catalyze SUMOylation and ubiquitylation[15,23-25]. Interestingly, KAP1 resides in distinct cellular compartments, including the pericentric and centromeric heterochromatin, euchromatin and cytoplasm[6,25,26], implicating its essential functions for different cellular activities. Taken together, the diverse function and regulated subcellular localization suggest that KAP1 impacts multiple aspects of biological processes and it warrants more rigorous investigations of this important protein in the future. In this review, we aim to discuss both the transcriptional and non-transcriptional functions of KAP1. The PTMs of KAP1 involved in these processes will be highlighted. Lastly, the clinical relevance of KAP1 in cancer will also be elaborated.

PROTEIN STRUCTURE AND PTMS OF KAP1

Given that the overall structure of KAP1 has been extensively reviewed previously[27] (and references therein) we will mainly focus on the PTMs of KAP1 and how these PTMs crosstalk to each other and affect the interaction between KAP1 and its partners. KAP1 is a member of TIF1 family, which includes four proteins, TIF1α, TIF1β, TIF1γ and TIF1δ. As the other members in the TIF1 family, KAP1 has an N-terminal tripartite motif (TRIM), RBCC domain, which is composed by a RING finger, 2 B-box zinc fingers and a coiled-coil region. In addition, KAP1 also shares a central TIF1 signature sequence (TSS), an HP1 binding domain (HP1BD), a C-terminal combination of PHD and bromodomain with the other TIF1 members. Different from the other TIF1 proteins, KAP1 does not have a nuclear receptor (NR) box[27] (Figure 2).

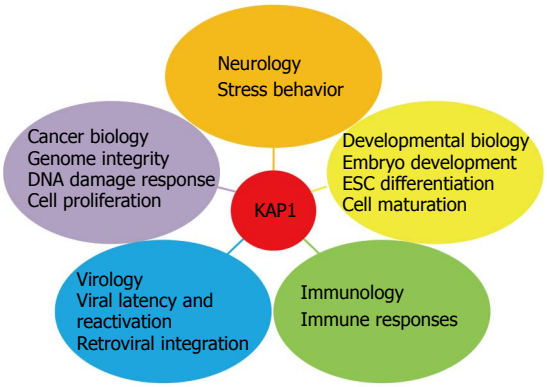

Figure 1 KRAB domain-associated protein 1 is involved in multiple aspects of cellular physiology. Neurology: *Kap1* knockout in mouse forebrain induces higher level of anxiety-like behavior. Developmental Biology: Several conditional *kap1* deletions impair normal cell development including embryonic stem cell (ESC) differentiation, spermatogenesis, erythropoiesis, and the development of T-cell and B-cell. Immunology: KAP1 is involved in immune responses by regulating T/B cell activity and immune tolerance. Virology: KAP1 is critical to suppress retroviral activation and prevent HIV integration. Cancer Biology: KAP1 is positively or negatively correlated with prognosis in different cancer types. The roles of KAP1 in maintaining genome stability, mediating DNA damage response and affecting cell proliferation *in vitro* imply its potential roles during tumorigenesis.

The RBCC domain of KAP1 interacts with various KRAB-ZFPs and is considered as an important region for the KAP1 recruitment to KRAB-ZFP binding sites across the genome[28,29]. It has been demonstrated that RBCC domain forms a homotrimer with a single KRAB domain[30]. Interestingly, a recent study suggests that KAP1 can still bind to promoter regions without RBCC domain, suggesting additional mechanisms that might contribute to the KAP1 recruitment to transcription factors on the promoter regions[31]. The TSS domain is adjacent to the RBCC domain, and is required for the transcriptional repressive activity of TIF1γ[32]. However, the function of TSS in KAP1 has yet to been defined.

The hydrophobic PxVxL pentapeptide is located at the central region of KAP1, namely HP1BD. HP1BD interacts with the chromoshadow domain of HP1 proteins and this KAP1-HP1 interaction is critical for the KAP1-mediated gene silencing[5,6,33,34]. It is believed that KAP1-HP1 complex plays a critical role in heterochromatin maintenance and gene silencing. In fact, HP1BD

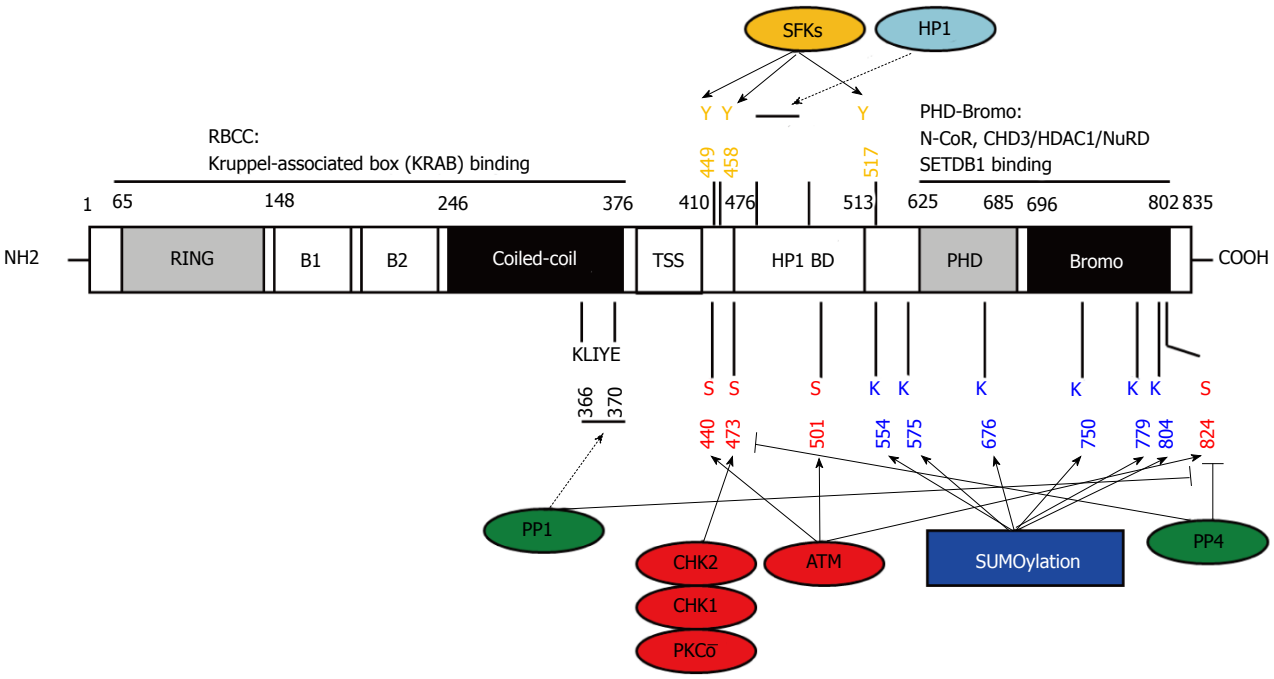

Figure 2 KAP1 structure, post-translational modifications and interacting proteins. KAP1 has multi-domains for protein-protein interaction and post-translational modification. RBCC: RING-B1-B2-coiled-coil; TSS: TIF signature sequence; HP1 BD: HP1 binding domain; PHD: Plant homeo domain. Numbers represent the sequence of amino acids; Blue: SUMOylation sites; Red: Serine phosphorylation sites targeted by the indicated kinases (shown in red) or antagonized by phosphatases (shown in green); Orange: Tyrosine phosphorylation sites targeted by the indicated kinase family; KLIYF: PP1 binding site; PxVxL: HP1 binding site; Dotted lines: Protein-protein interaction; SFKs: Src family kinases; HP1: Heterochromatin-associated protein 1; ATM: Ataxia-telangiectasia mutated; PP1: Protein phosphatase 1; CHD3/NuRD: Chromodomain helicase DNA binding protein 3/nucleosome remodeling deacetylase; HDAC: Histone deacetylase.

Table 1 Chromatin-associated factors/chromatin-remodeling enzymes interacting with KAP1

Chromatin-associated factors/ chromatin-remodeling enzymes	Consequences of binding with KAP1	Ref.
HP1	HP1-KAP1 interaction leads to transcriptional repression and has an essential role in development and cell differentiation. Phosphorylation at Ser-473 or Tyr-449, 458, 517 of KAP1 inhibits its interaction with HP1.	[5,6,33,43,46,53,94-97,105]
SETDB1	KAP1 binds to SETDB1 through SUMO:SIM interaction to methylate H3K9 at gene regulatory regions to achieve gene silencing.	[9,15,16,109]
N-CoR	N-CoR represses basal transcription by the recruitment of HDACs to deacetylate histones. KAP1 is involved in N-CoR-1 complex to mediate transcriptional repression.	[7]
CHD3 (Mi-2α)/NuRD	NuRD complex mediates chromatin remodelling and histone deacetylation *via* CHD3 (Mi-2α) and HDACs, respectively. KAP1 interacts with NuRD complex *via* PHD and bromodomain to alter the chromatin structure.	[8,21,37]
HDAC1	KAP1-HDAC1 complex interaction not only regulates histone modification but also non-histone protein deacetylation to exert a variety of different functions (also shown in Table 2).	[113]
SMARCAD1	SMARCAD1 mediates histone deacetylation and associates with KAP1-HDAC1 complex to regulate chromatin marks.	[58]
DNMT	KAP1 associates with DNMT to maintain DNA methylation at imprinting control region (also shown in Table 2).	[59,61]

HP1: Heterochromatin-associated protein 1; ATM: Ataxia-telangiectasia mutated; PP1: Protein phosphatase 1; CHD3/NuRD: Chromodomain helicase DNA binding protein 3/nucleosome remodeling deacetylase; HDAC: Histone deacetylase; N-CoR: Nuclear co-repressor; SETDB1: Bifurcated 1; DNMT: DNA methyltransferase.

is required for nuclear retention of KAP1 and disrupting KAP1-HP1 interaction reactivates the imprinted gene expression by perturbing histone and DNA methylation levels *in vivo*[31,35]. However, the detailed mechanism underlying the KAP1 recruitment to HP1 on heterochromatin remains to be elucidated.

The C-terminal PHD and bromodomain of KAP1 (PB domain) recognize histone tail and are also required for the KAP1-mediated gene silencing by recruiting histone modifiers. Specifically, KAP1 interacts with CHD3/NuRD complex, and histone methyltransferase, SET domain, bifurcated 1 (SETDB1)[8,9]. These observations suggest a model for KAP1-dependent recruitment of histone modifiers for histone methylation and heterochromatin formation to achieve gene silencing[27].

Notably, the carboxyl-terminus of KAP1 is subjected

to multiple types of PTM. Several studies have revealed that KAP1 is SUMOylated and the SUMOylation of KAP1 is required for its repressive function[15,16,18]. Within or adjacent to the PB domain, six lysines of 554, 575, 676, 750, 779 and 804 have been validated as SUMOylation sites, and the distinct SUMOylation combinations differentially affect the interaction between the bromodomain with SETDB1 and CHD3[15,16,18,28]. KAP1 also undergoes auto-SUMOylation[15]. The magnitude of KAP1 SUMOylation is balanced by deSUMOylases, sentrin specific peptidase 1 (SENP1), SENP7 and the phosphorylation status at serine-824 of KAP1[15,17,36,37].

The serine-824 of KAP1 is primarily phosphorylated by phosphoinositide 3 kinase-like protein kinases (PIKKs), including ataxia-telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), and DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs)[38]. This specific serine 824-phosphorylation is crucial for DDR in different aspects, for examples, the ATM-mediated KAP1 serine-824 phosphorylation is responsible for activating DNA damage checkpoints and chromatin relaxation[17,20], whereas ATR or DNA-PKcs presumably compensates for ATM-deficiency during DDR[39]. Our laboratory has demonstrated that protein phosphatase 1 (PP1) interacts with KAP1 through the PP1-binding motif in the coiled-coil region of KAP1 to dephosphorylate KAP1 at serine-824[36]. In addition, protein phosphatase 4 (PP4) also mediates the KAP1 dephosphorylation at serine-824 and another phosphorylation site at serine-473 upon DNA damage[40,41].

Many DNA damage-inducing agents render both KAP1 serine-824 phosphorylation and serine-473 phosphorylation through ATM-Chk2 or ATR-Chk1 pathways[42]. KAP1 serine-473 phosphorylation is also involved in efficient DNA repair and cell survival upon DNA damage[42-44]. However, unlike the phosphorylation at serine-824, the KAP1 serine-473 phosphorylation is diffusely localized in the nucleus instead of accumulating at damage sites and forming foci[44,45]. It remains to be established whether different KAP1 phosphorylation sites play distinct roles in response to DNA damage. Interestingly, the KAP1 serine-473 phosphorylation regulates cell cycle progression. A study demonstrated that PKCδ phosphorylates KAP1 at serine-473 during S phase of cell cycle. This event dampens the KAP1 and HP1γ interaction and de-represses cyclin A2 to promote S phase progression[46]. Conceivably, KAP1 serine-473 phosphorylation perturbs its association with HP1γ, thereby rendering pan-nuclear distribution. This may be due to the structural alteration of PxVxL domain, which is the HP1 binding site of KAP1. KAP1 mutant that loses its binding ability with HP1 indeed translocates to cytoplasm[31]. This implies that KAP1 serine-473 phosphorylation could function beyond gene regulation in cytoplasm. Results from several studies also suggest that KAP1 serine-473 phosphorylation is associated with immune response. First, KAP1 is constitutively phosphorylated at serine-473 but not serine-824 upon T cell receptor activation in thymocytes[47,48]. Second, Kaposi's Sarcoma-Associated Herpesvirus (KSHV) infection induces phosphorylation at KAP1 serine-473 in endothelial cells[49]. Many KAP1's partners, such as STAT1/3, NF-κB and IRF5/7[24,49-52], are involved in inflammation and immune response. Further exploring the role of KAP1 serine-473 phosphorylation in the immune responses induced by viral infection or other types of stress could be rewarding. More recently, Kubota *et al*[53] reported that tyrosines-449, 458 and 517 of KAP1 are phosphorylated by Src family kinases (SFKs). The phosphorylation of these tyrosine residues also interferes the interaction between KAP1 and HP1. As SFKs are involved in regulating a wide-range of oncogenic processes, including cell growth and differentiation, this finding implies that KAP1 plays a role in SFK-mediated oncogenic transformation.

KAP1 is also demonstrated to be acetylated and the level of KAP1 acetylation is downregulated by HDAC10[54]. Although HDAC10 regulates KAP1 transcriptional co-repressor activity, there is no direct evidence showing whether the acetylation affects KAP1-mediated transcriptional control. Studies on the regulation of KAP1 acetylation and whether KAP1 acetylation crosstalks with other types of KAP1 lysine-PTM are expected to provide additional insights into the roles of PTMs in governing KAP1 function.

TRANSCRIPTIONAL FUNCTIONS OF KAP1

KAP1 was originally identified to be associated with KRAB-ZFPs, suggesting a role of KAP1 in transcriptional control. In addition to the RBCC domain, there are also other domains allowing KAP1 to interact with a wide variety of proteins such as histone acetylases (HATs), HDACs, HMTs and DNA methyltransferases (DNMTs). Thus, KAP1 has the flexibility to regulate transcription through multiple mechanisms, possibly depending on its PTMs, location and specific binding partners (Figure 2, Tables 1 and 2).

Histone modification and chromatin remodeling

RBCC domain is required for the recruitment of KAP1 to KRAB-ZFPs, and KAP1-binding sites are enriched in the promoter regions of KRAB-ZFPs, suggesting an auto-regulation between KAP1 and KRAB-ZFPs[55]. However, a recent genome-wide study reports that KAP1 does not regulate the expression of those ZFPs and other genes that KAP1 is bound to their promoter regions. Instead, KAP1 regulates the expression of genes with KAP1 binding sites distant from their transcription start sites. Furthermore, deletion of the RBCC domain did not profoundly affect the binding of KAP1 to corresponding promoter regions[31]. Considering that KAP1 interacts with HP1 and other histone modifiers and is involved in long-range transcriptional repression[56], KAP1-mediated chromatin remodeling might contribute to the majority of the KAP1-mediated transcriptional repression.

In addition to the basal transcription, KAP1-mediated

Table 2 Transcription factors involved in KAP1-regulated gene expression

Transcription factor	KAP1 function	Ref.
Myc	KAP1 is involved in MM-1 and HDAC1 complex to suppress c-Myc transcription activity	[10,11]
ZNF160	KAP1 interacts with ZNF160 and recruits HDAC to downregulate TLR4 in intestinal epithelial cells	[79]
Oct3/4	KAP1 is potentially involved in regulating pluripotency of embryonic stem cells	[78]
E2F1	KAP1 suppresses E2F1 transcriptional activity and inhibits E2F1 acetylation in an HDAC1 dependent manner	[13]
p53	KAP1 is associated with MDM2-p53-HDAC1 complex and inhibits p53 acetylation and promotes p53 degradation	[75]
p53	MAGE proteins stabilize KAP1-p53 complex to decrease acetylation and promote degradation of p53	[80]
p53	KAP1 ubiquitinates p53 for proteasome degradation	[23]
IRF7	KAP1 sumolyates IRF7 and suppresses IRF7 transcriptional activity in IFN production	[24]
ZBRK1	KAP1 interacts with ZBRK1 to repress *Gadd45α* and *p21* gene expression	[16,17,69]
HIF-1α	VHLaK potentially recruits KAP1 to HIF-1α complex to suppress HIF-1α downstream gene expression	[72]
Nrf2	KAP1 interacts with Nrf2 and facilitates Nrf2 transactivation activity	[76]
STAT3	KAP1 interacts with STAT3 to suppress STAT3 transcriptional activity	[82]
STAT1	KAP1 interacts with STAT1 to suppress STAT1 transcriptional activity	[50]
ZNF689	ZNF689 potentially recruits KAP1 to suppress autophagy-gene-targeting miRNAs	[71]
FOXP3	KAP1 is recruited by FIK in FOXP3-FIK-KAP1 complex to suppress FOXP3-target genes	[74]
NFκB	KAP1 is associated with NFκB and negatively regulates it acetylation and transcriptional activity	[51]
SRY	KAP1 is recruited by KRAB-O to SRY binding sites for gene regulation	[68,73]
ZFP57	ZFP57 and KAP1 are associated with NP95-DNMT complex for maintaining DNA methylation at imprinting control region	[59,61,62]

VHLaK: VHL-associated KRAB-A domain-containing protein; HDAC: Histone deacetylase; DNMT: DNA methyltransferase.

chromatin remodeling also participates in the activated transcription. We have shown that the DNA damage-induced KAP1 serine-824 phosphorylation decreases SUMOylated KAP1 to reduce the H3K9 di- and tri-methylation, a mark enriched at repressed genes, and to increase H3K14 acetylation, a mark enriched at the active genes, on the promoter regions of KAP1-targeted genes, thereby relaxing the chromatin structure and activating the transcription of pro-arrest and pro-apoptotic genes, including *p21*, *Gadd45a*, *Bax*, *Noxa* and *Puma*[16,17,36]. Likewise, the phosphorylation of KAP1 serine-824 by the viral protein kinase induces chromatin remodeling to activate lytic genes to support KSHV lytic replication[57]. Notably, KAP1 mediates the chromatin remodeling within the promoter regions, but not the distal regions of its targeted genes upon DNA damage and viral reactivation[16,57]. It would be interesting to investigate how KAP1 mediates chromatin remodeling at distinct gene regulatory regions during basal and activated transcription. Furthermore, a study showing that KAP1 interacts with a SWI/SNIF-like, ATP-dependent chromatin remodeling protein, SMARCAD1, demonstrates that the KAP1-SMARCAD1 complex regulates global H3K9 trimethylation and H3/H4 deacetylation to maintain the silenced loci during DNA replication[58]. Whether this complex is also responsible for KAP1-mediated transcriptional repression during other circumstances remains unknown.

DNA methylation

Emerging evidence suggests that KAP1-containing complex is not only associated with histone modification, but also regulates DNA methylation. KAP1-mediated DNA methylation is important for genomic imprinting and epigenetic reprogramming during embryogenesis[59-63]. Studies have demonstrated that ZFP57, a KRAB-ZFP important for maintaining DNA methylation at the imprinted loci, recruits KAP1 to imprinting control regions (ICRs) to interact with NP95, a protein responsible for recruiting DNMTs to hemimethylated DNA, resulting in the maintenance of genomic imprinting at ICRs[59,61]. Although these results implicate that KAP1 might indirectly recruit DNMT to specific loci, it remains elusive which domain of KAP1 is responsible for the DNMT recruitment. Histone modification and DNA methylation are inter-dependent, and histone lysine methylation is involved in maintaining genomic imprinting at ICRs[64,65]. Whether KAP1 plays a role in the crosstalk between these two epigenetic regulation pathways warrants further investigation.

Transcriptional co-regulator

Although it is known that KAP1 can regulate gene expression by chromatin remodeling and DNA methylation as discussed in the previous sections, the specificity of KAP1-regulated genes under certain conditions is still largely unknown. Here, we reviewed how KAP1 regulates transcription factors on specific gene loci for gene regulation (Table 2).

KAP1 has no DNA binding domain but is recruited by a variety of different KRAB-ZFPs *via* its RBCC domain[29,66,67]. Therefore, KAP1 is believed to exert its transcriptional repression by associating with KRAB-ZFPs to specific promoter region of its regulating gene[29]. Although KRAB-ZFP is a large family containing over 400 genes encoding more than 700 predicted proteins, only a few studies directly address the role of KAP1 in regulating the KRAB-ZFP-mediated transcriptional repression[68]. In 1996, KAP1 was for the first time demonstrated to interact with KRAB domain of a human ZFP KOX1/ZNF10 to exert transcriptional repression[4]. Later, ZBRK1 was identified as a sequence specific KRAB-ZFP to recruit KAP1 to suppress *Gadd45a* and *p21* expression[16,17,69]. Our laboratory has demonstrated that KAP1 SUMOylation status dictates the ZBRK1-

KAP1-mediated gene repressive function by enhancing histone methylation without altering KAP1-ZBRK1 interaction[16,17]. Another KRAB-ZFP, ZNF160 has been shown to suppress TLR4 through the binding with KAP1 in intestinal epithelial cells for immune tolerance to commensal bacteria in gut[70]. More recently, ZNF689 is identified as a potential KRAB-ZFP for recruiting KAP1 to suppress autophagy-gene-targeting microRNAs (miRNAs). The KAP1-mediated repression of these autophagy-gene-targeting miRNA promotes mitophagy, a specific type of autophagy targeting mitochondria, during erythrocyte maturation[71].

Interestingly, proteins with KRAB domain but not DNA binding domain are also reported to being capable of serving as a bridge for KAP1-mediated gene repression[68,72-74]. The KRAB Only (KRAB-O) protein is one example to recruit KAP1 to the sex determination transcription factor SRY for regulating SRY-targeted genes[68,73]. VHL-associated KRAB-A domain-containing protein (VHLaK) promotes the formation of HIF-1α-VHLaK-KAP1 complex and potentially suppresses HIF-1α signaling[72]. FOXP3-interacting KRAB domain-containing protein (FIK) is another bridge protein to recruit KAP1 to the FOXP3 binding sites for suppressing FOXP3-target genes[74]. Other transcription factors without KRAB domain including Myc, Oct3/4, E2F1, p53, IRF5/7, Nrf2, STAT1/3 and NF-κB can also interact with KAP1 for gene regulation[10,13,24,49-52,75-78]. More investigation is required to understand whether the formation of these KAP1-transcription factor complexes relies on other bridge proteins.

Several studies have reported that KAP1 is capable of modulating the acetylation status of histones or transcription factors. In general, KAP1-mediated HDAC recruitment negatively regulates gene expression. While mainly serving for histone deacetylation and for heterochromatin maintenance[79], the KAP1-HDAC complex is also postulated to negatively regulate the acetylation level of the transcription factors. For example, KAP1 stimulates E2F1-HDAC1 complex formation to deacetylate E2F1, which suppresses E2F1-mediated apoptotic gene expression in response to DNA damage[13]. KAP1 is also involved in MDM2-p53-HDAC1 complex and promotes deacetylation and MDM2-mediated degradation of p53[75]. More recently, melanoma antigen (MAGE) family proteins, which are highly expressed in many tumors, have been shown to enhance the formation of KAP1-p53 complex and to reduce p53 acetylation[80]. Interestingly, another study shows that MAGE proteins enhance the ubiquitin E3 ligase activity of KAP1 to ubiquitylate p53 for degradation[23]. These studies provide a rationale for developing compounds blocking KAP1-MAGE interaction for anti-cancer purpose[81].

In addition to regulating the activity of deacetylase complex, KAP1 is reported to disrupt the interaction of NF-κB and p300, an acetyltransferase. By this mechanism, the acetylation level of NF-κB is reduced and the transcriptional activity of NF-κB is dampened[51]. Besides, the physical interaction between KAP1 and STAT family members has been identified, and KAP1 negatively regulates STAT1 and STAT3 signaling[50,82]. However, the molecular mechanism underlying this event remains unclear. Whether KAP1 regulates the PTMs of STAT family still needs to be examined.

NON-TRANSCRIPTIONAL FUNCTIONS OF KAP1

While extensive efforts have been made in understanding how KAP1 regulates transcription, less is known about its non-transcriptional functions. In fact, KAP1 has a critical, transcription-independent role in DNA repair processes. Additionally, emerging evidence shows that KAP1 possesses enzymatic activity required for multiple cellular processes. In the following sections, we aim to discuss the role of KAP1 as a signaling scaffold protein in DNA repair and its novel roles as SUMO and ubiquitin E3 ligases.

Signaling scaffold protein in DNA damage response

White and colleagues identified that the PIKK family members, in response to DNA damage, phosphorylate KAP1 at serine-824. The serine-824 phosphorylated KAP1 co-localizes with several DNA repair factors, including γH2AX, 53BP1 and TopBP1, implicating a role for KAP1 in DNA repair processes[20,38]. It was further demonstrated that the KAP1 serine-824 phosphorylation is responsible for ATM-mediated chromatin relaxation, a crucial step for DNA double-strand break (DSB) repair[20]. Several lines of evidence suggest that the function of KAP1 in DSB repair is likely to be associated with the chromatin complexity and cell cycle status. Approximately 25% of DSBs that are located within heterochromatin require ATM signaling for repair, and knockdown of KAP1 bypasses the repair defects caused by ATM inhibition, suggesting that KAP1 is a direct downstream effector in ATM-mediated heterochromatin repair[83]. Although it has not been directly demonstrated, the SUMOylation of KAP1 seems to be important for heterochromatin maintenance because the interaction between KAP1 and the nucleosome remodeler, CHD3 requires KAP1 SUMOylation, and the chromatin retention of CHD3 is critical for chromatin plasticity during DDR[15,21,37]. It has been shown that the ATM-mediated KAP1 serine- 824 phosphorylation perturbs the SUMO-dependent interaction of KAP1 and CHD3 at the carboxyl-terminus of KAP1, thereby resulting in the de-condensation of heterochromatin[21]. Another possibility would be the deSUMOylase, SENP7, negatively regulates the SUMOylation status of KAP1 to release CHD3 and promote chromatin relaxation[37]. However, how SENP7 is recruited to deSUMOylate KAP1 at the damage sites remains to be defined. It is also suggested that following the KAP1 phosphorylation-dependent chromatin relaxation, the KAP1-dependent heterochromatin reconstitution mediated by the release of ATM is a prerequisite for error-free homologous recombination (HR) repair[84]. It would be interesting to further delineate how KAP1 is involved in the heterochromatin reconstitution and whether the re-SUMOylation of

KAP1 is required for the reconstitution. In addition to its role in HR repair within heterochromatin, KAP1 has been shown to promote non-homologous end joining (NHEJ) repair, presumably within euchromatin[41]. Whether the chromatin localization of KAP1 determines its function in DSB repair is still unclear. Several studies have shown that HP1 is required for recruiting KAP1 to DNA damage sites for the repair within heterochromatin[44,85]. The disruption of HP1BD in KAP1 results in a defect in forming discrete serine-824 phosphorylated KAP1 foci, which have been considered as a critical signal for DSB repair[44]. How HP1 recruits KAP1 and whether KAP1 is responsible for HP1-mediated DSB repair warrant more detailed investigation.

Depletion of KAP1 is able to rescue the defects in NHEJ repair caused by ATM inhibition in G1 cells[83,86], whereas HR repair can be restored by knocking down KAP1 in ATM-inhibited G2 cells[87,88], indicating that the participation of KAP1 in specific DSB repair pathways is cell cycle-dependent. It is suggested that KAP1 serine-824 phosphorylation is enhanced by 53BP1 within heterochromatic regions and the concentrated phosphorylated KAP1 signal enables NHEJ repair[86]. On the other hand, a recent report shows that 53BP1 is required for enhancing KAP1 serine-824 phosphorylation and HR repair during G2-phase[89]. Collectively, these studies all indicate an important role of KAP1 in DSB repair. However, it is still unclear how cell cycle progression affects the role of KAP1 in selecting DSB repair pathway. Intriguingly, it has been observed that the retention of KAP1 on chromatin is largely reduced in G2-phase[87,90], suggesting that the association of KAP1 with chromatin is possibly regulated in a cell cycle-dependent manner and the chromatin retention of KAP1 may be a critical determinant that directs DSB pathway choice.

Taken together, chromatin remodeling could be one of the major functions served by KAP1 in DSB repair. Because KAP1 has multiple domains for protein-protein interaction, we speculate that KAP1 has additional roles, independent of chromatin remodeling, in DSB repair, such as the recruitment of members of DNA repair machinery.

Enzymatic activity

Recently, KAP1 has been found to possess SUMO E3 ligase activity *via* its PHD domain to recruit the SUMO-conjugating enzyme UBC9. This was first identified by demonstrating that KAP1 auto-SUMOylated its bromodomain to generate a repressive form of KAP1[15]. Later, another two proteins IRF7 and Vps34 have been identified as substrates of KAP1-mediated SUMOylation[24,25]. IRF7 is a transcription factor and master regulator of type I interferon-dependent immune responses. The SUMOylation of IRF7 by KAP1 RING finger domain reduces its transcription activity. Therefore, KAP1 could be a negative regulator of IRF7 and suppress IFN-based antiviral responses[24]. IRF7 is so far the only transcription factor identified as KAP1 target for SUMOylation. Several transcription factors are known to be SUMOylated and growing evidence suggests SUMOylation negatively regulates transcription[91]. Whether KAP1 mediates SUMOylation-dependent suppression of other transcription factors deserves another look.

More interestingly, KAP1 not only SUMOylates nuclear proteins but also targets a cytoplasmic protein, vacuolar protein-sorting(Vps) 34, which is crucial for autophagosome formation and plays a central role during autophagy. The SUMOylation of Vps34 enhances its binding to Beclin 1 and triggers autophagosome formation in the presence of acetylated HSP70. This study also suggests that the accumulation of KAP1 in the cytoplasm of cells treated with a pan-HDAC inhibitor, panobinostat *via* an unknown mechanism[25].

In addition to SUMO E3 ligase, the RING finger domain of KAP1 has ubiquitin E3 ligase activity. Studies have demonstrated that KAP1, in the presence of MAGE, ubiquitinates p53 and ZNF382 to facilitate their degradation[23,80,92]. This could be an additional mechanism for KAP1 to regulate gene expression. Taken together, recent progresses suggest a non-canonical function of KAP1, dependent on its SUMO and ubiquitin E3 ligase activity. These novel functions of KAP1 are not exclusively confined in the nucleus and might impact cellular physiology in response to various stresses by mechanisms beyond transcriptional regulation.

IMPLICATIONS OF KAP1 IN CELLULAR PHYSIOLOGY

KAP1 is involved in many aspects of cellular physiology (Figure 1). First, KAP1 exerts critical function during embryonic development because global *Kap1*-knockout causes embryonic lethality in mice due to the inability to undergo gastrulation[19] (Table 3). The function of KAP1 in maintaining pluripotency of embryonic stem cells (ESCs) has been demonstrated[78,93]. In addition, KAP1 is also required for ESC differentiation[94-97]. Studies using conditional *Kap1*-knockout mice show that KAP1 plays pivotal roles in different cellular maturation processes such as spermatogenesis, erythropoiesis, and the development of T-cell and B-cell (Table 3)[71,94,98-101]. Beyond regulating T-cell and B-cell differentiation, KAP1 also functions in immune response by additional mechanisms. For example, KAP1 is present in FOXP3-containing complex and facilitates the suppressor activity of regulatory T cells[74]. Moreover, KAP1 is involved in immunoglobulin class switch recombination[102]. Recent studies demonstrate that KAP1 associates with STAT1 and STAT3, master regulators of immune response, to negatively regulate their downstream signaling. These results suggest the involvement of KAP1 in immune response[50,51,82].

Using a mouse model, Jakobsson *et al*[103] show that conditional deletion of *Kap1* in adult forebrain caused higher level of anxiety-like activity, suggesting that Kap1 is a regulator for behavioral stress response. Although the molecular mechanism remains elusive, this could be due to the function of Kap1 in regulating gene expression in hippocampus. It is worth noting that among

Table 3 Mouse models illustrating the physiological functions of Kap1

Animal model	Phenotype	Ref.
Kap1 knockout mice	Embryonic lethal prior to gastulation	[19]
Hemato-specific Kap1 knockout mice	Impaired erythropoiesis	[71,100]
T-cell-specific Kap1 knockout mice	Defective T-cell differentiation	[48,98]
B-cell-specific Kap1 knockout mice	Defective B-cell differentiation	[99]
Tamoxifen-inducible-germ cell-lineage-specific Kap1 depletion mice	Impaired spermatogenesis	[101]
Liver-specific Kap1 knockout mice	Male-predominant steatosis and hepatic adenoma	[120]
Kap1 knockout in mice forebrain	Anxiety-like-behavior and cognitive impairments	[103]

these dysregulated genes in hippocampus, some are imprinted genes[103]. We speculate that Kap1 is also required for the maintenance of a set of specific imprinted genes because many recent studies have demonstrated that KAP1 mediates DNA methylation by recruiting DNMTs to ICRs during early embryogenesis[59,61,62].

More than maintaining normal cellular physiological functions, KAP1 also regulates several pathways in response to different stresses. As mentioned earlier, several studies have focused on the role of KAP1 in DDR. KAP1 is known to be phosphorylated at serine-824 by ATM upon DNA damage and to transduce downstream signaling[20,38]. For instance, ATM-mediated KAP1 phosphorylation leads to de-repression of *p21*, *Gadd45a*, *Bax*, *Puma*, and *Noxa*, causing cell cycle arrest and apoptosis[16,17,36]. Additionally, KAP1 is an ATM downstream mediator during DSB-induced heterochromatin relaxation, which facilitates DNA repair[20,21,83]. KAP1 also cooperates with MDM2 to suppress p53 signaling by promoting p53 degradation[75,104]. Thus, PTMs of KAP1 might be important in regulating p53 activity as well.

In contrast to its co-repressor function, KAP1 can also be a co-activator in specific circumstances. It has been reported that mouse Kap1 interacts with Nrf2 to enhance Nrf2-meidated-cytoprotective function in NIH3T3 cells in response to oxidative stress[76]. However, whether KAP1 exerts similar co-activator activity in human cells is not clear.

Some studies demonstrate that KAP1 mediates viral gene expression and plays a role during viral latency. Specifically, KAP1 is associated with KSHV lytic gene promoter to suppress lytic gene expression and in turn maintains virus latency[57]. Similar observation has also been made in retroviruses such as Murine Leukemia Virus (MLV) and human T-cell lymphotropic virus-1 (HTLV-1), that KAP1 restricts pro-viral gene activation[105-108]. Interestingly, recent studies show that retroelements derived from endogenous retroviruses (ERVs), which are extensively present in mammalian genome, can also be silenced by KAP1 to protect genome integrity in embryonic stem cells[109-112]. In addition, KAP1-HDAC1 complex deacetylates and inhibits human immunodeficiency virus (HIV) integrase activity, thereby reducing HIV infectivity and its integration to host genome[113].

CLINICAL RELEVANCE FOCUSED ON CANCER BIOLOGY

The clinical relevance of KAP1 in diseases remains elusive. Most reports were based on studies in cancers. Higher expression of KAP1 has been linked to pro-metastatic cervical cancer[114]. Moreover, the up-regulation of KAP1 could be a potential marker in colorectal cancer patients and higher level of KAP1 is correlated with poorer overall survival in patients with gastric cancer and thyroid carcinoma[115-119]. These clinical studies suggest that higher KAP1 level is linked to a poor prognosis in certain cancers. However, opposite conclusions were also reported. KAP1 overexpression is associated with better overall survival in early-stage lung cancer[14]. Furthermore, a mouse model showed that *Kap1*-depletion in liver increases male-predominant hepatic adenoma[120] (Table 2).

Based on the studies focusing on transcriptional regulation, KAP1 suppresses p53 transcription activity and has been proposed to be a target for anti-cancer therapy[75,104]. Nonetheless, KAP1 also suppresses activity of oncogenic transcription factors HIF-1α and STAT3[72,82]. *In vitro* studies using cancer cell lines also show divergent results. KAP1 has been shown to restrain cell growth in breast and lung cancer cells whereas it promotes melanoma cell growth and enhanced KAP1 activity mediates cervical cancer invasion[14,80,114]. Taken together, it is still inconclusive how KAP1 regulates tumorigenesis. Because KAP1 is a multi-faceted protein, it might have tissue-specific function. More study is definitely required to understand how KAP1 contributes to tumorigenesis in particular tissue and whether KAP1 could be a target for cancer therapy.

CONCLUSION

The functions of KAP1 in diverse cellular physiology have been studied for 18 years. However, the mechanisms of KAP1 in regulating these different cellular processes are still largely unclear. The best-characterized role of KAP1 is its co-repressor function in regulating KRAB-ZFP-mediated or other transcription factor-associated gene silencing. In the future, the identification of more KAP1-interacting transcription factors may help to elucidate how KAP1 regulates gene expression under specific condition. Clearly, KAP1 is critical for maintaining genome integrity as KAP1 modulates the dynamics of hetero- and euchromatin maintenance. In addition, DNA damage induces a robust and transient KAP1 serine-824 phosphorylation and KAP1 is involved in DNA repair pathway choice. However, the molecular mechanism for KAP1-mediated DNA repair requires more rigorous in-

vestigation.

KAP1 is subjected to several types of PTM. The phosphorylation at serine-824 and SUMOylation at several lysine residues of KAP1 have been linked to gene regulation. Whether these lysine residues are subjected to additional PTMs and if yes, what is the associated functional consequences need to be further elucidated. Moreover, it would be interesting to look at other PTM sites, such as the phosphorylation of serine-473. Whether the serine-473 phosphorylation affects the transcriptional repressor activity of KAP1 is still under investigation. Another interesting question is how KAP1 shuttles among different cellular compartments by PTMs. The serine-824 phosphorylation has distinct nuclear localization pattern from the serine-473 phosphorylation, thus suggesting that KAP1 phosphorylation may affect its subcellular localization. Intriguingly, KAP1 is not only present in nucleus, and has been observed to translocate from nucleus to cytoplasm[25]. How PTM affects its nucleus-cytoplasmic shuttling and potential chromatin extraction of KAP1 deserve additional investigation. Finally, considering that KAP1 possesses SUMO and ubiquitin E3 activities, it might regulate the function or fate of its SUMO- and ubiquitin-targets. In the future, studying the non-transcriptional function of KAP1 might help to further decipher its unrecognized face.

REFERENCES

1 **Le Douarin B**, Nielsen AL, Garnier JM, Ichinose H, Jeanmougin F, Losson R, Chambon P. A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. *EMBO J* 1996; **15**: 6701-6715 [PMID: 8978696]

2 **Friedman JR**, Fredericks WJ, Jensen DE, Speicher DW, Huang XP, Neilson EG, Rauscher FJ. KAP-1, a novel corepressor for the highly conserved KRAB repression domain. *Genes Dev* 1996; **10**: 2067-2078 [PMID: 8769649 DOI: 10.1101/gad.10.16.2067]

3 **Kim SS**, Chen YM, O'Leary E, Witzgall R, Vidal M, Bonventre JV. A novel member of the RING finger family, KRIP-1, associates with the KRAB-A transcriptional repressor domain of zinc finger proteins. *Proc Natl Acad Sci USA* 1996; **93**: 15299-15304 [PMID: 8986806 DOI: 10.1073/pnas.93.26.15299]

4 **Moosmann P**, Georgiev O, Le Douarin B, Bourquin JP, Schaffner W. Transcriptional repression by RING finger protein TIF1 beta that interacts with the KRAB repressor domain of KOX1. *Nucleic Acids Res* 1996; **24**: 4859-4867 [PMID: 9016654 DOI: 10.1093/nar/24.24.4859]

5 **Nielsen AL**, Ortiz JA, You J, Oulad-Abdelghani M, Khechumian R, Gansmuller A, Chambon P, Losson R. Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. *EMBO J* 1999; **18**: 6385-6395 [PMID: 10562550 DOI: 10.1093/emboj/18.22.6385]

6 **Ryan RF**, Schultz DC, Ayyanathan K, Singh PB, Friedman JR, Fredericks WJ, Rauscher FJ. KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Krüppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol Cell Biol* 1999; **19**: 4366-4378 [PMID: 10330177]

7 **Underhill C**, Qutob MS, Yee SP, Torchia J. A novel nuclear receptor corepressor complex, N-CoR, contains components of the mammalian SWI/SNF complex and the corepressor KAP-1. *J Biol Chem* 2000; **275**: 40463-40470 [PMID: 11013263 DOI: 10.1074/jbc.M007864200]

8 **Schultz DC**, Friedman JR, Rauscher FJ. Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. *Genes Dev* 2001; **15**: 428-443 [PMID: 11230151 DOI: 10.1101/gad.869501]

9 **Schultz DC**, Ayyanathan K, Negorev D, Maul GG, Rauscher FJ. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev* 2002; **16**: 919-932 [PMID: 11959841 DOI: 10.1101/gad.973302]

10 **Satou A**, Taira T, Iguchi-Ariga SM, Ariga H. A novel transrepression pathway of c-Myc. Recruitment of a transcriptional corepressor complex to c-Myc by MM-1, a c-Myc-binding protein. *J Biol Chem* 2001; **276**: 46562-46567 [PMID: 11585818 DOI: 10.1074/jbc.M104937200]

11 **Satou A**, Hagio Y, Taira T, Iguchi-Ariga SM, Ariga H. Repression of the c-fms gene in fibroblast cells by c-Myc-MM-1-TIF1beta complex. *FEBS Lett* 2004; **572**: 211-215 [PMID: 15304350 DOI: 10.1016/j.febslet.2004.07.034]

12 **Hagio Y**, Kimura Y, Taira T, Fujioka Y, Iguchi-Ariga SM, Ariga H. Distinct localizations and repression activities of MM-1 isoforms toward c-Myc. *J Cell Biochem* 2006; **97**: 145-155 [PMID: 16173081 DOI: 10.1002/jcb.20619]

13 **Wang C**, Rauscher FJ, Cress WD, Chen J. Regulation of E2F1 function by the nuclear corepressor KAP1. *J Biol Chem* 2007; **282**: 29902-29909 [PMID: 17704056 DOI: 10.1074/jbc.M704757200]

14 **Chen L**, Chen DT, Kurtyka C, Rawal B, Fulp WJ, Haura EB, Cress WD. Tripartite motif containing 28 (Trim28) can regulate cell proliferation by bridging HDAC1/E2F interactions. *J Biol Chem* 2012; **287**: 40106-40118 [PMID: 23060449 DOI: 10.1074/jbc.M112.380865]

15 **Ivanov AV**, Peng H, Yurchenko V, Yap KL, Negorev DG, Schultz DC, Psulkowski E, Fredericks WJ, White DE, Maul GG, Sadofsky MJ, Zhou MM, Rauscher FJ. PHD domain-mediated E3 ligase activity directs intramolecular sumoylation of an adjacent bromodomain required for gene silencing. *Mol Cell* 2007; **28**: 823-837 [PMID: 18082607 DOI: 10.1016/j.molcel.2007.11.012]

16 **Lee YK**, Thomas SN, Yang AJ, Ann DK. Doxorubicin down-regulates Kruppel-associated box domain-associated protein 1 sumoylation that relieves its transcription repression on p21WAF1/CIP1 in breast cancer MCF-7 cells. *J Biol Chem* 2007; **282**: 1595-1606 [PMID: 17079232 DOI: 10.1074/jbc.M606306200]

17 **Li X**, Lee YK, Jeng JC, Yen Y, Schultz DC, Shih HM, Ann DK. Role for KAP1 serine 824 phosphorylation and sumoylation/desumoylation switch in regulating KAP1-mediated transcriptional repression. *J Biol Chem* 2007; **282**: 36177-36189 [PMID: 17942393 DOI: 10.1074/jbc.M706912200]

18 **Mascle XH**, Germain-Desprez D, Huynh P, Estephan P, Aubry M. Sumoylation of the transcriptional intermediary factor 1beta (TIF1beta), the Co-repressor of the KRAB Multifinger proteins, is required for its transcriptional activity and is modulated by the KRAB domain. *J Biol Chem* 2007; **282**: 10190-10202 [PMID: 17298944 DOI: 10.1074/jbc.M611429200]

19 **Cammas F**, Mark M, Dollé P, Dierich A, Chambon P, Losson R. Mice lacking the transcriptional corepressor TIF1beta are defective in early postimplantation development. *Development* 2000; **127**: 2955-2963 [PMID: 10851139 DOI: 10.1016/j.neuron.2008.09.036]

20 **Ziv Y**, Bielopolski D, Galanty Y, Lukas C, Taya Y, Schultz DC, Lukas J, Bekker-Jensen S, Bartek J, Shiloh Y. Chromatin relaxation in response to DNA double-strand breaks is

modulated by a novel ATM- and KAP-1 dependent pathway. *Nat Cell Biol* 2006; **8**: 870-876 [PMID: 16862143 DOI: 10.1038/ncb1446]

21 **Goodarzi AA**, Kurka T, Jeggo PA. KAP-1 phosphorylation regulates CHD3 nucleosome remodeling during the DNA double-strand break response. *Nat Struct Mol Biol* 2011; **18**: 831-839 [PMID: 21642969 DOI: 10.1038/nsmb.2077]

22 **Liu B**, Wang Z, Ghosh S, Zhou Z. Defective ATM-Kap-1-mediated chromatin remodeling impairs DNA repair and accelerates senescence in progeria mouse model. *Aging Cell* 2013; **12**: 316-318 [PMID: 23173799 DOI: 10.1111/acel.12035]

23 **Doyle JM**, Gao J, Wang J, Yang M, Potts PR. MAGE-RING protein complexes comprise a family of E3 ubiquitin ligases. *Mol Cell* 2010; **39**: 963-974 [PMID: 20864041 DOI: 10.1016/j.molcel.2010.08.029]

24 **Liang Q**, Deng H, Li X, Wu X, Tang Q, Chang TH, Peng H, Rauscher FJ, Ozato K, Zhu F. Tripartite motif-containing protein 28 is a small ubiquitin-related modifier E3 ligase and negative regulator of IFN regulatory factor 7. *J Immunol* 2011; **187**: 4754-4763 [PMID: 21940674 DOI: 10.4049/jimmunol.1101704]

25 **Yang Y**, Fiskus W, Yong B, Atadja P, Takahashi Y, Pandita TK, Wang HG, Bhalla KN. Acetylated hsp70 and KAP1-mediated Vps34 SUMOylation is required for autophagosome creation in autophagy. *Proc Natl Acad Sci USA* 2013; **110**: 6841-6846 [PMID: 23569248 DOI: 10.1073/pnas.1217692110]

26 **Matsuda E**, Agata Y, Sugai M, Katakai T, Gonda H, Shimizu A. Targeting of Krüppel-associated box-containing zinc finger proteins to centromeric heterochromatin. Implication for the gene silencing mechanisms. *J Biol Chem* 2001; **276**: 14222-14229 [PMID: 11278721 DOI: 10.1074/jbc.M010663200]

27 **Iyengar S**, Farnham PJ. KAP1 protein: an enigmatic master regulator of the genome. *J Biol Chem* 2011; **286**: 26267-26276 [PMID: 21652716 DOI: 10.1074/jbc.R111.252569]

28 **Peng H**, Gibson LC, Capili AD, Borden KL, Osborne MJ, Harper SL, Speicher DW, Zhao K, Marmorstein R, Rock TA, Rauscher FJ. The structurally disordered KRAB repression domain is incorporated into a protease resistant core upon binding to KAP-1-RBCC domain. *J Mol Biol* 2007; **370**: 269-289 [PMID: 17512541 DOI: 10.1016/j.jmb.2007.03.047]

29 **Urrutia R**. KRAB-containing zinc-finger repressor proteins. *Genome Biol* 2003; **4**: 231 [PMID: 14519192 DOI: 10.1186/gb-2003-4-10-231]

30 **Peng H**, Begg GE, Schultz DC, Friedman JR, Jensen DE, Speicher DW, Rauscher FJ. Reconstitution of the KRAB-KAP-1 repressor complex: a model system for defining the molecular anatomy of RING-B box-coiled-coil domain-mediated protein-protein interactions. *J Mol Biol* 2000; **295**: 1139-1162 [PMID: 10653693 DOI: 10.1006/jmbi.1999.3402]

31 **Iyengar S**, Ivanov AV, Jin VX, Rauscher FJ, Farnham PJ. Functional analysis of KAP1 genomic recruitment. *Mol Cell Biol* 2011; **31**: 1833-1847 [PMID: 21343339 DOI: 10.1128/MCB.01331-10]

32 **Venturini L**, You J, Stadler M, Galien R, Lallemand V, Koken MH, Mattei MG, Ganser A, Chambon P, Losson R, de Thé H. TIF1gamma, a novel member of the transcriptional intermediary factor 1 family. *Oncogene* 1999; **18**: 1209-1217 [PMID: 10022127 DOI: 10.1038/onc.2012.501]

33 **Lechner MS**, Begg GE, Speicher DW, Rauscher FJ. Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: direct chromoshadow domain-KAP-1 corepressor interaction is essential. *Mol Cell Biol* 2000; **20**: 6449-6465 [PMID: 10938122 DOI: 10.1128/mcb.20.17.6449-6465.2000]

34 **Sripathy SP**, Stevens J, Schultz DC. The KAP1 corepressor functions to coordinate the assembly of de novo HP1-demarcated microenvironments of heterochromatin required for KRAB zinc finger protein-mediated transcriptional repression. *Mol Cell Biol* 2006; **26**: 8623-8638 [PMID: 16954381 DOI: 10.1128/MCB.00487-06]

35 **Riclet R**, Chendeb M, Vonesch JL, Koczan D, Thiesen HJ, Losson R, Cammas F. Disruption of the interaction between transcriptional intermediary factor 1{beta} and heterochromatin protein 1 leads to a switch from DNA hyper- to hypomethylation and H3K9 to H3K27 trimethylation on the MEST promoter correlating with gene reactivation. *Mol Biol Cell* 2009; **20**: 296-305 [PMID: 18923144 DOI: 10.1091/mbc.E08-05-0510]

36 **Li X**, Lin HH, Chen H, Xu X, Shih HM, Ann DK. SUMOylation of the transcriptional co-repressor KAP1 is regulated by the serine and threonine phosphatase PP1. *Sci Signal* 2010; **3**: ra32 [PMID: 20424263 DOI: 10.1126/scisignal.2000781]

37 **Garvin AJ**, Densham RM, Blair-Reid SA, Pratt KM, Stone HR, Weekes D, Lawrence KJ, Morris JR. The deSUMOylase SENP7 promotes chromatin relaxation for homologous recombination DNA repair. *EMBO Rep* 2013; **14**: 975-983 [PMID: 24018422 DOI: 10.1038/embor.2013.141]

38 **White DE**, Negorev D, Peng H, Ivanov AV, Maul GG, Rauscher FJ. KAP1, a novel substrate for PIKK family members, colocalizes with numerous damage response factors at DNA lesions. *Cancer Res* 2006; **66**: 11594-11599 [PMID: 17178852 DOI: 10.1158/0008-5472.CAN-06-4138]

39 **Tomimatsu N**, Mukherjee B, Burma S. Distinct roles of ATR and DNA-PKcs in triggering DNA damage responses in ATM-deficient cells. *EMBO Rep* 2009; **10**: 629-635 [PMID: 19444312 DOI: 10.1038/embor.2009.60]

40 **Lee DH**, Goodarzi AA, Adelmant GO, Pan Y, Jeggo PA, Marto JA, Chowdhury D. Phosphoproteomic analysis reveals that PP4 dephosphorylates KAP-1 impacting the DNA damage response. *EMBO J* 2012; **31**: 2403-2415 [PMID: 22491012 DOI: 10.1038/emboj.2012.86]

41 **Liu J**, Xu L, Zhong J, Liao J, Li J, Xu X. Protein phosphatase PP4 is involved in NHEJ-mediated repair of DNA double-strand breaks. *Cell Cycle* 2012; **11**: 2643-2649 [PMID: 22732494 DOI: 10.4161/Cc.20957]

42 **Hu C**, Zhang S, Gao X, Gao X, Xu X, Lv Y, Zhang Y, Zhu Z, Zhang C, Li Q, Wong J, Cui Y, Zhang W, Ma L, Wang C. Roles of Kruppel-associated Box (KRAB)-associated Co-repressor KAP1 Ser-473 Phosphorylation in DNA Damage Response. *J Biol Chem* 2012; **287**: 18937-18952 [PMID: 22496453 DOI: 10.1074/jbc.M111.313262]

43 **Bolderson E**, Savage KI, Mahen R, Pisupati V, Graham ME, Richard DJ, Robinson PJ, Venkitaraman AR, Khanna KK. Kruppel-associated Box (KRAB)-associated co-repressor (KAP-1) Ser-473 phosphorylation regulates heterochromatin protein 1β (HP1-β) mobilization and DNA repair in heterochromatin. *J Biol Chem* 2012; **287**: 28122-28131 [PMID: 22715096 DOI: 10.1074/jbc.M112.368381]

44 **White D**, Rafalska-Metcalf IU, Ivanov AV, Corsinotti A, Peng H, Lee SC, Trono D, Janicki SM, Rauscher FJ. The ATM substrate KAP1 controls DNA repair in heterochromatin: regulation by HP1 proteins and serine 473/824 phosphorylation. *Mol Cancer Res* 2012; **10**: 401-414 [PMID: 22205726 DOI: 10.1158/1541-7786.MCR-11-0134]

45 **Blasius M**, Forment JV, Thakkar N, Wagner SA, Choudhary C, Jackson SP. A phospho-proteomic screen identifies substrates of the checkpoint kinase Chk1. *Genome Biol* 2011; **12**: R78 [PMID: 21851590 DOI: 10.1186/gb-2011-12-8-r78]

46 **Chang CW**, Chou HY, Lin YS, Huang KH, Chang CJ, Hsu TC, Lee SC. Phosphorylation at Ser473 regulates heterochromatin protein 1 binding and corepressor function of TIF1beta/KAP1. *BMC Mol Biol* 2008; **9**: 61 [PMID: 18590578 DOI: 10.1186/1471-2199-9-61]

47 **Zhou XF**, Yu J, Chang M, Zhang M, Zhou D, Cammas F, Sun SC. TRIM28 mediates chromatin modifications at the TCRα enhancer and regulates the development of T and natural killer T cells. *Proc Natl Acad Sci USA* 2012; **109**: 20083-20088 [PMID: 23169648 DOI: 10.1073/pnas.1214704109]

48 **Chikuma S**, Suita N, Okazaki IM, Shibayama S, Honjo T. TRIM28 prevents autoinflammatory T cell development in vivo. *Nat Immunol* 2012; **13**: 596-603 [PMID: 22544392 DOI: 10.1038/ni.2293]

49 **King CA**. Kaposi's sarcoma-associated herpesvirus kaposin B induces unique monophosphorylation of STAT3 at serine 727 and MK2-mediated inactivation of the STAT3 transcriptional repressor TRIM28. *J Virol* 2013; **87**: 8779-8791 [PMID: 23740979 DOI: 10.1128/JVI.02976-12]

50 **Kamitani S**, Ohbayashi N, Ikeda O, Togi S, Muromoto R, Sekine Y, Ohta K, Ishiyama H, Matsuda T. KAP1 regulates type I interferon/STAT1-mediated IRF-1 gene expression. *Biochem Biophys Res Commun* 2008; **370**: 366-370 [PMID: 18381204 DOI: 10.1016/j.bbrc.2008.03.104]

51 **Kamitani S**, Togi S, Ikeda O, Nakasuji M, Sakauchi A, Sekine Y, Muromoto R, Oritani K, Matsuda T. Krüppel-associated box-associated protein 1 negatively regulates TNF-α-induced NF-κB transcriptional activity by influencing the interactions among STAT3, p300, and NF-κB/p65. *J Immunol* 2011; **187**: 2476-2483 [PMID: 21810609 DOI: 10.4049/jimmunol.1003243]

52 **Eames HL**, Saliba DG, Krausgruber T, Lanfrancotti A, Ryzhakov G, Udalova IA. KAP1/TRIM28: an inhibitor of IRF5 function in inflammatory macrophages. *Immunobiology* 2012; **217**: 1315-1324 [PMID: 22995936 DOI: 10.1016/j.imbio.2012.07.026]

53 **Kubota S**, Fukumoto Y, Aoyama K, Ishibashi K, Yuki R, Morinaga T, Honda T, Yamaguchi N, Kuga T, Tomonaga T, Yamaguchi N. Phosphorylation of KRAB-associated protein 1 (KAP1) at Tyr-449, Tyr-458, and Tyr-517 by nuclear tyrosine kinases inhibits the association of KAP1 and heterochromatin protein 1α (HP1α) with heterochromatin. *J Biol Chem* 2013; **288**: 17871-17883 [PMID: 23645696 DOI: 10.1074/jbc.M112.437756]

54 **Lai IL**, Lin TP, Yao YL, Lin CY, Hsieh MJ, Yang WM. Histone deacetylase 10 relieves repression on the melanogenic program by maintaining the deacetylation status of repressors. *J Biol Chem* 2010; **285**: 7187-7196 [PMID: 20032463 DOI: 10.1074/jbc.M109.061861]

55 **O'Geen H**, Squazzo SL, Iyengar S, Blahnik K, Rinn JL, Chang HY, Green R, Farnham PJ. Genome-wide analysis of KAP1 binding suggests autoregulation of KRAB-ZNFs. *PLoS Genet* 2007; **3**: e89 [PMID: 17542650 DOI: 10.1371/journal.pgen.0030089]

56 **Groner AC**, Meylan S, Ciuffi A, Zangger N, Ambrosini G, Dénervaud N, Bucher P, Trono D. KRAB-zinc finger proteins and KAP1 can mediate long-range transcriptional repression through heterochromatin spreading. *PLoS Genet* 2010; **6**: e1000869 [PMID: 20221260 DOI: 10.1371/journal.pgen.1000869]

57 **Chang PC**, Fitzgerald LD, Van Geelen A, Izumiya Y, Ellison TJ, Wang DH, Ann DK, Luciw PA, Kung HJ. Kruppel-associated box domain-associated protein-1 as a latency regulator for Kaposi's sarcoma-associated herpesvirus and its modulation by the viral protein kinase. *Cancer Res* 2009; **69**: 5681-5689 [PMID: 19584288 DOI: 10.1158/0008-5472.CAN-08-4570]

58 **Rowbotham SP**, Barki L, Neves-Costa A, Santos F, Dean W, Hawkes N, Choudhary P, Will WR, Webster J, Oxley D, Green CM, Varga-Weisz P, Mermoud JE. Maintenance of silent chromatin through replication requires SWI/SNF-like chromatin remodeler SMARCAD1. *Mol Cell* 2011; **42**: 285-296 [PMID: 21549307 DOI: 10.1016/j.molcel.2011.02.036]

59 **Quenneville S**, Verde G, Corsinotti A, Kapopoulou A, Jakobsson J, Offner S, Baglivo I, Pedone PV, Grimaldi G, Riccio A, Trono D. In embryonic stem cells, ZFP57/KAP1 recognize a methylated hexanucleotide to affect chromatin and DNA methylation of imprinting control regions. *Mol Cell* 2011; **44**: 361-372 [PMID: 22055183 DOI: 10.1016/j.molcel.2011.08.032]

60 **Quenneville S**, Turelli P, Bojkowska K, Raclot C, Offner S, Kapopoulou A, Trono D. The KRAB-ZFP/KAP1 system contributes to the early embryonic establishment of site-specific DNA methylation patterns maintained during development. *Cell Rep* 2012; **2**: 766-773 [PMID: 23041315 DOI: 10.1016/j.celrep.2012.08.043]

61 **Zuo X**, Sheng J, Lau HT, McDonald CM, Andrade M, Cullen DE, Bell FT, Iacovino M, Kyba M, Xu G, Li X. Zinc finger protein ZFP57 requires its co-factor to recruit DNA methyltransferases and maintains DNA methylation imprint in embryonic stem cells via its transcriptional repression domain. *J Biol Chem* 2012; **287**: 2107-2118 [PMID: 22144682 DOI: 10.1074/jbc.M111.322644]

62 **Takikawa S**, Wang X, Ray C, Vakulenko M, Bell FT, Li X. Human and mouse ZFP57 proteins are functionally interchangeable in maintaining genomic imprinting at multiple imprinted regions in mouse ES cells. *Epigenetics* 2013; **8**: 1268-1279 [PMID: 24135613]

63 **Messerschmidt DM**, de Vries W, Ito M, Solter D, Ferguson-Smith A, Knowles BB. Trim28 is required for epigenetic stability during mouse oocyte to embryo transition. *Science* 2012; **335**: 1499-1502 [PMID: 22442485 DOI: 10.1126/science.1216154]

64 **Cedar H**, Bergman Y. Linking DNA methylation and histone modification: patterns and paradigms. *Nat Rev Genet* 2009; **10**: 295-304 [PMID: 19308066 DOI: 10.1038/nrg2540]

65 **Kacem S**, Feil R. Chromatin mechanisms in genomic imprinting. *Mamm Genome* 2009; **20**: 544-556 [PMID: 19760321 DOI: 10.1007/s00335-009-9223-4]

66 **Itokawa Y**, Yanagawa T, Yamakawa H, Watanabe N, Koga H, Nagase T. KAP1-independent transcriptional repression of SCAN-KRAB-containing zinc finger proteins. *Biochem Biophys Res Commun* 2009; **388**: 689-694 [PMID: 19695231 DOI: 10.1016/j.bbrc.2009.08.065]

67 **Meylan S**, Groner AC, Ambrosini G, Malani N, Quenneville S, Zangger N, Kapopoulou A, Kauzlaric A, Rougemont J, Ciuffi A, Bushman FD, Bucher P, Trono D. A gene-rich, transcriptionally active environment and the pre-deposition of repressive marks are predictive of susceptibility to KRAB/KAP1-mediated silencing. *BMC Genomics* 2011; **12**: 378 [PMID: 21791101 DOI: 10.1186/1471-2164-12-378]

68 **Jin VX**, O'Geen H, Iyengar S, Green R, Farnham PJ. Identification of an OCT4 and SRY regulatory module using integrated computational and experimental genomics approaches. *Genome Res* 2007; **17**: 807-817 [PMID: 17567999 DOI: 10.1101/gr.6006107]

69 **Zheng L**, Pan H, Li S, Flesken-Nikitin A, Chen PL, Boyer TG, Lee WH. Sequence-specific transcriptional corepressor function for BRCA1 through a novel zinc finger protein, ZBRK1. *Mol Cell* 2000; **6**: 757-768 [PMID: 11090615 DOI: 10.1016/S1097-2765(00)00075-7]

70 **Takahashi K,** Sugi Y, Hosono A, and Kaminogawa S. Epigenetic Regulation of TLR4 Gene Expression in Intestinal Epithelial Cells for the Maintenance of Intestinal Homeostasis. *J Immunol* 2009; **183**: 6522-6529 [DOI: 10.4049/jimmunol.0901271]

71 **Barde I**, Rauwel B, Marin-Florez RM, Corsinotti A, Laurenti E, Verp S, Offner S, Marquis J, Kapopoulou A, Vanicek J, Trono D. A KRAB/KAP1-miRNA cascade regulates erythropoiesis through stage-specific control of mitophagy. *Science* 2013; **340**: 350-353 [PMID: 23493425 DOI: 10.1126/science.1232398]

72 **Li Z**, Wang D, Na X, Schoen SR, Messing EM, Wu G. The VHL protein recruits a novel KRAB-A domain protein to repress HIF-1alpha transcriptional activity. *EMBO J* 2003; **22**: 1857-1867 [PMID: 12682018 DOI: 10.1093/Emboj/Cdg173]

73 **Peng H**, Ivanov AV, Oh HJ, Lau YF, Rauscher FJ. Epigenetic gene silencing by the SRY protein is mediated by a KRAB-O protein that recruits the KAP1 co-repressor machinery. *J Biol Chem* 2009; **284**: 35670-35680 [PMID: 19850934 DOI: 10.1074/jbc.M109.032086]

74 **Huang C**, Martin S, Pfleger C, Du J, Buckner JH, Bluestone JA, Riley JL, Ziegler SF. Cutting Edge: a novel, human-specific interacting protein couples FOXP3 to a chromatin-remodeling complex that contains KAP1/TRIM28. *J Immunol* 2013; **190**: 4470-4473 [PMID: 23543754 DOI: 10.4049/jimmunol.1203561]

75 **Wang C**, Ivanov A, Chen L, Fredericks WJ, Seto E, Rauscher FJ, Chen J. MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. *EMBO J* 2005; **24**: 3279-3290 [PMID: 16107876 DOI: 10.1038/sj.emboj.7600791]

76 **Maruyama A**, Nishikawa K, Kawatani Y, Mimura J, Hosoya T, Harada N, Yamamato M, Itoh K. The novel Nrf2-interacting factor KAP1 regulates susceptibility to oxidative stress by promoting the Nrf2-mediated cytoprotective response. *Biochem J* 2011; **436**: 387-397 [PMID: 21382013 DOI: 10.1042/BJ20101748]

77 **Tian C**, Xing G, Xie P, Lu K, Nie J, Wang J, Li L, Gao M, Zhang L, He F. KRAB-type zinc-finger protein Apak specifically regulates p53-dependent apoptosis. *Nat Cell Biol* 2009; **11**: 580-591 [PMID: 19377469 DOI: 10.1038/ncb1864]

78 **Seki Y**, Kurisaki A, Watanabe-Susaki K, Nakajima Y, Nakanishi M, Arai Y, Shiota K, Sugino H, Asashima M. TIF-1beta regulates the pluripotency of embryonic stem cells in a phosphorylation-dependent manner. *Proc Natl Acad Sci USA* 2010; **107**: 10926-10931 [PMID: 20508149 DOI: 10.1073/pnas.0907601107]

79 **Takahashi K**, Sugi Y, Hosono A, Kaminogawa S. Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis. *J Immunol* 2009; **183**: 6522-6529 [PMID: 19846881 DOI: 10.4049/jimmunol.0901271]

80 **Yang B**, O'Herrin SM, Wu J, Reagan-Shaw S, Ma Y, Bhat KM, Gravekamp C, Setaluri V, Peters N, Hoffmann FM, Peng H, Ivanov AV, Simpson AJ, Longley BJ. MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. *Cancer Res* 2007; **67**: 9954-9962 [PMID: 17942928 DOI: 10.1158/0008-5472.CAN-07-1478]

81 **Bhatia N**, Yang B, Xiao TZ, Peters N, Hoffmann MF, Longley BJ. Identification of novel small molecules that inhibit protein-protein interactions between MAGE and KAP-1. *Arch Biochem Biophys* 2011; **508**: 217-221 [PMID: 21277283 DOI: 10.1016/j.abb.2011.01.007]

82 **Tsuruma R**, Ohbayashi N, Kamitani S, Ikeda O, Sato N, Muromoto R, Sekine Y, Oritani K, Matsuda T. Physical and functional interactions between STAT3 and KAP1. *Oncogene* 2008; **27**: 3054-3059 [PMID: 18037959 DOI: 10.1038/sj.onc.1210952]

83 **Goodarzi AA**, Noon AT, Deckbar D, Ziv Y, Shiloh Y, Löbrich M, Jeggo PA. ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. *Mol Cell* 2008; **31**: 167-177 [PMID: 18657500 DOI: 10.1016/j.molcel.2008.05.017]

84 **Geuting V**, Reul C, Löbrich M. ATM release at resected double-strand breaks provides heterochromatin reconstitution to facilitate homologous recombination. *PLoS Genet* 2013; **9**: e1003667 [PMID: 23935532 DOI: 10.1371/journal.pgen.1003667]

85 **Baldeyron C**, Soria G, Roche D, Cook AJ, Almouzni G. HP1alpha recruitment to DNA damage by p150CAF-1 promotes homologous recombination repair. *J Cell Biol* 2011; **193**: 81-95 [PMID: 21464229 DOI: 10.1083/jcb.201101030]

86 **Noon AT**, Shibata A, Rief N, Löbrich M, Stewart GS, Jeggo PA, Goodarzi AA. 53BP1-dependent robust localized KAP-1 phosphorylation is essential for heterochromatic DNA double-strand break repair. *Nat Cell Biol* 2010; **12**: 177-184 [PMID: 20081839 DOI: 10.1038/Ncb2017]

87 **Beucher A**, Birraux J, Tchouandong L, Barton O, Shibata A, Conrad S, Goodarzi AA, Krempler A, Jeggo PA, Löbrich M. ATM and Artemis promote homologous recombination of radiation-induced DNA double-strand breaks in G2. *EMBO J* 2009; **28**: 3413-3427 [PMID: 19779458 DOI: 10.1038/emboj.2009.276]

88 **Shibata A**, Conrad S, Birraux J, Geuting V, Barton O, Ismail A, Kakarougkas A, Meek K, Taucher-Scholz G, Löbrich M, Jeggo PA. Factors determining DNA double-strand break repair pathway choice in G2 phase. *EMBO J* 2011; **30**: 1079-1092 [PMID: 21317870 DOI: 10.1038/emboj.2011.27]

89 **Kakarougkas A**, Ismail A, Klement K, Goodarzi AA, Conrad S, Freire R, Shibata A, Lobrich M, Jeggo PA. Opposing roles for 53BP1 during homologous recombination. *Nucleic Acids Res* 2013; **41**: 9719-9731 [PMID: 23969417 DOI: 10.1093/nar/gkt729]

90 **Goodarzi AA**, Noon AT, Jeggo PA. The impact of heterochromatin on DSB repair. *Biochem Soc Trans* 2009; **37**: 569-576 [PMID: 19442252 DOI: 10.1042/BST0370569]

91 **Verger A**, Perdomo J, Crossley M. Modification with SUMO. A role in transcriptional regulation. *EMBO Rep* 2003; **4**: 137-142 [PMID: 12612601 DOI: 10.1038/sj.embor.embor738]

92 **Xiao TZ**, Bhatia N, Urrutia R, Lomberk GA, Simpson A, Longley BJ. MAGE I transcription factors regulate KAP1 and KRAB domain zinc finger transcription factor mediated gene repression. *PLoS One* 2011; **6**: e23747 [PMID: 21876767 DOI: 10.1371/journal.pone.0023747]

93 **Hu G**, Kim J, Xu Q, Leng Y, Orkin SH, Elledge SJ. A genome-wide RNAi screen identifies a new transcriptional module required for self-renewal. *Genes Dev* 2009; **23**: 837-848 [PMID: 19339689 DOI: 10.1101/gad.1769609]

94 **Cammas F**, Oulad-Abdelghani M, Vonesch JL, Huss-Garcia Y, Chambon P, Losson R. Cell differentiation induces TIF-1beta association with centromeric heterochromatin via an HP1 interaction. *J Cell Sci* 2002; **115**: 3439-3448 [PMID: 12154074]

95 **Herzog M**, Wendling O, Guillou F, Chambon P, Mark M, Losson R, Cammas F. TIF1β association with HP1 is essential for post-gastrulation development, but not for Sertoli cell functions during spermatogenesis. *Dev Biol* 2011; **350**: 548-558 [PMID: 21163256 DOI: 10.1016/j.ydbio.2010.12.014]

96 **Cammas F**, Herzog M, Lerouge T, Chambon P, Losson R. Association of the transcriptional corepressor TIF1beta with heterochromatin protein 1 (HP1): an essential role for progression through differentiation. *Genes Dev* 2004; **18**: 2147-2160 [PMID: 15342492 DOI: 10.1101/gad.302904]

97 **Cammas F,** Janoshazi A, Lerouge T, and Losson R. Dynamic and selective interactions of the transcriptional corepressor TIF1 beta with the heterochromatin protein HP1 isotypes during cell differentiation. *Differentiation* 2007; **75**: 627-637 [DOI: 10.1111/j.1432-0436.2007.00166.x]

98 **Santoni de Sio FR**, Barde I, Offner S, Kapopoulou A, Corsinotti A, Bojkowska K, Genolet R, Thomas JH, Luescher IF, Pinschewer D, Harris N, Trono D. KAP1 regulates gene networks controlling T-cell development and responsiveness. *FASEB J* 2012; **26**: 4561-4575 [PMID: 22872677 DOI: 10.1096/fj.12-206177]

99 **Santoni de Sio FR**, Massacand J, Barde I, Offner S, Corsinotti A, Kapopoulou A, Bojkowska K, Dagklis A, Fernandez M, Ghia P, Thomas JH, Pinschewer D, Harris N, Trono D. KAP1 regulates gene networks controlling mouse B-lymphoid cell differentiation and function. *Blood* 2012; **119**: 4675-4685 [PMID: 22452978 DOI: 10.1182/blood-2011-12-401117]

100 **Hosoya T**, Clifford M, Losson R, Tanabe O, Engel JD. TRIM28 is essential for erythroblast differentiation in the mouse. *Blood* 2013; **122**: 3798-3807 [PMID: 24092935 DOI: 10.1182/blood-2013-04-496166]

101 **Weber P**, Cammas F, Gerard C, Metzger D, Chambon P, Losson R, Mark M. Germ cell expression of the transcriptional co-repressor TIF1beta is required for the maintenance of spermatogenesis in the mouse. *Development* 2002; **129**: 2329-2337 [PMID: 11973266]

102 **Jeevan-Raj BP**, Robert I, Heyer V, Page A, Wang JH, Cammas F, Alt FW, Losson R, Reina-San-Martin B. Epigenetic tethering of AID to the donor switch region during immunoglobulin class switch recombination. *J Exp Med* 2011; **208**: 1649-1660 [PMID: 21746811 DOI: 10.1084/jem.20110118]

103 **Jakobsson J**, Cordero MI, Bisaz R, Groner AC, Busskamp V, Bensadoun JC, Cammas F, Losson R, Mansuy IM, Sandi C, Trono D. KAP1-mediated epigenetic repression in the forebrain modulates behavioral vulnerability to stress. *Neuron* 2008; **60**: 818-831 [PMID: 19081377]

104 **Okamoto K**, Kitabayashi I, Taya Y. KAP1 dictates p53 response induced by chemotherapeutic agents via Mdm2 interaction. *Biochem Biophys Res Commun* 2006; **351**: 216-222 [PMID: 17056014 DOI: 10.1016/j.bbrc.2006.10.022]

105 **Wolf D**, Cammas F, Losson R, Goff SP. Primer binding site-dependent restriction of murine leukemia virus requires HP1 binding by TRIM28. *J Virol* 2008; **82**: 4675-4679 [PMID: 18287239 DOI: 10.1128/JVI.02445-07]

106 **Wolf D**, Goff SP. TRIM28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. *Cell* 2007; **131**: 46-57 [PMID: 17923087 DOI: 10.1016/j.cell.2007.07.026]

107 **Wolf D**, Goff SP. Embryonic stem cells use ZFP809 to silence retroviral DNAs. *Nature* 2009; **458**: 1201-1204 [PMID: 19270682 DOI: 10.1038/Nature07844]

108 **Wolf D**, Hug K, Goff SP. TRIM28 mediates primer binding site-targeted silencing of Lys1,2 tRNA-utilizing retroviruses in embryonic cells. *Proc Natl Acad Sci USA* 2008; **105**: 12521-12526 [PMID: 18713861 DOI: 10.1073/pnas.0805540105]

109 **Matsui T**, Leung D, Miyashita H, Maksakova IA, Miyachi H, Kimura H, Tachibana M, Lorincz MC, Shinkai Y. Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. *Nature* 2010; **464**: 927-931 [PMID: 20164836 DOI: 10.1038/nature08858]

110 **Schlesinger S**, Lee AH, Wang GZ, Green L, Goff SP. Proviral silencing in embryonic cells is regulated by Yin Yang 1. *Cell Rep* 2013; **4**: 50-58 [PMID: 23810560 DOI: 10.1016/j.celrep.2013.06.003]

111 **Rowe HM**, Jakobsson J, Mesnard D, Rougemont J, Reynard S, Aktas T, Maillard PV, Layard-Liesching H, Verp S, Marquis J, Spitz F, Constam DB, Trono D. KAP1 controls endogenous retroviruses in embryonic stem cells. *Nature* 2010; **463**: 237-240 [PMID: 20075919 DOI: 10.1038/nature08674]

112 **Tan X**, Xu X, Elkenani M, Smorag L, Zechner U, Nolte J, Engel W, Pantakani DV. Zfp819, a novel KRAB-zinc finger protein, interacts with KAP1 and functions in genomic integrity maintenance of mouse embryonic stem cells. *Stem Cell Res* 2013; **11**: 1045-1059 [PMID: 23954693 DOI: 10.1016/j.scr.2013.07.006]

113 **Allouch A**, Di Primio C, Alpi E, Lusic M, Arosio D, Giacca M, Cereseto A. The TRIM family protein KAP1 inhibits HIV-1 integration. *Cell Host Microbe* 2011; **9**: 484-495 [PMID: 21669397 DOI: 10.1016/j.chom.2011.05.004]

114 **Lin LF**, Li CF, Wang WJ, Yang WM, Wang DD, Chang WC, Lee WH, Wang JM. Loss of ZBRK1 contributes to the increase of KAP1 and promotes KAP1-mediated metastasis and invasion in cervical cancer. *PLoS One* 2013; **8**: e73033 [PMID: 23991171 DOI: 10.1371/journal.pone.0073033]

115 **Yokoe T,** Toiyama Y, Okugawa Y, Tanaka K, Ohi M, Inoue Y, Mohri Y, Miki C, and Kusunoki M. KAP1 Is Associated With Peritoneal Carcinomatosis in Gastric Cancer. *Ann Surg Oncol* 2009; **17**: 821-828

116 **Hector S**, Chen H, Kijanka G, Murray F, Prehn JH. A reverse-ELISA for the detection of TRIM28/KAP1 serum autoantibodies in colorectal cancer patients. *Acta Oncol* 2012; **51**: 394-396 [PMID: 22268577 DOI: 10.3109/0284186X.2011.652742]

117 **Wang YY**, Li L, Zhao ZS, Wang HJ. Clinical utility of measuring expression levels of KAP1, TIMP1 and STC2 in peripheral blood of patients with gastric cancer. *World J Surg Oncol* 2013; **11**: 81 [PMID: 23548070 DOI: 10.1186/1477-7819-11-81]

118 **Martins MB**, Marcello MA, Morari EC, Cunha LL, Soares FA, Vassallo J, Ward LS. Clinical utility of KAP-1 expression in thyroid lesions. *Endocr Pathol* 2013; **24**: 77-82 [PMID: 23645532 DOI: 10.1007/s12022-013-9245-z]

119 **Kijanka G**, Hector S, Kay EW, Murray F, Cummins R, Murphy D, MacCraith BD, Prehn JH, Kenny D. Human IgG antibody profiles differentiate between symptomatic patients with and without colorectal cancer. *Gut* 2010; **59**: 69-78 [PMID: 19828471 DOI: 10.1136/gut.2009.178574]

120 **Bojkowska K**, Aloisio F, Cassano M, Kapopoulou A, Santoni de Sio F, Zangger N, Offner S, Cartoni C, Thomas C, Quenneville S, Johnsson K, Trono D. Liver-specific ablation of Krüppel-associated box-associated protein 1 in mice leads to male-predominant hepatosteatosis and development of liver adenoma. *Hepatology* 2012; **56**: 1279-1290 [PMID: 22684873 DOI: 10.1002/hep.25767]

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REVIEW

Mnk kinase pathway: Cellular functions and biological outcomes

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Abstract

The mitogen-activated protein kinase (MAPK) interacting protein kinases 1 and 2 (Mnk1 and Mnk2) play important roles in controlling signals involved in mRNA translation. In addition to the MAPKs (p38 or Erk), multiple studies suggest that the Mnk kinases can be regulated by other known kinases such as Pak2 and/or other unidentified kinases by phosphorylation of residues distinct from the sites phosphorylated by the MAPKs. Several studies have established multiple Mnk protein targets, including PSF, heterogenous nuclear ribonucleoprotein A1, Sprouty 2 and have lead to the identification of distinct biological functions and substrate specificity for the Mnk kinases. In this review we discuss the pathways regulating the Mnk kinases, their known substrates as well as the functional consequences of engagement of pathways controlled by Mnk kinases. These kinases play an important role in mRNA translation *via* their regulation of eukaryotic initiation factor 4E (eIF4E) and their functions have important implications in tumor biology as well as the regulation of drug resistance to anti-oncogenic therapies. Other studies have identified a role for the Mnk kinases in cap-independent mRNA translation, suggesting that the Mnk kinases can exert important functional effects independently of the phosphorylation of eIF4E. The role of Mnk kinases in inflammation and inflammation-induced malignancies is also discussed.

Key words: Mnk kinases; mRNA translation; Mitogen-activated protein kinase signaling; eIF4E phosphorylation; Drug resistance; Cytokine production; Cytokine signaling

Core tip: The Mnk kinases are important downstream targets of the Erk and p38 mitogen-activated protein kinase (MAPK) pathways and their activity can also be modulated by MAPK independent signals. The Mnk kinases play important roles in regulating mRNA translation and, because of this, are key mediators of oncogenic progression, drug resistance, production of pro-inflammatory cytokines and cytokine signaling. This review focuses on the pathways regulating the Mnk kinases, the substrates on the Mnk kinases as well as the biological functions of the Mnk kinases.

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INTRODUCTION

The Erk (extracellular regulated kinase) and p38 (mitogen-activated protein kinase) MAPK pathways are known to play important roles in mediating multiple biological processes including development, apoptosis, autophagy, oncogenesis, inflammation, *etc*[1]. Kinases that can be

phosphorylated by multiple MAPKs such as the MAPK interacting protein kinases (Mnks) can exert multiple biological functions due to their ability to respond to a wide range of external stimuli such as mitogens as well as stress inducers[1]. The Mnk kinase family includes Mnk1 and Mnk2 which were originally discovered in two independent screens as substrates for Erk1[2] and Erk2[3]. It is now well established that the Mnk kinases can be activated by either Erk or p38 MAPKs in response to multiple extracellular stimuli and phosphorylate their major downstream effector, the cap binding eukaryotic initiation factor 4E (eIF4E)[4].

Mnk1 and Mnk2 are serine/threonine kinases with substantial similarity in their coding sequences and motifs present in their structures[3]. Both kinases contain a N-terminal basic amino acid rich region that can mediate their localization; a catalytic domain similar to the serine/threonine kinases such as the Rsks, Ca21/calmodulin (CaM)-dependent kinases, Mapkap kinase-2 and Mapkap kinase-3 containing conserved MAPK phosphorylation sites; and an MAPK binding domain in their carboxyl terminus[3]. Mnk1 is activated in response to treatment with growth factors, ultraviolet (UV) radiation, mitogens and stress inducing agents such as anisomycin or sorbitol as well as by cytokines such as type Ⅰ and type Ⅱ interferons (IFNs), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, *etc.*[3-6]. In contrast Mnk2 exhibits high basal activity that is more resistant to the inhibition of Erk and p38 and these observations can be partially explained by differences in the C-terminal domains of the Mnk kinases[3,7].

A detailed look at the Mnk kinases has shown that both Mnk1 and Mnk2 undergo alternative splicing[8,9]. Mnk1 and Mnk2 transcripts are alternatively spliced giving rise to two distinct isoforms for each[8,9]. The b isoforms lack the MAPK binding C-terminal domain and therefore their activity is MAPK independent[8,10,11]. The b isoforms also lack a nuclear export sequence while still retaining the nuclear localization signal and therefore both Mnk1b and Mnk2b are preferentially localized to the nucleus and in PML bodies which also contain eIF4E[8,10]. While most of the studies on the Mnk kinases have focused on the Mnk1a and Mnk2a isoforms, evidence suggests that aberrant regulation of Mnk splicing can have important biological consequences. The splicing factor SF2/ASF which can function as a proto-oncogene in multiple human cancers can regulate Mnk2 splicing[12]. Overexpression of SF2/ASF was shown to result in the increased expression of the MAPK independent Mnk2b isoform, while knockdown of SF2/ASF attenuated the expression of Mnk2b[12]. This study suggests the need for a better understanding of the factors that regulate Mnk splicing as the preferential expression of the MAPK independent Mnk isoforms can have important biological implications.

POST TRANSCRIPTIONAL REGULATION OF MNK KINASES

Mnk kinase activity is mainly regulated by the upstream p38 and Erk MAPK pathways. The p38 MAPK pathway is activated by a variety of stress inducers such as osmotic shock, UV radiation, as well as cytokine and chemokine stimulation[13]; while engagement of the Erk MAPK pathway is primarily mediated by pro-growth stimuli such as growth factors and phorbol esters[1]. Thus the Mnk kinases can play a dual role in mediating cellular responses to stress as well as responses to mitogens in a context-specific manner. MAPK phosphorylation of Mnk1 results in the phosphorylation of Thr 209 and Thr 214 located in the T loop activation domain, whereas mouse Mnk1 is phosphorylated on Thr 197 and Thr 202[14].

Phosphorylation of Mnk1 has been shown to activate its kinase activity as well as to enhance its binding to the eukaryotic initiation factor 4G (eIF4G) which functions as a scaffolding protein[14,15]. Additionally Mnk1 mediated phosphorylation of eIF4E regulates its release from eIF4G[14]. eIF4G contains binding sites for the cap binding eIF4E and the poly A tail protein (PABP) at the N-terminus[16,17] while the C-terminal domain contains docking sites for eIF3, eIF4A and Mnk1[15,17]. eIF4G along with its binding partners and the small ribosomal subunits are important components of the 48S initiation complex required for translation initiation[18]. Studies suggest that Mnk1 is unable to interact with eIF4E in the absence of eIF4G and a mutant eIF4E lacking the ability to bind eIF4G is not a good Mnk1 substrate[15]. Additionally Mnk1 can interact with the eIF4G related translational repressor p97[15]. p97 which functions as a cap dependent and cap independent translation repressor has a 28% homology to the C-terminal of eIF4G and can interact with translation initiation factors such as eIF3, eIF4A but is unable to interact with the mRNA recruiting eIF4E[19]. Thus p97 may be a potential negative regulator of Mnk1 mediated phosphorylation of eIF4E [15]. Also PKCα which was initially believed to be a Mnk1 kinase is known to phosphorylate eIF4G on Ser 1186 facilitating its binding to Mnk1[20] and may potentially play an important role in regulating Mnk1 activity by indirectly controlling the phosphorylation of eIF4E. Mnk2 has also been shown to interact with eIF4G and to function as an eIF4E kinase[21]. Thus, regulation of the Mnk-eIF4G interaction can play an important role in regulating Mnk activity.

Other studies have suggested that phosphorylation of Mnk1 by the p21 activated kinase 2 (Pak2/γ-Pak) can negatively regulate its kinase activity[22]. Pak2 belongs to a family of serine/threonine kinases and is activated in response to stress inducing stimuli such as UV and ionizing radiation induced DNA damage, serum starvation, by the binding of the GTP bound small G protein cdc24 as well as by caspase 3 mediated cleavage[23]. Caspase 3 activated Pak2 mediated engagement of Mnk1 results in the phosphorylation of Thr 22 and Ser 27, residues that lie in the N-terminal domain of Mnk1 that can interact with eIF4G and thereby attenuates the affinity of Mnk1 towards eIF4G[22]. Additionally Pak2 mediated engagement of Mnk1 also attenuated Mnk1 mediated phosphorylation of eIF4G[22]. As the experiments conducted in this study were for the most part performed *in vitro*,

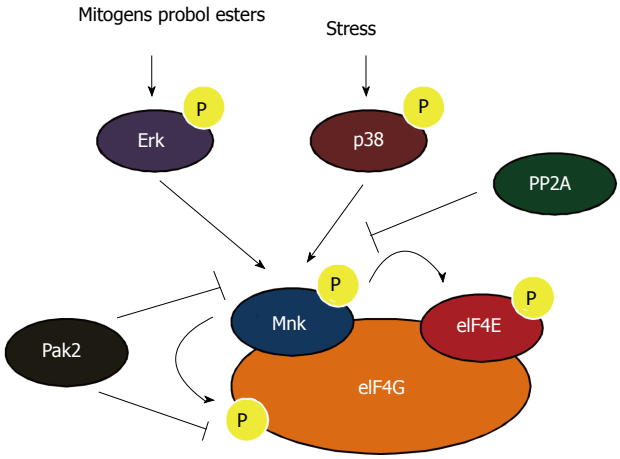

Figure 1 Regulation of Mnk kinases. The Mnk kinases are phosphorylated on Thr 197/202 by the p38 and Erk1/2 mitogen-activated protein kinases (MAPKs). They can associate with eIF4G and this interaction is essential for the efficient phosphorylation of their target eIF4E. The Mnk kinases are also known to phosphorylate eukaryotic initiation factor 4G (eIF4G) but its functional consequences remain to be determined. Pak2 can phosphorylate Mnk1 on Thr22/Ser27 resulting in decreased affinity for eIF4G and potentially interferes with Mnk1 mediated phosphorylation of eIF4E. Additionally Pak2 also phosphorylates eIF4G inhibiting its interaction with eIF4E. Protein phosphatase 2A (PP2A) is a phosphatase for Mnk1 and thereby negatively regulates Mnk kinase activity.

Pak2 mediated phosphorylation of Mnk1 did not affect Mnk1 mediated phosphorylation of eIF4E. Additionally Pak2 can also phosphorylate eIF4G at the eIF4E binding domain and compete with eIF4E to bind eIF4G, thereby exerting suppressive effects on cap dependent translation[24]. These results suggest that Mnk activity may be modulated independently of the MAPK pathway and may account for the observation that all stimuli that result in phosphorylation of Mnk1 do not result in activation of eIF4E on serine 209.

Mnk kinase activity can be negatively regulated by the protein phosphatase 2A (PP2A)[25]. Small interfering RNA mediated knockdown of PP2A or pharmacological inhibition of PP2A was found to result in increased phosphorylation of its direct target Mnk1 and subsequently increased phosphorylation of eIF4E[25]. Phosphorylation of eIF4E in response to PP2A inhibition leads to increased cap dependent translation of growth promoting mRNAs such as c-myc and Mcl-1[25].

Multiple studies have shown that Mnk2 has high basal activity that is mostly unresponsive to external stimuli. A study by Stead *et al*[26] showed that treatment of cells with rapamycin, the classic inhibitor of the mammalian target of rapamycin (mTOR), resulted in enhanced phosphorylation of eIF4E that was mediated by the enhanced activity of Mnk2 and not by Mnk1. The increase in Mnk2 activity was mediated by the decrease in phosphorylation of Mnk2 on Ser 437 by an unidentified mechanism[26]. These results suggest that Mnk2 activity may also be possibly modulated independently of the MAPK pathway. The regulation of Mnk kinases by upstream signaling proteins is summarized in Figure 1.

EFFECTORS OF THE MNK KINASES

The Mnk kinases function as serine/threonine kinases and are known to phosphorylate a number of downstream targets including eIF4E[3,21], hnRNPA1[27] and Sprouty2[28]. Additionally Mnk1 and Mnk2 can also exhibit substrate specificity[29], resulting in substrates that are unique to Mnk1 and Mnk2, respectively. Recent studies have surprising uncovered a kinase independent function for Mnk2 in negatively regulating eIF4G and p70S6K phosphorylations[30]. The proteins that regulate signaling downstream of the Mnk kinases are discussed in detail and are summarized in Figure 2.

eIF4E

A major and well characterized target of the Mnk kinases is the cap binding protein eIF4E. eIF4E is phosphorylated on Ser 209[31] by the Mnk kinases[3] but its role in regulating mRNA translation remains undetermined. Multiple biochemical studies have shown that phosphorylation of eIF4E reduces its affinity for the 5' m7G cap[32,33]. Based on X-ray crystallography data, Scheper *et al*[34] have speculated that the phosphate group on Ser 209 may negatively interact with the phosphate groups on the RNA backbone as well as the mRNA cap. They have put forth a model in which Mnk mediated phosphorylation of eIF4E after the formation of the pre-initiation translation complex leads to the release of eIF4E and thereby enables it to be available for another round of initiation of mRNA translation[34].

Studies based on the targeted deletion of Mnk1 and Mnk2 in mice have suggested that the expression of Mnk1 and/or Mnk2 and the phosphorylation of its target eIF4E is dispensable for survival[35]. Mice with a targeted deletion of Mnk1 and/or Mnk2 do not exhibit any developmental or reproductive defects[35]. Additionally the mouse studies also confirmed previous reports that Mnk1 is more sensitive to external stimuli as mitogen mediated eIF4E phosphorylation was defective in the Mnk1-/- cells, while basal eIF4E phosphorylation was attenuated in Mnk2-/- cells[35]. Mouse embryonic fibroblasts and adult tissues from mice lacking both Mnk1 and Mnk2 did not exhibit any basal or inducible eIF4E phosphorylation indicating that the Mnk kinases are key regulators of eIF4E phosphorylation[35]. Interestingly, cells from Mnk1 and Mnk2 deficient mice did not exhibit any defects in cap dependent translation or general protein synthesis, indicating that Mnk mediated phosphorylation of eIF4E is not critical under basal conditions but may be important during their activation with external stimuli[35].

Similarly knock-in mice expressing a mutant eIF4E (eIF4E S209A) which cannot be phosphorylated do not exhibit any developmental or viability defects[36]. These results suggest that while phosphorylation of eIF4E may not be critical for general mRNA translation, it may be

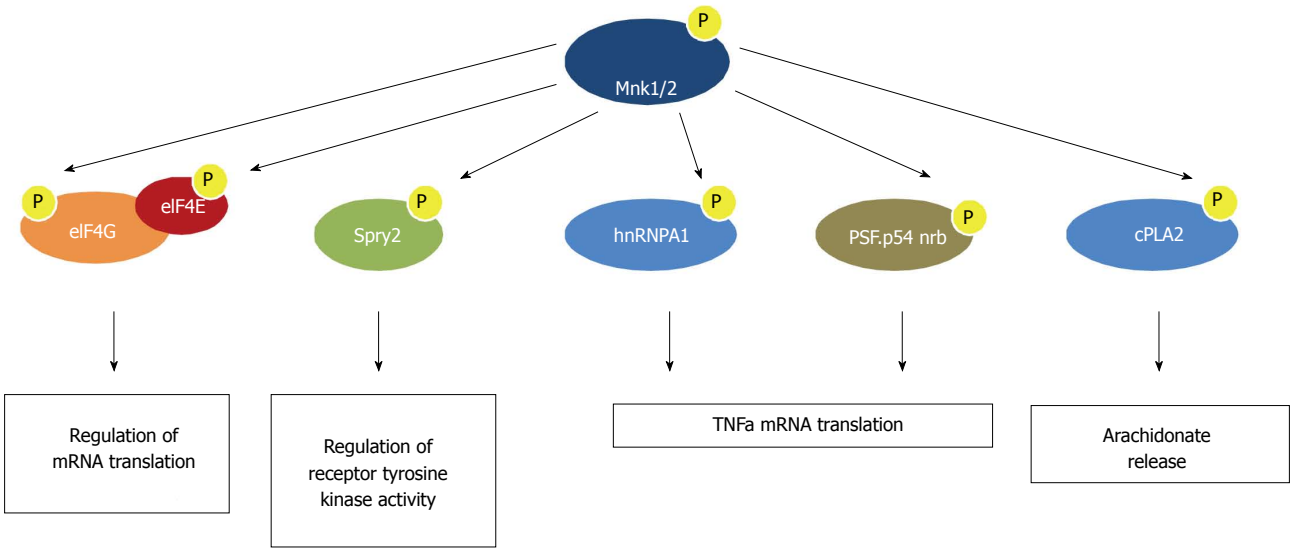

Figure 2 Effectors of the Mnk kinases. The Mnk kinases can regulate multiple biological processes by phosphorylating multiple substrates. Mnk mediated phosphorylation of eIF4E and eIF4G can play an important role in mediating cap dependent translation. The Mnk substrates hnRNPA1 and PSF play an important role in mediating the translation of AU rich elements containing mRNAs such as the TNF-α mRNA. The Mnk kinases also phosphorylate cPLA2 which plays an important role in arachidonate release from platelets. TNF-α: Tumor necrosis factor-α; eIF4G: Eukaryotic initiation factor 4G; hnRNPA1: Heterogenous nuclear ribonucleoprotein A1; cPLA2: Cytosolic phospholipase A2.

important for the translation of specific mRNAs, induced by specific stimuli. Phosphorylation of eIF4E is important for the translation of mRNAs containing 5' untranslated terminal regions (UTRs) with extensive secondary structure[37].

Besides its ability to bind capped mRNA, nuclear eIF4E can interact with a 100 nt eIF4E-sensitive element (4E-SE) region in the 3'UTRs of mRNAs and promote the nuclear export of the bound mRNA[38]. The phosphorylation of eIF4E on Ser 209 is required for its mRNA export activity, as well as transformation[39]. Mnk-mediated phosphorylation of eIF4E can facilitate the nuclear export of mRNAs such as HDM2[40], Cyclin D1[38] and other growth regulatory mRNAs[41].

Sprouty 2

Sprouty2 (Spry2) belongs family of proteins homologous to the Drosophila melanogaster Spry[42] that acts as a negative regulator of multiple receptor tyrosine kinase pathways[43,44] by negatively controlling the Erk MAPK pathway[45]. A study by DaSilva *et al*[28] showed that Mnk1 can phosphorylate Spry2 on Ser 112 and Ser 121 leading to increased stability of Spry2. Inhibition of Mnk activity resulted in increased tyrosine phosphorylation of Spry2 leading to increased binding of c-Cbl and promoting the polyubiquitination of Spry2; consequently resulting in a proteosome mediated decrease in Spry2 expression[28]. Additionally a mutant Spry2 (S112A and S121A) that cannot be phosphorylated by Mnk1 also increased proteosomal degradation of Spry2[28]. Mnk1 mediated stabilization of Spry2 was found to be functionally important for the antagonism of fibroblast growth factor (FGF) signaling by Spry2[28].

Another study showed that Mnk2 can regulate the phosphorylation of Spry2 on Ser 112 and Ser 121[46]. This study established that Mnk2-mediated phosphorylation of Spry2 increased its interaction with the E3 ubiquitin ligase NEDD4 and lead to increased proteosomal targeting of Spry2[46]. Additionally, small interfering RNA mediated silencing of Mnk2 attenuated Spry2 NEDD4 interactions and enhanced the ability of Spry2 to inhibit FGF signaling[46]. The results of the studies by DaSilva *et al*[28] and Edwin *et al*[46] are conflicting, but it is important to note that the studies were conducted in distinct biological cell lines. It is possible that Mnk kinases negatively or positively regulate Spry2 expression in a cell-specific manner, depending on the presence of additional regulatory cellular signals. More work focusing on the relevance of Mnk mediated phosphorylation of Spry2 is required to get a better understanding of the consequences of Spry2 phosphorylation by Mnk kinases.

Studies in our laboratory have previously shown that the Mnk kinases are activated by both type Ⅰ and type Ⅱ interferons (IFNs)[5,6]. IFNs are potent antiviral agents that also generate antiproliferative and antitumor responses[47,48]. Both type Ⅰ and type Ⅱ IFN mediated engagement of the Mnk kinases is important for regulating the inhibitory effects of IFNs on normal hematopoiesis by regulating the translation of specific IFN stimulated genes[5,6]. Importantly, engagement of Mnk kinases also play a critical role in mediating the anti-neoplastic effects of IFNs on primitive myeloproliferative neoplasm (MPN) precursors from patients with polycythemia vera[49]. Other work in our laboratory has shown that type Ⅰ IFNs can upregulate the expression of both Spry1 and Spry2 in a Mnk1 and Mnk2 dependent manner[50]. Data from mouse embryonic fibroblasts (MEFs) derived from mice with a targeted deletion of Spry1, Spry2 and Spry4 suggests

lack of Spry expression promotes IFN mediated antiviral responses[50]. The Spry 1, 2, 3 triple knockout MEFs exhibit enhanced activation of the p38 MAPK pathway in response to IFN treatment and, consequently, enhanced transcriptional activity and expression of the IFN stimulated gene ISG15[50]. Additionally knockdown of either Spry1, Sry2 or Spry4 was found to result in enhanced antileukemic effects of type I IFNs[50]. Thus, Mnk mediated phosphorylation of Spry proteins can have important biological consequences, but more work is required to elucidate the role of Mnk mediated phosphorylation of Spry proteins and its biological relevance in response to tyrosine kinase signaling.

hnRNPA1

The Mnk pathway plays an important role in production of TNF-α *via* its effector hnRNPA1. TNF-α is mainly secreted by activated macrophages and T lymphocytes and plays important roles in regulating inflammation[51]. Enhanced secretion of TNF-α is implicated in diseases such as rheumatoid arthritis, and inflammatory bowel disease[52], as well as in superantigen-induced septic shock[53]. Thus, the mechanisms regulating its expression have important clinical-translational and therapeutic relevance. The TNF-α mRNA is tightly regulated by the AU rich elements (AREs) present in the 3'UTR that regulate its nuclear cytoplasmic export[54], mRNA stability[55] as well as its mRNA translation[56]. TNF-α production in activated macrophages, as well as T cells, is regulated by the p38 and Erk MAPK pathways[57,58], consistent with the concept that their common downstream effectors, Mnk kinases may play an important role in TNF-α production.

Buxade *et al*[27] showed that inhibition of Mnk1 activity/expression results in attenuated production of TNF-α in T cells. In that study, overexpression of Mnk1 resulted in increased expression of a reporter construct tagged with the TNF-α 3'UTR suggesting that Mnk1 regulation of TNF-α may be mediated by the AREs[27]. Mnk1 was found to phosphorylated the TNF-α ARE binding protein hnRNPA1 on Ser 192 and Ser 310/311/312 resulting in the disassociation of hnRNPA1 from the TNF-α 3'UTR[27]. Thus, during T cell activation, activation of the MAPK cascade leads to the engagement of Mnk1 and the phosphorylation of its target hnRNPA1 and its disassociation from the TNF-α ARE, consequently promoting the translation of the TNF-α mRNA[27]. ARE elements have also been identified in mRNA encoding cytokines (GM-CSF, IL-3, IFNγ, *etc.*), proto-oncogenes (bcl, c-myc etc.) as well as in nuclear transcription factors (c-fos, c-jun, junB, *etc.*)[59] suggesting that the Mnk kinases can mediate the translation of multiple mRNAs independently of translation initiation complex.

Guil *et al*[60] showed that stress induced engagement of the Mnk kinases results in the phosphorylation of hnRNPA1 leading to its accumulation in stress granules. Depletion of hnRNPA1 or the Mnk kinases attenuates cell recovery following osmotic stress, suggesting that Mnk-mediated recruitment of hnNPA1 to stress granules plays an important role in regulating cell physiology possibly by controlling the expression of stress responsive mRNAs[60]. Many stress inducing stimuli can lead to senescence and this pathway[61,62] may potentially be mediated by the Mnk kinases due to their engagement by the stress activated p38 MAPK pathway. Mnk1 phosphorylation and expression is enhanced in senescent diploid human fibroblasts as compared to young fibroblasts[63]. In senescent cells, Mnk1 can phosphorylate hnRNPA1 leading to the cytoplasmic accumulation of hnRNPA1. Depletion of hnRNPA1 results in induction of senescence[64], suggesting that Mnk kinases may potentially regulate cellular senescence by regulating the cellular distribution of hnRNPA1.

PSF

Buxade *et al*[29] sought to identify novel substrates for the Mnk kinases. Using a proteomic approach, the researchers examined the ability of the Mnk kinases to phosphorylate proteins that could bind to a 5' cap resin[29]. They identified PSF [the PTB (polypyrimidine tract-binding protein)-associated splicing factor] as a potential Mnk substrate[29]. *In vitro* studies showed that the Mnk kinases could phosphorylate PSF on Ser 8 and Ser 283[29]. Remarkably, phosphorylation of PSF on Ser 8 was preferentially mediated by Mnk2 suggesting that Mnk1 and Mnk2 exhibit distinct substrate specificities[29]. PSF along with its partner p54 (nrb) was found to bind mRNAs containing AREs in their 3'UTR, and Mnk mediated phosphorylation of PSF was found to enhance its binding to the TNF-α mRNA containing AREs[29]. Notably, Mnk mediated phosphorylation of PSF did not affect the stability or the nuclear cytoplasmic localization of PSF or the bound TNF-α mRNA, but its effects on TNF-α mRNA translation were undetermined[29]. Thus another Mnk substrate can bind ARE elements in the 3'UTR of mRNAs again underscoring the role of Mnk kinases in mediating mRNA physiology independently of the cap translation initiation complex.

Cytosolic phospholipase A2

Cytosolic phospholipase A2 (cPLA2) is an enzyme activated by increased cytosolic calcium and catalyzes the release of arachidonate acid from glycerophospholipids to provide the precursor of the eicosanoids[65]. Eicosanoids are important secondary messenger molecules that play an important role in inflammation, immunity as well as regulation of the central nervous system[66]. Mnk1 was found to phosphorylate cPLA2 on Ser 727 resulting in the enhancement of its enzymatic activity[65]. Thrombin mediated platelet activation was found to result in Mnk1 mediated engagement of cPLA2 and arachidonate release[65]. Thus the Mnk kinases can play a role in regulating arachidonate acid release and thereby mediate eicosanoid signaling. Although no follow-up studies on the regulatory effects of the Mnk pathway on cPLA2 have been reported, further studies in that direction may provide important insights regarding the role of Mnk kinases

in various cellular and biological contexts.

Mnk2 specific interactions

The Mnk2 kinase was initially identified in a yeast two hybrid screen attempting to identify proteins that can interact with the ligand binding domain of the estrogen receptor β (ERβ)[9]. Only the nuclear Mnk2b isoform and not Mnk2a or Mnk1 was found to specifically interact with ERβ and not ERα[9]. Interestingly esterdiol treatment was found to augment Mnk2b binding to ERβ[9], but whether this interaction leads to the phosphorylation of ERβ or alters ERβ mediated transcription remains to be determined. Another study has reported that ERβ can be phosphorylated on Ser 105 by estradiol-mediated Erk1/2 activation or osmotic stress induced p38 MAPK activation and this phosphorylation was found to inhibit breast cancer migration and invasion[67]. These observations suggest that ERβ may be a potential substrate for the Mnk kinases.

Mnk2 has also been shown to phosphorylate plectin on Ser 4642[68]. Plectin is an ubiquitously expressed protein that can interact with microtubules, intermediate filaments and the actin microfilaments; and thereby plays an important role in regulating cellular responses to mechanical stress[69]. Mnk2 mediated plectin phosphorylation was found to attenuate plectin interactions with the intermediate filaments and reduced plectin phosphorylation was observed at sites of cell substrate contact that require a network of intermediate filaments[68]. These results suggest a potential role for Mnk kinases in mediating cytoskeletal integrity.

A study by Hu *et al*[30] showed that Mnk2 expression is augmented during muscle atrophy. Overexpression of Mnk2, but not Mnk1, was found to attenuate eIF4G phosphorylation on Ser 1108 and reduced basal p70 S6 kinase (p70S6K) phosphorylation at Thr 389 and Ser 371 in a kinase independent manner[30]. The serine-arginine rich protein kinase family members SRPK1, SRPK2 and SRPK3 were identified as the kinases that mediate eIF4G phosphorylation on Ser 1108[30]. Results from *in vivo* studies showed that dexamethasone treatment or starvation of Mnk2 knockout mice resulted in enhanced phosphorylation eIF4G Ser 1108 as compared to the wild type mice[30]. Mnk2 was found to selectively interact with the mammalian target of rapamycin complex 1 (mTORC1) , in a kinase independent manner and this interaction was essential to regulate Mnk2 mediated decreased phosphorylation of p70S6K[30]. As phosphorylation of eIF4G Ser 1108 and p70S6K Thr 389 and Ser 371 is associated with enhanced mRNA translation, these observations suggest Mnk2 may play an important role in negatively regulating protein synthesis during muscle atrophy[30]. These observations are consistent with other findings showing that overexpression of Mnk kinases can negatively regulate cap dependent translation[70] and suggest that Mnk mediated regulation of mRNA translation may be context dependent. Altogether, the available evidence indicates that Mnk1 and Mnk2 exhibit differing substrate specificities and, possibly, distinct biological functions. The functional differences between Mnk1 and Mnk2 need to further explored in future studies using both *in vitro* and *in vivo* approaches.

BIOLOGICAL FUNCTIONS OF THE MNK KINASES

There is extensive and definitive evidence that Mnk kinases regulate the phosphorylation and/or activity of proteins involved in diverse cellular functions. As a result of such effects, the Mnk kinases play important roles in cancer biology, development of drug resistance to cancer therapeutics, cap independent translation, as well in mediating pro-inflammatory cytokine production and cytokine signaling (Figure 3).

Role of Mnk kinases in tumorigenesis

eIF4E is known to be upregulated in a variety of human cancers and is linked to poor prognosis[71]. Additionally, overexpression of eIF4E in NIH-3T3 and rat 2 fibroblasts results in their oncogenic transformation[72]. As eIF4E is modulated by phosphorylation by Mnk kinases, Mnk kinases and phosphorylated eIF4E may have important roles in cancer biology (reviewed in[73]). Studies with mouse models using a rapid adoptive transfer strategy suggest that a constitutively active Mnk1 leads to increased eIF4E phosphorylation and promotes lymphomagenesis by preventing apoptosis and/or by upregulating mRNA translation of the anti-apoptotic Mcl-1[74].

Mouse embryonic fibroblasts derived from mice with a targeted deletion of both Mnk1 and Mnk2 are resistant to Ras mediated transformation[75]. Deletion of both Mnk1 and Mnk2 in a T-cell specific Pten null lymphoma model resulted in delayed tumorigenesis and lymphomas with an absence of eIF4E phosphorylation[75]. Additionally knock-in mice expressing a mutant eIF4E (S209A) that cannot be phosphorylated are resistant to oncogenic transformation by both c-myc and a constitutively active Ras[36]. Additionally the knock-in mice are resistant to Pten loss-induced prostate cancer and exhibit decreased expression of proteins involved in tumorigenesis such as vascular endothelial growth factor (VEGF) and matrix metalloprotease 3 (MMP3)[36]. Moreover, phosphorylated eIF4E positively correlates with progression to human prostate carcinoma[36]. Other studies have shown that inhibition of Mnk activity and the consequent decrease in the phosphorylation of eIF4E strongly attenuates the polysomal recruitment of terminal oligopyrimidine messenger RNAs (TOP mRNAs) and results in decreased expression of mRNAs involved in proliferation in prostate cancer[76].

The Mnk kinases are overexpressed in glioblastoma and inhibition of the Mnk kinases results in attenuated cell growth and increased sensitivity to rapamycin[77]. Additionally, inhibition of Mnk activity was found to attenuate mRNA translation of a subset of genes involved

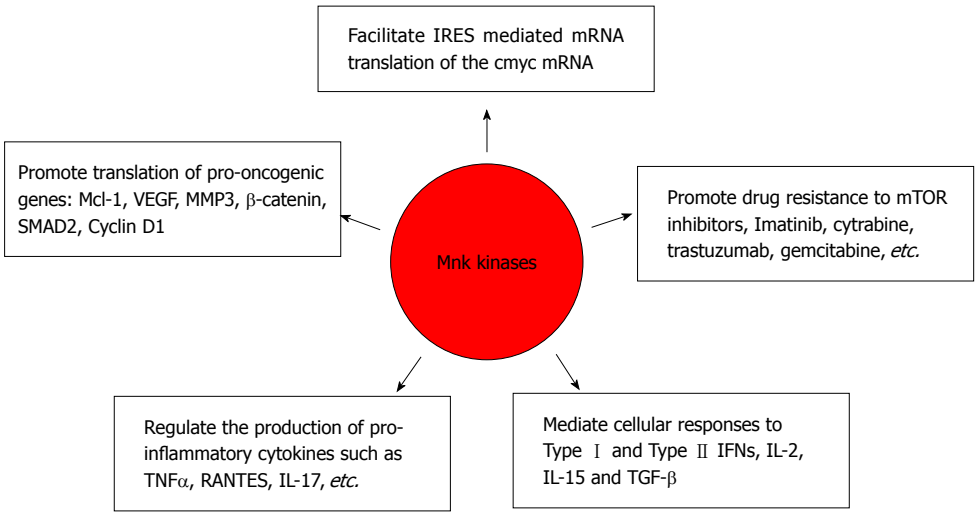

Figure 3 Biological functions of Mnk kinases. The Mnk kinases play an important role in multiple biological processes. Mnk1/2 can regulate tumor biology by mediating the translation of multiple genes that promote tumor growth and resistance to apoptosis. They also mediate resistance to chemotherapy as well as targeted therapy agents such as trastuzumab, imatinib, gemcitabine, *etc*. Mnk kinases are also implicated in regulating cap dependent translation of oncogenes as well as viral mRNA. Additionally the Mnk kinases play an important role in mediating the production of multiple pro-inflammatory cytokines such as TNF-α, RANTES and IL-17 and also mediate cellular responses to multiple cytokines such as Type Ⅰ and Type Ⅱ IFNs, IL-2, IL-15 and TGF-β. TNF-α: Tumor necrosis factor-α; IL: Interleukin; RANTES: Regulated upon activation normal T cell expressed and presumably secreted; TGF: Transforming growth factor; MMP3: Matrix metalloprotease 3; IRES: Internal ribosome entry sites; IFNs: Interferons.

in transforming growth factor β (TGFβ) signaling and regulation of signal transduction and induced cell cycle arrest[77]. A microarray analysis of polysomal mRNA revealed an important role for Mnk kinases in mediating the mRNA translation of SMAD2[77]. Importantly, SMAD2 expression positively correlated with Mnk1 expression in human glioblastoma patients and Mnk1 was found to play an important role in mediating TGFβ induced cell motility[77].

The phosphorylation of Mnk1 and Mnk2 is elevated in Her-2 over-expressing breast cancers and inhibition of Mnk activity can attenuate growth in soft agar[78]. Inhibition of Mnk activity in breast cancer cell lines exerts a cytostatic effect by downregulating the expression of Cyclin D1, one of the targets of phosphorylated eIF4E[79]. In breast cancer cell lines, the integrin α6β4 interaction leads to the engagement of the Mnk kinases in a p38 and Erk dependent manner and enhances VEGF mRNA translation[80].

The Mnk kinases are also known to play a role in hematological malignancies. Acute myeloid leukemia (AML) is often characterized by expression of different fusion proteins that account for leukemic transformation[81]. A microarray study demonstrated that MNK1 is post-translationally stabilized by PML-RAR alpha[82]. Notably, inhibition of Mnk1 activity/expression was found to enhance ATRA (all-trans retinoic acid) induced myeloid differentiation[82]. Another recent study has shown that chronic myeloid leukemia (CML) patients exhibiting blast crisis are characterized by enhanced Mnk-eIF4E phosphorylation consequently leading to augmented β-catenin protein synthesis as well as its nuclear translocation and activation[83]. These results suggest that inhibition of the Mnk kinases may have potential anti-leukemic properties.

Thus the Mnk kinases can play an important role in tumor progression and the development of Mnk inhibitors will have an important clinical applications.

Role of Mnk kinases in drug resistance

Mnk kinases can modulate multiple aspects of tumor biology and data from multiple studies suggest that they may also be involved in drug resistance by multiple mechanisms. Inhibition of mTOR by drugs such as temsirolimus (CCI-779), everolimus (RAD001), and ridaforolimus (AP-23573) has shown promising results in preclinical studies and are under investigation in cancer clinical trials[84]. Numerous studies in our laboratory as well as others have shown that rapamycin treatment of cancer cells results in the phosphorylation of the Mnk kinases as well as its target eIF4E[26,85] in a phosphoinositide 3-kinase (PI-3K) dependent manner[86]. In malignant hematopoietic cells, rapamycin treatment leads to a phosphorylation of Mnk1 and its target eIF4E, while simultaneous inhibition of both mTOR and Mnk kinases enhances the anti-leukemic effects of rapamycin[85]. Additionally Mnk1 inhibition has been shown to augment the anti-tumor effects of rapamycin in multiple human lung cancer cell lines[86]. In prostate cancer cells, inhibition of mTOR or the Mnk kinases results in distinct changes in translation initiation and the simultaneous inhibition both kinases exerts additive negative effects in the recruitment of TOP mRNAs and strong suppressive effects on cell cycle progression[76].

CML is characterized by the t(9; 22) translocation resulting in the constitutively active fusion oncogene bcr-abl, and its inhibition by imatinib mesylate (imatinib) results in a potent patient responses[87]. However, patients with late stage disease often develop resistance to imatinib resulting in decreased drug efficacy[88,89]. A study by Zhang *et al*[90] showed that simultaneous inhibition of the Mnk

kinases and imatinib treatment resulted in a synergistic enhancemet of the anti-leukemic effects of imatinib by augmenting its anti-proliferative and apoptotic effects. Inhibition of the Mnk kinases was found to attenuate polysomal mRNA recruitment by enhancing imatinib mediating inhibition of the pre-initiation complex eIF4F and by independently inhibiting the phosphorylation of the pre-initiation complex associated ribosomal protein S6 (rpS6)[90]. Additionally inhibition of the Mnk kinases has been found to also enhance the anti-leukemic effects of the chemotherapeutic drug cytarabine, currently in clinical use for the treatment of acute myeloid leukemia (AML)[85].

Breast cancers with overexpression of the oncogenic Her-2 are clinically treated with trastuzumab (herceptin), a monoclonal antibody targeting the ectodomain of Her-2[91]. Breast cancer patients that respond to trastuzumab often develop resistance within a year of initiation of treatment[92], underscoring a need to uncover the mechanisms contributing to drug resistance. The oncogenic Y-box-binding protein-1 (YB-1) can be phosphorylated by the p90 ribosomal S6 kinase as well as Akt promoting its nuclear translocation, upregulating the expression of the epidermal growth factor (EGFR), MET, PIK3CA and CD44 ultimately conferring trastuzumab resistance[93]. Using an unbiased chromatin immunoprecipitation sequencing approach to identify the transcriptional targets of YB-1, Astanehe *et al*[94] identified Mnk1 as a YB-1 transcriptional target. Mnk1 and Mnk2 were found to be overexpressed in trastuzumab resistant cell lines and depletion of Mnk1 was found to augment trastuzumab sensitivity[94]. Consistently, overexpression of Mnk1 was sufficient to confer trastuzumab resistance[94] suggesting a causative role for Mnk1 in the process.

Pancreatic ductal adenocarcinoma (PDAC) is clinically treated with the chemotherapeutic drug gemcitabine which results in marginal benefits when used as a single agent[95]. A study by Adesso *et al*[96], showed that eIF4E phosphorylation positively correlates with PDAC tumor grade and predicts a ppor prognosis. *In vitro* studies showed that gemcitabine treatment can induce eIF4E phosphorylation in a Mnk2 dependent and Mnk1 independent manner[96]. Gemcitabine was found to induce the expression of the oncogenic splicing factor serine/arginine rich splicing factor (SRSF1) which preferentially promoted the expression of the MAPK independent Mnk2b isoform with high basal activity[96]. Interestingly, inhibition of Mnk activity synergistically enhanced the anti-oncogenic effects of gemcitabine by promoting apoptosis suggesting an important role for Mnk2 and SRSF1 in mediating gemcitabine resistance[96].

Thus the Mnk kinases can regulate resistance to chemotherapy as well as targeted therapy in multiple cancer types. The clinical development of Mnk inhibitors may therefore play an important role in enhancing the efficacy of cancer therapeutics.

Role of Mnk kinases in cap independent translation

The role of Mnk kinases in cap dependent translation had been the subject of extensive work, but more recent evidence suggests that the Mnk kinases may also play an important role in mediating cap independent translation. Cap independent translation is mediated by the internal ribosome entry sites (IRES) in the 5'UTR of the target mRNAs[97,98]. The IRES elements possess complex secondary and tertiary structures that facilitate the interaction with the 40S ribosome in the absence of eIF4E and other translation initiation factors[99]. IRES elements can thereby facilitate mRNA translation when cap dependent translation is impaired in virus infected cells[100] or in malignant cells treated with drugs inhibiting cap dependent translation[101] .

Cap dependent translation is often dis-regulated in malignant cells and drugs inhibiting cap dependent translation are in common clinical use. Studies in multiple neoplastic cell types have suggested that cancer sensitivity to rapalogs is decreased by induction of the Akt pathway[102,103] subsequently resulting in IRES mediated translation of oncogenes such as VEGF[104], cyclin D1 and c-myc[105]. Interestingly, the IRES mediated translation of oncogenes is also regulated by the p38 and Erk MAPK pathways[105] suggesting a role for the Mnk kinases in controlling cap independent translation. A recent study by Shi *et al*[106] demonstrated that mTOR inhibition by rapamycin in multiple myeloma cells results in the activation of Mnk1. Inhibition of Mnk activity or expression was found to attenuate rapamycin induced upregulation of c-myc IRES activity[106]. Combination treatment of malignant cells with rapamycin and a Mnk inhibitor was found to abolish c-myc expression and enhanced the anti-oncogenic activity of rapamycin[106].

Additional evidence from viral studies also supports a role for the Mnk kinases in the regulation of IRES mediated translation. A study by Goetz *et al*[107] showed that replication and cytotoxicity of the prototype oncolytic poliovirus PVSRIPO in glioblastoma multiforme (GBM) results in the engagement of Mnk1 subsequently resulting in the enhanced cap independent translation of the viral RNA[107]. Taken together, these results suggest that Mnk kinases play important roles in regulating cap independent translation and more studies along this line are required to gain mechanistic insight into such effects.

Role of Mnk kinases in inflammation

MAPK pathways such as Erk and p38 have been shown to play important roles in modulating immune responses by mediating the production of cytokines that control the initiation of innate immunity; the activation of adaptive immunity; and by regulating cellular responses to cytokines involved in immune responses[108]. As Mnk kinases are effectors of MAPK pathways, these observations suggest that they may play important roles in mediating cytokine production at the translational level. Indeed pharmacological blockade of Mnk kinases was found to attenuate the production of pro-inflammatory cytokines such TNF-α, IL-6, and monocyte chemo-attractant protein-1 and enhanced the production of the anti-inflam-

matory cytokine IL-10 in macrophages stimulated with multiple Toll like receptor (TLR) agonists[109]. Also, data from multiple studies have shown that Mnk kinases play important roles in mediating the production of multiple pro-inflammatory cytokines such as TNF-α, RANTES and IL-17 and in mediating the cellular responses to Type I and Type II IFNs, IL-2, IL-15 and TGF-β (reviewed in[110]).

Most of the studies focusing on the role of the Mnk kinases in inflammation have utilized small interfering RNA mediated Mnk knockdown or pharmacological inhibitors of the Mnk kinases. Recently a study by Gorentla *et al*[111], examined the role of Mnk kinases in T cell development in mice with a targeted deletion of Mnk1 and Mnk2. This study showed that in mice lacking Mnk1 and Mnk2, T-cell receptor mediated Ser 209 phosphorylation of eIF4E in T cells was completely abolished[111]. Lack of of Mnk1 and Mnk2 expression in T cells had no influence on the development of conventional αβ T cells, regulatory T cells, or NKT (natural killer T cells)[111]. The Mnk1/2 double knockout mice also did not exhibit any deficiencies in CD8 T cell response to bacterial or viral infection[111]. Interestingly, while lack of the Mnk kinases does not inhibit Th1 and Th17 differentiation *in vitro*, immunization of mice with myelin oligodendrocyte glycoprotein peptide in complete Freund's adjuvant, an experimental model of autoimmune encephalomyelitis, resulted in attenuated production of IFNγ and IL-17 by CD4 T cells and attenuated differentiation of Th1 and Th17 cells[111]. Collectively, these results suggest that while the Mnk kinases are dispensable for normal T cell development and function, they may play important roles in regulating the cytokines required for T cell differentiation or antigen presenting cell (APC) activation pathways, and thereby modulate Th cell differentiation in an T cell extrinsic manner[111].

Another recent study focused on the role of the Mnk kinases in the generation of neutrophil responses. Neutrophils are involved in acute inflammatory response and secrete proinflammatory cytokines such as TNF-α, IL-1β, IL-8, IFNγ, IL-4, IL-10, *etc*.[112]. Mnk1 is phosphorylated in human neutrophils upon treatment with LPS or TNF-α[113]. Inhibition of the Mnk kinases in LPS or TNF-α stimulated human neutrophils was found to attenuate the secretion of CXCL8, CCL-3 and CCL4 while the mRNA levels of the cytokines were unaffected, Mnk inhibition also attenuated the anti-apoptotic effects of LPS and TNF-α[113]. Overexpression of a kinase active Mnk1 and not a kinase dead Mnk1 mutant was found to enhance LPS- and TNF-α- induced cytokine secretion[113]. Similarly the Mnk kinases play important roles in pro-inflamatory cytokine production in macrophages[109]. These studies further support the observation that the Mnk kinases are attractive targets for diseases associated with inflammation.

While pro-inflammatory cytokines play an important role in mediating an effective immune response to pathogens, their persistent enhanced expression is associated with multiple disorders such as auto-immune diseases[114], allergies[115], neurological disorders[116], sepsis[117], cardiovascular diseases[118], obesity[119] and cancer[120]. As the Mnk kinases represent a central node in regulating pro-inflammatory cytokine production, development of Mnk inhibitors will have important broad spectrum translational implications.

CONCLUSION

The Mnk kinases are regulated by the p38 and Erk MAPK pathways and their activity can also be modulated by other MAPK independent mechanisms. Multiple proteins such as those involved in mRNA translation (eIF4E, eIF4G), in TNF-α mRNA expression (hnRNPA1, PSF), in platelet activity (cPLA2) and in regulation of receptor tyrosine kinase activity (Spry2) are regulated by the Mnk kinases. As a result, the Mnk kinases can play important roles in controlling cap-dependent and -independent translation, participate in the pathophysiology of several malignant and inflammatory diseases and diminish responses to cancer therapeutics (Figure 3).

The above observations suggest that development of Mnk inhibitors can have broad spectrum clinical applications. Most of the studies discussed in this review used the Mnk inhibitor CGP57380 a low weight molecular compound identified from the Novartis Pharma compound collection that can inhibit both Mnk1 and Mnk2 activity[121]. The IC_{50} of CGP57380 against Mnk1 is seen at a concentration of 2.2 μmol/L[70], the concentration at which it can also inhibit the activity of other kinases such as casein kinase, MAP2K1 and BR serine/threonine-protein kinase 2[122]. As a result this compound cannot be used for *in vivo* studies and research mainly utilizing CGP57380 should be interpreted with caution. The antifungal agent cercosporamide is also reported to inhibit Mnk activity, although it exhibits higher specificity for Mnk2 as compared to Mnk1[123]. Importantly cercosporamide has been shown to exhibit anti-tumor effects in both *in vitro* and *in vivo* studies[123, 124]. More research efforts are needed to develop Mnk inhibitors that can be tested in clinical settings.

REFERENCES

1 **Roux PP**, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev* 2004; **68**: 320-344 [PMID: 15187187 DOI: 10.1128/MMBR.68.2.320-344.2004]

2 **Fukunaga R**, Hunter T. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J* 1997; **16**: 1921-1933 [PMID: 9155018 DOI: 10.1093/emboj/16.8.1921]

3 **Waskiewicz AJ**, Flynn A, Proud CG, Cooper JA. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J* 1997; **16**: 1909-1920 [PMID: 9155017 DOI: 10.1093/emboj/16.8.1909]

4 **Wang X**, Flynn A, Waskiewicz AJ, Webb BL, Vries RG, Baines IA, Cooper JA, Proud CG. The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP

kinase pathways. *J Biol Chem* 1998; **273**: 9373-9377 [PMID: 9545260]

5 **Joshi S**, Sharma B, Kaur S, Majchrzak B, Ueda T, Fukunaga R, Verma AK, Fish EN, Platanias LC. Essential role for Mnk kinases in type II interferon (IFNgamma) signaling and its suppressive effects on normal hematopoiesis. *J Biol Chem* 2011; **286**: 6017-6026 [PMID: 21149447 DOI: 10.1074/jbc.M110.197921]

6 **Joshi S**, Kaur S, Redig AJ, Goldsborough K, David K, Ueda T, Watanabe-Fukunaga R, Baker DP, Fish EN, Fukunaga R, Platanias LC. Type I interferon (IFN)-dependent activation of Mnk1 and its role in the generation of growth inhibitory responses. *Proc Natl Acad Sci USA* 2009; **106**: 12097-12102 [PMID: 19574459 DOI: 10.1073/pnas.0900562106]

7 **Parra JL**, Buxadé M, Proud CG. Features of the catalytic domains and C termini of the MAPK signal-integrating kinases Mnk1 and Mnk2 determine their differing activities and regulatory properties. *J Biol Chem* 2005; **280**: 37623-37633 [PMID: 16162500]

8 **O'Loghlen A**, González VM, Piñeiro D, Pérez-Morgado MI, Salinas M, Martín ME. Identification and molecular characterization of Mnk1b, a splice variant of human MAP kinase-interacting kinase Mnk1. *Exp Cell Res* 2004; **299**: 343-355 [PMID: 15350534]

9 **Slentz-Kesler K**, Moore JT, Lombard M, Zhang J, Hollingsworth R, Weiner MP. Identification of the human Mnk2 gene (MKNK2) through protein interaction with estrogen receptor beta. *Genomics* 2000; **69**: 63-71 [PMID: 11013076]

10 **Scheper GC**, Parra JL, Wilson M, Van Kollenburg B, Vertegaal AC, Han ZG, Proud CG. The N and C termini of the splice variants of the human mitogen-activated protein kinase-interacting kinase Mnk2 determine activity and localization. *Mol Cell Biol* 2003; **23**: 5692-5705 [PMID: 12897141 DOI: 10.1128/MCB.23.16.5692-5705.2003]

11 **Goto S**, Yao Z, Proud CG. The C-terminal domain of Mnk1a plays a dual role in tightly regulating its activity. *Biochem J* 2009; **423**: 279-290 [PMID: 19650764]

12 **Karni R**, de Stanchina E, Lowe SW, Sinha R, Mu D, Krainer AR. The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat Struct Mol Biol* 2007; **14**: 185-193 [PMID: 17310252]

13 **Zarubin T**, Han J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res* 2005; **15**: 11-18 [PMID: 15686620]

14 **Shveygert M**, Kaiser C, Bradrick SS, Gromeier M. Regulation of eukaryotic initiation factor 4E (eIF4E) phosphorylation by mitogen-activated protein kinase occurs through modulation of Mnk1-eIF4G interaction. *Mol Cell Biol* 2010; **30**: 5160-5167 [PMID: 20823271 DOI: 10.1128/MCB.00448-10]

15 **Pyronnet S**, Imataka H, Gingras AC, Fukunaga R, Hunter T, Sonenberg N. Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. *EMBO J* 1999; **18**: 270-279 [PMID: 9878069 DOI: 10.1093/emboj/18.1.270]

16 **Imataka H**, Gradi A, Sonenberg N. A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J* 1998; **17**: 7480-7489 [PMID: 9857202 DOI: 10.1093/emboj/17.24.7480]

17 **Lamphear BJ**, Kirchweger R, Skern T, Rhoads RE. Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. *J Biol Chem* 1995; **270**: 21975-21983 [PMID: 7665619]

18 **Rhoads RE**, Joshi B, Minich WB. Participation of initiation factors in the recruitment of mRNA to ribosomes. *Biochimie* 1994; **76**: 831-838 [PMID: 7880899]

19 **Imataka H**, Olsen HS, Sonenberg N. A new translational regulator with homology to eukaryotic translation initiation factor 4G. *EMBO J* 1997; **16**: 817-825 [PMID: 9049310 DOI: 10.1093/emboj/16.4.817]

20 **Dobrikov M**, Dobrikova E, Shveygert M, Gromeier M. Phosphorylation of eukaryotic translation initiation factor 4G1 (eIF4G1) by protein kinase C{alpha} regulates eIF4G1 binding to Mnk1. *Mol Cell Biol* 2011; **31**: 2947-2959 [PMID: 21576361 DOI: 10.1128/MCB.05589-11]

21 **Scheper GC**, Morrice NA, Kleijn M, Proud CG. The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells. *Mol Cell Biol* 2001; **21**: 743-754 [PMID: 11154262 DOI: 10.1128/MCB.21.3.743-754.2001]

22 **Orton KC**, Ling J, Waskiewicz AJ, Cooper JA, Merrick WC, Korneeva NL, Rhoads RE, Sonenberg N, Traugh JA. Phosphorylation of Mnk1 by caspase-activated Pak2/gamma-PAK inhibits phosphorylation and interaction of eIF4G with Mnk. *J Biol Chem* 2004; **279**: 38649-38657 [PMID: 15234964]

23 **Roig J**, Traugh JA. Cytostatic p21 G protein-activated protein kinase gamma-PAK. *Vitam Horm* 2001; **62**: 167-198 [PMID: 11345898]

24 **Ling J**, Morley SJ, Traugh JA. Inhibition of cap-dependent translation via phosphorylation of eIF4G by protein kinase Pak2. *EMBO J* 2005; **24**: 4094-4105 [PMID: 16281055]

25 **Li Y**, Yue P, Deng X, Ueda T, Fukunaga R, Khuri FR, Sun SY. Protein phosphatase 2A negatively regulates eukaryotic initiation factor 4E phosphorylation and eIF4F assembly through direct dephosphorylation of Mnk and eIF4E. *Neoplasia* 2010; **12**: 848-855 [PMID: 20927323]

26 **Stead RL**, Proud CG. Rapamycin enhances eIF4E phosphorylation by activating MAP kinase-interacting kinase 2a (Mnk2a). *FEBS Lett* 2013; **587**: 2623-2628 [PMID: 23831578]

27 **Buxadé M**, Parra JL, Rousseau S, Shpiro N, Marquez R, Morrice N, Bain J, Espel E, Proud CG. The Mnks are novel components in the control of TNF alpha biosynthesis and phosphorylate and regulate hnRNP A1. *Immunity* 2005; **23**: 177-189 [PMID: 16111636]

28 **DaSilva J**, Xu L, Kim HJ, Miller WT, Bar-Sagi D. Regulation of sprouty stability by Mnk1-dependent phosphorylation. *Mol Cell Biol* 2006; **26**: 1898-1907 [PMID: 16479008 DOI: 10.1128/MCB.26.5.1898-1907.2006]

29 **Buxadé M**, Morrice N, Krebs DL, Proud CG. The PSF. p54nrb complex is a novel Mnk substrate that binds the mRNA for tumor necrosis factor alpha. *J Biol Chem* 2008; **283**: 57-65 [PMID: 17965020]

30 **Hu SI**, Katz M, Chin S, Qi X, Cruz J, Ibebunjo C, Zhao S, Chen A, Glass DJ. MNK2 inhibits eIF4G activation through a pathway involving serine-arginine-rich protein kinase in skeletal muscle. *Sci Signal* 2012; **5**: ra14 [PMID: 22337810]

31 **Flynn A**, Proud CG. Serine 209, not serine 53, is the major site of phosphorylation in initiation factor eIF-4E in serum-treated Chinese hamster ovary cells. *J Biol Chem* 1995; **270**: 21684-21688 [PMID: 7665584]

32 **Scheper GC**, van Kollenburg B, Hu J, Luo Y, Goss DJ, Proud CG. Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. *J Biol Chem* 2002; **277**: 3303-3309 [PMID: 11723111]

33 **von Der Haar T**, Ball PD, McCarthy JE. Stabilization of eukaryotic initiation factor 4E binding to the mRNA 5′-Cap by domains of eIF4G. *J Biol Chem* 2000; **275**: 30551-30555 [PMID: 10887196]

34 **Scheper GC**, Proud CG. Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? *Eur J Biochem* 2002; **269**: 5350-5359 [PMID: 12423333]

35 **Ueda T**, Watanabe-Fukunaga R, Fukuyama H, Nagata S, Fukunaga R. Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development. *Mol Cell Biol* 2004; **24**: 6539-6549 [PMID: 15254222 DOI: 10.1128/MCB.24.15.6539-6549.2004]

36 **Furic L**, Rong L, Larsson O, Koumakpayi IH, Yoshida

K, Brueschke A, Petroulakis E, Robichaud N, Pollak M, Gaboury LA, Pandolfi PP, Saad F, Sonenberg N. eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. *Proc Natl Acad Sci USA* 2010; **107**: 14134-14139 [PMID: 20679199 DOI: 10.1073/pnas.1005320107]

37 **Koromilas AE**, Lazaris-Karatzas A, Sonenberg N. mRNAs containing extensive secondary structure in their 5′ noncoding region translate efficiently in cells overexpressing initiation factor eIF-4E. *EMBO J* 1992; **11**: 4153-4158 [PMID: 1396596]

38 **Culjkovic B**, Topisirovic I, Skrabanek L, Ruiz-Gutierrez M, Borden KL. eIF4E promotes nuclear export of cyclin D1 mRNAs via an element in the 3′UTR. *J Cell Biol* 2005; **169**: 245-256 [PMID: 15837800 DOI: 10.1083/jcb.200501019]

39 **Topisirovic I**, Ruiz-Gutierrez M, Borden KL. Phosphorylation of the eukaryotic translation initiation factor eIF4E contributes to its transformation and mRNA transport activities. *Cancer Res* 2004; **64**: 8639-8642 [PMID: 15574771]

40 **Phillips A**, Blaydes JP. MNK1 and EIF4E are downstream effectors of MEKs in the regulation of the nuclear export of HDM2 mRNA. *Oncogene* 2008; **27**: 1645-1649 [PMID: 17828301]

41 **Strudwick S**, Borden KL. The emerging roles of translation factor eIF4E in the nucleus. *Differentiation* 2002; **70**: 10-22 [PMID: 11963652]

42 **Hacohen N**, Kramer S, Sutherland D, Hiromi Y, Krasnow MA. sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell* 1998; **92**: 253-263 [PMID: 9458049]

43 **Mason JM**, Morrison DJ, Basson MA, Licht JD. Sprouty proteins: multifaceted negative-feedback regulators of receptor tyrosine kinase signaling. *Trends Cell Biol* 2006; **16**: 45-54 [PMID: 16337795]

44 **Kim HJ**, Bar-Sagi D. Modulation of signalling by Sprouty: a developing story. *Nat Rev Mol Cell Biol* 2004; **5**: 441-450 [PMID: 15173823]

45 **Cabrita MA**, Christofori G. Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis* 2008; **11**: 53-62 [PMID: 18219583]

46 **Edwin F**, Anderson K, Patel TB. HECT domain-containing E3 ubiquitin ligase Nedd4 interacts with and ubiquitinates Sprouty2. *J Biol Chem* 2010; **285**: 255-264 [PMID: 19864419 DOI: 10.1074/jbc.M109.030882]

47 **Borden EC**, Sen GC, Uze G, Silverman RH, Ransohoff RM, Foster GR, Stark GR. Interferons at age 50: past, current and future impact on biomedicine. *Nat Rev Drug Discov* 2007; **6**: 975-990 [PMID: 18049472]

48 **Platanias LC**. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* 2005; **5**: 375-386 [PMID: 15864272]

49 **Mehrotra S**, Sharma B, Joshi S, Kroczynska B, Majchrzak B, Stein BL, McMahon B, Altman JK, Licht JD, Baker DP, Eklund EA, Wickrema A, Verma A, Fish EN, Platanias LC. Essential role for the Mnk pathway in the inhibitory effects of type I interferons on myeloproliferative neoplasm (MPN) precursors. *J Biol Chem* 2013; **288**: 23814-23822 [PMID: 23814052 DOI: 10.1074/jbc.M113.476192]

50 **Sharma B**, Joshi S, Sassano A, Majchrzak B, Kaur S, Aggarwal P, Nabet B, Bulic M, Stein BL, McMahon B, Baker DP, Fukunaga R, Altman JK, Licht JD, Fish EN, Platanias LC. Sprouty proteins are negative regulators of interferon (IFN) signaling and IFN-inducible biological responses. *J Biol Chem* 2012; **287**: 42352-42360 [PMID: 23074222 DOI: 10.1074/jbc.M112.400721]

51 **Locksley RM**, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 2001; **104**: 487-501 [PMID: 11239407]

52 **Feldmann M**, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* 1996; **14**: 397-440 [PMID: 8717520]

53 **Miethke T**, Wahl C, Heeg K, Echtenacher B, Krammer PH, Wagner H. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. *J Exp Med* 1992; **175**: 91-98 [PMID: 1730929]

54 **Dumitru CD**, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 2000; **103**: 1071-1083 [PMID: 11163183]

55 **Kontoyiannis D**, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. *Immunity* 1999; **10**: 387-398 [PMID: 10204494]

56 **Han J**, Brown T, Beutler B. Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. *J Exp Med* 1990; **171**: 465-475 [PMID: 2303781]

57 **Buxadé M**, Ramírez-Alvarado M, Fernández-Troy N, MacKenzie S, Casaroli-Marano RP, Vilella R, Espel E. Integrating signals from T-cell receptor and serum by T cells enhance translation of tumour necrosis factor-alpha. *Immunology* 2001; **102**: 416-425 [PMID: 11328375 DOI: 10.1046/j.1365-2567.2001.01206.x]

58 **Hoffmeyer A**, Grosse-Wilde A, Flory E, Neufeld B, Kunz M, Rapp UR, Ludwig S. Different mitogen-activated protein kinase signaling pathways cooperate to regulate tumor necrosis factor alpha gene expression in T lymphocytes. *J Biol Chem* 1999; **274**: 4319-4327 [PMID: 9933633]

59 **Chen CY**, Shyu AB. AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci* 1995; **20**: 465-470 [PMID: 8578590]

60 **Guil S**, Long JC, Cáceres JF. hnRNP A1 relocalization to the stress granules reflects a role in the stress response. *Mol Cell Biol* 2006; **26**: 5744-5758 [PMID: 16847328 DOI: 10.1128/MCB.00224-06]

61 **Marusyk A**, Wheeler LJ, Mathews CK, DeGregori J. p53 mediates senescence-like arrest induced by chronic replicational stress. *Mol Cell Biol* 2007; **27**: 5336-5351 [PMID: 17515610 DOI: 10.1128/MCB.01316-06]

62 **Toussaint O**, Remacle J, Dierick JF, Pascal T, Frippiat C, Royer V, Magalhacs JP, Zdanov S, Chainiaux F. Stress-induced premature senescence: from biomarkers to likeliness of in vivo occurrence. *Biogerontology* 2002; **3**: 13-17 [PMID: 12014832]

63 **Ziaei S**, Shimada N, Kucharavy H, Hubbard K. MNK1 expression increases during cellular senescence and modulates the subcellular localization of hnRNP A1. *Exp Cell Res* 2012; **318**: 500-508 [PMID: 22227431 DOI: 10.1016/j.yexcr.2011.12.015]

64 **Shimada N**, Rios I, Moran H, Sayers B, Hubbard K. p38 MAP kinase-dependent regulation of the expression level and subcellular distribution of heterogeneous nuclear ribonucleoprotein A1 and its involvement in cellular senescence in normal human fibroblasts. *RNA Biol* 2009; **6**: 293-304 [PMID: 19430204]

65 **Hefner Y**, Borsch-Haubold AG, Murakami M, Wilde JI, Pasquet S, Schieltz D, Ghomashchi F, Yates JR, Armstrong CG, Paterson A, Cohen P, Fukunaga R, Hunter T, Kudo I, Watson SP, Gelb MH. Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases. *J Biol Chem* 2000; **275**: 37542-37551 [PMID: 10978317]

66 **Funk CD**. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001; **294**: 1871-1875 [PMID: 11729303]

67 **Lam HM**, Suresh Babu CV, Wang J, Yuan Y, Lam YW, Ho SM, Leung YK. Phosphorylation of human estrogen recep-

tor-beta at serine 105 inhibits breast cancer cell migration and invasion. *Mol Cell Endocrinol* 2012; **358**: 27-35 [PMID: 22370157 DOI: 10.1016/j.mce.2012.02.012]
68 **Bouameur JE**, Schneider Y, Begré N, Hobbs RP, Lingasamy P, Fontao L, Green KJ, Favre B, Borradori L. Phosphorylation of serine 4,642 in the C-terminus of plectin by MNK2 and PKA modulates its interaction with intermediate filaments. *J Cell Sci* 2013; **126**: 4195-4207 [PMID: 23843618 DOI: 10.1242/jcs.127779]
69 **Svitkina TM**, Verkhovsky AB, Borisy GG. Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. *J Cell Biol* 1996; **135**: 991-1007 [PMID: 8922382]
70 **Knauf U**, Tschopp C, Gram H. Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2. *Mol Cell Biol* 2001; **21**: 5500-5511 [PMID: 11463832 DOI: 10.1128/MCB.21.16.5500-5511.2001]
71 **De Benedetti A**, Harris AL. eIF4E expression in tumors: its possible role in progression of malignancies. *Int J Biochem Cell Biol* 1999; **31**: 59-72 [PMID: 10216944]
72 **Lazaris-Karatzas A**, Montine KS, Sonenberg N. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. *Nature* 1990; **345**: 544-547 [PMID: 2348862]
73 **Hou J**, Lam F, Proud C, Wang S. Targeting Mnks for cancer therapy. *Oncotarget* 2012; **3**: 118-131 [PMID: 22392765]
74 **Wendel HG**, Silva RL, Malina A, Mills JR, Zhu H, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Teruya-Feldstein J, Pelletier J, Lowe SW. Dissecting eIF4E action in tumorigenesis. *Genes Dev* 2007; **21**: 3232-3237 [PMID: 18055695 DOI: 10.1101/gad.1604407]
75 **Ueda T**, Sasaki M, Elia AJ, Chio II, Hamada K, Fukunaga R, Mak TW. Combined deficiency for MAP kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2) delays tumor development. *Proc Natl Acad Sci USA* 2010; **107**: 13984-13990 [PMID: 20679220 DOI: 10.1073/pnas.1008136107]
76 **Bianchini A**, Loiarro M, Bielli P, Busà R, Paronetto MP, Loreni F, Geremia R, Sette C. Phosphorylation of eIF4E by MNKs supports protein synthesis, cell cycle progression and proliferation in prostate cancer cells. *Carcinogenesis* 2008; **29**: 2279-2288 [PMID: 18809972]
77 **Grzmil M**, Morin P, Lino MM, Merlo A, Frank S, Wang Y, Moncayo G, Hemmings BA. MAP kinase-interacting kinase 1 regulates SMAD2-dependent TGF-β signaling pathway in human glioblastoma. *Cancer Res* 2011; **71**: 2392-2402 [PMID: 21406405]
78 **Chrestensen CA**, Shuman JK, Eschenroeder A, Worthington M, Gram H, Sturgill TW. MNK1 and MNK2 regulation in HER2-overexpressing breast cancer lines. *J Biol Chem* 2007; **282**: 4243-4252 [PMID: 17130135]
79 **Wheater MJ**, Johnson PW, Blaydes JP. The role of MNK proteins and eIF4E phosphorylation in breast cancer cell proliferation and survival. *Cancer Biol Ther* 2010; **10**: 728-735 [PMID: 20686366 DOI: 10.4161/cbt.10.7.12965]
80 **Korneeva NL,** Soung YH, Kim HI, Giordano A, Rhoads RE, Gram H, Chung J. Mnk mediates integrin α6β4-dependent eIF4E phosphorylation and translation of VEGF mRNA. *Mol Cancer Res* 2010; **8**: 1571-1578 [PMID: 21047768 DOI: 10.1158/1541-7786.MCR-10-0091]
81 **Müller-Tidow C**, Steffen B, Cauvet T, Tickenbrock L, Ji P, Diederichs S, Sargin B, Köhler G, Stelljes M, Puccetti E, Ruthardt M, deVos S, Hiebert SW, Koeffler HP, Berdel WE, Serve H. Translocation products in acute myeloid leukemia activate the Wnt signaling pathway in hematopoietic cells. *Mol Cell Biol* 2004; **24**: 2890-2904 [PMID: 15024077 DOI: 10.1128/MCB.24.7.2890-2904.2004]
82 **Worch J**, Tickenbrock L, Schwäble J, Steffen B, Cauvet T, Mlody B, Buerger H, Koeffler HP, Berdel WE, Serve H, Müller-Tidow C. The serine-threonine kinase MNK1 is post-translationally stabilized by PML-RARalpha and regulates differentiation of hematopoietic cells. *Oncogene* 2004; **23**: 9162-9172 [PMID: 15516979]
83 **Lim S**, Saw TY, Zhang M, Janes MR, Nacro K, Hill J, Lim AQ, Chang CT, Fruman DA, Rizzieri DA, Tan SY, Fan H, Chuah CT, Ong ST. Targeting of the MNK-eIF4E axis in blast crisis chronic myeloid leukemia inhibits leukemia stem cell function. *Proc Natl Acad Sci USA* 2013; **110**: E2298-E2307 [PMID: 23737503 DOI: 10.1073/pnas.1301838110]
84 **Brachmann S,** Fritsch C, Maira SM, García-Echeverría C. PI3K and mTOR inhibitors: a new generation of targeted anticancer agents. *Curr Opin Cell Biol* 2009; **21**: 194-198 [PMID: 19201591 DOI: 10.1016/j.ceb.2008.12.011]
85 **Altman JK**, Glaser H, Sassano A, Joshi S, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Tallman MS, Platanias LC. Negative regulatory effects of Mnk kinases in the generation of chemotherapy-induced antileukemic responses. *Mol Pharmacol* 2010; **78**: 778-784 [PMID: 20664001 DOI: 10.1124/mol.110.064642]
86 **Wang X**, Yue P, Chan CB, Ye K, Ueda T, Watanabe-Fukunaga R, Fukunaga R, Fu H, Khuri FR, Sun SY. Inhibition of mammalian target of rapamycin induces phosphatidylinositol 3-kinase-dependent and Mnk-mediated eukaryotic translation initiation factor 4E phosphorylation. *Mol Cell Biol* 2007; **27**: 7405-7413 [PMID: 17724079 DOI: 10.1128/MCB.00760-07]
87 **Simonsson B**, Grp IS. Beneficial effects of cytogenetic and molecular response on long-term outcome in patients with newly diagnosed chronic myeloid leukemia in chronic phase (CML-CP) treated with imatinib (IM): Update from the IRIS study. *Blood* 2005; **106**: 52a-52a
88 **Gorre ME,** Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science* 2001; **293**: 876-880 [PMID: 11423618 DOI: 10.1126/science.1062538]
89 **Shah NP,** Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, Sawyers CL. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* 2002; **2**: 117-125 [PMID: 12204532 DOI: 10.1016/S1535-6108(02)00096-X]
90 **Zhang M**, Fu W, Prabhu S, Moore JC, Ko J, Kim JW, Druker BJ, Trapp V, Fruehauf J, Gram H, Fan HY, Ong ST. Inhibition of polysome assembly enhances imatinib activity against chronic myelogenous leukemia and overcomes imatinib resistance. *Mol Cell Biol* 2008; **28**: 6496-6509 [PMID: 18694961 DOI: 10.1128/MCB.00477-08]
91 **Carter P**, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci USA* 1992; **89**: 4285-4289 [PMID: 1350088]
92 **Nahta R**, Esteva FJ. HER2 therapy: molecular mechanisms of trastuzumab resistance. *Breast Cancer Res* 2006; **8**: 215 [PMID: 17096862 DOI: 10.1186/bcr1612]
93 **Dhillon J**, Astanehe A, Lee C, Fotovati A, Hu K, Dunn SE. The expression of activated Y-box binding protein-1 serine 102 mediates trastuzumab resistance in breast cancer cells by increasing CD44+ cells. *Oncogene* 2010; **29**: 6294-6300 [PMID: 20802512]
94 **Astanehe A**, Finkbeiner MR, Krzywinski M, Fotovati A, Dhillon J, Berquin IM, Mills GB, Marra MA, Dunn SE. MKNK1 is a YB-1 target gene responsible for imparting trastuzumab resistance and can be blocked by RSK inhibition. *Oncogene* 2012; **31**: 4434-4446 [PMID: 22249268]
95 **Kern SE**, Shi C, Hruban RH. The complexity of pancreatic ductal cancers and multidimensional strategies for therapeutic targeting. *J Pathol* 2011; **223**: 295-306 [PMID: 21125682 DOI: 10.1002/path.2813]
96 **Adesso L,** Calabretta S, Barbagallo F, Capurso G, Pilozzi

E, Geremia R, Delle Fave G, Sette C. Gemcitabine triggers a pro-survival response in pancreatic cancer cells through activation of the MNK2/eIF4E pathway. *Oncogene* 2013; **32**: 2848-2857 [PMID: 22797067 DOI: 10.1038/onc.2012.306]

97 **Pelletier J,** Sonenberg N. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* 1988; **334**: 320-325 [PMID: 2839775 DOI: 10.1038/334320a0]

98 **Jang SK**, Kräusslich HG, Nicklin MJ, Duke GM, Palmenberg AC, Wimmer E. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J Virol* 1988; **62**: 2636-2643 [PMID: 2839690]

99 **Komar AA**, Hatzoglou M. Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states. *Cell Cycle* 2011; **10**: 229-240 [PMID: 21220943 DOI: 10.4161/cc.10.2.14472]

100 **Hanson PJ**, Zhang HM, Hemida MG, Ye X, Qiu Y, Yang D. IRES-Dependent Translational Control during Virus-Induced Endoplasmic Reticulum Stress and Apoptosis. *Front Microbiol* 2012; **3**: 92 [PMID: 22461781 DOI: 10.3389/fmicb.2012.00092]

101 **Holcík M.** Targeting translation for treatment of cancer--a novel role for IRES? *Curr Cancer Drug Targets* 2004; **4**: 299-311 [PMID: 15134536 DOI: 10.2174/1568009043333005]

102 **Wan X,** Harkavy B, Shen N, Grohar P, Helman LJ. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. *Oncogene* 2007; **26**: 1932-1940 [PMID: 17001314 DOI: 10.1038/sj.onc.1209990]

103 **Sun SY**, Rosenberg LM, Wang X, Zhou Z, Yue P, Fu H, Khuri FR. Activation of Akt and eIF4E survival pathways by rapamycin-mediated mammalian target of rapamycin inhibition. *Cancer Res* 2005; **65**: 7052-7058 [PMID: 16103051 DOI: 10.1158/0008-5472.CAN-05-0917]

104 **Frost P,** Shi Y, Hoang B, Lichtenstein A. AKT activity regulates the ability of mTOR inhibitors to prevent angiogenesis and VEGF expression in multiple myeloma cells. *Oncogene* 2007; **26**: 2255-2262 [PMID: 17016437 DOI: 10.1038/sj.onc.1210019]

105 **Shi Y**, Sharma A, Wu H, Lichtenstein A, Gera J. Cyclin D1 and c-myc internal ribosome entry site (IRES)-dependent translation is regulated by AKT activity and enhanced by rapamycin through a p38 MAPK- and ERK-dependent pathway. *J Biol Chem* 2005; **280**: 10964-10973 [PMID: 15634685]

106 **Shi Y**, Frost P, Hoang B, Yang Y, Fukunaga R, Gera J, Lichtenstein A. MNK kinases facilitate c-myc IRES activity in rapamycin-treated multiple myeloma cells. *Oncogene* 2013; **32**: 190-197 [PMID: 22370634 DOI: 10.1038/onc.2012.43]

107 **Goetz C**, Everson RG, Zhang LC, Gromeier M. MAPK signal-integrating kinase controls cap-independent translation and cell type-specific cytotoxicity of an oncolytic poliovirus. *Mol Ther* 2010; **18**: 1937-1946 [PMID: 20648000 DOI: 10.1038/mt.2010.145]

108 **Dong C,** Davis RJ, Flavell RA. MAP kinases in the immune response. *Annu Rev Immunol* 2002; **20**: 55-72 [PMID: 11861597 DOI: 10.1146/annurev.immunol.20.091301.131133]

109 **Rowlett RM,** Chrestensen CA, Nyce M, Harp MG, Pelo JW, Cominelli F, Ernst PB, Pizarro TT, Sturgill TW, Worthington MT. MNK kinases regulate multiple TLR pathways and innate proinflammatory cytokines in macrophages. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G452-G459 [PMID: 18032482 DOI: 10.1152/ajpgi.00077.2007]

110 **Joshi S**, Platanias LC. Mnk Kinases in Cytokine Signaling and Regulation of Cytokine Responses. *Biomol Concepts* 2012; **3**: 127-139 [PMID: 23710261 DOI: 10.1515/bmc-2011-0057]

111 **Gorentla BK**, Krishna S, Shin J, Inoue M, Shinohara ML, Grayson JM, Fukunaga R, Zhong XP. Mnk1 and 2 are dispensable for T cell development and activation but important for the pathogenesis of experimental autoimmune encephalomyelitis. *J Immunol* 2013; **190**: 1026-1037 [PMID: 23269249 DOI: 10.4049/jimmunol.1200026]

112 **Kasama T,** Miwa Y, Isozaki T, Odai T, Adachi M, Kunkel SL. Neutrophil-derived cytokines: potential therapeutic targets in inflammation. *Curr Drug Targets Inflamm Allergy* 2005; **4**: 273-279 [PMID: 16101533 DOI: 10.2174/1568010054022114]

113 **Fortin CF,** Mayer TZ, Cloutier A, McDonald PP. Translational control of human neutrophil responses by MNK1. *J Leukoc Biol* 2013; **94**: 693-703 [PMID: 23401599 DOI: 10.1189/jlb.0113012]

114 **Brennan FM,** Feldmann M. Cytokines in autoimmunity. *Curr Opin Immunol* 1992; **4**: 754-759 [PMID: 1466799 DOI: 10.1016/0952-7915(92)90057-L]

115 **Bachert C,** Hauser U, Prem B, Rudack C, Ganzer U. Proinflammatory cytokines in allergic rhinitis. *Eur Arch Otorhinolaryngol* 1995; **252** Suppl 1: S44-S49 [PMID: 7537566 DOI: 10.1007/BF02484434]

116 **Merrill JE**. Proinflammatory and antiinflammatory cytokines in multiple sclerosis and central nervous system acquired immunodeficiency syndrome. *J Immunother* (1991) 1992; **12**: 167-170 [PMID: 1445805]

117 **Netea MG,** van der Meer JW, van Deuren M, Kullberg BJ. Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? *Trends Immunol* 2003; **24**: 254-258 [PMID: 12738419 DOI: 10.1016/S1471-4906(03)00079-6]

118 **Dinarello CA,** Pomerantz BJ. Proinflammatory cytokines in heart disease. *Blood Purif* 2001; **19**: 314-321 [PMID: 11244192 DOI: 10.1159/000046960]

119 **Stienstra R,** Tack CJ, Kanneganti TD, Joosten LA, Netea MG. The inflammasome puts obesity in the danger zone. *Cell Metab* 2012; **15**: 10-18 [PMID: 22225872 DOI: 10.1016/j.cmet.2011.10.011]

120 **Arya M,** Patel HR, Williamson M. Chemokines: key players in cancer. *Curr Med Res Opin* 2003; **19**: 557-564 [PMID: 14594528 DOI: 10.1185/030079903125002216]

121 **Tschopp C,** Knauf U, Brauchle M, Zurini M, Ramage P, Glueck D, New L, Han J, Gram H. Phosphorylation of eIF-4E on Ser 209 in response to mitogenic and inflammatory stimuli is faithfully detected by specific antibodies. *Mol Cell Biol Res Commun* 2000; **3**: 205-211 [PMID: 10891393 DOI: 10.1006/mcbr.2000.0217]

122 **Bain J**, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P. The selectivity of protein kinase inhibitors: a further update. *Biochem J* 2007; **408**: 297-315 [PMID: 17850214 DOI: 10.1042/BJ20070797]

123 **Konicek BW,** Stephens JR, McNulty AM, Robichaud N, Peery RB, Dumstorf CA, Dowless MS, Iversen PW, Parsons S, Ellis KE, McCann DJ, Pelletier J, Furic L, Yingling JM, Stancato LF, Sonenberg N, Graff JR. Therapeutic inhibition of MAP kinase interacting kinase blocks eukaryotic initiation factor 4E phosphorylation and suppresses outgrowth of experimental lung metastases. *Cancer Res* 2011; **71**: 1849-1857 [PMID: 21233335 DOI: 10.1158/0008-5472.CAN-10-3298]

124 **Altman JK**, Szilard A, Konicek BW, Iversen PW, Kroczynska B, Glaser H, Sassano A, Vakana E, Graff JR, Platanias LC. Inhibition of Mnk kinase activity by cercosporamide and suppressive effects on acute myeloid leukemia precursors. *Blood* 2013; **121**: 3675-3681 [PMID: 23509154 DOI: 10.1182/blood-2013-01-477216]

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REVIEW

Critical role of bicarbonate and bicarbonate transporters in cardiac function

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Abstract

Bicarbonate is one of the major anions in mammalian tissues and extracellular fluids. Along with accompanying H^+, HCO_3^- is generated from CO_2 and H_2O, either spontaneously or *via* the catalytic activity of carbonic anhydrase. It serves as a component of the major buffer system, thereby playing a critical role in pH homeostasis. Bicarbonate can also be utilized by a variety of ion transporters, often working in coupled systems, to transport other ions and organic substrates across cell membranes. The functions of HCO_3^- and HCO_3^--transporters in epithelial tissues have been studied extensively, but their functions in heart are less well understood. Here we review studies of the identities and physiological functions of Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransporters of the *SLC4A* and *SLC26A* families in heart. We also present RNA Seq analysis of their cardiac mRNA expression levels. These studies indicate that *slc4a*3 (AE3) is the major Cl^-/HCO_3^- exchanger and plays a protective role in heart failure, and that *Slc4a4* (NBCe1) is the major Na^+/HCO_3^- cotransporter and affects action potential duration. In addition, previous studies show that HCO_3^- has a positive inotropic effect in the perfused heart that is largely independent of effects on intracellular Ca^{2+}. The importance of HCO_3^- in the regulation of contractility is supported by experiments showing that isolated cardiomyocytes exhibit sharply enhanced contractility, with no change in Ca^{2+} transients, when switched from Hepes-buffered to HCO_3^-- buffered solutions. These studies demonstrate that HCO_3^- and HCO_3^--handling proteins play important roles in the regulation of cardiac function.

Key words: SLC4; SLC26; Slc26a6; AE1; AE2; NBCn1

Core tip: Bicarbonate is one of the major anions in mammalian tissues and fluids. It plays a critical role in pH homeostasis and is utilized by various transporters to transport other ions and organic substrates across cell membranes. Here we review studies of the physiological functions of Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransporters in heart, present RNA Seq analysis of their cardiac mRNA expression levels, and show that bicarbonate is required for optimal contractility in isolated cardiac myocytes. These studies demonstrate that HCO_3^- and HCO_3^- handling proteins are abundant in heart and play important roles in the regulation of cardiac function.

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INTRODUCTION

In mammalian tissues, bicarbonate/CO_2 is the major extrinsic buffer system of both extracellular and intracellular fluids. HCO_3^-/CO_2 are likely to play a particularly important role in cardiac muscle[1,2], which relies almost entirely on oxidative metabolism and continuously converts large quantities of O_2 to CO_2. *In vivo*, HCO_3^- is usually formed by carbonic anhydrase-mediated hydration of CO_2[2,3], in a reaction that also generates H^+ ($CO_2 + H_2O \rightarrow H^+ + HCO_3^-$). As might be expected, cardiac myocytes express a variety of ion transporters that mediate extrusion of H^+ and either extrusion or uptake of HCO_3^-. This allows fine control of intracellular pH (pH_i) and coupling of H^+ and HCO_3^- transport to the transport of other ions, thereby affecting not only pH_i, but also cell volume and both cellular and systemic ion homeostasis[4-6].

H^+ and HCO_3^- are, in effect, transient ions[2] that can be used to transport other ions and organic substrates across cell membranes, both directly and by secondary active transport. These ion transporters include Cl^-/HCO_3^- exchangers, Na^+/HCO_3^- cotransporters (NBCs), and Na^+/H^+ exchangers (NHEs). The functions of the various acid-base transporters have been studied most extensively in epithelial tissues; however, they exhibit a surprising abundance and diversity in cardiac tissues. In this paper we review studies describing the identities, membrane locations, and functions of the major HCO_3^- transporters in heart. In addition, we report relative mRNA expression levels in mouse heart for members of the *SLC4A* and *SLC26A* anion transporter families[7-9], which include all of the known Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransporters. Finally, we discuss previous studies of the effects of HCO_3^- on the isolated heart[10] and correlate those results with new data using isolated cardiac myocytes. The available evidence shows that a diverse group of transporters are responsible for movements of HCO_3^- into and out of the heart and demonstrate that the presence of HCO_3^- has a major stimulatory effect on contractility that is, at least in part, independent of changes in intracellular Ca^{2+}.

IDENTIFICATION AND LOCATIONS OF CARDIAC HCO_3^- TRANSPORTERS

Cloning and hybridization analyses have led to the identification of three Cl^-/HCO_3^- exchangers of the *SLC4A* family in heart. These anion exchangers are termed AE1, AE2, and AE3 (Anion Exchanger 1, 2, and 3; gene symbols, *Slc4a1-3*), and one Cl^-/HCO_3^- exchanger of the *SLC26A* family, termed Slc26a6 or PAT1 (putative anion transporter 1). Among the known Na^+/HCO_3^- cotransporters (NBCs), which are members of the *SLC4A* family, the electrogenic NBCe1 and electroneutral NBCn1 (gene symbols, *Slc4a4* and *Slc4a7*) have been identified in heart. Excellent reviews of the *SLC4A* and *SLC26A*[7-9,11,12] families of transporters have been published recently and provide detailed information about specific isoforms, including their ion transport specificities and their physiological functions in various tissues.

AE1 is the band 3 Cl^-/HCO_3^- exchanger that is expressed most prominently in red blood cells[13] and also includes a kidney variant[14] derived from an alternative promoter in intron 4 of the erythrocyte transcription unit[15]. Erythrocyte AE1 plays major roles in maintaining the stability of the cytoskeleton[16] and in gas exchange[17]. Cardiac AE1 mRNA identified in rat heart is smaller than that of the erythroid and kidney variants[18]. It encodes a truncated protein based on immunoblot analyses, although the exact identity of the cardiac AE1 protein in rat heart has not been determined[19]. Immunofluorescence studies of rat heart suggest that truncated cardiac AE1 protein is restricted to intercalated discs[20]. Expression of AE1 is sharply reduced in adult mouse heart compared with its levels in fetal heart[21], consistent with the RNA Seq data discussed below. AE2 is expressed at low levels in heart[18,21,22], and AE2a, one of 4 variants derived from the use of alternative promoters[23], was the only variant detected[22]. The membrane location of AE2 in heart has not been determined. AE3 mRNAs are expressed at very high levels in heart[18,24] and encode both a full-length variant (AE3fl) that is expressed in brain and other tissues and a much more abundant cardiac variant[25-27]. The cardiac AE3 (AE3c) mRNA is derived from an alternative promoter located in intron 6 of the longer transcription unit and has a unique 73-amino acid sequence that replaces the first 270 amino acids of AE3fl[25]. In fetal mouse heart, AE3fl is the predominant form; however, in adult heart, AE3fl is largely restricted to the atria, while AE3c is the predominant form in ventricles[27]. In cardiac myocytes, AE3 protein has been localized to t-tubules and the sarcolemma, with apparent foci of expression at costameres[28].

The *SLC26A* family transports a broad range of anions, including sulfate, chloride, iodide, bicarbonate, oxalate, and formate, and some isoforms can function as anion channels[9]. The first member of this family shown to function as a Cl^-/HCO_3^- exchanger was *Slc26a3*[29]; however, it is primarily an epithelial transporter and is expressed at only low levels in adult heart[21]. Slc26a6 can mediate Cl^-/HCO_3^- exchange[30-32], which appears to be its major function in apical membranes of the intestine[33]. In the renal proximal tubule, however, Slc26a6 functions primarily as a Cl^-/formate and Cl^-/oxalate exchanger[34,35]. Slc26a6 also mediates Cl^-/OH^- exchange and has been proposed to serve both as a Cl^-/HCO_3^- exchanger and as a Cl^-/OH^- exchanger in heart[21,36]. Slc26a6 protein has been localized to the t-tubules and sarcolemma[28].

Prior to the molecular cloning of Na^+/HCO_3^- cotransporter (NBC) isoforms, both electroneutral[37,38] and electrogenic[39,40] Na^+/HCO_3^- cotransport activities had been identified in cardiac muscle. NBCe1 (gene symbol, *Slc4a4*), the first NBC to be cloned[41,42], is electrogenic. In kidney, NBCe1 mediates outward transport of 1 Na^+ and 3 HCO_3^- across the basolateral membrane of proximal tubule epithelial cells[41,42]. In most other tissues, including

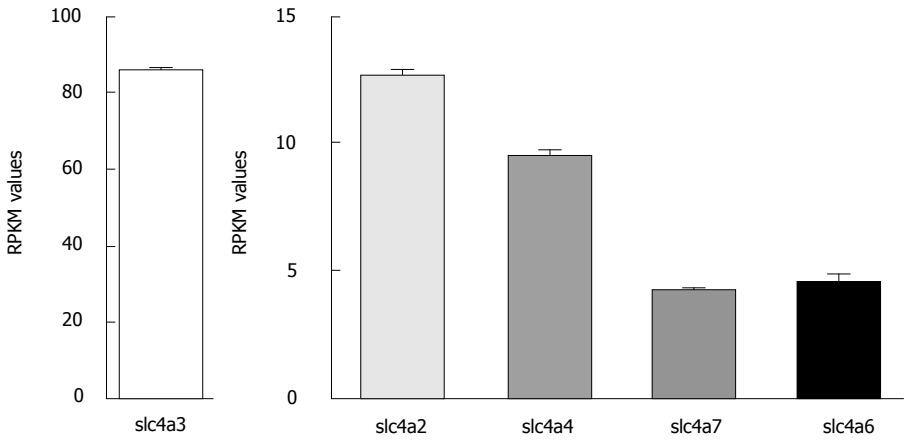

Figure 1 Relative expression levels of the major Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransporters in mouse heart. RPKM values ± SE as determined by RNA Seq analysis (see Table 1 legend) are shown for the most abundant known HCO_3^- transporters in wild-type FVB/N mouse hearts (n = 4). Note the difference in scale for AE3 and the other transporters.

heart, regulation *via* phosphorylation results in a stoichiometry of 1:2[43]. Cloning of the cardiac form of NBCe1 revealed that it has a different N-terminus than the kidney variant[44], which is derived from an alternative promoter and first exon. NBCn1 is electroneutral and transports Na^+ and HCO_3^- in a 1:1 ratio. It was cloned from rat smooth muscle[45] and skeletal muscle[46] and shown to be expressed in heart. A study using an antibody to the N-terminal sequence of an NBCn1 variant indicated that expression in heart was restricted to endothelial and smooth muscle cells[47]. However, NBCn1 transcripts undergo extensive alternative splicing, including a relatively cardiac-specific exon[48], and the use of alternative promoters that yield alternative N-termini[49]. Both NBCe1 and NBCn1 have been shown by Western blots to be expressed in cardiac myocytes[50]. Immunolocalization studies demonstrated that both isoforms are localized to t-tubules, lateral sarcolemma, and intercalated discs[50].

EXPRESSION LEVELS OF HCO_3^- TRANSPORTERS IN HEART

As discussed above, the major HCO_3^- transporters in the mammalian heart include both Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransporters of the *SLC4A* gene family and at least one Cl^-/HCO_3^- exchanger of the *SLC26A* family. Gene expression data for hearts of wild-type FVBN mice were determined by RNA Seq analysis[51], a powerful method for determining the expression levels of all known mRNAs in a tissue of interest[52,53]. Relative mRNA expression levels for the *SLC4A* and *SLC26A* families are shown in Table 1. Transcript levels are expressed as RPKM values (reads per kilobase of exon per million mapped reads), which normalizes expression to the length of the mRNA. Graphical representations of mRNA expression levels of the HCO_3^- transporters that have been identified in heart and appear to be expressed at physiologically relevant levels are shown in Figure 1.

Among the *SLC4A* transporters, the AE3 Cl^-/HCO_3^- exchanger was expressed at very high levels (RPKM = 85.7 ± 0.6), suggesting that it serves as the major HCO_3^- efflux mechanism in mouse cardiac myocytes. AE2 (RPKM = 12.57 ± 0.28) was expressed at lower levels than AE3, but its levels of expression were still greater than some of the other transporters. Although NBC4 (*Slc4a5* or NBCe2) was reported in human heart[54], it is not expressed at significant levels in mouse heart or in rat heart[55]. Of the two major Na^+/HCO_3^- cotransporters in mouse heart, NBCe1 (RPKM = 9.45 ± 0.25) was more abundant than NBCn1 (RPKM = 4.24 ± 0.05). By comparison, RPKM values for the NHE1 Na^+/H^+ exchanger[51], recognized as the major Na^+-dependent acid extruder in heart, were 9.10 ± 0.20.

Among the SLC26A transporters, Slc26a2, Slc26a6, and Slc26a10 were expressed most abundantly. However, among these three *SLC26A* transporters, the only known HCO_3^- transporter is Slc26a6 (RPKM = 4.56 ± 0.26). As discussed above, it has been shown to function as a Cl^-/HCO_3^- exchanger, but it also mediates Cl^-/OH^-, Cl^-/formate, and Cl^-/oxalate exchange[9]. Slc26a2 is a sulfate transporter and also transports Cl^- and oxalate[9]. The ion specificity of Slc26a10 has not been determined[9], but its high level of expression (RPKM = 31.66 ± 2.55) suggests that it plays an important role in mouse heart.

Some of the HCO_3^- transporters expressed at low levels (*e.g.*, the Slc4a8 Na^+-dependent Cl^-/HCO_3^- exchanger and Slc26a3 Cl^-/HCO_3^- exchanger) could still play important roles in heart, particularly if they were restricted to specialized regions of the heart or were expressed primarily in earlier stages of development or in cell-types other than cardiac myocytes. For example, Na^+-dependent Cl^-/HCO_3^- exchange, which could be due to the activity of Slc4a8[56], has been identified in both chicken embryonic cardiomyocytes[57] and in vascular endothelial cells[58].

PHYSIOLOGICAL FUNCTIONS OF CARDIAC HCO_3^- TRANSPORTERS

Cl^-/HCO_3^- Exchangers

Because of the high Cl^- concentrations of extracellular

Table 1 Relative mRNA levels for the Slc4a and Slc26a anion transporters in mouse heart

SLC4A family			*SLC26A* family		
Gene symbol	Transporter name and major function(s); alternate names	Average ± SE	Gene symbol	Transporter name and major function(s); alternate names	Average ± SE
Slc4a1	AE1 Cl^-/HCO_3^- exchanger; Band 3	0.2 ± 0.05	*Slc26a1*	SAT1 sulfate/anion exchanger; Slc26a1	0.34 ± 0.07
Slc4a2	AE2 Cl^-/HCO_3^- exchanger	12.57 ± 0.28	*Slc26a2*	DTDST sulfate/anion exchanger; Slc26a2	2.98 ± 0.17
Slc4a3	AE3 Cl^-/HCO_3^- exchanger	85.7 ± 0.64	*Slc26a3*	DRA $Cl^-/HCO3^-$, exchanger; Slc26a3	0.64 ± 0.05
Slc4a4	NBCe1 Na^+/HCO_3^- cotransporter; NBC1	9.45 ± 0.25	*Slc26a4*	Pendrin Cl^-/HCO_3^- exchanger; Slc26a4	0.02 ± 0.01
Slc4a5	NBCe2 Na^+/HCO_3^- cotransporter; NBC4	0.003 ± 0.002	*Slc26a6*	PAT1 Cl^-/HCO_3^-, Cl^-/formate exchanger; Slc26a6	4.56 ± 0.26
Slc4a7	NBCn1 Na^+/HCO_3^- cotransporter; NBC2; NBC3	4.24 ± 0.05	*Slc26a7*	Slc26a7 Cl^-/HCO_3^- exchanger, Cl^- channel; TAT1	0.15 ± 0.04
Slc4a8	NDCBE Na^+-driven Cl^-/HCO_3^- exchanger; Slc4a8	0.73 ± 0.04	*Slc26a9*	Slc26a9 Cl^-/HCO_3^- exchanger, Cl^- channel	0.01 ± 0.01
Slc4a9	AE4 Cl^-/HCO_3^- exchanger	0.02 ± 0.01	*Slc26a10*	Slc26a10, transporter function unknown	31.66 ± 2.55
Slc4a10	NBCn2 Na^+/HCO_3^- cotransporter	0.02 ± 0.003	*Slc26a11*	Slc26a11 anion exchanger, Cl^- channel; KBAT	1.15 ± 0.14

Relative mRNA expression levels were determined using RNA from 4-month-old male FVB/N mouse hearts (n = 4) as described previously[51]. Values are RPKM (reads per kilobase per million mapped reads) ± SE and are a measure of the relative abundance of specific gene transcripts[53]. For some Slc26a transporters, ion transport specificities are more complex than indicated; see Alper and Sharma[9].

fluids, electroneutral Cl^-/HCO_3^- exchangers mediate outward transport of HCO_3^- and inward transport of Cl^-. The direct effect of this activity is to reduce pH_i, thereby contributing to pH_i regulation[59], and to enhance Cl^--loading, which could affect Cl^- currents that in turn could affect action potentials or rhythmicity[60,61]. Also, when coupled with Na^+-dependent acid extrusion mechanisms, Cl^-/HCO_3^- exchange facilitates Na^+-loading, which can affect contractility as discussed below and may contribute to cardiac hypertrophy[2]. AE1, AE2, and AE3 are electroneutral, but Cl^-/HCO_3^- exchangers of the *SLC26A* family are reported to support both electroneutral or electrogenic anion exchange[9,12].

The physiological functions of AE1 in heart are unclear. An AE1 global knockout mouse has been shown to develop cardiac hypertrophy[28]. The investigators noted, however, that the levels of AE1 in the adult heart are relatively low (confirmed by the data in Table 1) and that null mutants exhibit severe hemolytic anemia and spherocytosis. They attributed the hypertrophy to the blood defect and concluded that one of the more abundant Cl^-/HCO_3^- exchangers, possibly AE3, was more likely to provide the HCO_3^- extrusion capability that has been proposed to balance Na^+-dependent acid extrusion *via* transporters such as the NHE1 Na^+/H^+ exchanger (discussed below). AE2 is a potential candidate for this activity as it is known to operate in concert with NHE1 on basolateral membranes of colonic epithelial cells[62]. AE2 is much less abundant than AE3 and its functions in heart have not been determined.

It has been suggested that one of the major functions of Cl^-/HCO_3^- exchange in heart is to counter the alkalinizing effects of Na^+/H^+ exchange. This would allow increased Na^+/H^+ exchange activity[2,63], which in turn would lead to increased Na^+-loading and Ca^{2+}-loading *via* reverse activity of the Na^+/Ca^{2+} exchanger[64]. Inhibition of Na^+/H^+ exchange reduces cardiac hypertrophy[4,65] and overexpression of an activated NHE1 Na^+/H^+ exchanger induces hypertrophy and increases cytosolic Na^+, Ca^{2+} transients, and contractility[66]. Studies have shown that the reduction in hypertrophy in spontaneously hypertensive rats in response to angiotensin II blockade involves reductions in both Na^+/H^+ exchange and Cl^-/HCO_3^- exchange[67]. Intracellular pH in NHE1-overexpressing myocytes was significantly higher when they were maintained in Hepes-buffered media than in CO_2/HCO_3^--buffered media[66]. This is consistent with the view[68,69] that Cl^-/HCO_3^- exchange balances the alkalinizing effects of Na^+/H^+ exchange, which would be expected to facilitate pH_i-neutral Na^+-loading *in vivo*.

The Cl^-/HCO_3^- exchanger that has been most heavily studied in heart is AE3. Its mRNA is expressed at much higher levels in heart than those of the other HCO_3^- transporters (see Table 1) and it has a cardiac specific variant[25-27], indicating that it serves a specialized function. Earlier studies showed that Cl^-/HCO_3^- exchange and Na^+/H^+ exchange were increased in the hypertrophic heart of spontaneously hypertensive rats[67] and that AE3fl mRNA was upregulated[70]. Although this might suggest that AE3fl accounts for the increased anion exchange activity, the investigators cautioned against this interpretation as AE3fl is expressed at low levels in the adult rat and mouse heart[19,25,27]. Treatment of papillary muscles with an inhibitory anti-AE3 antibody led to an increase in the slow-force response to stretch, which is dependent on Na^+/H^+ exchange, and caused a substantial reduction in Cl^-/HCO_3^- exchange, supporting the view that AE3 is the major Cl^-/HCO_3^- exchanger in cardiac muscle[71]. Analysis of pH_i in tissues treated with the anti-AE3 antibody indicated that AE3 is the major anion exchanger responsible for countering the alkalinizing effects of NHE1-mediated Na^+/H^+ exchange[71]. It has been suggested that activation of AE3 and NHE1 together might contribute to hypertrophy[72], but so far there is no direct proof of this hypothesis.

The initial studies of a gene-targeted AE3-null mouse

showed that the loss of AE3 did not impair cardiovascular performance *in vivo* under basal conditions or after β-adrenergic stimulation, and it also had no effect on ischemia-reperfusion injury using the Langendorff-perfused heart[6]. The latter finding was surprising as there is evidence that Cl^-/HCO_3^- exchange mediates some of the changes in pH_i and intracellular Cl^- that contribute to reperfusion injury[73]. Heart weight/body weight ratios were significantly reduced in null mutants relative to wild-type mice, consistent with the possibility that loss of AE3 activity might contribute to a reduction in hypertrophy. When AE3-null mice were crossed with an NKCC1 Na^+-K^+-$2Cl^-$ cotransporter-null mouse, which had normal contractility[74], the double mutant mice exhibited a contractility defect *in vivo* and in isolated myocytes, and Ca^{2+} extrusion mediated by the Na^+/Ca^{2+} exchanger was increased[6]. NKCC1 has been shown to provide substantial Na^+ influx in chick cardiac myocytes[75] and to affect Na^+/Ca^{2+} exchange in mouse astrocytes[76]. Therefore, it is possible that the additional loss of NKCC1 caused a reduction in Na^+-loading in AE3/NKCC1 double mutants, with subsequent effects on contractility.

The above studies were consistent with the possibility that loss of AE3 might protect against hypertrophy; however, they also showed that its absence can impair cardiac function under certain conditions. To test whether AE3-deficiency might protect against hypertrophy, AE3-null mice were crossed with a transgenic hypertrophic cardiomyopathy mouse model[77] carrying a Glu180Gly mutation in α-tropomyosin[78]. The additional loss of AE3 in the Glu180Gly mutant caused no decrease in the degree of hypertrophy and led to more rapid decompensation, heart failure, and death. Cardiac performance in response to β-adrenergic stimulation was severely impaired in double mutants. The double mutants exhibited more arrhythmic events as heart rates were increased by electrical pacing to assess force-frequency responses and Ca^{2+}-handling was also impaired. It was concluded that AE3 activity is needed for better preservation of cardiac function during heart failure and that it would not be an appropriate therapeutic target for cardiac arrhythmias or hypertrophy. In a more recent study[79] it was shown that hearts of AE3-null mice exhibit blunting of the force-frequency response when they are paced to higher heart rates *in vivo*. Phosphorylation of Akt, which plays a central role in mechanosensory signaling, was increased in paced AE3-null hearts and phosphorylation of adenosine 5'-monophosphate-activated protein kinase (AMPK) was reduced[79]. These data suggest that the increased susceptibility of AE3-null mice to decompensation in heart failure might be due impaired rate dependent inotropy, an insufficient response to biomechanical stress, and metabolic perturbations.

The functions of Slc26a6 in heart have not yet been determined. As discussed by Alper and Sharma[9], there is controversy about the electrogenicity of the Cl^-/HCO_3^- exchange activity of Slc26a6, which is the only known Cl^-/HCO_3^- exchanger of the *SLC26A* family expressed at significant levels in heart[21]. Some studies[80,81] reported electrogenic Cl^-/HCO_3^- exchange for Slc26a6 with a stoichiometry of $1Cl^-/2HCO_3^-$ and others[82] reported electroneutral exchange with a 1:1 ratio. In the latter study[82], the investigators could detect electrogenic Cl^-/oxalate transport mediated by Slc26a6 in oocytes but Cl^-/HCO_3^- and Cl^-/OH^- exchange appeared to be electroneutral. Cl^-/OH^- exchange in cardiac myocytes, which has been attributed to Slc26a6[21], is electroneutral[36], and Slc26a6-mediated Cl^-/formate exchange is electroneutral[83]. Regardless of whether Slc26a6-mediated Cl^-/HCO_3^- exchange is electrogenic, during most of the excitation-relaxation cycle, when the membrane potential is negative, it would transport HCO_3^- out of the cell. However, it is possible that reversal of electrogenic Cl^-/HCO_3^- exchange might occur at positive membrane potentials. The potential functions of electrogenic Slc26a6-mediated Cl^-/HCO_3^- exchange in heart have not been studied. Furthermore, it is not clear which of the various transport functions of Slc26a6 is the most important in heart.

Na^+/HCO_3^- Cotransporters

As discussed above, there is evidence for both electroneutral and electrogenic NBC activities in cardiac myocytes[37-40,84,85]. NBCe1 and NBCn1, along with NHE1, have been immunolocalized in rat myocytes[50]. These three transporters are the major Na^+-dependent alkalinizing mechanisms in cardiac myocytes. In the isolated perfused ferret heart, NBC and NHE activities contributed equally to recovery of pH_i, both after an acid load[86] and also during reperfusion following ischemia[87]. In the latter study, it was suggested that NBC-mediated Na^+ influx might contribute to Ca^{2+} overload and injury after reperfusion. Later studies using an inhibitory antibody showed that inhibition of NBCe1 protected against ischemia-reperfusion injury in the isolated rat heart[88]. Similarly, in rat ventricular myocytes subjected to anoxic conditions, simultaneous inhibition of NBC and NHE1 activities prevented hypercontracture induced by Ca^{2+}-overload during reoxygenation, whereas inhibition of either activity alone was insufficient[89]. This suggests that NBCe1, like the NHE1 Na^+/H^+ exchanger, can be a significant source of Na^+-loading, although the magnitude of Na^+-loading *via* NBCe1 has been estimated to be lower than that of Na^+/H^+ exchange[64]. Also, NHE1 has cardioprotective effects that appear to be independent of effects on Na^+ and Ca^{2+} loading[51,90].

NBCe1 is localized to t-tubules[50], along with the L-type Ca channel (LTCC) and NCX1 Na^+/Ca^{2+} exchanger[91], whereas NHE1 is expressed at highest levels in intercalated discs[50,92]. Thus, NBCe1 appears to be well situated to affect excitation-contraction coupling[93], particularly since it is electrogenic. In fact, a substantial NBC-mediated HCO_3^- current has been demonstrated beginning at -50 millivolts[84], and electrogenic NBC activity causes a shortening of the action potential duration (APD) and affects the resting membrane potential[40,94]. By shortening the APD, NBCe1 could reduce the open time of the LTCC and

with its location in the t-tubule it could reduce intraluminal (extracellular) pH and increase pH_i, both of which reduce LTCC-mediated Ca^{2+} currents[95]. Thus, while NBCe1 activity may serve as a Na^+-loading mechanism that could, in principle, contribute to Ca^{2+}-loading *via* reverse mode activity of the Na^+/Ca^{2+} exchanger, its effects on the APD and on LTCC activity might counteract this tendency. NBCn1 is also in t-tubules of ventricular myocytes[50] and because it is electroneutral it could operate throughout the excitation-contraction cycle.

Both NBCe1 and NBCn1, along with NHE1, were induced in rat heart during pressure overload hypertrophy[55]. After an acid load, the rate of pHi recovery *via* NBC and NHE activities were increased accordingly, and NBC activity in the physiological pH_i range was similar to that of NHE1[55]. Additional experiments[55] showed that when rats were subjected to pressure overload and then treated with losartan, an angiotensin Ⅱ AT_1 receptor antagonist, both hypertrophy and the induction of NBCe1 and NBCn1 were sharply reduced. The results suggest, but do not prove, that increased NBC activities contribute to the development of hypertrophy. NBCe1 mRNA and protein were also induced in human failing hearts[88] and in rat hearts following myocardial infarction[96]; however, in the latter study, treatment with an angiotensin II AT_1 receptor antagonist had no effect on NBCe1 expression. The effects of angiotensin Ⅱ on NBC activity in cardiac myocytes are complex as some studies report activation of NBC activity[97,98] and others report inhibition[99]. A more recent study showed that cardiac expression of both NBCn1 and NBCe1 were induced in spontaneously hypertensive rats in which angiotensin II plays a major role[100]. However, NBCe1 activity was reduced due to a reduction in its protein expression in t-tubule and sarcolemmal membranes; nevertheless, total NBC activity increased due to an increase in NBCn1 activity. The authors noted that a reduction in NBCe1 activity leads to an increase in APD, which is a common occurrence during cardiac hypertrophy[101], and that this would likely cause an increase in Ca^{2+}-influx *via* LTCC[102]. Thus, NBCe1 activity, rather than inhibition of its activity, may be cardioprotective in some disease conditions.

EFFECTS OF HCO_3^- ON CONTRACTILITY AND Ca^{2+} IN ISOLATED HEARTS

Given the abundance and diversity of HCO_3^- transporters in heart and the fact that HCO_3^- is part of the major buffer system in biological systems, it is surprising that there has been little reported work on the specific effects of HCO_3^- on contractility and Ca^{2+}-handling. In an interesting and important study, Fülöp *et al*[10] analyzed Langendorff-perfused guinea pig hearts in the presence of both Krebs solution buffered with CO_2/HCO_3^- and Tyrode solution buffered with HEPES[10]. Contractility in isolated hearts was significantly greater in Krebs solution than in Tyrode solution. However, when Tyrode solution was supplemented with CO_2/HCO_3^-, in the continuing presence of HEPES, contractility increased to the levels observed in Krebs solution. Changes in contractility were reversible as the buffers were switched between those containing CO_2/HCO_3^- or HEPES alone. Despite increased contractility, both the amplitude and duration of the Ca^{2+} transients were lower in solutions containing CO_2/HCO_3^- buffer, indicating that enhanced Ca^{2+} transients were not responsible for the enhanced contractility. Analyses of isolated trabeculae also revealed increased contractility in CO_2/HCO_3^- buffer, with faster times to peak tension and shorter relaxation times[10]. In purkinje fibers and papillary muscles, the duration of the action potential was reduced in the presence of CO_2/HCO_3^- buffer[10]. This finding is consistent with the proposed effects of NBCe1 activity on the action potential[103].

The reduced contractility in the isolated heart and isolated tissues in response to the absence of HCO_3^- may have been due to reduced pH_i or buffering power[10]. However, in wild-type myocytes used in a study of the effects of Na^+/H^+ exchange on Ca^{2+} and contractility, pH_i was the same in HEPES buffer as in buffer containing CO_2/HCO_3^-[66]. Also, in myocytes overexpressing an activated NHE1, the absence of CO_2/HCO_3^- led to an increase in pH_i[66]. These results suggest that a HCO_3^--dependent transport mechanisms, *i.e.*, Cl^-/HCO_3^- exchange, is needed to counter the alkalinizing effects of NHE1.

EFFECTS OF HCO_3^- ON CONTRACTILITY AND Ca^{2+} IN ISOLATED MYOCYTES

Although studies of cardiac myocyte mechanics and Ca^{2+} handling are commonly performed in HEPES buffered solution (Tyrode's solution), we are unaware of studies directly comparing the effects of the two buffer conditions. Therefore, we performed experiments to assess the effects of HEPES-buffered and HCO_3^--buffered solutions on both contraction of rat ventricular myocytes and Ca^{2+} transients. The concentrations of cations were identical for the two solutions, and their osmolarities were the same.

Switching from HEPES-buffered solution to HCO_3^--buffered solution had a bi-phasic effect on myocyte contraction, determined by measurements of cell shortening as described previously[104]. It first resulted in transient suppression of myocyte contraction, followed by reversal of suppression, and enhancement of contraction (Figure 2A). On average, the downward suppressive phase lasted about 50 s (Figure 2B), resulting in a peak suppression of cell shortening from 7.04% in control (*i.e.*, HEPES) to 4.35% (Figure 2C). This was followed by gradual stimulation of contraction; at steady-state, contraction was significantly increased to 11.39% (Figure 2C). Switching from HEPES-buffered Tyrode's solution to an isosmotic solution that contained both HEPES and HCO_3^- produced the same bi-phasic effect and reached the same steady-state increase in contractility. Thus, the stimulation of contraction by HCO_3^- in isolated cardiac myocytes was fully reversible, as observed previously in the isolated

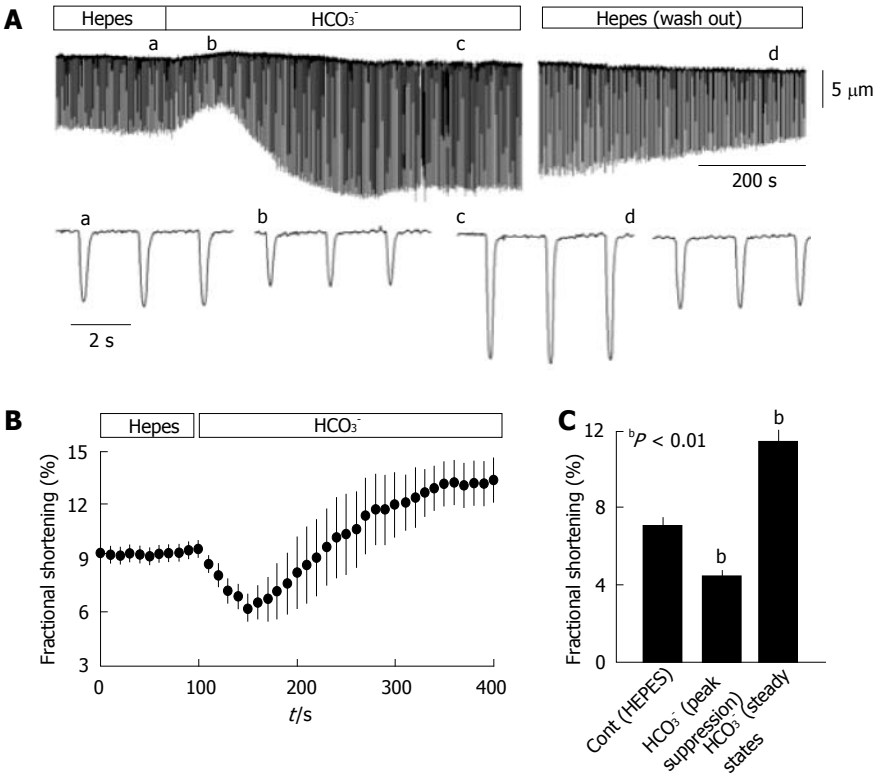

Figure 2 Isolated myocytes exhibit greater contractility in CO_2/HCO_3^- buffer than in HEPES buffer. Ventricular myocytes from rat hearts were enzymatically dissociated using Langendorff perfusion[104] and myocyte mechanics were analyzed at room temperature (24 ℃), with stimulation at 0.5 Hz as described previously[107]. Myocytes were switched between HEPES-buffered Tyrode's solution (in mmol/L: NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 10, and Na-HEPES 5; pH = 7.4; bubbled with 100% O_2) and HCO_3^--buffered Krebs solution (in mmol/L: NaCl 120, $NaHCO_3^-$ 25, KCl 4.2, KH_2PO_4 1.2, $MgCl_2$ 1, $CaCl_2$ 1.8, and glucose 10; pH = 7.4 when bubbled with 95% O_2 and 5% CO_2). A: Representative contraction tracing of a myocyte bathed in HEPES buffer, then switched to HCO_3^- buffer, and then returned to HEPES buffer; the lower tracings show an expanded scale for the indicated (a-d) time points. The lower panels show the time course of fractional shortening (B) and average fractional shortening (C) of myocytes (n = 19) in HEPES buffer and then switched to HCO_3^--containing buffer. Experiments were performed using myocytes from 3 hearts and statistical analysis was conducted using a paired t-test. Values are means ± SE.

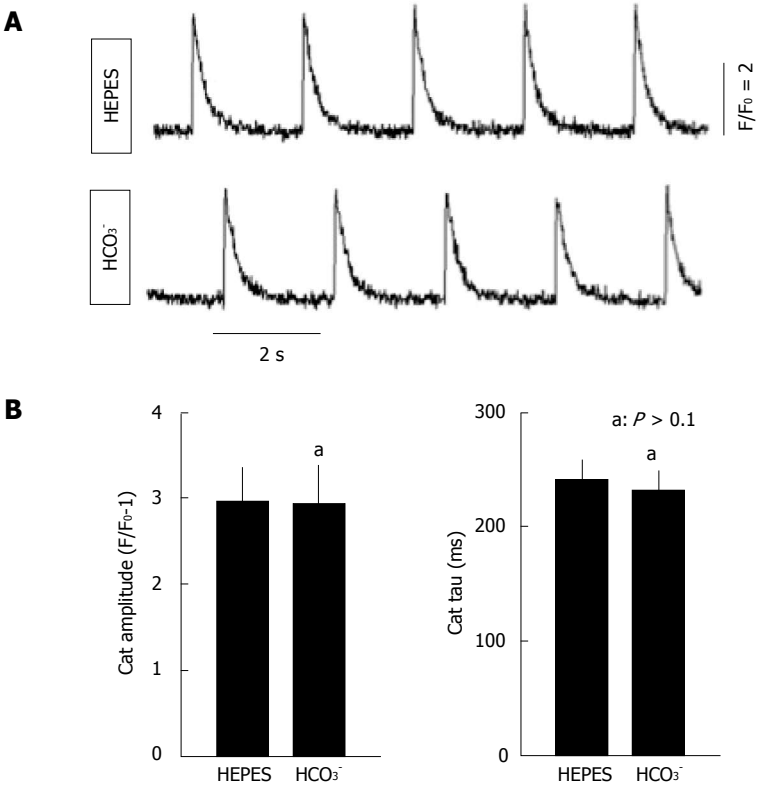

Figure 3 Ca^{2+} transient analysis in isolated rat myocytes bathed in CO_2/HCO_3^- buffer and in HEPES buffer. For recording of Ca^{2+} transients, isolated ventricular myocytes were loaded with fluo-4 acetoxymethyl ester (5 μmol/L, Molecular Probes, Eugene, OR) and activated with field stimulation at 0.5 Hz. Fluorescence signals were measured using a Nikon TE 2000 microscope and an InCyt Standard PM photometry system (Intracellular Imaging, Cincinnati, OH) as described previously[104]. A: Representative Ca^{2+} transients in HEPES and HCO_3^--containing buffers; B: Average Ca^{2+} transient (CaT) amplitudes and tau values, a measure of the rate of decay of the Ca^{2+} transient in HEPES and HCO_3^--containing buffers (n = 12). The same cells were imaged in both buffers. Myocytes were from the same preparations used in Figure 2, with statistical analyses performed using a paired t-test. No significant differences were observed.

heart[10].

In addition to affecting myocyte contractility, HCO_3^- also had a small but significant effect on myocyte length. Switching from HEPES to the HCO_3^--buffered solution transiently increased myocyte length by approximately 1% (data not shown). Such transient lengthening was independent of myocyte contraction, and was also observed in unpaced, quiescent myocytes. This suggests the possibility that cell volume was increased by the addition of HCO_3^-, which is reasonable given the role of Cl^-/HCO_3^- exchange in cell volume regulation[105,106].

Interestingly, the marked effect of HCO_3^- on myo-

cyte contraction was not accompanied by any detectable change in the Ca^{2+} transient. Figure 3A shows Ca^{2+} transients from the same myocyte in the presence of HEPES or HCO_3^- solutions. Switching from HEPES-buffered solutions to HCO_3^--buffered solutions altered neither the amplitude nor the time constant of the Ca^{2+} transient (Figure 3B). These data using isolated myocytes correlate well with the previous studies using isolated hearts[10]. Both sets of data show that HCO_3^- has a major effect on contractility, without any major effects on the amplitude of the Ca^{2+} transient.

CONCLUSION

The studies reviewed here show that the mammalian heart contains an abundance of HCO_3^- transporters, which include both Cl^-/HCO_3^- exchangers and Na^+/HCO_3^- cotransporters. Their most obvious function is regulation of pH_i, although it is possible that this is not their major function. This is particularly apparent in the case of the Cl^-/HCO_3^- exchangers because at the high frequencies occurring *in vivo*, cardiac myocytes generate a substantial acid load. Thus, there would appear to be little need to maintain a robust capacity for recovery from an alkaline load. Nevertheless, it is possible that the cardiac anion exchangers regulate the pH or electrolyte concentrations of sub-sarcolemmal or t-tubule microdomains. With regard to electrolyte homeostasis, coupling of Cl^-/HCO_3^- exchange and Na^+/HCO_3^- cotransport (or Na^+/H^+ exchange) can serve as a pH_i-neutral Na^+- and Cl^--loading mechanism, with Na^+ affecting Ca^{2+}-loading *via* Na^+/Ca^{2+} exchange. In addition to effects on Na^+-loading, electrogenic Na^+/HCO_3^- cotransport can affect the duration of the action potential[40,84,94] and, by affecting subsarcolemmal and t-tubular pH, it might also affect the activity of L-type Ca^{2+} channels[95]. Finally, the available data show that the presence of CO_2/HCO_3^- buffer has a major effect on contractility that cannot be readily explained by effects on Ca^{2+}-handling, thus suggesting that HCO_3^- homeostasis plays an important role in the regulation of cardiac contractility. The mechanism is not known, but it is conceivable that intracellular HCO_3^- concentrations affect myofibrillar function and that dynamic transporter-mediated HCO_3^- fluxes have a major effect on electrical and ionic properties of the myocyte. Further studies of the effects of HCO_3^- and the cardiac functions of each of the major HCO_3^- transporters will be needed to resolve these issues.

REFERENCES

1 **Vaughan-Jones RD**, Spitzer KW. Role of bicarbonate in the regulation of intracellular pH in the mammalian ventricular myocyte. *Biochem Cell Biol* 2002; **80**: 579-596 [PMID: 12440699]

2 **Casey JR**. Why bicarbonate? *Biochem Cell Biol* 2006; **84**: 930-939 [PMID: 17215880 DOI: 10.1139/o06-184]

3 **Spitzer KW**, Skolnick RL, Peercy BE, Keener JP, Vaughan-Jones RD. Facilitation of intracellular H(+) ion mobility by CO(2)/HCO(3)(-) in rabbit ventricular myocytes is regulated by carbonic anhydrase. *J Physiol* 2002; **541**: 159-167 [PMID: 12015427 DOI: 10.1113/jphysiol.2001.013268]

4 **Karmazyn M**, Kilić A, Javadov S. The role of NHE-1 in myocardial hypertrophy and remodelling. *J Mol Cell Cardiol* 2008; **44**: 647-653 [PMID: 18329039 DOI: 10.1016/j.yjmcc.2008.01.005]

5 **Vaughan-Jones RD**, Spitzer KW, Swietach P. Intracellular pH regulation in heart. *J Mol Cell Cardiol* 2009; **46**: 318-331 [PMID: 19041875 DOI: 10.1016/j.yjmcc.2008.10.024]

6 **Prasad V**, Bodi I, Meyer JW, Wang Y, Ashraf M, Engle SJ, Doetschman T, Sisco K, Nieman ML, Miller ML, Lorenz JN, Shull GE. Impaired cardiac contractility in mice lacking both the AE3 Cl-/HCO3- exchanger and the NKCC1 Na+-K+-2Cl- cotransporter: effects on Ca2+ handling and protein phosphatases. *J Biol Chem* 2008; **283**: 31303-31314 [PMID: 18779325 DOI: 10.1074/jbc.M803706200]

7 **Alper SL**. Molecular physiology and genetics of Na+-independent SLC4 anion exchangers. *J Exp Biol* 2009; **212**: 1672-1683 [PMID: 19448077 DOI: 10.1242/jeb.029454]

8 **Romero MF**, Chen AP, Parker MD, Boron WF. The SLC4 family of bicarbonate (HCO_3^-) transporters. *Mol Aspects Med* 2013; **34**: 159-182 [PMID: 23506864 DOI: 10.1007/s00424-003-1180-2]

9 **Alper SL**, Sharma AK. The SLC26 gene family of anion transporters and channels. *Mol Aspects Med* 2013; **34**: 494-515 [PMID: 23506885 DOI: 10.1016/j.mam.2012.07.009]

10 **Fülöp L**, Szigeti G, Magyar J, Szentandrássy N, Ivanics T, Miklós Z, Ligeti L, Kovács A, Szénási G, Csernoch L, Nánási PP, Bányász T. Differences in electrophysiological and contractile properties of mammalian cardiac tissues bathed in bicarbonate - and HEPES-buffered solutions. *Acta Physiol Scand* 2003; **178**: 11-18 [PMID: 12713510 DOI: 10.1046/j.1365-201X.2003.01114.x]

11 **Soleimani M**. SLC26 Cl-/HCO3- exchangers in the kidney: roles in health and disease. *Kidney Int* 2013; **84**: 657-666 [PMID: 23636174 DOI: 10.1038/ki.2013.138]

12 **Bonar PT**, Casey JR. Plasma membrane Cl^-/HCO_3^- exchangers: structure, mechanism and physiology. *Channels* (Austin) 2008; **2**: 337-345 [PMID: 19066446 DOI: 10.4161/chan.2.5.6899]

13 **Kopito RR**, Lodish HF. Primary structure and transmembrane orientation of the murine anion exchange protein. *Nature* 1985; **316**: 234-238 [PMID: 2410791 DOI: 10.1038/316234a0]

14 **Kudrycki KE**, Shull GE. Primary structure of the rat kidney band 3 anion exchange protein deduced from a cDNA. *J Biol Chem* 1989; **264**: 8185-8192 [PMID: 2722777]

15 **Kudrycki KE**, Shull GE. Rat kidney band 3 Cl-/HCO3- exchanger mRNA is transcribed from an alternative promoter. *Am J Physiol* 1993; **264**: F540-F547 [PMID: 8456965]

16 **Peters LL**, Shivdasani RA, Liu SC, Hanspal M, John KM, Gonzalez JM, Brugnara C, Gwynn B, Mohandas N, Alper SL, Orkin SH, Lux SE. Anion exchanger 1 (band 3) is required to prevent erythrocyte membrane surface loss but not to form the membrane skeleton. *Cell* 1996; **86**: 917-927 [PMID: 8808627 DOI: 10.1016/S0092-8674(00)80167-1]

17 **Bruce LJ**, Beckmann R, Ribeiro ML, Peters LL, Chasis JA, Delaunay J, Mohandas N, Anstee DJ, Tanner MJ. A band 3-based macrocomplex of integral and peripheral proteins in the RBC membrane. *Blood* 2003; **101**: 4180-4188 [PMID: 12531814 DOI: 10.1182/blood-2002-09-2824]

18 **Kudrycki KE**, Newman PR, Shull GE. cDNA cloning and tissue distribution of mRNAs for two proteins that are related to the band 3 Cl-/HCO3- exchanger. *J Biol Chem* 1990; **265**: 462-471 [PMID: 2294114]

19 **Richards SM**, Jaconi ME, Vassort G, Pucéat M. A spliced variant of AE1 gene encodes a truncated form of Band 3 in heart: the predominant anion exchanger in ventricular myocytes. *J Cell Sci* 1999; **112** (Pt 10): 1519-1528 [PMID: 10212146]

20 **Moura Lima PR**, Salles TS, Costa FF, Saad ST. alpha-cardiac actin (ACTC) binds to the band 3 (AE1) cardiac isoform.

J Cell Biochem 2003; **89**: 1215-1221 [PMID: 12898519 DOI: 10.1002/jcb.10561]
21 **Alvarez BV**, Kieller DM, Quon AL, Markovich D, Casey JR. Slc26a6: a cardiac chloride-hydroxyl exchanger and predominant chloride-bicarbonate exchanger of the mouse heart. *J Physiol* 2004; **561**: 721-734 [PMID: 15498800 DOI: 10.1113/jphysiol.2004.077339]
22 **Wang Z**, Schultheis PJ, Shull GE. Three N-terminal variants of the AE2 Cl-/HCO3- exchanger are encoded by mRNAs transcribed from alternative promoters. *J Biol Chem* 1996; **271**: 7835-7843 [PMID: 8631828]
23 **Medina JF**, Lecanda J, Acín A, Ciesielczyk P, Prieto J. Tissue-specific N-terminal isoforms from overlapping alternate promoters of the human AE2 anion exchanger gene. *Biochem Biophys Res Commun* 2000; **267**: 228-235 [PMID: 10623603 DOI: 10.1006/bbrc.1999.1951]
24 **Kopito RR**, Lee BS, Simmons DM, Lindsey AE, Morgans CW, Schneider K. Regulation of intracellular pH by a neuronal homolog of the erythrocyte anion exchanger. *Cell* 1989; **59**: 927-937 [PMID: 2686841 DOI: 10.1016/0092-8674(89)90615-6]
25 **Linn SC**, Kudrycki KE, Shull GE. The predicted translation product of a cardiac AE3 mRNA contains an N terminus distinct from that of the brain AE3 Cl-/HCO3- exchanger. Cloning of a cardiac AE3 cDNA, organization of the AE3 gene, and identification of an alternative transcription initiation site. *J Biol Chem* 1992; **267**: 7927-7935 [PMID: 1560021]
26 **Yannoukakos D**, Stuart-Tilley A, Fernandez HA, Fey P, Duyk G, Alper SL. Molecular cloning, expression, and chromosomal localization of two isoforms of the AE3 anion exchanger from human heart. *Circ Res* 1994; **75**: 603-614 [PMID: 7923606 DOI: 10.1161/01.RES.75.4.603]
27 **Linn SC**, Askew GR, Menon AG, Shull GE. Conservation of an AE3 Cl-/HCO3- exchanger cardiac-specific exon and promoter region and AE3 mRNA expression patterns in murine and human hearts. *Circ Res* 1995; **76**: 584-591 [PMID: 7895333 DOI: 10.1161/01.RES.76.4.584]
28 **Alvarez BV**, Kieller DM, Quon AL, Robertson M, Casey JR. Cardiac hypertrophy in anion exchanger 1-null mutant mice with severe hemolytic anemia. *Am J Physiol Heart Circ Physiol* 2007; **292**: H1301-H1312 [PMID: 17056673 DOI: 10.1152/ajpheart.00449.2006]
29 **Melvin JE**, Park K, Richardson L, Schultheis PJ, Shull GE. Mouse down-regulated in adenoma (DRA) is an intestinal Cl(-)/HCO(3)(-) exchanger and is up-regulated in colon of mice lacking the NHE3 Na(+)/H(+) exchanger. *J Biol Chem* 1999; **274**: 22855-22861 [PMID: 10428871 DOI: 10.1074/jbc.274.32.22855]
30 **Wang Z**, Petrovic S, Mann E, Soleimani M. Identification of an apical Cl(-)/HCO3(-) exchanger in the small intestine. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G573-G579 [PMID: 11842009 DOI: 10.1152/ajpgi.00338.2001]
31 **Petrovic S**, Ma L, Wang Z, Soleimani M. Identification of an apical Cl-/HCO-3 exchanger in rat kidney proximal tubule. *Am J Physiol Cell Physiol* 2003; **285**: C608-C617 [PMID: 12736136 DOI: 10.1152/ajpcell.00084.2003]
32 **Jiang Z**, Grichtchenko II, Boron WF, Aronson PS. Specificity of anion exchange mediated by mouse Slc26a6. *J Biol Chem* 2002; **277**: 33963-33967 [PMID: 12119287 DOI: 10.1074/jbc.M202660200]
33 **Simpson JE**, Schweinfest CW, Shull GE, Gawenis LR, Walker NM, Boyle KT, Soleimani M, Clarke LL. PAT-1 (Slc26a6) is the predominant apical membrane Cl-/HCO3- exchanger in the upper villous epithelium of the murine duodenum. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G1079-G1088 [PMID: 17170027 DOI: 10.1152/ajpgi.00354.2006]
34 **Knauf F**, Yang CL, Thomson RB, Mentone SA, Giebisch G, Aronson PS. Identification of a chloride-formate exchanger expressed on the brush border membrane of renal proximal tubule cells. *Proc Natl Acad Sci USA* 2001; **98**: 9425-9430 [PMID: 11459928 DOI: 10.1073/pnas.141241098]
35 **Aronson PS**. Ion exchangers mediating Na+, HCO3 - and Cl- transport in the renal proximal tubule. *J Nephrol* 2006; **19** Suppl 9: S3-S10 [PMID: 16736438]
36 **Niederer SA**, Swietach P, Wilson DA, Smith NP, Vaughan-Jones RD. Measuring and modeling chloride-hydroxyl exchange in the Guinea-pig ventricular myocyte. *Biophys J* 2008; **94**: 2385-2403 [PMID: 18055536 DOI: 10.1529/biophysj.107.118885]
37 **Dart C**, Vaughan-Jones RD. Na(+)-HCO3- symport in the sheep cardiac Purkinje fibre. *J Physiol* 1992; **451**: 365-385 [PMID: 1403816]
38 **Lagadic-Gossmann D**, Buckler KJ, Vaughan-Jones RD. Role of bicarbonate in pH recovery from intracellular acidosis in the guinea-pig ventricular myocyte. *J Physiol* 1992; **458**: 361-384 [PMID: 1302269]
39 **Camilión de Hurtado MC**, Alvarez BV, Pérez NG, Cingolani HE. Role of an electrogenic Na(+)-HCO3- cotransport in determining myocardial pHi after an increase in heart rate. *Circ Res* 1996; **79**: 698-704 [PMID: 8831493 DOI: 10.1161/01.RES.79.4.698]
40 **Aiello EA**, Petroff MG, Mattiazzi AR, Cingolani HE. Evidence for an electrogenic Na+-HCO3- symport in rat cardiac myocytes. *J Physiol* 1998; **512** (Pt 1): 137-148 [PMID: 9729624 DOI: 10.1111/j.1469-7793.1998.137bf.x]
41 **Romero MF**, Hediger MA, Boulpaep EL, Boron WF. Expression cloning and characterization of a renal electrogenic Na+/HCO3- cotransporter. *Nature* 1997; **387**: 409-413 [PMID: 9163427 DOI: 10.1038/387409a0]
42 **Burnham CE**, Amlal H, Wang Z, Shull GE, Soleimani M. Cloning and functional expression of a human kidney Na+: HCO3- cotransporter. *J Biol Chem* 1997; **272**: 19111-19114 [PMID: 9235899 DOI: 10.1074/jbc.272.31.19111]
43 **Gross E**, Kurtz I. Structural determinants and significance of regulation of electrogenic Na(+)-HCO(3)(-) cotransporter stoichiometry. *Am J Physiol Renal Physiol* 2002; **283**: F876-F887 [PMID: 12372762]
44 **Choi I**, Romero MF, Khandoudi N, Bril A, Boron WF. Cloning and characterization of a human electrogenic Na+-HCO-3 cotransporter isoform (hhNBC). *Am J Physiol* 1999; **276**: C576-C584 [PMID: 10069984]
45 **Choi I**, Aalkjaer C, Boulpaep EL, Boron WF. An electroneutral sodium/bicarbonate cotransporter NBCn1 and associated sodium channel. *Nature* 2000; **405**: 571-575 [PMID: 10850716 DOI: 10.1038/35014615]
46 **Pushkin A**, Abuladze N, Lee I, Newman D, Hwang J, Kurtz I. Cloning, tissue distribution, genomic organization, and functional characterization of NBC3, a new member of the sodium bicarbonate cotransporter family. *J Biol Chem* 1999; **274**: 16569-16575 [PMID: 10347222 DOI: 10.1074/jbc.274.23.16569]
47 **Damkier HH**, Nielsen S, Praetorius J. An anti-NH2-terminal antibody localizes NBCn1 to heart endothelia and skeletal and vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 2006; **290**: H172-H180 [PMID: 16126812 DOI: 10.1152/ajpheart.00713.2005]
48 **Cooper DS**, Lee HJ, Yang HS, Kippen J, Yun CC, Choi I. The electroneutral sodium/bicarbonate cotransporter containing an amino terminal 123-amino-acid cassette is expressed predominantly in the heart. *J Biomed Sci* 2006; **13**: 593-595 [PMID: 16547769 DOI: 10.1007/s11373-006-9078-6]
49 **Liu Y**, Qin X, Wang DK, Guo YM, Gill HS, Morris N, Parker MD, Chen LM, Boron WF. Effects of optional structural elements, including two alternative amino termini and a new splicing cassette IV, on the function of the sodium-bicarbonate cotransporter NBCn1 (SLC4A7). *J Physiol* 2013; **591**: 4983-5004 [PMID: 23959679 DOI: 10.1113/jphysi-

ol.2013.258673]
50 **Garciarena CD**, Ma YL, Swietach P, Huc L, Vaughan-Jones RD. Sarcolemmal localisation of Na+/H+ exchange and Na+-HCO3- co-transport influences the spatial regulation of intracellular pH in rat ventricular myocytes. *J Physiol* 2013; **591**: 2287-2306 [PMID: 23420656 DOI: 10.1113/jphysiol.2012.249664]
51 **Prasad V**, Lorenz JN, Miller ML, Vairamani K, Nieman ML, Wang Y, Shull GE. Loss of NHE1 activity leads to reduced oxidative stress in heart and mitigates high-fat diet-induced myocardial stress. *J Mol Cell Cardiol* 2013; **65**: 33-42 [PMID: 24080184 DOI: 10.1016/j.yjmcc.2013.09.013]
52 **Martin JA**, Wang Z. Next-generation transcriptome assembly. *Nat Rev Genet* 2011; **12**: 671-682 [PMID: 21897427 DOI: 10.1038/nrg3068]
53 **Malone JH**, Oliver B. Microarrays, deep sequencing and the true measure of the transcriptome. *BMC Biol* 2011; **9**: 34 [PMID: 21627854 DOI: 10.1186/1741-7007-9-34]
54 **Pushkin A**, Abuladze N, Newman D, Lee I, Xu G, Kurtz I. Cloning, characterization and chromosomal assignment of NBC4, a new member of the sodium bicarbonate cotransporter family. *Biochim Biophys Acta* 2000; **1493**: 215-218 [PMID: 10978526 DOI: 10.1016/S0167-4781(00)00149-4]
55 **Yamamoto T**, Shirayama T, Sakatani T, Takahashi T, Tanaka H, Takamatsu T, Spitzer KW, Matsubara H. Enhanced activity of ventricular Na+-HCO3- cotransport in pressure overload hypertrophy. *Am J Physiol Heart Circ Physiol* 2007; **293**: H1254-H1264 [PMID: 17416604 DOI: 10.1152/ajpheart.00964.2006]
56 **Grichtchenko II**, Choi I, Zhong X, Bray-Ward P, Russell JM, Boron WF. Cloning, characterization, and chromosomal mapping of a human electroneutral Na(+)-driven Cl-HCO3 exchanger. *J Biol Chem* 2001; **276**: 8358-8363 [PMID: 11133997 DOI: 10.1074/jbc.C000716200]
57 **Liu S**, Piwnica-Worms D, Lieberman M. Intracellular pH regulation in cultured embryonic chick heart cells. Na(+)-dependent Cl-/HCO3- exchange. *J Gen Physiol* 1990; **96**: 1247-1269 [PMID: 1962815]
58 **Sun B**, Vaughan-Jones RD, Kambayashi J. Two distinct HCO(-)(3)-dependent H(+) efflux pathways in human vascular endothelial cells. *Am J Physiol* 1999; **277**: H28-H32 [PMID: 10409178]
59 **Chiappe de Cingolani GE**, Ennis IL, Morgan PE, Alvarez BV, Casey JR, Camilión de Hurtado MC. Involvement of AE3 isoform of Na(+)-independent Cl(-)/HCO(3)(-) exchanger in myocardial pH(i) recovery from intracellular alkalization. *Life Sci* 2006; **78**: 3018-3026 [PMID: 16458934 DOI: 10.1016/j.lfs.2005.11.030]
60 **Hume JR**, Duan D, Collier ML, Yamazaki J, Horowitz B. Anion transport in heart. *Physiol Rev* 2000; **80**: 31-81 [PMID: 10617765]
61 **Vaughan-Jones RD**. Chloride activity and its control in skeletal and cardiac muscle. *Philos Trans R Soc Lond B Biol Sci* 1982; **299**: 537-548 [PMID: 6130545 DOI: 10.1098/rstb.1982.0150]
62 **Gawenis LR**, Bradford EM, Alper SL, Prasad V, Shull GE. AE2 Cl-/HCO3- exchanger is required for normal cAMP-stimulated anion secretion in murine proximal colon. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: G493-G503 [PMID: 20110461 DOI: 10.1152/ajpgi.00178.2009]
63 **Pérez NG**, Alvarez BV, Camilión de Hurtado MC, Cingolani HE. pHi regulation in myocardium of the spontaneously hypertensive rat. Compensated enhanced activity of the Na(+)-H+ exchanger. *Circ Res* 1995; **77**: 1192-1200 [PMID: 7586232 DOI: 10.1161/01.RES.77.6.1192]
64 **Garciarena CD**, Youm JB, Swietach P, Vaughan-Jones RD. H^+-activated Na^+ influx in the ventricular myocyte couples Ca^{2+}-signalling to intracellular pH. *J Mol Cell Cardiol* 2013; **61**: 51-59 [PMID: 23602948 DOI: 10.1016/j.yjmcc.2013.04.008]
65 **Yoshida H**, Karmazyn M. Na(+)/H(+) exchange inhibition attenuates hypertrophy and heart failure in 1-wk postinfarction rat myocardium. *Am J Physiol Heart Circ Physiol* 2000; **278**: H300-H304 [PMID: 10644613]
66 **Nakamura TY**, Iwata Y, Arai Y, Komamura K, Wakabayashi S. Activation of Na+/H+ exchanger 1 is sufficient to generate Ca2+ signals that induce cardiac hypertrophy and heart failure. *Circ Res* 2008; **103**: 891-899 [PMID: 18776042 DOI: 10.1161/CIRCRESAHA.108.175141]
67 **Ennis IL**, Alvarez BV, Camilión de Hurtado MC, Cingolani HE. Enalapril induces regression of cardiac hypertrophy and normalization of pHi regulatory mechanisms. *Hypertension* 1998; **31**: 961-967 [PMID: 9535421 DOI: 10.1161/01.HYP.31.4.961]
68 **Camilión de Hurtado MC**, Alvarez BV, Pérez NG, Ennis IL, Cingolani HE. Angiotensin II activates Na+-independent Cl--HCO3- exchange in ventricular myocardium. *Circ Res* 1998; **82**: 473-481 [PMID: 9506708 DOI: 10.1161/01.RES.82.4.473]
69 **Alvarez BV**, Fujinaga J, Casey JR. Molecular basis for angiotensin II-induced increase of chloride/bicarbonate exchange in the myocardium. *Circ Res* 2001; **89**: 1246-1253 [PMID: 11739292 DOI: 10.1161/hh2401.101907]
70 **Chiappe de Cingolani G**, Morgan P, Mundiña-Weilenmann C, Casey J, Fujinaga J, Camilión de Hurtado M, Cingolani H. Hyperactivity and altered mRNA isoform expression of the Cl(-)/HCO(3)(-) anion-exchanger in the hypertrophied myocardium. *Cardiovasc Res* 2001; **51**: 71-79 [PMID: 11399249 DOI: 10.1016/S0008-6363(01)00276-0]
71 **Cingolani HE**, Chiappe GE, Ennis IL, Morgan PG, Alvarez BV, Casey JR, Dulce RA, Pérez NG, Camilión de Hurtado MC. Influence of Na+-independent Cl--HCO3- exchange on the slow force response to myocardial stretch. *Circ Res* 2003; **93**: 1082-1088 [PMID: 14576196 DOI: 10.1161/01.RES.0000102408.25664.01]
72 **Alvarez BV**, Johnson DE, Sowah D, Soliman D, Light PE, Xia Y, Karmazyn M, Casey JR. Carbonic anhydrase inhibition prevents and reverts cardiomyocyte hypertrophy. *J Physiol* 2007; **579**: 127-145 [PMID: 17124262 DOI: 10.1113/jphysiol.2006.123638]
73 **Lai ZF**, Nishi K. Intracellular chloride activity increases in guinea pig ventricular muscle during simulated ischemia. *Am J Physiol* 1998; **275**: H1613-H1619 [PMID: 9815068]
74 **Meyer JW**, Flagella M, Sutliff RL, Lorenz JN, Nieman ML, Weber CS, Paul RJ, Shull GE. Decreased blood pressure and vascular smooth muscle tone in mice lacking basolateral Na(+)-K(+)-2Cl(-) cotransporter. *Am J Physiol Heart Circ Physiol* 2002; **283**: H1846-H1855 [PMID: 12384462 DOI: 10.1152/ajpheart.00083.2002]
75 **Frelin C**, Chassande O, Lazdunski M. Biochemical characterization of the Na+/K+/Cl- co-transport in chick cardiac cells. *Biochem Biophys Res Commun* 1986; **134**: 326-331 [PMID: 3947328 DOI: 10.1016/0006-291X(86)90566-8]
76 **Lenart B**, Kintner DB, Shull GE, Sun D. Na-K-Cl cotransporter-mediated intracellular Na+ accumulation affects Ca2+ signaling in astrocytes in an in vitro ischemic model. *J Neurosci* 2004; **24**: 9585-9597 [PMID: 15509746 DOI: 10.1523/JNEUROSCI.2569-04.2004]
77 **Prabhakar R**, Boivin GP, Grupp IL, Hoit B, Arteaga G, Solaro RJ, Wieczorek DF. A familial hypertrophic cardiomyopathy alpha-tropomyosin mutation causes severe cardiac hypertrophy and death in mice. *J Mol Cell Cardiol* 2001; **33**: 1815-1828 [PMID: 11603924 DOI: 10.1006/jmcc.2001.1445]
78 **Al Moamen NJ**, Prasad V, Bodi I, Miller ML, Neiman ML, Lasko VM, Alper SL, Wieczorek DF, Lorenz JN, Shull GE. Loss of the AE3 anion exchanger in a hypertrophic cardiomyopathy model causes rapid decompensation and heart failure. *J Mol Cell Cardiol* 2011; **50**: 137-146 [PMID: 21056571

DOI: 10.1016/j.yjmcc.2010.10.028]
79 **Prasad V**, Lorenz JN, Lasko VM, Nieman ML, Al Moamen NJ, Shull GE. Loss of the AE3 Cl(-)/HCO(-) 3 exchanger in mice affects rate-dependent inotropy and stress-related AKT signaling in heart. *Front Physiol* 2013; **4**: 399 [PMID: 24427143 DOI: 10.3389/fphys.2013.00399]
80 **Ko SB**, Shcheynikov N, Choi JY, Luo X, Ishibashi K, Thomas PJ, Kim JY, Kim KH, Lee MG, Naruse S, Muallem S. A molecular mechanism for aberrant CFTR-dependent HCO(3)(-) transport in cystic fibrosis. *EMBO J* 2002; **21**: 5662-5672 [PMID: 12411484 DOI: 10.1093/emboj/cdf580]
81 **Shcheynikov N**, Wang Y, Park M, Ko SB, Dorwart M, Naruse S, Thomas PJ, Muallem S. Coupling modes and stoichiometry of Cl-/HCO3- exchange by slc26a3 and slc26a6. *J Gen Physiol* 2006; **127**: 511-524 [PMID: 16606687 DOI: 10.1085/jgp.200509392]
82 **Chernova MN**, Jiang L, Friedman DJ, Darman RB, Lohi H, Kere J, Vandorpe DH, Alper SL. Functional comparison of mouse slc26a6 anion exchanger with human SLC26A6 polypeptide variants: differences in anion selectivity, regulation, and electrogenicity. *J Biol Chem* 2005; **280**: 8564-8580 [PMID: 15548529 DOI: 10.1074/jbc.M411703200]
83 **Ohana E**, Shcheynikov N, Yang D, So I, Muallem S. Determinants of coupled transport and uncoupled current by the electrogenic SLC26 transporters. *J Gen Physiol* 2011; **137**: 239-251 [PMID: 21282402 DOI: 10.1085/jgp.201010531]
84 **Yamamoto T**, Swietach P, Rossini A, Loh SH, Vaughan-Jones RD, Spitzer KW. Functional diversity of electrogenic Na+-HCO3- cotransport in ventricular myocytes from rat, rabbit and guinea pig. *J Physiol* 2005; **562**: 455-475 [PMID: 15550467 DOI: 10.1113/jphysiol.2004.071068]
85 **Vaughan-Jones RD**, Villafuerte FC, Swietach P, Yamamoto T, Rossini A, Spitzer KW. pH-Regulated Na(+) influx into the mammalian ventricular myocyte: the relative role of Na(+)-H(+) exchange and Na(+)-HCO Co-transport. *J Cardiovasc Electrophysiol* 2006; **17** Suppl 1: S134-S140 [PMID: 16686668 DOI: 10.1111/j.1540-8167.2006.00394.x]
86 **Grace AA**, Kirschenlohr HL, Metcalfe JC, Smith GA, Weissberg PL, Cragoe EJ, Vandenberg JI. Regulation of intracellular pH in the perfused heart by external HCO3- and Na(+)-H+ exchange. *Am J Physiol* 1993; **265**: H289-H298 [PMID: 8393626]
87 **Vandenberg JI**, Metcalfe JC, Grace AA. Mechanisms of pHi recovery after global ischemia in the perfused heart. *Circ Res* 1993; **72**: 993-1003 [PMID: 8386598 DOI: 10.1161/01.RES.72.5.993]
88 **Khandoudi N**, Albadine J, Robert P, Krief S, Berrebi-Bertrand I, Martin X, Bevensee MO, Boron WF, Bril A. Inhibition of the cardiac electrogenic sodium bicarbonate cotransporter reduces ischemic injury. *Cardiovasc Res* 2001; **52**: 387-396 [PMID: 11738055 DOI: 10.1016/S0008-6363(01)00430-8]
89 **Schäfer C**, Ladilov YV, Siegmund B, Piper HM. Importance of bicarbonate transport for protection of cardiomyocytes against reoxygenation injury. *Am J Physiol Heart Circ Physiol* 2000; **278**: H1457-H1463 [PMID: 10775122]
90 **Schäfer C**, Ladilov YV, Schäfer M, Piper HM. Inhibition of NHE protects reoxygenated cardiomyocytes independently of anoxic Ca(2+) overload and acidosis. *Am J Physiol Heart Circ Physiol* 2000; **279**: H2143-H2150 [PMID: 11045947]
91 **Thomas MJ**, Sjaastad I, Andersen K, Helm PJ, Wasserstrom JA, Sejersted OM, Ottersen OP. Localization and function of the Na+/Ca2+-exchanger in normal and detubulated rat cardiomyocytes. *J Mol Cell Cardiol* 2003; **35**: 1325-1337 [PMID: 14596789 DOI: 10.1016/j.yjmcc.2003.08.005]
92 **Petrecca K**, Atanasiu R, Grinstein S, Orlowski J, Shrier A. Subcellular localization of the Na+/H+ exchanger NHE1 in rat myocardium. *Am J Physiol* 1999; **276**: H709-H717 [PMID: 9950874]
93 **Aronsen JM**, Swift F, Sejersted OM. Cardiac sodium transport and excitation-contraction coupling. *J Mol Cell Cardiol* 2013; **61**: 11-19 [PMID: 23774049 DOI: 10.1016/j.yjmcc.2013.06.003]
94 **Villa-Abrille MC**, Petroff MG, Aiello EA. The electrogenic Na+/HCO3- cotransport modulates resting membrane potential and action potential duration in cat ventricular myocytes. *J Physiol* 2007; **578**: 819-829 [PMID: 17138608 DOI: 10.1113/jphysiol.2006.120170]
95 **Saegusa N**, Moorhouse E, Vaughan-Jones RD, Spitzer KW. Influence of pH on Ca^{2+} current and its control of electrical and Ca^{2+} signaling in ventricular myocytes. *J Gen Physiol* 2011; **138**: 537-559 [PMID: 22042988 DOI: 10.1085/jgp.201110658]
96 **Sandmann S**, Yu M, Kaschina E, Blume A, Bouzinova E, Aalkjaer C, Unger T. Differential effects of angiotensin AT1 and AT2 receptors on the expression, translation and function of the Na+-H+ exchanger and Na+-HCO3- symporter in the rat heart after myocardial infarction. *J Am Coll Cardiol* 2001; **37**: 2154-2165 [PMID: 11419902 DOI: 10.1016/S0735-1097(01)01287-6]
97 **Kohout TA**, Rogers TB. Angiotensin II activates the Na+/HCO3- symport through a phosphoinositide-independent mechanism in cardiac cells. *J Biol Chem* 1995; **270**: 20432-20438 [PMID: 7657618 DOI: 10.1074/jbc.270.35.20432]
98 **De Giusti VC**, Garciarena CD, Aiello EA. Role of reactive oxygen species (ROS) in angiotensin II-induced stimulation of the cardiac Na+/HCO3- cotransport. *J Mol Cell Cardiol* 2009; **47**: 716-722 [PMID: 19646989 DOI: 10.1016/j.yjmcc.2009.07.023]
99 **De Giusti VC**, Orlowski A, Aiello EA. Angiotensin II inhibits the electrogenic Na+/HCO3- cotransport of cat cardiac myocytes. *J Mol Cell Cardiol* 2010; **49**: 812-818 [PMID: 20692267 DOI: 10.1016/j.yjmcc.2010.07.018]
100 **Orlowski A**, Ciancio MC, Caldiz CI, De Giusti VC, Aiello EA. Reduced sarcolemmal expression and function of the NBCe1 isoform of the Na^+-HCO_3^- cotransporter in hypertrophied cardiomyocytes of spontaneously hypertensive rats: role of the renin-angiotensin system. *Cardiovasc Res* 2014; **101**: 211-219 [PMID: 24253522 DOI: 10.1093/cvr/cvt255]
101 **Lebeche D**, Kaprielian R, Hajjar R. Modulation of action potential duration on myocyte hypertrophic pathways. *J Mol Cell Cardiol* 2006; **40**: 725-735 [PMID: 16600293 DOI: 10.1016/j.yjmcc.2006.01.018]
102 **Carmeliet E**. Action potential duration, rate of stimulation, and intracellular sodium. *J Cardiovasc Electrophysiol* 2006; **17** Suppl 1: S2-S7 [PMID: 16686677 DOI: 10.1111/j.1540-8167.2006.00378.x]
103 **De Giusti VC**, Orlowski A, Villa-Abrille MC, de Cingolani GE, Casey JR, Alvarez BV, Aiello EA. Antibodies against the cardiac sodium/bicarbonate co-transporter (NBCe1) as pharmacological tools. *Br J Pharmacol* 2011; **164**: 1976-1989 [PMID: 21595652 DOI: 10.1111/j.1476-5381.2011.01496.x]
104 **Yan S**, Chen Y, Dong M, Song W, Belcher SM, Wang HS. Bisphenol A and 17β-estradiol promote arrhythmia in the female heart via alteration of calcium handling. *PLoS One* 2011; **6**: e25455 [PMID: 21980463 DOI: 10.1371/journal.pone.0025455]
105 **Mason MJ**, Smith JD, Garcia-Soto JJ, Grinstein S. Internal pH-sensitive site couples Cl-(-)HCO3- exchange to Na+-H+ antiport in lymphocytes. *Am J Physiol* 1989; **256**: C428-C433 [PMID: 2537575]
106 **Jiang L**, Chernova MN, Alper SL. Secondary regulatory volume increase conferred on Xenopus oocytes by expression of AE2 anion exchanger. *Am J Physiol* 1997; **272**: C191-C202 [PMID: 9038825]
107 **Belcher SM**, Chen Y, Yan S, Wang HS. Rapid estrogen receptor-mediated mechanisms determine the sexually di-

morphic sensitivity of ventricular myocytes to 17β-estradiol and the environmental endocrine disruptor bisphenol A. *Endocrinology* 2012; **153**: 712-720 [PMID: 22166976 DOI: 10.1210/en.2011-1772]

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REVIEW

FoxO3a and disease progression

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Abstract

The Forkhead box O (FoxO) family has recently been highlighted as an important transcriptional regulator of crucial proteins associated with the many diverse functions of cells. So far, FoxO1, FoxO3a, FoxO4 and FoxO6 proteins have been identified in humans. Although each FoxO family member has its own role, unlike the other FoxO families, FoxO3a has been extensively studied because of its rather unique and pivotal regulation of cell proliferation, apoptosis, metabolism, stress management and longevity. FoxO3a alteration is closely linked to the progression of several types of cancers, fibrosis and other types of diseases. In this review, we will examine the function of FoxO3a in disease progression and also explore FoxO3a's regulatory mechanisms. We will also discuss FoxO3a as a potential target for the treatment of several types of disease.

Key words: Forkhead box O; Cell proliferation; Apoptosis; Stress; Aging

Core tip: Forkhead box O (FoxO)3a has recently been highlighted as a critical protein that regulates numerous cell functions from proliferation/apoptosis to stress-resistance and aging. FoxO3a has been found to be deregulated in several diseases and FoxO3a targeting approaches are currently underway to treat various types of cancers. This review will describe the current concept of FoxO3a's pathological role in various diseases and elucidate the regulatory mechanisms involved. It will also provide the clinical significance and strategies to target FoxO3a to limit the progression of human diseases.

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INTRODUCTION

Forkhead box O (FoxO) transcription factors are the human homologues of the *C. elegans* transcription factor DAF-16 and share a highly conserved 110-amino acid DNA binding domain, forkhead box or winged-helix domain[1,2]. Forkhead box proteins comprise more than 100 members in humans, classified from FOXA to FOXR[3-5]. Members of class O share the characteristic of being regulated by the insulin/PI3K/Akt signaling pathway[4]. Four principal members of the mammalian FoxO subfamily, FoxO1, FoxO3a, FoxO4 and FoxO6 have been previously described[3]. Although they seem to bind a common set of DNA sites, FoxO6 is mainly specific to neurons, while the other 3 FoxO family members are expressed in most tissues. These FoxO members are linked to cell survival, cellular proliferation and DNA damage repair response[5,6]. Among them, FoxO3a has recently been studied extensively as a crucial protein that is involved in the regulation of several essential cellular functions (see page 349). Prior studies have shown that FoxO3a functions as a tumor suppressor by regulating expression of genes in-

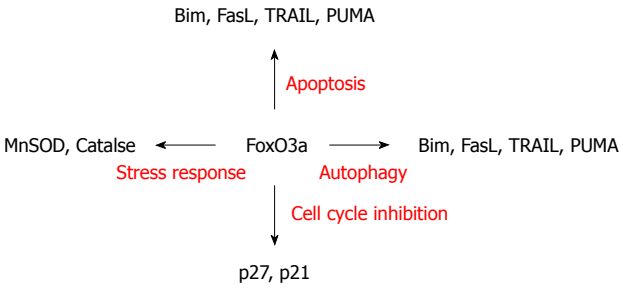

Figure 1 Forkhead box O3a target genes. Forkhead box O (FoxO)3a transcriptionally activates several target genes. FoxO3a binds to the promoter of apoptosis inducing genes, such as *Bim*, *FasL* and *TRAIL*, and to the promoter of cell cycle inhibitors, such as p27 and p21. FoxO3a also activates autophagy genes Gabarapl1, ATG12, etc. A recent study showed that FoxO3a also participates in the activation of stress response genes, such as MnSOD and catalase in response to oxidative stress.

volved in apoptosis, cell cycle arrest, oxidative stress resistance and autophagy[3,7-9] (Figure 1). In general, FoxO3a is known to suppress cell cycle progression and promote cell death. Thus, it has been thought that FoxO3a can be an important target to inhibit cancer cell progression. However, recent studies have discovered other functions of FoxO3a, such as stress response and longevity, as described on page 349. FoxO3a alteration is also linked to many different types of disease. Interestingly, FoxO3a increases autophagy to protect cells from environmental stresses[10,11]. Thus, under this situation, unlike the general concept of FoxO3a's role, FoxO3a potentially has a protective role in maintaining a cell's homeostasis. Perhaps the most interesting feature of FoxO3a is its biological role associated with longevity (page 349). Based on this, it becomes clear that FoxO3a has diverse roles in response to many environmental stimuli and these recent findings certainly change our view on the previous roles of FoxO3a. Therefore, from the perspective of disease progression, it is imperative to define the potential role of FoxO3a in cells and elucidate how alteration of FoxO3a is linked to the development of several types of disease.

FOXO3A STRUCTURE

Recent technologies have revealed that the primary structure of FoxO3a contains highly conserved residues of the helix H3 (motif NXXRHXXS/T), which is the main DNA recognition element that binds into a major groove, which comprises the majority of the direct base-specific contacts[1,6]. Recent studies further revealed that FoxO proteins recognize two consensus sequences, 5'-GTAAA(T/C)AA-3' known as the Daf-16 family member-binding element[6,7] and 5'-(C/A)(A/C)AAA(C/T)AA-3' known as the insulin-responsive sequence (IRE)[8,9]. Crystal structure revealed that the recognition helix H3 docked perpendicular to the major groove making extensive contacts with the DNA[7]. FoxO3a contains several crucial domains[12] (Figure 2) such as a nuclear localization signal (NLS), a nuclear export signal (NES) and a transactivation domain (TA).

FOXO3A REGULATORY MECHANISMS

Phosphorylation and dephosphorylation

FoxO3a is regulated by posttranslational modifications such as phosphorylation, acetylation and ubiquitination, each of which affects the transcriptional activity of FoxO proteins[11-16] (Figure 2). The potency of FoxO3a is carefully regulated by phosphorylation. The phosphorylation of FoxO3a by several kinases is well established. Among them, protein kinase B (Akt) is an important kinase that directly phosphorylates FoxOs. In the case of FoxO3a, T32, S253 and S315 residues are phosphorylated by Akt and, in particular, the phosphorylation of S253 is a crucial residue regulating the nuclear/cytoplasmic shuttling of FoxO3a. For example, when cells are cultured in the presence of growth factors or insulin, FoxO3a is phosphorylated by Akt and mainly localized to the cytoplasm, which prevents its transcriptional activity. The phosphorylation event of FoxO3a by Akt facilitates FoxO3a interaction with the 14-3-3 nuclear export protein, further preventing nuclear re-import by concealing nuclear localization signals[13]. Furthermore, the phosphorylation of FoxO3a by activated Akt promotes an association with an ubiquitin E3 ligase, subsequently polyubiquitinating FoxO3a, which facilitates FoxO3a degradation by proteasomes[13-17]. Thus, the activation of Akt is thought to be critical in FoxO3a regulation. However, in some tumors, FoxO3a remains in the cytoplasm even in the absence of active Akt[14]. It has been found that IkB kinase (IKK) phosphorylates FoxO3a at serine 644, thereby inhibiting its transcriptional activity in an Akt-independent manner[15]. The phosphorylation of FoxO3a by IKK also leads to its cytoplasmic localization, although the underlying export mechanism is not understood. The insulin/IGF-1 and integrin-dependent signaling pathways activate Akt *via* PTEN suppression which phosphorylates FoxO3a, thereby rendering it functionally inactive. In contrast, FoxO3a is localized to the nucleus to activate its target genes when growth factors or serum are deprived. Additionally, serum and glucocorticoid regulated kinase (SGK), casein kinase 1 (CK1), dual specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A), janus N-terminal kinase (JNK), mitogen-activated protein kinases (MAPKs), IkappaB kinase (IKKβ), mammalian sterile 20-like kinase 1 (MST1) and AMP activated protein kinase (AMPK) are also known to regulate FoxO3a and other family members[18-23] by phosphorylating multiple residues. Interestingly, SGK1 is transcriptionally up-regulated in response to a variety of external stimuli, including growth factors. SGK1 is also known to phosphorylate the pivotal ser 253 residue, which triggers its location to the cytoplasm, thereby inhibiting its function[19]. In contrast, AMPK activates FoxO3a function. 6 threonine/serine residues (T179, S399, S413, S555, S588 and S626) in mammalian FoxO3a are found to be phosphorylated by AMPK[24,25]. Mutation of these phosphorylation residues to alanine severely impairs its function, yet it does not alter its ability to bind to cognate

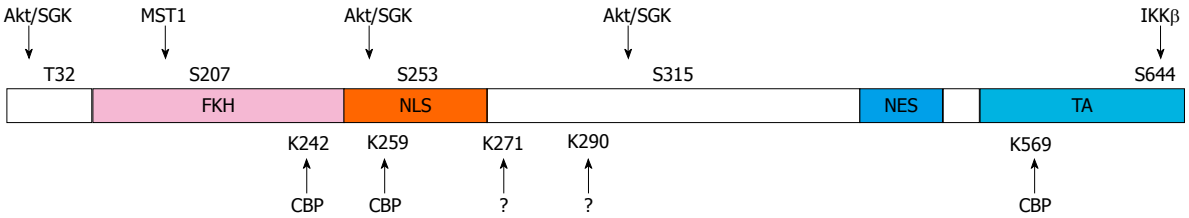

Figure 2 Major phosphorylation and acetylation residues of FoxO3a. Post-translational modification sites of FoxO3a. Shown are sites of serine/threonine phosphorylation by Akt/SGK, MST1, IKKβ or the residues acetylated by CBP or unidentified acetyl transferases (?) on FoxO3a domains[12]. FKH: Forkhead DNA binding domain; NLS: Nuclear localization signal; NES: Nuclear export sequence; TA: Transactivation domain; Akt: Protein kinase B; MST1: Mammalian sterile 20 like kinase-1; CBP: The cyclic–AMP responsive element binding (CREB) binding protein, IKKβ: Ikβ kinase; SGK: Serum-and glucocorticoid-induced protein kinase.

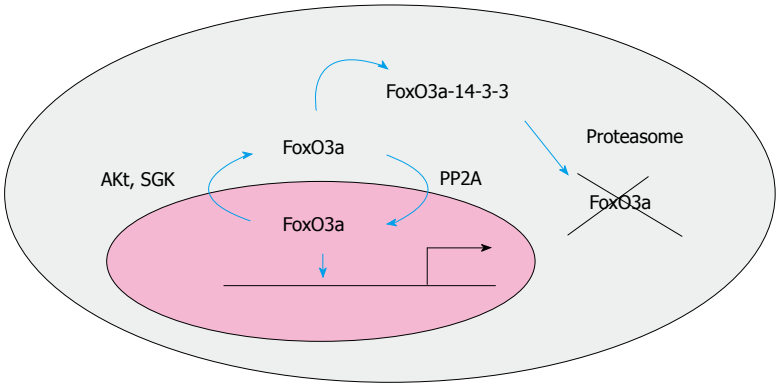

Figure 3 Forkhead box O3a localization by phosphorylation and dephosphorylation. Forkhead box O (FoxO)3a becomes translocated to the cytoplasm when phosphorylated on ser 253 residue by Akt or SGK. FoxO3a is then bound to 14-3-3 and this interaction promotes its degradation by the proteasome. In contrast, FoxO3a is dephosphorylated by protein phosphatase-2A and this opposite event facilitates its relocation into the nucleus, thereby activating its target genes. SGK: Serum-and glucocorticoid-induced protein kinase; Akt: Protein kinase B.

sequences or to participate in nucleocytoplasmic shuttling depending on external cues[25]. Likewise, JNK also phosphorylates FoxO3a, activating FoxO3a function by enhancing its location into the nucleus which subsequently increases its transcriptional activity[18,22].

Unlike kinases, very few phosphatases have been found to regulate FoxO3a. One particular phosphatase, protein phosphatase-2A (PP2A), has been shown to regulate FoxO3a function. Nho *et al*[26] showed that when fibroblasts attach to 2D type collagen coated plates, PP2A activity is suppressed, which facilitates FoxO3a inactivation by enhanced Akt, promoting fibroblast proliferation. But the over-expression of PP2A reverses this inactivation and increases dephosphorylated FoxO3a, thereby suppressing their proliferation. Singh *et al*[24] also demonstrated that FoxO3a interacts with PP2A C/A subunits in HeLA cells, dephosphorylating its T32/S253 residues, which subsequently inhibits the interaction of the 14-3-3 protein to FoxO3a by Akt. This study showed that PP2A is required for the reactivation of FoxO3a by promoting its translocation to the nucleus (Figure 3). Interestingly, recent studies also showed that the adenovirus E1A stabilizes FoxO3a by inducing the expression of PP2A/C, which suppresses βTrCP-mediated degradation of FoxO3a[25]. Thus, these studies clearly suggest that the imbalance between kinases and phosphatase(s) can greatly affect a cell's fate by curbing FoxO3a function and the alteration of these kinases and phosphatases are directly linked to certain disease progression.

Ubiquitin proteasome degradation

As we briefly described above, FoxO3a degradation is also an important step to regulate its function. The single molecule RING-finger E3 ligase murine double minute 2 (MDM2) promotes ubiquitination of FoxO3a as well as FoxO1 and FoxO4, facilitating their degradation[27]. Intriguingly, knockout or knockdown of MDM2 alone increases FoxO3a protein levels. This effect was shown to be mediated by MDM2-induced polyubiquitination of FoxO proteins[27,28], whereas another study showed that MDM2 catalyzes multiple monoubiquitination of FoxO4 rather than polyubiquitination[28]. When FoxO3a is located to the cytoplasm by Akt, FoxO3a becomes ubiquitinated and this event triggers a proteasome-dependent degradation process. Like MDM2, FoxO3a phosphorylation by IKK also leads to its ubiquitination and degradation[15]. Thus, these studies document that FoxO3a localization in the cytoplasm not only deactivates FoxO3a function but also becomes a crucial step leading to FoxO3a degradation.

Acetylation, transcriptional regulation, microRNA and others

Acetylation also plays an important role in regulating FoxO3a. Oxidative stress triggers FoxO3a acetylation/deacetylation and affects the localization of FoxO3a. For example, protein acetylase CREB binding protein (CBP)[29-31], p300[32,33] and deacetylase Sirt are known to modulate FoxO3a function[34-38], although a precise mechanism describing the effects of acetylation and deacetylation is not known. A recent piece of evidence suggests that the FoxO family is also regulated by microRNA. mir155, mir96 and mir21 are thought to directly regulate FoxO3a, while mir205 regulates FoxO3a *via* its

upstream target PTEN[39-43]. FoxO3a is also known to be regulated by a transcription factor. E2F-1 can bind to the promoter region of FoxO1 and FoxO3a, thereby regulating FoxO3a at the mRNA level[44]. FoxO3a mRNAs are modulated as a function of age in rat muscle, peaking at 6 and 23 mo, suggesting that FoxO3a may also affect longevity in mammals[45].

FOXO3A FUNCTION

Cell proliferation and apoptosis

Perhaps the two most significant cellular processes that are regulated by FoxO transcription factor are the suppression of cell cycle progression and the promotion of apoptosis[46-50]. FoxO3a activation increases cell cycle inhibitor proteins p21 and p27, both of which subsequently suppress G1 to S cell cycle transition[51-54]. Although p27 is transcriptionally regulated by FoxO3a *via* the PI3K/Akt-dependent axis, it has been shown that p27 is also regulated *via* the FoxO3a/NF-κB/c-Myc-dependent pathway. Chandramohan *et al*[55] showed that in WEHI 231 cells, the suppression of PI3K activity promotes a decrease in c-Myc dependent p27 expression *via* NF-κB inhibition. Since NF-κB is frequently altered in many types of cancers and NF-κB transcriptionally activates *c-Myc* gene expression, this finding suggests that p27 is reciprocally regulated by FoxO3a and c-Myc. A recent study further suggests that FoxO3a inhibits NF-κB function and that the alteration of FoxO3a is associated with hyper-proliferative helper T cells, cigarette smoke-induced inflammation, airspace enlargement and chronic obstructive pulmonary disease[56,57]. Likewise, FoxO3a also increases several target genes, such as Bim, TRAIL, PUMA and Fas ligand, which all promote cell apoptosis. For example, FoxO3a directly binds to the promoter region of Bim, causing sympathetic neuron cell death[44]. The activation of the transcription factor FoxO3a led to increased TRAIL transcription and induction of G1 arrest in the absence of v-Abl inhibition; this effect could be inhibited by the expression of a constitutively active Akt mutant in BCR-Abl-transformed human cells. Ghaffari *et al*[49] also demonstrated that cytokine and BCR-Abl suppression of TRAIL transcription is mediated through phosphorylation and inhibition of the FoxO3a transcription factor. This study showed that BCR-Abl-induced inhibition of TRAIL transcription is linked to the tumorigenicity in chronic myeloid leukemia[50]. FoxO3a is also associated with the regulation of PUMA and Noxa proteins in lymphoid and neuroblastoma cells, respectively[58,59]. Thus, these findings clearly demonstrate that FoxO3a-dependent cell cycle arrest and apoptosis induction are important for tumor suppression (Table 1) and further indicate that the pathological alteration of FoxO3a can potentially contribute to the acquisition of uncontrolled cell proliferation and an apoptosis-resistant cell phenotype.

Stress resistant effect

The most recent discovery regarding FoxO3a's function is that it is also associated with stress response and longevity. In contrast to FoxO3a's better known functions of inhibiting cell proliferation and promoting apoptosis as described above, FoxO3a also participates in protecting cells when exposed to unfavorable conditions. This seemingly contradictory effect of FoxO3a has been observed in various cell models and it has been found that the reactive oxygen species (ROS) are linked to the activation of FoxO3a to protect cells from a stress inducing environment[60,61]. In *C. elegans,* DAF-16 is thought to regulate 230 genes on the ablated germ cell line background and most of these genes are related to the resistance of external stress[62,63]. Deregulated ROS induce apoptosis and are associated with various diseases and aging. Sirtuin-1 (Sirt1) decreases ROS levels and promotes cell survival under oxidative stress conditions. Interestingly, FoxO3a and other FoxO family members increase superoxide dismutase (SOD) and protect cells from oxidative stress in a Sirt1-dependent manner[34,38]. A Sirt1/FoxO3a-dependent cell regulatory function that has been linked to stress management was previously studied. Brunet *et al* showed that Sirt1 and FoxO3a form a complex in cells in response to oxidative stress and Sirt1 increases the ability of FoxO3a to induce cell cycle arrest and resistance to oxidative stress but inhibited FoxO3a's function to induce cell death[38]. These results showed that FoxO3a deacetylation by Sirt1 in response to ROS can be an important self defense mechanism to detoxifying harmful reactive molecules, further suggesting that Sirt1 is linked to protect cells from a stress inducing environment by tipping FoxO dependent response away from apoptosis and toward stress resistance[38]. Studies also found that Sirt3, which belongs to class Ⅲ of HDACs, is linked to the resistance of stress inducing environments by detoxifying ROS. The role of Sirt3 and FoxO3a function is particularly well described in myocytes[64]. At the cellular level, when cardiomyocytes are exposed to stressful stimuli, Sirt3 levels are elevated, which subsequently deacetylase FoxO3a and facilitate its location into the nucleus to activate anti-oxidant genes[65]. Among them, catalase (Cat) and manganese superoxide dismutase (MnSOD) are direct targets of detoxifying enzymes by FoxO3a. Thus, the increased level of Cat and MsSoD by FoxO3a activation may efficiently and effectively manage ROS, which can be beneficial for reducing stress induced by ROS. Interestingly, a prior study found a potential FoxO activator as a way to protect cells from oxidative stress. Resveratrol, a polyphenolic flavonoid abundant in red wine with potent antioxidant activity, is known to up-regulate the FoxO family and block caspase 3, 8, and 9 activation, protecting photoreceptor cells from oxidative stress[66]. Thus, it is believed that when cells are exposed to a stress inducing environment, FoxO3a protects cells by utilizing SOD, catalase, *etc*., and this action is ultimately beneficial to cells. Given the fact that FoxO3a is linked to stress response and cells utilize FoxO3a to respond to ROS, it is a plausible scenario that the activation of FoxO3a under stress inducing conditions triggers the cell's defense sys-

Table 1 FoxO3a target genes in various cell types

FoxO3a target genes	Cell types
Bim	Neuron cells[48]
TRAIL	Bcr/Abl transformed cells[57]
TRAIL	Chronic myeloid leukemia[46]
PUMA	Lymphoid cells[58]
Noxa	Neuroblastoma[59]
FasL	Glomerular mesangial cells[102]
p27, Caveolin-1	Glomerular mesangial cells[102]
p21	Glomerular mesangial cells[102]

Shown are previously known FoxO3a target genes that regulate cell proliferation and apoptosis in different cell types.

tem, which can protect cells from harmful environments.

Longevity

However, perhaps the most intriguing recent discovery in FoxO3a function is that the *FoxO3a* gene is associated with aging. Because FoxO3a is regulated by insulin-IGF1 signaling (IIS) which influences metabolism and lifespan in model organisms[67], FoxO3a had been proposed to be an ideal candidate to study longevity as the link between FoxO3a and longevity that has previously been described. Willcox *et al*[68] described 3 single nucleotide polymorphisms (SNPs) in the *FoxO3a* gene that were statistically significantly associated with longevity and different aging phenotypes in a sample of long-lived Americans of Japanese ancestry. Furthermore, Flachsbart *et al*[69] found that not only were certain FoxO3a variants very common in 90 year olds, they were even more common in 100 year olds, emphasizing the importance of genetics for aging well. It becomes clear that increases in cellular ROS levels are known to be associated with aging[70-75]. Increased cellular oxidative stress regulates FoxO post-translational modifications and the activation of the FoxO family has been shown to regulate cellular oxidative-stress resistance[76-81]. Interestingly, to support these findings, recent studies suggest a possibility that Sirt3 and FoxO3a have been linked to an extended life span in humans[75-78,82].

FOXO3A IN CLINICAL APPLICATION

FoxO3, FoxO1 and FoxO4 are present at chromosomal translocation break points in cells of rhabdomyosarcomas and acute myeloid leukemia. Among the FoxO family, FoxO3a has been shown to be deregulated in several tumor types, including breast cancer[83-85], prostate cancer[86-88], glioblastoma[89] and leukemia[90,91]. Therefore, FoxO3a has been targeted as a way to treat several types of cancers. Interestingly, Akt, IKK and Erk are three commonly activated oncogenic kinases in human cancers and all three kinases target FoxO3a in an identical manner to inhibit its tumor suppressor function[92]. All three kinase-mediated phosphorylations stimulate FoxO3a ubiquitination, resulting in its proteasomal degradation. Thus, a FoxO3a targeting approach *via* the modulation of above kinases is currently underway. For example, the chemotherapeutic drugs paclitaxel[93] and KP372-1 (a multiple kinase inhibitor)[30], currently used in the treatment of breast carcinoma, activate FoxO3a by reducing Akt activity. Doxorubicin activates FoxO3a to induce the expression of the multidrug resistance gene ABCB1 (MDR1) in K562 doxorubicin-sensitive leukemic cells[94]. Imatinib activates FoxO3a and induces Bim-dependent apoptosis through inhibition of BCR-ABL in chronic myeloid leukemia[95]. Imatinib also induces erythroid differentiation through repressing ID1 gene transcription by FoxO3a activation[96]. BMS-345541, a selective IKK inhibitor, promotes apoptosis in T-cell acute lymphoblastic leukemia (T-ALL) cell lines[97]. Several pieces of evidence in recent years further suggest that a FoxO3a targeting approach may be helpful for the treatment of other types of human diseases. For example, FoxO3a causes the induction of apoptosis in prostate cancer cells *via* up-regulating PUMA[98]. Low levels of FoxO3a may link to chemotherapy resistance in liver cancer and FoxO3a appears to present antitumor properties in hepatocellular carcinoma[99-101]. FoxO3a also plays a role in the neuroprotective effect of the erythropoietin (EPO) role in Parkinson's disease *via* Akt[102]. Thus, all these studies indicate that as our knowledge for FoxO3a targeting approaches continuously develop, the clinical application of FoxO3 is potentially promising to limit the progression of human diseases in the future.

FUTURE APPLICATION OF FOXO3A

FoxO3a has recently been recognized as a promising therapeutic target to treat cancers and other types of diseases. To improve therapeutic outcomes, FoxO3a-dependent chemosensitization is being currently tested. Studies suggest that precise FoxO3a regulation is essential for homeostasis and if there is deregulation of FoxO3a by environmental factors, such as chronic exposure to ROS or genetic/epigenetic alteration, this pathological condition can directly lead to abnormal proliferation or changes in apoptotic signals, which subsequently are responsible for disease progression. In particular, age-dependent FoxO3a modulation is an interesting concept to help understand the pathogenesis of certain types of disease models. If FoxO3a is a crucial protein mainly deregulated by aging, maintaining optimum FoxO3a activity in a patient's specific clinical condition can be beneficial to minimize age-dependent disease. For example, the preservation of optimum FoxO3a activity using drugs such as paclitaxel may be helpful for patients with age-related diseases. Clearly, more studies are required to elucidate FoxO3a's function as an effective and useful target capable of preventing or limiting the progression of diseases without clinical compromise.

REFERENCES

1 **Clark KL**, Halay ED, Lai E, Burley SK. Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. *Nature* 1993; **364**: 412-420 [PMID: 8332212 DOI:

10.1038/364412a0]
2 **Arden KC**. Multiple roles of FOXO transcription factors in mammalian cells point to multiple roles in cancer. *Exp Gerontol* 2006; **41**: 709-717 [PMID: 16806782 DOI: 10.1016/j.exger.2006.05.015]
3 **Huang H**, Tindall DJ. Dynamic FoxO transcription factors. *J Cell Sci* 2007; **120**: 2479-2487 [PMID: 17646672 DOI: 10.1242/jcs.001222]
4 **Dillin A**, Crawford DK, Kenyon C. Timing requirements for insulin/IGF-1 signaling in C. elegans. *Science* 2002; **298**: 830-834 [PMID: 12399591 DOI: 10.1126/science.1074240]
5 **Monsalve M**, Olmos Y. The complex biology of FOXO. *Curr Drug Targets* 2011; **12**: 1322-1350 [PMID: 21443460 DOI: 10.2174/138945011796150307]
6 **Biggs WH**, Cavenee WK, Arden KC. Identification and characterization of members of the FKHR (FOX O) subclass of winged-helix transcription factors in the mouse. *Mamm Genome* 2001; **12**: 416-425 [PMID: 11353388 DOI: 10.1007/s003350020002]
7 **Furuyama T**, Nakazawa T, Nakano I, Mori N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* 2000; **349**: 629-634 [PMID: 10880363 DOI: 10.1042/0264-6021:]
8 **Hall RK**, Yamasaki T, Kucera T, Waltner-Law M, O'Brien R, Granner DK. Regulation of phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein-1 gene expression by insulin. The role of winged helix/forkhead proteins. *J Biol Chem* 2000; **275**: 30169-30175 [PMID: 10913147 DOI: 10.1074/jbc.M004898200]
9 **Hribal ML**, Nakae J, Kitamura T, Shutter JR, Accili D. Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *J Cell Biol* 2003; **162**: 535-541 [PMID: 12925703 DOI: 10.1083/jcb.200212107]
10 **Warr MR**, Binnewies M, Flach J, Reynaud D, Garg T, Malhotra R, Debnath J, Passegué E. FOXO3A directs a protective autophagy program in haematopoietic stem cells. *Nature* 2013; **494**: 323-327 [PMID: 23389440 DOI: 10.1038/nature11895]
11 **Xia N**, Strand S, Schlufter F, Siuda D, Reifenberg G, Kleinert H, Förstermann U, Li H. Role of SIRT1 and FOXO factors in eNOS transcriptional activation by resveratrol. *Nitric Oxide* 2013; **32**: 29-35 [PMID: 23583951 DOI: 10.1016/j.niox.2013.04.001]
12 **Greer EL**, Brunet A. FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* 2005; **24**: 7410-7425 [PMID: 16288288 DOI: 10.1038/sj.onc.1209086]
13 **Plas DR**, Thompson CB. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. *J Biol Chem* 2003; **278**: 12361-12366 [PMID: 12517744 DOI: 10.1074/jbc.M213069200]
14 **Brunet A**, Park J, Tran H, Hu LS, Hemmings BA, Greenberg ME. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol* 2001; **21**: 952-965 [PMID: 11154281 DOI: 10.1128/MCB.21.3.952-965.2001]
15 **Hu MC**, Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H, Kobayashi R, Hung MC. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* 2004; **117**: 225-237 [PMID: 15084260 DOI: 10.1016/S0092-8674(04)00302-2]
16 **Vogt PK**, Jiang H, Aoki M. Triple layer control: phosphorylation, acetylation and ubiquitination of FOXO proteins. *Cell Cycle* 2005; **4**: 908-913 [PMID: 15917664 DOI: 10.4161/cc.4.7.1796]
17 **Fu W**, Ma Q, Chen L, Li P, Zhang M, Ramamoorthy S, Nawaz Z, Shimojima T, Wang H, Yang Y, Shen Z, Zhang Y, Zhang X, Nicosia SV, Zhang Y, Pledger JW, Chen J, Bai W. MDM2 acts downstream of p53 as an E3 ligase to promote FOXO ubiquitination and degradation. *J Biol Chem* 2009; **284**: 13987-14000 [PMID: 19321440 DOI: 10.1074/jbc.M901758200]
18 **Sunters A**, Madureira PA, Pomeranz KM, Aubert M, Brosens JJ, Cook SJ, Burgering BM, Coombes RC, Lam EW. Paclitaxel-induced nuclear translocation of FOXO3a in breast cancer cells is mediated by c-Jun NH2-terminal kinase and Akt. *Cancer Res* 2006; **66**: 212-220 [PMID: 16397234 DOI: 10.1158/0008-5472.CAN-05-1997]
19 **Huang H**, Tindall DJ. Regulation of FOXO protein stability via ubiquitination and proteasome degradation. *Biochim Biophys Acta* 2011; **1813**: 1961-1964 [PMID: 21238503 DOI: 10.1016/j.bbamcr.2011.01.007]
20 **Chatterjee A**, Chatterjee U, Ghosh MK. Activation of protein kinase CK2 attenuates FOXO3a functioning in a PML-dependent manner: implications in human prostate cancer. *Cell Death Dis* 2013; **4**: e543 [PMID: 23492774 DOI: 10.1038/cddis.2013.63]
21 **Gao J**, Yang X, Yin P, Hu W, Liao H, Miao Z, Pan C, Li N. The involvement of FoxO in cell survival and chemosensitivity mediated by Mirk/Dyrk1B in ovarian cancer. *Int J Oncol* 2012; **40**: 1203-1209 [PMID: 22159921 DOI: 10.3892/ijo.2011.1293]
22 **Wang X**, Chen WR, Xing D. A pathway from JNK through decreased ERK and Akt activities for FOXO3a nuclear translocation in response to UV irradiation. *J Cell Physiol* 2012; **227**: 1168-1178 [PMID: 21604264 DOI: 10.1002/jcp.22839]
23 **Clavel S**, Siffroi-Fernandez S, Coldefy AS, Boulukos K, Pisani DF, Dérijard B. Regulation of the intracellular localization of Foxo3a by stress-activated protein kinase signaling pathways in skeletal muscle cells. *Mol Cell Biol* 2010; **30**: 470-480 [PMID: 19917721 DOI: 10.1128/MCB.00666-09]
24 **Nho RS**, Kahm J. beta1-Integrin-collagen interaction suppresses FoxO3a by the coordination of Akt and PP2A. *J Biol Chem* 2010; **285**: 14195-14209 [PMID: 20223831 DOI: 10.1074/jbc.M109.052845]
25 **Singh A**, Ye M, Bucur O, Zhu S, Tanya Santos M, Rabinovitz I, Wei W, Gao D, Hahn WC, Khosravi-Far R. Protein phosphatase 2A reactivates FOXO3a through a dynamic interplay with 14-3-3 and AKT. *Mol Biol Cell* 2010; **21**: 1140-1152 [PMID: 20110348 DOI: 10.1091/mbc.E09-09-0795]
26 **Chiacchiera F**, Simone C. The AMPK-FoxO3A axis as a target for cancer treatment. *Cell Cycle* 2010; **9**: 1091-1096 [PMID: 20190568 DOI: 10.4161/cc.9.6.11035]
27 **Milkiewicz M**, Roudier E, Doyle JL, Trifonova A, Birot O, Haas TL. Identification of a mechanism underlying regulation of the anti-angiogenic forkhead transcription factor FoxO1 in cultured endothelial cells and ischemic muscle. *Am J Pathol* 2011; **178**: 935-944 [PMID: 21281824 DOI: 10.1016/j.ajpath.2010.10.042]
28 **Brenkman AB**, de Keizer PL, van den Broek NJ, Jochemsen AG, Burgering BM. Mdm2 induces mono-ubiquitination of FOXO4. *PLoS One* 2008; **3**: e2819 [PMID: 18665269 DOI: 10.1371/journal.pone.0002819]
29 **Wang F**, Marshall CB, Li GY, Yamamoto K, Mak TW, Ikura M. Synergistic interplay between promoter recognition and CBP/p300 coactivator recruitment by FOXO3a. *ACS Chem Biol* 2009; **4**: 1017-1027 [PMID: 19821614 DOI: 10.1021/cb900190u]
30 **Zeng Z**, Samudio IJ, Zhang W, Estrov Z, Pelicano H, Harris D, Frolova O, Hail N, Chen W, Kornblau SM, Huang P, Lu Y, Mills GB, Andreeff M, Konopleva M. Simultaneous inhibition of PDK1/AKT and Fms-like tyrosine kinase 3 signaling by a small-molecule KP372-1 induces mitochondrial dysfunction and apoptosis in acute myelogenous leukemia. *Cancer Res* 2006; **66**: 3737-3746 [PMID: 16585200 DOI: 10.1158/0008-5472.CAN-05-1278]
31 **Wang F**, Marshall CB, Yamamoto K, Li GY, Gasmi-Seabrook GM, Okada H, Mak TW, Ikura M. Structures of KIX do-

main of CBP in complex with two FOXO3a transactivation domains reveal promiscuity and plasticity in coactivator recruitment. *Proc Natl Acad Sci USA* 2012; **109**: 6078-6083 [PMID: 22474372 DOI: 10.1073/pnas.1119073109]
32 **Emerling BM**, Weinberg F, Liu JL, Mak TW, Chandel NS. PTEN regulates p300-dependent hypoxia-inducible factor 1 transcriptional activity through Forkhead transcription factor 3a (FOXO3a). *Proc Natl Acad Sci USA* 2008; **105**: 2622-2627 [PMID: 18268343 DOI: 10.1073/pnas.0706790105]
33 **Senf SM**, Sandesara PB, Reed SA, Judge AR. p300 Acetyltransferase activity differentially regulates the localization and activity of the FOXO homologues in skeletal muscle. *Am J Physiol Cell Physiol* 2011; **300**: C1490-C1501 [PMID: 21389279 DOI: 10.1152/ajpcell.00255.2010]
34 **Hori YS**, Kuno A, Hosoda R, Horio Y. Regulation of FOXOs and p53 by SIRT1 modulators under oxidative stress. *PLoS One* 2013; **8**: e73875 [PMID: 24040102 DOI: 10.1371/journal.pone.0073875]
35 **Kim MJ**, Ahn K, Park SH, Kang HJ, Jang BG, Oh SJ, Oh SM, Jeong YJ, Heo JI, Suh JG, Lim SS, Ko YJ, Huh SO, Kim SC, Park JB, Kim J, Kim JI, Jo SA, Lee JY. SIRT1 regulates tyrosine hydroxylase expression and differentiation of neuroblastoma cells via FOXO3a. *FEBS Lett* 2009; **583**: 1183-1188 [PMID: 19285077 DOI: 10.1016/j.febslet.2009.03.007]
36 **Ni HM**, Du K, You M, Ding WX. Critical role of FoxO3a in alcohol-induced autophagy and hepatotoxicity. *Am J Pathol* 2013; **183**: 1815-1825 [PMID: 24095927 DOI: 10.1016/j.ajpath.2013.08.011]
37 **Lai L**, Yan L, Gao S, Hu CL, Ge H, Davidow A, Park M, Bravo C, Iwatsubo K, Ishikawa Y, Auwerx J, Sinclair DA, Vatner SF, Vatner DE. Type 5 adenylyl cyclase increases oxidative stress by transcriptional regulation of manganese superoxide dismutase via the SIRT1/FoxO3a pathway. *Circulation* 2013; **127**: 1692-1701 [PMID: 23536361 DOI: 10.1161/CIRCULATIONAHA.112.001212]
38 **Brunet A**, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 2004; **303**: 2011-2015 [PMID: 14976264 DOI: 10.1126/science.1094637]
39 **Babar IA**, Czochor J, Steinmetz A, Weidhaas JB, Glazer PM, Slack FJ. Inhibition of hypoxia-induced miR-155 radiosensitizes hypoxic lung cancer cells. *Cancer Biol Ther* 2011; **12**: 908-914 [PMID: 22027557 DOI: 10.4161/cbt.12.10.17681]
40 **Lin H**, Dai T, Xiong H, Zhao X, Chen X, Yu C, Li J, Wang X, Song L. Unregulated miR-96 induces cell proliferation in human breast cancer by downregulating transcriptional factor FOXO3a. *PLoS One* 2010; **5**: e15797 [PMID: 21203424 DOI: 10.1371/journal.pone.0015797]
41 **Ling N**, Gu J, Lei Z, Li M, Zhao J, Zhang HT, Li X. microRNA-155 regulates cell proliferation and invasion by targeting FOXO3a in glioma. *Oncol Rep* 2013; **30**: 2111-2118 [PMID: 23970205]
42 **Wang K**, Li PF. Foxo3a regulates apoptosis by negatively targeting miR-21. *J Biol Chem* 2010; **285**: 16958-16966 [PMID: 20371612 DOI: 10.1074/jbc.M109.093005]
43 **Cai J**, Fang L, Huang Y, Li R, Yuan J, Yang Y, Zhu X, Chen B, Wu J, Li M. miR-205 targets PTEN and PHLPP2 to augment AKT signaling and drive malignant phenotypes in non-small cell lung cancer. *Cancer Res* 2013; **73**: 5402-5415 [PMID: 23856247 DOI: 10.1158/0008-5472.CAN-13-0297]
44 **Nowak K**, Killmer K, Gessner C, Lutz W. E2F-1 regulates expression of FOXO1 and FOXO3a. *Biochim Biophys Acta* 2007; **1769**: 244-252 [PMID: 17482685 DOI: 10.1016/j.bbaexp.2007.04.001]
45 **Furuyama T**, Yamashita H, Kitayama K, Higami Y, Shimokawa I, Mori N. Effects of aging and caloric restriction on the gene expression of Foxo1, 3, and 4 (FKHR, FKHRL1, and AFX) in the rat skeletal muscles. *Microsc Res Tech* 2002; **59**: 331-334 [PMID: 12424797 DOI: 10.1002/jemt.10213]
46 **Rathbone CR**, Booth FW, Lees SJ. FoxO3a preferentially induces p27Kip1 expression while impairing muscle precursor cell-cycle progression. *Muscle Nerve* 2008; **37**: 84-89 [PMID: 17894357 DOI: 10.1002/mus.20897]
47 **Roy SK**, Chen Q, Fu J, Shankar S, Srivastava RK. Resveratrol inhibits growth of orthotopic pancreatic tumors through activation of FOXO transcription factors. *PLoS One* 2011; **6**: e25166 [PMID: 21980390 DOI: 10.1371/journal.pone.0025166]
48 **Gilley J**, Coffer PJ, Ham J. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J Cell Biol* 2003; **162**: 613-622 [PMID: 12913110 DOI: 10.1083/jcb.200303026]
49 **Wilson MK**, McWhirter SM, Amin RH, Huang D, Schlissel MS. Abelson virus transformation prevents TRAIL expression by inhibiting FoxO3a and NF-kappaB. *Mol Cells* 2010; **29**: 333-341 [PMID: 20213318 DOI: 10.1007/s10059-010-0029-8]
50 **Ghaffari S**, Jagani Z, Kitidis C, Lodish HF, Khosravi-Far R. Cytokines and BCR-ABL mediate suppression of TRAIL-induced apoptosis through inhibition of forkhead FOXO3a transcription factor. *Proc Natl Acad Sci USA* 2003; **100**: 6523-6528 [PMID: 12750477 DOI: 10.1073/pnas.0731871100]
51 **van den Heuvel AP**, Schulze A, Burgering BM. Direct control of caveolin-1 expression by FOXO transcription factors. *Biochem J* 2005; **385**: 795-802 [PMID: 15458387 DOI: 10.1042/BJ20041449]
52 **Kops GJ**, Medema RH, Glassford J, Essers MA, Dijkers PF, Coffer PJ, Lam EW, Burgering BM. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol Cell Biol* 2002; **22**: 2025-2036 [PMID: 11884591 DOI: 10.1128/MCB.22.7.2025-2036.2002]
53 **Hauck L**, Harms C, Grothe D, An J, Gertz K, Kronenberg G, Dietz R, Endres M, von Harsdorf R. Critical role for FoxO3a-dependent regulation of p21CIP1/WAF1 in response to statin signaling in cardiac myocytes. *Circ Res* 2007; **100**: 50-60 [PMID: 17158337 DOI: 10.1161/01.RES.0000254704.92532.b9]
54 **Miyauchi H**, Minamino T, Tateno K, Kunieda T, Toko H, Komuro I. Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. *EMBO J* 2004; **23**: 212-220 [PMID: 14713953 DOI: 10.1038/sj.emboj.7600045]
55 **Chandramohan V**, Jeay S, Pianetti S, Sonenshein GE. Reciprocal control of Forkhead box O 3a and c-Myc via the phosphatidylinositol 3-kinase pathway coordinately regulates p27Kip1 levels. *J Immunol* 2004; **172**: 5522-5527 [PMID: 15100294]
56 **Lin L**, Hron JD, Peng SL. Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity* 2004; **21**: 203-213 [PMID: 15308101 DOI: 10.1016/j.immuni.2004.06.016]
57 **Hwang JW**, Rajendrasozhan S, Yao H, Chung S, Sundar IK, Huyck HL, Pryhuber GS, Kinnula VL, Rahman I. FOXO3 deficiency leads to increased susceptibility to cigarette smoke-induced inflammation, airspace enlargement, and chronic obstructive pulmonary disease. *J Immunol* 2011; **187**: 987-998 [PMID: 21690325 DOI: 10.4049/jimmunol.1001861]
58 **You H**, Pellegrini M, Tsuchihara K, Yamamoto K, Hacker G, Erlacher M, Villunger A, Mak TW. FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. *J Exp Med* 2006; **203**: 1657-1663 [PMID: 16801400 DOI: 10.1084/jem.20060353]
59 **Obexer P**, Geiger K, Ambros PF, Meister B, Ausserlechner MJ. FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells. *Cell Death Differ* 2007; **14**: 534-547 [PMID: 16888645 DOI: 10.1038/sj.cdd.4402017]
60 **Sengupta A**, Molkentin JD, Paik JH, DePinho RA, Yutzey KE. FoxO transcription factors promote cardiomyocyte

survival upon induction of oxidative stress. *J Biol Chem* 2011; **286**: 7468-7478 [PMID: 21159781 DOI: 10.1074/jbc.M110.179242]

61 **Liu JW**, Chandra D, Rudd MD, Butler AP, Pallotta V, Brown D, Coffer PJ, Tang DG. Induction of prosurvival molecules by apoptotic stimuli: involvement of FOXO3a and ROS. *Oncogene* 2005; **24**: 2020-2031 [PMID: 15674333 DOI: 10.1038/sj.onc.1208385]

62 **Murphy CT**, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, Ahringer J, Li H, Kenyon C. Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. *Nature* 2003; **424**: 277-283 [PMID: 12845331 DOI: 10.1038/nature01789]

63 **McCormick M**, Chen K, Ramaswamy P, Kenyon C. New genes that extend Caenorhabditis elegans' lifespan in response to reproductive signals. *Aging Cell* 2012; **11**: 192-202 [PMID: 22081913 DOI: 10.1111/j.1474-9726.2011.00768.x]

64 **Sengupta A**, Molkentin JD, Yutzey KE. FoxO transcription factors promote autophagy in cardiomyocytes. *J Biol Chem* 2009; **284**: 28319-28331 [PMID: 19696026 DOI: 10.1074/jbc.M109.024406]

65 **Sundaresan NR**, Gupta M, Kim G, Rajamohan SB, Isbatan A, Gupta MP. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest* 2009; **119**: 2758-2771 [PMID: 19652361]

66 **Huang W**, Li G, Qiu J, Gonzalez P, Challa P. Protective effects of resveratrol in experimental retinal detachment. *PLoS One* 2013; **8**: e75735 [PMID: 24040416 DOI: 10.1371/journal.pone.0075735]

67 **Li M**, Chiu JF, Gagne J, Fukagawa NK. Age-related differences in insulin-like growth factor-1 receptor signaling regulates Akt/FOXO3a and ERK/Fos pathways in vascular smooth muscle cells. *J Cell Physiol* 2008; **217**: 377-387 [PMID: 18615585 DOI: 10.1002/jcp.21507]

68 **Willcox BJ**, Donlon TA, He Q, Chen R, Grove JS, Yano K, Masaki KH, Willcox DC, Rodriguez B, Curb JD. FOXO3A genotype is strongly associated with human longevity. *Proc Natl Acad Sci USA* 2008; **105**: 13987-13992 [PMID: 18765803 DOI: 10.1073/pnas.0801030105]

69 **Flachsbart F**, Caliebe A, Kleindorp R, Blanché H, von Eller-Eberstein H, Nikolaus S, Schreiber S, Nebel A. Association of FOXO3A variation with human longevity confirmed in German centenarians. *Proc Natl Acad Sci USA* 2009; **106**: 2700-2705 [PMID: 19196970 DOI: 10.1073/pnas.0809594106]

70 **Morris BJ**. A forkhead in the road to longevity: the molecular basis of lifespan becomes clearer. *J Hypertens* 2005; **23**: 1285-1309 [PMID: 15942449 DOI: 10.1097/01.hjh.0000173509.45363.dd]

71 **Eelen G**, Verlinden L, Meyer MB, Gijsbers R, Pike JW, Bouillon R, Verstuyf A. 1,25-Dihydroxyvitamin D3 and the aging-related forkhead box O and sestrin proteins in osteoblasts. *J Steroid Biochem Mol Biol* 2013; **136**: 112-119 [PMID: 22989484 DOI: 10.1016/j.jsbmb.2012.09.011]

72 **Kops GJ**, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* 2002; **419**: 316-321 [PMID: 12239572 DOI: 10.1038/nature01036]

73 **Giordano S**, Darley-Usmar V, Zhang J. Autophagy as an essential cellular antioxidant pathway in neurodegenerative disease. *Redox Biol* 2014; **2**: 82-90 [PMID: 24494187 DOI: 10.1016/j.redox.2013.12.013]

74 **El Assar M**, Angulo J, Rodríguez-Mañas L. Oxidative stress and vascular inflammation in aging. *Free Radic Biol Med* 2013; **65**: 380-401 [PMID: 23851032 DOI: 10.1016/j.freeradbiomed.2013.07.003]

75 **Liochev SI**. Reactive oxygen species and the free radical theory of aging. *Free Radic Biol Med* 2013; **60**: 1-4 [PMID: 23434764 DOI: 10.1016/j.freeradbiomed.2013.02.011]

76 **van der Horst A**, Burgering BM. Stressing the role of FoxO proteins in lifespan and disease. *Nat Rev Mol Cell Biol* 2007; **8**: 440-450 [PMID: 17522590 DOI: 10.1038/nrm2190]

77 **Chen IC**, Chiang WF, Chen PF, Chiang HC. STRESS-responsive deacetylase SIRT3 is up-regulated by areca nut extract-induced oxidative stress in human oral keratinocytes. *J Cell Biochem* 2014; **115**: 328-339 [PMID: 24339251 DOI: 10.1002/jcb.24667]

78 **Zhang S**, Zhao Y, Xu M, Yu L, Zhao Y, Chen J, Yuan Y, Zheng Q, Niu X. FoxO3a modulates hypoxia stress induced oxidative stress and apoptosis in cardiac microvascular endothelial cells. *PLoS One* 2013; **8**: e80342 [PMID: 24278276 DOI: 10.1371/journal.pone.0080342]

79 **Raju I**, Kannan K, Abraham EC. FoxO3a Serves as a Biomarker of Oxidative Stress in Human Lens Epithelial Cells under Conditions of Hyperglycemia. *PLoS One* 2013; **8**: e67126 [PMID: 23805295 DOI: 10.1371/journal.pone.0067126]

80 **Chen SJ**, Zhang W, Tong Q, Conrad K, Hirschler-Laszkiewicz I, Bayerl M, Kim JK, Cheung JY, Miller BA. Role of TRPM2 in cell proliferation and susceptibility to oxidative stress. *Am J Physiol Cell Physiol* 2013; **304**: C548-C560 [PMID: 23302782 DOI: 10.1152/ajpcell.00069.2012]

81 **Li Z**, Zhang H, Chen Y, Fan L, Fang J. Forkhead transcription factor FOXO3a protein activates nuclear factor κB through B-cell lymphoma/leukemia 10 (BCL10) protein and promotes tumor cell survival in serum deprivation. *J Biol Chem* 2012; **287**: 17737-17745 [PMID: 22474286 DOI: 10.1074/jbc.M111.291708]

82 **Tseng AH**, Shieh SS, Wang DL. SIRT3 deacetylates FOXO3 to protect mitochondria against oxidative damage. *Free Radic Biol Med* 2013; **63**: 222-234 [PMID: 23665396 DOI: 10.1016/j.freeradbiomed.2013.05.002]

83 **Yang JY**, Chang CJ, Xia W, Wang Y, Wong KK, Engelman JA, Du Y, Andreeff M, Hortobagyi GN, Hung MC. Activation of FOXO3a is sufficient to reverse mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor chemoresistance in human cancer. *Cancer Res* 2010; **70**: 4709-4718 [PMID: 20484037 DOI: 10.1158/0008-5472.CAN-09-4524]

84 **Chen J**, Gomes AR, Monteiro LJ, Wong SY, Wu LH, Ng TT, Karadedou CT, Millour J, Ip YC, Cheung YN, Sunters A, Chan KY, Lam EW, Khoo US. Constitutively nuclear FOXO3a localization predicts poor survival and promotes Akt phosphorylation in breast cancer. *PLoS One* 2010; **5**: e12293 [PMID: 20808831 DOI: 10.1371/journal.pone.0012293]

85 **Lam M**, Carmichael AR, Griffiths HR. An aqueous extract of Fagonia cretica induces DNA damage, cell cycle arrest and apoptosis in breast cancer cells via FOXO3a and p53 expression. *PLoS One* 2012; **7**: e40152 [PMID: 22761954 DOI: 10.1371/journal.pone.0040152]

86 **Shukla S**, Bhaskaran N, Maclennan GT, Gupta S. Deregulation of FoxO3a accelerates prostate cancer progression in TRAMP mice. *Prostate* 2013; **73**: 1507-1517 [PMID: 23765843 DOI: 10.1002/pros.22698]

87 **Shukla S**, Bhaskaran N, Babcook MA, Fu P, Maclennan GT, Gupta S. Apigenin inhibits prostate cancer progression in TRAMP mice via targeting PI3K/Akt/FoxO pathway. *Carcinogenesis* 2014; **35**: 452-460 [PMID: 24067903 DOI: 10.1093/carcin/bgt316]

88 **Chen Q**, Ganapathy S, Singh KP, Shankar S, Srivastava RK. Resveratrol induces growth arrest and apoptosis through activation of FOXO transcription factors in prostate cancer cells. *PLoS One* 2010; **5**: e15288 [PMID: 21179458 DOI: 10.1371/journal.pone.0015288]

89 **Sunayama J**, Sato A, Matsuda K, Tachibana K, Watanabe E, Seino S, Suzuki K, Narita Y, Shibui S, Sakurada K, Kayama T, Tomiyama A, Kitanaka C. FoxO3a functions as a key integrator of cellular signals that control glioblastoma stem-like cell differentiation and tumorigenicity. *Stem Cells* 2011; **29**:

1327-1337 [PMID: 21793107]
90 **Ruvolo PP**. The Herculean task of killing cancer cells: suppression of FOXO3A in acute leukemia involves a hydra of multiple survival kinases. *Cell Cycle* 2012; **11**: 2589 [PMID: 22751430 DOI: 10.4161/cc.21233]
91 **Wang W**, Li NN, Du Y, Lv FF, Lin GQ. FoxO3a and nilotinib-induced erythroid differentiation of CML-BC cells. *Leuk Res* 2013; **37**: 1309-1314 [PMID: 23915976 DOI: 10.1016/j.leukres.2013.07.001]
92 **Yang W**, Dolloff NG, El-Deiry WS. ERK and MDM2 prey on FOXO3a. *Nat Cell Biol* 2008; **10**: 125-126 [PMID: 18246039 DOI: 10.1038/ncb0208-125]
93 **Sunters A**, Fernández de Mattos S, Stahl M, Brosens JJ, Zoumpoulidou G, Saunders CA, Coffer PJ, Medema RH, Coombes RC, Lam EW. FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. *J Biol Chem* 2003; **278**: 49795-49805 [PMID: 14527951 DOI: 10.1074/jbc.M309523200]
94 **Hui RC**, Francis RE, Guest SK, Costa JR, Gomes AR, Myatt SS, Brosens JJ, Lam EW. Doxorubicin activates FOXO3a to induce the expression of multidrug resistance gene ABCB1 (MDR1) in K562 leukemic cells. *Mol Cancer Ther* 2008; **7**: 670-678 [PMID: 18347152 DOI: 10.1158/1535-7163.MCT-07-0397]
95 **Naka K**, Hoshii T, Muraguchi T, Tadokoro Y, Ooshio T, Kondo Y, Nakao S, Motoyama N, Hirao A. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature* 2010; **463**: 676-680 [PMID: 20130650 DOI: 10.1038/nature08734]
96 **Birkenkamp KU**, Essafi A, van der Vos KE, da Costa M, Hui RC, Holstege F, Koenderman L, Lam EW, Coffer PJ. FOXO3a induces differentiation of Bcr-Abl-transformed cells through transcriptional down-regulation of Id1. *J Biol Chem* 2007; **282**: 2211-2220 [PMID: 17132628 DOI: 10.1074/jbc.M606669200]
97 **Buontempo F**, Chiarini F, Bressanin D, Tabellini G, Melchionda F, Pession A, Fini M, Neri LM, McCubrey JA, Martelli AM. Activity of the selective IκB kinase inhibitor BMS-345541 against T-cell acute lymphoblastic leukemia: involvement of FOXO3a. *Cell Cycle* 2012; **11**: 2467-2475 [PMID: 22713244 DOI: 10.4161/cc.20859]
98 **Dey P**, Ström A, Gustafsson JÅ. Estrogen receptor β upregulates FOXO3a and causes induction of apoptosis through PUMA in prostate cancer. *Oncogene* 2014; **33**: 4213-4225 [PMID: 24077289 DOI: 10.1038/onc.2013.384]
99 **Jia Y**, Mo SJ, Feng QQ, Zhan ML, OuYang LS, Chen JC, Ma YX, Wu JJ, Lei WL. EPO-dependent activation of PI3K/Akt/FoxO3a signalling mediates neuroprotection in in vitro and in vivo models of Parkinson's disease. *J Mol Neurosci* 2014; **53**: 117-124 [PMID: 24390959 DOI: 10.1007/s12031-013-0208-0]
100 **Carbajo-Pescador S**, Mauriz JL, García-Palomo A, González-Gallego J. FoxO proteins: regulation and molecular targets in liver cancer. *Curr Med Chem* 2014; **21**: 1231-1246 [PMID: 24372208]
101 **Liang C**, Chen W, Zhi X, Ma T, Xia X, Liu H, Zhang Q, Hu Q, Zhang Y, Bai X, Liang T. Serotonin promotes the proliferation of serum-deprived hepatocellular carcinoma cells via upregulation of FOXO3a. *Mol Cancer* 2013; **12**: 14 [PMID: 23418729 DOI: 10.1186/1476-4598-12-14]
102 **Ghosh Choudhury G**, Lenin M, Calhaun C, Zhang JH, Aboud HE. PDGF inactivates forkhead family transcription factor by activation of Akt in glomerular mesangial cells. *Cell Signal* 2003; **15**: 161-170 [PMID: 12464387 DOI: 10.1016/S0898-6568(02)00057-8]

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REVIEW

Matrix metalloproteinases and gastrointestinal cancers: Impacts of dietary antioxidants

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Abstract

The process of carcinogenesis is tightly regulated by antioxidant enzymes and matrix degrading enzymes, namely, matrix metalloproteinases (MMPs). Degradation of extracellular matrix (ECM) proteins like collagen, proteoglycan, laminin, elastin and fibronectin is considered to be the prerequisite for tumor invasion and metastasis. MMPs can degrade essentially all of the ECM components and, most MMPs also substantially contribute to angiogenesis, differentiation, proliferation and apoptosis. Hence, MMPs are important regulators of tumor growth both at the primary site and in distant metastases; thus the enzymes are considered as important targets for cancer therapy. The implications of MMPs in cancers are no longer mysterious; however, the mechanism of action is yet to be explained. Herein, our major interest is to clarify how MMPs are tied up with gastrointestinal cancers. Gastrointestinal cancer is a variety of cancer types, including the cancers of gastrointestinal tract and organs, *i.e.*, esophagus, stomach, biliary system, pancreas, small intestine, large intestine, rectum and anus. The activity of MMPs is regulated by its endogenous inhibitor tissue inhibitor of metalloproteinase (TIMP) which bind MMPs with a 1:1 stoichiometry. In addition, RECK (reversion including cysteine-rich protein with kazal motifs) is a membrane bound glycoprotein that inhibits MMP-2, -9 and -14. Moreover, α2-macroglobulin mediates the uptake of several MMPs thereby inhibit their activity. Cancerous conditions increase intrinsic reactive oxygen species (ROS) through mitochondrial dysfunction leading to altered protease/anti-protease balance. ROS, an index of oxidative stress is also involved in tumorigenesis by activation of different MAP kinase pathways including MMP induction. Oxidative stress is involved in cancer by changing the activity and expression of regulatory proteins especially MMPs. Epidemiological studies have shown that high intake of fruits that rich in antioxidants is associated with a lower cancer incidence. Evidence indicates that some antioxidants inhibit the growth of malignant cells by inducing apoptosis and inhibiting the activity of MMPs. This review is discussed in six subchapters, as follows.

Key words: Gastrointestinal cancer; Matrix metalloproteinase; Tissue inhibitor of matrix metalloproteinases; Reactive oxygen species; Antioxidants

Core tip: Matrix metalloproteinases (MMPs), a group of zinc dependent endopeptidases, substantially contribute to extra cellular remodelling, angiogenesis, cellular differentiation, proliferation and apoptosis. MMPs are also important regulators of tumor growth both at the primary site and in distant metastasis; thus the enzymes are considered as important targets for cancer therapy. This review describes the roles and regulation of different MMPs and their subsequent actions over different gastrointestinal cancers both in epigenetic and cellular level. Furthermore, this review summarizes the current state of knowledge of dietary antioxidants in preventing gastrointestinal cancer progression as well as mechanism of action.

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EPIDEMIOLOGY AND GENETIC BASIS OF GASTRIC CANCERS

As far the incidence rate is concerned, gastric cancer holds the fourth position among the most common cancers in men and fifth in women, from a worldwide perspective. The death rate goes hand to hand with lung cancer, the most frequent cancer globally[1]. Approximately, one million cases of gastric cancer were reported in the year 2008, which accounted for almost 8% of all cancerous incidents throughout the world[1]. Regions of Asia, eastern Europe, South America were highlighted in the reports as the most affected continents[2]. Carcinogenesis in the gastrointestinal tract, accounts for marked geographic variations in incidence and shows morphological heterogeneity. Histologically, gastric cancers are mainly of two types, diffuse and localized intestinal types. Poorly differentiated cancer cells, scattered within the stromal cells diagnosed as diffuse-type gastric cancer (DGC), whereas tubular gland like structures formed by the cancer cells having a few stromal components give rise to intestinal-type gastric cancer (IGC). Recognized as a familial disease many years ago, hereditary diffuse gastric cancer has now been identified as an autosomal dominant cancer susceptibility syndrome. This familial disease was probably most elegantly demonstrated in the family of Napoleon Bonaparte[3,4].

Chromosomal anomalies leading to gastrointestinal cancers

Aneuploidy of chromosomes 4, 8, 17 and 20 in gastric cancer were reported in several studies. Researchers have been trying to identify the precise stages known from Correa's pathway, where these chromosomal anomalies arise. Chromosome 4 and 20 were found to be amplified with the deletion of chromosome 17(p53) in multiple known progressive stages of carcinogenesis including normal gastric mucosa as well as metaplasia, dysplasia and cancer. A significant increase in the levels of aneuploidy was also reported with disease severity. Moreover, in some cases significant positive association was observed between chromosome 4 amplification and infection with *Helicobacter pylori* (*H. pylori*). In the same study, a similar kind of aneuploid condition was induced *in vitro* by exposing a human cell line to hydrogen peroxide suggesting that *H. pylori* induces gastric cancer with the help of reactive oxygen species (ROS) mediated chromosomal aneuploidy[5].

In 1988, Correa proposed the stages of human gastric cancer progression using several stages including gastritis, metaplasia, dysplasia, carcinoma, *etc.* In a study by Sugai *et al*[6] chromosomal allelic losses were tested of multiple cancer related chromosomal loci (1p, 3p, 4p, 5q, 8p, 9p, 13p, 17p, 18q and 22q). In addition, microsatellite instability (MSI) and overexpression of p53 protein were checked in all tumor samples. A prominent 3p allelic loss was observed in the cases of gastric phenotypes, whereas 5q allelic loss was highly associated to the intestinal phenotypes. Both loss of heterozygosity and microsatellite instability were observed in the genetic profiles of the mixed phenotypes. Allelic losses of 5q, 3p and 18q loci were consistent in intra-mucosal carcinomas and allelic losses of 17p, 1p and 9p were associated to submucosal carcinomas, all leading to loss of heterozygosity. MSI was observed only in 6 out of 31 cases of mixed phenotype gastric cancers, while p53 overexpression is observed in most of the cases of differentiated gastric carcinomas[6].

Specific gene mutations and their contributions

In most cases, the molecular expression of several biological markers show no link between the young ($\leqslant$ 45 years) and the aged ($\geqslant$ 45 years) patients, suggesting that early onset of gastric cancers possess different expression patterns of several important biomolecules[7]. Early onset gastric cancer patients may be more susceptible to the genetic factors but these individuals account for 10% or even less of all gastric cancer patients throughout the world[8,9]. Only10% of early onset gastric cancer cases belong to the inherited gastric cancer predisposition syndromes, but the genetic events taking place in the background of these remain largely unclear till date.

Development of tumors, often result from defects in several signaling pathways, including tyrosine phosphorylation, which occurs through the combined actions of protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs). About 26% of colorectal cancers and a minute fraction of gastric cancers were reported to have mutations in the *PTP* genes. Mutated PTP originally are tumor suppressor genes, which regulate pathways associated with cell growth and differentiation. Wang *et al*[10] uncovered 83 such somatic mutations by mutational analysis of the six tyrosine phosphatase gene superfamily namely (PTPRF, PTPRG, PTPRT, PTPN3, PTPN13, and PTPN14). Production of truncated proteins lacking phosphatase activity was due to 15 mutations, which were nonsense, frame shift or splice site mutations. Reduced phosphatase activity was resulted in 5 missense mutations in PTPRT. Restoration of wild-type PTPRT expression in human cancer cells tended to cease cell growth[10].

Several studies reported the involvement of different biomolecules for the cause of gastric cancer. E-cadherin, p53, cycloxygenase-2 (COX-2), trefoil factor-1 (TFF-1), β-catenin, p16, c-myc, *etc.*, are some of the known molecules. Significant difference in the expression of these markers and some other molecules are found, namely c-jun, HuR, C/EBP-β, *etc.*, in early onset of gastric cancer, as well as regular gastric cancer patients. TFF-1 was overexpressed with a comparatively lower level of COX-2 in the early onset gastric cancer, whereas COX-2 overex-

pression and loss of TFF1 was found in regular cancers. Surprisingly, overexpression of COX-2, C/EBP-β in intestinal type gastric cancer was observed[7].

Risk factors for familial and non-familial gastrointestinal cancers

Gastrointestinal carcinomas like, esophageal adenocarcinoma, gastroesophageal junctional adenocarcinoma, *etc.*, often originate from Barrett's esophagus (BE), a chronic gastroesophageal reflux disease[11,12]. When BE and its associated diseases occur in families, they are collectively included within a syndrome named Familial Barrett's Esophagus, categorized as a complex genetic disease[13,14].

The onset of adenocarcinomas is thought to be determined by a combined effect of genetic variation and distinct environmental factors. Chak *et al*[15] determined the relationship between risk factors and the age of onset of these cancers. Family history of BE/cancer occurrence, gastroesophageal reflux symptoms, obesity (defined as body mass index > 30) and other risk factors were assessed in a total of 356 gastroesophageal adenocarcinoma patients. This study reports that both familial and non-familial cancers arise at similar ages, but obesity is associated with a comparatively earlier age of onset.

Appropriate clinical counseling based on the genetics of gastrointestinal cancers always depends on well substantiated data reflecting the risk factors existing through a family. The estimated risk of gastric cancer within a family, however, may differ widely from one another. A group of researchers from Sweden used the updated Swedish Family-Cancer Database to investigate the familial risks of gastric cancer in 5358 patients among the offspring and 36486 patients among the parents. In this investigation, 133 families were identified having one parent and one offspring recognized as patients of gastric cancer, whereas 20 families had two affected offspring. The standardized incidence ratio (SIR) for the families was 1.63, when the parents displayed gastric cancer and the same was 2.93 in the families where the siblings had the disease. Cancer in the corpus (main body of the stomach) was related to high sibling risk (SIR = 7.28). Whenever gastric cancer was diagnosed in the parents, the SIR for cancer in the cardia (the area joining stomach and esophagus) was 1.54. In most cases, upper stomach cancer did show a particular association to esophageal adenocarcinoma. Histological analysis revealed an increase of signet ring cells in cancers. Among the factors, giving rise to high sibling risk in the case of corpus cancer, *H. pylori* infection may be an important one. The association of upper stomach cancer and esophageal adenocarcinoma in families may also lead to important clues on the aetiology of both diseases[16].

Chromatin remodeling and epigenetic modifications as etiological factors

Various carcinogenic pathways and environmental factors may contribute to the aetiologies of gastric cancers[17]. Several genes as well as some of their mutations were identified in a study by exome sequencing of 22 gastric cancer samples. In this way, genes participating in chromatin remodeling were most commonly found to be mutated, leading to alterations in specific pathway. Protein deficiency of AT-rich interactive domain-containing protein 1A (ARID1A) were observed in 83% of gastric cancers with MSI, 73% of those with Epstein-Barr virus (EBV) infection and 11% of those that were not infected with EBV and microsatellite stable. A small division of the disease may arise due to *TP53* (gene encoding p53) mutations, as well as other genetic alterations and modified pathways. Occurrence of these mutations shows a negative correlation with mutations in *TP53*. The significance of chromatin remodeling is highlighted in the context of gastric cancers, which also reveal some new genomic landscapes[18].

Overexpression of claudin-4 (CLDN4), a protein involved in tight junctions is known to be associated to gastric cancers. Increased expression of CLDN4 on the membrane enhances the barrier like function of tight junctions which tends to prevent the migration and invasion of gastric cancer cells, without affecting cell growth. The epigenetic regulation of CLDN4 overexpression and its clinical significance as potential therapeutic targets was reported by Kwon *et al*[19]. DNA hypomethylation parallels to CLDN4 upregulation in both cancerous and non-cancerous gastric tissues. In normal gastric tissues, bivalent histone modifications often lead to repression of CLDN4 expression, whereas loss of repressive histone methylations results in upregulation of CLDN4 in gastric cancer cells[19].

Methylation level of long interspersed element-1 (LINE-1) is associated with esophagus gastric as well as colon cancer progression and prognosis[20]; this helps in assessing tumor heterogeneity and drug efficacy for the personalized treatment of patients with gastrointestinal cancers. Okada *et al*[21] documented that promoter methylation rate of seven genes *TP73*, *BLU*, *FSD1*, *BCL7A*, *MARK1*, *SCRN1*, and *NKX3.1* are higher in EBV-associated gastric carcinomas compare to EBV-negative gastric carcinoma signify the viral-mediated epigenetic alteration in cancer. Report suggested that *H. pyroli* infection induced promoter methylations of THBS1 and GATA-4 gene in the early stages of chronic gastritis and gastric cancer development[22]. Jin *et al*[23,24] reported the enhanced rate of promoter methylation of a transmembrane glycoprotein endoglin and Ras-related associated with diabetes gene in human ESCC. Also, TIMP-3 hypermethylation contributes to the downregulation TIMP-3 protein expression in ESCC and is associated with poor patient survival[25]. Poplineau *et al*[26] reported that a DNA hypomethylation agent enhanced upregulation of MMP-1 gene expression and triggered tumor cell invasion. In contrast, treatment with S-adenosylmethionine, a methyl donor, resulted in activation of TIMP-2 and significant downregulation of MMP-2 and MT1-MMP gene in colorectal cancer[27]. Prognostic values of promoter hypermethylation in patients with gastric cancer

Table 1 Major matrix metalloproteinases studied in cancer biology

Collagenase	Gelatinase	Stromolysin	Matrilysin	Membrane type MMPs	Others
MMP-1	MMP-2	MMP-3	MMP-7	MMP-14	MMP-12
MMP-8	MMP-9	MMP-10	MMP-26	MMP-15	MMP-20
MMP-13		MMP-11		MMP-16	MMP-28
MMP-18		MMP-19		MMP-17	
				MMP-24	
				MMP-25	

MMP: Matrix metalloproteinases.

documented that patients with higher stage of colorectal cancer possess a higher concentration of methylated APC, TIMP-3 and hMLH1 in the serum[28]. Wang *et al*[29] reported a frequent hypermethylation of RASSF1A gene promoter in gastric and colon cancer and predicted its utility as a diagnostic marker.

ELEVATED INDUCTION OF MMPS IN GASTROINTESTINAL CANCERS

The MMPs are comprised of a family of endopeptidases, which can cleave almost every component of the extra cellular matrix (ECM) proteins. It is documented that many non-ECM proteins can also be cleaved by selected MMPs. Structurally, they all have a zinc ion in the catalytic domain and their activity is dependent on divalent ions, mainly, Zn^{2+} and Ca^{2+}[30,31]. There are about 27 different MMPs discovered so far and they are subdivided in groups according to substrate specificity and structural integrity (Table 1). Induction and expression of MMPs are regulated at the level of transcription and translation, respectively. Further complexity of MMPs is the activation from zymogen to active enzyme and, secondly, the mRNA stability of few MMPs play critical role. ProMMPs are converted to active MMPs by intra-molecular cleavage of cysteine bridge between thiol group at the prodomain and Zn^{2+} near the catalytic site. The overall activity depends on the availability of the substrate as well as inhibitor in pericellular space, though a high concentration of MMPs exists near the plasma membrane.

Cancer progression can be explained in six major steps: self-support in growth signals; resistance to growth-inhibitory signals; reduced apoptosis; uncontrolled replication; sustained angiogenesis; and tissue invasion followed by metastasis[32]. Considerable evidence has demonstrated that disease progression in experimental animal models of cancer invasion and metastasis correlate with enhanced secretion of specific MMPs by tumor cells and/or stromal cells. Gastrointestinal cancer can be subdivided into different types, *e.g.*, cancers of upper digestive tract, esophageal cancer, gastric cancer, pancreatic cancer, liver cancer, gallbladder cancer and others like MALT lymphoma, gastrointestinal stromal tumors, cancer of the biliary tree, cancer of the lower digestive tract, colorectal and gastrointestinal carcinoid tumor.

Role of MMPs in esophageal and gastric cancer

Role of MMPs in gastrointestinal cancer has been well studied. IHC analysis of tumor biopsy samples suggest the expression of MMP-1 in 24% of oesophageal cancers, while MMP-2 and MMP-9 in 78% and 70% of samples respectively[33]. Similarly, studies revealed that MMP-13 is localized predominantly in tumor cells; and the presence of MMP-13 together with MT1-MMP is implicated in determining tumor aggressiveness of human oesophageal carcinomas. Etoh *et al*[34] found a significant correlation in survival period for subjects with the expression of MMP-13 and MT1-MMP in tumor tissue. Moreover, the activities of MMP-2, -3, -9, and -10 enzymes were detected in each of the 24 cancer cases. MMP overexpression was reported in tumors in comparison to normal tissue; having elevated levels of the activated form of MMP-3 and -10 in tumors. In addition, MMP-3 and -10 mRNA levels were significantly higher in tumors than paired normal tissues in both the stromal and epithelial component of tissues[35].

One of the important features of the malignant phenotype in both colorectal and gastric cancer is the overexpression of MMP-2 and -9 as well as activation of proMMP-2 to active MMP-2. Expression of MMP-2, -1 and -9 was found in 94%, 73% and 70% respectively in gastric specimens when studied in 74 patients. Conversely, MMP-3 was only present in 27% of tumors while MMP-1 and -9 were present mostly in all intestinal phenotypes of gastric cancer. In addition to MMPs, TIMP-1 and TIMP-2 were detected in approximately 50% of gastric tumors. Progression of gastric cancer is associated with MMP-13 expression along with its coexpression with MT1-MMP and/or MMP-2 that may have a synergistic effect in the progression of the disease[36]. The expression of TIMP-3 was significantly higher than that of MMP-3, and MMP-3/TIMP-3 was lower in gastric cancer tissue at the early stages (n = 18) than in that of the advanced stage group (n = 26) ($P < 0.05$)[37]. MMP-7 expression has been found to be prognostic marker for metastasis of gastric carcinoma because MMP-7 mRNA as well as protein was pronounced in aggressiveness carcinoma tissues.

Role of specific MMPs in colorectal cancer

The critical event in the process of cancer invasion and metastasis is the degradation of the ECM surrounding

the tumor tissue[38]. This ECM is degraded by the action of a set of proteases, in which several types of MMPs play major role, of which MMP-2 and -9 are most prominent. The basement membrane which prevents an invading epithelial tumor is mainly made up of type IV collagen, which is substrate of MMP-2 and -9. The event of basement membrane degradation promotes epithelial tumor invasion. Higher levels of MMP-1, -2, -3, -7, -9, -13, and MT1-MMP expression have been documented in human colorectal. Murray *et al*[33] demonstrated that MMP-1 in colorectal cancer specimens was linked to a poor prognosis of the disease. This study was later confirmed by performing IHC, FISH, and RT-PCR on 142 samples of colorectal carcinomas[39]. The latent form of MMP-2, *i.e.*, proMMP-2, is expressed in significant levels almost in all normal tissues. *MMP-2* acts as the 'house-keeping' gene due to its importance in normal cellular physiology. While active *MMP-2* is found in neoplastic tissues, it is lacking in most normal tissues. Parsons *et al*[40] in 1992, were the first who described the role of MMP-2 in colorectal cancer and the ratio of MMP-2 to proMMP-2 was 20 fold higher in colorectal cancer specimens in comparison to non-malignant biopsies as judged by gelatin zymography. Parsons *et al*[40] demonstrated increased expression of proMMP-9 in colorectal cancer. The increased activity of proMMP-9 from inflammatory cells may cause an early change in progression from adenoma to carcinoma, when colonic adenoma is compared to normal mucosa. Increased co-expression of MMP-3 and MMP-9 has been found in colorectal tumors. Co-expression of uPA with MMP-9 in colorectal cancers is responsible for the activation of plasminogen to plasmin[41]. Plasmin stimulates proMMP-3 to active MMP-3 which in turn promotes proMMP-9 to active MMP-9, thus, resulting in colorectal cancer progression[41]. Excess MMP-9 expression in colorectal cancer contributes to the inflammation related to neoplasms but not to aggressive tumors[42]. Low levels of microsatellite instability and poor prognosis is observed with increased expression of MMP-3 in colorectal cancer. Moreover 90% of colonic adenocarcinomas demonstrated high levels of MMP-7 expression. Studies on surgically resected colorectal cancer specimens elucidated the clinical importance of MMP-7 expression in this cancer type. It demonstrated that overexpression of MMP-7 in colorectal cancer (measured by IHC and in situ hybridization) directly relates to nodal or distant metastasis[43,44]. On the contrary, MMP-12 overexpression is associated with increased survival in colorectal cancer because of its influence as protective factor presumably by inhibiting tumor angiogenesis[43]. In fact, inhibition of tumor growth with upregulation of MMP-12, also known as macrophage metalloprotease, is well accepted. It was reported by Dong *et al*[45] that macrophages capable of producing MMP-12 in tumors that are responsible for increased production of angiostatin, an inhibitor of tumor neovascularization[45,46]. High expression of MMP-13 results in poor survival of colon cancer patients. Colorectal tumor biopsy specimens were examined for the identification of MMP-13 by Leeman *et al*[47]. MMP-13 was found in 91% of cases and was localized to cytosol of tumor tissues. Significantly higher activity of MMP-13 was observed in malignant than the non-malignant tissues. Moreover, plasmin, MT1-MMP and MMP-2 are key molecules in the production of active MMP-13. Active MMP-13 was found to be responsible for activation of MMP-9 during cancer progression[48].

MMPs polymorphism in tumor formation

Unlike classical oncogenes, MMPs are not upregulated by gene amplification or mutations. The increased MMP expression in tumours is mainly due to transcriptional changes rather than genetic alterations. The only two reported genetic alterations in cancer cells are translocation of the *MMP-23* gene in neuroblastoma[49] and amplification of the *MT5-MMP* gene[50]. Polymorphisms in MMP promoters also affect gene transcription and influence cancer susceptibility (Figure 1). The estimated number of single nucleotide polymorphisms (SNPs) in the human genome is 10 million, while only a small part of these polymorphisms are functionally relevant. The differences in allele transcription caused by polymorphisms in the MMP promoters are subtle compared with the overexpression that arises from the amplification of oncogenes. Most of the functional SNPs are located in the promoter region of the *MMP-1*,*-2*,*-3*,*-9* and *-7* genes that are associated with gastric cancer risk.

MMP1-1607 1G/2G polymorphism was found to be associated with gastric cancer risk as presence of extra guanine (2G) creates a binding site for Ets-1 transcription factor that enhances transcription of MMP-1. Bradbury *et al*[51] reported an elevated esophageal cancer risk in 1G/2G and 2G/2G carrier. MMP-1 protein expression is higher in tumors from gastric cancer patients who carry the 2G allele not 1G homozygotes[52]. Moreover, 2G homozygotes are more likely to develop invasive tumors. Dey *et al*[52] reported that MMP-1 promoter polymorphism is significantly associated with lower stomach tumor formation. MMP-1 -1607 1G/2G polymorphism is also involved with colon cancer risks.

MMP-2 polymorphism was investigated mainly in the promoter region, *e.g.*, MMP-2 -1306 C/T, -735 C/T, -790T/G, -955A/C, and -1575G/A in the context of gastrointestinal cancer risk[53]. Studies reported association of gastrointestinal cancer risk with -1306 C/T and -735 C/T polymorphic site worldwide. Price *et al*[54] characterized genetic variants in the human MMP-2 -1306 C/T allele-specific transcriptional regulation. The common C>T transition at -1306 disrupts a Sp1-type promoter site (CCACC box), leading to lower promoter activity with the T allele[54]. On the other hand, G to A substitution at the MMP-2 -1575 site reduces gene expression due to a reduction of estrogen receptor-α binding to A allele[55]. Fruh *et al*[56] found the presence of CC allele at MMP-2 -1306 position in *H. pylori* infected individuals which provide protection against esophagus adenocarcinoma. Studies also reported that presence of CC allele at

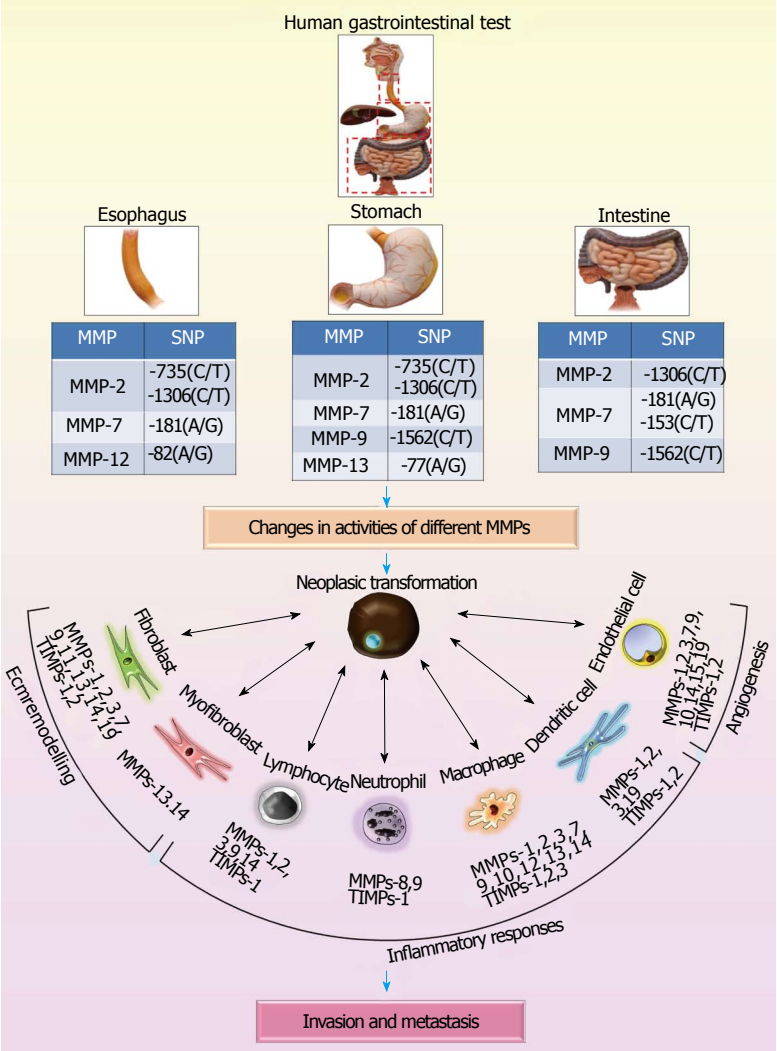

Figure 1 Matrix metalloproteinases polymorphism in gastrointestinal cancers. Single nucleotide polymorphism (SNP) for matrix metalloproteinases (MMP) genes in gastrointestinal organs (*e.g.*, esophagus, stomach and intestine) of human has been reported. These SNPs are involved in changing MMPs activities in neoplastic transformation of gastric tissues in cancer patients. In addition to cancerous cells, the secretion of MMPs by fibroblasts, myofibroblasts, lymphocytes, neutrophils, macrophages, dendritic cells and endothelial cells has been documented. Both MMPs and tissue inhibitor of metalloproteinases (TIMPs) are important in regulation of extracellular matrix (ECM) remodeling, inflammatory responses and angiogenesis for cancer invasion and metastasis.

-1306 site increases the risk of ESCC, although Chen *et al*[54,57] did not observe any positive association of MMP-2 -1306 C/T polymorphism with ESCC in Mongolian population suggests differences in genetic susceptibility between Han-ethnic Chinese and the Mongolian population. However, both positive and negative influences of MMP-2 -1306 C/T polymorphism with gastric cancer in Asian and Caucasian population were reported[58]. Studies were performed to evaluate the association of MMP-2 -1306 C/T polymorphism with colon cancer risk[59]. Langer *et al*[53,60] reported that presence of CC or CT genotype enhances the survival rate of colon cancer patients. There is also a significant association of MMP-2 -735 C/T polymorphism with esophageal cancer risks[54].

MMP-9 over expression is associated with almost all the hallmark steps of cancer progression that make MMP-9 an ideal candidate gene for genetic association studies. Functional polymorphisms, *e.g.*, MMP-9 promoter (-90CA(n), -1562C/T) as well as structural region (R279Q, P574R, R668Q) polymorphism were studied, assuming it might influence the *MMP-9* gene expression or protein activity. *MMP-9* polymorphism and gastrointestinal cancer risk is apparent in both Chinese and Caucasian populations[61,62]. In a hospital based case control study in Chinese population, *MMP-9* polymorphism in individual carrying RR genotype at P574R have increased risk of ESCC while R279Q and R668Q polymorphism has no association with cancer risk. In contrast Fang *et al*[63] reported that individuals having RR genotype at R279Q site have enhanced risk towards colon cancer. Tang *et al*[62] showed R279Q and P574R polymorphism were associated with lymph node metastasis of gastric cancer. Positive association of MMP-9 -1562 C/T polymorphism and colon cancer risk has been reported by Woo *et al*[61] in Korean population. Other studies reported a higher incident of lymph node metastasis in gastric and colon cancer patients having CC genotype at -1562 bp[64,65].

Association of SNPs in MMP-3 promoter and gastrointestinal cancer has been investigated in MMP-3 -1171 6A/5A site, since transcription repressor bind strongly with 6A allele leading to reduced gene expression. Bradbury *et al*[51] suggested a positive association of EA risk with 6A/5A or 5A/5A carrier. In addition, Zhang *et al*[64] found a higher ESCC risk among smokers having the 5A allele and reported that an elevated risk of lymph node metastasis in patients having 5A allele instead of the 6A allele. Interestingly, Dey *et al*[52] reported that the frequency of homozygotes for the 6A allele is lower in

gastric cancer patients than in controls of eastern Indian population[66]. On contrary, only one study performed in Japanese population that showed higher incidents of colon cancer in individuals having 6A/6A genotype.

Two common functional MMP-7 SNPs (-181A/G, -153C/T) are believed to control gene expression in several diseases, including gastrointestinal cancer[67-69]. MMP-7 up-regulation was significantly related to the promoter activity variation of the –181A/G alleles. Jormsjö *et al*[68] reported that the expression and promoter activity of the MMP7 -181G allele was higher in G over A and, attributed to the formation of a putative binding site (NGAAN) for a heat-shock transcription factor to G-allele. On the contrary, Richards *et al*[69] reported that elevated plasma MMP-7 level in AA genotype was governed by the G to A transition in -181 bp resulting in higher binding for the forkhead box A2 transcription factor to AA genotype. Studies reported a positive association of MMP-7 -181A/G polymorphism in esophagus, and gastric adenocarcinoma in Chinese population with an increased gastric cancer risk in G allele carrier[70]. However, in contrast, Kubbent *et al*[58] reported more AA and less AG in gastric cancer group. Moreover, MMP-7, -181 A/G and -153 C/T polymorphism is also significantly associated with colon cancer risk[71].

REGULATION OF MMPS IN GASTROINTESTINAL CANCER

An understanding of the MMP regulation in different cellular processes, *e.g.*, apoptosis, angiogenesis, invasion, metastasis as well as immune function is important for early prognosis and better therapeutics of gastrointestinal cancers. The regulation of MMPs goes awry at any or all cellular processes during cancer development[30]. The regulatory mechanisms shared among different cellular processes might control the invasive property of cells. The presence of MMP-1, -2, -3, -9, and MT1-MMP mRNA and protein in gastric and colorectal cancer tissues are evident from IHC and FISH assay. It is also known that MMPs are produced by inflammatory and fibroblast cells, in the vicinity of cancer cells. Among various signaling pathways, mitogen-activated protein kinases (MAPKs) pathways are important in the regulation of MMP induction as most of MMP promoters contain AP-1 and NFκB-binding sites, the downstream target of MAPK pathways. NFκB and AP-1 activity are significantly enhanced during cancer progression. JNK pathway induces MMP expression through activation as well as nuclear translocation of multiple transcription factors such as Jun D, ATF and most of the MMP promoter contain putative-binding sites for these DNA-binding proteins[72]. It is now conceivable that the function of MMPs is not only confined to invasion and metastasis steps of cancer but they also facilitate initial phases of cancer development. In cancer, special emphasis has been placed on the degradation of type IV collagen, a major protein component of basement membranes that can be cleaved by MMP-2 and -9. Disease progression in experimental animal models of cancer invasion and metastasis correlate with enhanced secretion of specific MMPs by tumor cells and/or stromal cells. Specific MMPs appear to have different functions depending on the stage of cancer and tissue type.

MMPs regulate apoptosis

MMPs especially, MMP-3, -7, -9 and -11 regulate apoptosis by degrading matrix protein. MMPs have both apoptotic and anti-apoptotic actions on endothelial and epithelial cells by cleaving adhesion molecule, *e.g.*, VE-cadherin[73], PECAM-1[74] and E-cadherin[75]. Detachment of adhesion molecules from the membrane is prerequisite for apoptosis to occur. Degradation of laminin by MMP-3 is another example of enhanced apoptosis in mammary epithelial cells possibly by degrading laminin[76]. MMP-7 releases the Fas ligand from the membrane which then induces apoptosis of neighboring cells, or decreases cancer-cell apoptosis, depending on the system[77]. MMPs might also negatively regulate cancer-cell growth, by means of activation of TGF-β or generation of pro-apoptotic molecules such as Fas ligand or TNF-α. By producing heparin-binding epidermal growth factor (HBEGF) from the latent form, *i.e.*, pro-HBEGF, MMP-7 promotes cell survival which is opposes the apoptosis *via* tyrosine kinase-mediated pathway. Moreover, MMP-11 also inhibits cancer cell apoptosis, as indicated by Wu *et al*[78], who showed that over expression of MMP-11 decreases spontaneous apoptosis in tumor xenografts. In contrast, MMP11-null mice show a higher rate of apoptosis compared to wild-type when challenged with cancer cells. MMP-11 inhibits apoptosis by the mechanism of releasing IGFs, known to can act as survival factors[79]. Although MMP-9 and -11 decrease cancer cell apoptosis, they increase apoptosis during development[78,80].

MMPs regulate angiogenesis

Angiogenesis, the formation of new capillaries from pre-existing vessels, is associated with several physiological processes as well as pathological conditions. Vascular endothelial growth factor (VEGF) is a mitogen for endothelial cells and is required for tumor-induced angiogenesis. The activation of MMPs is governed by VEGF and then activated MMPs degrade collagen and ECM proteins of basement membrane thereby aiding in the migration of endothelial cells. Cleavage of collagen type I allow endothelial cells to invade the tumor stroma during vessel formation and MMP promote the process by degrading collagen[81]. MMP-2, -9 and -14 directly regulate angiogenesis, and MMP-19 might also be important as it is expressed in blood vessel[82]. Furthermore, reduced MMP-2 expression resulted in decreased angiogenesis in cancer cells in chicken chorioallantoic membrane model. Tumor angiogenesis is significantly inhibited in mice deficient in MMP-2 in comparison with wild type mice[83]. Cleavage of collagen type IV by MMP-2 exposes a cryptic, αvβ3 integrin binding site within the collagen that

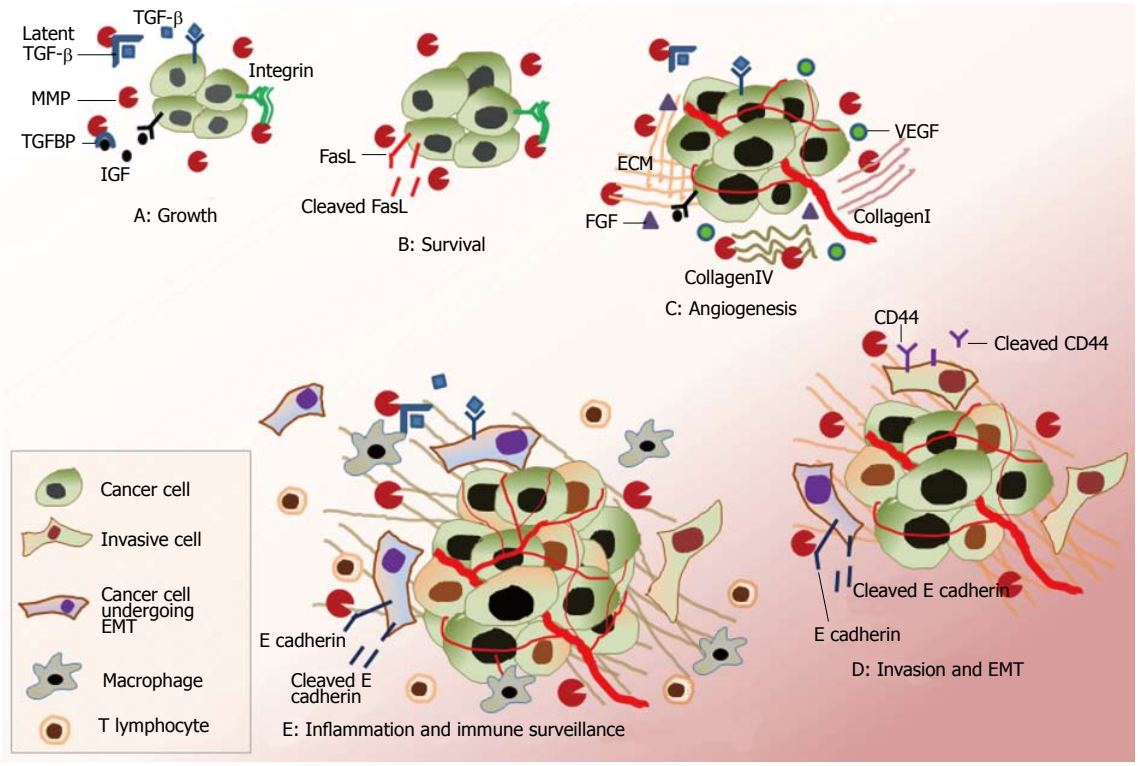

Figure 2 Roles of matrix metalloproteinases in cancer progression. The matrix metalloproteinases (MMPs) play complex but important roles during different stages of cancer progression. A and B: Growth and survival. MMP modulates cellular growth by cleaving different cellular components. It promotes cellular growth by releasing IGF from insulin growth factor-binding protein (IGF-BP). MMP-7 promotes cell survival by resisting apoptosis through cleaving Fas ligand (FasL). MMP modulates integrin signalling by regulating the extracellular matrix (ECM), which regulates growth. MMP activates transforming growth factor-β (TGF-β) from its latent TGF-β complex, which plays important roles in tumour development; C: Angiogenesis. MMP promotes angiogenesis through recruitment of VEGF, FGF. Angiogenesis is further promoted by degradation of extracellular component like collagen I, IV, fibrin, *etc*., which also act as pro-angiogenic factors; D: Invasion and epithelial to mesenchymal transition (EMT). MMP modulates invasion by degrading specific cellular components, including E-cadherin and CD-44. MMP is involved in mesenchymal transition through cleavage of E-cadherin and modulating TGF-β signaling. MMP-3 is directly involved in EMT, whereas MMP-9 has roles in differentiation; E: Inflammation and immune surveillance. MMPs also regulate immune reactions against the cancer cells. MMP-mediated TGF-β activation inhibits T lymphocyte proliferation. MMPs also modulate cancer-cell sensitivity to natural killer cells and leukocyte accumulation by cleaving different chemokines and cytokine families. VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factors.

helps in migration of endothelial cells both *in vitro* and *in vivo* model[84].

MMP-9 is a key player for pathological angiogenesis as revealed by experiments done in transgenic models of tumour progression, in the K14-HPV16 skin cancer model[85]. In contrast, the angiogenesis process is unaffected by MMP-2 in skin cancer. Both MMP-14 and MMP-9 null mice have impaired angiogenesis during development[86]. Cleavage of plasminogen by MMP-2, -3, -7, -9 and -12 generates angiostatin[87], and MMP-3, -9, -12, -13 and -20 are involved in the generation of endostatin, a C-terminal fragment of the basement membrane collagen type XVIII[45,88]. Both angiostatin and endostatin reduce endothelial cell proliferation[46]; endostatin also inhibits endothelial cell invasion by acting as an inhibitor of MMP-14 and -2[89]. By degrading fibrin matrix of blood vessels, MMP-14 promotes cell invasion and thus increase angiogenesis. In contrast, MMP-12 inhibits tumor angiogenesis by inhibiting endothelial cell invasion *via* a different pathway that mediated by urokinase-type plasminogen activator receptor.

MMPs regulate invasion and metastasis

One of the foremost and major steps in invasion is migration of cancer cells from the site of origin to the docking site. The cleavage of ECM is essential for detachment of cancerous cells from neighboring. Cleavage of laminin-5 by MMP-2 and -14 generates a cryptic site that facilitates cell migration[90]. This is supported by the fact of colocalization of laminin-5 and MMP-14 in human cancer specimen and abundance of degraded laminin in tumor tissues[91]. In addition, the main receptor for hyaluronan, CD44 is cleaved by MMP-14, thus, extracellular domain is released, thereby facilitating tissue invasion (Figure 2). Moreover, cell migration is hampered when the cleavage site is mutated[92]. In addition to binding to the ECM, CD44 also binds MMP-9, thereby localizing the enzyme to the cell surface that promotes tumor invasion and angiogenesis, as confirmed by overexpression of the extracellular domain of CD44 and suppression of tumor invasiveness[93]. Thus, cancer cell migration is regulated by cycles of MMP activity or localized MMP activity, not by continuously high activity. During metas-

tasis, cancer cells first cross the epithelial basement membrane and invade the surrounding stroma, followed by invasion to blood or lymphatic vessels; then extravasate to new tissues and establish growth of new proliferating cells in new tissues. The role of MMPs in metastasis was evidenced by *in vitro* invasion assays and *in vivo* xenograft metastasis assays. Overexpression of MMP-2, -3, -13 and -14 promotes invasion as documented through either optic nerve explants or cell culture in matrigel[94-96]. In addition, metastasis is reduced in the MMP-2 and MMP-9 null mice as compared with wild-type mice. There is no linear relationship between MMP-2 expression and cell invasion, rather cells expressing intermediate levels of active MMP-2 are the most invasive. The localization of specific MMPs to specialized surfaces on the cell membrane is necessary for their ability to promote invasion. Endothelial cell-adhesion molecule E-cadherin is associated with cancer progression, as it is cleaved by MMP-3 or -7[97]. The released fragment of E-cadherin promotes tumor cell invasion in a paracrine manner *in vitro*[98,99]. MMP-2, -9 and -14 are known to localize to invadopodia. Moreover, MMP-2 is recruited to invadopodia by either binding to α5β3 integrin[100] or by binding to MMP-14. MMP-14 is recruited to invadopodia by means of its transmembrane and cytoplasmic domains. Overexpression of MMP-14 increases the number of cancer cells in an experimental metastasis assay. Furthermore, the docking of cancer cells at the secondary site, the late events in the metastatic process also involves MMP activity.

MMPs and the immune responses to cancer

Inflammatory reactions by tumour-specific cytotoxic T lymphocytes, natural killer cells, neutrophils and macrophages are a key mechanism of cellular carcinoma[101]. The immune system is capable of recognizing and attacking cancer cells, while cancer cells somehow escape immune surveillance. MMPs are involved in the escape mechanisms. Although the immune response helps to delay tumour progression, chronic inflammation is also associated with various cancers including cancers those of gastric mucosa, large bowel, and liver[101]. In animal models, mast cells, neutrophils and macrophages are contributors to the progression of cancer. Inflammatory cells synthesize several MMPs, including -9, -12 and -14 and stimulate cancer progression. Indeed, MMP-9 null mice are less prone to skin cancer[85]. Especially, MMP-9 can cleave interleukin-2 receptor (IL-2R)-α and thereby suppress the proliferation of the T lymphocytes[102]. MMP-9 also can act on IL-8, and, thus increases the activity by several folds. MMP-2 cleaves the monocyte chemoattractant protein-3, and the cleaved fragment not only is inactivated but also becomes an antagonist to the receptors[103]. Furthermore, CXCL12 (also known as stromal-cell-derived factor 1) is cleaved and inactivated by MMP-1, -3, -9, -13 and -14[104]. CXCL12 is a ligand for the CXC chemokine receptor 4 (CXCR4) on leukocytes. Inhibition of the binding of CXCL12 to CXCR4 greatly reduces metastasis to lung and lymph nodes in breast cancer[105]. MMP-11 acts on α1-proteinase-inhibitor and the cleaved product altered sensitivity of tumor cells towards natural killer cells[106]. Moreover, few MMPs also activate TGF-β an important inhibitor of the T-lymphocyte response against tumors[107]. MMPs play indirect roles in proliferation of cancer cells by acting on growth factors that entangled into ECM (Figure 3). First, membrane-bound precursors of some growth factors, *e.g.*, TGF-β, are released by MMPs or ADAMs[107]. Second, degradation of growth factors by MMPs makes them available in pericellular space, *e.g.*, MMPs can cleave IGF-BP to release IGF[108]. Finally, cell proliferation by growth factors occurs through integrin signaling.

MAJOR IMPACT OF OXIDATIVE STRESS ON GASTROINTESTINAL DISEASES

For many years, researchers have recognized ROS only as causative factor in pathological processes, although the opinion has now changed. ROS were shown to meet the criteria for signalling molecules and they have significant roles in biological functions. This section deals with involvement of ROS in physiological and pathological processes and, subsequent responses in cancer cells under oxidative stress.

Generation of ROS in biological systems

ROS are often byproducts of mitochondrial function that are constantly generated and eliminated from physiological systems. ROS are oxygen containing reactive species, that may contain an unpaired electron, *e.g.*, superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) and/or non-radical molecules, such as hydrogen peroxide (H_2O_2). $O_2^{\cdot -}$ are formed by chemical reduction of molecular oxygen, by electrons that escape from complex Ⅰ and Ⅲ of electron transport chain and $O_2^{\cdot -}$ is than dismutated to H_2O_2. Nearly 2% of molecular oxygen consumed during mitochondrial respiration ends up as $O_2^{\cdot -}$[109,110]. Apart from mitochondria, ROS can be generated from a family of trans-membrane proteins, known as NADPH oxidases (Nox). These enzymes catalyse NADPH dependent reduction of molecular oxygen to $O_2^{\cdot -}$[111].

Increased ROS generation can induce lipid peroxidation and protein oxidation, hampering normal cellular processes. In addition, ROS can target mitochondrial DNA (mDNA) more effectively due to its proximity and lack of protective histones and limited repair mechanism. mDNA does not contain any introns and encodes for 13 respiratory complexes essential for electron transport chain and oxidative phosphorylation. Mutation of mDNA results in aberrant mitochondrial proteins during ATP generation; this induces further ROS production. Moreover, ROS cause nuclear DNA damage through DNA oxidation (by formation of 8-oxo-G, 8-oxo-dG), which contributes to mutation[109].

Cancer cells are known to be metabolically active and require elevated amount of energy to support their functionality. To meet their ATP need, cancer cells exhibit an increased dependency to glycolysis which slows down

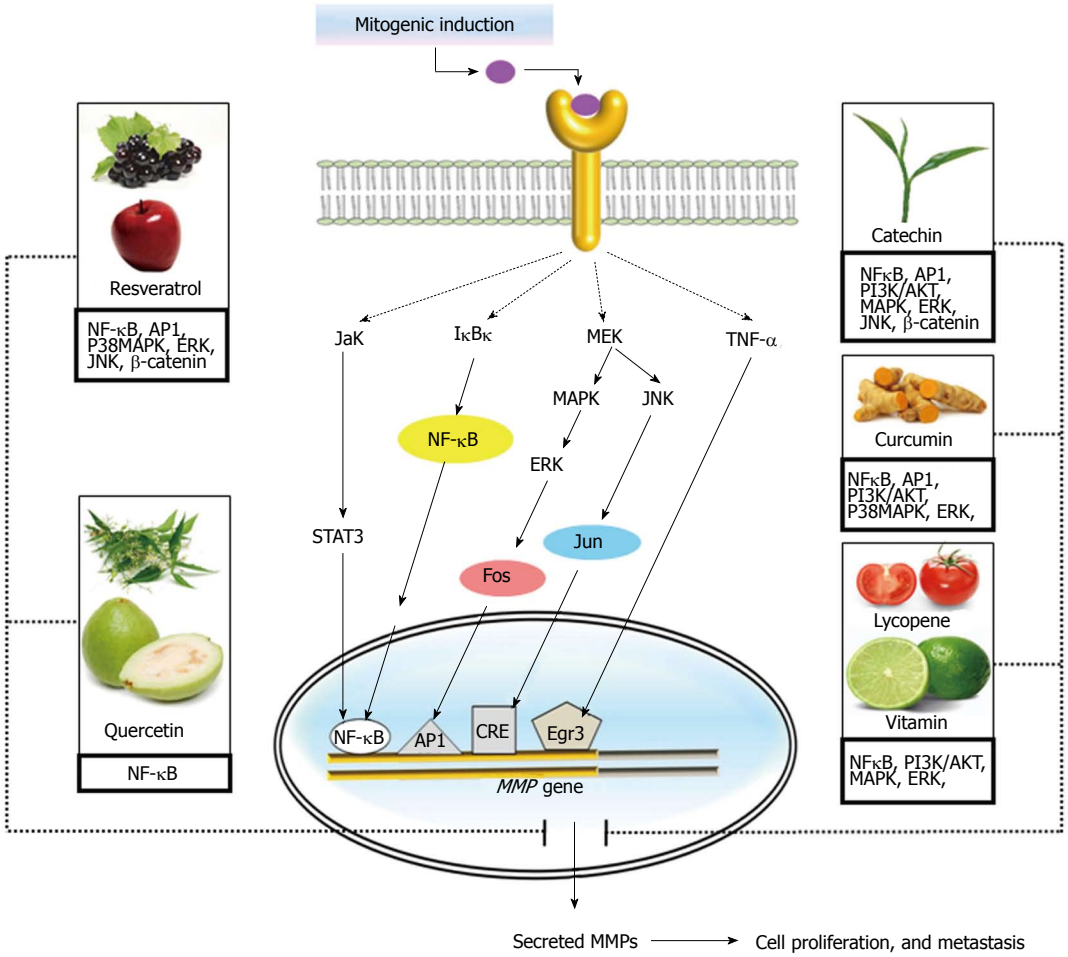

Figure 3 Role of dietary antioxidants in modulating matrix metalloproteinases action. Different ingredients in diet possess antioxidant activity. They act on various signalling pathways and transcription factors which modulate the synthesis and secretion of matrix metalloproteinases (MMPs). Therefore regulates MMP-dependent cellular processes, including proliferation and metastasis. TNF: Tumor necrosis factor; MAPK: Mitogen-activated protein kinases; MEK: Methyl ethyl ketone.

mitochondrial energy production. This phenomenon of stimulation of glycolysis in cancer cells that inhibit mitochondrial respiration, is known as "Crabtree effect"[112]. However, inhibition of glycolysis in tumor cells may result impairment of mitochondrial oxidative phosphorylation and subsequently associated with hypoxia and higher amounts of ROS generation and accumulation of ROS-mediated reaction products[113].

Oxidative stress and gastrointestinal diseases

Oxidative stress plays important roles in pathogenesis of gastro-intestinal diseases, which include mucosal damage, gastro-intestinal ulcers, and cancer. Although gastric ulcer can be generated by different factors, *e.g.*, non-steroidal anti-inflammatory drugs (NSAIDs), thermal stress, ethanol, and *H. pylori* infection, leading to oxidative damage through free radical generation (specially OH·) and subsequent apoptotic responses of gastric mucosa[114]. In addition, gastro-intestinal diseases are associated to increased oxidative stress and oxidants levels, such as glutathione, lipid peroxidation, myeloperoxidases, protein carbonyl, *etc*[115]. Furthermore, pathogens are directly involved in aggravating oxidative stress; for example, complete eradication of *H. pylori* is reported to attenuate oxidative stress in gastric mucosa[116].

Although certain types of gastro-intestinal inflammations, like ulcerative colitis, hepatitis, *H. pylori* infection, are more prone to develop cancer, the reasons are still not well elucidated. Inflammation and subsequent elevated oxidative stress might be the factors for aggravating chronic inflammation and inducing malignant transformation. Transgenic mice expressing hepatitis B protein in liver develop chronic hepatitis with elevated levels of 8-oxo-dG, leading to hepatocellular carcinoma[117]. It is well accepted that inflammation is always accompanied with elevated oxidative stress in cancer. Gastric cancer patients (with normal renal and hepatic functions) are found to have significantly increased lipid peroxidation levels[118]. Gastric carcinoma patients have significantly higher myeloperoxidase activity than controls, both before and after operation, although total antioxidant status was decreased post-operation[119]. Gastric cancers are also associated with augmented protein oxidation, although no differences are found in oxidative stress parameters and antioxidant enzyme activities between anti-*H. pylori* IgG positive and negative gastric cancer patients[120].

Cellular responses in cancer cells under oxidative stress

ROS, such as $O_2^{\cdot}$ and H_2O_2, have roles in proliferation and cellular growth that contribute to the development of cancer. Cancer cells also exhibit different cellular responses under oxidative stress, which include senescence, autophagy and apoptotic responses; although it is still not well understood, how these cellular pathways assume priority to become activated under particular conditions. H_2O_2 can directly modulate autophagic responses during nutritional starvation through Atg4 (autophagin-1) expression and accumulation of LC3-PE on the autophagosomal membrane[121]. In addition, ROS-induced autophagy is dependent on constitutive expression of Atg, baclin, hypoxia induced factor-1 (HIF-1) and BNIP3[122]. Mutations in *ATG* genes (like *ATG2B, ATG5, ATG9B,* and *ATG12*) are involved in gastric and colorectal carcinomas and may contribute to cancer development by deregulating the autophagy process. Moreover, increased autophagy (through upregulation of Atg5 and Atg7) is also involved in *in vitro* malignant transformation by K-Ras[123]. ROS and ROS-mediated signalling pathways are also involved in senescence responses. Overexpression of p21, along with increased ROS production, directly induces the senescence phenotype; while inhibition of p21-mediated ROS accumulation can rescue cells from senescence[124]. In addition, sub-lethal doses of H_2O_2 can induce senescence-like growth arrest at the G1 stage *via* up regulation of p53 and p21. Anti-apoptotic Bcl-2 family members antagonise ROS generation during apoptosis. Moreover, ROS generation is accompanied by cellular apoptosis due to lipid peroxidation and mitochondrial depolarization.

Increased ROS production in cancer cells is associated with constant activation of transcription factors including NFκB and AP-1. Moreover, a recent study found that activation of TLR4 promotes gastric cancer by increasing mitochondrial ROS generation through NFκB activation[125]. Oncogenic signals are involved in ROS generation that promote metastasis in gastric cancer[126]. The oncogene c-myc has been reported to develop genetic instability, DNA damage and mitigation of p53 function through ROS production[127]. RAS2 mutation promotes oxidative stress by restricting mitochondrial respiration into non-phosphorylating state. K-Ras is also involved in malignant transformation through ROS mediated JNK activation[123]. ROS and Rac1b are involved in MMP-3 mediated epithelial to mesenchymal transition (EMT). ROS also stimulate the expression of Snail and cause damage to DNA and subsequent genomic instability[128]. Reports have suggested a possible association of Nox and Nox-mediated ROS generation for carcinogenesis[111]. Nox1 is involved in *H. pylori*-induced gastric cancer and in angiogenic responses for tumour formation[129,130]. Nox2 is involved in phagocytosis; Nox4 controls cell survival in different cancers, including gastric, colon and pancreatic cancers[111,131]. Increased ROS can cause detachment of JNK associated glutathione-S-transferase (GST)-π and thus facilitates JNK activation. In addition, H_2O_2 mediated JNK activation also causes downregulation of JNK phosphatases[132]. Mice that have an inactive c-Jun that lacks JNK phosphorylation site or deficient in JNK displayed reduced tumor development[133,134]. Moreover, increased ROS productions in oncogenically transformed cells potentiate JNK and p38 MAP kinases activation[135].

Role of endogenous antioxidants during oxidative injury

Endogenous antioxidants are essential for maintenance and neutralization of perturbed oxidative free radical status. Superoxide dismutases (SOD) are important antioxidants, as Mn-dependent SOD (Mn-SOD) null mice cannot survive after birth[136]. Heterozygous Mn-SOD null mice can survive, however, show higher incidences of lymphomas and adenocarcinomas[137]. In addition, Zn-dependent SOD (Zn-SOD) knockdown mice develop hepatic cancer in late stages of life[138]. Mice lacking catalase also exhibit elevated cancer incidences[139]. Glutathione peroxidases (GPx) also have significant roles as antioxidants. Simultaneous knock out of GPx-1 and -2 in mice leads to gastrointestinal cancer[140].

Clinical publications have reported different levels of endogenous antioxidants in gastrointestinal cancers. GST, GPx and SOD activities are reported to be significantly elevated in colorectal cancers, than adjacent normal tissues[141]. Stomach adenocarcinoma and esophageal squamous cell carcinoma show significantly increased Mn-SOD expression as compared to noncancerous cells[142]. In clinical studies with gastric and colorectal cancer, GPx, SOD, glucose-6-phosphate dehydrogenase (G6PD), malonaldehyde and glutathione reductase were found elevated in the malignant phenotype[143]. However, because of the sustained oxidative stress conditions, these antioxidants are insufficient in cancer, eventually resulting in decreased antioxidant levels in several cancers[144]. Moreover, modulated expressions of Mn-SOD are reported due to mutations in the promoter region, abnormal methylation, loss of heterozygosity or mutation in the coding sequences[145]. These differential results of cellular antioxidant SOD cause metabolic chaos, due to different types and grades of malignancy[146].

DIETARY ANTIOXIDANTS AS A MODULATOR OF MMPs IN GASTROINTESTINAL CANCER

Human diet contains a mixture of oxidants and antioxidants substances. Dietary and endogenous antioxidants are important for cellular protection by reacting and/or eliminating oxidizing free radicals. The question of whether antioxidant supplements might protect against cancer has drawn much attention since the mid '80s and different antioxidants were extensively studied thereafter, although the results of the investigations are mixed and contradictory. Antioxidant and endogeneous redox enzymes act as the first-line defense against ROS in all

cellular compartments and also extracellularly. The most important of these enzymes include SOD, GPx, catalase and peroxiredoxins. The specific role of above enzymes in carcinogenesis is still unambiguous since their roles in ROS detoxification are well known. It is noteworthy that we do not yet fully understand the chemopreventive role of phytochemicals as antioxidants, or as modulators of other processes related to carcinogenesis. In this section, we highlight the effects of dietary antioxidants in prevention of cancers with particular emphasis on the regulation of MMPs activity.

Tea polyphenol and catechin

Tea, derived from the plant *Camellia sinensis*, is the most consumed beverage worldwide and it is grown in over 30 countries around the world, exclusively in the subtropical and tropical zones[147]. It is processed in different ways in different parts of the world to produce green, black, or Oolong tea. Both green and black teas have been studied for their health benefits, particularly for prevention and treatment of inflammatory diseases as well as cancer. Green tea is rich in polyphenolic substances, which include flavonoid, flavanols, and flavinidols all of which have antioxidant properties. The most common flavonal in tea is catechin. The most active and abundant catechin in green tea is epigallactocatechin-3-gallate (EGCG) that has been shown to inhibit cancer cell growth *in vitro* and *in vivo*. In black tea the major polyphenols are theaflavin and thearubigin.

During the last decade several epidemiological studies have linked tea consumption, especially green tea to a reduced risk of cancer in humans. Morse *et al*[148] documented the beneficial effects of the polyphenol fractions of green tea, the polyphenol fractions of black tea, *i.e.*, EGCG and theaflavins against N-nitrosomethylbenzylamine (NMBA)-induce esophageal cancer in rat. Wang *et al*[149] investigated both protective and therapeutic effects of green tea and black tea extract on esophageal tumorigenesis in rats. A population-based case-control study in Shanghai indicated that tea consumption was strongly associated with reduced colorectal cancer incident. Green tea polyphenol supplementation during the initiation or postinitiation period significantly lowered azoxymethane-induced tumor incidence in rats[150]. In addition, catechin and EGCG reduced colon tumor incidence in a 1, 2-dimethylhydrazine (DMH)-induced intestinal cancer.

Green tea polyphones were shown to prevent cancer cell proliferation and invasion. EGCG has been shown to inhibit NFκB activity in human colon cancer cells[151]. Several studies indicate that chemopreventive properties of EGCG can also mediated by inhibition of MMP induction. EGCG inhibited the PMA-induced cell invasiveness and MMP-9 expression in human gastric cancer adenocarcinoma (AGS) cells[152]. Fassina *et al*[152] documented that EGCG (25-100 μmol/L) inhibits the MMP-2 and -9 in endothelial cells. EGCG inhibited the activity and expression of MT1MMP, a protein responsible for the activation of MMP-2 as examined by Annabi *et al*[153]. Onoda *et al*[154] found that gastric cancer cell lines, *e.g.*, MKN-1, MKN-28, MKN-45, NUGC-3 and TMK-1 are sensitive to EGCG treatment with NUGC-3 being the most sensitive. Furthermore, EGCG suppresses Met signaling in HCT116 human colon cancer cells[155]. In another study, EGCG may exert at least part of its anticancer effect by inhibiting angiogenesis through blocking the induction of VEGF and binding to its receptors. EGCG has been shown to affect MMP-2 and -9 activities both directly and indirectly in endothelial cells thereby inhibiting or delaying cancer invasion and metastasis. Concanavalin A-induced activation of MMP-2 and activity of MT1-MMP has been reduced by EGCG[156]. Catechin, another major component of tea, prevents vascular smooth muscle cell invasion by inhibiting MT1-MMP activity and MMP-2 expression[157]. Black tea polyphenols inhibit DMH-induced colorectal cancer by inhibiting MMP-7 induction *via* Wnt/β-catenin pathway[158]. Hwang *et al*[159] showed the apoptotic effect of EGCG in HT-29 colon cancer cells that was mediated by AMPK signaling. Hence, catechin and EGCG exert strong anticancer activity by targeting transcription factors like NFκB, and AP-1 which involve in the regulation of mainly MMP-2, -9 and -7 activities. Specifically, EGCG regulates angiogenesis and apoptosis *via* changing expression of VEGF, uPA, IGF-1, EGFR, cell cycle regulatory proteins and in turn affects NFκB, PI3-K/Akt, ERK, JNK, Ras/Raf/MAPK and AP-1 signaling pathways, thereby acting as chemopreventive agent[160].

Curcumin

Curcuma longa (Zingiberaceae family) rhizomes have been traditionally used in the south Asian countries for the treatment of a variety of inflammatory conditions and different diseases including carcinomas[161]. The pharmacological properties of curcumin are attributed mainly to the curcumin (diferuloylmethane)-(1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-hepadiene-3,5-dione) a hydrophobic polyphenol present in the rhizome. Curcumin is a potent antioxidant that acts as a free radical scavenger[162]. Curcumin possesses a wide range of pharmacological activities including anti-inflammatory, chemo-preventive, and antimicrobial and wound healing effects[162-164]. Curcumin which is also known as turmeric, has been shown to exhibit dose dependent chemo-preventive effects in several gastrointestinal cancers including colon, duodenal, stomach, esophageal and oral carcinogenesis[165,166]. *In vivo* and *in vitro* studies have demonstrated curcumin' s ability to inhibit carcinogenesis at three stages: tumor promotion, angiogenesis and tumor growth[167]. These protective effects of curcumin are attributed mainly to its antioxidant properties and investigated for the purpose of developing novel drugs. It reduces carcinogen-induced tumorigenesis in the fore-stomach and N-ethyl-N'-nitro-nitrosoguanidine-induced duodenal tumors. Lower incidences of bowel cancer in Indians, possibly due to the use of turmeric during food preparation. The molecular basis of anti-carcinogenic and chemopreventive effects

of curcumin is targeted to transcription factors, growth regulators, adhesion molecules, apoptotic genes, angiogenesis regulators and cellular signaling molecules[166].

Koo *et al*[168] showed that curcumin and 5-fluorouracil (5-FU) additively inhibited the growth of gastric carcinoma cells. In another study, curcumin reversed the multidrug resistance of a human gastric carcinoma cell line[169]. Curcumin exhibited both preventive and therapeutic effects on the incidence and multiplicity of fore-stomach tumors induced by benzopyrene in mice[170]. A dietary supplementation of 0.15% curcumin reduces intestinal tumor formation in $Min^{-/-}$ mice by 63%. Curcumin induces apoptosis and prevents adenoma development in the intestinal tract in mice[171]. Tetrahydrocurcumin (THC) significantly decreases DMH-induced colon carcinogenesis[172]. Shpitz *et al*[173] showed that curcumin and celecoxib synergistically inhibit colorectal cancer progression in DMH-induced rat model. Several studies documented the inhibitory effects of curcumin on AOM-induced colon cancer[174]. Curcumin inhibits the expression of MMP-9 both *in vitro* and *in vivo* and thereby inhibits tumor invasion and metastasis. Bimonte *et al*[175] reported that curcumin inhibits the expression of MMP-9 in orthotopically implanted pancreatic tumors. Curcumin causes a significant reduction of tumor volume, and MMP-9 activity in a xenografted model[176]. Curcumin also reduces the expression of major MMPs *via* reduced NFκB activity and AP-1 transcription[176]. Lin *et al*[177] reported that curcumin inhibits SK-Hep-1 hepatocellular carcinoma cell invasion *via* suppression of MMP-9 secretion. Curcumin prevents human colon cancer, colo-205 cells migration through the inhibition of NFκB/p65 and downregulation of cyclooxygenase-2 and MMP-2 expression[178]. Lin *et al*[179] reported that curcumin inhibits SDF-1α-induced invasion of human esophageal carcinoma cells by down regulating MMP-2 promoter activity as well as suppressing the formation of lipid raft-associated Rac1/PI3K/Akt signaling complexes. In conclusion, curcumin appears to have a significant potential in the treatment of multiple diseases that are due to oxidative stress. Thus, various inflammatory pathways ultimately act on MMP transcription or expression during prevention of various types of cancer by curcumin.

Resveratrol

Resveratrol (trans-3,4',5-trihydroxystilbene), a non-flavonoid polyphenolic antioxidant, has attracted considerable attention due to its anti-oxidant, anti-cancer, and anti-inflammatory properties. It is in abundance in grapes and grape products such as wine, moderately abundant in blueberries, and sparsely abundant in other plants. Resveratrol is a scavenger of hydroxyl, superoxide, and other radicals and thus acts as a potent antioxidant. It protects against ROS-mediated lipid peroxidation in cell membranes and DNA damage. Resveratrol enhances the expression and/or the activity of drug metabolizing phase I/II enzymes such as Mn-SOD, GST, cytochrome P450 reductase, quinine oxidoreductase, NAD(P)H: quinone oxidoreductase (NQO1), quinone reductase (QR), heme oxygenase-1 (HO-1), glutamate cysteine ligase (GCL); thereby protecting against oxidative DNA damages[180]. Several studies demonstrated that resveratrol exhibits strong chemopreventive effects in various experimentally induced tumor models as well as inhibits proliferation and induces apoptosis of various cancerous or transformed cells. Oral or intra-peritoneal administration of resveratrol decreases expressions of COX-1, COX-2, and PGE2 reducing the number and size of esophageal tumors in N-nitrosomethylbenzylamine-induce rat tumor model[181]. Similarly, oral administration of resveratrol limits the formation of aberrant crypt foci and tumors in the colon of rats that are treated with chemical carcinogen, *i.e.*, 1,2-dimethylhydrazine (DMH) or azoxymethane. Resveratrol prevented the formation of colon tumors and reduced the formation of small intestinal tumors by 70% in $APC^{-/+}$ mice[182]. Resveratrol inhibits MMP-9 expression and invasion of human hepatocellular carcinoma cells. Resveratrol modulates all three MAP kinases namely ERK1/2, JNK, and p38 MAPK. Resveratrol impairs the expression of EMT-related genes (E-cadherin, N-cadherin, vimentin, MMP-2, and -9) in pancreatic cancer cells and inhibits proliferation, migration, and invasion[183]. Ji *et al*[184] demonstrated that resveratrol possesses chemopreventive effects in HCT116 CRC through inhibition of MMP-7 *via* Wnt/β-catenin signalling pathway. Weng *et al*[185] suggested that resveratrol and its related methoxy analogue MR-3 might exert anti-invasive activity against hepatoma cells through regulation of MMP-2 and -9 as well as TIMPs. Harikumar *et al*[186] reported that resveratrol can enhance chemopreventive activity of gemcitabine *in vitro* and *in vivo* mouse model of pancreatic cancer. In summary, anticancer activity of resveratrol is augmented by upregulation of TIMP and downregulation of MMP-9 expression.

Quercetin

Quercetin or 3,5,7,3',4'-tetrahydroxyflavone is a flavonoid found abundantly in plant-derived foods and has been shown to possess several health beneficial activities including anti-tumor, anti-inflammation and anti-proliferation; it has recently gained attention due to its potential anticancer activity. As an antioxidant it possesses the most potent ROS scavenging activity and provides protection against the development of variety of cancers by ameliorating ROS-mediated cellular damages[187]. Moreover, it reduces the level of oxidative enzymes, such as xanthine oxidase (XOD), lipoxygenase and NADPH oxidase, thereby preventing free radical-induced cellular damage[187]. *In vivo* studies have been performed to depict the chemopreventive properties of quercetin on different cancers. Volate *et al*[188] found that food supplementation of approximately 3% quercetin exerts significantly beneficial effects by decreasing precancerous lesions through induction of cellular. Dihal *et al*[189] reported that quercetin inhibits azoxymethane-induced colorectal carcinogenesis in F344 rats. Quercetin was found to be protective

against hepatocarcinoma that was generated by N-nitrosodiethylamine and, was accompanied by the maintenance of a correct intracellular oxidant/antioxidant status. Quercetin prevents 4-nitroquinoline 1-oxide-induced oral carcinogenesis during the initiation/post initiation phases of carcinogen treatment. A limited number of experiments were conducted to investigate the effects of quercetin in the regulation of MMP activity in gastrointestinal cancer. It has been found that quercetin supplementation did not alter MMP-2 or TIMP-2 gene transcription or plasma protein levels but TIMP-1 gene expression and plasma protein levels decreased significantly. Recently, Lai *et al*[190] reported that migration and invasion of SAS human oral cancer was inhibited by quercetin *via* downregulation of MMP-2 and -9 in a NFκB dependent pathway. They also showed significant reduction of the MMP-7, -10 protein levels by quercetin treatment.

Other promising dietary antioxidants

The roles of few more dietary antioxidants *e.g.*, melatonin, lycopene, retinoic acid, vitamin C and vitamin E in prevention of cancer through regulation of MMPs are discussed below. Melatonin is a naturally occurring antioxidant synthesized mainly by the pineal gland of vertebrates and also found in many edible plant products[191]. Recent research documents that consumption of tropical fruit enhances the serum melatonin level as well as raises the antioxidant capacity of blood serum[191,192]. Melatonin retards the development of cancer in different animal models and possesses strong anti-proliferative and pro-apoptotic effects in various cancer cells. Sharman *et al*[193] reported that long term exposure to dietary melatonin reduces tumor number and size in aged male mice. In addition, melatonin inhibits the growth of murine gastric carcinoma cells by upregulation of p21, Bax and down regulation of Bcl-2. Decreased expressions of melatonin receptor in various cancers also suggest the importance of melatonin signaling in cancer development[194]. Hong *et al*[195] reported that melatonin treatment induces apoptosis, autophagy, and senescence in human colorectal cancer cells. A few report suggested that melatonin prevents gastrointestinal cancer development by modulation of MMP functions. Melatonin induces apoptosis and reduces invasiveness of HepG2 cells *in vitro* through TIMP-1 upregulation and attenuation of MMP-9 activity *via* NFκB signal pathway[196]. Rudra *et al*[192] reported that melatonin reduces MMP-9 activity in AGS cell line and binds directly to its active site.

Two separate studies reported the chemo-preventive effects of lycopene in human colon cancer. Tang *et al*[197] evaluated the chemo-preventive effects of lycopene and fish oil in a mouse xenograft model of colon cancer. They found that inhibition of tumor growth and progression by the augmenting p21 (CIP1/WAF1) and p27 (Kip1) expression, and suppression of MMP-7, MMP-9, COX-2 and PGE2, PCNA, β-catenin, cyclin D1 and c-Myc proteins[197]. *In vitro* study by Lin *et al*[198] suggested the inhibitory effects of lycopene on tumor progression, where cell invasion was inhibited by down regulation of MMP-7 expression *via* blocking MAPK/ERK and PI3K/Akt signaling pathways. Adachi *et al*[199] reported that naturally occurring retinoid, all-trans retinoic acid (ATRA), 9-cis retinoic acid and 13-cis retinoic acid are helpful in the prevention and therapy of colon cancer. ATRA prevents tumor invasion in mice and inhibits *in vitro* invasion of colon cancer cells by down regulation of MMP-7 expression. Moreover, Park *et al*[200] reported that retinol reduces the invasive potential of retinoic acid resistance colon cancer cells by decreasing MMP-1,-2,-7,-9 expressions and activity. Retinol reduces the metastatic potential of colon cancer cells *via* down regulation of MMP induction in a retinoic acid receptor-independent mechanism. β-ionone, the derivative product of carotenoids, is the precursor of vitamin A also possesses anti-proliferative activity in cancer cells[201]. Liu *et al*[202] found that that γ-tocotrienol inhibit gastric cancer cell (SGC-7901) proliferation by reducing MMP-1 and -2 activity *via* modulating the expression of their inhibitor TIMP-1 and TIMP-2. Dietary supplementation of naturally occurring antioxidants, ascorbic acid reduces the size of colon xenograft cancer by downregulation of MMP-9 and VEGF in nude mice[203]. Vitamin E (γ-tocotrienol) effectively inhibits the growth of human gastric cancer in a xenograft mouse model. γ-tocotrienol inhibited the proliferation of gastric cancer cell lines, *via* inhibiting the NFκB mediated up-regulation of MMP-9 and VEGF[204].

CONCLUSION

The immense complexity of cancer disease is not yet fully characterized despite numerous advances in modern molecular biology. Complexity in cancer cells arise from heterogeneity of tumor microenvironment, inflammatory stimuli, immune responses, diet effects as well as intestinal micobiota. All these factors determine whether the fate of cancer cells undergo apoptosis or proliferation or even develop resistance to drugs. Cancer is a multifactorial disease, which varies from patient to patient. Even the complexity lies in a certain tumor cells that may refer to tumors with diverse genetics. In fact, every cancer types is unique. Hence, intramolecular heterogeneity poses another dimension during cancer progression. Significant intra-tumor heterogeneity is present in many patients, thus drug resistance may develop. Sequencing technology is used to monitor clonal dynamics of cancer cells. In this context, careful attention should be given to detect minor clones of clinical significance. Tumor heterogenity was documented by the Cancer Genome Atlas and the Cancer Genome Analysis projects.

In the future, personalised cancer medicine may be possible by accounting for both interpatient and intrapatient heterogeneity. Additionally, new therapeutic strategies are important for targeting cellular conditions like cellular senescence rather than targeting a particular biomolecule. Given the complexity of cancer, it is unlikely universal therapeutic strategy will be employed

for different cancer types and stages. The MMP family of enzymes occupies a major importance in the field of gastric cancer research. Literature in last two decades of MMP biochemistry and cancer biology supports the possibility of particular MMP in particular types of cancer, including gastrointestinal cancer. Both basic and applied research is needed to decipher the mechanisms of cancer progression and regulatory roles of particular MMP associated with different cancer types. Drug discovery efforts have uncovered pharmacological inhibitors of different MMPs. Specific MMP inhibitors at a specific dose would be an important achievement to treat particularly gastric cancer and to halt to the progression of these diseases.

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REFERENCES

1 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]
2 **Wadhwa R**, Song S, Lee JS, Yao Y, Wei Q, Ajani JA. Gastric cancer-molecular and clinical dimensions. *Nat Rev Clin Oncol* 2013; **10**: 643-655 [PMID: 24061039 DOI: 10.1038/nrclinonc]
3 **Murakami T**. Pathomorphological diagnosis. Definition and gross classification of early gastric cancer. *Gann Monogr Cancer Res* 1971: 11
4 **Sokoloff B**. Predisposition to cancer in the bonaparte family. *Am J Surgery* 1938; **40**: 673-678
5 **Williams L**, Jenkins GJ, Doak SH, Fowler P, Parry EM, Brown TH, Griffiths AP, Williams JG, Parry JM. Fluorescence in situ hybridisation analysis of chromosomal aberrations in gastric tissue: the potential involvement of Helicobacter pylori. *Br J Cancer* 2005; **92**: 1759-1766 [PMID: 15827559 DOI: 10.1038/sj.bjc.6602533]
6 **Sugai T**, Habano W, Uesugi N, Jao YF, Nakamura S, Abe K, Takagane A, Terashima M. Three independent genetic profiles based on mucin expression in early differentiated-type gastric cancers--a new concept of genetic carcinogenesis of early differentiated-type adenocarcinomas. *Mod Pathol* 2004; **17**: 1223-1234 [PMID: 15154009 DOI: 10.1038/sj.bjc.6602533]
7 **Milne AN**, Carvalho R, Morsink FM, Musler AR, de Leng WW, Ristimäki A, Offerhaus GJ. Early-onset gastric cancers have a different molecular expression profile than conventional gastric cancers. *Mod Pathol* 2006; **19**: 564-572 [PMID: 16474375 DOI: 10.1038/modpathol]
8 **Kokkola A**, Sipponen P. Gastric carcinoma in young adults. *Hepatogastroenterology* 2001; **48**: 1552-1555 [PMID: 11813570]
9 **Kikuchi S**, Nakajima T, Nishi T, Kobayashi O, Konishi T, Inaba Y, Wada O, Satou H, Ishibashi T, Ichikawa S, Okamoto N, Hirata T, Kubo T, Sato N, Miki K, Myoga A. Association between family history and gastric carcinoma among young adults. *Jpn J Cancer Res* 1996; **87**: 332-336 [PMID: 8641962]
10 **Wang Z**, Shen D, Parsons DW, Bardelli A, Sager J, Szabo S, Ptak J, Silliman N, Peters BA, van der Heijden MS, Parmigiani G, Yan H, Wang TL, Riggins G, Powell SM, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE. Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science* 2004; **304**: 1164-1166 [PMID: 15155950 DOI: 10.1126/science]
11 **Shaheen N**, Ransohoff DF. Gastroesophageal reflux, barrett esophagus, and esophageal cancer: scientific review. *JAMA* 2002; **287**: 1972-1981 [PMID: 11960540 DOI: 10.1001/jama.287.15.1972]
12 **Lagergren J**, Bergström R, Lindgren A, Nyrén O. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. *N Engl J Med* 1999; **340**: 825-831 [PMID: 10080844 DOI: 10.1056/NEJM199903183401101]
13 **Chak A**, Ochs-Balcom H, Falk G, Grady WM, Kinnard M, Willis JE, Elston R, Eng C. Familiality in Barrett's esophagus, adenocarcinoma of the esophagus, and adenocarcinoma of the gastroesophageal junction. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1668-1673 [PMID: 16985029 DOI: 10.1158/1055-9965]
14 **Sappati Biyyani RS**, Chessler L, McCain E, Nelson K, Fahmy N, King J. Familial trends of inheritance in gastro esophageal reflux disease, Barrett's esophagus and Barrett's adenocarcinoma: 20 families. *Dis Esophagus* 2007; **20**: 53-57 [PMID: 17227311 DOI: 10.1111/j.1442-2050]
15 **Chak A**, Falk G, Grady WM, Kinnard M, Elston R, Mittal S, King JF, Willis JE, Kondru A, Brock W, Barnholtz-Sloan J. Assessment of familiality, obesity, and other risk factors for early age of cancer diagnosis in adenocarcinomas of the esophagus and gastroesophageal junction. *Am J Gastroenterol* 2009; **104**: 1913-1921 [PMID: 19491834 DOI: 10.1038/ajg.2009.241]
16 **Hemminki K**, Sundquist J, Ji J. Familial risk for gastric carcinoma: an updated study from Sweden. *Br J Cancer* 2007; **96**: 1272-1277 [PMID: 17406355 DOI: 10.1038/sj.bjc.6603722]
17 **Bosman F**, Carneiro F, Hruban R, Theise N. Who classification of tumours of the digestive system iarc. The International Agency for Research on Cancer, 2010: 3
18 **Wang K**, Kan J, Yuen ST, Shi ST, Chu KM, Law S, Chan TL, Kan Z, Chan AS, Tsui WY, Lee SP, Ho SL, Chan AK, Cheng GH, Roberts PC, Rejto PA, Gibson NW, Pocalyko DJ, Mao M, Xu J, Leung SY. Exome sequencing identifies frequent mutation of ARID1A in molecular subtypes of gastric cancer. *Nat Genet* 2011; **43**: 1219-1223 [PMID: 22037554 DOI: 10.1038/ng.982]
19 **Kwon MJ**, Kim SH, Jeong HM, Jung HS, Kim SS, Lee JE, Gye MC, Erkin OC, Koh SS, Choi Y-L, Park CK, Shin YK. Claudin-4 overexpression is associated with epigenetic derepression in gastric carcinoma. *Lab Invest* 2011; **91**: 1652-1667 [PMID: 21844869 DOI: 10.1038/ labinvest. 2011.117]
20 **Baba Y**, Watanabe M, Murata A, Shigaki H, Miyake K, Ishimoto T, Iwatsuki M, Iwagami S, Yoshida N, Oki E, Sakamaki K, Nakao M, Baba H. LINE-1 hypomethylation, DNA copy number alterations, and CDK6 amplification in esophageal squamous cell carcinoma. *Clin Cancer Res* 2014; **20**: 1114-1124 [PMID: 24423610 DOI: 10.1158/1078-0432.CCR-13-1645]
21 **Okada T**, Nakamura M, Nishikawa J, Sakai K, Zhang Y, Saito M, Morishige A, Oga A, Sasaki K, Suehiro Y, Hinoda Y, Sakaida I. Identification of genes specifically methylated in Epstein-Barr virus-associated gastric carcinomas. *Cancer Sci* 2013; **104**: 1309-1314 [PMID: 23829175 DOI: 10.1111/cas.12228]
22 **Alvarez MC**, Ladeira MS, Scaletsky IC, Pedrazzoli J, Ribeiro ML. Methylation pattern of THBS1, GATA-4, and HIC1 in pediatric and adult patients infected with Helicobacter pylori. *Dig Dis Sci* 2013; **58**: 2850-2857 [PMID: 23765259 DOI: 10.1007/s10620-013-2742-6]
23 **Jin Z**, Zhao Z, Cheng Y, Dong M, Zhang X, Wang L, Fan X, Feng X, Mori Y, Meltzer SJ. Endoglin promoter hypermethylation identifies a field defect in human primary esophageal cancer. *Cancer* 2013; **119**: 3604-3609 [PMID: 23893879 DOI: 10.1002/cncr.28276]
24 **Jin Z**, Feng X, Jian Q, Cheng Y, Gao Y, Zhang X, Wang L, Zhang Y, Huang W, Fan X, Chen S, Yu H, Zhao Z, Dong M, Liu J, Mori Y, Meltzer SJ. Aberrant methylation of the Ras-

related associated with diabetes gene in human primary esophageal cancer. *Anticancer Res* 2013; **33**: 5199-5203 [PMID: 24222170]

25 **Ninomiya I**, Kawakami K, Fushida S, Fujimura T, Funaki H, Takamura H, Kitagawa H, Nakagawara H, Tajima H, Kayahara M, Ohta T. Quantitative detection of TIMP-3 promoter hypermethylation and its prognostic significance in esophageal squamous cell carcinoma. *Oncol Rep* 2008; **20**: 1489-1495 [PMID: 19020732 DOI: 10.3892/or_00000170]

26 **Poplineau M**, Schnekenburger M, Dufer J, Kosciarz A, Brassart-Pasco S, Antonicelli F, Diederich M, Trussardi-Régnier A. The DNA hypomethylating agent, 5-aza-2'-deoxycytidine, enhances tumor cell invasion through a transcription-dependent modulation of MMP-1 expression in human fibrosarcoma cells. *Mol Carcinog* 2013 [PMID: 24038389 DOI: 10.1002/mc.22071]

27 **Hussain Z**, Khan MI, Shahid M, Almajhdi FN. S-adenosylmethionine, a methyl donor, up regulates tissue inhibitor of metalloproteinase-2 in colorectal cancer. *Genet Mol Res* 2013; **12**: 1106-1118 [PMID: 23661436 DOI: 10.4238/2013.April.10.6]

28 **Leung WK**, To KF, Chu ES, Chan MW, Bai AH, Ng EK, Chan FK, Sung JJ. Potential diagnostic and prognostic values of detecting promoter hypermethylation in the serum of patients with gastric cancer. *Br J Cancer* 2005; **92**: 2190-2194 [PMID: 15942635 DOI: 10.1038/sj.bjc.6602636]

29 **Wang YC**, Yu ZH, Liu C, Xu LZ, Yu W, Lu J, Zhu RM, Li GL, Xia XY, Wei XW, Ji HZ, Lu H, Gao Y, Gao WM, Chen LB. Detection of RASSF1A promoter hypermethylation in serum from gastric and colorectal adenocarcinoma patients. *World J Gastroenterol* 2008; **14**: 3074-3080 [PMID: 18494062 DOI: 10.3748/wjg.14.3074]

30 **Egeblad M**, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002; **2**: 161-174 [PMID: 11990853 DOI: 10.1038/nrc745]

31 **Tezvergil-Mutluay A**, Agee KA, Hoshika T, Carrilho M, Breschi L, Tjäderhane L, Nishitani Y, Carvalho RM, Looney S, Tay FR, Pashley DH. The requirement of zinc and calcium ions for functional MMP activity in demineralized dentin matrices. *Dent Mater* 2010; **26**: 1059-1067 [PMID: 20688380 DOI: 10.1016/j.dental.2010.07.006]

32 **Susnow N**, Zeng L, Margineantu D, Hockenbery DM. Bcl-2 family proteins as regulators of oxidative stress. *Semin Cancer Biol* 2009; **19**: 42-49 [PMID: 19138742 DOI: 10.1016/j.semcancer.2008.12.002]

33 **Murray GI**, Duncan ME, O'Neil P, McKay JA, Melvin WT, Fothergill JE. Matrix metalloproteinase-1 is associated with poor prognosis in oesophageal cancer. *J Pathol* 1998; **185**: 256-261 [PMID: 9771478 DOI: 10.1002/(SICI)1096-9896]

34 **Etoh T**, Inoue H, Yoshikawa Y, Barnard GF, Kitano S, Mori M. Increased expression of collagenase-3 (MMP-13) and MT1-MMP in oesophageal cancer is related to cancer aggressiveness. *Gut* 2000; **47**: 50-56 [PMID: 10861264 DOI: 10.1136/gut.47.1.50]

35 **Mukherjee S**, Roth MJ, Dawsey SM, Yan W, Rodriguez-Canales J, Erickson HS, Hu N, Goldstein AM, Taylor PR, Richardson AM, Tangrea MA, Chuaqui RF, Emmert-Buck MR. Increased matrix metalloproteinase activation in esophageal squamous cell carcinoma. *J Transl Med* 2010; **8**: 91 [PMID: 20920372 DOI: 10.1186/1479-5876-8-91]

36 **Elnemr A**, Yonemura Y, Bandou E, Kinoshita K, Kawamura T, Takahashi S, Tochiori S, Endou Y, Sasaki T. Expression of collagenase-3 (matrix metalloproteinase-13) in human gastric cancer. *Gastric Cancer* 2003; **6**: 30-38 [PMID: 12673424 DOI: 10.1007/s101200300004]

37 **Liu HQ**, Song S, Wang JH, Zhang SL. Expression of MMP-3 and TIMP-3 in gastric cancer tissue and its clinical significance. *Oncol Lett* 2011; **2**: 1319-1322 [PMID: 22848309 DOI: 10.3892/ol.2011.399]

38 **Liabakk N-B**, Talbot I, Smith RA, Wilkinson K, Balkwill F. Matrix metalloprotease 2 (mmp-2) and matrix metalloprotease 9 (mmp-9) type iv collagenases in colorectal cancer. *Cancer Res* 1996; **56**: 190-196

39 **Shiozawa J**, Ito M, Nakayama T, Nakashima M, Kohno S, Sekine I. Expression of matrix metalloproteinase-1 in human colorectal carcinoma. *Mod Pathol* 2000; **13**: 925-933 [PMID: 11007031]

40 **Parsons SL**, Watson SA, Collins HM, Griffin NR, Clarke PA, Steele RJ. Gelatinase (MMP-2 and -9) expression in gastrointestinal malignancy. *Br J Cancer* 1998; **78**: 1495-1502 [PMID: 9836483]

41 **Inuzuka K**, Ogata Y, Nagase H, Shirouzu K. Significance of coexpression of urokinase-type plasminogen activator, and matrix metalloproteinase 3 (stromelysin) and 9 (gelatinase B) in colorectal carcinoma. *J Surg Res* 2000; **93**: 211-218 [PMID: 11027463 DOI: 10.1006/jsre.2000.5952]

42 **Nielsen BS**, Timshel S, Kjeldsen L, Sehested M, Pyke C, Borregaard N, Danø K. 92 kDa type IV collagenase (MMP-9) is expressed in neutrophils and macrophages but not in malignant epithelial cells in human colon cancer. *Int J Cancer* 1996; **65**: 57-62 [PMID: 8543396 DOI: 10.1002/(SICI)1097-0215]

43 **Zucker S**, Vacirca J. Role of matrix metalloproteinases (MMPs) in colorectal cancer. *Cancer Metastasis Rev* 2004; **23**: 101-117 [PMID: 15000152 DOI: 10.1023/A: 1025867130437]

44 **Adachi Y**, Yamamoto H, Itoh F, Hinoda Y, Okada Y, Imai K. Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. *Gut* 1999; **45**: 252-258 [PMID: 10403738 DOI: 10.1136/gut.45.2.252]

45 **Dong Z**, Kumar R, Yang X, Fidler IJ. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. *Cell* 1997; **88**: 801-810 [PMID: 9118223 DOI: 10.1016/S0092-8674(00)81926-1]

46 **O'Reilly MS**, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994; **79**: 315-328 [PMID: 7525077 DOI: 10.1016/0092-8674(94)90200-3]

47 **Leeman MF**, McKay JA, Murray GI. Matrix metalloproteinase 13 activity is associated with poor prognosis in colorectal cancer. *J Clin Pathol* 2002; **55**: 758-762 [PMID: 12354802 DOI: 10.1136/jcp.55.10.758]

48 **Knäuper V**, Will H, López-Otin C, Smith B, Atkinson SJ, Stanton H, Hembry RM, Murphy G. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. Evidence that MT1-MMP (MMP-14) and gelatinase a (MMP-2) are able to generate active enzyme. *J Biol Chem* 1996; **271**: 17124-17131 [PMID: 8663255]

49 **Gururajan R**, Lahti JM, Grenet J, Easton J, Gruber I, Ambros PF, Kidd VJ. Duplication of a genomic region containing the Cdc2L1-2 and MMP21-22 genes on human chromosome 1p36.3 and their linkage to D1Z2. *Genome Res* 1998; **8**: 929-939 [PMID: 9750192 DOI: 10.1101/gr.8.9.929]

50 **Llano E**, Pendás AM, Freije JP, Nakano A, Knäuper V, Murphy G, López-Otin C. Identification and characterization of human MT5-MMP, a new membrane-bound activator of progelatinase a overexpressed in brain tumors. *Cancer Res* 1999; **59**: 2570-2576 [PMID: 10363975]

51 **Bradbury PA**, Zhai R, Hopkins J, Kulke MH, Heist RS, Singh S, Zhou W, Ma C, Xu W, Asomaning K, Ter-Minassian M, Wang Z, Su L, Christiani DC, Liu G. Matrix metalloproteinase 1, 3 and 12 polymorphisms and esophageal adenocarcinoma risk and prognosis. *Carcinogenesis* 2009; **30**: 793-798 [PMID: 19321798 DOI: 10.1093/carcin/bgp065]

52 **Dey S**, Ghosh N, Saha D, Kesh K, Gupta A, Swarnakar S. Matrix metalloproteinase-1 (MMP-1) Promoter polymorphisms are well linked with lower stomach tumor formation in eastern Indian population. *PLoS One* 2014; **9**: e88040 [PMID: 24505369 DOI: 10.1371/journal.pone.0088040]

53 **Langers AM**, Verspaget HW, Hommes DW, Sier CF. Single-

nucleotide polymorphisms of matrix metalloproteinases and their inhibitors in gastrointestinal cancer. *World J Gastrointest Oncol* 2011; **3**: 79-98 [PMID: 21731908 DOI: 10.4251/wjgo.v3.i6.79]

54 **Chen XB**, Chen GL, Liu JN, Yang JZ, Yu DK, Lin DX, Tan W. [Genetic polymorphisms in STK15 and MMP-2 associated susceptibility to esophageal cancer in Mongolian population]. *Zhonghua Yu Fang Yi Xue Za Zhi* 2009; **43**: 559-564 [PMID: 19954064]

55 **Harendza S**, Lovett DH, Panzer U, Lukacs Z, Kuhnl P, Stahl RA. Linked common polymorphisms in the gelatinase a promoter are associated with diminished transcriptional response to estrogen and genetic fitness. *J Biol Chem* 2003; **278**: 20490-20499 [PMID: 12657623 DOI: 10. 10.1074/jbc.M211536200]

56 **Früh M**, Zhou W, Zhai R, Su L, Heist RS, Wain JC, Nishioka NS, Lynch TJ, Shepherd FA, Christiani DC, Liu G. Polymorphisms of inflammatory and metalloproteinase genes, Helicobacter pylori infection and the risk of oesophageal adenocarcinoma. *Br J Cancer* 2008; **98**: 689-692 [PMID: 18253117 DOI: 10.1038/sj.bjc.6604234]

57 **Li Y**, Sun DL, Duan YN, Zhang XJ, Wang N, Zhou RM, Chen ZF, Wang SJ. Association of functional polymorphisms in MMPs genes with gastric cardia adenocarcinoma and esophageal squamous cell carcinoma in high incidence region of North China. *Mol Biol Rep* 2010; **37**: 197-205 [PMID: 19562509 DOI: 10.1007/s11033-009-9593-4]

58 **Kubben FJ**, Sier CF, Meijer MJ, van den Berg M, van der Reijden JJ, Griffioen G, van de Velde CJ, Lamers CB, Verspaget HW. Clinical impact of MMP and TIMP gene polymorphisms in gastric cancer. *Br J Cancer* 2006; **95**: 744-751 [PMID: 16940985 DOI: 10.1038/sj.bjc.6603307]

59 **Saeed HM**, Alanazi MS, Parine NR, Shaik J, Semlali A, Alharbi O, Azzam N, Aljebreen A, Almadi M, Shalaby MA. Matrix metalloproteinase-2 (-1306 c& gt; t) promoter polymorphism and risk of colorectal cancer in the Saudi population. *Asian Pac J Cancer Prev* 2013; **14**: 6025-6030 [PMID: 24289619]

60 **Langers AM**, Sier CF, Hawinkels LJ, Kubben FJ, van Duijn W, van der Reijden JJ, Lamers CB, Hommes DW, Verspaget HW. MMP-2 geno-phenotype is prognostic for colorectal cancer survival, whereas MMP-9 is not. *Br J Cancer* 2008; **98**: 1820-1823 [PMID: 18506186 DOI: 10.1038/sj.bjc.6604380]

61 **Woo M**, Park K, Nam J, Kim JC. Clinical implications of matrix metalloproteinase-1, -3, -7, -9, -12, and plasminogen activator inhibitor-1 gene polymorphisms in colorectal cancer. *J Gastroenterol Hepatol* 2007; **22**: 1064-1070 [PMID: 17608852 DOI: 10.1111/j.1440-1746.2006]

62 **Tang Y**, Zhu J, Chen L, Chen L, Zhang S, Lin J. Associations of matrix metalloproteinase-9 protein polymorphisms with lymph node metastasis but not invasion of gastric cancer. *Clin Cancer Res* 2008; **14**: 2870-2877 [PMID: 18451255 DOI: 10.1158/1078-0432]

63 **Fang WL**, Liang WB, He H, Zhu Y, Li SL, Gao LB, Zhang L. Association of matrix metalloproteinases 1, 7, and 9 gene polymorphisms with genetic susceptibility to colorectal carcinoma in a Han Chinese population. *DNA Cell Biol* 2010; **29**: 657-661 [PMID: 20662554 DOI: 10.1089/dna]

64 **Matsumura S**, Oue N, Nakayama H, Kitadai Y, Yoshida K, Yamaguchi Y, Imai K, Nakachi K, Matsusaki K, Chayama K, Yasui W. A single nucleotide polymorphism in the MMP-9 promoter affects tumor progression and invasive phenotype of gastric cancer. *J Cancer Res Clin Oncol* 2005; **131**: 19-25 [PMID: 15565457 DOI: 10.1007/s00432-004-0621-4]

65 **Xing LL**, Wang ZN, Jiang L, Zhang Y, Xu YY, Li J, Luo Y, Zhang X. Matrix metalloproteinase-9-1562C& gt; T polymorphism may increase the risk of lymphatic metastasis of colorectal cancer. *World J Gastroenterol* 2007; **13**: 4626-4629 [PMID: 17729419]

66 **Dey S**, Stalin S, Gupta A, Saha D, Kesh K, Swarnakar S. Matrix metalloproteinase3 gene promoter polymorphisms and their haplotypes are associated with gastric cancer risk in eastern Indian population. *Mol Carcinog* 2012; **51** Suppl 1: E42-E53 [PMID: 22121090 DOI: 10.1002/mc.21837]

67 **Ke P**, Wu ZD, Wen HS, Ying MX, Long HC, Qing LG. Current evidence on associations between the MMP-7 (-181A& gt; G) polymorphism and digestive system cancer risk. *Asian Pac J Cancer Prev* 2013; **14**: 2269-2272 [PMID: 23725125]

68 **Jormsjö S**, Whatling C, Walter DH, Zeiher AM, Hamsten A, Eriksson P. Allele-specific regulation of matrix metalloproteinase-7 promoter activity is associated with coronary artery luminal dimensions among hypercholesterolemic patients. *Arterioscler Thromb Vasc Biol* 2001; **21**: 1834-1839 [PMID: 11701474 DOI: 10.1161/hq1101.098229]

69 **Richards TJ**, Park C, Chen Y, Gibson KF, Peter Di Y, Pardo A, Watkins SC, Choi AM, Selman M, Pilewski J, Kaminski N, Zhang Y. Allele-specific transactivation of matrix metalloproteinase 7 by FOXA2 and correlation with plasma levels in idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2012; **302**: L746-L754 [PMID: 22268124 DOI: 10.1152/ajplung.00319.2011]

70 **Zhang J**, Jin X, Fang S, Wang R, Li Y, Wang N, Guo W, Wang Y, Wen D, Wei L, Dong Z, Kuang G. The functional polymorphism in the matrix metalloproteinase-7 promoter increases susceptibility to esophageal squamous cell carcinoma, gastric cardiac adenocarcinoma and non-small cell lung carcinoma. *Carcinogenesis* 2005; **26**: 1748-1753 [PMID: 15930031 DOI: 10.1093/carcin/bgi144]

71 **Ghilardi G**, Biondi ML, Erario M, Guagnellini E, Scorza R. Colorectal carcinoma susceptibility and metastases are associated with matrix metalloproteinase-7 promoter polymorphisms. *Clin Chem* 2003; **49**: 1940-1942 [PMID: 14578330 DOI: 10.1373/clinchem.2003.018911]

72 **Cirillo G**, Casalino L, Vallone D, Caracciolo A, De Cesare D, Verde P. Role of distinct mitogen-activated protein kinase pathways and cooperation between Ets-2, ATF-2, and Jun family members in human urokinase-type plasminogen activator gene induction by interleukin-1 and tetradecanoyl phorbol acetate. *Mol Cell Biol* 1999; **19**: 6240-6252 [PMID: 10454570]

73 **Herren B**, Levkau B, Raines EW, Ross R. Cleavage of beta-catenin and plakoglobin and shedding of VE-cadherin during endothelial apoptosis: evidence for a role for caspases and metalloproteinases. *Mol Biol Cell* 1998; **9**: 1589-1601 [PMID: 9614196 DOI: 10.1091/mbc.9.6.1589]

74 **Ilan N**, Mohsenin A, Cheung L, Madri JA. PECAM-1 shedding during apoptosis generates a membrane-anchored truncated molecule with unique signaling characteristics. *FASEB J* 2001; **15**: 362-372 [PMID: 11156952 DOI: 10.1096/fj.00-0372com]

75 **Steinhusen U**, Weiske J, Badock V, Tauber R, Bommert K, Huber O. Cleavage and shedding of E-cadherin after induction of apoptosis. *J Biol Chem* 2001; **276**: 4972-4980 [PMID: 11076937 DOI: 10.1074/jbc.M006102200]

76 **Sympson CJ**, Talhouk RS, Alexander CM, Chin JR, Clift SM, Bissell MJ, Werb Z. Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J Cell Biol* 1994; **125**: 681-693 [PMID: 8175886]

77 **Powell WC**, Fingleton B, Wilson CL, Boothby M, Matrisian LM. The metalloproteinase matrilysin proteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. *Curr Biol* 1999; **9**: 1441-1447 [PMID: 10607586 DOI: 10.1016/S0960-9822(00)80113-X]

78 **Wu E**, Mari BP, Wang F, Anderson IC, Sunday ME, Shipp MA. Stromelysin-3 suppresses tumor cell apoptosis in a murine model. *J Cell Biochem* 2001; **82**: 549-555 [PMID: 11500932 DOI: 10.1002/jcb.1181]

79 **Baserga R**. The contradictions of the insulin-like growth

factor 1 receptor. *Oncogene* 2000; **19**: 5574-5581 [PMID: 11114737]
80 **Swarnakar S**, Paul S, Singh LP, Reiter RJ. Matrix metalloproteinases in health and disease: regulation by melatonin. *J Pineal Res* 2011; **50**: 8-20 [PMID: 20964709]
81 **Seandel M**, Noack-Kunnmann K, Zhu D, Aimes RT, Quigley JP. Growth factor-induced angiogenesis in vivo requires specific cleavage of fibrillar type I collagen. *Blood* 2001; **97**: 2323-2332 [PMID: 11290594 DOI: 10.1182/blood.V97.8.2323]
82 **Kolb C**, Mauch S, Peter HH, Krawinkel U, Sedlacek R. The matrix metalloproteinase RASI-1 is expressed in synovial blood vessels of a rheumatoid arthritis patient. *Immunol Lett* 1997; **57**: 83-88 [PMID: 9232430 DOI: 10.1016/S0165-2478(97)00057-6]
83 **Itoh T**, Tanioka M, Yoshida H, Yoshioka T, Nishimoto H, Itohara S. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res* 1998; **58**: 1048-1051 [PMID: 9500469]
84 **Xu J**, Rodriguez D, Petitclerc E, Kim JJ, Hangai M, Moon YS, Davis GE, Brooks PC. Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. *J Cell Biol* 2001; **154**: 1069-1079 [PMID: 11535623 DOI: 10.1083/jcb.200103111]
85 **Coussens LM**, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 2000; **103**: 481-490 [PMID: 11081634]
86 **Zhou Z**, Apte SS, Soininen R, Cao R, Baaklini GY, Rauser RW, Wang J, Cao Y, Tryggvason K. Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I. *Proc Natl Acad Sci USA* 2000; **97**: 4052-4057 [PMID: 10737763 DOI: 10.1073/pnas.060037197]
87 **Cornelius LA**, Nehring LC, Harding E, Bolanowski M, Welgus HG, Kobayashi DK, Pierce RA, Shapiro SD. Matrix metalloproteinases generate angiostatin: effects on neovascularization. *J Immunol* 1998; **161**: 6845-6852 [PMID: 9862716]
88 **Gorrin-Rivas MJ**, Arii S, Furutani M, Mizumoto M, Mori A, Hanaki K, Maeda M, Furuyama H, Kondo Y, Imamura M. Mouse macrophage metalloelastase gene transfer into a murine melanoma suppresses primary tumor growth by halting angiogenesis. *Clin Cancer Res* 2000; **6**: 1647-1654 [PMID: 10815882]
89 **Kim YM**, Jang JW, Lee OH, Yeon J, Choi EY, Kim KW, Lee ST, Kwon YG. Endostatin inhibits endothelial and tumor cellular invasion by blocking the activation and catalytic activity of matrix metalloproteinase. *Cancer Res* 2000; **60**: 5410-5413 [PMID: 11034081]
90 **Giannelli G**, Falk-Marzillier J, Schiraldi O, Stetler-Stevenson WG, Quaranta V. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science* 1997; **277**: 225-228 [PMID: 9211848 DOI: 10.1126/science.277.5323.225]
91 **Koshikawa N**, Giannelli G, Cirulli V, Miyazaki K, Quaranta V. Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. *J Cell Biol* 2000; **148**: 615-624 [PMID: 10662785 DOI: 10.1083/jcb.148.3.615]
92 **Kajita M**, Itoh Y, Chiba T, Mori H, Okada A, Kinoh H, Seiki M. Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. *J Cell Biol* 2001; **153**: 893-904 [PMID: 11381077 DOI: 10.1083/jcb.153.5.893]
93 **Yu Q**, Stamenkovic I. Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. *Genes Dev* 1999; **13**: 35-48 [PMID: 9887098 DOI: 10.1101/gad.13.1.35]
94 **Beliën AT**, Paganetti PA, Schwab ME. Membrane-type 1 matrix metalloprotease (MT1-MMP) enables invasive migration of glioma cells in central nervous system white matter. *J Cell Biol* 1999; **144**: 373-384 [PMID: 9922462 DOI: 10.1083/jcb.144.2.373]
95 **Deryugina EI**, Luo GX, Reisfeld RA, Bourdon MA, Strongin A. Tumor cell invasion through matrigel is regulated by activated matrix metalloproteinase-2. *Anticancer Res* 1997; **17**: 3201-3210 [PMID: 9413149]
96 **Ala-Aho R**, Johansson N, Baker AH, Kähäri VM. Expression of collagenase-3 (MMP-13) enhances invasion of human fibrosarcoma HT-1080 cells. *Int J Cancer* 2002; **97**: 283-289 [PMID: 11774278 DOI: 10.1002/ijc.1619]
97 **Noë V**, Fingleton B, Jacobs K, Crawford HC, Vermeulen S, Steelant W, Bruyneel E, Matrisian LM, Mareel M. Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci* 2001; **114**: 111-118 [PMID: 11112695]
98 **Sternlicht MD**, Lochter A, Sympson CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ, Werb Z. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 1999; **98**: 137-146 [PMID: 10428026 DOI: 10.1016/S0092-8674(00)81009-0]
99 **Lochter A**, Galosy S, Muschler J, Freedman N, Werb Z, Bissell MJ. Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J Cell Biol* 1997; **139**: 1861-1872 [PMID: 9412478 DOI: 10.1083/jcb.139.7.1861]
100 **Brooks PC**, Strömblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP, Cheresh DA. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* 1996; **85**: 683-693 [PMID: 8646777 DOI: 10.1016/S0092-8674(00)81235-0]
101 **Coussens LM**, Werb Z. Inflammatory cells and cancer: think different! *J Exp Med* 2001; **193**: F23-F26 [PMID: 11257144 DOI: 10.1084/jem.193.6.F23]
102 **Sheu BC**, Hsu SM, Ho HN, Lien HC, Huang SC, Lin RH. A novel role of metalloproteinase in cancer-mediated immunosuppression. *Cancer Res* 2001; **61**: 237-242 [PMID: 11196168]
103 **McQuibban GA**, Gong JH, Wong JP, Wallace JL, Clark-Lewis I, Overall CM. Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* 2002; **100**: 1160-1167 [PMID: 12149192]
104 **McQuibban GA**, Gong JH, Tam EM, McCulloch CA, Clark-Lewis I, Overall CM. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 2000; **289**: 1202-1206 [PMID: 10947989 DOI: 10.1126/science.289.5482.1202]
105 **Müller A**, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001; **410**: 50-56 [PMID: 11242036 DOI: 10.1038/35065016]
106 **Kataoka H**, Uchino H, Iwamura T, Seiki M, Nabeshima K, Koono M. Enhanced tumor growth and invasiveness in vivo by a carboxyl-terminal fragment of alpha1-proteinase inhibitor generated by matrix metalloproteinases: a possible modulatory role in natural killer cytotoxicity. *Am J Pathol* 1999; **154**: 457-468 [PMID: 10027404 DOI: 10.1016/S0002-9440(10)65292-3]
107 **Yu Q**, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 2000; **14**: 163-176 [PMID: 10652271 DOI: 10.1101/gad.14.2.163]
108 **Mañes S**, Mira E, Barbacid MM, Ciprés A, Fernández-Resa P, Buesa JM, Mérida I, Aracil M, Márquez G, Martínez-A C. Identification of insulin-like growth factor-binding protein-1 as a potential physiological substrate for human stromelysin-3. *J Biol Chem* 1997; **272**: 25706-25712 [PMID: 9325295]
109 **Murphy MP**. How mitochondria produce reactive oxygen species. *Biochem J* 2009; **417**: 1-13 [PMID: 19061483 DOI: 10.1042/BJ20081386]
110 **Gogvadze V**, Orrenius S, Zhivotovsky B. Mitochondria

in cancer cells: what is so special about them? *Trends Cell Biol* 2008; **18**: 165-173 [PMID: 18296052 DOI: 10.1016/j.tcb.2008.01.006]
111 **Meitzler JL**, Antony S, Wu Y, Juhasz A, Liu H, Jiang G, Lu J, Roy K, Doroshow JH. NADPH oxidases: a perspective on reactive oxygen species production in tumor biology. *Antioxid Redox Signal* 2014; **20**: 2873-2889 [PMID: 24156355 DOI: 10.1089/ars.2013.5603]
112 **Ibsen KH**. The Crabtree effect: a review. *Cancer Res* 1961; **21**: 829-841 [PMID: 13717358]
113 **Wu M**, Neilson A, Swift AL, Moran R, Tamagnine J, Parslow D, Armistead S, Lemire K, Orrell J, Teich J, Chomicz S, Ferrick DA. Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am J Physiol Cell Physiol* 2007; **292**: C125-C136 [PMID: 16971499 DOI: 10.1152/ajpcell.00247.2006]
114 **Ding SZ**, Minohara Y, Fan XJ, Wang J, Reyes VE, Patel J, Dirden-Kramer B, Boldogh I, Ernst PB, Crowe SE. Helicobacter pylori infection induces oxidative stress and programmed cell death in human gastric epithelial cells. *Infect Immun* 2007; **75**: 4030-4039 [PMID: 17562777 DOI: 10.1128/IAI.00172-07]
115 **Ganguly K**, Swarnakar S. Chronic gastric ulceration causes matrix metalloproteinases-9 and -3 augmentation: alleviation by melatonin. *Biochimie* 2012; **94**: 2687-2698 [PMID: 22959068 DOI: 10.1016/j.biochi.2012.08.004]
116 **Banerjee A**, Mukhopadhyay AK, Paul S, Bhattacharyya A, Swarnakar S. Unveiling the intricacies of helicobacter pylori-induced gastric inflammation: T helper cells and matrix metalloproteinases at a crossroad. Current topics in Gastritis-2012 Mozsik G, editor. *InTech* 2013 [DOI: 10.5772/46056]
117 **Hagen TM**, Huang S, Curnutte J, Fowler P, Martinez V, Wehr CM, Ames BN, Chisari FV. Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1994; **91**: 12808-12812 [PMID: 7809125]
118 **Reddy EP**, Reddy VS, Chandra Mouli K, Rao P. Physiological antioxidant system and oxidative stress in stomach cancer patients with normal renal and hepatic function. *Online J Health Allied Sci* 2010: 8
119 **Czygier M**, Kamocki Z, Ławicki S, Szmitkowski M. [The plasma level of myeloperoxidase (MPO) and total antioxidant status (TAS) in gastric cancer patients after surgery]. *Przegl Lek* 2010; **67**: 443-445 [PMID: 21387751]
120 **Noyan T**, Guducuoglu H, Ilhan M. A study of oxidative stress parameters in anti-helicobacter pylorus immunoglobulin g positive and negative gastric cancer patients. *Yonsei Med J* 2009; **50**: 677-682 [PMID: 19881972 DOI: 10.3349/ymj.2009.50.5.677]
121 **Huang J**, Lam GY, Brumell JH. Autophagy signaling through reactive oxygen species. *Antioxid Redox Signal* 2011; **14**: 2215-2231 [PMID: 20874258 DOI: 10.1089/ars.2010.3554]
122 **Gibson SB**. A matter of balance between life and death: targeting reactive oxygen species (ROS)-induced autophagy for cancer therapy. *Autophagy* 2010; **6**: 835-837 [PMID: 20818163 DOI: 10.4161/auto.6.7.13335]
123 **Kim MJ**, Woo SJ, Yoon CH, Lee JS, An S, Choi YH, Hwang SG, Yoon G, Lee SJ. Involvement of autophagy in oncogenic K-Ras-induced malignant cell transformation. *J Biol Chem* 2011; **286**: 12924-12932 [PMID: 21300795 DOI: 10.1074/jbc.M110.138958]
124 **Macip S**, Igarashi M, Fang L, Chen A, Pan ZQ, Lee SW, Aaronson SA. Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. *EMBO J* 2002; **21**: 2180-2188 [PMID: 11980715 DOI: 10.1093/emboj/21.9.2180]
125 **Yuan X**, Zhou Y, Wang W, Li J, Xie G, Zhao Y, Xu D, Shen L. Activation of TLR4 signaling promotes gastric cancer progression by inducing mitochondrial ROS production. *Cell Death Dis* 2013; **4**: e794 [PMID: 24030146 DOI: 10.1038/cddis.2013.334]
126 **Liu L**, Sun L, Zhao P, Yao L, Jin H, Liang S, Wang Y, Zhang D, Pang Y, Shi Y, Chai N, Zhang H, Zhang H. Hypoxia promotes metastasis in human gastric cancer by up-regulating the 67-kDa laminin receptor. *Cancer Sci* 2010; **101**: 1653-1660 [PMID: 20491781 DOI: 10.1111/j.1349-7006]
127 **Vafa O**, Wade M, Kern S, Beeche M, Pandita TK, Hampton GM, Wahl GM. c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. *Mol Cell* 2002; **9**: 1031-1044 [PMID: 12049739 DOI: 10.1016/S1097-2765(02)00520-8]
128 **Radisky DC**, Levy DD, Littlepage LE, Liu H, Nelson CM, Fata JE, Leake D, Godden EL, Albertson DG, Nieto MA, Werb Z, Bissell MJ. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* 2005; **436**: 123-127 [PMID: 16001073 DOI: 10.1038/nature03688]
129 **Tominaga K**, Kawahara T, Sano T, Toida K, Kuwano Y, Sasaki H, Kawai T, Teshima-Kondo S, Rokutan K. Evidence for cancer-associated expression of NADPH oxidase 1 (Nox1)-based oxidase system in the human stomach. *Free Radic Biol Med* 2007; **43**: 1627-1638 [PMID: 18037128 DOI: 10.1016/j.freeradbiomed.2007.08.029]
130 **Arbiser JL**, Petros J, Klafter R, Govindajaran B, McLaughlin ER, Brown LF, Cohen C, Moses M, Kilroy S, Arnold RS, Lambeth JD. Reactive oxygen generated by Nox1 triggers the angiogenic switch. *Proc Natl Acad Sci USA* 2002; **99**: 715-720 [PMID: 11805326]
131 **Block K**, Gorin Y. Aiding and abetting roles of NOX oxidases in cellular transformation. *Nat Rev Cancer* 2012; **12**: 627-637 [PMID: 22918415 DOI: 10.1038/nrc3339]
132 **Chen YR**, Shrivastava A, Tan TH. Down-regulation of the c-Jun N-terminal kinase (JNK) phosphatase M3/6 and activation of JNK by hydrogen peroxide and pyrrolidine dithiocarbamate. *Oncogene* 2001; **20**: 367-374 [PMID: 11313966]
133 **Chen N**, Nomura M, She QB, Ma WY, Bode AM, Wang L, Flavell RA, Dong Z. Suppression of skin tumorigenesis in c-Jun NH(2)-terminal kinase-2-deficient mice. *Cancer Res* 2001; **61**: 3908-3912 [PMID: 11358804]
134 **Behrens A**, Jochum W, Sibilia M, Wagner EF. Oncogenic transformation by ras and fos is mediated by c-Jun N-terminal phosphorylation. *Oncogene* 2000; **19**: 2657-2663 [PMID: 10851065]
135 **Benhar M**, Dalyot I, Engelberg D, Levitzki A. Enhanced ROS production in oncogenically transformed cells potentiates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase activation and sensitization to genotoxic stress. *Mol Cell Biol* 2001; **21**: 6913-6926 [PMID: 11564875 DOI: 10.1128/MCB.21.20.6913-6926.2001]
136 **Li Y**, Huang TT, Carlson EJ, Melov S, Ursell PC, Olson JL, Noble LJ, Yoshimura MP, Berger C, Chan PH, Wallace DC, Epstein CJ. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 1995; **11**: 376-381 [PMID: 7493016 DOI: 10.1038/ng1295-376]
137 **Van Remmen H**, Ikeno Y, Hamilton M, Pahlavani M, Wolf N, Thorpe SR, Alderson NL, Baynes JW, Epstein CJ, Huang TT, Nelson J, Strong R, Richardson A. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. *Physiol Genomics* 2003; **16**: 29-37 [PMID: 14679299]
138 **Elchuri S**, Oberley TD, Qi W, Eisenstein RS, Jackson Roberts L, Van Remmen H, Epstein CJ, Huang T-T. Cuznsod deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life. *Oncogene* 2004; **24**: 367-380 [PMID: 15531919 DOI: 10.1038/sj.onc.1208207]
139 **Ishii K**, Zhen LX, Wang DH, Funamori Y, Ogawa K, Taketa K. Prevention of mammary tumorigenesis in acatalasemic

mice by vitamin E supplementation. *Jpn J Cancer Res* 1996; **87**: 680-684 [PMID: 8698615]
140 **Chu FF**, Esworthy RS, Chu PG, Longmate JA, Huycke MM, Wilczynski S, Doroshow JH. Bacteria-induced intestinal cancer in mice with disrupted Gpx1 and Gpx2 genes. *Cancer Res* 2004; **64**: 962-968 [PMID: 14871826 DOI: 10.1158/0008-5472.CAN-03-2272]
141 **Kanbagli O**, Ozdemirler G, Bulut T, Yamaner S, Aykaç-Toker G, Uysal M. Mitochondrial lipid peroxides and antioxidant enzymes in colorectal adenocarcinoma tissues. *Jpn J Cancer Res* 2000; **91**: 1258-1263 [PMID: 11123424]
142 **Janssen AM**, Bosman CB, van Duijn W, Oostendorp-van de Ruit MM, Kubben FJ, Griffioen G, Lamers CB, van Krieken JH, van de Velde CJ, Verspaget HW. Superoxide dismutases in gastric and esophageal cancer and the prognostic impact in gastric cancer. *Clin Cancer Res* 2000; **6**: 3183-3192 [PMID: 10955802]
143 **Scibior D**, Skrzycki M, Podsiad M, Czeczot H. Glutathione level and glutathione-dependent enzyme activities in blood serum of patients with gastrointestinal tract tumors. *Clin Biochem* 2008; **41**: 852-858 [PMID: 18394427 DOI: 10.1016/j.clinbiochem.2008.03.005]
144 **Oberley LW**, Bize IB, Sahu SK, Leuthauser SW, Gruber HE. Superoxide dismutase activity of normal murine liver, regenerating liver, and H6 hepatoma. *J Natl Cancer Inst* 1978; **61**: 375-379 [PMID: 210289]
145 **Hernandez-Saavedra D**, McCord JM. Paradoxical effects of thiol reagents on Jurkat cells and a new thiol-sensitive mutant form of human mitochondrial superoxide dismutase. *Cancer Res* 2003; **63**: 159-163 [PMID: 12517793]
146 **Hwang TS**, Choi HK, Han HS. Differential expression of manganese superoxide dismutase, copper/zinc superoxide dismutase, and catalase in gastric adenocarcinoma and normal gastric mucosa. *Eur J Surg Oncol* 2007; **33**: 474-479 [PMID: 17129702]
147 **Yang CS**, Landau JM. Effects of tea consumption on nutrition and health. *J Nutr* 2000; **130**: 2409-2412 [PMID: 11015465]
148 **Morse MA**, Kresty LA, Steele VE, Kelloff GJ, Boone CW, Balentine DA, Harbowy ME, Stoner GD. Effects of theaflavins on N-nitrosomethylbenzylamine-induced esophageal tumorigenesis. *Nutr Cancer* 1997; **29**: 7-12 [PMID: 9383778 DOI: 10.1080/01635589709514595]
149 **Yang G**, Zheng W, Xiang YB, Gao J, Li HL, Zhang X, Gao YT, Shu XO. Green tea consumption and colorectal cancer risk: a report from the Shanghai Men's Health Study. *Carcinogenesis* 2011; **32**: 1684-1688 [PMID: 21856996 DOI: 10.1093/carcin/bgr186]
150 **Caderni G**, De Filippo C, Luceri C, Salvadori M, Giannini A, Biggeri A, Remy S, Cheynier V, Dolara P. Effects of black tea, green tea and wine extracts on intestinal carcinogenesis induced by azoxymethane in F344 rats. *Carcinogenesis* 2000; **21**: 1965-1969 [PMID: 11062155 DOI: 10.1093/carcin/21.11.1965]
151 **Peng G**, Dixon DA, Muga SJ, Smith TJ, Wargovich MJ. Green tea polyphenol (-)-epigallocatechin-3-gallate inhibits cyclooxygenase-2 expression in colon carcinogenesis. *Mol Carcinog* 2006; **45**: 309-319 [PMID: 16508969 DOI: 10.1002/mc.20166]
152 **Kim HS**, Kim MH, Jeong M, Hwang YS, Lim SH, Shin BA, Ahn BW, Jung YD. EGCG blocks tumor promoter-induced MMP-9 expression via suppression of MAPK and AP-1 activation in human gastric AGS cells. *Anticancer Res* 2004; **24**: 747-753 [PMID: 15161022]
153 **Annabi B**, Lachambre MP, Bousquet-Gagnon N, Page M, Gingras D, Beliveau R. Green tea polyphenol (-)-epigallocatechin 3-gallate inhibits MMP-2 secretion and MT1-MMP-driven migration in glioblastoma cells. *Biochim Biophys Acta* 2002; **1542**: 209-220 [PMID: 11853893 DOI: 10.1016/S0167-4889(01)00187-2]
154 **Onoda C**, Kuribayashi K, Nirasawa S, Tsuji N, Tanaka M, Kobayashi D, Watanabe N. (-)-Epigallocatechin-3-gallate induces apoptosis in gastric cancer cell lines by down-regulating survivin expression. *Int J Oncol* 2011; **38**: 1403-1408 [PMID: 21344159 DOI: 10.3892/ ijo.2011.951]
155 **Larsen CA**, Dashwood RH. (-)-Epigallocatechin-3-gallate inhibits Met signaling, proliferation, and invasiveness in human colon cancer cells. *Arch Biochem Biophys* 2010; **501**: 52-57 [PMID: 20361925 DOI: 10.1016/j.abb.2010.03.017]
156 **Cheng XW**, Kuzuya M, Nakamura K, Liu Z, Di Q, Hasegawa J, Iwata M, Murohara T, Yokota M, Iguchi A. Mechanisms of the inhibitory effect of epigallocatechin-3-gallate on cultured human vascular smooth muscle cell invasion. *Arterioscler Thromb Vasc Biol* 2005; **25**: 1864-1870 [PMID: 16051878 DOI: 10.1161/01.ATV.0000179675.49619.9b]
157 **El Bedoui J**, Oak MH, Anglard P, Schini-Kerth VB. Catechins prevent vascular smooth muscle cell invasion by inhibiting MT1-MMP activity and MMP-2 expression. *Cardiovasc Res* 2005; **67**: 317-325 [PMID: 15885676 DOI: 10.1016/j.cardiores.2005.03.017]
158 **Patel R**, Ingle A, Maru GB. Polymeric black tea polyphenols inhibit 1,2-dimethylhydrazine induced colorectal carcinogenesis by inhibiting cell proliferation via Wnt/beta-catenin pathway. *Toxicol Appl Pharmacol* 2008; **227**: 136-146 [PMID: 18037152 DOI: 10.1016/j.taap.2007.10.009]
159 **Hwang JT**, Ha J, Park IJ, Lee SK, Baik HW, Kim YM, Park OJ. Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway. *Cancer Lett* 2007; **247**: 115-121 [PMID: 16797120 DOI: 10.1016/j.canlet.2006.03.030]
160 **Shankar S**, Ganapathy S, Srivastava RK. Green tea polyphenols: biology and therapeutic implications in cancer. *Front Biosci* 2007; **12**: 4881-4899 [PMID: 17569617]
161 **Ammon HP**, Wahl MA. Pharmacology of Curcuma longa. *Planta Med* 1991; **57**: 1-7 [PMID: 2062949 DOI: 10.1055/s-2006-960004]
162 **Barzegar A**, Moosavi-Movahedi AA. Intracellular ROS protection efficiency and free radical-scavenging activity of curcumin. *PLoS One* 2011; **6**: e26012 [PMID: 22016801]
163 **Swarnakar S**, Ganguly K, Kundu P, Banerjee A, Maity P, Sharma AV. Curcumin regulates expression and activity of matrix metalloproteinases 9 and 2 during prevention and healing of indomethacin-induced gastric ulcer. *J Biol Chem* 2005; **280**: 9409-9415 [PMID: 15615723 DOI: 10.1074/jbc.M413398200]
164 **Jana S**, Paul S, Swarnakar S. Curcumin as anti-endometriotic agent: implication of MMP-3 and intrinsic apoptotic pathway. *Biochem Pharmacol* 2012; **83**: 797-804 [PMID: 22227273 DOI: 10.1016/j.bcp.2011.12.030]
165 **Rajasekaran SA**. Therapeutic potential of curcumin in gastrointestinal diseases. *World J Gastrointest Pathophysiol* 2011; **2**: 1-14 [PMID: 21607160 DOI: 10.4291/wjgp.v2.i1.1]
166 **Aggarwal BB**, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res* 2003; **23**: 363-398 [PMID: 12680238]
167 **Nagpal M**, Sood S. Role of curcumin in systemic and oral health: An overview. *J Nat Sci Biol Med* 2013; **4**: 3-7
168 **Koo JY**, Kim HJ, Jung KO, Park KY. Curcumin inhibits the growth of AGS human gastric carcinoma cells in vitro and shows synergism with 5-fluorouracil. *J Med Food* 2004; **7**: 117-121 [PMID: 15298755 DOI: 10.1089/1096620041224229]
169 **Tang XQ**, Bi H, Feng JQ, Cao JG. Effect of curcumin on multidrug resistance in resistant human gastric carcinoma cell line SGC7901/VCR. *Acta Pharmacol Sin* 2005; **26**: 1009-1016 [PMID: 16038636 DOI: 10.1111/j.1745-7254]
170 **Anand P**, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB. Curcumin and cancer: an "old-age" disease with an "age-old" solution. *Cancer Lett* 2008; **267**: 133-164 [PMID: 18462866 DOI: 10.1016/j.canlet.2008.03.025]
171 **Perkins S**, Verschoyle RD, Hill K, Parveen I, Threadgill MD, Sharma RA, Williams ML, Steward WP, Gescher AJ. Che-

mopreventive efficacy and pharmacokinetics of curcumin in the min/+ mouse, a model of familial adenomatous polyposis. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 535-540 [PMID: 12050094]

172 **Kim JM**, Araki S, Kim DJ, Park CB, Takasuka N, Baba-Toriyama H, Ota T, Nir Z, Khachik F, Shimidzu N, Tanaka Y, Osawa T, Uraji T, Murakoshi M, Nishino H, Tsuda H. Chemopreventive effects of carotenoids and curcumins on mouse colon carcinogenesis after 1,2-dimethylhydrazine initiation. *Carcinogenesis* 1998; **19**: 81-85 [PMID: 9472697 DOI: 10.1093/carcin/19.1.81]

173 **Shpitz B**, Giladi N, Sagiv E, Lev-Ari S, Liberman E, Kazanov D, Arber N. Celecoxib and curcumin additively inhibit the growth of colorectal cancer in a rat model. *Digestion* 2006; **74**: 140-144 [PMID: 17228149 DOI: 10.1159/000098655]

174 **Huang MT**, Lou YR, Ma W, Newmark HL, Reuhl KR, Conney AH. Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. *Cancer Res* 1994; **54**: 5841-5847 [PMID: 7954412]

175 **Bimonte S**, Barbieri A, Palma G, Luciano A, Rea D, Arra C. Curcumin inhibits tumor growth and angiogenesis in an orthotopic mouse model of human pancreatic cancer. *Biomed Res Int* 2013; **2013**: 810423 [PMID: 24324975 DOI: 10.1155/2013/810423]

176 **Kunnumakkara AB**, Diagaradjane P, Guha S, Deorukhkar A, Shentu S, Aggarwal BB, Krishnan S. Curcumin sensitizes human colorectal cancer xenografts in nude mice to gamma-radiation by targeting nuclear factor-kappaB-regulated gene products. *Clin Cancer Res* 2008; **14**: 2128-2136 [PMID: 18381954]

177 **Lin LI**, Ke YF, Ko YC, Lin JK. Curcumin inhibits SK-Hep-1 hepatocellular carcinoma cell invasion in vitro and suppresses matrix metalloproteinase-9 secretion. *Oncology* 1998; **55**: 349-353 [PMID: 9663426]

178 **Su CC**, Chen GW, Lin JG, Wu LT, Chung JG. Curcumin inhibits cell migration of human colon cancer colo 205 cells through the inhibition of nuclear factor kappa B /p65 and down-regulates cyclooxygenase-2 and matrix metalloproteinase-2 expressions. *Anticancer Res* 2006; **26**: 1281-1288 [PMID: 16619535]

179 **Lin ML**, Lu YC, Chen HY, Lee CC, Chung JG, Chen SS. Suppressing the formation of lipid raft-associated Rac1/PI3K/Akt signaling complexes by curcumin inhibits SDF-1α-induced invasion of human esophageal carcinoma cells. *Mol Carcinog* 2014; **53**: 360-379 [PMID: 23192861 DOI: 10.1002/mc.21984]

180 **Goswami SK**, Das DK. Resveratrol and chemoprevention. *Cancer Lett* 2009; **284**: 1-6 [PMID: 19261378 DOI: 10.1016/j.canlet.2009]

181 **Li ZG**, Hong T, Shimada Y, Komoto I, Kawabe A, Ding Y, Kaganoi J, Hashimoto Y, Imamura M. Suppression of N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumorigenesis in F344 rats by resveratrol. *Carcinogenesis* 2002; **23**: 1531-1536 [PMID: 12189197 DOI: 10.1093/carcin/23.9.1531]

182 **Schneider Y**, Duranton B, Gossé F, Schleiffer R, Seiler N, Raul F. Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis. *Nutr Cancer* 2001; **39**: 102-107 [PMID: 11588890 DOI: 10.1207/S15327914nc391_14]

183 **Li W**, Ma J, Ma Q, Li B, Han L, Liu J, Xu Q, Duan W, Yu S, Wang F, Wu E. Resveratrol inhibits the epithelial-mesenchymal transition of pancreatic cancer cells via suppression of the PI-3K/Akt/NF-κB pathway. *Curr Med Chem* 2013; **20**: 4185-4194 [PMID: 23992306]

184 **Ji Q**, Liu X, Fu X, Zhang L, Sui H, Zhou L, Sun J, Cai J, Qin J, Ren J, Li Q. Resveratrol inhibits invasion and metastasis of colorectal cancer cells via MALAT1 mediated Wnt/β-catenin signal pathway. *PLoS One* 2013; **8**: e78700 [PMID: 24244343 DOI: 10.1371/journal.pone.0078700]

185 **Weng CJ**, Wu CF, Huang HW, Wu CH, Ho CT, Yen GC. Evaluation of anti-invasion effect of resveratrol and related methoxy analogues on human hepatocarcinoma cells. *J Agric Food Chem* 2010; **58**: 2886-2894 [PMID: 20131808 DOI: 10.1021/jf904182y]

186 **Harikumar KB**, Kunnumakkara AB, Sethi G, Diagaradjane P, Anand P, Pandey MK, Gelovani J, Krishnan S, Guha S, Aggarwal BB. Resveratrol, a multitargeted agent, can enhance antitumor activity of gemcitabine in vitro and in orthotopic mouse model of human pancreatic cancer. *Int J Cancer* 2010; **127**: 257-268 [PMID: 19908231 DOI: 10.1002/ijc.25041]

187 **Gibellini L**, Pinti M, Nasi M, Montagna JP, De Biasi S, Roat E, Bertoncelli L, Cooper EL, Cossarizza A. Quercetin and cancer chemoprevention. *Evid Based Complement Alternat Med* 2011; **2011**: 591356 [PMID: 21792362 DOI: 10.1093/ecam/neq053]

188 **Volate SR**, Davenport DM, Muga SJ, Wargovich MJ. Modulation of aberrant crypt foci and apoptosis by dietary herbal supplements (quercetin, curcumin, silymarin, ginseng and rutin). *Carcinogenesis* 2005; **26**: 1450-1456 [PMID: 15831530 DOI: 10.1093/carcin/bgi089]

189 **Dihal AA**, de Boer VC, van der Woude H, Tilburgs C, Bruijntjes JP, Alink GM, Rietjens IM, Woutersen RA, Stierum RH. Quercetin, but not its glycosidated conjugate rutin, inhibits azoxymethane-induced colorectal carcinogenesis in F344 rats. *J Nutr* 2006; **136**: 2862-2867 [PMID: 17056814]

190 **Lai WW**, Hsu SC, Chueh FS, Chen YY, Yang JS, Lin JP, Lien JC, Tsai CH, Chung JG. Quercetin inhibits migration and invasion of SAS human oral cancer cells through inhibition of NF-κB and matrix metalloproteinase-2/-9 signaling pathways. *Anticancer Res* 2013; **33**: 1941-1950 [PMID: 23645742]

191 **Reiter RJ**, Tan DX, Manchester LC, Simopoulos AP, Maldonado MD, Flores LJ, Terron MP. Melatonin in edible plants (phytomelatonin): Identification, concentrations, bioavailability and proposed functions. *World Rev Nutr Diet* 2007; **97**: 211-230 [PMID: 17167296]

192 **Rudra DS**, Pal U, Maiti NC, Reiter RJ, Swarnakar S. Melatonin inhibits matrix metalloproteinase-9 activity by binding to its active site. *J Pineal Res* 2013; **54**: 398-405 [PMID: 23330737 DOI: 10.1111/jpi.12034]

193 **Xu L**, Liu H, Zhang H, Wang RX, Song J, Zhou RX. Growth-inhibitory activity of melatonin on murine foregastric carcinoma cells in vitro and the underlying molecular mechanism. *Anat Rec* (Hoboken) 2013; **296**: 914-920 [PMID: 23564716 DOI: 10.1002/ar.22689]

194 **Nemeth C**, Humpeler S, Kallay E, Mesteri I, Svoboda M, Rögelsperger O, Klammer N, Thalhammer T, Ekmekcioglu C. Decreased expression of the melatonin receptor 1 in human colorectal adenocarcinomas. *J Biol Regul Homeost Agents* 2011; **25**: 531-542 [PMID: 22217986]

195 **Hong Y**, Won J, Lee Y, Lee S, Park K, Chang KT, Hong Y. Melatonin treatment induces interplay of apoptosis, autophagy, and senescence in human colorectal cancer cells. *J Pineal Res* 2014; **56**: 264-274 [PMID: 24484372 DOI: 10.1111/jpi.12119]

196 **Ordoñez R**, Carbajo-Pescador S, Prieto-Dominguez N, García-Palomo A, González-Gallego J, Mauriz JL. Inhibition of matrix metalloproteinase-9 and nuclear factor kappa B contribute to melatonin prevention of motility and invasiveness in HepG2 liver cancer cells. *J Pineal Res* 2014; **56**: 20-30 [PMID: 24117795 DOI: 10.1111/jpi.12092]

197 **Tang FY**, Pai MH, Kuo YH, Wang XD. Concomitant consumption of lycopene and fish oil inhibits tumor growth and progression in a mouse xenograft model of colon cancer. *Mol Nutr Food Res* 2012; **56**: 1520-1531 [PMID: 22961879 DOI: 10.1002/mnfr.201200098]

198 **Lin MC**, Wang FY, Kuo YH, Tang FY. Cancer chemopreventive effects of lycopene: suppression of MMP-7 expression

and cell invasion in human colon cancer cells. *J Agric Food Chem* 2011; **59**: 11304-11318 [PMID: 21923160 DOI: 10.1021/jf202433f]

199 **Adachi Y**, Itoh F, Yamamoto H, Iku S, Matsuno K, Arimura Y, Imai K. Retinoic acids reduce matrilysin (matrix metalloproteinase 7) and inhibit tumor cell invasion in human colon cancer. *Tumour Biol* 2001; **22**: 247-253 [PMID: 11399950]

200 **Park EY**, Wilder ET, Lane MA. Retinol inhibits the invasion of retinoic acid-resistant colon cancer cells in vitro and decreases matrix metalloproteinase mRNA, protein, and activity levels. *Nutr Cancer* 2007; **57**: 66-77 [PMID: 17516864 DOI: 10.1080/01635580701268238]

201 **Tanaka T**, Shnimizu M, Moriwaki H. Cancer chemoprevention by carotenoids. *Molecules* 2012; **17**: 3202-3242 [PMID: 22418926 DOI: 10.3390/molecules17033202]

202 **Liu HK**, Wang Q, Li Y, Sun WG, Liu JR, Yang YM, Xu WL, Sun XR, Chen BQ. Inhibitory effects of gamma-tocotrienol on invasion and metastasis of human gastric adenocarcinoma SGC-7901 cells. *J Nutr Biochem* 2010; **21**: 206-213 [PMID: 19195866 DOI: 10.1016/j.jnutbio]

203 **Roomi MW**, Ivanov V, Kalinovsky T, Niedzwiecki A, Rath M. In vivo antitumor effect of ascorbic acid, lysine, proline and green tea extract on human colon cancer cell HCT 116 xenografts in nude mice: evaluation of tumor growth and immunohistochemistry. *Oncol Rep* 2005; **13**: 421-425 [PMID: 15706410]

204 **Manu KA**, Shanmugam MK, Ramachandran L, Li F, Fong CW, Kumar AP, Tan P, Sethi G. First evidence that γ-tocotrienol inhibits the growth of human gastric cancer and chemosensitizes it to capecitabine in a xenograft mouse model through the modulation of NF-κB pathway. *Clin Cancer Res* 2012; **18**: 2220-2229 [PMID: 22351692 DOI: 10.1158/1078-0432.CCR-11-2470]

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ORIGINAL ARTICLE

Effects of acute doxorubicin treatment on hepatic proteome lysine acetylation status and the apoptotic environment

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Abstract

AIM: To determine if doxorubicin (Dox) alters hepatic proteome acetylation status and if acetylation status was associated with an apoptotic environment.

METHODS: Doxorubicin (20 mg/kg; Sigma, Saint Louis, MO; *n* = 8) or NaCl (0.9%; *n* = 7) was administered as an intraperitoneal injection to male F344 rats, 6-wk of age. Once animals were treated with Dox or saline, all animals were fasted until sacrifice 24 h later.

RESULTS: Dox treatment decreased proteome lysine acetylation likely due to a decrease in histone acetyltransferase activity. Proteome deacetylation may likely not be associated with a proapoptotic environment. Dox did not increase caspase-9, -8, or -3 activation nor poly (adenosine diphosphate-ribose) polymerase-1 cleavage. Dox did stimulate caspase-12 activation, however, it likely did not play a role in apoptosis induction.

CONCLUSION: Early effects of Dox involve hepatic proteome lysine deacetylation and caspase-12 activation under these experimental conditions.

Key words: Sirtuin 1; Sirtuin 3; Caspase; Apoptosis; Acetylation; Histone deacetylase; Histone acetyltransferase

Core tip: Doxorubicin (Dox) is an effective chemotherapeutic agent, but known to cause cardiotoxicity and hepatotoxicity. Cellular stress can alter proteome acetylation status in various experimental models, which has been associated with a proapoptotic environment. The effects of Dox on hepatic lysine acetylation status has not been studied. The study revealed five interesting findings that open the door for new areas of investigation: (1) Dox induces proteome lysine deacetylation; (2) lysine deacetylation is, at least in part, due to a decrease in histone acetyltransferase activity; (3) lysine deacetylation is likely not associated with an apoptotic environment; (4) Dox-induced hepatic injury is associated with caspase-12 activation; and (5) caspase-12 activation is not involved in apoptosis induction. These results may in the future translate to lysine acetylation homeostasis and/or caspase-12 as therapeutic targets.

Dirks-Naylor AJ, Kouzi SA, Bero JD, Tran NTK, Yang S, Mabolo R. Effects of acute doxorubicin treatment on hepatic proteome lysine acetylation status and the apoptotic environment. *World J Biol Chem* 2014; 5(3): 377-386 Available from: URL: http://www.wjgnet.com/1949-8454/full/v5/i3/377.htm DOI: http://dx.doi.org/10.4331/wjbc.v5.i3.377

INTRODUCTION

Doxorubicin (Dox) is a highly effective therapeutic agent

in treating various cancers, but proven to cause cardiotoxicity. Mechanisms of cardiotoxicity have not been distinctly defined, but published literature has suggested that the induction of oxidative stress, apoptosis and mitochondrial dysfunction are involved[1-3]. In addition to proven toxicity in cardiac tissue, hepatic tissue reveals molecular and morphological signs of toxicity[4-9]. Since oxidative stress has been shown to play a role in both cardiac and hepatic toxicity, the use of antioxidants as a preventative measure has been widely studied. However, supplementation with antioxidants in clinical trials have only shown limited protection[10]. Thus, additional research is essential to determine additional mechanisms involved in Dox-induced toxicities. Therefore, the aim of this study was to determine the role of proteome lysine acetylation dyshomeostasis and apoptosis in Dox-induced hepatic toxicity.

Lysine acetylation is a common posttranslational modification[11]. Lysine acetylation regulates function of histones, proteins affecting the ubiquitin-proteosome system, transcription factors, cytoskeletal components, energy generating enzymes, and oxidative stress defense proteins[11]. In various experimental models, cellular stress has been shown to alter proteome lysine acetylation status, causing deacetylation, and thereby affecting the apoptotic environment[12]. Moreover, normalization of the lysine acetylation status has been shown to prevent stimulation of apoptotic pathways[12]. The effects of Dox on proteome acetylation status is unknown, thus, we aimed to make this determination. We hypothesized that Dox-induced cellular injury and stress would lead to lysine deacetylation and may contribute to Dox-induced toxicity. Acetylation status is determined by activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs)[11,13]. Thus, we also investigated potential mechanisms of deacetylation by assessing general HDAC activity and expression of sirtuins, one of several known classes of HDACs, as well as HAT activity.

Secondly, we aimed to determine if the proteome lysine acetylation status was associated with a proapoptotic environment, as shown in other experimental models. Apoptosis can be activated by a variety of specific signaling pathways (*e.g.*, receptor-mediated, mitochondrial-mediated, endoplasmic reticulum (ER)-mediated, *etc.*), depending on the initiating stimulus, and is associated with a distinct apical caspase[14]. These apical caspases converge on caspase-3, which is considered the central executioner of apoptosis. Activation of caspase-3 is responsible for the demise of the cell and most of the characteristic morphology associated with apoptosis, such as DNA fragmentation, cell shrinkage, and membrane blebbing. Thus, we assessed the content and activation of apical caspases and the activity of caspase-3 to determine if the proteome acetylation status was associated with the apoptotic environment in this experimental model. Furthermore, the effects of Dox on apical caspases and their role in hepatic toxicity have not been previously studied.

MATERIALS AND METHODS

Animals and experimental design

The institutional Research Review Board approved all protocols and procedures. Male F344 rats, 6-weeks of age, were purchased from Charles River (Wilmington, MA). The rats were arbitrarily separated into groups and were housed in a light controlled and temperature (22 ℃ ± 2 ℃) controlled facility. Doxorubicin (20 mg/kg; Sigma, Saint Louis, MO; n = 8) or NaCl (0.9%; n = 7) was injected intraperitoneal (IP). The dose of doxorubicin used in this study is equivalent to clinical doses used in humans that are pharmacologically scaled for use in rats[15,16]. Food and water was withheld from all animals during the experimental period (24 h). Doxorubicin administration can cause up to an approximate 70% decrease in both food and water intake within several hours of administration and typically persists for numerous days[17]. All other investigations examining the effects of Dox on the liver in laboratory animals did not control for differences in food and water intake, thus the Dox group was nutrient deficient while the control group received plenty of food and water. As a result, it was not possible to distinguish between the effects of Dox and the effects of anorexia. Anorexia has been shown to alter hepatic oxidative stress, autophagy, mitochondrial morphology, survival signaling, and many more processes[18-21]. Thus, to control for Dox-induced anorexia we fasted all animals. After exposure to ether, rats were sacrificed by dislocation of the cervical spine. Tissues were excised immediately following sacrifice, saline rinsed and frozen in liquid nitrogen. Tissues were stored at -80 ℃ until analysis.

Western analysis

Liver samples were homogenized (Power Gen 125, Fisher Scientific, Pittsburgh, PA) in ice-cold phosphate-buffered saline (2.68 mmol/L KCl , 1.75 mmol/L KH_2PO_4, 137 mmol/L NaCl, 10 mmol/L Na_2HPO_4, 5 mmol/L EDTA). Ten uL/mL of Halt Phosphatase Inhibitor Cocktail and 10 uL/mL of Halt Protease Inhibitor Cocktail (Pierce Biochemicals, Rockford, IL) was added to the buffer immediately before homogenization. The homogenate was centrifuged at 660 × g at 4 ℃ for 10 min. The supernatant was used for biochemical analysis. The Bicinchoninic Acid Protein Assay Kit (Sigma, Saint Louis, MO) was used to assess protein concentration. Samples were run in quadruplicate.

Protein (proteome) lysine acetylation and protein content of sirtuin 1 (Sirt1), sirtuin 3 (Sirt3), poly (ADP-ribose) polymerase-1 (PARP-1), and procaspase-1, -8, -9, -12 were determined by standard wet Western blot analysis. Proteins (50 ug) were separated on tris/glycine 4%-20% separating polyacrylamide PAGEr Gold Precast Gels (Lonza, Rockland, ME) under denaturing conditions and transferred to nitrocellulose membranes. Membranes were blocked in PBS blocking solution containing 5.0% powdered milk for one hour at room temperature. Membranes were incubated in primary antibody overnight

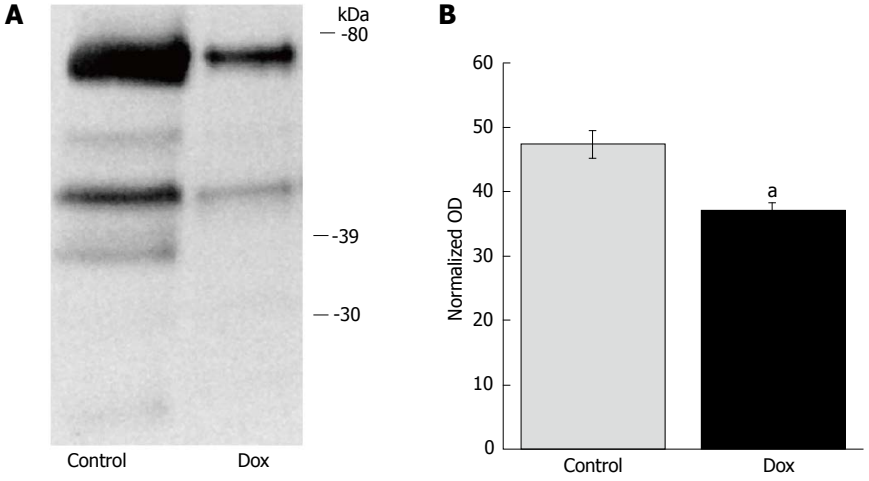

Figure 1 Proteome lysine acetylation status. A: Representative Western blot showing doxorubicin (Dox)-induced proteome lysine deacetylation; B: Graphical representation of OD units determined by whole lane analysis and normalized to Ponceau staining. $^{a}P < 0.050$, control *vs* Dox.

at 4 ℃ (dilution of 1:1000; antibodies were purchased from Santa Cruz Biotechnology, INC, Santa Cruz, CA, sc-137254, sc-7150, sc-56036, sc-166320, sc-81663, sc-21747, sc-271014, sc-15404, sc-99143, sc-32268). Membranes were incubated with secondary HRP-linked antibody, with a dilution of 1:10000, for two hours shaking at room temperature. Bands of interest were imaged using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biochemicals, Rockford, IL) and the Kodak IS4000R Imaging System (Carestream Health, Inc., New Haven, CT). To assess equal loading of protein, ponceau staining (Pierce Biochemicals, Rockford, IL) of the nitrocellulose membranes was used. Whole lane analysis for each sample was used to determine densitometry of Ponceau staining. Data are presented as arbitrary units of densitometry calculated by subtracting the background intensity from the mean intensity of each band. Arbitrary OD for each band was normalized to the densitometry of Ponceau staining of each lane to account for variances in loading. The utilization of Ponceau staining as a reproducible alternative to actin in assessing equal loading has been validated [22].

HDAC activity

An HDAC Colorimetric Assay (BioVision, Milpitas, CA) was purchased to determine HDAC activity. The manufacturer's instructions were followed. Samples were run in triplicate. Data are expressed as arbitrary OD/protein concentration as determined by the BCA Assay.

HAT activity

Histone acetyltransferse activity was measured using a HAT Colorimetric Assay Kit (BioVision, Milpitas, CA). The manufacturer's instructions were followed. Samples were run in triplicate. Data are expressed as arbitrary OD/protein concentration as determined by the BCA Assay.

Caspase-3 activity

A Caspase-3 Colorimetric Assay Kit (BioVision, Milpitas, CA) was used to determine caspase-3 activity. The manufacturer's instructions were followed. Samples were run in triplicate. Data are expressed as arbitrary OD/protein concentration as determined by the BCA Assay.

Statistical analysis

For statistical analysis, a Student's *t*-test was used. $P < 0.05$ was deemed statistically significant. Data is presented as mean ± SEM. For all analysis the $n = 8$ for the Dox group and $n = 7$ for the control group.

RESULTS

Proteome lysine acetylation status

Since proteome lysine deacetylation has been associated with pro-apoptotic environment and cellular injury in alternative experimental models, we determined the effects of Dox on acetylation status in the liver. Dox promoted proteome lysine deacetylation of treated animals (42.88 ± 2.06 *vs* 32.03 ± 1.00, $P = 0.002$; control *vs* Dox, respectively; Figure 1). Proteins of molecular weight between 30-80 kDa appear to be most affected, as we did not observe protein bands detected by the lysine acetylation antibody above or below these molecular weights. These results suggest that early effects of acute Dox toxicity involve proteome lysine deacetylation.

Protein content of Sirt1 and Sirt3

Sirtuins are a class of HDACs that have recently been under intense investigation, in part, due to their potential role in longevity[23]. To investigate the potential mechanism of Dox-induced deacetylation, we investigated the effects of Dox on the expression of Sirt1 and Sirt3, two of the most studied lysine deacetylases in the sirtuin family. Despite significant deacetylation, Sirt1 content was not affected by Dox treatment (128.57 ± 6.10 *vs* 122.89 ± 4.09, control *vs* Dox, respectively, $P = 0.44$) nor Sirt3 (27.14 ± 2.69 *vs* 28.00 ± 2.28, control *vs* Dox, respectively, $P = 0.81$; Figure 2).

Interestingly, a prominent band of approximately 45 kDa was detected in the liver with the Sirt1 antibody. Dox treatment increased the content of this species compared to control (139. 0 ± 4.25 *vs* 107.86 ± 4.06, respectively, $P = 0.0001$). It is possible that this species is

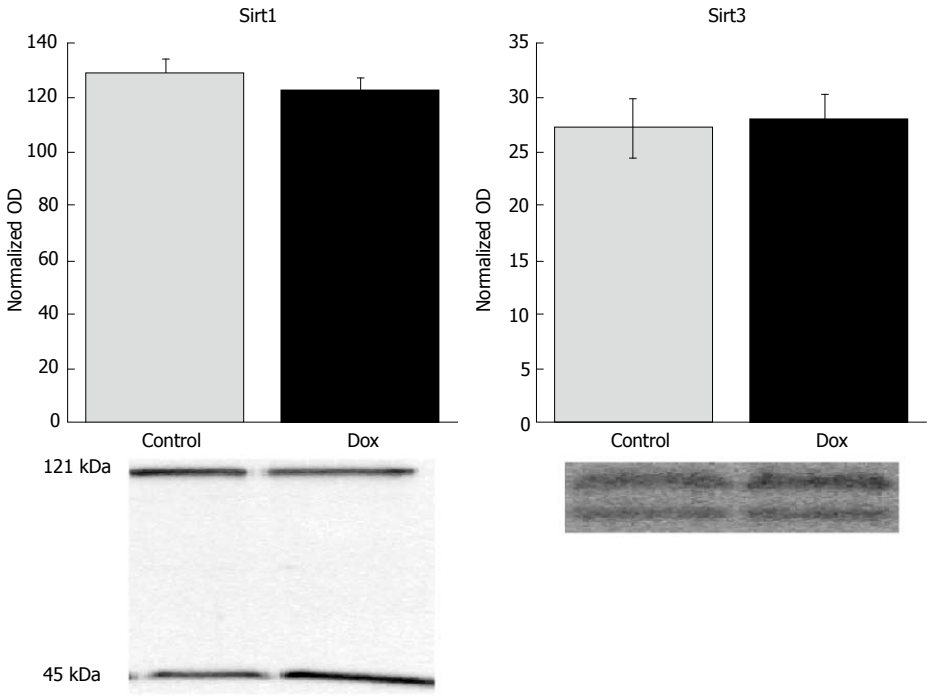

Figure 2 Protein content of Sirt1 and Sirt3. Doxorubicin (Dox) did not affect the expression of Sirt1 or Sirt 3 in liver of treated animals. Representative Western blot shows 120 kDa Sirt1. A 45 kDa species detected by the Sirt1 antibody is also shown. Sirt: Sirtuin.

a cleaved fragment of Sirt1. A recent study has shown, in chondrocytes, that Sirt1 can be cleaved into a 75 kDa fragment that plays a role in regulation of apoptosis[24]. The antibody used in this study does not target the 75 kDa fragment, however, the species detected may be the approximately 45 kDa remnant of the cleaved Sirt1.

HDAC activity

We also assessed general HDAC activity to determine if deacetylases might be responsible for the Dox-induced proteome lysine deacetylation. We did not observe a difference between Dox treated and control animals (0.120 ± 0.020 *vs* 0.151 ± 0.017, respectively, $P = 0.29$; Figure 3A). Thus, the data suggests that HDACs may not be responsible for the observed proteome lysine deacetylation in Dox treated animals.

HAT activity

Since the proteome deacetylation did not appear to be due to increased HDAC activity, we assessed HAT activity. Dox decreased hepatic HAT activity (0.209 ± 0.006 *vs* 0.189 ± 0.004, $P = 0.017$, Figure 3B). Thus, the data suggest that the Dox-induced proteome deacetylation is, at least in part, due to decreased HAT activity in livers of treated animals.

Expression and activation of apical caspases

Caspase-9: To evaluate the influence of Dox treatment on the stimulation of apoptosis mediated by mitochondria, we determined the content of procaspase-9 and the presence of any cleavage products representing the active caspase. Dox treatment increased procaspase-9 content in the liver (46.86 ± 1.66 *vs* 56.22 ± 1.96, control *vs* Dox, respectively, $P = 0.003$; Figure 4). No cleavage products were detected. The results suggest that activation of the mitochondrial-mediated apoptotic pathway may not contribute to early mechanisms of hepatic toxicity.

Caspase-8: To evaluate the influence of Dox treatment on the stimulation of receptor-mediated apoptosis, we determined the content of procaspase-8 and any potential cleavage products. Dox treatment did not affect content of procaspase-8 or its activation (33.81 ± 2.38 *vs* 33.99 ± 2.40, control *vs* Dox, respectively, $P = 0.96$; Figure 5). There was no statistical difference in the presence of lower molecular weight bands between treatment groups (data not shown). The results suggest that activation of caspase-8 by receptor-mediated mechanisms may not contribute to early mechanisms causing acute hepatic toxicity.

Caspase-12: Caspase-12 has been implicated in stimulating apoptosis in response to ER dysfunction and calcium dyshomeostasis[25-27], therefore, we assessed the content of procaspase-12 and any potential cleavage products. Hepatic procaspase-12 (50 kDa) content in Dox treated animals tended to be lower than those of control animals (38.66 ± 5.82 *vs* 56.0 ± 6.98, respectively) , however statistical significance was not reached ($P = 0.075$). The content of the cleavage product of caspase-12 (40 kDa) was significantly increased by Dox treatment (78.11 ± 7.73 *vs* 43.0 ± 5.91, Dox *vs* control, respectively, $P = 0.004$; Figure 6).

Caspase-1: Caspase-1 is an inflammatory caspase involved in the processing of proinflammatory cytokines.

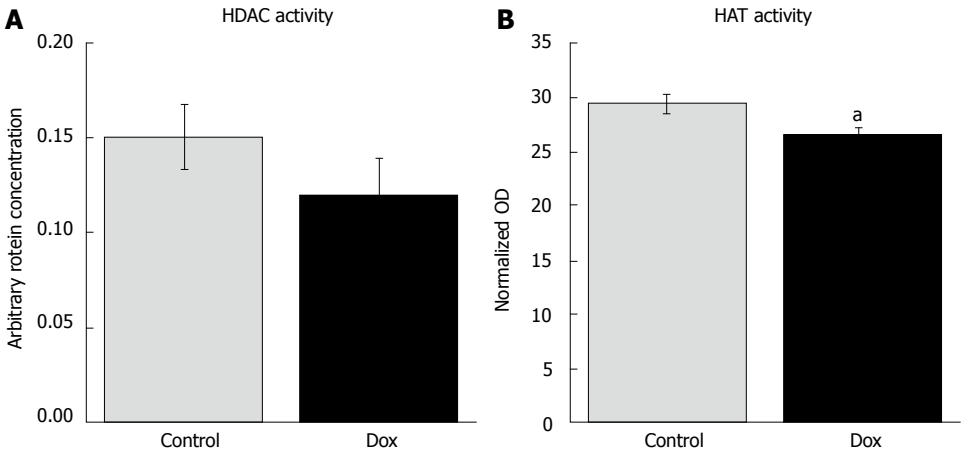

Figure 3 Histone deacetylase and histone acetyltransferase activity. A: Conventional histone deacetylase (HDAC) activity was not affected by doxorubicin (Dox) treatment; B: Dox decreased hepatic histone acetyltransferase (HAT) activity. $^{a}P < 0.050$, 0.209 ± 0.006 *vs* 0.189 ± 0.004.

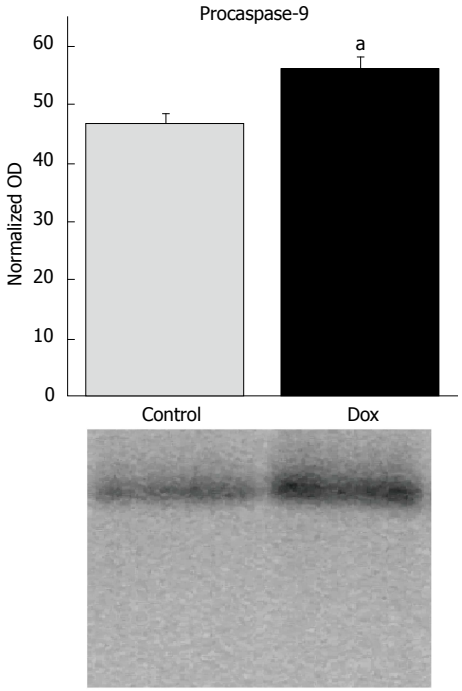

Figure 4 Protein content of procaspase-9. Doxorubicin (Dox) increased the hepatic expression of procaspase-9. Representative Western blots show the band corresponding to procaspase-9; no bands of lower molecular weight representing cleavage products were present. $^{a}P < 0.050$, control *vs* Dox.

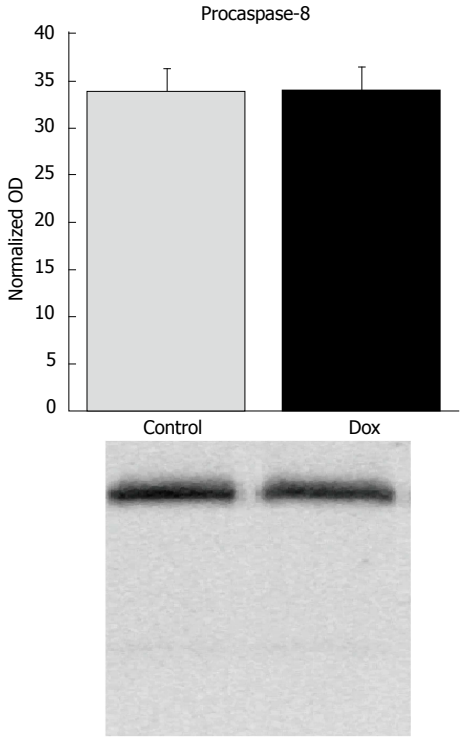

Figure 5 Protein content of procaspase-8. Doxorubicin (Dox) did not affect hepatic content of procaspase-8. Furthermore, there was no difference in the presence of lower molecular weight bands between treatment groups.

Aside from its implicated role in apoptosis, caspase-12 has been shown to play a role in the inflammatory process[28]. Caspase-12 is a dominant negative effector of caspase-1. Since we observed processing of caspase-12, we assessed expression and activation of caspase-1 to determine if processing of caspase-12 affected expression or activation of caspase-1. Dox treatment did not affect expression of procaspase-1 (52.9 ± 10.33 *vs* 38.2 ± 6.23, control *vs* Dox, respectively, $P = 0.22$). There was no statistical difference in the presence of lower molecular weight bands between treatment groups (data not shown), suggesting that Dox did not lead to its activation (Figure 7).

Markers of apoptosis: Caspase-3 is considered the central executioner of apoptosis and cleaves a plethora of substrates which lead to the demise of the cell and to the classical morphological characteristics of apoptosis, such as DNA fragmentation. Thus, we measured caspase-3 activity to assess activation of apoptosis. Dox did not affect the activity of caspase-3 activity in treated animals (0.193 ± 0.061) compared to control animals (0.134 ± 0.016, $P = 0.45$, Figure 8A). PARP-1 is an enzyme involved in DNA repair. Upon stimulation of apoptosis, PARP-1 is cleaved by various proteases, including caspase-3, in order to disengage the repair process. Thus, PARP-1 cleavage

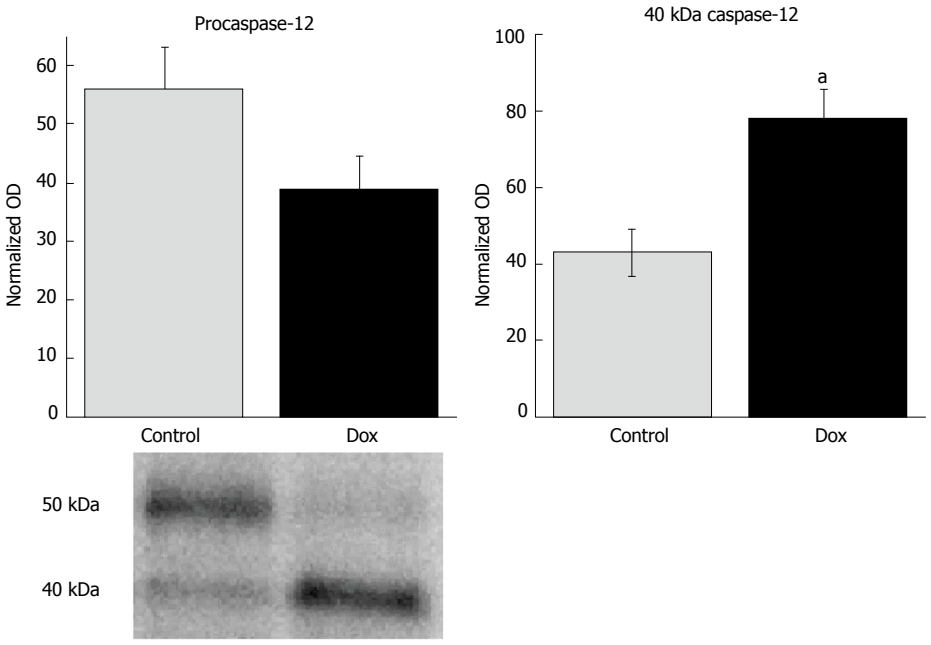

Figure 6 Protein content of procaspase-12 and 40 kDa cleavage product. Doxorubicin (Dox) treatment did not significantly affect the hepatic content of procaspase-12. However, Dox did induce activation of caspase-12 leading to increased content of the 40 kDa cleavage product. Representative Western blot shows the 50 kDa procaspase-12 and the 40 kDa cleaved product. [a]$P < 0.050$, Dox *vs* control.

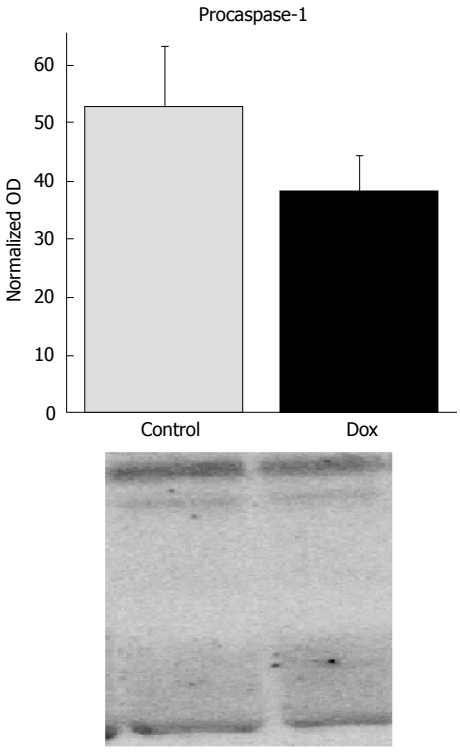

Figure 7 Protein content of procaspase-1. Doxorubicin (Dox) treatment did not affect the hepatic content of procaspase-1. Furthermore, there was no difference in the presence of lower molecular weight bands between treatment groups.

is a commonly used marker of apoptosis. In the current study, Dox treatment did not lead to the cleavage and inactivation of PARP-1. We analyzed PARP-1 and all cleavage products, but only included the data for the 89 kDa cleavage product since this is the product produced *via* cleavage by caspase-3 (88.86 ± 5.83 *vs* 91.1 ± 8.59, control *vs* Dox, respectively, $P = 0.84$, Figure 8B and C). The results suggest that apoptosis did not occur in response to acute Dox treatment, at least 24 h post injection under these experimental conditions. Figure 9 shows Ponceau staining of the samples used in all of the representative Western blots.

DISCUSSION

Recently, it has been shown that cellular stress can cause cellular deacetylation of nuclear and cytoplasmic proteins and that normalization of the acetylation status can protect cells from injury and death[12,29]. Furthermore, caloric restriction, an intervention shown to provoke protection against cellular insults and to increase longevity, increases acetylation status of the mitochondrial proteome in the liver, heart, and kidney[30]. However, the effects were tissue specific in that caloric restriction did not alter acetylation status of the mitochondrial proteome in the brain and caused deacetylation in brown adipose tissue[30]. Since proteome lysine deacetylation may be a marker of cellular stress in various tissues, we investigated the effects of Dox on proteome lysine acetylation status in the liver which was previously unknown. It was found that acute Dox treatment promoted lysine deacetylation which is consistent with the notion that an imbalance in acetylation status favoring deacetylation may signify cellular stress in the liver. Proteins most affected by Dox appear to be those with a molecular weight between 30-80 kDa.

Acetylation status is determined by the activities of HATs and HDACs. Sirt1 and Sirt3 are members of the

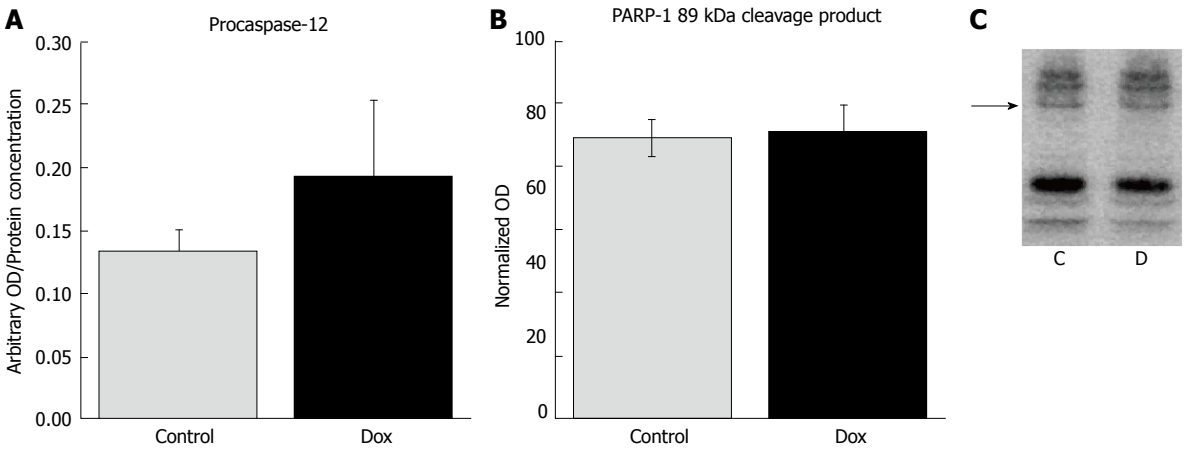

Figure 8 Markers of apoptosis. A: Caspase-3 activity was not affected by Dox treatment; B: Graphical representation of results for the 89 kDa cleavage product of poly (ADP-ribose) polymerase-1 (PARP-1); C: Representative Western blot of PARP-1 cleavage. The arrow indicates the 89 kDa cleavage product. C: Control; D: Dox.

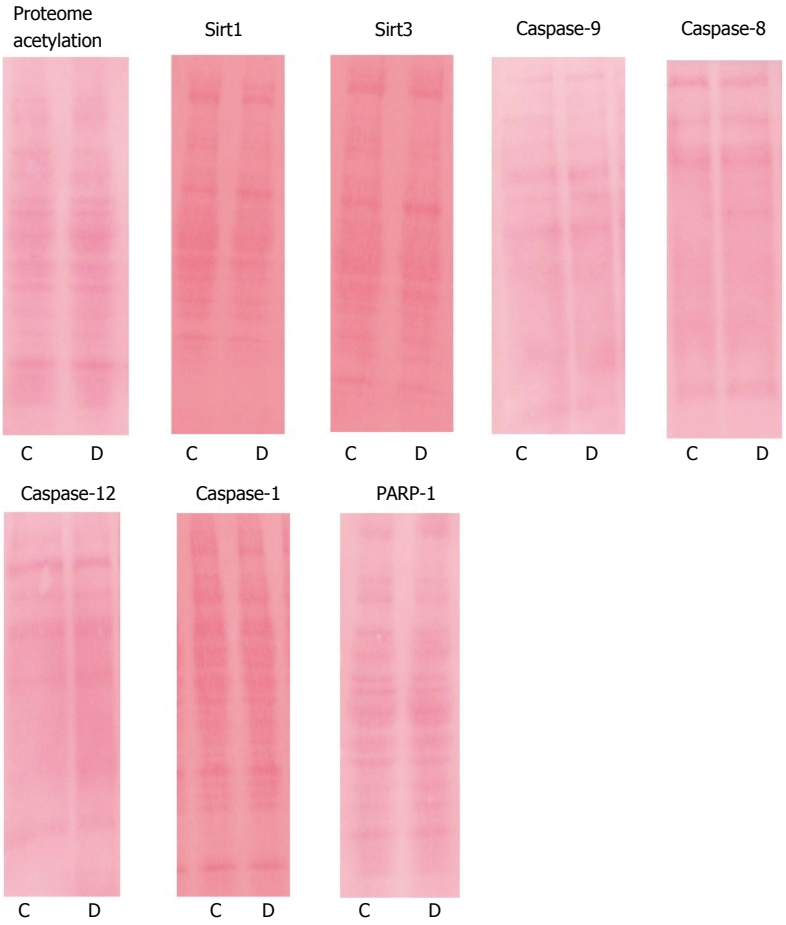

Figure 9 Ponceau staining. Ponceau staining of samples shown in Western blots photos. C: Control; D: Dox.

class Ⅲ HDACs, nicotinamide adenine dinucleotide (NAD^+)-dependent enzymes, and are the most studied of the seven sirtuins[13]. Sirt1 primarily targets nuclear and some cytoplasmic proteins while Sirt3 targets mitochondrial proteins. Although acute Dox treatment induced proteome lysine deacetylation, Dox did not affect the protein content of Sirt1 or Sirt3 nor the overall HDAC activity. However, it did decrease HAT activity. These results suggest that proteome lysine deacetylation may not be due to a generalized increase in HDAC activity and may rather be due to a decrease in HAT activity.

No studies have previously reported the influence of Dox administration on protein levels of Sirt1 or Sirt3. However, a previous study reported that Dox administration increased the mRNA content of Sirt1 in the heart of treated mice[2]. The discrepancy could be due to tissue specific differences or differences in experimental design. A reduction in food intake and low nutrient availability

increases the expression of Sirt1, as well as Sirt3[31-33]. To control for changes in food intake, we fasted both the control and treatment groups. Zhang *et al* did not control for Dox-induced changes in food intake and thus it cannot be determined if changes in Sirt1 expression are a direct effect of Dox or an indirect effect of anorexia.

Western analysis of Sirt1 revealed two prominent bands of 120 kDa and 45 kDa in the liver. The 120 kDa band corresponds to the expected molecular weight of Sirt1. It is unknown what the 45 kDa species is at this time. However, since this species was responsive to Dox treatment we searched the literature for possibilities. Three studies by the same group reported that Sirt1 can be cleaved under inflammatory conditions into a 75 kDa fragment in chondrocytes[24,34,35]. This fragment is void of deacetylation activity but shown to associate with cytochrome c on the mitochondrial membrane and to prevent apoptosis[24]. The cleavage of human Sirt1 occurs at amino acid residue 533 with the N-terminus being the 75 kDa fragment[34]. Our C-terminus Sirt1 antibody does not detect the N-terminus 75 kDa fragment, but may be detecting the C-terminus 45 kDa fragment. The content of this fragment increased with Dox treatment in the liver, possibly representing an increase in Sirt1 cleavage as a mechanism to prevent apoptosis in stressed hepatocytes. Future investigation is required to identify this Dox-responsive species.

To determine if proteome deacetylation was associated with a proapoptotic environment, as shown in other experimental models, we assessed the content and activation of several apical caspases associated with specific apoptotic stimuli. Activity of caspase-3 and cleavage of PARP-1 were also assessed. To our knowledge, this is the first investigation on the effects of acute Dox administration on the hepatic expression and activation of apical caspases. Our data suggests that acute Dox treatment is not likely associated with apoptosis induction. Dox administration did not stimulate the activation of caspase-9, caspase-8, caspase-3 or induce cleavage of PARP-1 in the liver under these experimental conditions. However, Dox treatment did increase the activation of caspase-12. Caspase-12 has been implicated in both apoptosis and inflammation. Early work suggested that in response to ER stress and calcium dyshomeostasis, caspase-12 can be activated by m-calpain and then induces apoptosis *via* cleavage of caspase-9 with consequent activation of caspase-3[25-27]. Our data suggests that caspase-12 may not be involved in apoptosis induction since we did not detect an increase in caspase-9 cleavage, caspase-3 activity or PARP-1 cleavage. In fact, more recent reports suggest that caspase-12 is incapable of cleaving caspase-9 or caspase-3. It was reported that the catalytic activity of caspase-12 was confined to autoprocessing and was unable to cleave other substrates[28,36]. Thus, caspase-12 may be playing a role in the inflammatory process rather than apoptosis. Caspase-12 has been shown to be an inhibitor of caspase-1. Caspase-12 binds procaspase-1 and prevents its activation and consequent cytokine processing[28]. Both procaspase-12 and the processed form of caspase-12 have been found to be a part of the caspase-1 inhibitory complex[28]. However, the role of caspase-12 cleavage in regulating the inflammatory response is unknown at this time, but has been suggested as a means for temporal limitation of the inhibitory effect of caspase-12[28]. The effects of Dox on the expression and activation of caspase-1 were assessed, but we did not observe an effect. Procaspase-12 has also been shown to have an inhibitory effect on NF-κB[37]. Thus, autoprocessing of caspase-12 may lead to decreased suppression of NF-κB and increased expression of anti-apoptotic proteins helping to protect the liver from Dox-induced apoptosis. This hypothesis is speculative and further studies are required to discern the role of caspase-12 in Dox-induced injury. In summary, lysine deacetylation likely is not associated with an apoptotic environment. However, a limitation is the lack of deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) to verify the lack of apoptosis induction. With this limitation, it cannot be conclusively ruled out that apoptosis independent of caspase activation or PARP-1 cleavage did not occur.

Although, our results suggest that Dox does not likely induce apoptosis, at least at this early time point under these experimental conditions, a previous study reported that Dox may induce apoptosis in the liver *via* the accumulation of p53, decreased expression of Bcl-xL, and cytochrome c release from mitochondria[5]. Cytochrome c release stimulates apoptosis *via* caspase-9 activation, however, they did not assess caspase-9 activity. Our results differ from Patel *et al*[5] in that we found that Dox does not increase caspase-9 activation. In the current study, we report that Dox increases the content of procaspase-9 with no evidence of cleavage products. We have also found that Dox decreases the activity of caspase-9 and mitophagy in hepatic tissue of these animals (data will be published elsewhere). We hypothesize that the decreased caspase-9 activity may be due to increased mitophagy, by the elimination of damaged mitochondria susceptible to cytochrome c release[38] and thus may be a protective response to an initial insult induced by Dox. Our results may contrast from Patel et al. due to differences in experimental design as there were several relevant disparities [5]. First, Patel *et al*[5] used an extremely high dose of Dox (60 mg/kg) which was three times the dose used in the current study. Secondly, our animals were fasted to control for the anorexic effect of Dox, while Patel *et al*[5] did not account for this variable. Therefore, the Dox animals were severely deficient in nutrients while the control animals were not. Thirdly, Patel *et al*[5] assessed Dox induced hepatic damage 48 h after treatment, while we examined toxicity at 24 h post treatment. It is possible that hepatic apoptosis may occur at a later time point due to the indirect effects of progressing peripheral organ damage, such as progression of heart failure. Animals treated with Dox usually show signs of heart failure within days of treatment which varies depending on the dose administered. Heart failure is known to cause liver damage[39].

Alternatively, complete fasting (compared to a 50%-70% reduction) of the animals had an unintended protective effect against Dox-induced hepatic apoptosis. Recently, it was shown that fasting prior to Dox treatment, but then fed ad libitum upon treatment, protected against cardiac toxicity by means of autophagy induction[40]. The absence of an ad-libitum fed Dox group and control group does not allow the determination of the effects of fasting on the variables assessed in our study. Yet, the objective was simply to eliminate this external variable. More studies are necessary to determine if fasting extends similar protection against hepatotoxicity.

Early effects of acute Dox treatment include altered proteome acetylation homeostasis favoring lysine deacetylation. The mechanism of proteome lysine deacetylation likely involves decreased activity of HATs, rather than increased activity of HDACs. In our experimental model, lysine deacetylation does not appear to be associated with an apoptotic environment. Acute Dox treatment did not increase cleavage of caspase-9, cleavage of caspase-8, cleavage of PARP-1, or caspase-3 activity. Dox treatment did stimulate caspase-12 activation, however, its activation does not appear to play a role in apoptosis induction. Thus, early mechanisms of hepatic toxicity may not involve induction of apoptosis, at least under these experimental conditions. The liver may be able to engage an early protective response against an initial Dox-induced insult. Further damage to the liver, which may include apoptosis, may occur at later time points as damage to peripheral organs progresses and may be an indirect effect of Dox. Alternatively, fasting of the animals in our experimental design may have provoked a protective response which prevented apoptosis. Further studies are required to discern the role of proteome lysine deacetylation, as well as the role of caspase-12, in Dox-induced hepatic toxicity. It is not clear whether lysine deacetylation and caspase-12 activation contribute to toxicity or play a role in a protective response. Clarification will elucidate the potential of acetylation homeostasis and caspase-12 as therapeutic targets against Dox-induced toxicity.

COMMENTS

Background

Doxorubicin-induced toxicity is a major cause of morbidity and mortality in cancer survivors. Thus, mechanisms of toxicity is essential to elucidate in order to prevent doxorubicin-induced tissue damage in vital organs.

Research frontiers

Proteome lysine deacetylation has been shown to be associated with cellular injury in various experimental models. Normalization of the acetylation status has been shown to prevent cell death. However, the role of altered proteome lysine acetylation status in doxorubicin-induced toxicity has not been previously studied.

Innovations and breakthroughs

Doxorubicin treatment has been found to alter the hepatic proteome acetylation status. However, the modulation of the acetylation status was not associated with apoptosis, as shown in other experimental models. The results lay the groundwork for future studies to determine the role of proteome lysine deacetylation in doxorubicin-induced toxicity. Furthermore, caspase-12 was identified as having a role in doxorubicin-induced hepatic toxicity, which may be either pathological or protective.

Applications

Proteome lysine acetylation and caspase-12 may be potential therapeutic targets to prevent doxorubicin-induced toxicity.

Terminology

Acetylation is a common posttranslational modification in which an acetyl group is covalently attached to a protein. Apoptosis is cellular suicide or programmed cell death.

Peer Review

The authors describe the evaluation of the effect of doxorubicin on proteome acetylation status and found that decreased proteome lysine acetylation was associated with decreased activity of histone deacetylases. Overall, the paper is well written.

REFERENCES

1 **Pointon AV**, Walker TM, Phillips KM, Luo J, Riley J, Zhang SD, Parry JD, Lyon JJ, Marczylo EL, Gant TW. Doxorubicin in vivo rapidly alters expression and translation of myocardial electron transport chain genes, leads to ATP loss and caspase 3 activation. *PLoS One* 2010; **5**: e12733 [PMID: 20856801 DOI: 10.1371/journal.pone.0012733]

2 **Zhang S**, Liu X, Bawa-Khalfe T, Lu LS, Lyu YL, Liu LF, Yeh ET. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. *Nat Med* 2012; **18**: 1639-1642 [PMID: 23104132 DOI: 10.1038/nm.2919]

3 **Childs AC**, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C. Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2:Bax ratio. *Cancer Res* 2002; **62**: 4592-4598 [PMID: 12183413]

4 **Koçkar MC**, Nazıroğlu M, Celik O, Tola HT, Bayram D, Koyu A. N-acetylcysteine modulates doxorubicin-induced oxidative stress and antioxidant vitamin concentrations in liver of rats. *Cell Biochem Funct* 2010; **28**: 673-677 [PMID: 21104935 DOI: 10.1002/cbf.1707]

5 **Patel N**, Joseph C, Corcoran GB, Ray SD. Silymarin modulates doxorubicin-induced oxidative stress, Bcl-xL and p53 expression while preventing apoptotic and necrotic cell death in the liver. *Toxicol Appl Pharmacol* 2010; **245**: 143-152 [PMID: 20144634 DOI: 10.1016/j.taap.2010.02.002]

6 **el-Missiry MA**, Othman AI, Amer MA, Abd el-Aziz MA. Attenuation of the acute adriamycin-induced cardiac and hepatic oxidative toxicity by N-(2-mercaptopropionyl) glycine in rats. *Free Radic Res* 2001; **35**: 575-581 [PMID: 11767415 DOI: 10.1080/10715760100301581]

7 **Kalender Y**, Yel M, Kalender S. Doxorubicin hepatotoxicity and hepatic free radical metabolism in rats. The effects of vitamin E and catechin. *Toxicology* 2005; **209**: 39-45 [PMID: 15725512 DOI: 10.1016/j.tox.2004.12.003]

8 **Henninger C**, Huelsenbeck J, Huelsenbeck S, Grösch S, Schad A, Lackner KJ, Kaina B, Fritz G. The lipid lowering drug lovastatin protects against doxorubicin-induced hepatotoxicity. *Toxicol Appl Pharmacol* 2012; **261**: 66-73 [PMID: 22712078 DOI: 10.1016/j.taap.2012.03.012]

9 **Yagmurca M**, Bas O, Mollaoglu H, Sahin O, Nacar A, Karaman O, Songur A. Protective effects of erdosteine on doxorubicin-induced hepatotoxicity in rats. *Arch Med Res* 2007; **38**: 380-385 [PMID: 17416283 DOI: 10.1016/j.arcmed.2007.01.007]

10 **Ladas EJ**, Jacobson JS, Kennedy DD, Teel K, Fleischauer A, Kelly KM. Antioxidants and cancer therapy: a systematic review. *J Clin Oncol* 2004; **22**: 517-528 [PMID: 14752075 DOI: 10.1200/JCO.2004.03.086]

11 **Kim SC**, Sprung R, Chen Y, Xu Y, Ball H, Pei J, Cheng T, Kho Y, Xiao H, Xiao L, Grishin NV, White M, Yang XJ, Zhao Y. Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Mol Cell* 2006; **23**: 607-618 [PMID: 16916647 DOI: 10.1016/j.molcel.2006.06.026]

12 **Li Y**, Alam HB. Creating a pro-survival and anti-inflammatory phenotype by modulation of acetylation in models of

hemorrhagic and septic shock. *Adv Exp Med Biol* 2012; **710**: 107-133 [PMID: 22127890 DOI: 10.1007/978-1-4419-5638-5_11]
13 **Suzuki T**. 'Explorative Study on Isoform-Selective Histone Deacetylase Inhibitors'. *Chem Pharm Bull* (Tokyo) 2009; **57**: 897-906 [DOI: 10.1248/cpb.57.897]
14 **Chowdhury I**, Tharakan B, Bhat GK. Caspases - an update. *Comp Biochem Physiol B Biochem Mol Biol* 2008; **151**: 10-27 [PMID: 18602321 DOI: 10.1016/j.cbpb.2008.05.010]
15 **Freireich EJ**, Gehan EA, Rall DP, Schmidt LH, Skipper HE. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer Chemother Rep* 1966; **50**: 219-244 [PMID: 4957125]
16 **Glass B**, Kloess M, Bentz M, Schlimok G, Berdel WE, Feller A, Trümper L, Loeffler M, Pfreundschuh M, Schmitz N. Dose-escalated CHOP plus etoposide (MegaCHOEP) followed by repeated stem cell transplantation for primary treatment of aggressive high-risk non-Hodgkin lymphoma. *Blood* 2006; **107**: 3058-3064 [PMID: 16384932 DOI: 10.1182/blood-2005-04-1570]
17 **Gilliam LA**, Ferreira LF, Bruton JD, Moylan JS, Westerblad H, St Clair DK, Reid MB. Doxorubicin acts through tumor necrosis factor receptor subtype 1 to cause dysfunction of murine skeletal muscle. *J Appl Physiol* (1985) 2009; **107**: 1935-1942 [PMID: 19779154 DOI: 10.1152/japplphysiol.00776.2009]
18 **Sorensen M**, Sanz A, Gómez J, Pamplona R, Portero-Otín M, Gredilla R, Barja G. Effects of fasting on oxidative stress in rat liver mitochondria. *Free Radic Res* 2006; **40**: 339-347 [PMID: 16517498 DOI: 10.1080/10715760500250182]
19 **Marczuk-Krynicka D**, T Hryniewiecki, J Piatek, and J Paluszak. 'The Effect of Brief Food Withdrawal on the Level of Free Radicals and Other Parameters of Oxidative Status in the Liver'. *Med Sci Monit* 2003; **9**: 131-135
20 **Zhang YK**, Wu KC, Klaassen CD. Genetic activation of Nrf2 protects against fasting-induced oxidative stress in livers of mice. *PLoS One* 2013; **8**: e59122 [PMID: 23527105 DOI: 10.1371/journal.pone.0059122]
21 **Rambold AS**, Kostelecky B, Elia N, Lippincott-Schwartz J. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. *Proc Natl Acad Sci USA* 2011; **108**: 10190-10195 [PMID: 21646527 DOI: 10.1073/pnas.1107402108]
22 **Romero-Calvo I**, Ocón B, Martínez-Moya P, Suárez MD, Zarzuelo A, Martínez-Augustin O, de Medina FS. Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Anal Biochem* 2010; **401**: 318-320 [PMID: 20206115 DOI: 10.1016/j.ab.2010.02.036]
23 **Houtkooper RH**, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. *Nat Rev Mol Cell Biol* 2012; **13**: 225-238 [PMID: 22395773]
24 **Oppenheimer H**, Gabay O, Meir H, Haze A, Kandel L, Liebergall M, Gagarina V, Lee EJ, Dvir-Ginzberg M. 75-kd sirtuin 1 blocks tumor necrosis factor α-mediated apoptosis in human osteoarthritic chondrocytes. *Arthritis Rheum* 2012; **64**: 718-728 [PMID: 21987377 DOI: 10.1002/art.33407]
25 **Nakagawa T**, Yuan J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J Cell Biol* 2000; **150**: 887-894 [PMID: 10953012 DOI: 10.1083/jcb.150.4.887]
26 **Nakagawa T**, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 2000; **403**: 98-103 [PMID: 10638761 DOI: 10.1038/47513]
27 **Morishima N**, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem* 2002; **277**: 34287-34294 [PMID: 12097332 DOI: 10.1074/jbc.M204973200]
28 **Roy S**, Sharom JR, Houde C, Loisel TP, Vaillancourt JP, Shao W, Saleh M, Nicholson DW. Confinement of caspase-12 proteolytic activity to autoprocessing. *Proc Natl Acad Sci USA* 2008; **105**: 4133-4138 [PMID: 18332441 DOI: 10.1073/pnas.0706658105]
29 **Li Y**, Alam HB. Modulation of acetylation: creating a pro-survival and anti-inflammatory phenotype in lethal hemorrhagic and septic shock. *J Biomed Biotechnol* 2011; **2011**: 523481 [PMID: 21403879]
30 **Schwer B**, Eckersdorff M, Li Y, Silva JC, Fermin D, Kurtev MV, Giallourakis C, Comb MJ, Alt FW, Lombard DB. Calorie restriction alters mitochondrial protein acetylation. *Aging Cell* 2009; **8**: 604-606 [PMID: 19594485 DOI: 10.1111/j.1474-9726.2009.00503.x]
31 **Noriega LG**, Feige JN, Canto C, Yamamoto H, Yu J, Herman MA, Mataki C, Kahn BB, Auwerx J. CREB and ChREBP oppositely regulate SIRT1 expression in response to energy availability. *EMBO Rep* 2011; **12**: 1069-1076 [PMID: 21836635 DOI: 10.1038/embor.2011.151]
32 **Palacios OM**, Carmona JJ, Michan S, Chen KY, Manabe Y, Ward JL, 3rd, L. J. Goodyear, and Q. Tong, 'Diet and Exercise Signals Regulate Sirt3 and Activate Ampk and Pgc-1alpha in Skeletal Muscle'. *Aging* (Albany NY) 2009; **1**: 771-83
33 **Hayashida S**, Arimoto A, Kuramoto Y, Kozako T, Honda S, Shimeno H, Soeda S. Fasting promotes the expression of SIRT1, an NAD+ -dependent protein deacetylase, via activation of PPARalpha in mice. *Mol Cell Biochem* 2010; **339**: 285-292 [PMID: 20148352 DOI: 10.1007/s11010-010-0391-z]
34 **Dvir-Ginzberg M**, Gagarina V, Lee EJ, Booth R, Gabay O, Hall DJ. Tumor necrosis factor α-mediated cleavage and inactivation of SirT1 in human osteoarthritic chondrocytes. *Arthritis Rheum* 2011; **63**: 2363-2373 [PMID: 21305533 DOI: 10.1002/art.30279]
35 **Gabay O**, Oppenhiemer H, Meir H, Zaal K, Sanchez C, Dvir-Ginzberg M. Increased apoptotic chondrocytes in articular cartilage from adult heterozygous SirT1 mice. *Ann Rheum Dis* 2012; **71**: 613-616 [PMID: 22258484 DOI: 10.1136/ard.2011.200504]
36 **Lee HJ**, Lee SH, Park SH, Sharoar MG, Shin SY, Lee JS, Cho B, Park IS. Purification of catalytically active caspase-12 and its biochemical characterization. *Arch Biochem Biophys* 2010; **502**: 68-73 [PMID: 20646990 DOI: 10.1016/j.abb.2010.07.013]
37 **Labbé K**, Miu J, Yeretssian G, Serghides L, Tam M, Finney CA, Erdman LK, Goulet ML, Kain KC, Stevenson MM, Saleh M. Caspase-12 dampens the immune response to malaria independently of the inflammasome by targeting NF-kappaB signaling. *J Immunol* 2010; **185**: 5495-5502 [PMID: 20876354 DOI: 10.4049/jimmunol.1002517]
38 **Yang Y**, Xing D, Zhou F, Chen Q. Mitochondrial autophagy protects against heat shock-induced apoptosis through reducing cytosolic cytochrome c release and downstream caspase-3 activation. *Biochem Biophys Res Commun* 2010; **395**: 190-195 [PMID: 20361931 DOI: 10.1016/j.bbrc.2010.03.155]
39 **Alvarez AM**, Mukherjee D. Liver abnormalities in cardiac diseases and heart failure. *Int J Angiol* 2011; **20**: 135-142 [PMID: 22942628 DOI: 10.1055/s-0031-1284434]
40 **Kawaguchi T**, Takemura G, Kanamori H, Takeyama T, Watanabe T, Morishita K, Ogino A, Tsujimoto A, Goto K, Maruyama R, Kawasaki M, Mikami A, Fujiwara T, Fujiwara H, Minatoguchi S. Prior starvation mitigates acute doxorubicin cardiotoxicity through restoration of autophagy in affected cardiomyocytes. *Cardiovasc Res* 2012; **96**: 456-465 [PMID: 22952253 DOI: 10.1093/cvr/cvs282]

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ORIGINAL ARTICLE

Apoptosis induced by Fas signaling does not alter hepatic hepcidin expression

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Abstract

AIM: To determine the regulation of human hepcidin (*HAMP*) and mouse hepcidin (*hepcidin-1* and *hepcidin-2*) gene expression in the liver by apoptosis using *in vivo* and *in vitro* experimental models.

METHODS: For the induction of the extrinsic apoptotic pathway, HepG2 cells were treated with various concentrations of CH11, an activating antibody for human Fas receptor, for 12 h. Male C57BL/6NCR and C57BL/6J strains of mice were injected intraperitoneally with sublethal doses of an activating antibody for mouse Fas receptor, Jo2. The mice were anesthetized and sacrificed 1 or 6 h after the injection. The level of apoptosis was quantified by caspase-3 activity assay. Liver injury was assessed by measuring the levels of ALT/AST enzymes in the serum. The acute phase reaction in the liver was examined by determining the expression levels of *IL-6* and *SAA3* genes by SYBR green quantitative real-time PCR (qPCR). The phosphorylation of transcription factors, Stat3, Smad4 and NF-κB was determined by western blotting. Hepcidin gene expression was determined by Taqman qPCR. The binding of transcription factors to *hepcidin-1* promoter was studied using chromatin immunoprecipitation (ChIP) assays.

RESULTS: The treatment of HepG2 cells with CH11 induced apoptosis, as shown by the significant activation of caspase-3 ($P < 0.001$), but did not cause any significant changes in *HAMP* expression. Short-term (1 h) Jo2 treatment (0.2 μg/g *b.w.*) neither induced apoptosis and acute phase reaction nor altered mRNA expression of mouse *hepcidin-1* in the livers of C57BL/6NCR mice. In contrast, 6 h after Jo2 injection, the livers of C57BL/6NCR mice exhibited a significant level of apoptosis ($P < 0.001$) and an increase in SAA3 ($P < 0.023$) and IL-6 ($P < 0.005$) expression in the liver. However, mRNA expression of *hepcidin-1* in the liver was not significantly altered. Despite the Jo2-induced phosphorylation of Stat3, no occupancy of *hepcidin-1* promoter by Stat3 was observed, as shown by ChIP assays. Compared to C57BL/6NCR mice, Jo2 treatment (0.2 μg/g *b.w.*) of C57BL/6J strain mice for 6 h induced a more prominent activation of apoptosis, liver injury and acute phase reaction. Similar to C57BL/6NCR mice, the level of liver *hepcidin-1* mRNA expression in the livers of C57BL/6J mice injected with a sublethal dose of Jo2 (0.2 μg/g *b.w.*) remained unchanged. The injection of C57BL/6J mice with a higher dose of Jo2 (0.32 μg/g *b.w.*) did not also alter hepatic hepcidin expression.

CONCLUSION: Our findings suggest that human or mouse hepcidin gene expression is not regulated by apoptosis induced *via* Fas receptor activation in the liver.

Key words: Iron metabolism; Jo2; CH11; Extrinsic

apoptosis; Stat3

Core tip: Apoptosis and Fas signaling participate in the pathogenesis of various liver diseases. Iron also contributes to liver injury. Changes in the expression of hepcidin, a key iron regulatory hormone, has been reported in various liver diseases. Recently, apoptosis has been implicated in the regulation of hepcidin. This study investigates effector caspase activation and apoptosis induced by Fas receptor signaling and its relationship to hepatic hepcidin expression.

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INTRODUCTION

Apoptosis is involved in the pathogenesis of various liver diseases[1]. Hepatocyte apoptosis can be activated *via* the extrinsic apoptotic pathway through the binding of ligands to death receptors such as Fas, TNF receptor 1 and TRAIL receptor 2. Upon ligand binding, the receptor will trimerize and the C-terminal death domain will recruit Fas-associated protein with death domain to form death-inducing signaling complex (DISC), which subsequently recruits procaspase-8 and induces its self-cleavage and activation. Activated caspase-8 can directly cleave and activate caspase-3, the executioner caspase, which is responsible for the cleavage of target proteins to execute apoptosis. Caspase-3 activation is frequently used as a marker for apoptosis. Flice-Inhibitory Protein Long form (FLIPL) blocks apoptosis by inhibiting the recruitment and autoproteolytic cleavage of procaspase-8. In addition, in hepatocytes, the signal from death receptor can be amplified through the mitochondrial (intrinsic) apoptotic pathway. Activated caspase-8 can cleave Bcl-2 family protein, Bid. Truncated Bid (tBid) activates proapoptotic Bcl-2 family proteins, and induces permeabilization of the mitochondrial outer membrane and the leakage of the mitochondrial content including cytochrome c. Cytochrome c forms a complex with apoptotic peptidase activating factor 1, recruits and activates caspase-9, which subsequently cleaves caspase-3 and executes apoptosis.

A role for apoptosis has been suggested in the regulation of hepcidin[2,3]. Hepcidin, an antimicrobial peptide synthesized primarily by the liver, is the central regulator of iron metabolism. It is synthesized as an 84 amino acid precursor peptide, which is then cleaved to its 25 amino acid biologically active circulatory form. Unlike humans, who have one copy of hepcidin gene (*HAMP*), mice have two hepcidin genes, *hepcidin-1* and *hepcidin-2*. Similar to *HAMP*, *hepcidin-1* is involved in the regulation of iron homeostasis but the function of *hepcidin-2* is unknown. Hepcidin exerts its regulatory function by blocking the uptake and export of dietary iron from the intestine and the release of iron from macrophages. Hepcidin achieves this by binding to ferroportin, the only known iron exporter, and causing its internalization and degradation *via* the lysosomal pathway. The suppression of hepcidin expression in the liver therefore leads to systemic iron overload whereas its induction causes iron deficiency and anemia.

Weizer-Stern *et al*[4] have demonstrated that p53, a tumor suppressor and inducer of apoptosis, participates in the regulation of hepcidin. In their study, a putative p53 response element on hepcidin gene promoter has been identified and validated by chromatin immunoprecipitation assays. Over-expression of p53 in hepatoma cells has been shown to induce hepcidin gene transcription and conversely, the silencing of p53 resulted in down-regulation of hepcidin expression[4]. It is however unclear whether p53-mediated apoptosis is involved in the regulation of hepatic hepcidin expression[4]. On the other hand, Li *et al*[5] have suggested a role for Fas signaling in the regulation of hepcidin expression in tissue culture cells and female mouse livers. A lethal dose of anti-Fas activating antibody, Jo2 has been reported to exert an immediate stimulatory and a late suppression effect on hepcidin mRNA expression in the liver[5]. Although a relationship between FLIPL, IL-6, Stat3 and hepcidin expression has been shown, they did not however establish a direct correlation between apoptosis and hepcidin. Besides committing cell death, Fas induced DISC formation also participates in the activation of cell signaling pathways, including IL-6 and NF-κB[6]. Of note, hepcidin expression is regulated by various signaling pathways. As an acute phase protein, hepcidin is stimulated by endotoxin and inflammatory cytokine signaling[7-9]. The effect of IL-6 is mediated through the activation of Jak/Stat pathway and the binding of Stat3 to hepcidin gene promoter[10,11]. As an iron regulatory protein, hepcidin is also regulated by the signals from iron sensors, such as bone morphogenetic protein 6 (BMP6)[12-14]. The BMP receptor-specific Smad pathway (*via* the phosphorylation of transcription factors, Smad1/5/8) has been shown to be involved in the up-regulation of hepcidin transcription. BMP6 knockout mice exhibit iron overload and reduced hepcidin expression[15-17]. Similarly, mice lacking the expression of the common Smad protein, Smad4 exhibit iron overload and a dramatic decrease in the expression of hepcidin in the liver[18]. In addition, growth factors such as epidermal growth factor and hepatocyte growth factor suppress the expression of hepcidin by inhibiting the signaling of the BMP-Smad pathway[19].

The aim of this study is to investigate the causal relationship between Fas-signaling-induced effector caspase activation and apoptosis, and the regulation of human and mouse hepcidin gene transcription. These studies will help us to further understand the regulation and the role of hepcidin in liver diseases.

Table 1 Taqman probe and primer sequences for quantitative real-time PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Taqman probe
HAMP	TGCCCATGTTCCAGAGGC	CCGCAGCAGAAAATGCAGAT	AAGGAGGCGAGACACCCACTTCCC
Human Gapdh	TGAAGGTCGGAGTCAACGG	AGAGTTAAAAGCAGCCCTGGTG	TTTGGTCGTATTGGGCGCCTGG
Hepcidin-1	TGCAGAAGAGAAGGAAGAGAGACA	CACACTGGGAATTGTTACAGCATT	CAACTTCCCCATCTGCATCTTCTGCTGT
Hepcidin-2	GCGATCCCAATGCAGAAGAG	TGTTACAGCACTGACAGCAGAATC	AGGAAGAGAGACATCAACTTCCCCATCTGC
Mouse Gapdh	TCACTGGCATGGCCTTCC	GGCGGCACGTCAGATCC	TTCCTACCCCCAATGTGTCCGTCG

Table 2 Primer sequences for SYBR green quantitative real-time PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Mouse SAA3	GCCTGGGCTGCTAAAGTCAT	TGCTCCATGTCCCGTGAAC
Mouse IL-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
Mouse Gapdh	GTGGAGATTGTTGCCATCAACGA	CCCATTCTCGGCCTTGACTGT

MATERIALS AND METHODS

Cell culture experiments

HepG2 human hepatoma cells were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with glutamine and 10% fetal calf serum (Atlantic Biologicals). 1.3×10^6 or 3.9×10^6 cells were seeded in 25 cm^2 or 75 cm^2 flasks, respectively 24 h prior to experiments. Cells were exposed to an activating antibody, which is specific for human Fas (clone CH11, Millipore) at different concentrations for 12 h.

Animal experiments

Animal experiments were approved by the Animal Ethics Committee at the University of Nebraska Medical Center. C57BL/6J (the Jackson Laboratory) and C57BL/6NCR (NIH) strain male mice were maintained on a standard chow diet. 6 wk to 8 wk old male mice were injected intraperitoneally (*i.p.*) with an activating antibody, which is specific for mouse Fas (clone Jo2, BD Biosciences), at 0.2 μg or 0.32 μg per gram of body weight (*b.w.*). As control for *i.p.* injections, which per se might cause an acute phase reaction, a group of mice were injected with similar volume of 0.9% NaCl. All mice were sacrificed 1 or 6 h following injections.

RNA isolation, cDNA synthesis, and real-time quantitative PCR analysis

RNA isolation, cDNA synthesis and quantitative PCR (qPCR) were performed, as described previously[20]. The sequence of Taqman fluorescent probe [5′6-(FAM); 3′ (TAMRA-Q)] and primers, and SYBR green primers are shown in Tables 1 and 2.

Detection of apoptosis

Caspase-3 activity assays were performed with Ac-DEVD-AMC caspase-3 fluorogenic substrate (BD Biosciences) as described previously[21].

Measurement of liver enzymes

Blood collected from the right atrium of the anesthetized animal before sacrifice and allowed to coagulate at room temperature for 30 min was centrifuged at 1300 × *g* for 10 min to remove blood cells. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) liver enzyme levels in mice sera were measured at Clinical Chemistry Laboratory at University of Nebraska Medical Center.

Western Blotting

The isolation of nuclear lysates from the livers and western blotting were performed, as described previously[22]. Anti-phospo-Stat3, anti-Stat3, anti-phospho-Smad 1/5, anti-p65 and Gapdh primary antibodies, and secondary antibodies were obtained commercially (Santa Cruz, Cell Signaling).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed, as described previously[22,23]. Briefly, chromatin, isolated from formalin-fixed livers, was sheared by sonication and an aliquot was saved as total input DNA. Sheared chromatin was incubated with anti-Stat3, anti-Smad4 antibodies or control IgG and protein A-conjugated agarose beads (Santa Cruz, Cell signaling). Eluted DNA and total input DNA were subsequently analyzed by PCR using primers (sense: 5′- gccatactgaaggcactga -3′; antisense: 5′- gtgtggtg-gctgtctagg -3′) to amplify a 358bp region of the mouse *hepcidin-1* promoter.

Statistical analysis

SPSS software was used for statistical analysis. The significance of difference between two groups was determined by Student's *t*-test. A value of $P < 0.05$ was accepted as statistically significant.

RESULTS

Apoptosis and human hepcidin gene (HAMP) expression

CH11 antibody treatment for 12 h induced apoptosis in HepG2 cells in a concentration dependent manner, as confirmed with caspase-3 activity assay. A significant induction of caspase-3 activity was observed at 50

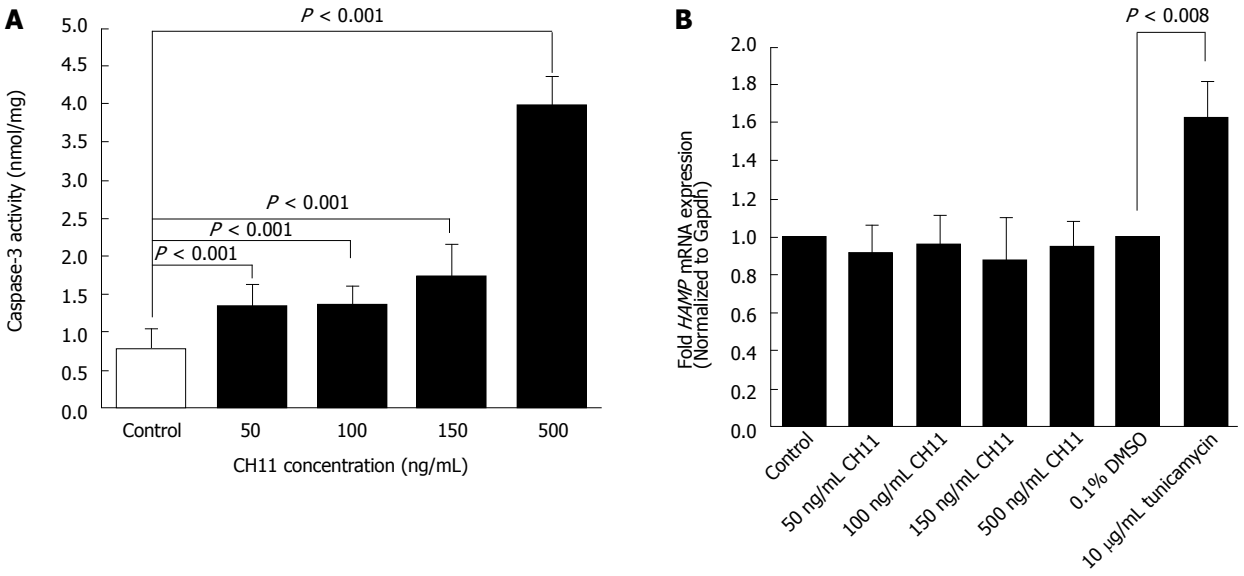

Figure 1 Caspase-3 activity and human hepcidin gene (*HAMP*) expression: HepG2 cells were treated with 50, 100, 150 and 500 ng/mL of CH11 antibody or solvent (control) for 12 h. A: Induction of apoptosis was confirmed by measuring caspase-3 activity, as described in the 'Materials and Methods' section. Caspase-3 activity was expressed as nanomole of flurogenic substrate cleaved per milligram of cell lysate protein; B: cDNA, synthesized from RNA isolated from CH11-treated, tunicamycin-treated and respective solvent-treated cells, was employed as a template in Taqman qPCR assays to determine *HAMP* mRNA expression, as described in Methods. *HAMP* expression in CH11-treated cells was expressed as fold expression of that in control cells.

ng/mL CH11 concentration, which increased four-fold with a 500 ng/mL concentration (Figure 1A). The level of *HAMP* mRNA expression in CH11-treated HepG2 cells was similar to that of control cells, as determined by qPCR (Figure 1B). HepG2 cells treated with 10 μg/mL of tunicamycin, a known inducer of hepcidin gene expression, for 8 h were used as the positive control (Figure 1B). These findings strongly suggest that the Fas-mediated apoptotic pathway does not alter *HAMP* transcription.

The effect of short-term (1 h) Jo2 treatment on apoptosis, acute phase response and mouse hepcidin gene expression in the livers of C57BL/6NCR mice

Since activation of human Fas did not alter human hepcidin gene expression, we performed experiments with activating antibody specific to mouse Fas, Jo2. C57BL/6NCR mice were injected with Jo2 antibody (0.2 μg/g *b.w.*) or NaCl (control), and sacrificed 1 h later. No significant increase in caspase-3 activity was observed in Jo2-injected mice compared to control mice (Figure 2A). The activation of acute phase reaction in the livers of these mice was evaluated by determining the levels of IL-6 and SAA3 mRNA expression by qPCR. Similar to caspase-3 activity, no significant changes were observed with the expression of these acute phase reaction genes (Figure 2B and C). The mRNA expression of *hepcidin-1* in mice livers was also unaltered by Jo2 exposure, as confirmed by qPCR (Figure 2D). However, 1 h Jo2 treatment induced a small but significant increase in *hepcidin-2* mRNA expression (Figure 2E).

The effect of longer (6 h) Jo2 treatment on apoptosis, acute phase response and mouse hepcidin gene expression in the livers of C57BL/6NCR mice

To further study the effect of Fas-mediated apoptosis, C57BL/6NCR mice were sacrificed 6 h after Jo2 or NaCl injections. A significant increase in caspase-3 activity was observed in the livers of Jo2-injected mice (Figure 3A). Similarly, the expression of acute phase reaction markers, IL-6 and SAA3 were also elevated in mice exposed to Jo2 for 6 h compared to control mice (Figures 3B and C). The levels of *hepcidin-1* and *hepcidin-2* mRNA expression in mice treated with Jo2 for 6 h were not significantly different from that in control mice (Figures 3D and E).

Jo2-mediated Stat3, Smad1/5 activation and hepcidin-1 promoter occupancy

The cytokine, IL-6 is known to activate hepcidin transcription *via* the Jak/Stat3 signaling pathway[10,11].We therefore investigated the activation of Stat3 in mice injected with Jo2 antibody (0.2 μg/g *b.w.*) or NaCl and sacrificed 1 or 6 h later. Six hours, but not 1 h of Jo2 treatment, was sufficient to induce the phosphorylation of Stat3 in the livers of mice, compared to respective control mice (Figure 4). Despite the activation of Stat3, we did not observe any significant changes in Stat3 binding *to hepcidin-1* promoter in Jo2-treated mice compared to control mice, as determined by ChIP assays (Figure 5).

NF-κB is one of the important transcription factors activated by Fas ligand binding. NF-κB activates the transcription of inflammatory cytokines including IL-6[1]. We therefore investigated the phosphorylation of the p65 subunit of NF-κB in mice treated with Jo2 for 1 or 6 h time periods. In contrast to Stat3, Jo2 induced a fast and transient activation of NF-κB. The phosphorylation of p65 in the liver was observed within 1 h after Jo2 injection and was absent at 6 h after Jo2 exposure (Figure 4).

Besides Jak/Stat3 pathway, hepcidin is also regulated by bone morphogenetic protein 6 (BMP6) and Smad

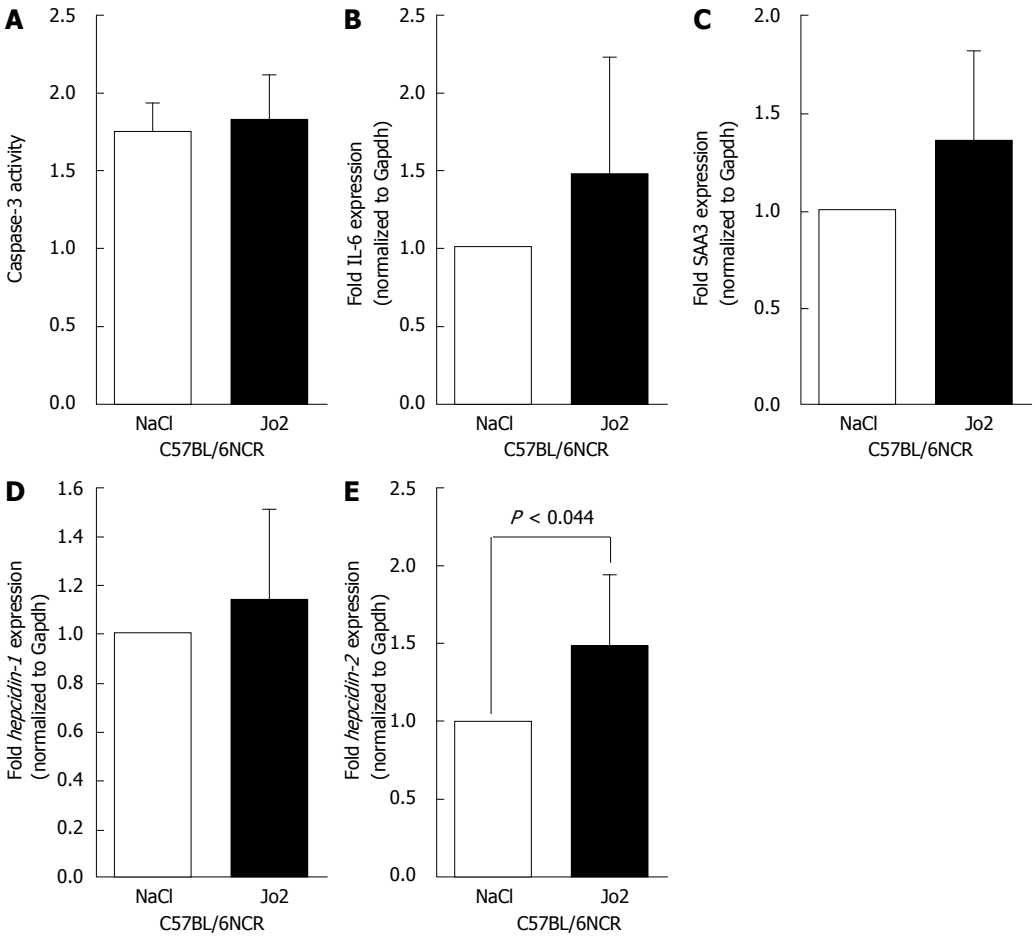

Figure 2 Caspase-3 activity, the expression of acute phase response and mouse hepcidin genes in the liver: C57BL/6NCR strain male mice, which were injected with Jo2 antibody (0.2 μg/g *b.w.*) or saline (as control), were sacrificed 1 h later. A: Caspase-3 activity was measured, as described in the 'Materials and Methods' section. B-E: *IL-6* (B), *SAA3* (C), *hepcidin-1* (D) and *hepcidin-2* (E) gene expression was determined by qPCR, and mRNA expression in Jo2-treated mice expressed as fold change of that in control mice.

pathway. This pathway has also been suggested to play a negative role in growth factor-induced regulation of hepcidin expression in the liver[19]. We therefore determined the activation of transcription factors, Smad1 and Smad5, which are activated downstream of BMP signaling pathway. Similar to NF-κB, Jo2 treatment induced an early and transient activation of Smad1/5 in the liver. The induction in Smad1/5 phosphorylation observed by 1 h Jo2 exposure was significantly weakened by 6 h after Jo2 injection (Figure 4). The binding of Smad4, the common mediator of Smad signaling, to mouse *hepcidin-1* promoter was also examined by ChIP assays. No significant increase in Smad4 occupancy of *hepcidin-1* promoter region harboring a Smad4 binding site was observed at 6 h after Jo2 injection, as compared to controls (Figure 5).

The effect of longer (6 h) Jo2 treatment on apoptosis, acute phase response and mouse hepcidin gene expression in the livers of C57BL/6J mice

In order to investigate the effect of Jo2 further, we employed a substrain of C57BL/6 mice. Of note, C57BL/6NCR and C57/BL/6J strains exhibit substantial genetic differences[24]. Compared to that observed with C57BL/6NCR, C57BL/6J mice treated with Jo2 (0.2 μg/g *b.w.*) for 6 h exhibited a significantly higher elevation of caspase-3 activity (Figure 6A). Similar robust activation was also observed with the expression of acute phase marker genes, *IL-6* and *SAA3* (Figures 6B and C). However, despite stronger apoptosis and acute phase reactions, Jo2 treatment did not induce any significant changes in the expression of both *hepcidin-1* and *hepcidin-2* in the livers of C57BL/6J mice, as was the case with C57BL/6NCR mice (compare Figures 6D, E and3D, E). Furthermore, the treatment of C57BL/6J mice with an even higher concentration of Jo2 (0.32 μg/g *b.w.*) did not induce any changes in the mRNA level of *hepcidin-1*. (Figure 6D). However, the treatment with 0.32 μg/g of Jo2 induced a significant suppression of *hepcidin-2* mRNA expression (Figure 6E).

The effect of Jo2 on liver enzymes in C57BL/6J and C57BL/6NCR mice

Jo2-induced apoptosis and acute phase reaction was stronger in the livers of C57BL/6J mice, compared to C57BL/6NCR mice. We therefore measured the serum levels of liver enzymes, ALT and AST, which is a com-

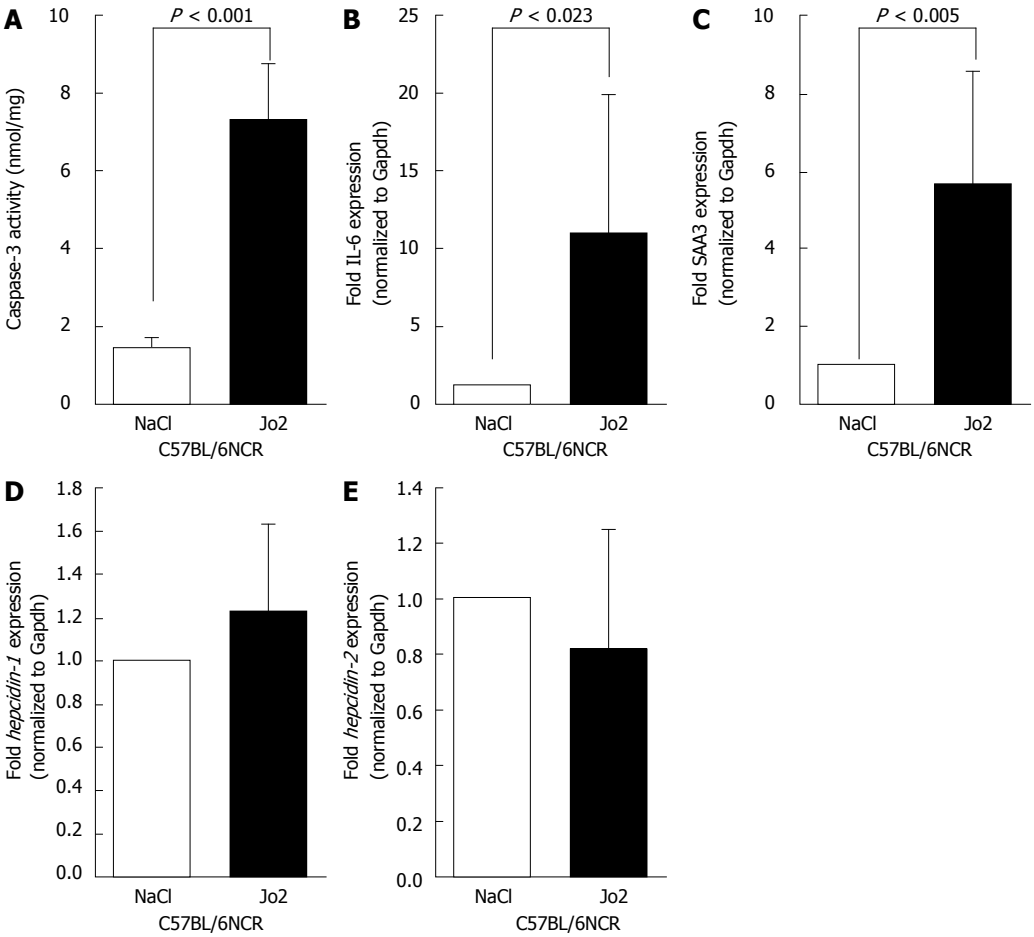

Figure 3 The effect of longer Jo2 treatment on the level of apoptosis, acute phase response and hepcidin gene expression: C57BL/6NCR male mice, which were injected with Jo2 (0.2 μg/g *b.w.*) or saline (control), were sacrificed 6 h later. A-C: Cell lysates and RNA isolated from the livers were employed in caspase-3 assays (A) or to synthesize cDNA as a template for SYB green qPCR assays to determine *IL-6* (B) or *SAA3* (C) gene expression; D, E: *Hepcidin-1* (D) and *hepcidin-2* (E) mRNA expression was determined by Taqman qPCR. Gene expression in Jo2-injected mice was expressed as fold change of that in control mice.

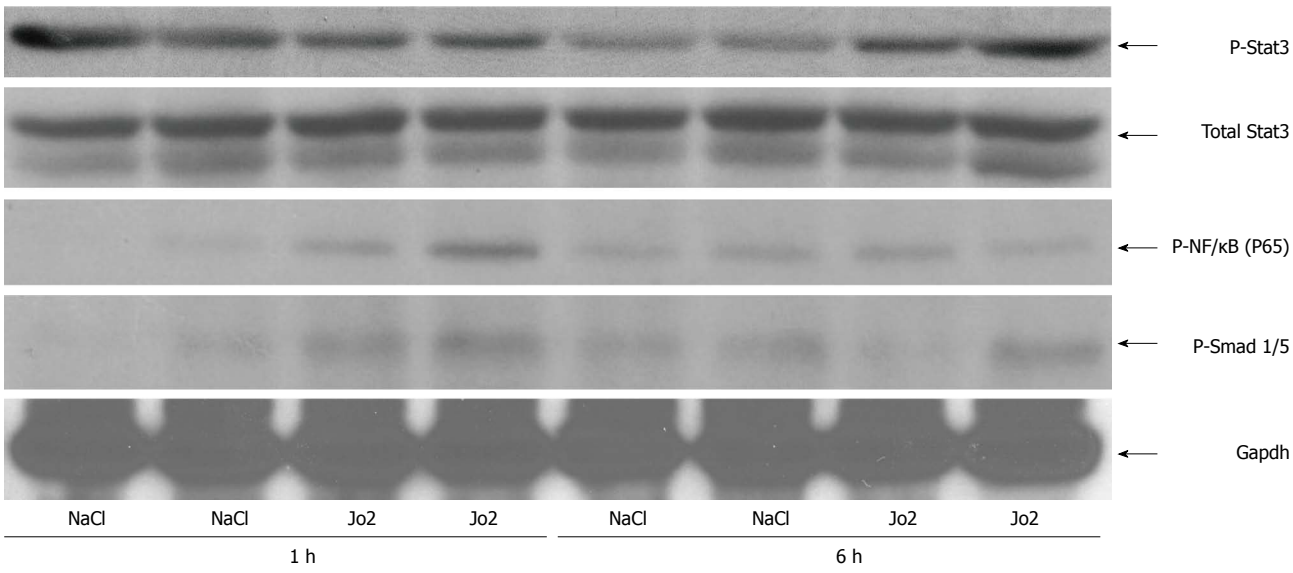

Figure 4 Phosphorylation of Stat3, NF-κB (P65) and Smad 1/5 in the liver. Whole cell lysates prepared from the livers of C57BL/6NCR mice injected with Jo2 (0.2 μg/g *b.w.*) and sacrificed after 1 or 6 h were employed for western blotting using anti-phospho--Stat3 (P-Stat3), anti-total Stat3, anti-phospho-P65 (P65) and anti-phospho-Smad1/5 antibodies, as described in Material and Methods. Anti-Gapdh antibody was used as protein loading control.

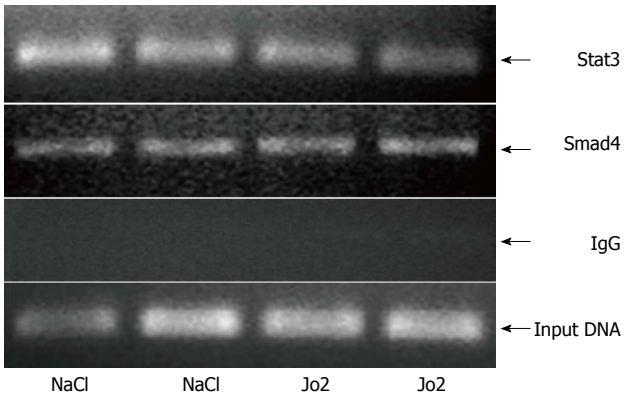

Figure 5 Chromatin immunoprecipitation assays. The binding of Stat3 or Smad4 to the *hepcidin-1* promoter in the livers of C57BL/6NCR mice, which were injected with Jo2 (0.2 μg/g *b.w.*) or saline (control) and sacrificed 6 h later, was determined by ChIP assays, as described in Material and Methods. Total input DNA was used as control to evaluate the amount of chromatin.

monly used diagnostic test to determine liver function and injury. Jo2 injected at a concentration of 0.2 μg/g did not cause a significant elevation of serum ALT or AST levels in C57BL6/NCR mice (Figure 7). However, the sera of C57BL/6J mice injected with 0.2 μg/g of Jo2, exhibited a dramatic increase in both ALT and AST levels, compared to controls (Figure 7). The injection of C57BL/6J mice with a higher dose of Jo2 (0.32 μg/g) induced further increase in serum ALT and AST levels (data not shown).

DISCUSSION

Apoptosis is one of the key factors which contribute to the pathogenesis of many liver diseases[1,25,26]. Apoptosis not only causes hepatocyte death directly but also induces inflammation and hepatic fibrosis[27,28]. The inhibition of caspase enzymes *via* known caspase inhibitors has been shown to effectively alleviate hepatocyte apoptosis and tissue damage in animal models of liver injury[29]. Due to its highly reactive nature and the liver serving as the major storage organ for it, iron is considered an important secondary risk factor in the progression of various liver diseases[30,31]. Therefore, it is of great importance to understand the interaction between apoptosis and iron metabolism. Since hepcidin is the central regulator of iron homeostasis and is primarily synthesized in the liver, this study investigated the effect of apoptosis on the regulation of hepcidin expression in the liver. Previously, Weizer-Stern *et al*[4] have elegantly demonstrated that p53, a tumor suppressor gene and an inducer of apoptosis, elevates human hepcidin gene transcription in HepG2 cells by binding to the corresponding response elements in hepcidin gene promoter. They have also reported that the overexpression of p53 blunts the stimulatory effect of IL-6 on hepcidin gene expression. Although indirect, these findings, for the first time, suggested a relationship between apoptosis and hepcidin and thereby the regulation of iron metabolism. However, due to various reasons, the *in vivo* relevance of this potential interaction is unclear. First, apoptosis signaling in cancer cell lines is frequently distorted and secondly, forced overexpression of p53 might have caused artificial effects. However, in a recent study, Li *et al*[5] have investigated the relationship between Fas -activated apoptosis signaling and expression of hepcidin gene expression. Fas activation decreased both mouse and human hepcidin mRNA expression *in vitro*. They have also shown that Balb/C3 female mice injected with lethal dose of Jo2 antibody (which killed mice within 4 h) exhibit a biphasic regulation of mouse hepcidin mRNA expression in the liver, namely an immediate elevation (within 0.5-1 h) followed by a suppression (within 4 h). They suggested that these changes in hepcidin expression correlates with the changes in FLIPL and IL-6 expression as well as the activation of the transcription factors, NF-κB, and Stat3. The knock-down or over-expression of FLIPL exerted a negative and a positive effect, respectively, on hepcidin expression. Based on their data, Li *et al*[5] have proposed a model suggesting that the stimulatory effect of Fas on hepcidin expression is achieved *via* IL-6 and Stat3, which themselves are activated by FLIPL and NF-κB. However, Li *et al*[5] did not confirm the presence (and the level) of apoptosis in the livers of Jo2-injected mice. Hence, it is unclear whether Fas-mediated apoptosis is directly involved in the regulation of hepcidin gene expression in the liver.

In our current study, we examined the effect of Fas signaling on hepatic hepcidin gene expression both *in vivo* and *in vitro*. In *in vitro* studies, the effect of CH11, an activating antibody specific for human Fas, was evaluated on hepcidin expression in HepG2 hepatoma cells. Even though CH11 induced apoptosis in a concentration dependent manner, as confirmed by the increased caspase-3 activity, the expression of human hepcidin gene was not significantly altered in these cells. Although we cannot exclude the possibility that Fas-mediated signaling in HepG2 hepatoma cells might be different than primary human hepatocytes, our findings strongly suggest that hepcidin gene expression in hepatocytes does not correlate with the significant induction of caspase activation. Of note, the liver is composed of various cell types and it is therefore feasible that not only hepatocytes, but other cells such as Kupffer cells, might be involved in the regulation of hepcidin gene by apoptosis. Hence, an *in vivo* experimental model whereby male C57BL/6 mice are injected with Jo2 antibody to specifically activate Fas-mediated apoptosis was employed to study hepcidin gene expression in whole liver. Male mice were chosen for these studies because unlike humans, female mice express higher levels of hepcidin compared to male mice[32]. Sublethal concentrations of Jo2 antibody were chosen for our experiments because based on the reports in the literature, this dose of Jo2 is more suitable for studies, which investigate the activation of Fas-mediated apoptosis in liver diseases[33]. Accordingly, we observed no lethality under our experimental conditions. Mice with short-term (1 h) Jo2 treatment did not display any significant

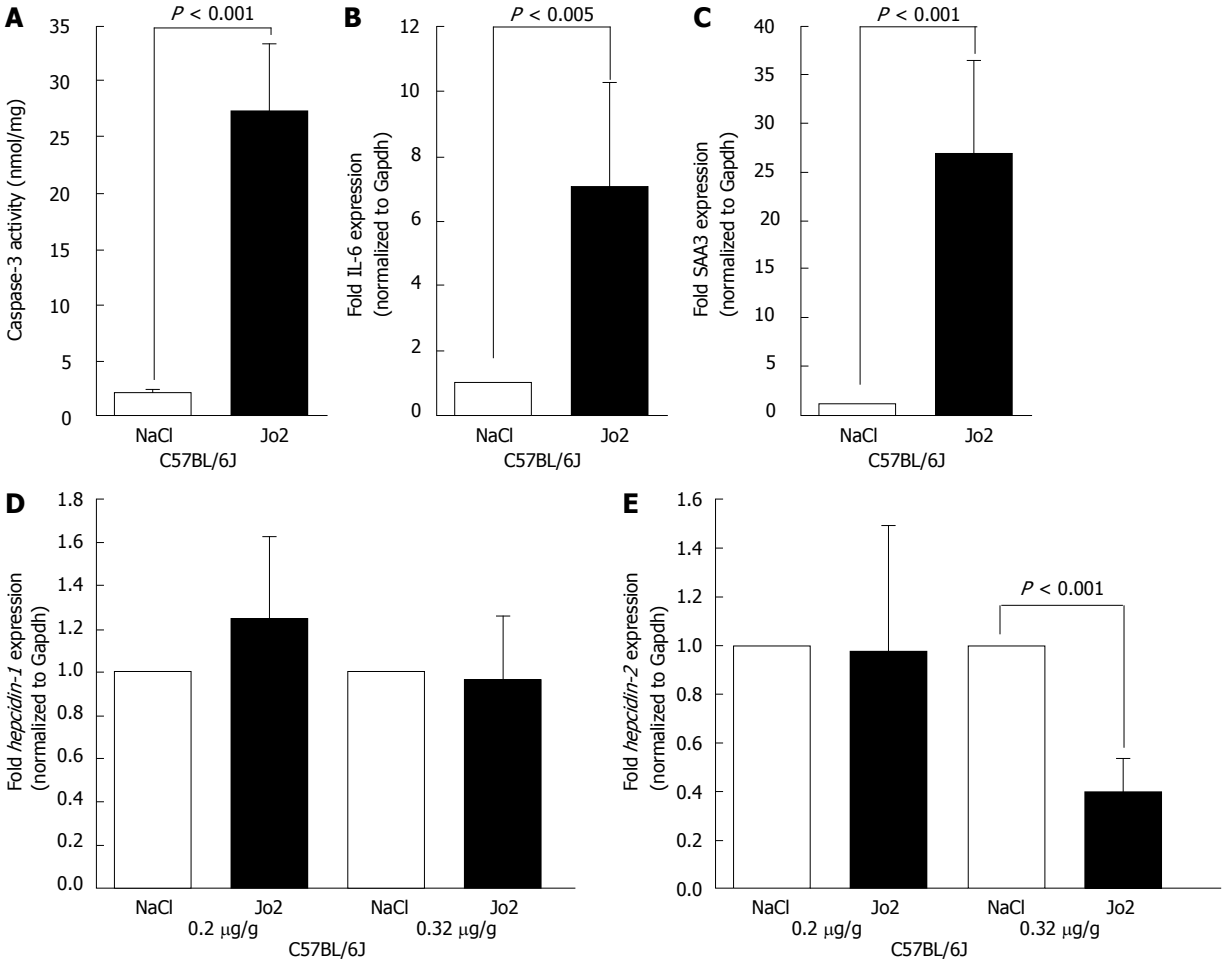

Figure 6 The effect of Jo2 on apoptosis, acute phase response and hepcidin gene expression in C57BL/6J mice. C57BL/6J male mice (n = 21) were injected with 0.2 μg/g *b.w.* and 0.32 μg/g *b.w.* Jo2 or saline and sacrificed 6 h later. Cell lysates and RNA isolated from the livers were employed in caspase-3 assays (A) or to synthesize cDNA as a template for SYB green qPCR assays to determine *IL-6* (B) or *SAA3* (C) gene expression. *Hepcidin-1* (D) and *hepcidin-2* (E) mRNA expression was determined by Taqman qPCR. Gene expression in Jo-2 injected mice was expressed as fold change of that in control mice.

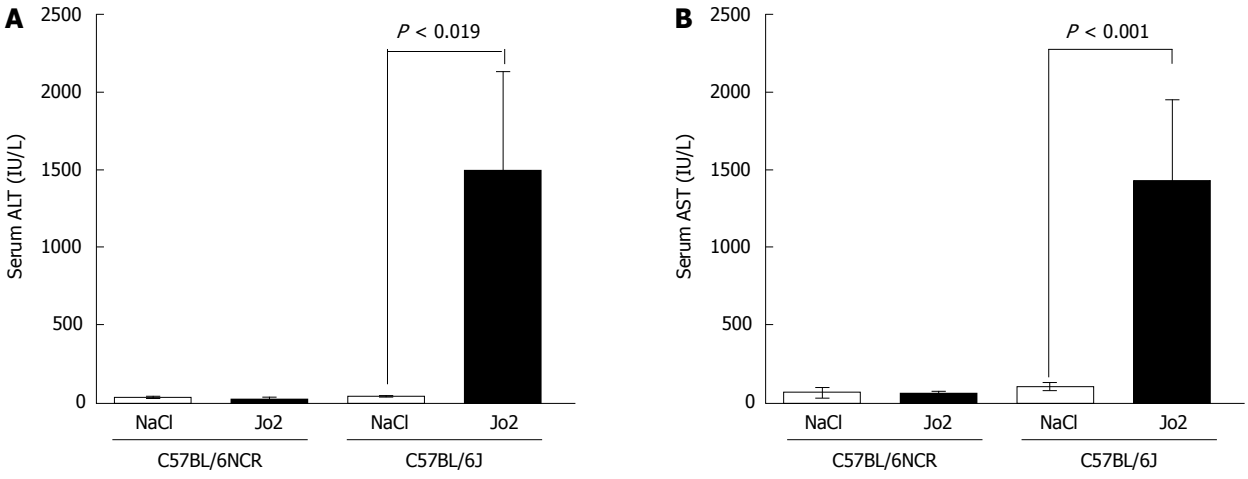

Figure 7 Comparison of serum liver enzyme levels in Jo2-injected C57BL/6J and C57BL/6NCR mice. Mice injected with Jo2 (0.2 μg/g *b.w.*) were sacrificed 6 h later. Serum ALT (A) and AST (B) enzymes were measured, as described in "Materials and Methods".

changes both in the activity of caspase-3 enzyme and the expression of acute phase marker genes, *IL-6* and *SAA3*, strongly suggesting the absence of apoptosis and acute phase responses in the livers of these mice. The macroscopic appearance of the livers from short-term Jo2-injected mice was also similar to those in control mice (data not shown). Further, the expression level of mouse *hepcidin-1* was not affected in the livers of these mice. In

contrast, longer treatment with Jo2 (6 h) significantly induced apoptosis and acute phase reaction. Concurrently, the livers of these mice displayed macroscopic differences such as a darker color suggesting the presence of hepatic hemorrhage and liver injury (data not shown). Interestingly, these changes did not correlate with the level of *hepcidin-1* mRNA expression in the liver. Similar to short-term, the longer treatment of mice with Jo2 did not alter the level of hepcidin gene expression.

It is well known that hepcidin gene transcription is strongly stimulated by IL-6 and Stat3 pathway. Since, we have shown that long-term, but not short-term, Jo2 treatment can induce acute phase reactions in the liver, the phosphorylation status of Stat3 was investigated to confirm its activation. In accordance with our acute phase gene expression findings, we observed Stat3 phosphorylation following 6 h, but not 1 h of Jo2 treatment. Taken together, our findings show that the activation of IL-6/Stat3 axis by Fas is not sufficient to induce *hepcidin-1* transcription and strongly suggest the presence of inhibitory mechanisms. This is also supported by our ChIP findings, which show that the occupancy of *hepcidin-1* promoter by Stat3 is similar in the livers of both Jo2-treated and control mice despite the differences in the activation status of Stat3. In contrast to Stat3, the phosphorylation of NF-κB was observed with short-term, but not long-term, Jo2 treatment. NF-κB is involved in inflammatory cytokine production in the liver including IL-6[26]. It is therefore possible that Jo-2-mediated early phase activation of NF-κB subsequently facilitates the induction of IL-6 transcription and consequent activation of Stat3, which was observed in the livers of mice with longer (6 h) Jo2 treatment.

Hepcidin is also activated by BMP/Smad pathway and Smad4 knockout mice display reduced hepcidin expression[18]. However, 6 h Jo2 administration did not significantly alter the phosphorylation of Smad1/5 proteins, which are transcription factors known to be activated by BMP pathway. Growth factors have been shown to suppress the signaling of BMP/Smad pathway and its stimulatory effect on hepcidin gene expression in the liver[19]. Of note, liver injury is known to stimulate the expression of growth factors as part of the liver regeneration process. It is therefore feasible that Fas-induced liver injury might suppress Smad activation and thereby counteract the stimulation of *hepcidin-1* transcription by Stat3. However, it should also be noted that despite significant differences in the level of liver injury, acute phase response and apoptosis, both C57BL/6J and C57BL/6NCR mice (under similar experimental conditions) did not display any significant changes in liver *hepcidin-1* expression. This suggests that mechanisms other than growth factors and inhibitory Smads might play a role in this process. Jo2-induced liver damage accompanied by DNA damage and the activation of p53 might be involved since p53 has been shown to suppress the stimulatory effect of IL-6 on hepcidin gene expression[4]. Furthermore, we observed differential regulation of *hepcidin-2* expression by Jo2 in the liver. Since the function of *hepcidin-2* is unknown, the significance of this finding and its potential role in liver injury and disease will be addressed in future studies.

In conclusion, Fas ligand-induced signaling and apoptosis do not play a significant role in the regulation of human (*HAMP*) or mouse (*hepcidin-1)* hepatic hepcidin gene expression, as shown by *in vitro* and *in vivo* experimental systems using human or mouse Fas receptor-activating antibodies, CH11 or Jo2 , respectively. The induction of extrinsic apoptotic pathway *via* Fas receptor signaling, as confirmed by effector caspase activation, significantly induced the phosphorylation and activation of the transcription factor, Stat3 in the liver. Stat3 is well known to be involved in inflammation-mediated elevation of hepcidin expression. However, no significant binding of Stat3 to hepcidin gene promoter was observed, as confirmed by chromatin immunoprecipitation studies. Our findings strongly suggest that the activation of Stat3 by Fas signaling-mediated apoptosis is not sufficient to stimulate hepcidin transcription in the liver. Using different strains of mice, we were also able to confirm that the severity of Fas-induced apoptosis (acute phase reaction or tissue injury) does not correlate with its effect on hepcidin gene expression in the liver. Interestingly, Jo2 treatment induced changes in *hepcidin-2* expression in a time-dependent manner but the function of this mouse gene is as yet unknown.

COMMENTS

Background

Apoptosis is widely observed and participates in the pathogenesis of various liver diseases. Iron, due to its highly reactive nature as a transitional metal, acts as a secondary risk factor in various liver diseases. Hepcidin, a small peptide primarily synthesized in the liver, is the central regulator of iron metabolism. Hepcidin maintains iron homeostasis by inhibiting iron absorption by the enterocytes in the duodenum and iron release by the macrophages of reticuloendothelial system. Hepcidin expression in the liver has been shown to be modulated by iron, inflammation and hypoxia but a direct role of apoptosis in hepcidin regulation has not been elucidated.

Research frontiers

Iron plays a role in liver injury and the transcriptional regulation of hepcidin gene in liver diseases has been highlighted by recent studies from various laboratories. A better understanding of the regulation of hepcidin and thereby iron homeostasis in the pathogenesis of liver diseases might facilitate the development of novel diagnosis and treatment strategies.

Innovations and breakthroughs

P53, a tumor suppressor and inducer of apoptosis, has been reported to promote hepcidin gene transcription through direct binding to its promoter. An independent study has recently suggested the involvement of Fas signaling in the regulation of liver hepcidin expression. However, a causal relationship between Fas-mediated effector caspase activation and apoptosis, and the regulation of hepcidin gene transcription has not been demonstrated. This study addressed this question by quantifying the level of Fas-induced apoptosis by caspase-3 activity assays and correlating it to both human and mouse hepcidin gene expression. In addition, different strains of mice with significant variances in their response to Fas treatment were also employed. Collectively, the authors' findings clearly demonstrate the lack of correlation between Fas-mediated apoptosis and hepatic hepcidin gene transcription.

Applications

The findings of this study, which strongly suggest that Fas-mediated apoptosis is not involved in the regulation of hepcidin expression, will further our understanding of the pathogenesis of liver diseases associated with increases in he-

patic iron content. Furthermore, the authors' findings, which demonstrate strain-specific responses to anti-Fas treatment, highlights the importance of choosing optimal mice strains for studies with Fas signaling in the liver.

Terminology

Apoptosis, also known as programmed cell death, is characterized by the sequential activation of a series of caspases, which also serve as markers for apoptosis. The pathogenesis of many liver diseases involve apoptotic pathways. Hepcidin is a small antimicrobial peptide synthesized mainly by the hepatocytes. It regulates iron homeostasis by binding to and inducing the degradation of the only known iron exporter, ferroportin.

Peer review

In this paper, Lu *et al* aimed to determine the regulation of human hepcidin (*HAMP*) and mouse hepcidin (*hepcidin-1* and *hepcidin-2*) gene expression in the liver by apoptosis using *in vivo* and *in vitro* experimental models. The role of hepcidin in liver fibrosis, *via* apoptosis, has emerged in recent years. Given the goal of achieving an explanation about the role of hepcidin is a growing concern, the analysis is justified and the aim of the study is clinically relevant. It is a well-designed study and the conclusions are consistent with the results.

REFERENCES

1 **Schattenberg JM**, Galle PR, Schuchmann M. Apoptosis in liver disease. *Liver Int* 2006; **26**: 904-911 [PMID: 16953829 DOI: 10.1111/j.1478-3231.2006.01324.x]

2 **Ganz T**. Hepcidin and iron regulation, 10 years later. *Blood* 2011; **117**: 4425-4433 [PMID: 21346250 DOI: 10.1182/blood-2011-01-258467]

3 **Ganz T**, Nemeth E. Hepcidin and disorders of iron metabolism. *Annu Rev Med* 2011; **62**: 347-360 [PMID: 20887198 DOI: 10.1146/annurev-med-050109-142444]

4 **Weizer-Stern O**, Adamsky K, Margalit O, Ashur-Fabian O, Givol D, Amariglio N, Rechavi G. Hepcidin, a key regulator of iron metabolism, is transcriptionally activated by p53. *Br J Haematol* 2007; **138**: 253-262 [PMID: 17593032 DOI: 10.1111/j.1365-2141.2007.06638.x]

5 **Li X**, Xu F, Karoopongse E, Marcondes AM, Lee K, Kowdley KV, Miao CH, Trobridge GD, Campbell JS, Deeg HJ. Allogeneic transplantation, Fas signaling, and dysregulation of hepcidin. *Biol Blood Marrow Transplant* 2013; **19**: 1210-1219 [PMID: 23707854 DOI: 10.1016/j.bbmt.2013.05.012]

6 **Guicciardi ME**, Gores GJ. Life and death by death receptors. *FASEB J* 2009; **23**: 1625-1637 [PMID: 19141537 DOI: 10.1096/fj.08-111005]

7 **Villarroel P**, Le Blanc S, Arredondo M. Interleukin-6 and lipopolysaccharide modulate hepcidin mRNA expression by HepG2 cells. *Biol Trace Elem Res* 2012; **150**: 496-501 [PMID: 23065424 DOI: 10.1007/s12011-012-9522-6]

8 **Nemeth E**, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood* 2003; **101**: 2461-2463 [PMID: 12433676 DOI: 10.1182/blood-2002-10-3235]

9 **Nemeth E**, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T. IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. *J Clin Invest* 2004; **113**: 1271-1276 [PMID: 15124018 DOI: 10.1172/JCI200420945]

10 **Verga Falzacappa MV**, Vujic Spasic M, Kessler R, Stolte J, Hentze MW, Muckenthaler MU. STAT3 mediates hepatic hepcidin expression and its inflammatory stimulation. *Blood* 2007; **109**: 353-358 [PMID: 16946298 DOI: 10.1182/blood-2006-07-033969]

11 **Pietrangelo A**, Dierssen U, Valli L, Garuti C, Rump A, Corradini E, Ernst M, Klein C, Trautwein C. STAT3 is required for IL-6-gp130-dependent activation of hepcidin in vivo. *Gastroenterology* 2007; **132**: 294-300 [PMID: 17241879 DOI: 10.1053/j.gastro.2006.10.018]

12 **Babitt JL**, Huang FW, Wrighting DM, Xia Y, Sidis Y, Samad TA, Campagna JA, Chung RT, Schneyer AL, Woolf CJ, Andrews NC, Lin HY. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat Genet* 2006; **38**: 531-539 [PMID: 16604073 DOI: 10.1038/ng1777]

13 **Truksa J**, Peng H, Lee P, Beutler E. Bone morphogenetic proteins 2, 4, and 9 stimulate murine hepcidin 1 expression independently of Hfe, transferrin receptor 2 (Tfr2), and IL-6. *Proc Natl Acad Sci USA* 2006; **103**: 10289-10293 [PMID: 16801541 DOI: 10.1073/pnas.0603124103]

14 **Lin L**, Valore EV, Nemeth E, Goodnough JB, Gabayan V, Ganz T. Iron transferrin regulates hepcidin synthesis in primary hepatocyte culture through hemojuvelin and BMP2/4. *Blood* 2007; **110**: 2182-2189 [PMID: 17540841 DOI: 10.1182/blood-2007-04-087593]

15 **Shanmugam NK**, Cherayil BJ. Serum-induced up-regulation of hepcidin expression involves the bone morphogenetic protein signaling pathway. *Biochem Biophys Res Commun* 2013; **441**: 383-386 [PMID: 24157792 DOI: 10.1016/j.bbrc.2013.10.065]

16 **Andriopoulos B**, Corradini E, Xia Y, Faasse SA, Chen S, Grgurevic L, Knutson MD, Pietrangelo A, Vukicevic S, Lin HY, Babitt JL. BMP6 is a key endogenous regulator of hepcidin expression and iron metabolism. *Nat Genet* 2009; **41**: 482-487 [PMID: 19252486 DOI: 10.1038/ng.335]

17 **Meynard D**, Kautz L, Darnaud V, Canonne-Hergaux F, Coppin H, Roth MP. Lack of the bone morphogenetic protein BMP6 induces massive iron overload. *Nat Genet* 2009; **41**: 478-481 [PMID: 19252488 DOI: 10.1038/ng.320]

18 **Wang RH**, Li C, Xu X, Zheng Y, Xiao C, Zerfas P, Cooperman S, Eckhaus M, Rouault T, Mishra L, Deng CX. A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. *Cell Metab* 2005; **2**: 399-409 [PMID: 16330325 DOI: 10.1016/j.cmet.2005.10.010]

19 **Goodnough JB**, Ramos E, Nemeth E, Ganz T. Inhibition of hepcidin transcription by growth factors. *Hepatology* 2012; **56**: 291-299 [PMID: 22278715 DOI: 10.1002/hep.25615]

20 **Harrison-Findik DD**, Klein E, Crist C, Evans J, Timchenko N, Gollan J. Iron-mediated regulation of liver hepcidin expression in rats and mice is abolished by alcohol. *Hepatology* 2007; **46**: 1979-1985 [PMID: 17763462 DOI: 10.1002/hep.21895]

21 **Harrison-Findik DD**, Lu S, Zmijewski EM, Jones J, Zimmerman MC. Effect of alcohol exposure on hepatic superoxide generation and hepcidin expression. *World J Biol Chem* 2013; **4**: 119-130 [PMID: 24340135 DOI: 10.4331/wjbc.v4.i4.119]

22 **Gerjevic LN**, Liu N, Lu S, Harrison-Findik DD. Alcohol Activates TGF-Beta but Inhibits BMP Receptor-Mediated Smad Signaling and Smad4 Binding to Hepcidin Promoter in the Liver. *Int J Hepatol* 2012; **2012**: 459278 [PMID: 22121494 DOI: 10.1155/2012/459278]

23 **Weinmann AS**, Bartley SM, Zhang T, Zhang MQ, Farnham PJ. Use of chromatin immunoprecipitation to clone novel E2F target promoters. *Mol Cell Biol* 2001; **21**: 6820-6832 [PMID: 11564866 DOI: 10.1128/MCB.21.20.6820-6832.2001]

24 **Mekada K**, Abe K, Murakami A, Nakamura S, Nakata H, Moriwaki K, Obata Y, Yoshiki A. Genetic differences among C57BL/6 substrains. *Exp Anim* 2009; **58**: 141-149 [PMID: 19448337 DOI: 10.1538/expanim.58.141]

25 **Rust C**, Gores GJ. Apoptosis and liver disease. *Am J Med* 2000; **108**: 567-574 [PMID: 10806286 DOI: 10.1016/S0002-9343(00)00370-3]

26 **Chakraborty JB**, Oakley F, Walsh MJ. Mechanisms and biomarkers of apoptosis in liver disease and fibrosis. *Int J Hepatol* 2012; **2012**: 648915 [PMID: 22567408 DOI: 10.1155/2012/648915]

27 **Canbay A**, Taimr P, Torok N, Higuchi H, Friedman S, Gores GJ. Apoptotic body engulfment by a human stellate cell line is profibrogenic. *Lab Invest* 2003; **83**: 655-663 [PMID: 12746475 DOI: 10.1097/01.LAB.0000069036.63405.5C]

28 **Canbay A**, Feldstein A, Baskin-Bey E, Bronk SF, Gores GJ. The caspase inhibitor IDN-6556 attenuates hepatic injury and fibrosis in the bile duct ligated mouse. *J Pharmacol Exp*

Ther 2004; **308**: 1191-1196 [PMID: 14617689 DOI: 10.1124/jpet.103.060129]
29 **Natori S**, Higuchi H, Contreras P, Gores GJ. The caspase inhibitor IDN-6556 prevents caspase activation and apoptosis in sinusoidal endothelial cells during liver preservation injury. *Liver Transpl* 2003; **9**: 278-284 [PMID: 12619025 DOI: 10.1053/jlts.2003.50019]
30 **Fargion S**, Valenti L, Fracanzani AL. Beyond hereditary hemochromatosis: new insights into the relationship between iron overload and chronic liver diseases. *Dig Liver Dis* 2011; **43**: 89-95 [PMID: 20739232 DOI: 10.1016/j.dld.2010.07.006]
31 **Datz C**, Felder TK, Niederseer D, Aigner E. Iron homeostasis in the metabolic syndrome. *Eur J Clin Invest* 2013; **43**: 215-224 [PMID: 23289518 DOI: 10.1111/eci.12032]
32 **Vujic Spasic M**, Kiss J, Herrmann T, Kessler R, Stolte J, Galy B, Rathkolb B, Wolf E, Stremmel W, Hentze MW, Muckenthaler MU. Physiologic systemic iron metabolism in mice deficient for duodenal Hfe. *Blood* 2007; **109**: 4511-4517 [PMID: 17264297 DOI: 10.1182/blood-2006-07-036186]
33 **Haga S**, Terui K, Zhang HQ, Enosawa S, Ogawa W, Inoue H, Okuyama T, Takeda K, Akira S, Ogino T, Irani K, Ozaki M. Stat3 protects against Fas-induced liver injury by redox-dependent and -independent mechanisms. *J Clin Invest* 2003; **112**: 989-998 [PMID: 14523036 DOI: 10.1172/JCI17970]

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REVIEW

Insight into the mechanisms and functions of spliceosomal snRNA pseudouridylation

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Abstract

Pseudouridines (Ψs) are the most abundant and highly conserved modified nucleotides found in various stable RNAs of all organisms. Most Ψs are clustered in regions that are functionally important for pre-mRNA splicing. Ψ has an extra hydrogen bond donor that endows RNA molecules with distinct properties that contribute significantly to RNA-mediated cellular processes. Experimental data indicate that spliceosomal snRNA pseudouridylation can be catalyzed by both RNA-dependent and RNA-independent mechanisms. Recent work has also demonstrated that pseudouridylation can be induced at novel positions under stress conditions, suggesting a regulatory role for Ψ.

Key words: Pre-mRNA splicing; U2 snRNA; Box H/ACA ribonucleoprotein; Pseudouridine; Induced RNA modification

Core tip: Pseudouridines (Ψs) are the most abundant and highly conserved modified nucleotides identified in various stable RNAs of all organisms. Most Ψs are clustered in regions that are functionally important for pre-mRNA splicing. Ψ has an extra hydrogen bond donor that endows RNA molecules with distinct properties that contribute significantly to RNA-mediated cellular processes. Experimental data indicate that spliceosomal snRNA pseudouridylation can be catalyzed by both RNA-dependent and RNA-independent mechanisms. Recent work has also demonstrated that pseudouridylation can be induced at novel positions under stress conditions, suggesting a regulatory role for Ψ.

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INTRODUCTION

Many eukaryotic genes consist of blocks of coding sequences (exons) separated by blocks of noncoding sequences, termed introns[1]. Introns are removed from a primary transcript (pre-mRNA) by a process called pre-mRNA splicing. This process is carried out by a huge complex called the spliceosome, which comprises about 300 proteins and 5 small RNAs[2]. The five small RNAs are uridine-rich, and are thus called U small nuclear RNAs (U1, U2, U4, U5 and U6 snRNAs) (Figure 1)[3,4]. In eukaryotic cells, U snRNAs exist as RNA-protein complexes called small nuclear ribonucleoproteins (snRNPs). During spliceosome assembly, snRNPs are sequentially recruited onto a pre-mRNA substrate, resulting in the formation of several short stretches of RNA-RNA duplexes that play key roles in recognizing, specifying and catalyzing the two successive chemical reactions (Figure 2)[5-9].

First, the 5' splice site is recognized by the U1 snRNP

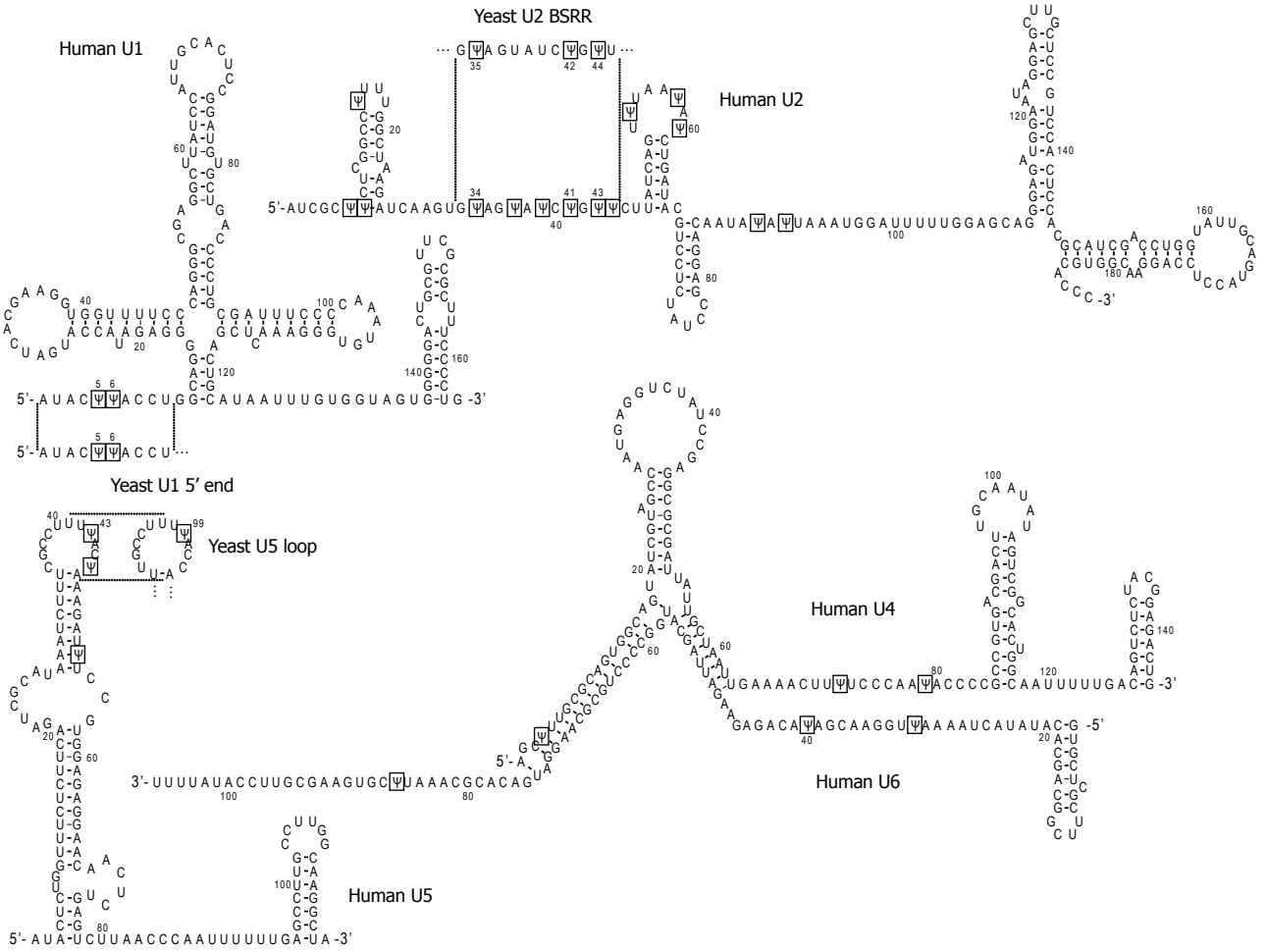

Figure 1 Primary sequences and secondary structures of human spliceosomal snRNAs (U1, U2, U4, U5 and U6). Pseudouridines (Ψ) are boxed. The sequences of yeast snRNAs where the Ψs have their counterparts in human snRNAs (the 5' end region of U1, branch site recognition region (BSRR) of U2, and loop region of U5) are also shown. The structures are predicted by the "multifold" program and are consistent with the genetic/biochemical mapping data.

through base pairing interactions[10-12], resulting in the formation of the commitment complex or the early (E) complex. Second, U2 snRNP binds, again *via* base-pairing interactions, to the branch site, and forms a pre-splicing complex, namely complex A[7,13,14]. Base-pairing between the U2 snRNA and the branch site bulges out the branch point nucleotide (typically an adenosine residue), which is thus made available for the first chemical reaction[15-17] (see below). Next, the U4/U6.U5 tri-snRNP particle, in which U4 and U6 are extensively base-paired with each other, joins the A complex, leading to the formation of complex B1[18-21]. Subsequently, a series of RNA-RNA interaction rearrangements occur, resulting in the release of U1 and U4 snRNPs and hence the formation of complex B2 or the spliceosome. In the newly formed spliceosome, U5 contacts the splice sites and U6 base-pairs with both the 5' splice site and U2[22], thus forming the active site for the first chemical reaction, in which the 2'-OH group of the bulged out branch nucleotide adenosine attacks the 5' splice site. This generates the 5' exon and the 2/3 lariat intermediate. Immediately after the first chemical reaction, additional conformational changes occur, leading to the formation of complex C and the initiation of the second chemical reaction, where the liberated 3'-OH of the 5' exon attacks the phosphate group of the 3' splice site. The second chemical reaction results in the release of the lariat intron as well as the ligation of the exons (mRNA) (Figure 2)[2,23]. Finally, the mRNA product is released, and the U2, U5, and U6 snRNPs are disassembled and recycled for further rounds of pre-mRNA splicing[24,25].

It is notable that all five spliceosomal snRNAs are extensively posttranscriptionally modified[26-29]. Pseudouridine (Ψ), the C5-glycoside isomer of uridine, is the most abundant in these RNAs. For example, there are 14 Ψs out of 189 nucleotides of vertebrate U2 snRNA, accounting for approximately 60% of the total modifications and approximately 7% of the total nucleotides[30-32]. Strikingly, the majority of the Ψs are present in regions that are functionally important for pre-mRNA splicing, including the regions involved in RNA-RNA interactions in the splicing complexes/spliceosome (Figure 1).

Because of its unique structural and chemical properties and its proven biological importance, Ψ has begun to receive increasing research attention. However, due to the difficulty of developing effective assays and experimental systems, there had been little progress in research on RNA pseudouridylation until fairly recently. In the

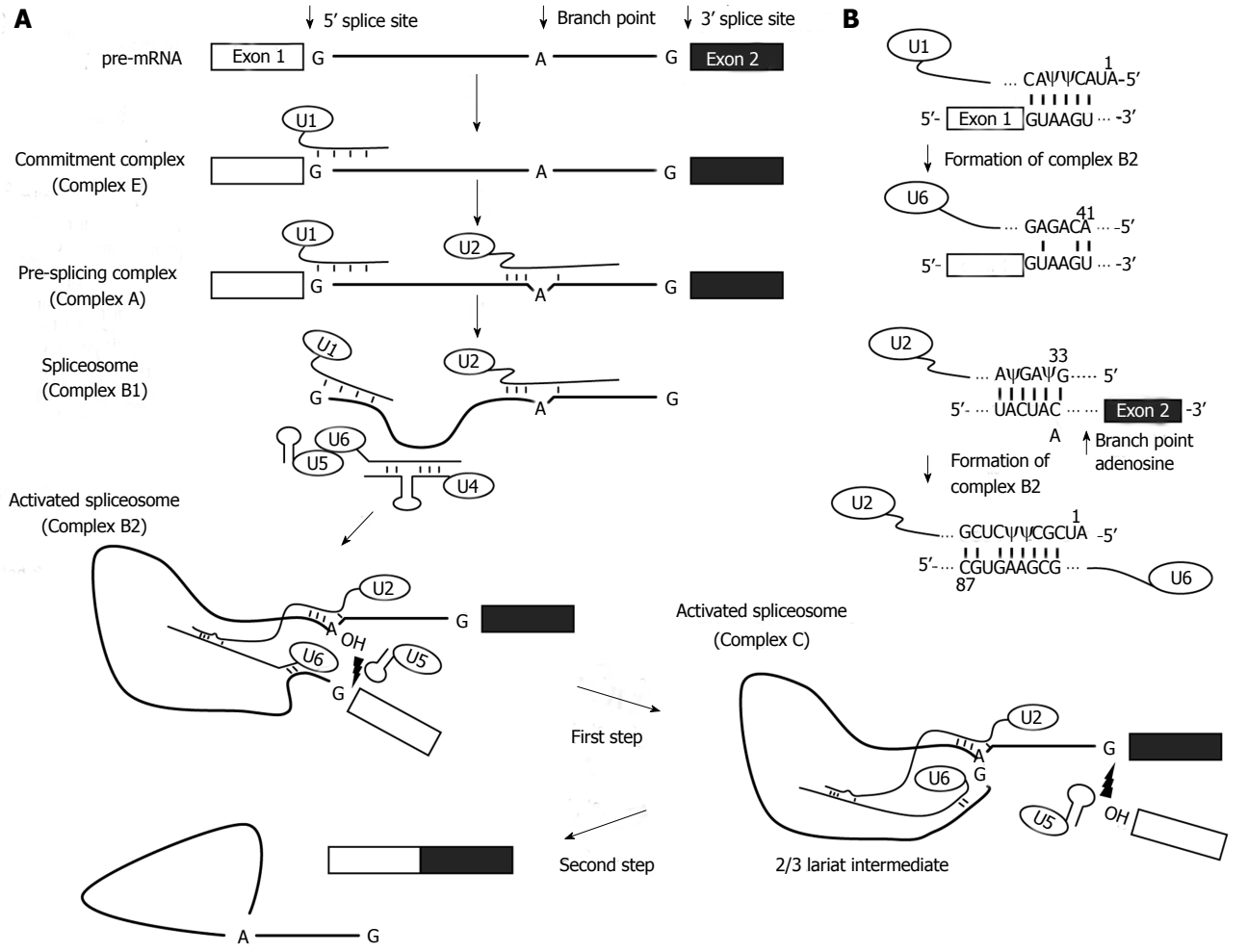

Figure 2 The splicing reaction. A: Steps of the spliceosome-mediated splicing reaction. The thick lines represent the intron and the boxes are exons. The short lines between RNA strands represent watson-crick base-pairing interactions. The 2'-OH groups of branch point adenosine and the cut-off 5' exon are pictured in the activated spliceosomes. The lightning symbols depict a nucleophilic attack that causes a transesterification reaction; B: Putative RNA-RNA hybrids formed during the splicing reaction.

past approximately 17 years, however, several laboratories have made several remarkable discoveries[33-38] underscoring the notion that Ψs in U snRNAs are not just bystanders in the process of pre-mRNA splicing, but that they are active participants in spliceosome assembly and splicing. This review discusses the mechanisms and functions of spliceosomal snRNA pseudouridylation, focusing on the most extensively studied U2 snRNA.

ΨS ARE IMPORTANT FOR RNA FUNCTION

Ψs are abundant, conserved, and reside in important regions of snRNAs

Ψ was first detected as an unknown nucleotide more than 60 years ago[39] and soon afterward it was identified as 5-ribosyluracil, an isomer of uridine (1-ribosyluracil)[40]. Since its discovery, Ψ has been found in various stable RNAs (including rRNAs[41-45], tRNAs[46-49], and snRNAs[26-31,50]) of all organisms, and now it has been known as the most abundant modified nucleotide. Besides being abundant, Ψs are highly conserved across species especially in functionally important regions of snRNAs. For example, both vertebrate and yeast U1 snRNAs contain two Ψs at the 5' end region (Ψ5 and Ψ6) known to recognize and base-pair with the 5' splice site during spliceosome assembly (Figure 1 and Table 1). Three of the six Ψs (Ψ34, Ψ41 and Ψ43) in the vertebrate U2 branch site recognition region (BSRR), which is involved in base-pairing with the pre-mRNA branch site, have their counterparts in yeast U2 (corresponding to Ψ35, Ψ42, and Ψ44, respectively). Likewise, one of the Ψs (Ψ43) in the conserved loop of vertebrate U5, which participates in interacting with the 5' and 3' exon sequences, is also present in yeast U5 snRNA at the equivalent site (Ψ99). Ψs are also found in the U4-U6 duplex regions as well as in other regions of U6 that are important for function. In other instances, some of these Ψs are conserved in plant snRNAs[51] and in minor-class snRNAs (U4atac and U12)[52]. The phylogenetic conservation as well as the strategic location of Ψs clearly suggests that they play functionally important roles in pre-mRNA splicing.

Characteristics of Ψ

Ψ is converted from its isomer, uridine (U) (Figure 3).

Table 1 Pseudouridylation sites within yeast and human spliceosomal snRNAs

Organism	snRNA	Position	Catalyst	Ref.
Yeast	U1	Ψ5	Cbf5	[52], Unpublished data Yu Lab
		Ψ6	Cbf5	[52], Unpublished data Yu Lab
	U2	Ψ35	Pus7	[52,70]
		Ψ42	H/ACA RNP (snR81)	[52,83]
		Ψ44	Pus1	[52]
		(Ψ56; induced)	Pus7	[87]
		(Ψ93; induced)	H/ACA RNP (snR81)	[87]
	U5	Ψ99	NR	[52]
Human	U1	Ψ5	H/ACA RNP (ACA47)	[81, 88]
		Ψ6	H/ACA RNP (U109)	[88,89]
	U2	Ψ6	NR	
		Ψ7	H/ACA RNP (U100)	[43,79]
		Ψ15	NR	
		Ψ34	H/ACA RNP (U92)	[90, 91]
		Ψ37	H/ACA RNP (ACA45)	[81,91]
		Ψ39	H/ACA RNP (ACA26)	[81,91]
		Ψ41	H/ACA RNP (ACA45)	[81,91]
		Ψ43	NR	
		Ψ44	H/ACA RNP (U92)	[90,91]
		Ψ54	H/ACA RNP (U93)	[30,43,91,92]
		Ψ58	NR	
		Ψ60	NR	[93]
		Ψ89	H/ACA RNP (ACA35)	[81,91]
		Ψ91	NR	
	U4	Ψ4	NR	
		Ψ72	NR	
		Ψ79	NR	
	U5	Ψ43	H/ACA RNP (ACA57)	[81,94]
		Ψ46	H/ACA RNP (U85)	[80,94]
		Ψ53	H/ACA RNP (U93)	[30,91,92]
	U6	Ψ31	H/ACA RNP (ACA65)	[43]
		Ψ40	H/ACA RNP (ACA12)	[79,81,95]
		Ψ86	H/ACA RNP (ACA65)	[79]
	U12	Ψ19	H/ACA RNP (ACA68)	[43,96]
		Ψ28	H/ACA RNP (ACA66)	[43,96]
	U4atac	Ψ12	NR	[96]
	U6atac	Ψ83	NR	[96]

First, the glycosidic bond of U (N1-C1') is broken. The uracil base then rotates 180° along the N3-C6 axis, allowing the formation of a new carbon-carbon (C5-C1') bond between the base and the sugar[53,54]. As a result, the modified uridine, or Ψ, has an extra hydrogen bond donor at its non Watson-Crick edge, which can interact with its own phosphate backbone to form a rigid RNA structure[55-61]. Ψ can also contribute to stabilization of an RNA chain or an RNA-RNA interaction through alteration of RNA local structure or through enhancement of base stacking[62]. In this regard, it is reported that the Ψ-A pair is more stable than the U-A pair[63,64]. Thus, U-to-Ψ conversion endows the modified uridine (Ψ) with chemical properties that are distinct from those of uridine and all other known nucleotides.

Given that they are phylogenetically conserved, that they are clustered in functional regions, and that they have distinct chemical properties, Ψs are expected to affect the function of the RNA in which they reside. Extensive researches carried out over the past 15 years have indeed demonstrated that Ψs have the potential to impact numerous aspects of RNA biology, including structure, thermal stability, and biochemical interactions. Below, we discuss the function of U2 snRNA pseudouridylation.

FUNCTIONS OF U2 SNRNA PSEUDOURIDYLATION

U2 snRNA contains the most Ψs among all known snRNAs (*e.g.*, human U2 snRNA contains 13 Ψs), and for this reason, U2 snRNA pseudouridylation has been the most extensively studied. Three experimental systems have been fairly extensively used to study the function of U2 pseudouridylation, and they are discussed below.

The mammalian cell-free system

Over 20 years ago, Jeffery Patton carried out the first functional analysis of U2 snRNA modification[65,66]. He found that *in vitro* synthesized U2 snRNA could be efficiently pseudouridylated in HeLa cell S100 extracts[66]. He also demonstrated that the incorporation of 5-fluorouridine (5FU) into U2 snRNA site-specifically blocked U2 snRNA pseudouridylation and that the 5FU-containing U2 snRNP (free of Ψs) is more prone to salt-induced dissociation when compared with U2 snRNP containing regular nucleotides (pseudouridylated)[66]. These results suggested that U2 snRNA lacking Ψs was disadvantaged in snRNP assembly, implying that Ψs contribute to snRNP biogenesis.

A decade later (in 2004), the Lührmann group provided direct experimental evidence for the functional importance of U2 snRNA pseudouridylation in pre-mRNA splicing[67]. In this study, they depleted endogenous U2 snRNP from splicing extracts derived from HeLa cells using affinity selection with oligonucleotides complementary to U2 snRNA. Then they reconstituted the U2 snRNP *in vitro* using synthesized U2 snRNA. The reconstituted U2 snRNP was added to the U2-depleted extracts, and its ability to support pre-mRNA splicing was then assayed. Their results indicated that the three Ψs located within the 5' end region (Ψ6, Ψ7 and Ψ15) exhibited cumulative effects on U2 function. Specifically, they are required for the E complex formation. Together, the data obtained from mammalian *in vitro* systems have clearly suggested that Ψs in U2 snRNA play important roles in snRNP biogenesis and pre-mRNA splicing.

The Xenopus oocyte reconstitution system

A more detailed and systematic analysis of the effects of U2 snRNA pseudouridylation on pre-mRNA splicing was conducted in *Xenopus* oocytes[35,68,69]. In this experimental system, an endogenous snRNA can be specifically and nearly completely depleted upon injection of an antisense DNA oligonucleotide. Specifically, the DNA oligonucleotide, once injected, forms a duplex with its target snRNA, thus triggering an endogenous RNase H activity, which degrades the snRNA (the RNA strand of the RNA-DNA hybrid). Four hours later, the injected DNA

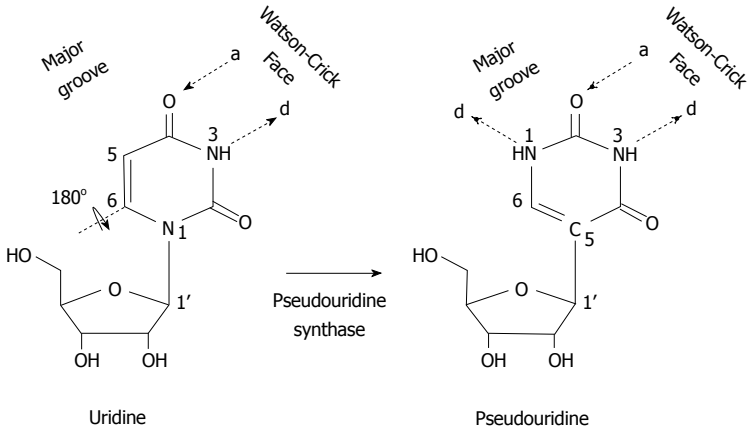

Figure 3 Schematic representation of uridine-to- pseudouridine isomerization. Pseudouridine is a rotational isomer of uridine, in which the N-C glycosidic bond is broken to form the C-C bond. This results in the creation of an extra hydrogen bond donor (d), while the number of hydrogen bond acceptors (a) is unchanged.

oligonucleotide itself is degraded by an endogenous DNase activity. Following the depletion of the endogenous snRNA, exogenously-derived snRNAs can then be injected, allowing an accurate measurement of capabilities of these injected snRNAs in restoring functional snRNP and pre-mRNA splicing activity.

To examine U2 pseudouridylation, an antisense U2 DNA oligonucleotide is injected. After the endogenous U2 snRNA is depleted, *in vitro* transcribed U2 (unmodified), cellular U2 (completely modified), or chimeric U2 snRNAs (partially modified) are injected into the U2 snRNA-depeleted *Xenopus* oocytes. After a short period of reconstitution (approximately 3.5 h), snRNP biogenesis and pre-mRNA splicing activity are analyzed.

Using this system, Yu *et al*[35] demonstrated that while unmodified *in vitro* transcribed U2 snRNA was unable to rescue splicing in U2 snRNA-depleted oocytes, cellularly-derived (modified) U2 effectively restored splicing activity, suggesting that U2 modifications, including many pseudouridines, are functionally important for splicing. Using chimeric U2 snRNAs derived from the combination of cellular (modified) and *in vitro* transcribed U2 (unmodified), Yu *et al*[35] further dissected U2 modifications and identified the Ψs that reside within the 5' end region of U2 to be important for splicing. Using anti-snRNP immunoprecipitation and glycerol gradient sedimentation, they also demonstrated that unmodified U2 snRNA was unable to form functional 17S snRNP, and that, consequently, U2 snRNA lacking Ψ was unable to participate in spliceosome assembly.

Surprisingly, however, while modifications in the 5' end region of U2 snRNA were shown to be required for both snRNP biogenesis and pre-mRNA splicing, the six Ψs in the BSRR (nucleotides 33-46) were not identified as functionally significant under these conditions[35]. Zhao *et al*[68] later found that Ψ formation occurred much faster in the BSRR than in the 5' region of U2 snRNA. Indeed, soon after it was injected into the nuclei of *Xenopus* oocytes, *in vitro* transcribed U2 became pseudouridylated in the BSRR although pseudouridylation had not yet occurred in the 5' end region, thus suggesting that the functionality of the Ψs in the U2 BSRR cannot be analyzed under these conditions. To overcome this problem, Zhao *et al*[69] employed 5FU-containing U2 to site-specifically inhibit Ψ formation in the U2 BSRR. Somewhat expectedly, U2 snRNAs lacking Ψs only in the BSRR failed to support pre-mRNA splicing. Taken together, these results indicate that virtually all Ψs tested in the *Xenopus* oocyte system are required for snRNP biogenesis and pre-mRNA splicing.

The yeast genetic system

The yeast system has also been used to study U2 pseudouridylation. There are a total of three Ψs (Ψ35, Ψ42 and Ψ44) in *Saccharomyces cerevisiae* (*S. cerevisiae*) U2, all of which are located in the BSRR (Figure 1). About a decade ago, all three pseudouridylases responsible for the formation of the three Ψs in yeast U2 were identified (see below), making it possible to carry out genetic experiments to analyze the function of yeast U2 pseudouridylation.

Pus7 catalyzes the formation of Ψ35, which interacts with the nucleotide next to the pre-mRNA branch point adenosine during pre-mRNA splicing. Interestingly, the *pus7* deletion strain, although still viable, displayed reduced growth rates under conditions of high salt or when grown in competition with wild-type yeast strains[70] (unpublished data). To examine the functional role of Ψ35 in more detail, Yang *et al*[37] used a synthetic lethal screen, and found that, interestingly, a combination of *pus7* deletion (loss of Ψ35) and a U2 point mutation at position 40 (U40G or U40Δ) resulted in a temperature-sensitive growth defect phenotype. They further demonstrated that pre-mRNA accumulated in the mutant strain under restrictive conditions, indicating that Ψ35 in the U2 BSRR contributes to pre-mRNA splicing in *S. cerevisiae*.

Recently, *Pus1* and *snR81* (pseudouridylases responsible for the formation of Ψ44 and Ψ42, respectively) were also deleted, either individually or in combination, from the yeast genome. The resulting strains were tested for their ability to support pre-mRNA splicing. These mutant strains exhibited splicing-deficient phenotype (Wu and Yu, unpublished data). Taken together, the data generated thus far strongly suggest that all three Ψs within yeast U2 snRNA play a role in pre-mRNA splicing. These results are consistent with the results obtained from the Xenopus oocyte microinjection system (see above).

Structural analyses

In recent years, various biophysical techniques have been

used to investigate the structural aspects of U2 snRNA pseudouridylation. Specifically, in 2001, Berglund *et al*[71] solved, at 2.18-Å resolution, a crystal structure of a self-complementary RNA modeled after the yeast U2 snRNA-branch site duplex in the absence of Ψ. Surprisingly, the adenosine adjacent to the expected branch point adenosine was bulged out, despite the fact that, in mammalian cell extracts, either of these adenosines was able to serve as the nucleophile that attacks the 5' splice site during pre-mRNA splicing. Subsequently, Newby and Greenbaum determined solution NMR structures of the yeast U2 snRNA-branch site duplex with or without Ψ35 in the U2 strand[60,62]. They showed that the presence of the Ψ35 in the U2 strand induced a structural change where the branch point adenine base bulged out of the duplex and the nucleophile (the 2'-OH of the adenosine) was placed in an accessible position for the first step of splicing[60].

More recently, Lin *et al*[72] reported the 1.57-Å resolution crystal structure of the U2 snRNA-branch site duplex in the presence of Ψ35 in the U2 strand. They observed an extra-helical branch point adenosine in which its 2'-OH was prominently exposed and available for attack on the 5' splice site. Thus, biophysical data have provided detailed structural information indicating that Ψ35 is somehow capable of altering the structure of the duplex, thereby making the 2'-OH group of the branch-point adenosine available for the first step of splicing.

MECHANISMS OF SPLICEOSOMAL SNRNA PSEUROURIDYLATION

Box H/ACA RNP-catalyzed (RNA-dependent) mechanism

In 1997, the Ni *et al*[73] and Ganot *et al*[74] demonstrated that box H/ACA RNAs, one of the two major families of small nucleolar RNAs, function as guide RNAs that direct site-specific synthesis of Ψ in rRNA. Box H/ACA RNAs exist in the cell as RNA-protein complexes (box H/ACA snRNPs). Each of the complexes consists of one unique box H/ACA RNA and four common core proteins, Cbf5 (NAP57 or Dyskerin in mammals/humans), Nhp2, Gar1, and Nop10. Each box H/ACA RNA forms a conserved hairpin-hinge-hairpin-tail structure, including a conserved H box in the hinge region and a conserved ACA box in the tail region (Figure 4). Each of the two hairpins in the box H/ACA RNA contains an internal loop (pseudouridylation pocket), which serves as a guide that base pairs with the target RNA to place the target uridine precisely at the base of the upper stem where Cbf5, a catalytic component of box H/ACA RNP, catalyzes the U-to-Ψ conversion[75,76] (Figure 4).

The discovery of the mechanism of box H/ACA RNA-guided rRNA pseudouridylation generated great interest in searching for additional box H/ACA RNAs. Both computational methods and experimental approaches were developed, resulting in the discovery of hundreds of new box H/ACA RNAs in several different organisms[43,77-79]. Interestingly, a number of guide sequences exhibited complementarity with spliceosomal snRNAs, suggesting that the box H/ACA RNAs may also guide pseudouridylation of snRNAs[80,81]. To experimentally verify this hypothesis, several laboratories tested the guide activity of newly identified snRNA-specific box H/ACA RNAs using several independent systems. For example, Zhao *et al*[82] demonstrated that a *Xenopus* box H/ACA RNA containing two putative pseudouridylation pockets was indeed able to direct U2 snRNA pseudouridylation at two different sites (positions 34 and 44 by the 5' pocket and the 3' pocket, respectively) in *Xenopus* oocytes. Jády *et al*[80] showed that U85, a special type of mammalian box H/ACA small nucleolar RNP, specifically directed pseudouridylation of U5 snRNA at position 46. Ma *et al*[83] reported that one of the yeast box H/ACA RNAs, snR81 RNA, guided Ψ42 formation in yeast U2 snRNA.

The fact that box H/ACA RNAs are able to direct spliceosomal snRNA pseudoruidylation in various organisms strongly suggests that RNA-dependent pseudouridylation is a major (if not the only) mechanism for Ψ formation in spliceosomal snRNAs. In this regard, a large number of box H/ACA RNAs have been identified, and upon inspection of their guide sequences, many of them are predicted to be specific for spliceosomal snRNAs.

Stand-alone protein-catalyzed (RNA-independent) mechanism

At a time when it was widely believed that box H/ACA RNA-dependent mechanism was responsible for Ψ formation in spliceosomal snRNAs, the Branlant lab reported that Pus1, a stand-alone protein pseudouridylase known to catalyze tRNA pseudouridylation, was also responsible for the formation of Ψ44 in *S. cerevisiae* U2 snRNA[52]. By using purified Pus1 and *in vitro* synthesized U2 snRNA, they showed that Pus1 catalyzed Ψ44 formation in yeast U2 snRNA. They also showed that deletion of *PUS1* resulted in the loss of Ψ44 in yeast U2 snRNA.

This was the first report demonstrating that spliceosomal snRNA pseudorudylation is catalyzed by an RNA-independent mechanism. Here, a stand-alone protein enzyme is responsible for both substrate recognition and catalysis. This mechanism is remarkably different from the RNA-dependent mechanism, in which a guide RNA is used to recognize the substrate and a catalytic protein component Cbf5 catalyzes the isomerization reaction.

Using a singly radiolabled U2 snRNA substrate to screen a yeast GST-ORF fusion library[84], Ma *et al*[70] subsequently identified YOR243c, a previously uncharacterized ORF, as a stand-alone pseudouridylase responsible for Ψ35 formation in yeast U2 snRNA. YOR243c was subsequently renamed Pseudouridine Synthase 7, *PUS7*. A BLAST search identified the *Pus7* homologs in many organisms, including *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and humans. Surprisingly, however, these homologs have no significant sequence or domain similarities to any known members of Ψ synthase families (TruA, TruB, RluA and RsuA families)[70]. Thus, *Pus7* represented a novel family of Ψ synthases present in many different organisms. Shortly after the

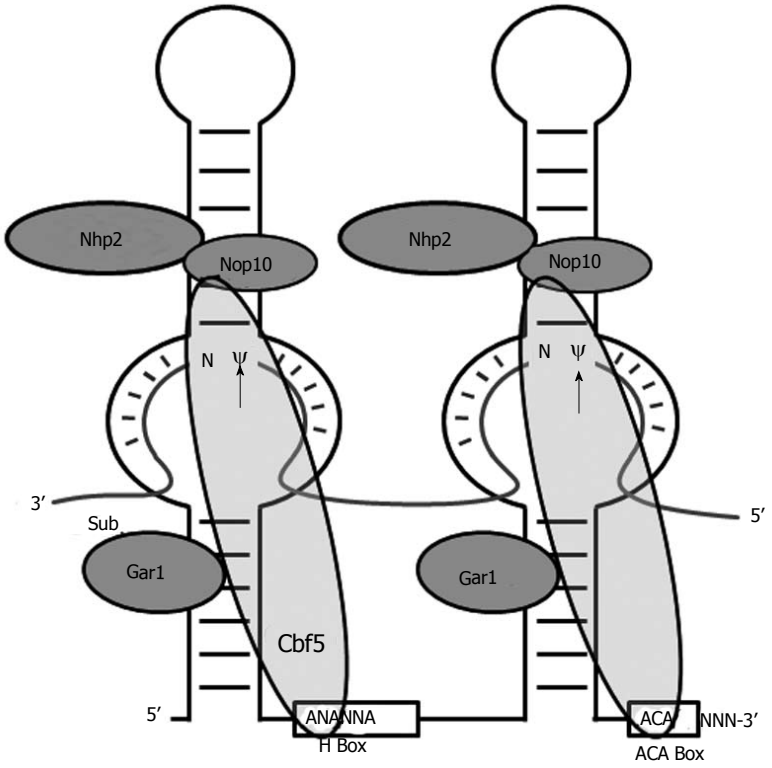

Figure 4 Schematic depiction of box H/ACA RNA. The core components of a box H/ACA RNP, a box H/ACA RNA and four proteins (Nhp2, Nop 10, Gar1 and Cbf5), are shown. An RNA substrate paired with the two internal loops of the box H/ACA RNA is also shown. The arrows indicate the target nucleotides for pseudouridylation. The H box (5'-ANANNA-3') and ACA box (5'-ACA-3') are indicated.

identification of *Pus7*, its *Escherichia coli* homolog, *TruD*, was identified[85]. Thus, yeast Pus7 and its homologs in other organisms have been classified as members of the TruD Ψ synthase family.

From an evolutionary point of view, it is interesting that yeast U2 pseudouridylation is catalyzed by both RNA-dependent (snR81 box H/ACA RNA for Ψ42) and RNA-independent (Pus7 for Ψ35 and Pus1 for Ψ44) mechanisms, whereas pseudouridylation of higher eukaryotic snRNAs is (or at least is widely believed to be) catalyzed exclusively by RNA-dependent mechanism. If it is true that the RNA-dependent mechanism evolved from the RNA-independent mechanism[86], the co-existence of the two mechanisms in yeast would suggest that snR81 box H/ACA RNP responsible for Ψ42 formation has evolved. However, Pus7 and Pus1 responsible for Ψ35 and Ψ44, respectively, might never have evolved (or evolved but were subsequently lost from the genome) in yeast. All box H/ACA RNPs responsible for spliceosomal snRNA pseudouridylation have evolved in higher eukaryotes.

Inducible snRNA pseudouridylation

Until recently all Ψs identified in RNAs have been considered constitutive modifications. In 2011, Wu *et al*[87] demonstrated for the first time that changes in growth conditions induce U2 pseudouridylation at novel sites, which, in turn, influences splicing. In this study, they exposed yeast cells to a widely used stress-nutrient deprivation (growing cells to saturation or using nutrient-depleted media), and subsequently isolated RNAs from stressed cells for pseudouridylation assays. Remarkably, they detected two novel Ψs (at positions 56 and 93) in U2 snRNA isolated from stressed cells. When the cells were exposed to another widely used stress-heat shock, they also detected Ψ56 (but not Ψ93). These two positions, 56 and 93, had previously been identified as unmodified uridines in yeast U2 snRNA.

Further analyses showed that the stand-alone protein Pus7, which is responsible for Ψ35 formation in U2, catalyzes Ψ56 formation, and that the box H/ACA RNA snR81, which directs pseudouridylation of U2 at position 42 and of 25S rRNA at position 1051[77], guides Ψ93 formation; in the latter case, position 1051 (constitutive) of 25S rRNA and position 93 (inducible) of U2 share a common pseudouridylation guide-the 3' pseudouridylation pocket of snR81 (the 5' pocket of snR81 is responsible for Ψ42 formation) (Figure 5). Interestingly, the sequences surrounding U56 and U93 are similar but not identical to the sequences surrounding the constitutively pseudouridylated target sites, Ψ35 of yeast U2 and Ψ1051 of 25S rRNA, respectively[87], suggesting that the inducibility of U2 pseudouridylation at positions 56 and 93 can be attributed to their imperfect substrate sequences or imperfect enzyme-substrate interactions. Indeed, Wu *et al*[87] subsequently showed that imperfect base-pairing interactions (two mismatches) between the guide sequence of snR81 and the target sequence of U2 (at position 93) were necessary for induced pseudouridylation.

CONCLUSION

It has been more than 60 years since Ψ was reported, and more than 15 years since the box H/ACA RNA family was discovered. Over the years (the last 15-20 years in particular), remarkable progress has been made towards elucidating the mechanism and function of spliceosomal snRNA pseudouridylation. However, the detailed molec-

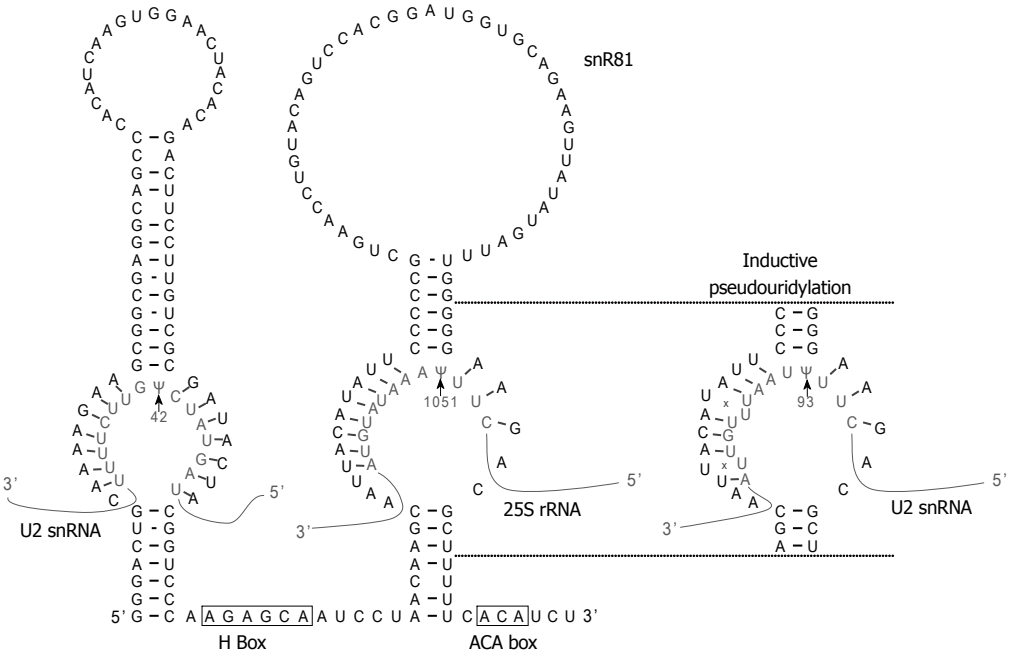

Figure 5 Constitutive and induced pseudouridylation by snR81 box H/ACA ribonucleoprotein. The sequence and structure of snR81 box H/ACA RNA is shown. As arrows indicate, the internal loop (pseudouridylation pocket) within the 5' hairpin is specific for Ψ42 (constitutive) of U2 snRNA, and the internal loop within the 3' hairpin is specific for Ψ1051 (constitutive) of 25S rRNA. Under stress coditions, the 3' pseudouridylation pocket becomes capable of directing the formation of Ψ93 (inducible) of U2 snRNA. As shown by "x", there are two U-U mismatches between the 3' pocket and the U2 sequence flanking position 93.

ular mechanisms of how Ψs affect pre-mRNA splicing remain unclear. With regard to the mechanisms of spliceosomal snRNA pseudouridylation, especially induced Ψ formation, there are still a number of unanswered questions. The concept that Ψ formation can be induced challenges the current paradigm that snRNA modifications are constitutive, and therefore further demonstration of the regulatability of spliceosomal snRNA pseudouridylation will significantly advance our understanding of spliceosomal snRNA modification and function. It is anticipated that the pace of snRNA pseudouridylation research (and RNA modification research in general) will quicken.

ACKNOWLEDGEMENTS

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REFERENCES

1 **Crick F**. Split genes and RNA splicing. *Science* 1979; **204**: 264-271 [PMID: 373120]
2 **Jurica MS**, Moore MJ. Pre-mRNA splicing: awash in a sea of proteins. *Mol Cell* 2003; **12**: 5-14 [PMID: 12887888]
3 **Tarn WY**, Steitz JA. Pre-mRNA splicing: the discovery of a new spliceosome doubles the challenge. *Trends Biochem Sci* 1997; **22**: 132-137 [PMID: 9149533]
4 **Staley JP**, Guthrie C. Mechanical devices of the spliceosome: motors, clocks, springs, and things. *Cell* 1998; **92**: 315-326 [PMID: 9476892]
5 **Bindereif A**, Green MR. An ordered pathway of snRNP binding during mammalian pre-mRNA splicing complex assembly. *EMBO J* 1987; **6**: 2415-2424 [PMID: 2959470]
6 **Konarska MM**, Sharp PA. Interactions between small nuclear ribonucleoprotein particles in formation of spliceosomes. *Cell* 1987; **49**: 763-774 [PMID: 2953438]
7 **Michaud S**, Reed R. An ATP-independent complex commits pre-mRNA to the mammalian spliceosome assembly pathway. *Genes Dev* 1991; **5**: 2534-2546 [PMID: 1836445]
8 **Wassarman DA**, Steitz JA. Interactions of small nuclear RNA's with precursor messenger RNA during in vitro splicing. *Science* 1992; **257**: 1918-1925 [PMID: 1411506]
9 **Karijolich J**, Yu YT. Spliceosomal snRNA modifications and their function. *RNA Biol* 2010; **7**: 192-204 [PMID: 20215871]
10 **Ruby SW**, Abelson J. An early hierarchic role of U1 small nuclear ribonucleoprotein in spliceosome assembly. *Science* 1988; **242**: 1028-1035 [PMID: 2973660]
11 **Seraphin B**, Rosbash M. Identification of functional U1 snRNA-pre-mRNA complexes committed to spliceosome assembly and splicing. *Cell* 1989; **59**: 349-358 [PMID: 2529976]
12 **Séraphin B**, Rosbash M. The yeast branchpoint sequence is not required for the formation of a stable U1 snRNA-pre-mRNA complex and is recognized in the absence of U2 snRNA. *EMBO J* 1991; **10**: 1209-1216 [PMID: 1827069]
13 **Parker R**, Siliciano PG, Guthrie C. Recognition of the TACTAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. *Cell* 1987; **49**: 229-239 [PMID: 3552247]
14 **Zhuang Y**, Weiner AM. A compensatory base change in human U2 snRNA can suppress a branch site mutation. *Genes Dev* 1989; **3**: 1545-1552 [PMID: 2612904]
15 **Zhuang YA**, Goldstein AM, Weiner AM. UACUAAC is the preferred branch site for mammalian mRNA splicing. *Proc Natl Acad Sci USA* 1989; **86**: 2752-2756 [PMID: 2704744]
16 **Woolford JL**. Nuclear pre-mRNA splicing in yeast. *Yeast* 1989; **5**: 439-457 [PMID: 2694677]
17 **Berglund JA**, Chua K, Abovich N, Reed R, Rosbash M. The splicing factor BBP interacts specifically with the pre-mRNA branchpoint sequence UACUAAC. *Cell* 1997; **89**: 781-787 [PMID: 9182766]
18 **Ruby SW**, Abelson J. Pre-mRNA splicing in yeast. *Trends Genet* 1991; **7**: 79-85 [PMID: 2031287]
19 **Sawa H**, Abelson J. Evidence for a base-pairing interaction

between U6 small nuclear RNA and 5′ splice site during the splicing reaction in yeast. *Proc Natl Acad Sci USA* 1992; **89**: 11269-11273 [PMID: 1333604]
20 **Lesser CF**, Guthrie C. Mutations in U6 snRNA that alter splice site specificity: implications for the active site. *Science* 1993; **262**: 1982-1988 [PMID: 8266093]
21 **Stevens SW**, Barta I, Ge HY, Moore RE, Young MK, Lee TD, Abelson J. Biochemical and genetic analyses of the U5, U6, and U4/U6 x U5 small nuclear ribonucleoproteins from Saccharomyces cerevisiae. *RNA* 2001; **7**: 1543-1553 [PMID: 11720284]
22 **Madhani HD**, Guthrie C. A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. *Cell* 1992; **71**: 803-817 [PMID: 1423631]
23 **Turner IA**, Norman CM, Churcher MJ, Newman AJ. Roles of the U5 snRNP in spliceosome dynamics and catalysis. *Biochem Soc Trans* 2004; **32**: 928-931 [PMID: 15506927]
24 **Brow DA**. Allosteric cascade of spliceosome activation. *Annu Rev Genet* 2002; **36**: 333-360 [PMID: 12429696]
25 **Licht K**, Medenbach J, Lührmann R, Kambach C, Bindereif A. 3′-cyclic phosphorylation of U6 snRNA leads to recruitment of recycling factor p110 through LSm proteins. *RNA* 2008; **14**: 1532-1538 [PMID: 18567812 DOI: 10.1261/rna.1129608]
26 **Wu G**, Yu AT, Kantartzis A, Yu YT. Functions and mechanisms of spliceosomal small nuclear RNA pseudouridylation. *Wiley Interdiscip Rev RNA* 2011; **2**: 571-581 [PMID: 21957045]
27 **Yu AT**, Ge J, Yu YT. Pseudouridines in spliceosomal snRNAs. *Protein Cell* 2011; **2**: 712-725 [PMID: 21976061]
28 **Ge J**, Yu YT. RNA pseudouridylation: new insights into an old modification. *Trends Biochem Sci* 2013; **38**: 210-218 [PMID: 23391857 DOI: 10.1016/j.tibs.2013.01.002]
29 **Yu YT**, Terns RM, Terns MP. Mechanisms and functions of RNA-guided RNA modification. In: Fine-Tuning of RNA-Functions by Modification and Editing. Grosjean H, editor. New York: Springer-Verlag Press, 2005: 223-262
30 **Reddy R**, Busch H. Small nuclear RNAs: RNA sequences, structure, and modifications. In: Birnsteil ML, ed. Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. Heidelberg: Springer-Verlag Press, 1988: 1-37
31 **Massenet S**, Mougin A, Branlant C. Posttranscriptional modifications in the U small nuclear RNAs. In: Grosjean H, ed. Modification and Editingof RNA. Washington, D.C: ASM Press, 1998: 201-208
32 **Yu YT**, Scharl EC, Smith CM, Steitz JA. The growing world of small nuclear ribonucleoproteins. In: Gesteland RF, Cech TR, Atkins JF, eds. The RNA World. New York: Cold Spring Harbor Laboratory Press, 1999: 487-524
33 **Ségault V**, Will CL, Sproat BS, Lührmann R. In vitro reconstitution of mammalian U2 and U5 snRNPs active in splicing: Sm proteins are functionally interchangeable and are essential for the formation of functional U2 and U5 snRNPs. *EMBO J* 1995; **14**: 4010-4021 [PMID: 7664740]
34 **Bakin AV**, Ofengand J. Mapping of pseudouridine residues in RNA to nucleotide resolution. *Methods Mol Biol* 1998; **77**: 297-309 [PMID: 9770678]
35 **Yu YT**, Shu MD, Steitz JA. Modifications of U2 snRNA are required for snRNP assembly and pre-mRNA splicing. *EMBO J* 1998; **17**: 5783-5795 [PMID: 9755178 DOI: 10.1093/emboj/17.19.5783]
36 **Zhao X**, Yu YT. Detection and quantitation of RNA base modifications. *RNA* 2004; **10**: 996-1002 [PMID: 15146083 DOI: 10.1261/rna.7110804]
37 **Yang C**, McPheeters DS, Yu YT. Psi35 in the branch site recognition region of U2 small nuclear RNA is important for pre-mRNA splicing in Saccharomyces cerevisiae. *J Biol Chem* 2005; **280**: 6655-6662 [PMID: 15611063]
38 **Dai Q**, Fong R, Saikia M, Stephenson D, Yu YT, Pan T, Piccirilli JA. Identification of recognition residues for ligation-based detection and quantitation of pseudouridine and N6-methyladenosine. *Nucleic Acids Res* 2007; **35**: 6322-6329 [PMID: 17881375 DOI: 10.1093/nar/gkm657]
39 **Cohn WE**, Volkin E. Nucleoside-5′-Phosphates from Ribonucleic Acid. *Nature* 1951; **167**: 483-484 [DOI: 10.1038/167483a0]
40 **Cohn WE**. 5-Ribosyl uracil, a carbon-carbon ribofuranosyl nucleoside in ribonucleic acids. *Biochim Biophys Acta* 1959; **32**: 569-571 [PMID: 13811055]
41 **Branlant C**, Krol A, Machatt MA, Pouyet J, Ebel JP, Edwards K, Kössel H. Primary and secondary structures of Escherichia coli MRE 600 23S ribosomal RNA. Comparison with models of secondary structure for maize chloroplast 23S rRNA and for large portions of mouse and human 16S mitochondrial rRNAs. *Nucleic Acids Res* 1981; **9**: 4303-4324 [PMID: 6170936]
42 **Maden BE**. The numerous modified nucleotides in eukaryotic ribosomal RNA. *Prog Nucleic Acid Res Mol Biol* 1990; **39**: 241-303 [PMID: 2247610]
43 **Schattner P**, Barberan-Soler S, Lowe TM. A computational screen for mammalian pseudouridylation guide H/ACA RNAs. *RNA* 2006; **12**: 15-25 [PMID: 16373490 DOI: 10.1261/rna.2210406]
44 **Ofengand J,** Fournier MJ. The pseudouridine residues of rRNA: number, location, biosynthesis, and function. In: Grosjean H, eds. Modification and Editing of RNA. Washington, D.C: ASM Press; 1998: 229-253
45 **Bachellerie JP**, Cavaille J. Small nucleolar RNAs guide the ribose methylations of eukaryotic rRNAs. In: Grosjean H, Benne R, eds. Modification and Editing of RNA. Washington, D.C: ASM Press, 1998: 255-272
46 **Björk GR.** Biosynthesis and function of modified nucleosides in tRNA. In: Soll D, RajBhandary U, eds. tRNA: Structure, Biosynthesis, and Function. Washington, D.C: ASM Press, 1995: 165-205
47 **Grosjean H**, Sprinzl M, Steinberg S. Posttranscriptionally modified nucleosides in transfer RNA: their locations and frequencies. *Biochimie* 1995; **77**: 139-141 [PMID: 7541252]
48 **Sprinzl M**, Horn C, Brown M, Ioudovitch A, Steinberg S. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res* 1998; **26**: 148-153 [PMID: 9399820]
49 **Hopper AK**, Phizicky EM. tRNA transfers to the limelight. *Genes Dev* 2003; **17**: 162-180 [PMID: 12533506]
50 **Stephenson D**, Karijolich J, Yu YT. Functional roles of spliceosomal snRNA modifications in pre-mRNA splicing. In: Smith H, ed. RNA and DNA Editing: Molecular Mechanisms and Their Integration into BiologicalSystems. Hoboken, NJ: Wiley-Interscience, 2008: 175-189
51 **Solymosy F,** Pollák T. Uridylate-Rich Small Nuclear RNAs (UsnRNAs), Their Genes and Pseudogenes, and UsnRNPs in Plants: Structure and Function. A Comparative Approach. *Crit Rev Plant Sci* 1993; **12:** 275-369 [DOI: 10.1080/07352689309701904]
52 **Massenet S**, Motorin Y, Lafontaine DL, Hurt EC, Grosjean H, Branlant C. Pseudouridine mapping in the Saccharomyces cerevisiae spliceosomal U small nuclear RNAs (snRNAs) reveals that pseudouridine synthase pus1p exhibits a dual substrate specificity for U2 snRNA and tRNA. *Mol Cell Biol* 1999; **19**: 2142-2154 [PMID: 10022901]
53 **Kammen HO**, Marvel CC, Hardy L, Penhoet EE. Purification, structure, and properties of Escherichia coli tRNA pseudouridine synthase I. *J Biol Chem* 1988; **263**: 2255-2263 [PMID: 3276686]
54 **Gu X**, Liu Y, Santi DV. The mechanism of pseudouridine synthase I as deduced from its interaction with 5-fluorouracil-tRNA. *Proc Natl Acad Sci USA* 1999; **96**: 14270-14275 [PMID: 10588695]
55 **Westhof E**, Dumas P, Moras D. Hydration of transfer RNA molecules: a crystallographic study. *Biochimie* 1988; **70**: 145-165 [PMID: 3134940]

56 **Arnez JG**, Steitz TA. Crystal structure of unmodified tRNA(Gln) complexed with glutaminyl-tRNA synthetase and ATP suggests a possible role for pseudo-uridines in stabilization of RNA structure. *Biochemistry* 1994; **33**: 7560-7567 [PMID: 8011621]
57 **Davis DR**. Stabilization of RNA stacking by pseudouridine. *Nucleic Acids Res* 1995; **23**: 5020-5026 [PMID: 8559660]
58 **Auffinger P**, Westhof E. RNA hydration: three nanoseconds of multiple molecular dynamics simulations of the solvated tRNA(Asp) anticodon hairpin. *J Mol Biol* 1997; **269**: 326-341 [PMID: 9199403]
59 **Charette M**, Gray MW. Pseudouridine in RNA: what, where, how, and why. *IUBMB Life* 2000; **49**: 341-351 [PMID: 10902565]
60 **Newby MI**, Greenbaum NL. Sculpting of the spliceosomal branch site recognition motif by a conserved pseudouridine. *Nat Struct Biol* 2002; **9**: 958-965 [PMID: 12426583]
61 **Kolev NG**, Steitz JA. In vivo assembly of functional U7 snRNP requires RNA backbone flexibility within the Sm-binding site. *Nat Struct Mol Biol* 2006; **13**: 347-353 [PMID: 16547514]
62 **Newby MI**, Greenbaum NL. A conserved pseudouridine modification in eukaryotic U2 snRNA induces a change in branch-site architecture. *RNA* 2001; **7**: 833-845 [PMID: 11424937]
63 **Hall KB**, McLaughlin LW. Properties of pseudouridine N1 imino protons located in the major groove of an A-form RNA duplex. *Nucleic Acids Res* 1992; **20**: 1883-1889 [PMID: 1579489]
64 **Newby MI**, Greenbaum NL. Investigation of Overhauser effects between pseudouridine and water protons in RNA helices. *Proc Natl Acad Sci USA* 2002; **99**: 12697-12702 [PMID: 12242344 DOI: 10.1073/pnas.202477199]
65 **Patton JR**. Multiple pseudouridine synthase activities for small nuclear RNAs. *Biochem J* 1993; **290** (Pt 2): 595-600 [PMID: 8452551]
66 **Patton JR**. Ribonucleoprotein particle assembly and modification of U2 small nuclear RNA containing 5-fluorouridine. *Biochemistry* 1993; **32**: 8939-8944 [PMID: 8364039]
67 **Dönmez G**, Hartmuth K, Lührmann R. Modified nucleotides at the 5′ end of human U2 snRNA are required for spliceosomal E-complex formation. *RNA* 2004; **10**: 1925-1933 [PMID: 15525712 DOI: 10.1261/rna.7186504]
68 **Zhao X**, Yu YT. Pseudouridines in and near the branch site recognition region of U2 snRNA are required for snRNP biogenesis and pre-mRNA splicing in Xenopus oocytes. *RNA* 2004; **10**: 681-690 [PMID: 15037777 DOI: 10.1261/rna.5159504]
69 **Zhao X**, Yu YT. Incorporation of 5-fluorouracil into U2 snRNA blocks pseudouridylation and pre-mRNA splicing in vivo. *Nucleic Acids Res* 2007; **35**: 550-558 [PMID: 17169984 DOI: 10.1093/nar/gkl1084]
70 **Ma X**, Zhao X, Yu YT. Pseudouridylation (Psi) of U2 snRNA in S. cerevisiae is catalyzed by an RNA-independent mechanism. *EMBO J* 2003; **22**: 1889-1897 [PMID: 12682021 DOI: 10.1093/emboj/cdg191]
71 **Berglund JA**, Rosbash M, Schultz SC. Crystal structure of a model branchpoint-U2 snRNA duplex containing bulged adenosines. *RNA* 2001; **7**: 682-691 [PMID: 11350032]
72 **Lin Y**, Kielkopf CL. X-ray structures of U2 snRNA-branchpoint duplexes containing conserved pseudouridines. *Biochemistry* 2008; **47**: 5503-5514 [PMID: 18435545 DOI: 10.1021/bi7022392]
73 **Ni J**, Tien AL, Fournier MJ. Small nucleolar RNAs direct site-specific synthesis of pseudouridine in ribosomal RNA. *Cell* 1997; **89**: 565-573 [PMID: 9160748]
74 **Ganot P**, Bortolin ML, Kiss T. Site-specific pseudouridine formation in preribosomal RNA is guided by small nucleolar RNAs. *Cell* 1997; **89**: 799-809 [PMID: 9182768]
75 **Meier UT**, Blobel G. NAP57, a mammalian nucleolar protein with a putative homolog in yeast and bacteria. *J Cell Biol* 1994; **127**: 1505-1514 [PMID: 7798307]
76 **Zebarjadian Y**, King T, Fournier MJ, Clarke L, Carbon J. Point mutations in yeast CBF5 can abolish in vivo pseudouridylation of rRNA. *Mol Cell Biol* 1999; **19**: 7461-7472 [PMID: 10523634]
77 **Schattner P**, Decatur WA, Davis CA, Ares M, Fournier MJ, Lowe TM. Genome-wide searching for pseudouridylation guide snoRNAs: analysis of the Saccharomyces cerevisiae genome. *Nucleic Acids Res* 2004; **32**: 4281-4296 [PMID: 15306656 DOI: 10.1093/nar/gkh768]
78 **Schattner P**, Brooks AN, Lowe TM. The tRNAscan-SE, snoscan and snoGPS web servers for the detection of tRNAs and snoRNAs. *Nucleic Acids Res* 2005; **33**: W686-W689 [PMID: 15980563 DOI: 10.1093/nar/gki366]
79 **Hüttenhofer A**, Kiefmann M, Meier-Ewert S, O'Brien J, Lehrach H, Bachellerie JP, Brosius J. RNomics: an experimental approach that identifies 201 candidates for novel, small, non-messenger RNAs in mouse. *EMBO J* 2001; **20**: 2943-2953 [PMID: 11387227 DOI: 10.1093/emboj/20.11.2943]
80 **Jády BE**, Kiss T. A small nucleolar guide RNA functions both in 2′-O-ribose methylation and pseudouridylation of the U5 spliceosomal RNA. *EMBO J* 2001; **20**: 541-551 [PMID: 11157760 DOI: 10.1093/emboj/20.3.541]
81 **Kiss AM**, Jády BE, Bertrand E, Kiss T. Human box H/ACA pseudouridylation guide RNA machinery. *Mol Cell Biol* 2004; **24**: 5797-5807 [PMID: 15199136 DOI: 10.1128/MCB.24.13.5797-5807.2004]
82 **Zhao X**, Li ZH, Terns RM, Terns MP, Yu YT. An H/ACA guide RNA directs U2 pseudouridylation at two different sites in the branchpoint recognition region in Xenopus oocytes. *RNA* 2002; **8**: 1515-1525 [PMID: 12515384]
83 **Ma X**, Yang C, Alexandrov A, Grayhack EJ, Behm-Ansmant I, Yu YT. Pseudouridylation of yeast U2 snRNA is catalyzed by either an RNA-guided or RNA-independent mechanism. *EMBO J* 2005; **24**: 2403-2413 [PMID: 15962000 DOI: 10.1038/sj.emboj.7600718]
84 **Phizicky EM**, Martzen MR, McCraith SM, Spinelli SL, Xing F, Shull NP, Van Slyke C, Montagne RK, Torres FM, Fields S, Grayhack EJ. Biochemical genomics approach to map activities to genes. *Methods Enzymol* 2002; **350**: 546-559 [PMID: 12073335]
85 **Kaya Y**, Ofengand J. A novel unanticipated type of pseudouridine synthase with homologs in bacteria, archaea, and eukarya. *RNA* 2003; **9**: 711-721 [PMID: 12756329 DOI: 10.1261/rna.5230603]
86 **Lafontaine DL**, Tollervey D. Birth of the snoRNPs: the evolution of the modification-guide snoRNAs. *Trends Biochem Sci* 1998; **23**: 383-388 [PMID: 9810226]
87 **Wu G**, Xiao M, Yang C, Yu YT. U2 snRNA is inducibly pseudouridylated at novel sites by Pus7p and snR81 RNP. *EMBO J* 2011; **30**: 79-89 [PMID: 21131909 DOI: 10.1038/emboj.2010.316]
88 **Reddy R**, Henning D, Busch H. Pseudouridine residues in the 5′-terminus of uridine-rich nuclear RNA I (U1 RNA). *Biochem Biophys Res Commun* 1981; **98**: 1076-1083 [PMID: 7225127]
89 **Gu AD**, Zhou H, Yu CH, Qu LH. A novel experimental approach for systematic identification of box H/ACA snoRNAs from eukaryotes. *Nucleic Acids Res* 2005; **33**: e194 [PMID: 16361266 DOI: 10.1093/nar/gni185]
90 **Darzacq X**, Jády BE, Verheggen C, Kiss AM, Bertrand E, Kiss T. Cajal body-specific small nuclear RNAs: a novel class of 2′-O-methylation and pseudouridylation guide RNAs. *EMBO J* 2002; **21**: 2746-2756 [PMID: 12032087 DOI: 10.1093/emboj/21.11.2746]
91 **Shibata H**, Ro-Choi TS, Reddy R, Choi YC, Henning D, Busch H. The primary nucleotide sequence of nuclear U-2 ribonucleic acid. The 5′-terminal portion of the molecule. *J Biol Chem* 1975; **250**: 3909-3920 [PMID: 165188]
92 **Kiss AM**, Jády BE, Darzacq X, Verheggen C, Bertrand E, Kiss T. A Cajal body-specific pseudouridylation guide RNA is composed of two box H/ACA snoRNA-like domains.

Nucleic Acids Res 2002; **30**: 4643-4649 [PMID: 12409454]
93 **Deryusheva S**, Choleza M, Barbarossa A, Gall JG, Bordonné R. Post-transcriptional modification of spliceosomal RNAs is normal in SMN-deficient cells. *RNA* 2012; **18**: 31-36 [PMID: 22124016 DOI: 10.1261/rna.030106.111]
94 **Krol A**, Gallinaro H, Lazar E, Jacob M, Branlant C. The nuclear 5S RNAs from chicken, rat and man. U5 RNAs are encoded by multiple genes. *Nucleic Acids Res* 1981; **9**: 769-787 [PMID: 6164980]
95 **Epstein P**, Reddy R, Henning D, Busch H. The nucleotide sequence of nuclear U6 (4.7 S) RNA. *J Biol Chem* 1980; **255**: 8901-8906 [PMID: 6773955]
96 **Massenet S**, Branlant C. A limited number of pseudouridine residues in the human atac spliceosomal UsnRNAs as compared to human major spliceosomal UsnRNAs. *RNA* 1999; **5**: 1495-1503 [PMID: 10580478]

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REVIEW

New insight in expression, transport, and secretion of brain-derived neurotrophic factor: Implications in brain-related diseases

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Abstract

Brain-derived neurotrophic factor (BDNF) attracts increasing attention from both research and clinical fields because of its important functions in the central nervous system. An adequate amount of BDNF is critical to develop and maintain normal neuronal circuits in the brain. Given that loss of BDNF function has been reported in the brains of patients with neurodegenerative or psychiatric diseases, understanding basic properties of BDNF and associated intracellular processes is imperative. In this review, we revisit the gene structure, transcription, translation, transport and secretion mechanisms of BDNF. We also introduce implications of BDNF in several brain-related diseases including Alzheimer's disease, Huntington's disease, depression and schizophrenia.

Key words: Brain-derived neurotrophic factor; Transcription; Transport; Secretion; Neurodegenerative disorders; Psychiatric disorders

Core tip: Brain-derived neurotrophic factor (BDNF) plays essential roles in the central nervous system (CNS) through a specific tropomyosin-related kinase receptor B (TrkB), contributing to neuronal survival, neurite outgrowth, and synaptic function. It is critically required for normal development and functions of the brain. A number of reports have shown an importance of BDNF in the pathophysiology of brain-associated diseases. This review describes molecular mechanisms underlying gene expression, transport, and secretion of BDNF and its function in the CNS. We further introduce recent findings on the possible involvement of BDNF in the pathophysiology of neurodegenerative and psychiatric diseases, and the potential effectiveness of enhancing BDNF/TrkB system for therapeutic applications.

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INTRODUCTION

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin (NT) family, was purified in 1982 from pig brain as a cell survival-promoting factor for sensory neurons[1] about 30 years after the discovery of nerve-growth factor (NGF)[2]. In the past three decades, two other neurotrophins, neurotrophin 3 (NT3) and neurotrophin 4 (NT4), have been identified in the mammalian brain and their function in neuronal survival, neurite outgrowth, and synaptic plasticity in the nervous system extensively investigated[3]. Each neurotrophin has a common receptor p75NTR and a specific tropomyosin-related kinase receptor (Trk); TrkA for NGF, TrkB for BDNF and NT4, TrkC for NT3. All neurotrophins are synthesized as 32 kD precursor proteins called pro-neurotrophins that are intra- and extracellularly cleaved to produce the mature neurotrophin form[4-6]. Mature neurotrophins bind to Trk receptors with high affinity while pro-neurotrophins preferentially bind to p75NTR[4-6]. Neurotrophins exert their biological function through several signaling pathways activated by Trk receptors or p75NTR after binding of neurotrophins. Binding of the BDNF dimer to TrkB induces dimerization and autophosphorylation of TrkB, resulting in sequential phosphorylation in three intracellular signaling pathways; Mitogen-activated protein kinase/extracellular signal-regulated protein kinase, phospholipase Cγ (PLCγ), and phosphatidylinositol 3-kinase (PI3K) pathways[7-10].

BDNF is the most studied and characterized neurotrophin in the central nervous system (CNS) and has received remarkable attention from clinicians because of its importance in the development and maintenance of normal brain functions. This is especially important, as growing evidence suggests a role of BDNF in the pathophysiology of brain-associated illnesses including both neurodegenerative and psychiatric diseases[11,12]. Appropriate intracellular processes including transcription from the *BDNF* gene, translation to protein, BDNF protein sorting to secretory vesicles, BDNF-containing vesicle transport, and BDNF secretion are essential to achieve normal BDNF functions as well as to activate the signaling pathways after TrkB phosphorylation. As decreased expression levels of BDNF in several brain regions are evident in postmortem studies of patients with the brain-related diseases[11,12], precise knowledge about *BDNF* gene expression is critical. Specifically, the responsible gene of Huntington's disease, huntingtin, has been shown to directly regulate intracellular transport of BDNF[13,14]. Furthermore, we recently reported impaired BDNF secretion and TrkB signaling in a cellular model of schizophrenia[15].

In this article, we review both converting (from gene to mature protein) and spatial regulation (transport and secretion) processes of BDNF. We will also highlight recent findings suggesting implications of BDNF in the pathophysiology of the brain-related diseases.

BDNF GENE STRUCTURE

BDNF mRNA and protein are abundantly expressed in the hippocampus, cerebral cortex, amygdala, and cerebellum in the mammalian brain[16-19]. Although BDNF is widely expressed in other tissues such as the heart, kidney, lung and testis, BDNF expression is higher in the brain than any other tissue during development[20]. It is also known that BDNF expression is dramatically increased in the rodent visual cortex with a peak at postnatal days 20-40 when visual plasticity is high[21,22].

The *BDNF* gene consists of at least eight 5' exons (exon I -Ⅷ) with each respective promoter and one 3' exon (exon Ⅸ) encoding BDNF protein in both human and rodent[23-25] (Figure 1). Pruunsild *et al*[25] recently identified two human-specific exons named exons Vh and Ⅷh. Exon Vh has a specific promoter while exon Ⅷh is not linked to an independent promoter. Transcription of the *BDNF* gene is initiated at each of 5' noncoding exons that are spliced onto the pre-proBDNF protein-coding 3' exon Ⅸ[24,25]. Rodent exon Ⅰ and human exons Ⅰ, Ⅶ and Ⅷ of the *BDNF* gene contain the ATG sequence from which translation could be started, which could produce the distinct pre-proBDNF proteins with longer amino acid sequences at the N-terminal end of the protein[24,25]. Other exons are untranslated exons, and translation of the transcripts (mRNAs) containing these exons only starts from the ATG sequence located at the exon Ⅸ[25]. Moreover, transcription of the BDNF gene terminates at two alternative polyadenylation sites in exon Ⅸ, giving rise to two distinct populations of mRNA with either short (approximately 0.35 kb) or long (approximately 2.85 kb) 3' untranslated regions (3' UTRs)[26,27]. To produce an identical pre-proBDNF protein, the evolutionary change in *BDNF* gene structure has remained a complex transcription mechanism that results in the expression of multiple BDNF mRNA variants. The diversity of BDNF mRNA leads to different neuronal distribution. The short 3' UTR BDNF mRNA variant is restricted to the cell body in hippocampal neurons while the long 3' UTR mRNAs are also observed in dendrites[27], indicating a specific dendritic transport system for the long 3' UTR BDNF mRNA and local dendritic translation.

TRANSCRIPTIONAL REGULATION IN *BDNF* GENE

Using multiple promoters in BDNF transcription allows for appropriate responses to intracellular processes and varying extracellular environments. Membrane depolarization is a well-known neuronal gene expression regulator, and more than 300 gene transcriptions have been identified as activity-dependent in neurons[28]. The transcription of BDNF in neurons is positively regulated by membrane depolarization induced by seizures[29], sensory stimuli[30-32] and activation of glutamate receptors such as N-methyl-D-aspartate (NMDA) receptors[33-35], suggesting its important function in experience-dependent modifications of neural circuits and brain development. Neuronal activity stimulates transcription initiation of the BDNF gene, which is regulated by elevation of intracellular Ca^{2+} concentration *via* NMDA receptors (NMDAR)

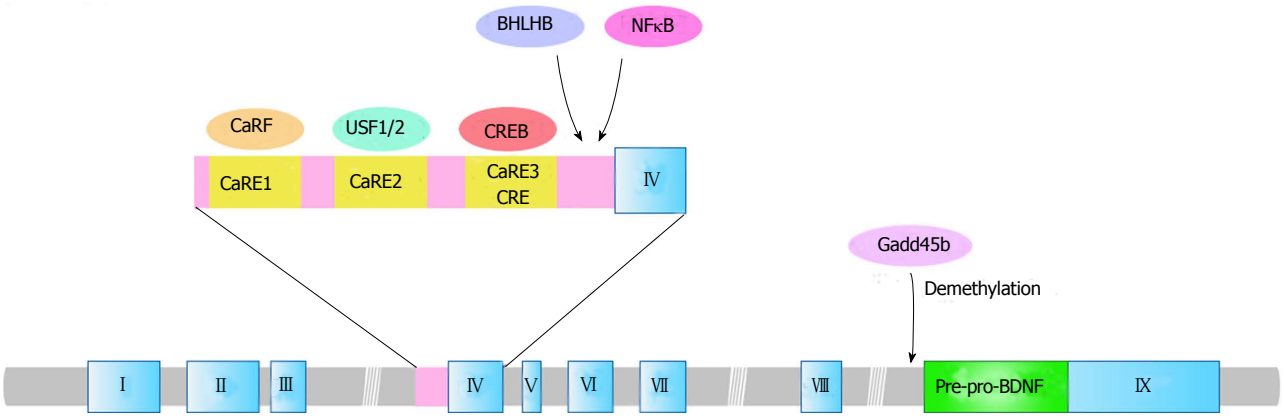

Figure 1 Schematic illustration of the activity-dependent regulators for Brain-derived neurotrophic factor gene transcription. A variety of transcriptional factors and regulators participate in the activity-induced transcription of *BDNF*. *BDNF* gene structure and Ca^{2+}-dependent regulation in *BDNF* exon Ⅳ and epigenetic regulation at the exon Ⅸ promoter region are presented. We referred to the description by Zheng *et al*[38], also see[180]. BDNF: Brain-derived neurotrophic factor; CaRF: Calcium responsive factor; USF: Upstream stimulatory factors; BHLHB: Basic helix-loop-helix; CREB: Cyclic AMP-responsive element binding protein; NFκB: Nuclear factor-κB.

or L-type voltage-gated calcium channels (L-VGCC)[35]. The activity- and Ca^{2+}-dependent transcription of the BDNF gene occurs predominantly through exons I and Ⅳ. The promoter region of BDNF exon Ⅳ contains three Ca^{2+}-response elements CaRE1, CaRE2 (also known as USF-binding element, UBE) and CaRE3 (also known as calcium response element, CRE)[36-38] (Figure 1). CaRE1 is located at 64-73 bp upstream of the transcription initiation site in exon Ⅳ. CaRE2 and CaRE3 are 43-52 bp and 29-36 bp upstream, respectively[38]. Regulation of exon Ⅳ transcription *via* CaRE3 has been extensively investigated. Cyclic AMP-responsive element binding protein (CREB), which is phosphorylated at multiple sites by calcium/calmodulin (CaM)-dependent protein kinases, cAMP-dependent protein kinase A (PKA) and MAPK cascades binds to CaRE3/CRE to activate the promoter[37-39]. Knock-in mice with a subtle mutation at the CREB binding site in endogenous CaRE3/CRE showed a disrupted sensory experience-dependent increase of the BDNF exon Ⅳ transcript in the cortex[40]. Greenberg and colleagues identified novel transcription regulators for BDNF exon Ⅳ such as CaRF (calcium responsive factor) and USFs (upstream stimulatory factors) for CaRE1 and CaRE2, respectively[36,41] (Figure 1). CaRF knockout mice showed reduced exon Ⅳ mRNA levels in the cerebral cortex with normal levels in the hippocampus and striatum, suggesting brain region-specific regulation of CaRF-CaRE1 at the BDNF promoter Ⅳ[42]. Recently, Zheng *et al*[37] revealed intriguing Ca^{2+}-dependent activation mechanisms of each CaRE, using cultured cortical neurons at DIV 11. Ca^{2+} entry through NMDAR or L-VGCC stimulated only CaRE1 and CaRE3 but not CaRE2, and CaRE1 and CaRE3 were activated *via* different sets of protein kinases depending on the route of calcium entry[37]. For example, CaRF is essential only for the L-VGCC-induced, not the NMDAR-induced, transcription of BDNF exon Ⅳ[37,38], indicating that other transcriptional regulators stimulate the NMDAR-dependent activation of CaRE1. Furthermore, the L-VGCC-dependent CaRE1 activation depends on protein kinase A (PKA), calcium/calmodulin-dependent protein kinase I (CaMKI), and CaMKIV, while CaRF activation requires MEK, PI3K and CaMKII[37,38]. On the other hand, CaRE3 activity is regulated by Ca^{2+} influx through both L-VGCC and NMDAR. MEK, PI3K, and PKA activity are required in both the L-VGCC - and NMDA-induced CaRE3 activation[37,38]. CaM and CaMKIV are needed in the L-VGCC-induced CaRE3 activation while CaMKI and CaMKIV are required in the NMDAR-mediated CaRE3 activation[37,38]. Although CaREs play a central role in the regulation of BDNF promoter IV, there are also other possible regulators for the promoter including basic helix-loop-helix B2 (BHLHB2) and nuclear factor-κB (NF-κB)[43,44] (Figure 1). Furthermore, neuronal activity stimulates BDNF promoter I as well as promoter IV. Several transcription factor binding sites such as CREB, USFs, myocyte enhancer factor 2D, and NF-κB have been suggested to stimulate BDNF promoter I [45-47], though the regulatory mechanisms have not been elucidated. Futhermore, Timmusk and colleagues have identified a cis-element PasRE (bHLH-PAS transcription factor response element) in both promoter I and Ⅳ. They also demonstrated that transcription factors ARNT2 (aryl hydrocarbon receptor nuclear translocator 2, a basic helix-loop-helix (bHLH)-PAS transcription factor) and NPAS4 (neuronal PAS domain protein 4) bind to PasRE (bHLH-PAS transcription factor response element) in both promoters, which is required for full induction of neuronal activity-dependent transcription from each exon[48,49].

Epigenetic regulations in transcription of the *BDNF* gene have also been reported. Decreased methylation of cytosine residues in CpG dinucleotides (CpG) of BDNF promoter Ⅳ, at least in part, mediates neuronal activity-dependent induction of BDNF transcription. Methyl-CpG-binding protein 2 (MeCP2), a member of the methyl-CpG-binding protein family, binds to methylated DNA in the BDNF promoter Ⅳ region and functions as a transcriptional regulator[50]. It is still controversial whether MeCP2 functions as an activator or a repressor for BDNF transcription. Because MeCP2 protein is released from the BDNF promoter Ⅳ region in response to neuronal activity-induced reduction of CpG methyla-

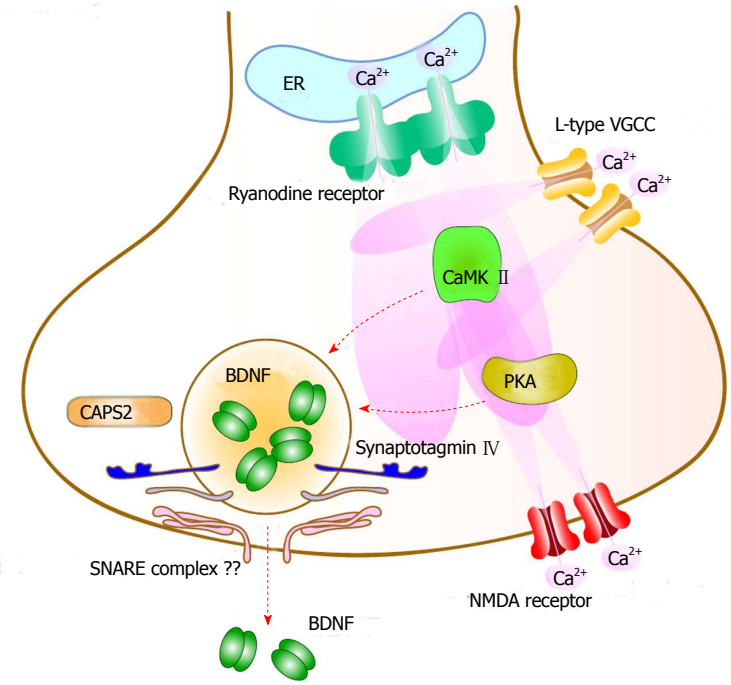

Figure 2 Schematic illustration of activity-dependent brain-derived neurotrophic factor secretion. A possible mechanism of intracellular Ca^{2+}-dependent secretion of BDNF suggested in literature. Ca^{2+} influx through NMDA receptor/L-type VGCC and subsequent Ca^{2+} release of internal stores via ryanodine receptors are required for secretion. CaMKⅡ, PKA, synaptotagmin Ⅳ, and CAPS2 also critically contribute to the membrane fusion process of BDNF-containing vesicles. VGCC: L-type voltage-gated calcium channels; CaMKⅡ: calcium/calmodulin-dependent protein kinase Ⅱ; PKA: protein kinase A; CAPS2: Ca^{2+}-dependent activator protein for secretion 2; BDNF: Brain-derived neurotrophic factor.

tion, it had been recognized as a transcriptional repressor for the *BDNF* gene[50]. However, both BDNF mRNA and protein levels were decreased in MeCP2-null mice[51], suggesting a possible repressive function of MeCP2 on BDNF expression. Furthermore, a combination mechanism of MeCP2 and CREB in BDNF transcription[52], and an additional function of MeCP2 other than transcriptional regulation, has also been indicated[3]. Recent findings demonstrated a novel epigenetic regulation in BDNF exon IX. Gadd45b, a neural immediate early gene, promotes activity-dependent demethylation in the BDNF exon IX promoter region[53] (Figure 1). Considering that electric stimulation-induced BDNF transcription was dramatically reduced in Gadd45b knock-out mice and that Gadd45b interacts and demethylates the promoter region of BDNF exon IX encoding pre-pro-BDNF[53], a gating function of Gadd45b in neuronal activity-dependent BDNF gene transcription was indicated.

BDNF TRANSPORT AND SECRETION

The secretion process of neurotrophins involves either the constitutive or regulated pathway, depending on whether secretion occurs spontaneously or in response to neuronal activity, respectively. Unlike other neurotrophins including NGF and NT-3 that are mainly secreted by the constitutive pathway, BDNF seems to be preferentially sorted into the regulated pathway[54-56]. As described above, the 32 kDa precursor form of BDNF (pre-pro-BDNF) is translated at the rough endoplasmic reticulum (ER) and then proteolytically cleaved to generate the 13 kDa mature form of BDNF. The pre-pro-BDNF is conveyed to the Golgi apparatus and sorted into the membrane stacks of the trans-Golgi network (TGN) where BDNF-containing dense core vesicles (DCVs) bud off. The pro-region of BDNF is implicated in the sorting step of BDNF into secretory vesicles[57,58]. A single nucleotide polymorphism (SNP) at nucleotide 196 in the pro-region of the human BDNF gene that produces an amino acid substitution from valine to methionine (val-66met) has a negative effect on the sorting of BDNF into vesicles[57]. The BDNF pro-region also binds to the lipid-raft-associated sorting receptor carboxypeptidase E (CPE) in the TGN, which is an important step for sorting into secretory vesicles[58]. Furthermore, a transmembrane protein sortilin which mainly resides in the membrane of the Golgi apparatus interacts with the pro-region of BDNF and regulates its sorting. BDNF vesicular sorting was impaired in pheochromocytoma PC12 cells that express the luminal domain sortilin lacking the transmembrane and cytoplasmic domains[59].

BDNF-containing vesicles are transported to secretion sites by motor protein complexes in polarized neurons, although the subcellular localization of BDNF secretion sites in neurons of the adult CNS is still controversial. Transport and secretion of BDNF-containing vesicles into axons and dendrites have been observed in cultured cortical and hippocampal neurons by visualization using exogenously transfected fluorescent protein-tagged BDNF[56,60,61], while knock-in mice expressing Myc-tagged BDNF showed specific localization of BDNF-containing vesicles at presynaptic terminals in the adult (8-wk-old) hippocampus[62]. Intriguingly, the Golgi apparatus present in dendrites was revealed in cultured hippocampal neurons and adult rat hippocampus, with fluorescent protein-tagged BDNF localized to the dendritic Golgi, indicating a local BDNF secretory pathway as well as local translation in dendrites[63,64]. Although BDNF transport properties are still not fully elucidated, bidirectional (anterograde and retrograde) trafficking of BDNF-containing vesicles in axons and dendrites have been reported in cultured neurons[56,65,66]. Furthermore,

the anterograde BDNF vesicle transport is microtubule-based with a motor protein kinesin and a coordinator dynactin[13,66]. Several important studies revealed that huntingtin, a polyglutamine-containing protein associated with Huntington disease (HD), and CPE are also involved in the motor protein complex for BDNF-containing vesicle transport and affect properties such as velocity and direction[13,14,65,66].

Evidence suggests that BDNF can be secreted from the cell soma, axon and dendrites of cultured cortical and hippocampal neurons in a neuronal activity (depolarization)-dependent manner[67,68] (Figure 2). Lessmann *et al*[68] clearly showed that activity-dependent BDNF secretion requires Ca^{2+} influx *via* ionotropic glutamate receptors or L-type VGCC and subsequent amplification of the initial Ca^{2+} elevation through Ca^{2+}-induced Ca^{2+} release from internal stores (the endoplasmic reticulum) *via* ryanodine receptors. Activation of Trk receptors or Na^{+} channels, on the other hand, is not directly associated with BDNF secretion[68-70]. They further revealed that activation of both CaMK Ⅱ and PKA were also required for secretion[67,70] (Figure 2). Considering the shared signaling pathways between the activity-dependent secretion and transcription of BDNF, decreased BDNF levels after secretion may be restored immediately with simultaneous induction of BDNF transcription. Although molecular mechanisms underlying the vesicle fusion step in BDNF secretion remain unclear, two proteins are suggested to regulate this step: Synaptotagmin Ⅳ and Ca^{2+}-dependent activator protein for secretion 2 (CAPS2). Synaptotagmin Ⅳ, a soluble NSF attachment protein receptor (SNARE) complex binding protein localized to BDNF containing vesicles, negatively regulates BDNF secretion at both axons and dendrites in hippocampal neurons[71]. It was also reported that CAPS2, a DCV-associated protein, enhanced activity-dependent BDNF secretion efficiency[72,73]. However, it is still unclear whether BDNF-containing vesicles use SNARE-dependent membrane fusion similar to synaptic vesicle exocytosis.

It is a matter of continuous debate where and how pre-pro-BDNF is converted to the mature form of BDNF in the CNS. It was previously concluded that pro-neurotrophins are cleaved by proteases such as furin and pro-protein convertases (PCs) in TGN or in DCVs prior to secretion[74]. Several recent studies, however, showed that hippocampal neurons secreted a considerable amount of pro-BDNF that was subsequently processed extracellularly to mature-BDNF by plasmin or matrix metalloproteinases[6,75]. More recently, immediate intracellular conversion of pro-BDNF to mature-BDNF along with co-localization of the cleaved pro-peptide region and mature-BDNF in secretory vesicles in hippocampal neurons were confirmed[62,76]. Resolving the discrepancy of whether neurons secrete pro-BDNF is a very important issue because pro-BDNF preferentially binds to p75NTR rather than TrkB, which in turn causes cell death or synaptic depression in neurons[77,78]. Where pro-neurotrophin cleavage takes place and which proteinases participate in the process might be dependent upon the developmental stage of neurons or the location of BDNF translation (the cell soma or dendrites)[79].

PHYSIOLOGICAL ROLES OF BDNF IN THE CNS

Neurons exhibit several distinct features such as a polarized structure, inter-connected circuits, and electrical activity. Many studies have revealed a variety of BDNF functions associated with these features in the developing and mature brain. BDNF acts as a neurite outgrowth and elongation factor, pro-survival factor, and synaptic regulator in the CNS. BDNF promotes axon initiation through two distinct signaling pathways. BDNF induces TrkB-dependent local elevation and stabilization of cAMP/PKA activity that are essential for axon initiation in undifferentiated neurites of hippocampal neurons[80,81]. Furthermore, BDNF/TrkB-induced Akt phosphorylation reduces GSK-3 activation which in turn decreases production of the active form of collapsin response mediator protein-2 that plays a critical role in microtubule assembly during axon elongation and branching in rat hippocampal neurons[82,83]. BDNF also promotes axon elongation and branching of sensory neurons both *in vitro* and *in vivo*[83,84]. A BDNF concentration gradient produced by a BDNF-containing micropipette demonstrated an attractive turning effect on the axonal growth cone in cultured *Xenopus* spinal neurons[83,84]. Interestingly, application of specific inhibitors for cAMP or PKA resulted in repulsive turning in the same BDNF gradient, indicating the critical role of cAMP levels to determine the growth cone's response between attraction and repulsion to the same BDNF gradient[83]. A comprehensive study on BDNF function in dendritic growth done by Wirth *et al*[85] revealed that overexpressed BDNF affected pyramidal cells by increasing length and number of apical dendritic segments in layer Ⅵ, and basal dendrites in layer Ⅴ in rat cortical slice cultures. It is important that such BDNF-promoted dendritogenesis and dendrite growth were observed only in BDNF-overexpressed neurons themselves, suggesting the autocrine action of BDNF[85]. Studies using primary cultured neurons have revealed the importance of MAP kinase and PI3 kinase activation *via* TrkB phosphorylation to promote BDNF-dependent dendritic growth[86,87]. It is also important to note that acute elevation in BDNF concentration promotes total growth of dendrites and the number of primary dendrites while gradual BDNF elevation increases branching number[88].

BDNF also plays a role in synapse formation and stabilization[89]. TrkB knockout mice showed a significant decline in the number of hippocampal synaptic structures[90]. TrkB conditional-mutant mice in which TrkB is deleted in the cerebellum resulted in a reduction of inhibitory synapses[90]. BDNF as a regulator of synaptogenesis *in vivo* was also confirmed in *Xenopus* optic axons[91]. Using the time-lapse imaging technique with GFP-tagged synaptobrevin Ⅱ as a marker for functional synapses, BDNF-induced retinal ganglion cell axon arborization and synaptogenesis were shown[91]. Furthermore, neu-

tralization of endogenous BDNF by function-blocking antibodies for BDNF rapidly (within 2 h) dismantled pre-existing GFP-synaptobrevin clusters, suggesting its importance in synaptic stabilization[92]. Exogenously applied BDNF induced functional excitatory and inhibitory synapse formation in cultured rat hippocampal neurons[93] and increased excitatory synapse numbers in rat hippocampal slices[94]. On the other hand, there are lines of evidence showing synaptogenic function of BDNF and TrkB in inhibitory GABAergic connections in the visual cortex[95-97], cerebellum[98], organotypic slice cultures of the rat hippocampus[99] and organotypic cerebellar cultures of mice[100]. BDNF serves as an important modulator for synaptic connections in mature neuronal circuits as well as in developing neuronal circuits. BDNF modulates synaptic efficacy and synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) in the developed CNS (for reviews, see[3,101,102]). For example, acute and chronic application of exogenous BDNF potentiates synaptic efficacy by increasing glutamate transmission in rat brain slices of the hippocampus[103], visual cortex[104], and hippocampal dissociated cultures[105,106]. Modulation of NMDA receptor function[107,108] and ion channels including Na_v[109], $K_v1.3$[110], and TRPC3 channels[111] by BDNF contributes to enhancement of excitatory synaptic efficacy. BDNF also promotes spine growth[112,113] and membrane insertion of NMDA[114] and AMPA[115] receptors at the postsynaptic cites of excitatory synapses. Contrary to its enhancing action on excitatory synapses, BDNF has been reported to modulate the efficacy of GABAergic synapse transmission both presynaptically and postsynaptically in a bi-directional manner. Presynaptic GABA transmission was suppressed by acute BDNF application in hippocampal slices[116] as well as chronic application in hippocampal cultures[117], while chronic BDNF in cultured hippocampal neurons potentiates presynaptic GABA transmission[118]. BDNF shows a bi-directional modulation also on postsynaptic $GABA_A$ receptor function. BDNF potentiated[119] and depressed[120,121] GABAergic postsynaptic currents through modulating $GABA_A$ receptor functions and regulating membrane insertion of $GABA_A$ receptors. Interestingly, BDNF decreased and increased inhibitory postsynaptic currents (IPSCs) when recorded from excitatory and inhibitory neurons, respectively[122]. Furthermore, IPSCs amplitude was increased by acute BDNF treatment in hippocampal slices obtained from postnatal day 6 rats whereas the opposite effect was observed in slices from postnatal day 14 rats[123]. These data indicate the complicated function of BDNF, especially in the modulation of basic properties of developed GABAergic synapses, depending on the region in the brain, stage of maturity, and duration of exposure to BDNF.

It has been frequently reported that synaptic BDNF is involved in LTP and LTD. LTP was first identified in the rat hippocampus as a long-lasting activity-dependent synaptic modification[124,125], and it is the leading hypothesis of the mechanism underlying experience-dependent learning and memory. Important roles of BDNF/TrkB signaling in specific high-frequency electrical stimuli (tetanus or Theta burst stimulation)-induced LTP in brain slices prepared from the hippocampus and cortex have been shown (for reviews, see[102,126]). A severe impairment in hippocampal LTP was confirmed in independent lines of BDNF knockout and heterozygous mice[127]. Interestingly, in heterozygous mice in which BDNF expression in the hippocampus is suppressed to approximately 60% of wild type levels, the same degree of impairment was found in LTP as that of knockout mice. Furthermore, supplementation of exogenous BDNF rescued this impairment, suggesting that BDNF plays a critical role in hippocampal LTP and a threshold level of BDNF is essential to assure LTP induction[128]. Of note, acute hippocampus-specific deletion of the *BDNF* gene in adult mice showed impaired novel object recognition and spatial learning in the Morris water maze[129]. These mice also demonstrated reduced extinction of conditioned fear in spite of normal acquisition and expression fear, suggesting a critical role for BDNF in hippocampal-dependent cognitive function and memory extinction[129]. TrkB knockout mice also exhibited impaired hippocampal LTP, illustrating the importance of TrkB in hippocampal-dependent learning involved in spatial memory tasks[130]. Mutant TrkB knock-in mice carrying a point mutation in the kinase domain of TrkB demonstrated that PLCγ-dependent activation of CREB and CaMKIV phosphorylation are critical factors for BDNF/TrkB-dependent hippocampal LTP[131]. Recently, mammalian target of rapamycin (mTOR) was also identified as a contributor to BDNF/TrkB-dependent hippocampal LTP[132]. Considering that PI3K and Akt are the upstream regulators of mTOR, the PI3K-AKT-mTOR pathway is thought to play a significant role in BDNF-dependent LTP. The PI3K-AKT pathway was also shown to enhance dendritic transport of PSD-95 to postsynaptic sites depending on BDNF/TrkB signaling[113], suggesting its involvement in persistent structural changes of spines after LTP induction. In contrast to mature BDNF, pro-BDNF mainly plays a role in LTD which is induced by low frequency stimulation (LFS). It has been reported that pro-BDNF enhanced NMDA receptor (NR2B)-dependent LTD in the hippocampus through p75NTR activation[78]. Application of pro-BDNF facilitated NR2B-mediated hippocampal LTD only in the wild type, but not in p75NTR knockout mice[78]. Interestingly, other forms of synaptic plasticity such as NMDA receptor-dependent LTP and NMDA receptor-independent LTD were intact in p75NTR knockout mice[78], suggesting a specific role of pro-BDNF in hippocampal LTD. Considering that p75NTR activation negatively affects dendrite complexity and spine density in hippocampal neurons[133], secreted pro-BDNF, which escapes processing by proteinases, modulates neurite morphology and synaptic plasticity in the opposite way as mature BDNF. Taken together, BDNF is deeply associated with neuronal connectivity through modulating the development of neural circuitry and regulating synaptic efficacy throughout the CNS. Therefore, impairment of BDNF function in the devel-

oping and mature brain is implicated in many psychiatric and neurodegenerative diseases.

CRITICAL BDNF GENE POLYMORPHISM

A critical non-synonymous SNP in the human BDNF gene was first reported in 2003[57], involving an amino acid substitution at valine 66 to methionine (Val66Met)[57]. The BDNF Val66Met polymorphism predominantly affects the sorting process of synthesized BDNF into secretory vesicles[57], resulting in reduction in activity-dependent BDNF secretion[12,57]. Inconsistent with critical BDNF roles in hippocampal LTP, met allele carriers ($BDNF^{met}$ carriers) showed impaired performance in episodic memory[57,134], verbal memory[135], and cognitive performance[136]. However, it is still controversial whether carrying the met allele causes significant reduction in human hippocampal volume despite the essential roles of BDNF in neurite development[12,137]. A mouse model of the polymorphism $BDNF^{met/met}$ knock-in mice showed an approximately 15% reduction in hippocampal volume and dendritic arbor complexity[138]. Cultured neurons from $BDNF^{met/met}$ knock-in mice exhibited normal levels of total BDNF protein but significantly reduced amounts of activity-dependent secretion of BDNF[138]. Interestingly, the $BDNF^{met/met}$ knock-in mice showed anxiety-related behaviors that did not improve with chronic antidepressant fluoxetine treatment[138]. Detailed electrophysiological analysis of hippocampal slices of $BDNF^{met/met}$ knock-in mice revealed normal basal glutamatergic transmission but reduced NMDA receptor-mediated current and NMDA receptor-dependent LTP[139]. It is clear that further investigation of BDNF transport and secretion processes are important for more detailed understanding of the neurotrophin and for the development of BDNF-based therapy for patients with brain-related diseases.

IMPLICATION OF BDNF IN BRAIN DISEASES

BDNF has been implicated in multiple brain-related diseases, as it plays a critical role in neuronal development, survival, and synaptic function. Although studies show involvement of BDNF in the pathophysiology of multiple brain-related illnesses such as bipolar disorder[140], Parkinson's disease[140], stroke, epilepsy[141], eating disorders[142], and substance use[142], we review four neuropsychiatric illnesses associated with BDNF dysfunction.

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder with noticeable impairment of cognitive function. Patients with AD show progressive loss of synapses and neurons especially in the entorhinal cortex and hippocampus, which causes a loss of their ability to acquire new memories[11,143-145]. A wide range of evidence exists due to brain cohort differences[146], though decreased expression levels of BDNF protein and mRNA have been consistently reported in the hippocampus and cortex of individuals with AD[147-151] as well as in serum[152] (Table 1). Furthermore, firm conclusions regarding involvement of the Val66Met polymorphism on disease onset or progression of AD have not been made (for review, see[140]). On the other hand, Nagahara *et al*[153] recently reported that BDNF gene delivery to the entorhinal cortex ameliorated entorhinal cortical and hippocampal neuronal degeneration in a mouse model of AD. Mice in this model expressed the human amyloid precursor protein (APP) with mutations and exhibited AD-like pathology such as cortical plaques, progressive cell loss in the entorhinal cortex and cognitive decline by 6-7 mo after birth[153,154]. Direct injection of lentiviral vector constitutively expressing BDNF into the entorhinal cortex of the mice after disease onset reversed synapse loss in both the entorhinal cortex and hippocampus, and restored learning and memory deficits[153]. Such *BDNF* gene delivery was similarly effective to the reduced memory function in normally aged (24-mo-old) rats and primates[153]. Because the entorhinal cortex is a primary input to the hippocampus, the rescued synaptic loss in the hippocampus of these model animals could be attributed to BDNF transport from the cortical region. Other AD mouse models with amyloid-β overproduction caused by APP mutations exhibited significant reduction in cortical BDNF mRNA[155]. Hippocampal BDNF protein is reduced in the 5XFAD transgenic mice expressing five familial AD mutant forms of human APP, with memory deficits rescued by a specific TrkB agonist, 7,8-dihydroxyflavone (7,8-DHF), without affecting endogenous BDNF levels[156]. Furthermore, neural stem cell (NSC) transplantation also improved cognitive function in AD model mice *via* BDNF secreted by NSC[157]. BDNF-based therapy is increasingly expected to ameliorate the symptoms of AD.

HUNTINGTON DISEASE

Huntington's disease (HD) is a neurodegenerative and autosomal dominant disease caused by repeats of the CAG trinucleotide in the huntingtin (htt) gene, which produces abnormal htt proteins with polyglutamine expansion (polyQ). Degeneration of striatal neurons is thought to induce progressive psychiatric, cognitive and motor dysfunction[158]. Possible link between HD and BDNF function has been reported. Given that substantial levels of BDNF protein are detectable in adult rodent striatum while TrkB mRNA, but not BDNF mRNA, is present along with the fact that cortical neurons projecting to the striatum contain high levels of BDNF mRNA, most striatal BDNF is anterogradely transported from the cortex[159]. It was revealed that wild-type htt but not mutant (polyQ) htt stimulated transcription from BDNF exon Ⅱ in the cerebral cortex[160,161]. Postmortem studies have revealed reduced BDNF mRNA and protein levels in the cortex[162,163], caudate-putamen[163,164], striatum[163], cerebellum[163], and substantia nigra[163] in patients with

HD. Decreased serum BDNF levels in patients was also reported[165]. The mouse models of HD expressing exon 1 of the human HD gene with expanded polyQ region recapitulate many of the features of human HD such as progressive behavioral deficit, impaired motor functions, cognitive decline, and premature death[166-169]. Reduced expression of BDNF was confirmed in the cortex[166,167] and striatum[168,169] of three HD mouse models (R6/1, R6/2, and YAC128). The antidepressant sertraline increased BDNF protein levels in the hippocampus and striatum, improving lifespan and motor performance, and ameliorated brain atrophy in R6/2 HD mice[170]. Genetically overexpressed BDNF in the cerebral cortex and striatum of YAC128 HD mice alleviated loss of striatal neurons and motor dysfunction, and improved procedural learning[168]. BDNF overexpression in the striatum improved cortico-striatal connectivity and motor function in R6/2 HD mice[167]. Furthermore, 7,8-DHF and its derivative 4'-dimethylamino-7,8- dihydroxyflavone (4'-DMA-7,8-DHF) extended survival, ameliorated brain atrophy, and improved motor deficits in N171-82Q HD mice[171]. Importantly, forebrain-specific BDNF-knock-out mice (Emx-BDNF KO mice) show a similar phenotype to that observed in the mouse models of HD[172,173]. In contrast to evidence suggesting involvement of BDNF in the pathophysiology of HD, no association between the Val-66Met polymorphism of the *BDNF* gene and onset/progression of HD has been reported[174-178].

Gauthier *et al*[13] have unveiled an important htt function in BDNF transport. Mutant htt reduced post-Golgi microtubule-based axonal transport velocity of BDNF-containing vesicles while wild-type htt accelerated transport velocity in cultured cortical neurons[13]. These findings suggest that mutations in htt lead to decreased amounts of BDNF in the striatum by inhibiting both BDNF transcription and axonal transport of BDNF-containing vesicles in cortical neurons. Furthermore, the direction of transport was regulated by phosphorylation of htt at serine 421[14]. Phosphorylated htt recruited motor protein kinesin-1 to BDNF-containing vesicles to facilitate anterograde transport, though wild-type htt enhances both anterograde and retrograde transport efficiency in cortical neurons. In contrast, retrograde transport was increased in the absence of htt phosphorylation at the serine 421 site[14]. Interestingly, mutant htt impairs the post-Golgi transport only in val-BDNF-containing vesicles but not in met-BDNF, which in turn produces a reduction in the amount of activity-dependent secretion of val-BDNF in cell lines[179]. Severely reduced BDNF levels in R6/1 HD mice hippocampus and striatum were rescued by environmental enrichment[169]. The enrichment, consisting of small cardboard boxes, small open wooden boxes, cylindrical cardboard tunnels, and folded sheets of paper prevented body weight loss and ameliorated motor symptoms of the mice[169]. Considering that the striatum itself does not produce BDNF and receives BDNF supply from the cortex, such enrichment may compensate by attenuating the mutant htt-induced impairment of anterograde transport of BDNF to the striatum[169,170]. Given the functional interaction between BDNF and htt, exogenous supplementation of BDNF or TrkB agonists to the striatum would be the primary choice for the treatment of HD patients.

DEPRESSION

Several lines of evidence implicate BDNF in the pathophysiology of psychiatric disorders[10-12,180]. Specifically, reduced BDNF expression levels and impaired BDNF function have been reported in patients with depression and schizophrenia[181-184]. Reduced BDNF[185-187] or TrkB[188] levels in the prefrontal cortex[185,186,188], hippocampus[185,186], amygdala[187], and serum[189-191] have been demonstrated in patients with depression, especially in suicide victims[185,186,189]. Considering that BDNF protein levels were unchanged in hippocampal and cortical tissue of suicide subjects who had been treated with antidepressants[186], increased levels of BDNF in these brain regions may be facilitated by treatment with antidepressants. Decreased serum BDNF levels have been also reported in patients with depression[189-191], which was recovered by antidepressant drug treatment[191-193]. Increased expression of BDNF or TrkB mRNA in the cerebellum[194] was also shown after chronic antidepressant treatment in subjects with depression. Decreased BDNF and TrkB expression levels are also evident in rodent models of depression. A variety of stressors on rodents such as social defeat[195], restraint (immobilization)[196,197], maternal deprivation[198], and glucocorticoid administration[196,199-201] can reduce BDNF and TrkB expression levels in the hippocampus, cortex, and amygdala. In the normal rat brain, hippocampal and cortical BDNF/TrkB levels were increased after administration of tricyclic antidepressants (TCA; including imipramine, desipramine)[202-204], selective serotonin reuptake inhibitors (SSRI; fluoxetine, paroxetine, sertraline)[202,204-206], noradrenergic and specific serotonergic antidepressants (NaSSA; mirtazapine, mianserin)[202,207], and monoamine oxidase inhibitors (MAOIs; tranylcypromine)[202-204]. Electroconvulsive seizures also significantly induced BDNF expression in the cortex[202,208] and hippocampus[202,209-211]. 7,8-DHF, a specific TrkB agonist, and its O-methylated metabolites showed an antidepressant-like effect in the forced swimming and tail suspension tests[212]. Of note, direct BDNF infusion into the rat midbrain induced antidepressant-like effects[213]. Interestingly, TrkB phosphorylation was also increased by antidepressants in the rat brain[214]. An antidepressant imipramine, however, induced TrkB phosphorylation even in the conditional BDNF knock-out mice, suggesting the antidepressant-induced TrkB activation might be BDNF-independent[215]. Despite compelling evidence of BDNF contribution to depression onset and symptoms, meta-analysis with inconsistent results from genetic association studies of the BDNF val66met polymorphism revealed no significant association between the val66met polymorphism and depression[216]. It must be noted, however,

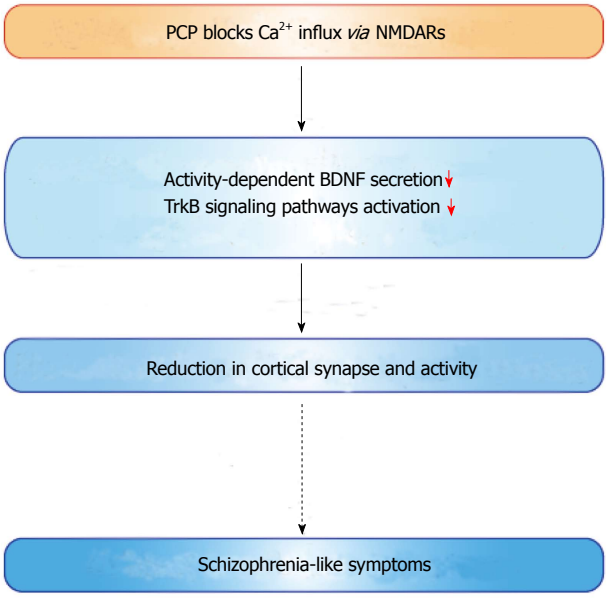

Figure 3 Molecular mechanisms of phencyclidine-induced synaptic loss as a cellular model of schizophrenia. Phencyclidine decreased the number of synaptic sites in cultured cortical neurons through blockade of Ca^{2+} influx *via* NMDARs and resultant suppression of BDNF secretion. The impairment in BDNF secretion reduced TrkB activation and resulted in decreased synaptic connectivity[15]. BDNF: Brain-derived neurotrophic factor; NMDAR: N-methyl-D-aspartate receptors; PCP: Phencyclidine.

that the polymorphism may affect the development of depression more in men than in women[216].

Chronic stress-induced hypothalamic pituitary-adrenal (HPA) axis abnormalities result in increased serum levels of glucocorticoids that are implicated in the pathogenesis of depression[10,217,218]. The HPA axis is an important endocrinological coping mechanism against stressful stimuli that includes release of hormones such as corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN), CRH-trigged adrenocorticotropic hormone (ACTH) from the anterior pituitary, and ACTH-induced glucocorticoids from the adrenal glands. We recently reported that the glucocorticoid receptor (GR), which has both genomic and non-genomic properties in cortical neurons, requires TrkB interaction for full activation of the BDNF-induced PLCγ pathway[219]. Chronic treatment with glucocorticoids decreased GR protein levels, and inhibited TrkB-GR interaction and resultant BDNF-induced glutamate release *via* the PLCγ pathway[219]. On the other hand, Jeanneteau *et al*[220] showed TrkB activation by acute administration of glucocorticoids in cortical neurons. The activation of TrkB by glucocorticoids promoted neuronal survival through the transcriptional function of GR and Akt signaling pathway activation[220]. They also revealed that BDNF/TrkB signaling regulates GR transcriptional activity by stimulating GR phosphorylation at S155 and S287[221,222]. These results suggest a possible involvement of the impaired reciprocal interaction between BDNF and the HPA axis in the pathogenesis of depressive disorders. Taken together, BDNF-based therapy for depressed patients could target GR function or the direct activation of TrkB[12].

SCHIZOPHRENIA

Approximately 1% of the world population is affected by schizophrenia. This psychiatric condition manifests in three major ways: Positive symptoms (hallucinations, delusions, thought and movement disorders), negative symptoms (flat affect, lack of pleasure and ability to begin and sustain planned activities), and cognitive symptoms (impaired ability to understand and use information, and problems with "working memory")[223]. Several brain regions have been implicated in the pathophysiology of this illness. Studies on postmortem brain tissue of schizophrenia patients demonstrates that the overall number of neurons in the prefrontal cortex is not decreased[224]. However, reduced synaptophysin (presynaptic protein) immunoreactivity and dendritic spine density of pyramidal cells were observed in the cortex[225,226], suggesting synaptic dysfunction in the pathogenesis of the disease. Because symptoms usually start between ages 16 and 30 with only rare cases reported after age 45[227], deficits in synaptic maturation and overall brain development may be heavily involved in disease onset. Expression levels of BDNF or TrkB have been investigated in brains of patients with schizophrenia. Whether or not BDNF levels are altered in brain tissue or serum of patients with schizophrenia is a controversial topic[184,228,229]. Some postmortem studies demonstrated elevated BDNF protein levels in the hippocampal and cortical tissue in schizophrenic patients[230,231], while decreased BDNF levels have also been shown in these brain regions[231-234] (Table 1). Reduced BDNF mRNA and protein levels in serum of patients with schizophrenia have been consistently reported (Table 1). There are some reports showing that clozapine treatment increased serum BDNF mRNA levels in patients[235,236]. A meta-analysis examining blood BDNF levels in schizophrenia found a moderate effect of reduced BDNF in patents with schizophrenia compared with controls[237]. Furthermore, val/met and met/met BDNF genotypes were reported in a meta-analysis to increase the risk of schizophrenia[142]. Decreased BDNF mRNA and protein levels have also been confirmed in animal models of schizophrenia induced by phencyclidine (PCP)[238], MK-801[239] or ibotenic acid[240]. In contrast to human patients, antipsychotic administration in rodents significantly reduces BDNF expression levels (Table 1). Second-generation antipsychotics such as risperidone and olanzapine, in addition to first-generation antipsychotics such as haloperidol and chlorpromazine, all tend to reduce BDNF protein levels in the rat cortex, hippocampus, and striatum[241-243] (Table 1). Although it is challenging to approach the molecular mechanisms underlying the pathophysiology of schizophrenia, PCP, a psychomimetic drug, is known to produce beneficial animal and cellular models for schizophrenia. PCP acts as a non-competitive NMDA receptor blocker and generates schizophrenia-like behavioral changes in humans and rodents[244,245]. Interestingly, sub-chronic PCP (2 mg/kg, *i.p.* twice daily for 7 d followed by 6 wk drug-free) administration decreased BDNF mRNA levels in many brain

Table 1 Implications of BDNF in brain-related diseases

BDNF expression levels in brain diseases and animal models	Possible involvement of BDNF in therapies
Alzheimer's disease (patients)	
Decreased expression levels of BDNF mRNA	
Hippocampus: ref.[147,148]	
Cortex: ref.[148,149]	
Decreased expression levels of BDNF protein	
Serum: ref.[152]	
Hippocampus: ref.[150]	
Cortex: ref.[150,151]; no change ref.[247]	
Alzheimer's disease (animal models)	Alzheimer's disease (animal models)
Decreased expression levels of BDNF mRNA	*BDNF* gene delivery ameliorated age-related cognitive impairment in aged primates and rats. ref.[153]
Cortex: Aβ transgenic strains (APPNLh and TgCRND8) ref.[155]	7, 8-DHF improved memory deficits in 5XFAD transgenic mouse. ref.[156]
Decreased expression levels of BDNF protein	
Hippocumpus: 5XFAD transgenic mouse ref.[156]	
Huntington disease (patients)	
Decreased expression levels of BDNF mRNA	
Serum: ref.[165]	
Cortex: ref.[162]	
Caudate: decreased TrkB mRNA ref.[162]	
Decreased expression levels of BDNF protein	
Cortex: ref.[163,165]	
Striatum: ref.[163]	
Cerebellum: ref.[163]	
Substantia nigra: ref.[163]	
Caudate and Putamen: ref.[164]	
Huntington disease (animal models)	Huntington disease (animal models)
Decreased expression levels of BDNF mRNA	BDNF overexpression improved motor function in R6/2 mice. ref.[167]
Cortex: R6/2 mice, ref.[166,167]	BDNF overexpression ameliorated YAC128 mice phenotype. ref.[168]
Decreased expression levels of BDNF protein	Environmental enrichment-increased BDNF improved the phenotype in R6/1 mice. ref.[169]
Striatum: YAC128 mice, ref.[168]; R6/1 mice ref.[169]	Sertraline-increased BDNF improved the phenotype in R6/2 mice. ref.[170]
	7, 8-DHF or 4'-DMA-7,8-DHF improved motor deficits in N171-82Q mice. ref.[171]
Depression (patients)	Depression (patients)
Decreased expression levels of BDNF mRNA	Antidepressants increased BDNF in serum. ref.[191-193,248]
Hippocampus: ref.[185] (suicidal)	Antidepressants increased BDNF and TrkB in the cerebellum. ref.[194]
Cortex: ref.[185] (suicidal), decreased TrkB mRNA ref.[188]	
Amygdala: ref.[187]	
Decreased expression levels of BDNF protein	
Serum: ref.[189-191]	
Hippocampus ref.[185] (suicidal)[186] (suicidal)	
Cortex: ref.[185] (suicidal)[186] (suicidal)	
Depression (animal models)	Depression (animal models)
Decreased expression levels of BDNF mRNA	Antidepressants increased BDNF in the rat brain
Hippocampus: social defeat stress, ref.[195]; restrain stress, ref.[196,197] (also TrkB); maternal deprivation, ref.[198]; dexamethasone, ref.[199]; corticosterone, ref.[196,200,201]; foot shock stress, ref.[249]	Tricyclic antidepressants (TCA; including imipramine, desipramine), ref.[202-204] Selective serotonin reuptake inhibitors (SSRI; fluoxetine, paroxetine, sertraline), ref.[202,204-206,251] Noradrenergic and specific serotonergic antidepressants (NaSSA; mirtazapine, mianserin), ref.[202,207] Monoamine oxidase inhibitors (MAOIs; tranylcypromine), ref.[202-204]
Cortex: social defeat stress, ref.[195]; maternal deprivation, ref.[198]; dexamethasone, ref.[199]; cold swim stress, ref.[250]	Electroconvulsive seizures induced BDNF expression. ref.[202,208-211] 7,8-DHF showed an antidepressant-like effect. ref.[212] Direct BDNF infusion into the rat midbrain induced antidepressant-like effects. ref.[213]
Amygdala: social defeat stress, ref.[193]	

Decreased expression levels of BDNF protein Hippocampus: maternal deprivation, ref.[198]; corticosterone, ref.[200,201]; cold swim stress, ref.[250]	
Schizophrenia (patients) Decreased expression levels of BDNF mRNA Serum: ref.[236,252]; decreased TrkB mRNA ref.[253] Cortex: ref.[122,233,254,255]; decreased TrkB mRNA ref.[255]; incrased trunkated TrkB mRNA ref.[256] Serum: ref.[235,257-260] Hippocampus: ref.[231]; abnormal expression of TrkB ref.[261]; increased BDNF mRNA ref.[230] Cortex: ref.[232-234]; increase BDNF mRNA ref.[230,231] Cerebrospinal fluid ref.[234]	Schizophrenia (patients) Clozapine treatment increased serum BDNF mRNA levels. ref.[235,236]
Schizophrenia (animal models) Decreased expression levels of BDNF mRNA Hippoaumpus: phencyclidine, ref.[238]; MK-801 ref.[239]; ibotenic acid lesions, ref.[240] Cortex: phencyclidine, ref.[238] Amygdala: phencyclidine, ref.[238]	Schizophrenia (animal models) Mk-801-induced decline in hippocampal BDNF mRNA was normalized by olanzapine, but exacerbated by haloperidol. ref.[238] Haloperidol or risperidone decreased BDNF protein in the rat frontal cortex and hippocampus. ref.[241] Haloperidol decreased BDNF and TrkB protein in the rat hippocampus. ref.[242] Risperidon, olanzapine, haloperidol, and chlorpromazine decreased BDNF levels in the rat striatum and hippocampus. ref.[243] Haloperidol down-regulated BDNF mRNA expression in the rat hippocampus. ref.[262]

BDNF: Brain-derived neurotrophic factor.

regions including the cortical, hippocampal, and amygdaloid regions in adult rats[238], while higher doses of PCP (10 mg/kg, *i.p.* for 14 d) increased BDNF levels in the hippocampus and entorhinal cortex of rat pups[246]. We recently demonstrated that PCP suppressed the activity-dependent secretion of BDNF in cultured cortical neurons through blockade of Ca^{2+} influx *via* NMDA receptors[15] (Figure 3). Decreased BDNF secretion subsequently caused a reduction in the activation of TrkB-dependent signaling pathways and resultant synaptic loss in cortical neurons[15], which is consistent with the observation that patients demonstrate reduced cortical synaptic structure[225-227]. Because of the multifactorial etiology of schizophrenia, whether the BDNF-based approach would be effective or not is unclear in this disease. Ultimately, the multiple, essential roles of BDNF in synaptic function, however, could influence future therapy for these patients.

CONCLUSION

Many efforts are being dedicated to understanding the intracellular processes of BDNF to develop BDNF-based effective treatments for the brain-related diseases. Although brain diseases are associated with an overall decrease in BDNF function, varying BDNF levels in specific brain regions may affect symptom attenuation. As BDNF does not cross the blood-brain barrier, researchers are challenged to develop *BDNF* gene or protein delivery methods to the CNS[11]. Molecules that increase BDNF or TrkB expression levels, TrkB phosphorylation or membrane insertion, or conversion from pro-BDNF to mature BDNF are important and novel treatment options[12]. Even if BDNF itself is not the main gene responsible for the particular brain disease, BDNF-based treatments may provide valuable symptom relief. It is also critical to elucidate the basic molecular mechanisms of BDNF expression, transport, and secretion so that new therapeutic approaches may be developed to treat these debilitating neuropsychiatric conditions.

REFERENCES

1 **Barde YA**, Edgar D, Thoenen H. Purification of a new neurotrophic factor from mammalian brain. *EMBO J* 1982; **1**: 549-553 [PMID: 7188352]
2 **Cohen S**, Levi-Montalcini R, Hamburger V. A nerve growth-stimulating factor isolated from sarcom as 37 and 180. *Proc Natl Acad Sci USA* 1954; **40**: 1014-1018 [PMID: 16589582]
3 **Park H**, Poo MM. Neurotrophin regulation of neural circuit development and function. *Nat Rev Neurosci* 2013; **14**: 7-23 [PMID: 23254191 DOI: 10.1038/nrn3379]
4 **Lu B**. Pro-region of neurotrophins: role in synaptic modulation. *Neuron* 2003; **39**: 735-738 [PMID: 12948441]
5 **Chao MV**. Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat Rev Neurosci* 2003; **4**: 299-309 [PMID: 12671646]
6 **Lu B**, Pang PT, Woo NH. The yin and yang of neurotrophin action. *Nat Rev Neurosci* 2005; **6**: 603-614 [PMID: 16062169]
7 **Huang EJ**, Reichardt LF. Trk receptors: roles in neuronal sig-

nal transduction. *Annu Rev Biochem* 2003; **72**: 609-642 [PMID: 12676795 DOI: 10.1146/annurev.biochem.72.121801.161629]
8 **Minichiello L**. TrkB signalling pathways in LTP and learning. *Nat Rev Neurosci* 2009; **10**: 850-860 [PMID: 19927149]
9 **Russo SJ**, Mazei-Robison MS, Ables JL, Nestler EJ. Neurotrophic factors and structural plasticity in addiction. *Neuropharmacology* 2009; **56** Suppl 1: 73-82 [PMID: 18647613 DOI: 10.1016/j.neuropharm.2008.06.059]
10 **Numakawa T**, Adachi N, Richards M, Chiba S, Kunugi H. Brain-derived neurotrophic factor and glucocorticoids: reciprocal influence on the central nervous system. *Neuroscience* 2013; **239**: 157-172 [PMID: 23069755 DOI: 10.1016/j.neuroscience.2012.09.073]
11 **Nagahara AH**, Tuszynski MH. Potential therapeutic uses of BDNF in neurological and psychiatric disorders. *Nat Rev Drug Discov* 2011; **10**: 209-219 [PMID: 21358740 DOI: 10.1038/nrd3366]
12 **Lu B**, Nagappan G, Guan X, Nathan PJ, Wren P. BDNF-based synaptic repair as a disease-modifying strategy for neurodegenerative diseases. *Nat Rev Neurosci* 2013; **14**: 401-416 [PMID: 23674053 DOI: 10.1038/nrn3505]
13 **Gauthier LR**, Charrin BC, Borrell-Pagès M, Dompierre JP, Rangone H, Cordelières FP, De Mey J, MacDonald ME, Lessmann V, Humbert S, Saudou F. Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. *Cell* 2004; **118**: 127-138 [PMID: 15242649]
14 **Colin E**, Zala D, Liot G, Rangone H, Borrell-Pagès M, Li XJ, Saudou F, Humbert S. Huntingtin phosphorylation acts as a molecular switch for anterograde/retrograde transport in neurons. *EMBO J* 2008; **27**: 2124-2134 [PMID: 18615096 DOI: 10.1038/emboj.2008.133]
15 **Adachi N**, Numakawa T, Kumamaru E, Itami C, Chiba S, Iijima Y, Richards M, Katoh-Semba R, Kunugi H. Phencyclidine-induced decrease of synaptic connectivity via inhibition of BDNF secretion in cultured cortical neurons. *Cereb Cortex* 2013; **23**: 847-858 [PMID: 22467667 DOI: 10.1093/cercor/bhs074]
16 **Ernfors P**, Wetmore C, Olson L, Persson H. Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* 1990; **5**: 511-526 [PMID: 2206535]
17 **Hofer M**, Pagliusi SR, Hohn A, Leibrock J, Barde YA. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO J* 1990; **9**: 2459-2464 [PMID: 2369898]
18 **Conner JM**, Lauterborn JC, Yan Q, Gall CM, Varon S. Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. *J Neurosci* 1997; **17**: 2295-2313 [PMID: 9065491]
19 **Yan Q**, Rosenfeld RD, Matheson CR, Hawkins N, Lopez OT, Bennett L, Welcher AA. Expression of brain-derived neurotrophic factor protein in the adult rat central nervous system. *Neuroscience* 1997; **78**: 431-448 [PMID: 9145800]
20 **Katoh-Semba R**, Takeuchi IK, Semba R, Kato K. Distribution of brain-derived neurotrophic factor in rats and its changes with development in the brain. *J Neurochem* 1997; **69**: 34-42 [PMID: 9202291 DOI: 10.1046/j.1471-4159.1997.69010034.x]
21 **Gorba T**, Klostermann O, Wahle P. Development of neuronal activity and activity-dependent expression of brain-derived neurotrophic factor mRNA in organotypic cultures of rat visual cortex. *Cereb Cortex* 1999; **9**: 864-877 [PMID: 10601005 DOI: 10.1093/cercor/9.8.864]
22 **Patz S**, Wahle P. Developmental changes of neurotrophin mRNA expression in the layers of rat visual cortex. *Eur J Neurosci* 2006; **24**: 2453-2460 [PMID: 17100834 DOI: 10.1111/j.1460-9568.2006.05126.x]
23 **Liu QR**, Lu L, Zhu XG, Gong JP, Shaham Y, Uhl GR. Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. *Brain Res* 2006; **1067**: 1-12 [PMID: 16376315]
24 **Aid T**, Kazantseva A, Piirsoo M, Palm K, Timmusk T. Mouse and rat BDNF gene structure and expression revisited. *J Neurosci Res* 2007; **85**: 525-535 [PMID: 17149751 DOI: 10.1002/jnr.21139]
25 **Pruunsild P**, Kazantseva A, Aid T, Palm K, Timmusk T. Dissecting the human BDNF locus: bidirectional transcription, complex splicing, and multiple promoters. *Genomics* 2007; **90**: 397-406 [PMID: 17629449 DOI: 10.1016/j.ygeno.2007.05.004]
26 **Timmusk T**, Palm K, Metsis M, Reintam T, Paalme V, Saarma M, Persson H. Multiple promoters direct tissue-specific expression of the rat BDNF gene. *Neuron* 1993; **10**: 475-489 [PMID: 8461137]
27 **An JJ**, Gharami K, Liao GY, Woo NH, Lau AG, Vanevski F, Torre ER, Jones KR, Feng Y, Lu B, Xu B. Distinct role of long 3' UTR BDNF mRNA in spine morphology and synaptic plasticity in hippocampal neurons. *Cell* 2008; **134**: 175-187 [PMID: 18614020 DOI: 10.1016/j.cell.2008.05.045]
28 **Lin Y**, Bloodgood BL, Hauser JL, Lapan AD, Koon AC, Kim TK, Hu LS, Malik AN, Greenberg ME. Activity-dependent regulation of inhibitory synapse development by Npas4. *Nature* 2008; **455**: 1198-1204 [PMID: 18815592 DOI: 10.1038/nature07319]
29 **Isackson PJ**, Huntsman MM, Murray KD, Gall CM. BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: temporal patterns of induction distinct from NGF. *Neuron* 1991; **6**: 937-948 [PMID: 2054188]
30 **Castrén E**, Zafra F, Thoenen H, Lindholm D. Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. *Proc Natl Acad Sci USA* 1992; **89**: 9444-9448 [PMID: 1409655 DOI: 10.1073/pnas.89.20.9444]
31 **Bozzi Y**, Pizzorusso T, Cremisi F, Rossi FM, Barsacchi G, Maffei L. Monocular deprivation decreases the expression of messenger RNA for brain-derived neurotrophic factor in the rat visual cortex. *Neuroscience* 1995; **69**: 1133-1144 [PMID: 8848102]
32 **Rocamora N**, Welker E, Pascual M, Soriano E. Upregulation of BDNF mRNA expression in the barrel cortex of adult mice after sensory stimulation. *J Neurosci* 1996; **16**: 4411-4419 [PMID: 8699252]
33 **Zafra F**, Hengerer B, Leibrock J, Thoenen H, Lindholm D. Activity dependent regulation of BDNF and NGF mRNAs in the rat hippocampus is mediated by non-NMDA glutamate receptors. *EMBO J* 1990; **9**: 3545-3550 [PMID: 2170117]
34 **Zafra F**, Castrén E, Thoenen H, Lindholm D. Interplay between glutamate and gamma-aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons. *Proc Natl Acad Sci USA* 1991; **88**: 10037-10041 [PMID: 1658793]
35 **Zafra F**, Lindholm D, Castrén E, Hartikka J, Thoenen H. Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *J Neurosci* 1992; **12**: 4793-4799 [PMID: 1281495]
36 **Tao X**, West AE, Chen WG, Corfas G, Greenberg ME. A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. *Neuron* 2002; **33**: 383-395 [PMID: 11832226]
37 **Zheng F**, Zhou X, Luo Y, Xiao H, Wayman G, Wang H. Regulation of brain-derived neurotrophic factor exon IV transcription through calcium responsive elements in cortical neurons. *PLoS One* 2011; **6**: e28441 [PMID: 22174809 DOI: 10.1371/journal.pone.0028441]
38 **Zheng F**, Zhou X, Moon C, Wang H. Regulation of brain-derived neurotrophic factor expression in neurons. *Int J Physiol Pathophysiol Pharmacol* 2012; **4**: 188-200 [PMID: 23320132]
39 **Koppel I**, Timmusk T. Differential regulation of Bdnf expression in cortical neurons by class-selective histone deacety-

lase inhibitors. *Neuropharmacology* 2013; **75**: 106-115 [PMID: 23916482 DOI: 10.1016/j.neuropharm.2013.07.015]
40 **Hong EJ**, McCord AE, Greenberg ME. A biological function for the neuronal activity-dependent component of Bdnf transcription in the development of cortical inhibition. *Neuron* 2008; **60**: 610-624 [PMID: 19038219 DOI: 10.1016/j.neuron.2008.09.024]
41 **Chen WG**, West AE, Tao X, Corfas G, Szentirmay MN, Sawadogo M, Vinson C, Greenberg ME. Upstream stimulatory factors are mediators of Ca2+-responsive transcription in neurons. *J Neurosci* 2003; **23**: 2572-2581 [PMID: 12684442]
42 **McDowell KA**, Hutchinson AN, Wong-Goodrich SJ, Presby MM, Su D, Rodriguiz RM, Law KC, Williams CL, Wetsel WC, West AE. Reduced cortical BDNF expression and aberrant memory in Carf knock-out mice. *J Neurosci* 2010; **30**: 7453-7465 [PMID: 20519520 DOI: 10.1523/JNEUROSCI.3997-09.2010]
43 **Jiang X**, Tian F, Du Y, Copeland NG, Jenkins NA, Tessarollo L, Wu X, Pan H, Hu XZ, Xu K, Kenney H, Egan SE, Turley H, Harris AL, Marini AM, Lipsky RH. BHLHB2 controls Bdnf promoter 4 activity and neuronal excitability. *J Neurosci* 2008; **28**: 1118-1130 [PMID: 18234890 DOI: 10.1523/JNEUROSCI.2262-07.2008]
44 **Kairisalo M**, Korhonen L, Sepp M, Pruunsild P, Kukkonen JP, Kivinen J, Timmusk T, Blomgren K, Lindholm D. NF-kappaB-dependent regulation of brain-derived neurotrophic factor in hippocampal neurons by X-linked inhibitor of apoptosis protein. *Eur J Neurosci* 2009; **30**: 958-966 [PMID: 19735291 DOI: 10.1111/j.1460-9568.2009.06898.x]
45 **Tabuchi A**, Sakaya H, Kisukeda T, Fushiki H, Tsuda M. Involvement of an upstream stimulatory factor as well as cAMP-responsive element-binding protein in the activation of brain-derived neurotrophic factor gene promoter I. *J Biol Chem* 2002; **277**: 35920-35931 [PMID: 12114522 DOI: 10.1074/jbc.M204784200]
46 **Lubin FD**, Ren Y, Xu X, Anderson AE. Nuclear factor-kappa B regulates seizure threshold and gene transcription following convulsant stimulation. *J Neurochem* 2007; **103**: 1381-1395 [PMID: 17727632 DOI: 10.1111/j.1471-4159.2007.04863.x]
47 **Flavell SW**, Kim TK, Gray JM, Harmin DA, Hemberg M, Hong EJ, Markenscoff-Papadimitriou E, Bear DM, Greenberg ME. Genome-wide analysis of MEF2 transcriptional program reveals synaptic target genes and neuronal activity-dependent polyadenylation site selection. *Neuron* 2008; **60**: 1022-1038 [PMID: 19109909 DOI: 10.1016/j.neuron.2008.11.029]
48 **Koppel I**, Aid-Pavlidis T, Jaanson K, Sepp M, Pruunsild P, Palm K, Timmusk T. Tissue-specific and neural activity-regulated expression of human BDNF gene in BAC transgenic mice. *BMC Neurosci* 2009; **10**: 68 [PMID: 19555478 DOI: 10.1186/1471-2202-10-68]
49 **Pruunsild P**, Sepp M, Orav E, Koppel I, Timmusk T. Identification of cis-elements and transcription factors regulating neuronal activity-dependent transcription of human BDNF gene. *J Neurosci* 2011; **31**: 3295-3308 [PMID: 21368041 DOI: 10.1523/JNEUROSCI.4540-10.2011]
50 **Chen WG**, Chang Q, Lin Y, Meissner A, West AE, Griffith EC, Jaenisch R, Greenberg ME. Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science* 2003; **302**: 885-889 [PMID: 14593183 DOI: 10.1126/science.1086446]
51 **Chang Q**, Khare G, Dani V, Nelson S, Jaenisch R. The disease progression of Mecp2 mutant mice is affected by the level of BDNF expression. *Neuron* 2006; **49**: 341-348 [PMID: 16446138]
52 **Chahrour M**, Jung SY, Shaw C, Zhou X, Wong ST, Qin J, Zoghbi HY. MeCP2, a key contributor to neurological disease, activates and represses transcription. *Science* 2008; **320**: 1224-1229 [PMID: 18511691 DOI: 10.1126/science.1153252]
53 **Ma DK**, Jang MH, Guo JU, Kitabatake Y, Chang ML, Pow-Anpongkul N, Flavell RA, Lu B, Ming GL, Song H. Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis. *Science* 2009; **323**: 1074-1077 [PMID: 19119186 DOI: 10.1126/science.1166859]
54 **Goodman LJ**, Valverde J, Lim F, Geschwind MD, Federoff HJ, Geller AI, Hefti F. Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. *Mol Cell Neurosci* 1996; **7**: 222-238 [PMID: 8726105]
55 **Farhadi HF**, Mowla SJ, Petrecca K, Morris SJ, Seidah NG, Murphy RA. Neurotrophin-3 sorts to the constitutive secretory pathway of hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with brain-derived neurotrophic factor. *J Neurosci* 2000; **20**: 4059-4068 [PMID: 10818141]
56 **Adachi N**, Kohara K, Tsumoto T. Difference in trafficking of brain-derived neurotrophic factor between axons and dendrites of cortical neurons, revealed by live-cell imaging. *BMC Neurosci* 2005; **6**: 42 [PMID: 15969745 DOI: 10.1186/1471-2202-6-42]
57 **Egan MF**, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, Zaitsev E, Gold B, Goldman D, Dean M, Lu B, Weinberger DR. The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* 2003; **112**: 257-269 [PMID: 12553913]
58 **Lou H**, Kim SK, Zaitsev E, Snell CR, Lu B, Loh YP. Sorting and activity-dependent secretion of BDNF require interaction of a specific motif with the sorting receptor carboxypeptidase e. *Neuron* 2005; **45**: 245-255 [PMID: 15664176]
59 **Chen ZY**, Ieraci A, Teng H, Dall H, Meng CX, Herrera DG, Nykjaer A, Hempstead BL, Lee FS. Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. *J Neurosci* 2005; **25**: 6156-6166 [PMID: 15987945 DOI: 10.1523/JNEUROSCI.1017-05.2005]
60 **Matsuda N**, Lu H, Fukata Y, Noritake J, Gao H, Mukherjee S, Nemoto T, Fukata M, Poo MM. Differential activity-dependent secretion of brain-derived neurotrophic factor from axon and dendrite. *J Neurosci* 2009; **29**: 14185-14198 [PMID: 19906967 DOI: 10.1523/JNEUROSCI.1863-09.2009]
61 **Jakawich SK**, Nasser HB, Strong MJ, McCartney AJ, Perez AS, Rakesh N, Carruthers CJ, Sutton MA. Local presynaptic activity gates homeostatic changes in presynaptic function driven by dendritic BDNF synthesis. *Neuron* 2010; **68**: 1143-1158 [PMID: 21172615 DOI: 10.1016/j.neuron.2010.11.034]
62 **Dieni S**, Matsumoto T, Dekkers M, Rauskolb S, Ionescu MS, Deogracias R, Gundelfinger ED, Kojima M, Nestel S, Frotscher M, Barde YA. BDNF and its pro-peptide are stored in presynaptic dense core vesicles in brain neurons. *J Cell Biol* 2012; **196**: 775-788 [PMID: 22412021 DOI: 10.1083/jcb.201201038]
63 **Horton AC**, Rácz B, Monson EE, Lin AL, Weinberg RJ, Ehlers MD. Polarized secretory trafficking directs cargo for asymmetric dendrite growth and morphogenesis. *Neuron* 2005; **48**: 757-771 [PMID: 16337914]
64 **Horton AC**, Ehlers MD. Dual modes of endoplasmic reticulum-to-Golgi transport in dendrites revealed by live-cell imaging. *J Neurosci* 2003; **23**: 6188-6199 [PMID: 12867502]
65 **Park JJ**, Cawley NX, Loh YP. A bi-directional carboxypeptidase E-driven transport mechanism controls BDNF vesicle homeostasis in hippocampal neurons. *Mol Cell Neurosci* 2008; **39**: 63-73 [PMID: 18573344 DOI: 10.1016/j.mcn.2008.05.016]
66 **Kwinter DM**, Lo K, Mafi P, Silverman MA. Dynactin regulates bidirectional transport of dense-core vesicles in the axon and dendrites of cultured hippocampal neurons. *Neuroscience* 2009; **162**: 1001-1010 [PMID: 19497353 DOI: 10.1016/j.neuroscience.2009.05.038]
67 **Edelmann E**, Lessmann V, Brigadski T. Pre- and postsynaptic twists in BDNF secretion and action in synaptic plasticity. *Neuropharmacology* 2014; **76** Pt C: 610-627 [PMID: 23791959 DOI: 10.1016/j.neuropharm.2013.05.043]

68 **Lessmann V**, Brigadski T. Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. *Neurosci Res* 2009; **65**: 11-22 [PMID: 19523993 DOI: 10.1016/j.neures.2009.06.004]

69 **Hartmann M**, Heumann R, Lessmann V. Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. *EMBO J* 2001; **20**: 5887-5897 [PMID: 11689429 DOI: 10.1093/emboj/20.21.5887]

70 **Kolarow R**, Brigadski T, Lessmann V. Postsynaptic secretion of BDNF and NT-3 from hippocampal neurons depends on calcium calmodulin kinase II signaling and proceeds via delayed fusion pore opening. *J Neurosci* 2007; **27**: 10350-10364 [PMID: 17898207 DOI: 10.1523/JNEUROSCI.0692-07.2007]

71 **Dean C**, Liu H, Dunning FM, Chang PY, Jackson MB, Chapman ER. Synaptotagmin-IV modulates synaptic function and long-term potentiation by regulating BDNF release. *Nat Neurosci* 2009; **12**: 767-776 [PMID: 19448629 DOI: 10.1038/nn.2315]

72 **Sadakata T**, Furuichi T. Developmentally regulated Ca2+-dependent activator protein for secretion 2 (CAPS2) is involved in BDNF secretion and is associated with autism susceptibility. *Cerebellum* 2009; **8**: 312-322 [PMID: 19238500 DOI: 10.1007/s12311-009-0097-5]

73 **Shinoda Y**, Sadakata T, Nakao K, Katoh-Semba R, Kinameri E, Furuya A, Yanagawa Y, Hirase H, Furuichi T. Calcium-dependent activator protein for secretion 2 (CAPS2) promotes BDNF secretion and is critical for the development of GABAergic interneuron network. *Proc Natl Acad Sci USA* 2011; **108**: 373-378 [PMID: 21173225 DOI: 10.1073/pnas.1012220108]

74 **Seidah NG**, Benjannet S, Pareek S, Savaria D, Hamelin J, Goulet B, Laliberte J, Lazure C, Chrétien M, Murphy RA. Cellular processing of the nerve growth factor precursor by the mammalian pro-protein convertases. *Biochem J* 1996; **314** (Pt 3): 951-960 [PMID: 8615794]

75 **Pang PT**, Teng HK, Zaitsev E, Woo NT, Sakata K, Zhen S, Teng KK, Yung WH, Hempstead BL, Lu B. Cleavage of proBDNF by tPA/plasmin is essential for long-term hippocampal plasticity. *Science* 2004; **306**: 487-491 [PMID: 15486301 DOI: 10.1126/science.1100135]

76 **Matsumoto T**, Rauskolb S, Polack M, Klose J, Kolbeck R, Korte M, Barde YA. Biosynthesis and processing of endogenous BDNF: CNS neurons store and secrete BDNF, not pro-BDNF. *Nat Neurosci* 2008; **11**: 131-133 [PMID: 18204444 DOI: 10.1038/nn2038]

77 **Lee R**, Kermani P, Teng KK, Hempstead BL. Regulation of cell survival by secreted proneurotrophins. *Science* 2001; **294**: 1945-1948 [PMID: 11729324 DOI: 10.1126/science.1065057]

78 **Woo NH**, Teng HK, Siao CJ, Chiaruttini C, Pang PT, Milner TA, Hempstead BL, Lu B. Activation of p75NTR by proBDNF facilitates hippocampal long-term depression. *Nat Neurosci* 2005; **8**: 1069-1077 [PMID: 16025106 DOI: 10.1038/nn1510]

79 **Barker PA**. Whither proBDNF? *Nat Neurosci* 2009; **12**: 105-106 [PMID: 19172162 DOI: 10.1038/nn0209-105]

80 **Shelly M**, Cancedda L, Lim BK, Popescu AT, Cheng PL, Gao H, Poo MM. Semaphorin3A regulates neuronal polarization by suppressing axon formation and promoting dendrite growth. *Neuron* 2011; **71**: 433-446 [PMID: 21835341 DOI: 10.1016/j.neuron.2011.06.041]

81 **Cheng PL**, Song AH, Wong YH, Wang S, Zhang X, Poo MM. Self-amplifying autocrine actions of BDNF in axon development. *Proc Natl Acad Sci USA* 2011; **108**: 18430-18435 [PMID: 22025720 DOI: 10.1073/pnas.1115907108]

82 **Yoshimura T**, Kawano Y, Arimura N, Kawabata S, Kikuchi A, Kaibuchi K. GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* 2005; **120**: 137-149 [PMID: 15652488 DOI: 10.1016/j.cell.2004.11.012]

83 **Song HJ**, Ming GL, Poo MM. cAMP-induced switching in turning direction of nerve growth cones. *Nature* 1997; **388**: 275-279 [PMID: 9230436]

84 **Cohen-Cory S**, Fraser SE. Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. *Nature* 1995; **378**: 192-196 [PMID: 7477323 DOI: 10.1038/378192a0]

85 **Wirth MJ**, Brun A, Grabert J, Patz S, Wahle P. Accelerated dendritic development of rat cortical pyramidal cells and interneurons after biolistic transfection with BDNF and NT4/5. *Development* 2003; **130**: 5827-5838 [PMID: 14573511 DOI: 10.1242/dev.00826]

86 **Miller FD**, Kaplan DR. Signaling mechanisms underlying dendrite formation. *Curr Opin Neurobiol* 2003; **13**: 391-398 [PMID: 12850225 DOI: 10.1016/S0959-4388(03)00072-2]

87 **Dijkhuizen PA**, Ghosh A. BDNF regulates primary dendrite formation in cortical neurons via the PI3-kinase and MAP kinase signaling pathways. *J Neurobiol* 2005; **62**: 278-288 [PMID: 15514998 DOI: 10.1002/neu.20100]

88 **Ji Y**, Lu Y, Yang F, Shen W, Tang TT, Feng L, Duan S, Lu B. Acute and gradual increases in BDNF concentration elicit distinct signaling and functions in neurons. *Nat Neurosci* 2010; **13**: 302-309 [PMID: 20173744 DOI: 10.1038/nn.2505]

89 **Vicario-Abejón C**, Owens D, McKay R, Segal M. Role of neurotrophins in central synapse formation and stabilization. *Nat Rev Neurosci* 2002; **3**: 965-974 [PMID: 12461553]

90 **Martínez A**, Alcántara S, Borrell V, Del Río JA, Blasi J, Otal R, Campos N, Boronat A, Barbacid M, Silos-Santiago I, Soriano E. TrkB and TrkC signaling are required for maturation and synaptogenesis of hippocampal connections. *J Neurosci* 1998; **18**: 7336-7350 [PMID: 9736654]

91 **Alsina B**, Vu T, Cohen-Cory S. Visualizing synapse formation in arborizing optic axons in vivo: dynamics and modulation by BDNF. *Nat Neurosci* 2001; **4**: 1093-1101 [PMID: 11593233]

92 **Hu B**, Nikolakopoulou AM, Cohen-Cory S. BDNF stabilizes synapses and maintains the structural complexity of optic axons in vivo. *Development* 2005; **132**: 4285-4298 [PMID: 16141221]

93 **Vicario-Abejón C**, Collin C, McKay RD, Segal M. Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. *J Neurosci* 1998; **18**: 7256-7271 [PMID: 9736647]

94 **Tyler WJ**, Pozzo-Miller LD. BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. *J Neurosci* 2001; **21**: 4249-4258 [PMID: 11404410]

95 **Huang ZJ**, Kirkwood A, Pizzorusso T, Porciatti V, Morales B, Bear MF, Maffei L, Tonegawa S. BDNF regulates the maturation of inhibition and the critical period of plasticity in mouse visual cortex. *Cell* 1999; **98**: 739-755 [PMID: 10499792 DOI: 10.1016/S0092-8674(00)81509-3]

96 **Kohara K**, Yasuda H, Huang Y, Adachi N, Sohya K, Tsumoto T. A local reduction in cortical GABAergic synapses after a loss of endogenous brain-derived neurotrophic factor, as revealed by single-cell gene knock-out method. *J Neurosci* 2007; **27**: 7234-7244 [PMID: 17611276 DOI: 10.1523/JNEUROSCI.1943-07.2007]

97 **Abidin I**, Eysel UT, Lessmann V, Mittmann T. Impaired GABAergic inhibition in the visual cortex of brain-derived neurotrophic factor heterozygous knockout mice. *J Physiol* 2008; **586**: 1885-1901 [PMID: 18238806 DOI: 10.1113/jphysiol.2007.148627]

98 **Rico B**, Xu B, Reichardt LF. TrkB receptor signaling is required for establishment of GABAergic synapses in the cerebellum. *Nat Neurosci* 2002; **5**: 225-233 [PMID: 11836532 DOI: 10.1038/nn808]

99 **Marty S**, Wehrlé R, Sotelo C. Neuronal activity and brain-derived neurotrophic factor regulate the density of inhibitory synapses in organotypic slice cultures of postnatal hippocampus. *J Neurosci* 2000; **20**: 8087-8095 [PMID: 11050130]

100 **Seil FJ**, Drake-Baumann R. TrkB receptor ligands promote activity-dependent inhibitory synaptogenesis. *J Neurosci* 2000; **20**: 5367-5373 [PMID: 10884321]

101 **Lu Y**, Christian K, Lu B. BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? *Neurobiol Learn Mem* 2008; **89**: 312-323 [PMID: 17942328 DOI: 10.1016/j.nlm.2007.08.018]

102 **Gottmann K**, Mittmann T, Lessmann V. BDNF signaling in the formation, maturation and plasticity of glutamatergic and GABAergic synapses. *Exp Brain Res* 2009; **199**: 203-234 [PMID: 19777221 DOI: 10.1007/s00221-009-1994-z]

103 **Kang H**, Schuman EM. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science* 1995; **267**: 1658-1662 [PMID: 7886457 DOI: 10.1126/science]

104 **Carmignoto G**, Pizzorusso T, Tia S, Vicini S. Brain-derived neurotrophic factor and nerve growth factor potentiate excitatory synaptic transmission in the rat visual cortex. *J Physiol* 1997; **498** (Pt 1): 153-164 [PMID: 9023775]

105 **Lessmann V**, Gottmann K, Heumann R. BDNF and NT-4/5 enhance glutamatergic synaptic transmission in cultured hippocampal neurones. *Neuroreport* 1994; **6**: 21-25 [PMID: 7703418]

106 **Levine ES**, Dreyfus CF, Black IB, Plummer MR. Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. *Proc Natl Acad Sci USA* 1995; **92**: 8074-8077 [PMID: 7644540]

107 **Suen PC**, Wu K, Levine ES, Mount HT, Xu JL, Lin SY, Black IB. Brain-derived neurotrophic factor rapidly enhances phosphorylation of the postsynaptic N-methyl-D-aspartate receptor subunit 1. *Proc Natl Acad Sci USA* 1997; **94**: 8191-8195 [PMID: 9223337]

108 **Lin SY**, Wu K, Levine ES, Mount HT, Suen PC, Black IB. BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities. *Brain Res Mol Brain Res* 1998; **55**: 20-27 [PMID: 9645956 DOI: 10.1016/S0169-328X(97)00349-5]

109 **Lesser SS**, Sherwood NT, Lo DC. Neurotrophins differentially regulate voltage-gated ion channels. *Mol Cell Neurosci* 1997; **10**: 173-183 [PMID: 9532579 DOI: 10.1006/mcne.1997.0656]

110 **Tucker K**, Fadool DA. Neurotrophin modulation of voltage-gated potassium channels in rat through TrkB receptors is time and sensory experience dependent. *J Physiol* 2002; **542**: 413-429 [PMID: 12122142 DOI: 10.1113/jphysiol.2002.017376]

111 **Li HS**, Xu XZ, Montell C. Activation of a TRPC3-dependent cation current through the neurotrophin BDNF. *Neuron* 1999; **24**: 261-273 [PMID: 10677043 DOI: 10.1016/S0896-6273(00)80838-7]

112 **Ji Y**, Pang PT, Feng L, Lu B. Cyclic AMP controls BDNF-induced TrkB phosphorylation and dendritic spine formation in mature hippocampal neurons. *Nat Neurosci* 2005; **8**: 164-172 [PMID: 15665879 DOI: 10.1038/nn1381]

113 **Yoshii A**, Constantine-Paton M. BDNF induces transport of PSD-95 to dendrites through PI3K-AKT signaling after NMDA receptor activation. *Nat Neurosci* 2007; **10**: 702-711 [PMID: 17515902 DOI: 10.1038/nn1903]

114 **Caldeira MV**, Melo CV, Pereira DB, Carvalho RF, Carvalho AL, Duarte CB. BDNF regulates the expression and traffic of NMDA receptors in cultured hippocampal neurons. *Mol Cell Neurosci* 2007; **35**: 208-219 [PMID: 17428676 DOI: 10.1016/j.mcn.2007.02.019]

115 **Itami C**, Kimura F, Kohno T, Matsuoka M, Ichikawa M, Tsumoto T, Nakamura S. Brain-derived neurotrophic factor-dependent unmasking of "silent" synapses in the developing mouse barrel cortex. *Proc Natl Acad Sci USA* 2003; **100**: 13069-13074 [PMID: 14557544 DOI: 10.1073/pnas.2131948100]

116 **Frerking M**, Malenka RC, Nicoll RA. Brain-derived neurotrophic factor (BDNF) modulates inhibitory, but not excitatory, transmission in the CA1 region of the hippocampus. *J Neurophysiol* 1998; **80**: 3383-3386 [PMID: 9862938]

117 **Bolton MM**, Pittman AJ, Lo DC. Brain-derived neurotrophic factor differentially regulates excitatory and inhibitory synaptic transmission in hippocampal cultures. *J Neurosci* 2000; **20**: 3221-3232 [PMID: 10777787]

118 **Baldelli P**, Novara M, Carabelli V, Hernández-Guijo JM, Carbone E. BDNF up-regulates evoked GABAergic transmission in developing hippocampus by potentiating presynaptic N- and P/Q-type Ca2+ channels signalling. *Eur J Neurosci* 2002; **16**: 2297-2310 [PMID: 12492424 DOI: 10.1046/j.1460-9568.2002.02313.x]

119 **Jovanovic JN**, Thomas P, Kittler JT, Smart TG, Moss SJ. Brain-derived neurotrophic factor modulates fast synaptic inhibition by regulating GABA(A) receptor phosphorylation, activity, and cell-surface stability. *J Neurosci* 2004; **24**: 522-530 [PMID: 14724252 DOI: 10.1523/JNEUROSCI.3606-03.2004]

120 **Tanaka T**, Saito H, Matsuki N. Inhibition of GABAA synaptic responses by brain-derived neurotrophic factor (BDNF) in rat hippocampus. *J Neurosci* 1997; **17**: 2959-2966 [PMID: 9096132]

121 **Brünig I**, Penschuck S, Berninger B, Benson J, Fritschy JM. BDNF reduces miniature inhibitory postsynaptic currents by rapid downregulation of GABA(A) receptor surface expression. *Eur J Neurosci* 2001; **13**: 1320-1328 [PMID: 11298792 DOI: 10.1046/j.0953-816x.2001.01506.x]

122 **Wardle RA**, Poo MM. Brain-derived neurotrophic factor modulation of GABAergic synapses by postsynaptic regulation of chloride transport. *J Neurosci* 2003; **23**: 8722-8732 [PMID: 14507972]

123 **Mizoguchi Y**, Ishibashi H, Nabekura J. The action of BDNF on GABA(A) currents changes from potentiating to suppressing during maturation of rat hippocampal CA1 pyramidal neurons. *J Physiol* 2003; **548**: 703-709 [PMID: 12640007 DOI: 10.1113/jphysiol.2003.038935]

124 **Bliss TV**, Lomo T. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol* 1973; **232**: 331-356 [PMID: 4727084]

125 **Malenka RC**, Bear MF. LTP and LTD: an embarrassment of riches. *Neuron* 2004; **44**: 5-21 [PMID: 15450156 DOI: 10.1016/j.neuron.2004.09.012]

126 **Panja D**, Bramham CR. BDNF mechanisms in late LTP formation: A synthesis and breakdown. *Neuropharmacology* 2014; **76** Pt C: 664-676 [PMID: 23831365 DOI: 10.1016/j.neuropharm.2013.06.024]

127 **Patterson SL**, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER. Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* 1996; **16**: 1137-1145 [PMID: 8663990 DOI: 10.1016/S0896-6273(00)80140-3]

128 **Pozzo-Miller LD**, Gottschalk W, Zhang L, McDermott K, Du J, Gopalakrishnan R, Oho C, Sheng ZH, Lu B. Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. *J Neurosci* 1999; **19**: 4972-4983 [PMID: 10366630]

129 **Heldt SA**, Stanek L, Chhatwal JP, Ressler KJ. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. *Mol Psychiatry* 2007; **12**: 656-670 [PMID: 17264839 DOI: 10.1038/sj.mp.4001957]

130 **Minichiello L**, Korte M, Wolfer D, Kühn R, Unsicker K, Cestari V, Rossi-Arnaud C, Lipp HP, Bonhoeffer T, Klein R. Essential role for TrkB receptors in hippocampus-mediated learning. *Neuron* 1999; **24**: 401-414 [PMID: 10571233 DOI: 10.1016/S0896-6273(00)80853-3]

131 **Minichiello L**, Calella AM, Medina DL, Bonhoeffer T, Klein R, Korte M. Mechanism of TrkB-mediated hippocampal long-term potentiation. *Neuron* 2002; **36**: 121-137 [PMID: 12367511 DOI: 10.1016/S0896-6273(02)00942-X]

132 **Tang SJ**, Reis G, Kang H, Gingras AC, Sonenberg N, Schuman EM. A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. *Proc Natl Acad Sci USA* 2002; **99**: 467-472 [PMID: 11756682 DOI: 10.1073/pnas.012605299]

133 **Zagrebelsky M**, Holz A, Dechant G, Barde YA, Bonhoeffer T, Korte M. The p75 neurotrophin receptor negatively modulates dendrite complexity and spine density in hippocampal neurons. *J Neurosci* 2005; **25**: 9989-9999 [PMID: 16251447 DOI: 10.1523/JNEUROSCI.2492-05.2005]

134 **Dempster E**, Toulopoulou T, McDonald C, Bramon E, Walshe M, Filbey F, Wickham H, Sham PC, Murray RM, Collier DA. Association between BDNF val66 met genotype and episodic memory. *Am J Med Genet B Neuropsychiatr Genet* 2005; **134B**: 73-75 [PMID: 15719396 DOI: 10.1002/ajmg.b.30150]

135 **Ho BC**, Milev P, O'Leary DS, Librant A, Andreasen NC, Wassink TH. Cognitive and magnetic resonance imaging brain morphometric correlates of brain-derived neurotrophic factor Val66Met gene polymorphism in patients with schizophrenia and healthy volunteers. *Arch Gen Psychiatry* 2006; **63**: 731-740 [PMID: 16818862 DOI: 10.1001/archpsyc.63.7.731]

136 **Miyajima F**, Ollier W, Mayes A, Jackson A, Thacker N, Rabbitt P, Pendleton N, Horan M, Payton A. Brain-derived neurotrophic factor polymorphism Val66Met influences cognitive abilities in the elderly. *Genes Brain Behav* 2008; **7**: 411-417 [PMID: 17973920 DOI: 10.1111/j.1601-183X.2007.00363.x]

137 **Molendijk ML**, Bus BA, Spinhoven P, Kaimatzoglou A, Oude Voshaar RC, Penninx BW, van IJzendoorn MH, Elzinga BM. A systematic review and meta-analysis on the association between BDNF val(66)met and hippocampal volume--a genuine effect or a winners curse? *Am J Med Genet B Neuropsychiatr Genet* 2012; **159B**: 731-740 [PMID: 22815222 DOI: 10.1002/ajmg.b.32078]

138 **Chen ZY**, Jing D, Bath KG, Ieraci A, Khan T, Siao CJ, Herrera DG, Toth M, Yang C, McEwen BS, Hempstead BL, Lee FS. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science* 2006; **314**: 140-143 [PMID: 17023662 DOI: 10.1126/science.1129663]

139 **Ninan I**, Bath KG, Dagar K, Perez-Castro R, Plummer MR, Lee FS, Chao MV. The BDNF Val66Met polymorphism impairs NMDA receptor-dependent synaptic plasticity in the hippocampus. *J Neurosci* 2010; **30**: 8866-8870 [PMID: 20592208 DOI: 10.1523/JNEUROSCI.1405-10.2010]

140 **Zuccato C**, Cattaneo E. Brain-derived neurotrophic factor in neurodegenerative diseases. *Nat Rev Neurol* 2009; **5**: 311-322 [PMID: 19498435 DOI: 10.1038/nrneurol.2009.54]

141 **Kim JM**, Stewart R, Kim SW, Yang SJ, Shin IS, Kim YH, Yoon JS. BDNF genotype potentially modifying the association between incident stroke and depression. *Neurobiol Aging* 2008; **29**: 789-792 [PMID: 17222482 DOI: 10.1016/j.neurobiolaging.2006.11.021]

142 **Gratacòs M**, González JR, Mercader JM, de Cid R, Urretavizcaya M, Estivill X. Brain-derived neurotrophic factor Val66Met and psychiatric disorders: meta-analysis of case-control studies confirm association to substance-related disorders, eating disorders, and schizophrenia. *Biol Psychiatry* 2007; **61**: 911-922 [PMID: 17217930 DOI: 10.1016/j.biopsych.2006.08.025]

143 **Selkoe DJ**. Alzheimer's disease is a synaptic failure. *Science* 2002; **298**: 789-791 [PMID: 12399581 DOI: 10.1126/science.1074069]

144 **Braak H**, Braak E. Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol* 1991; **82**: 239-259 [PMID: 1759558]

145 **Masliah E**, Mallory M, Hansen L, DeTeresa R, Alford M, Terry R. Synaptic and neuritic alterations during the progression of Alzheimer's disease. *Neurosci Lett* 1994; **174**: 67-72 [PMID: 7970158]

146 **Tapia-Arancibia L**, Aliaga E, Silhol M, Arancibia S. New insights into brain BDNF function in normal aging and Alzheimer disease. *Brain Res Rev* 2008; **59**: 201-220 [PMID: 18708092 DOI: 10.1016/j.brainresrev.2008.07.007]

147 **Phillips HS**, Hains JM, Armanini M, Laramee GR, Johnson SA, Winslow JW. BDNF mRNA is decreased in the hippocampus of individuals with Alzheimer's disease. *Neuron* 1991; **7**: 695-702 [PMID: 1742020]

148 **Connor B**, Young D, Yan Q, Faull RL, Synek B, Dragunow M. Brain-derived neurotrophic factor is reduced in Alzheimer's disease. *Brain Res Mol Brain Res* 1997; **49**: 71-81 [PMID: 9387865]

149 **Holsinger RM**, Schnarr J, Henry P, Castelo VT, Fahnestock M. Quantitation of BDNF mRNA in human parietal cortex by competitive reverse transcription-polymerase chain reaction: decreased levels in Alzheimer's disease. *Brain Res Mol Brain Res* 2000; **76**: 347-354 [PMID: 10762711]

150 **Hock C**, Heese K, Hulette C, Rosenberg C, Otten U. Region-specific neurotrophin imbalances in Alzheimer disease: decreased levels of brain-derived neurotrophic factor and increased levels of nerve growth factor in hippocampus and cortical areas. *Arch Neurol* 2000; **57**: 846-851 [PMID: 10867782 DOI: 10.1001/archneur.57.6.846]

151 **Narisawa-Saito M**, Wakabayashi K, Tsuji S, Takahashi H, Nawa H. Regional specificity of alterations in NGF, BDNF and NT-3 levels in Alzheimer's disease. *Neuroreport* 1996; **7**: 2925-2928 [PMID: 9116211]

152 **Laske C**, Stransky E, Leyhe T, Eschweiler GW, Maetzler W, Wittorf A, Soekadar S, Richartz E, Koehler N, Bartels M, Buchkremer G, Schott K. BDNF serum and CSF concentrations in Alzheimer's disease, normal pressure hydrocephalus and healthy controls. *J Psychiatr Res* 2007; **41**: 387-394 [PMID: 16554070 DOI: 10.1016/j.jpsychires.2006.01.014]

153 **Nagahara AH**, Merrill DA, Coppola G, Tsukada S, Schroeder BE, Shaked GM, Wang L, Blesch A, Kim A, Conner JM, Rockenstein E, Chao MV, Koo EH, Geschwind D, Masliah E, Chiba AA, Tuszynski MH. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nat Med* 2009; **15**: 331-337 [PMID: 19198615 DOI: 10.1038/nm.1912]

154 **Mucke L**, Masliah E, Yu GQ, Mallory M, Rockenstein EM, Tatsuno G, Hu K, Kholodenko D, Johnson-Wood K, McConlogue L. High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J Neurosci* 2000; **20**: 4050-4058 [PMID: 10818140]

155 **Peng S**, Garzon DJ, Marchese M, Klein W, Ginsberg SD, Francis BM, Mount HT, Mufson EJ, Salehi A, Fahnestock M. Decreased brain-derived neurotrophic factor depends on amyloid aggregation state in transgenic mouse models of Alzheimer's disease. *J Neurosci* 2009; **29**: 9321-9329 [PMID: 19625522 DOI: 10.1523/JNEUROSCI.4736-08.2009]

156 **Devi L**, Ohno M. 7,8-dihydroxyflavone, a small-molecule TrkB agonist, reverses memory deficits and BACE1 elevation in a mouse model of Alzheimer's disease. *Neuropsychopharmacology* 2012; **37**: 434-444 [PMID: 21900882 DOI: 10.1038/npp.2011.191]

157 **Blurton-Jones M**, Kitazawa M, Martinez-Coria H, Castello NA, Müller FJ, Loring JF, Yamasaki TR, Poon WW, Green KN, LaFerla FM. Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proc Natl Acad Sci USA* 2009; **106**: 13594-13599 [PMID: 19633196 DOI: 10.1073/pnas.0901402106]

158 **Reiner A**, Albin RL, Anderson KD, D'Amato CJ, Penney JB, Young AB. Differential loss of striatal projection neurons in Huntington disease. *Proc Natl Acad Sci USA* 1988; **85**: 5733-5737 [PMID: 2456581]

159 **Altar CA**, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay RM, Wiegand SJ. Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature* 1997; **389**: 856-860 [PMID: 9349818]

160 **Zuccato C**, Tartari M, Crotti A, Goffredo D, Valenza M, Conti L, Cataudella T, Leavitt BR, Hayden MR, Timmusk T, Rigamonti D, Cattaneo E. Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nat Genet* 2003; **35**: 76-83 [PMID: 12881722]

161 **Zuccato C**, Cattaneo E. Role of brain-derived neurotrophic factor in Huntington's disease. *Prog Neurobiol* 2007; **81**: 294-330 [PMID: 17379385]

162 **Zuccato C**, Marullo M, Conforti P, MacDonald ME, Tartari M, Cattaneo E. Systematic assessment of BDNF and its receptor levels in human cortices affected by Huntington's disease. *Brain Pathol* 2008; **18**: 225-238 [PMID: 18093249 DOI: 10.1111/

j.1750-3639.2007.00111.x]
163 **Seo H**, Sonntag KC, Isacson O. Generalized brain and skin proteasome inhibition in Huntington's disease. *Ann Neurol* 2004; **56**: 319-328 [PMID: 15349858 DOI: 10.1002/ana.20207]
164 **Ferrer I**, Goutan E, Marín C, Rey MJ, Ribalta T. Brain-derived neurotrophic factor in Huntington disease. *Brain Res* 2000; **866**: 257-261 [PMID: 10825501 DOI: 10.1016/S0006-8993(00)02237-X]
165 **Ciammola A**, Sassone J, Cannella M, Calza S, Poletti B, Frati L, Squitieri F, Silani V. Low brain-derived neurotrophic factor (BDNF) levels in serum of Huntington's disease patients. *Am J Med Genet B Neuropsychiatr Genet* 2007; **144B**: 574-577 [PMID: 17427191 DOI: 10.1002/ajmg.b.30501]
166 **Zuccato C**, Liber D, Ramos C, Tarditi A, Rigamonti D, Tartari M, Valenza M, Cattaneo E. Progressive loss of BDNF in a mouse model of Huntington's disease and rescue by BDNF delivery. *Pharmacol Res* 2005; **52**: 133-139 [PMID: 15967378 DOI: 10.1016/j.phrs.2005.01.001]
167 **Giralt A**, Carretón O, Lao-Peregrin C, Martín ED, Alberch J. Conditional BDNF release under pathological conditions improves Huntington's disease pathology by delaying neuronal dysfunction. *Mol Neurodegener* 2011; **6**: 71 [PMID: 21985529 DOI: 10.1186/1750-1326-6-71]
168 **Xie Y**, Hayden MR, Xu B. BDNF overexpression in the forebrain rescues Huntington's disease phenotypes in YAC128 mice. *J Neurosci* 2010; **30**: 14708-14718 [PMID: 21048129 DOI: 10.1523/JNEUROSCI.1637-10.2010]
169 **Spires TL**, Grote HE, Varshney NK, Cordery PM, van Dellen A, Blakemore C, Hannan AJ. Environmental enrichment rescues protein deficits in a mouse model of Huntington's disease, indicating a possible disease mechanism. *J Neurosci* 2004; **24**: 2270-2276 [PMID: 14999077 DOI: 10.1523/JNEUROSCI.1658-03.2004]
170 **Peng Q**, Masuda N, Jiang M, Li Q, Zhao M, Ross CA, Duan W. The antidepressant sertraline improves the phenotype, promotes neurogenesis and increases BDNF levels in the R6/2 Huntington's disease mouse model. *Exp Neurol* 2008; **210**: 154-163 [PMID: 18096160 DOI: 10.1016/j.expneurol.2007.10.015]
171 **Jiang M**, Peng Q, Liu X, Jin J, Hou Z, Zhang J, Mori S, Ross CA, Ye K, Duan W. Small-molecule TrkB receptor agonists improve motor function and extend survival in a mouse model of Huntington's disease. *Hum Mol Genet* 2013; **22**: 2462-2470 [PMID: 23446639 DOI: 10.1093/hmg/ddt098]
172 **Baquet ZC**, Gorski JA, Jones KR. Early striatal dendrite deficits followed by neuron loss with advanced age in the absence of anterograde cortical brain-derived neurotrophic factor. *J Neurosci* 2004; **24**: 4250-4258 [PMID: 15115821 DOI: 10.1523/JNEUROSCI.3920-03.2004]
173 **Strand AD**, Baquet ZC, Aragaki AK, Holmans P, Yang L, Cleren C, Beal MF, Jones L, Kooperberg C, Olson JM, Jones KR. Expression profiling of Huntington's disease models suggests that brain-derived neurotrophic factor depletion plays a major role in striatal degeneration. *J Neurosci* 2007; **27**: 11758-11768 [PMID: 17959817 DOI: 10.1523/JNEUROSCI.2461-07.2007]
174 **Schumacher J**, Jamra RA, Becker T, Ohlraun S, Klopp N, Binder EB, Schulze TG, Deschner M, Schmäl C, Höfels S, Zobel A, Illig T, Propping P, Holsboer F, Rietschel M, Nöthen MM, Cichon S. Evidence for a relationship between genetic variants at the brain-derived neurotrophic factor (BDNF) locus and major depression. *Biol Psychiatry* 2005; **58**: 307-314 [PMID: 16005437 DOI: 10.1016/j.biopsych.2005.04.006]
175 **Kishikawa S**, Li JL, Gillis T, Hakky MM, Warby S, Hayden M, MacDonald ME, Myers RH, Gusella JF. Brain-derived neurotrophic factor does not influence age at neurologic onset of Huntington's disease. *Neurobiol Dis* 2006; **24**: 280-285 [PMID: 16962786 DOI: 10.1016/j.nbd.2006.07.008]
176 **Mai M**, Akkad AD, Wieczorek S, Saft C, Andrich J, Kraus PH, Epplen JT, Arning L. No association between polymorphisms in the BDNF gene and age at onset in Huntington disease. *BMC Med Genet* 2006; **7**: 79 [PMID: 17096834 DOI: 10.1186/1471-2350-7-79]
177 **Metzger S**, Bauer P, Tomiuk J, Laccone F, Didonato S, Gellera C, Mariotti C, Lange HW, Weirich-Schwaiger H, Wenning GK, Seppi K, Melegh B, Havasi V, Balikó L, Wieczorek S, Zaremba J, Hoffman-Zacharska D, Sulek A, Basak AN, Soydan E, Zidovska J, Kebrdlova V, Pandolfo M, Ribaï P, Kadasi L, Kvasnicova M, Weber BH, Kreuz F, Dose M, Stuhrmann M, Riess O. Genetic analysis of candidate genes modifying the age-at-onset in Huntington's disease. *Hum Genet* 2006; **120**: 285-292 [PMID: 16847693 DOI: 10.1007/s00439-006-0221-2]
178 **Alberch J**, López M, Badenas C, Carrasco JL, Milà M, Muñoz E, Canals JM. Association between BDNF Val66Met polymorphism and age at onset in Huntington disease. *Neurology* 2005; **65**: 964-965 [PMID: 16186551]
179 **del Toro D**, Canals JM, Ginés S, Kojima M, Egea G, Alberch J. Mutant huntingtin impairs the post-Golgi trafficking of brain-derived neurotrophic factor but not its Val66Met polymorphism. *J Neurosci* 2006; **26**: 12748-12757 [PMID: 17151278 DOI: 10.1523/JNEUROSCI.3873-06.2006]
180 **Numakawa T**, Yokomaku D, Richards M, Hori H, Adachi N, Kunugi H. Functional interactions between steroid hormones and neurotrophin BDNF. *World J Biol Chem* 2010; **1**: 133-143 [PMID: 21540998 DOI: 10.4331/wjbc.v1.i5.133]
181 **Duman RS**, Monteggia LM. A neurotrophic model for stress-related mood disorders. *Biol Psychiatry* 2006; **59**: 1116-1127 [PMID: 16631126 DOI: 10.1016/j.biopsych.2006.02.013]
182 **Lee BH**, Kim YK. The roles of BDNF in the pathophysiology of major depression and in antidepressant treatment. *Psychiatry Investig* 2010; **7**: 231-235 [PMID: 21253405 DOI: 10.4306/pi.2010.7.4.231]
183 **Angelucci F**, Brenè S, Mathé AA. BDNF in schizophrenia, depression and corresponding animal models. *Mol Psychiatry* 2005; **10**: 345-352 [PMID: 15655562 DOI: 10.1038/sj.mp.4001637]
184 **Favalli G**, Li J, Belmonte-de-Abreu P, Wong AH, Daskalakis ZJ. The role of BDNF in the pathophysiology and treatment of schizophrenia. *J Psychiatr Res* 2012; **46**: 1-11 [PMID: 22030467 DOI: 10.1016/j.jpsychires.2011.09.022]
185 **Dwivedi Y**, Rizavi HS, Conley RR, Roberts RC, Tamminga CA, Pandey GN. Altered gene expression of brain-derived neurotrophic factor and receptor tyrosine kinase B in postmortem brain of suicide subjects. *Arch Gen Psychiatry* 2003; **60**: 804-815 [PMID: 12912764 DOI: 10.1001/archpsyc.60.8.804]
186 **Karege F**, Vaudan G, Schwald M, Perroud N, La Harpe R. Neurotrophin levels in postmortem brains of suicide victims and the effects of antemortem diagnosis and psychotropic drugs. *Brain Res Mol Brain Res* 2005; **136**: 29-37 [PMID: 15893584]
187 **Guilloux JP**, Douillard-Guilloux G, Kota R, Wang X, Gardier AM, Martinowich K, Tseng GC, Lewis DA, Sibille E. Molecular evidence for BDNF- and GABA-related dysfunctions in the amygdala of female subjects with major depression. *Mol Psychiatry* 2012; **17**: 1130-1142 [PMID: 21912391 DOI: 10.1038/mp.2011.113]
188 **Tripp A**, Oh H, Guilloux JP, Martinowich K, Lewis DA, Sibille E. Brain-derived neurotrophic factor signaling and subgenual anterior cingulate cortex dysfunction in major depressive disorder. *Am J Psychiatry* 2012; **169**: 1194-1202 [PMID: 23128924 DOI: 10.1176/appi.ajp.2012.12020248]
189 **Kim YK**, Lee HP, Won SD, Park EY, Lee HY, Lee BH, Lee SW, Yoon D, Han C, Kim DJ, Choi SH. Low plasma BDNF is associated with suicidal behavior in major depression. *Prog Neuropsychopharmacol Biol Psychiatry* 2007; **31**: 78-85 [PMID: 16904252 DOI: 10.1016/j.pnpbp.2006.06.024]
190 **Karege F**, Perret G, Bondolfi G, Schwald M, Bertschy G, Aubry JM. Decreased serum brain-derived neurotrophic factor levels in major depressed patients. *Psychiatry Res* 2002; **109**: 143-148 [PMID: 11927139]
191 **Shimizu E**, Hashimoto K, Okamura N, Koike K, Komatsu N, Kumakiri C, Nakazato M, Watanabe H, Shinoda N, Okada S, Iyo M. Alterations of serum levels of brain-derived neu-

rotrophic factor (BDNF) in depressed patients with or without antidepressants. *Biol Psychiatry* 2003; **54**: 70-75 [PMID: 12842310]
192 **Sen S**, Duman R, Sanacora G. Serum brain-derived neurotrophic factor, depression, and antidepressant medications: meta-analyses and implications. *Biol Psychiatry* 2008; **64**: 527-532 [PMID: 18571629 DOI: 10.1016/j.biopsych.2008.05.005]
193 **Chen B**, Dowlatshahi D, MacQueen GM, Wang JF, Young LT. Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. *Biol Psychiatry* 2001; **50**: 260-265 [PMID: 11522260]
194 **Bayer TA**, Schramm M, Feldmann N, Knable MB, Falkai P. Antidepressant drug exposure is associated with mRNA levels of tyrosine receptor kinase B in major depressive disorder. *Prog Neuropsychopharmacol Biol Psychiatry* 2000; **24**: 881-888 [PMID: 11041531 DOI: 10.1016/S0278-5846(00)00115-9]
195 **Pizarro JM**, Lumley LA, Medina W, Robison CL, Chang WE, Alagappan A, Bah MJ, Dawood MY, Shah JD, Mark B, Kendall N, Smith MA, Saviolakis GA, Meyerhoff JL. Acute social defeat reduces neurotrophin expression in brain cortical and subcortical areas in mice. *Brain Res* 2004; **1025**: 10-20 [PMID: 15464739 DOI: 10.1016/j.brainres.2004.06.085]
196 **Smith MA**, Makino S, Kvetnansky R, Post RM. Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J Neurosci* 1995; **15**: 1768-1777 [PMID: 7891134]
197 **Ueyama T**, Kawai Y, Nemoto K, Sekimoto M, Toné S, Senba E. Immobilization stress reduced the expression of neurotrophins and their receptors in the rat brain. *Neurosci Res* 1997; **28**: 103-110 [PMID: 9220467 DOI: 10.1016/S0168-0102(97)00030-8]
198 **Roceri M**, Hendriks W, Racagni G, Ellenbroek BA, Riva MA. Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. *Mol Psychiatry* 2002; **7**: 609-616 [PMID: 12140784 DOI: 10.1038/sj.mp.4001036]
199 **Barbany G**, Persson H. Regulation of Neurotrophin mRNA Expression in the Rat Brain by Glucocorticoids. *Eur J Neurosci* 1992; **4**: 396-403 [PMID: 12106347 DOI: 10.1111/j.1460-9568.1992.tb00888.x]
200 **Schaaf MJ**, de Jong J, de Kloet ER, Vreugdenhil E. Downregulation of BDNF mRNA and protein in the rat hippocampus by corticosterone. *Brain Res* 1998; **813**: 112-120 [PMID: 9824681 DOI: 10.1016/S0006-8993(98)01010-5]
201 **Jacobsen JP**, Mørk A. Chronic corticosterone decreases brain-derived neurotrophic factor (BDNF) mRNA and protein in the hippocampus, but not in the frontal cortex, of the rat. *Brain Res* 2006; **1110**: 221-225 [PMID: 16876769 DOI: 10.1016/j.brainres.2006.06.077]
202 **Nibuya M**, Morinobu S, Duman RS. Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J Neurosci* 1995; **15**: 7539-7547 [PMID: 7472505]
203 **Russo-Neustadt A**, Beard RC, Cotman CW. Exercise, antidepressant medications, and enhanced brain derived neurotrophic factor expression. *Neuropsychopharmacology* 1999; **21**: 679-682 [PMID: 10516964 DOI: 10.1016/S0893-133X(99)00059-7]
204 **Balu DT**, Hoshaw BA, Malberg JE, Rosenzweig-Lipson S, Schechter LE, Lucki I. Differential regulation of central BDNF protein levels by antidepressant and non-antidepressant drug treatments. *Brain Res* 2008; **1211**: 37-43 [PMID: 18433734 DOI: 10.1016/j.brainres.2008.03.023]
205 **Coppell AL**, Pei Q, Zetterström TS. Bi-phasic change in BDNF gene expression following antidepressant drug treatment. *Neuropharmacology* 2003; **44**: 903-910 [PMID: 12726822 DOI: 10.1016/S0028-3908(03)00077-7]
206 **De Foubert G**, Carney SL, Robinson CS, Destexhe EJ, Tomlinson R, Hicks CA, Murray TK, Gaillard JP, Deville C, Xhenseval V, Thomas CE, O'Neill MJ, Zetterström TS. Fluoxetine-induced change in rat brain expression of brain-derived neurotrophic factor varies depending on length of treatment. *Neuroscience* 2004; **128**: 597-604 [PMID: 15381288 DOI: 10.1016/j.neuroscience.2004.06.054]
207 **Rogóz Z**, Skuza G, Legutko B. Repeated treatment with mirtazepine induces brain-derived neurotrophic factor gene expression in rats. *J Physiol Pharmacol* 2005; **56**: 661-671 [PMID: 16391422]
208 **Angelucci F**, Aloe L, Jiménez-Vasquez P, Mathé AA. Electroconvulsive stimuli alter the regional concentrations of nerve growth factor, brain-derived neurotrophic factor, and glial cell line-derived neurotrophic factor in adult rat brain. *J ECT* 2002; **18**: 138-143 [PMID: 12394532]
209 **Smith MA**, Zhang LX, Lyons WE, Mamounas LA. Anterograde transport of endogenous brain-derived neurotrophic factor in hippocampal mossy fibers. *Neuroreport* 1997; **8**: 1829-1834 [PMID: 9223060]
210 **Altar CA**, Whitehead RE, Chen R, Wörtwein G, Madsen TM. Effects of electroconvulsive seizures and antidepressant drugs on brain-derived neurotrophic factor protein in rat brain. *Biol Psychiatry* 2003; **54**: 703-709 [PMID: 14512210]
211 **Altar CA**, Laeng P, Jurata LW, Brockman JA, Lemire A, Bullard J, Bukhman YV, Young TA, Charles V, Palfreyman MG. Electroconvulsive seizures regulate gene expression of distinct neurotrophic signaling pathways. *J Neurosci* 2004; **24**: 2667-2677 [PMID: 15028759 DOI: 10.1523/JNEUROSCI.5377-03.2004]
212 **Liu X**, Qi Q, Xiao G, Li J, Luo HR, Ye K. O-methylated metabolite of 7,8-dihydroxyflavone activates TrkB receptor and displays antidepressant activity. *Pharmacology* 2013; **91**: 185-200 [PMID: 23445871 DOI: 10.1159/000346920]
213 **Siuciak JA**, Lewis DR, Wiegand SJ, Lindsay RM. Antidepressant-like effect of brain-derived neurotrophic factor (BDNF). *Pharmacol Biochem Behav* 1997; **56**: 131-137 [PMID: 8981620 DOI: 10.1016/S0091-3057(96)00169-4]
214 **Saarelainen T**, Hendolin P, Lucas G, Koponen E, Sairanen M, MacDonald E, Agerman K, Haapasalo A, Nawa H, Aloyz R, Ernfors P, Castrén E. Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. *J Neurosci* 2003; **23**: 349-357 [PMID: 12514234]
215 **Rantamäki T**, Vesa L, Antila H, Di Lieto A, Tammela P, Schmitt A, Lesch KP, Rios M, Castrén E. Antidepressant drugs transactivate TrkB neurotrophin receptors in the adult rodent brain independently of BDNF and monoamine transporter blockade. *PLoS One* 2011; **6**: e20567 [PMID: 21666748 DOI: 10.1371/journal.pone.0020567]
216 **Verhagen M**, van der Meij A, van Deurzen PA, Janzing JG, Arias-Vásquez A, Buitelaar JK, Franke B. Meta-analysis of the BDNF Val66Met polymorphism in major depressive disorder: effects of gender and ethnicity. *Mol Psychiatry* 2010; **15**: 260-271 [PMID: 18852698 DOI: 10.1038/mp.2008.109]
217 **de Kloet ER**, Joëls M, Holsboer F. Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 2005; **6**: 463-475 [PMID: 15891777 DOI: 10.1038/nrn1683]
218 **Pariante CM**, Lightman SL. The HPA axis in major depression: classical theories and new developments. *Trends Neurosci* 2008; **31**: 464-468 [PMID: 18675469 DOI: 10.1016/j.tins.2008.06.006]
219 **Numakawa T**, Kumamaru E, Adachi N, Yagasaki Y, Izumi A, Kunugi H. Glucocorticoid receptor interaction with TrkB promotes BDNF-triggered PLC-gamma signaling for glutamate release via a glutamate transporter. *Proc Natl Acad Sci USA* 2009; **106**: 647-652 [PMID: 19126684 DOI: 10.1073/pnas.0800888106]
220 **Jeanneteau F**, Garabedian MJ, Chao MV. Activation of Trk neurotrophin receptors by glucocorticoids provides a neuroprotective effect. *Proc Natl Acad Sci USA* 2008; **105**: 4862-4867 [PMID: 18347336 DOI: 10.1073/pnas.0709102105]
221 **Lambert WM**, Xu CF, Neubert TA, Chao MV, Garabedian MJ, Jeanneteau FD. Brain-derived neurotrophic factor signaling rewrites the glucocorticoid transcriptome via glucocorticoid receptor phosphorylation. *Mol Cell Biol* 2013; **33**: 3700-3714 [PMID: 23878391 DOI: 10.1128/MCB.00150-13]

222 **Jeanneteau F**, Chao MV. Are BDNF and glucocorticoid activities calibrated? *Neuroscience* 2013; **239**: 173-195 [PMID: 23022538 DOI: 10.1016/j.neuroscience.2012.09.017]

223 The National Institute of Mental Health (NIMH) website. Available from: URL: http://www.nimh.nih.gov/health/topics/schizophrenia/index.shtml

224 **Akbarian S**, Kim JJ, Potkin SG, Hagman JO, Tafazzoli A, Bunney WE, Jones EG. Gene expression for glutamic acid decarboxylase is reduced without loss of neurons in prefrontal cortex of schizophrenics. *Arch Gen Psychiatry* 1995; **52**: 258-266 [PMID: 7702443 DOI: 10.1001/archpsyc.1995.03950160008002]

225 **Glantz LA**, Lewis DA. Reduction of synaptophysin immunoreactivity in the prefrontal cortex of subjects with schizophrenia. Regional and diagnostic specificity. *Arch Gen Psychiatry* 1997; **54**: 660-669 [PMID: 9236550 DOI: 10.1001/archpsyc.1997.01830190088009]

226 **Glantz LA**, Lewis DA. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. *Arch Gen Psychiatry* 2000; **57**: 65-73 [PMID: 10632234 DOI: 10.1001/archpsyc.57.1.65]

227 **Knable MB**, Barci BM, Webster MJ, Meador-Woodruff J, Torrey EF. Molecular abnormalities of the hippocampus in severe psychiatric illness: postmortem findings from the Stanley Neuropathology Consortium. *Mol Psychiatry* 2004; **9**: 609-620, 544 [PMID: 14708030 DOI: 10.1038/sj.mp.4001471]

228 **Vasic N**, Connemann BJ, Wolf RC, Tumani H, Brettschneider J. Cerebrospinal fluid biomarker candidates of schizophrenia: where do we stand? *Eur Arch Psychiatry Clin Neurosci* 2012; **262**: 375-391 [PMID: 22173848 DOI: 10.1007/s00406-011-0280-9]

229 **Vinogradov S**, Fisher M, Holland C, Shelly W, Wolkowitz O, Mellon SH. Is serum brain-derived neurotrophic factor a biomarker for cognitive enhancement in schizophrenia? *Biol Psychiatry* 2009; **66**: 549-553 [PMID: 19368899 DOI: 10.1016/j.biopsych.2009.02.017]

230 **Takahashi M**, Shirakawa O, Toyooka K, Kitamura N, Hashimoto T, Maeda K, Koizumi S, Wakabayashi K, Takahashi H, Someya T, Nawa H. Abnormal expression of brain-derived neurotrophic factor and its receptor in the corticolimbic system of schizophrenic patients. *Mol Psychiatry* 2000; **5**: 293-300 [PMID: 10889532 DOI: 10.1038/sj.mp.4000718]

231 **Durany N**, Michel T, Zöchling R, Boissl KW, Cruz-Sánchez FF, Riederer P, Thome J. Brain-derived neurotrophic factor and neurotrophin 3 in schizophrenic psychoses. *Schizophr Res* 2001; **52**: 79-86 [PMID: 11595394]

232 **Weickert CS**, Hyde TM, Lipska BK, Herman MM, Weinberger DR, Kleinman JE. Reduced brain-derived neurotrophic factor in prefrontal cortex of patients with schizophrenia. *Mol Psychiatry* 2003; **8**: 592-610 [PMID: 12851636 DOI: 10.1038/sj.mp.4001308]

233 **Hashimoto T**, Bergen SE, Nguyen QL, Xu B, Monteggia LM, Pierri JN, Sun Z, Sampson AR, Lewis DA. Relationship of brain-derived neurotrophic factor and its receptor TrkB to altered inhibitory prefrontal circuitry in schizophrenia. *J Neurosci* 2005; **25**: 372-383 [PMID: 15647480 DOI: 10.1523/JNEUROSCI.4035-04.2005]

234 **Issa G**, Wilson C, Terry AV, Pillai A. An inverse relationship between cortisol and BDNF levels in schizophrenia: data from human postmortem and animal studies. *Neurobiol Dis* 2010; **39**: 327-333 [PMID: 20451611 DOI: 10.1016/j.nbd.2010.04.017]

235 **Grillo RW**, Ottoni GL, Leke R, Souza DO, Portela LV, Lara DR. Reduced serum BDNF levels in schizophrenic patients on clozapine or typical antipsychotics. *J Psychiatr Res* 2007; **41**: 31-35 [PMID: 16546213 DOI: 10.1016/j.jpsychires.2006.01.005]

236 **Lee AH**, Lange C, Ricken R, Hellweg R, Lang UE. Reduced brain-derived neurotrophic factor serum concentrations in acute schizophrenic patients increase during antipsychotic treatment. *J Clin Psychopharmacol* 2011; **31**: 334-336 [PMID: 21508862 DOI: 10.1097/JCP.0b013e31821895c1]

237 **Green MJ**, Matheson SL, Shepherd A, Weickert CS, Carr VJ. Brain-derived neurotrophic factor levels in schizophrenia: a systematic review with meta-analysis. *Mol Psychiatry* 2011; **16**: 960-972 [PMID: 20733577 DOI: 10.1038/mp.2010.88]

238 **Snigdha S**, Neill JC, McLean SL, Shemar GK, Cruise L, Shahid M, Henry B. Phencyclidine (PCP)-induced disruption in cognitive performance is gender-specific and associated with a reduction in brain-derived neurotrophic factor (BDNF) in specific regions of the female rat brain. *J Mol Neurosci* 2011; **43**: 337-345 [PMID: 20852970 DOI: 10.1007/s12031-010-9447-5]

239 **Fumagalli F**, Molteni R, Roceri M, Bedogni F, Santero R, Fossati C, Gennarelli M, Racagni G, Riva MA. Effect of antipsychotic drugs on brain-derived neurotrophic factor expression under reduced N-methyl-D-aspartate receptor activity. *J Neurosci Res* 2003; **72**: 622-628 [PMID: 12749027 DOI: 10.1002/jnr.10609]

240 **Ashe PC**, Chlan-Fourney J, Juorio AV, Li XM. Brain-derived neurotrophic factor (BDNF) mRNA in rats with neonatal ibotenic acid lesions of the ventral hippocampus. *Brain Res* 2002; **956**: 126-135 [PMID: 12426055 DOI: 10.1016/S0006-8993(02)03176-1]

241 **Angelucci F**, Mathé AA, Aloe L. Brain-derived neurotrophic factor and tyrosine kinase receptor TrkB in rat brain are significantly altered after haloperidol and risperidone administration. *J Neurosci Res* 2000; **60**: 783-794 [PMID: 10861791 DOI: 10.1002/1097-4547(20000615)60:]

242 **Parikh V**, Khan MM, Mahadik SP. Olanzapine counteracts reduction of brain-derived neurotrophic factor and TrkB receptors in rat hippocampus produced by haloperidol. *Neurosci Lett* 2004; **356**: 135-139 [PMID: 14746882 DOI: 10.1016/j.neulet.2003.10.079]

243 **Pillai A**, Terry AV, Mahadik SP. Differential effects of long-term treatment with typical and atypical antipsychotics on NGF and BDNF levels in rat striatum and hippocampus. *Schizophr Res* 2006; **82**: 95-106 [PMID: 16442781 DOI: 10.1016/j.schres.2005.11.021]

244 **Morris BJ**, Cochran SM, Pratt JA. PCP: from pharmacology to modelling schizophrenia. *Curr Opin Pharmacol* 2005; **5**: 101-106 [PMID: 15661633]

245 **Javitt DC**, Zukin SR. Recent advances in the phencyclidine model of schizophrenia. *Am J Psychiatry* 1991; **148**: 1301-1308 [PMID: 1654746]

246 **Takahashi M**, Kakita A, Futamura T, Watanabe Y, Mizuno M, Sakimura K, Castren E, Nabeshima T, Someya T, Nawa H. Sustained brain-derived neurotrophic factor up-regulation and sensorimotor gating abnormality induced by postnatal exposure to phencyclidine: comparison with adult treatment. *J Neurochem* 2006; **99**: 770-780 [PMID: 16903871 DOI: 10.1111/j.1471-4159.2006.04106.x]

247 **Hock C**, Heese K, Müller-Spahn F, Hulette C, Rosenberg C, Otten U. Decreased trkA neurotrophin receptor expression in the parietal cortex of patients with Alzheimer's disease. *Neurosci Lett* 1998; **241**: 151-154 [PMID: 9507943 DOI: 10.1016/S0304-3940(98)00019-6]

248 **Gervasoni N**, Aubry JM, Bondolfi G, Osiek C, Schwald M, Bertschy G, Karege F. Partial normalization of serum brain-derived neurotrophic factor in remitted patients after a major depressive episode. *Neuropsychobiology* 2005; **51**: 234-238 [PMID: 15897674 DOI: 10.1159/000085725]

249 **Rasmusson AM**, Shi L, Duman R. Downregulation of BDNF mRNA in the hippocampal dentate gyrus after re-exposure to cues previously associated with footshock. *Neuropsychopharmacology* 2002; **27**: 133-142 [PMID: 12093587 DOI: 10.1016/S0893-133X(02)00286-5]

250 **Roceri M**, Cirulli F, Pessina C, Peretto P, Racagni G, Riva MA. Postnatal repeated maternal deprivation produces age-dependent changes of brain-derived neurotrophic factor expression in selected rat brain regions. *Biol Psychiatry* 2004; **55**: 708-714 [PMID: 15038999 DOI: 10.1016/j.biopsych.2003.12.011]

251 **Russo-Neustadt AA**, Alejandre H, Garcia C, Ivy AS, Chen

MJ. Hippocampal brain-derived neurotrophic factor expression following treatment with reboxetine, citalopram, and physical exercise. *Neuropsychopharmacology* 2004; **29**: 2189-2199 [PMID: 15199375 DOI: 10.1038/sj.npp.1300514]

252 **Xiu MH**, Hui L, Dang YF, Hou TD, Zhang CX, Zheng YL, Chen da C, Kosten TR, Zhang XY. Decreased serum BDNF levels in chronic institutionalized schizophrenia on long-term treatment with typical and atypical antipsychotics. *Prog Neuropsychopharmacol Biol Psychiatry* 2009; **33**: 1508-1512 [PMID: 19720106 DOI: 10.1016/j.pnpbp.2009.08.011]

253 **Hung YY**, Huang TL. Lower serum tropomyosin receptor kinase B levels in patients with schizophrenia. *Biomed J* 2013; **36**: 132-136 [PMID: 23806883 DOI: 10.4103/2319-4170.113229]

254 **Pillai A**. Decreased expression of Sprouty2 in the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder: a correlation with BDNF expression. *PLoS One* 2008; **3**: e1784 [PMID: 18335055 DOI: 10.1371/journal.pone.0001784]

255 **Weickert CS**, Ligons DL, Romanczyk T, Ungaro G, Hyde TM, Herman MM, Weinberger DR, Kleinman JE. Reductions in neurotrophin receptor mRNAs in the prefrontal cortex of patients with schizophrenia. *Mol Psychiatry* 2005; **10**: 637-650 [PMID: 15940304 DOI: 10.1038/sj.mp.4001678]

256 **Wong J**, Rothmond DA, Webster MJ, Weickert CS. Increases in two truncated TrkB isoforms in the prefrontal cortex of people with schizophrenia. *Schizophr Bull* 2013; **39**: 130-140 [PMID: 21795612 DOI: 10.1093/schbul/sbr070]

257 **Toyooka K**, Asama K, Watanabe Y, Muratake T, Takahashi M, Someya T, Nawa H. Decreased levels of brain-derived neurotrophic factor in serum of chronic schizophrenic patients. *Psychiatry Res* 2002; **110**: 249-257 [PMID: 12127475 DOI: 10.1016/S0165-1781(02)00127-0]

258 **Tan YL**, Zhou DF, Cao LY, Zou YZ, Zhang XY. Decreased BDNF in serum of patients with chronic schizophrenia on long-term treatment with antipsychotics. *Neurosci Lett* 2005; **382**: 27-32 [PMID: 15911116 DOI: 10.1016/j.neulet.2005.02.054]

259 **Palomino A**, Vallejo-Illarramendi A, González-Pinto A, Aldama A, González-Gómez C, Mosquera F, González-García G, Matute C. Decreased levels of plasma BDNF in first-episode schizophrenia and bipolar disorder patients. *Schizophr Res* 2006; **86**: 321-322 [PMID: 16829047 DOI: 10.1016/j.schres.2006.05.028]

260 **Jindal RD**, Pillai AK, Mahadik SP, Eklund K, Montrose DM, Keshavan MS. Decreased BDNF in patients with antipsychotic naïve first episode schizophrenia. *Schizophr Res* 2010; **119**: 47-51 [PMID: 20096541 DOI: 10.1016/j.schres.2009.12.035]

261 **Iritani S**, Niizato K, Nawa H, Ikeda K, Emson PC. Immunohistochemical study of brain-derived neurotrophic factor and its receptor, TrkB, in the hippocampal formation of schizophrenic brains. *Prog Neuropsychopharmacol Biol Psychiatry* 2003; **27**: 801-807 [PMID: 12921913 DOI: 10.1016/S0278-5846(03)00112-X]

262 **Bai O**, Chlan-Fourney J, Bowen R, Keegan D, Li XM. Expression of brain-derived neurotrophic factor mRNA in rat hippocampus after treatment with antipsychotic drugs. *J Neurosci Res* 2003; **71**: 127-131 [PMID: 12478621 DOI: 10.1002/jnr.10440]

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MINIREVIEWS

P2X receptors: New players in cancer pain

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Abstract

Pain is unfortunately a quite common symptom for cancer patients. Normally pain starts as an episodic experience at early cancer phases to become chronic in later stages. In order to improve the quality of life of oncological patients, anti-cancer treatments are often accompanied by analgesic therapies. The P2X receptor are adenosine triphosphate (ATP) gated ion channels expressed by several cells including neurons, cancer and immune cells. Purinergic signaling through P2X receptors recently emerged as possible common pathway for cancer onset/growth and pain sensitivity. Indeed, tumor microenvironment is rich in extracellular ATP, which has a role in both tumor development and pain sensation. The study of the different mechanisms by which P2X receptors favor cancer progression and relative pain, represents an interesting challenge to design integrated therapeutic strategies for oncological patients. This review summarizes recent findings linking P2X receptors and ATP to cancer growth, progression and related pain. Special attention has been paid to the role of P2X2, P2X3, P2X4 and P2X7 in the genesis of cancer pain and to the function of P2X7 in tumor growth and metastasis. Therapeutic implications of the administration of different P2X receptor blockers to alleviate cancer-associated pain sensations contemporarily reducing tumor progression are also discussed.

Key words: Cancer; Pain; Adenosine triphosphate; P2X2; P2X3; P2X2/3; P2X4; P2X7

Core tip: Cancer pain is an increasing emergency as the number of oncological patients and survivors tends to growth. Oncological patients will greatly benefit of new therapies combining anti-tumor effects with a reduction of pain perception. This review gives an overview of latest literature on P2X receptors role in tumor progression and different types of cancer pain. The potential of P2X receptors as therapeutic targets in tumor is also discussed.

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INTRODUCTION

Half of all oncological patients present pain symptoms[1]. This percentage arise in individuals undergoing cancer treatment getting worse, as tumor progress through its advanced stages[2-4]. Pain sensitization is also frequent in long-term cancer survivors following curative treatment[2,3]. Moreover, pain experience of oncological patients ranges from moderate to severe[3]. The most common pain locations in all cancer patients are back, abdomen and hips. Although there are many studies reporting an increased pain prevalence in cancer types involving head, bone, gynecological and gastrointestinal sites[2], other meta-analysis found no significant relation-

ship between pain and cancer type[3].

A growing body of literature attributed a crucial role in pain sensitization and transduction to adenosine triphosphate (ATP) and purinergic P2X receptors[5]. Moreover, P2X antagonists were successful in reducing neuropathic pain[6]. Furthermore, recent reports highlighted the role of P2X receptors in cancer development and progression[7,8]. Thus, it is tempting to speculate a possible P2X-dependent mechanism affecting both tumor growth and cancer pain. P2X receptors are ATP gated ion channels that, upon ligand stimulation, cause Na^+ and Ca^{2+} cellular influx and K^+ efflux. The P2X family includes seven transmembrane proteins (P2X1-7) ranging in length from the 362 amino acids of P2X6 to the 595 of P2X7[9]. Single P2X subunits assemble as homo or hetero-trimers, with each subunit formed by N and C terminal intracellular tails, two membrane spanning domains and a long extracellular loop including the ATP binding site[9,10]. Several cell types including neuronal, immune and cancer cells express P2X receptors. The role of ATP and its receptors in pain sensation is well accepted and diverse kind of pain have been associated to activation of different P2X receptors, *i.e.*, acute pain with sensory neurons-P2X3, neuropathic pain with glial cells-P2X4, inflammatory pain with immune cells-P2X7[11].

Here we give an overview of different forms of cancer pain, identifying the role of P2X receptors in each of them.

DIFFERENT TYPES OF PAIN IN CANCER

Although the sensation of pain, at different levels, is a common experience in various pathologies including cancer, it is very difficult to catalog it. Responding to this need the International Association for the Study of Pain (IASP) classified Chronic Pain originally in 1986, and later on in 1994. The IASP system of classification considers the main site of pain, the system affected, the pattern of occurrence, the time since pain onset, and etiology (http://www.iasp-pain.org). In 2010, Woolf[12] proposed another way to classify pain in three main classes: nociceptive, inflammatory, and neuropathic.

Nociceptive pain can be associated with peripheral nerve sensitization and defined as a high-threshold pain only activated in the presence of intense stimuli such as thermal, mechanical and chemical. These stimuli are transduced and encoded to the brain by nociceptive neurons, whose cell bodies are located in both the dorsal root ganglia (DRG) or the trigeminal ganglia[12,13]. Inflammatory pain is spontaneous pain arising by activation of the immune system due to tissue injury or infection[12,13]. The inflammatory response is normally protective towards pathogens, however, when deregulated can cause pathological processes, such as autoimmune diseases and pain[14]. Neuropathic pain is defined by IASP (http://www.iasp-pain.org) as a pain "caused by a lesion or disease of the somatosensory nervous system"; thus, a nerve injury could determine a peripheral sensitization, characterized by an altered sensitivity at the peripheral and central nervous system (PNS and CNS) level[15]. Infections and inflammation are other potential causes for neuropathic pain[16].

Considering different cancer types, patients' experiences are ascribable to all the three subclasses of pain. In fact, cancer patients' pain sensations are characterized by different sensory abnormalities or a combination of them: hypersensitivity or hyposensitivity; paresthesia (abnormal sensation that is not painful or unpleasant), dysaesthesia (unpleasant abnormal sensation); allodynia (pain produced by normally non-painful stimuli); or hyperalgesia (increased response to a stimulus that is normally painful)[4].

Cancer-related pain is present at any stage of cancer progression, with different intensity of frequency, and it is progressively increasing[17]. Initially cancer pain is the direct consequence of primary or metastatic tumor masses themselves, and then arises as side effect of treatments to eradicate cancer[1-3,15]. Pain perception could be considered also a useful alarm: most of nociceptive syndromes are easily discovered, and in some cases, their diagnosis could precede that of the neoplasia. On the other hand cancer associated-neuropathic syndromes are highly variable by individual[18]. Considering the impact of pain in patients' quality of life, it is not possible to define it as a simple side effect of tumor progression, for this reason a great interest is arising about the specific origin and mechanism of cancer pain (Figure 1).

Based on cancer stage and treatment, it is possible to define three diverse classes of cancer pain.

Tumor mass-induced pain

Cancer itself is the first source of pain: the extension and invasiveness of primary/metastatic cancer masses generates obstruction, infiltration, or peripheral nerve sensitization inducing nociceptive or neuropathic pain[4,19].

Bone cancer pain is an explicative example: it is caused by tumors arising in bone as osteosarcomas[20], or it is a consequence of skeletal metastases of lung, breast and prostate tumors[21]. When tumor cells infiltrate into bone, different processes begin including bone resorption, compression and damage of the sensory fibers, infiltration of immune cells followed by release of inflammatory cytokines[22]. Thus, cancer bone pain can be defined as nociceptive and inflammatory pain. Furthermore, neuropathic and inflammatory pain pathway activation occurs even at the spinal cord level[23].

Pain induced by anti-cancer treatments

Oncologic patients also suffer pain induced not directly by cancer itself, but by the different treatments that they are undergoing, *i.e.*, chemotherapy, radiation or surgery[18]. These pain experiences belong principally to nociceptive and neuropathic pain syndromes.

Surgery represents one of the most used therapeutic approaches to eradicate cancer. Thus, it is quite common that removal of tumor masses causes nerve sensitization or damage, inducing nociceptive pain. In case of amputation, patients develop multiple form of discomfort in the

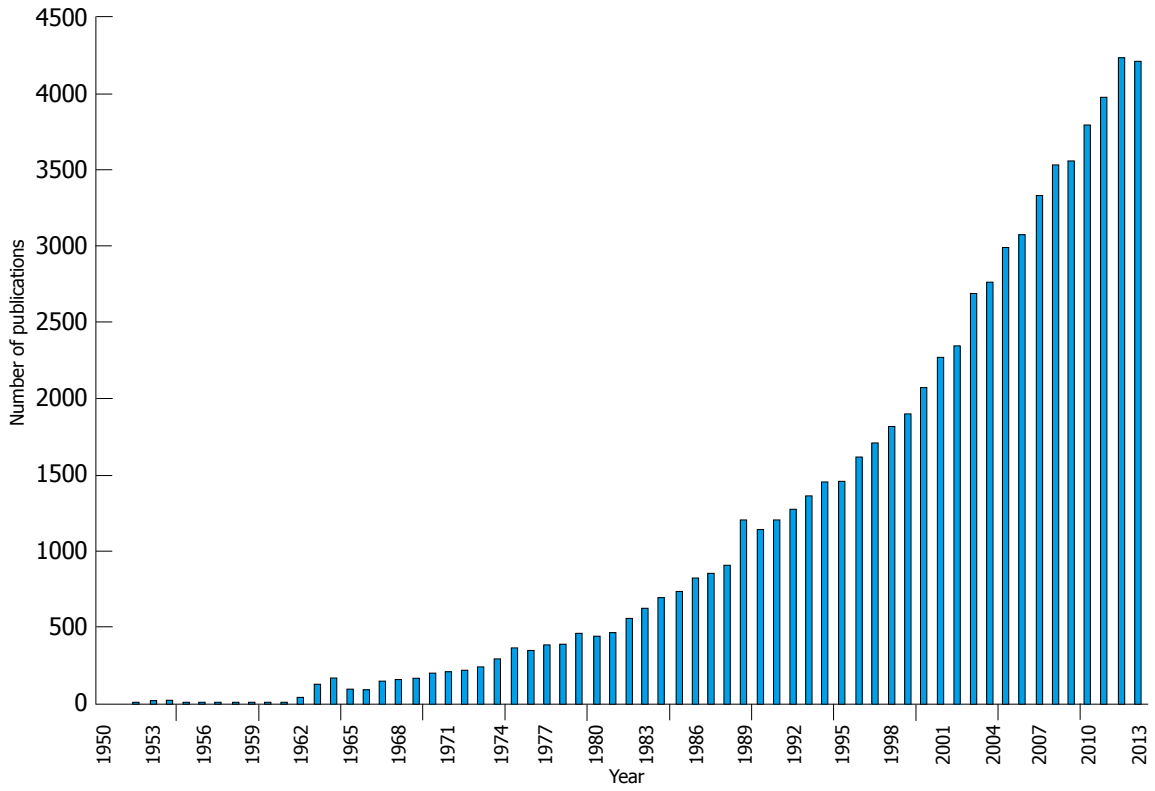

Figure 1 **Number of publications per year with keywords "cancer pain".** From MEDLINE®/PubMed®, a database of the United States National Library of Medicine.

missing limb. Both painful and non-painful sensation are defined as phantom limb phenomenon: a painful sensation in the residual portion of the amputated limb (stump pain) is associated with a painless sensation originating in the limb that no longer exists (phantom sensation)[24]. In this particular form of pain, the pathophysiology is not clearly defined, as it is not clear whether this is a phenomenon univocally dependent on PNS or CNS[24]. However, it was demonstrated that DRG neurons are a critical source of ectopic impulse discharge[25].

Anti-tumor chemotherapeutic agents are known to cause pain[26]. Chemotherapy-induced neuropathy, that causes pain increase in drug-dose-dependent manner[4], include neuropathic, non-neuropathic[27] and inflammatory pain, inflicting damage both on sensory neurons and satellite glial cells in DRG[28].

Even radiotherapy causes pain as side effect. For example, in breast cancer, radiotherapy successfully decreases mortality but can determine a long-lasting breast pain[29]. The administration of opioids is a good approach to prevent predictable pain due to radiotherapy in bone cancer patients[30].

Chronic non-cancer pain in cancer patients

Chronic non-cancer pain is a debilitative problem that compromises every aspect of patients' life. Normally, it could be defined as a nociceptive or neuropathic pain, lasting longer than the expected period of healing. Chronic pain sensation is due by persistent stimulation, changes or damages to the CNS or PNS, or both[31]. There is not a common therapeutic approach for these different types of patients' pain, because the pain type and severity is a result of genotype, and administered treatment has to be customized[31].

ATP AND P2X RECEPTORS IN TUMOR

A growing body of evidence indicates ATP and purinergic signaling as key players in both cancer and pain[32-34]. ATP is released in physiologic conditions by healthy cells exerting beneficial effects *via* several mechanisms for example acting as fast excitatory neurotransmitter or playing long-term (trophic) roles in cell proliferation, growth and development[5,35,36]. Nonetheless, high levels of ATP can have detrimental effects as this nucleotide acts as pain stimulus and is involved in different inflammatory and non-inflammatory pathologies, including cancer[9,37]. *In vivo* measure of extracellular ATP in tumor microenvironment allowed to estimate a cancer surrounding concentration of the nucleotide in the micromolar range[38]. Tumor cells can actively secrete ATP[39] or release it as consequence of spontaneous or chemotherapy-induced cell death[40]. Platelets are an extra source of ATP in tumor context, and thanks to release of the nucleotide they facilitate cancer cell extravasation and spreading[41]. Another, good candidate for ATP secretion is its own receptor P2X7 as its stimulation with the synthetic agonist BZ-ATP caused release of ATP requiring P2X7 large pore opening[42,43]. Once in the cancer milieu, ATP acts as growth promoting and pro-metastatic factor[7,8], neuronal and immune-cells stimulus[5,44] and main source of the immunosuppressant adenosine[45].

Several studies implicated ATP-receptor P2X7 in cell proliferation under nutrient deprivation conditions, similar to those present in cancer microenvironment, such as growth factors and glucose reduced availability[43,46-48]. Moreover, it was recently demonstrated that P2X7 facilitates tumor engraftment, growth and vascularization[7] and that P2X7 blockade or silencing effectively reduces tumor growth in animal models of colon cancer, melanoma and neuroblastoma[7,49]. P2X and P2Y receptors expression has been reported both in tumor and tumor-associated cells in several cancers (for a recent review see[34]). However, and in depth characterization of the role of ATP in the crosstalk among tumor and immune or neuronal surrounding cells is still missing.

P2X IN CANCER PAIN

Extracellular ATP triggers pain perception through activation of different P2X receptors. Indeed, P2X receptors are essential players in nociceptive, inflammatory and pathological pain transduction[38,50,51]. All the different compartments involved in pain transduction, sensory ganglia, DRG and spinal cord, are characterized by high expression of P2X receptors in excitable and non-excitable cells[51].

Different studies demonstrated the involvement of ATP and P2X receptors in cancer pain[22]. The use of diverse animal pain models allowed identifying P2X receptors as mediators of cancer pain perception trough DRG and spinal cord[28,52,53].

As mentioned before, one of the most studied cancer pain models is bone cancer: many common tumors preferentially metastasize to multiple bones and bone cancer patients suffer of moderate to severe chronic pain[20,54]. Interestingly, up-regulation of P2X3 receptor was founded in DRG of animals experiencing bone cancer pain[52,53,55]. Homomeric P2X3 and heteromeric P2X2/3 receptors are the main responsible for ATP evoked nociceptive pain[56,57], and were accordingly involved in bone cancer pain perception[55]. Recently, Liu *et al*[52] (2013) showed that , in rat bone cancer, DRG neurons-P2X3 receptor is functionally up-regulated by the neuronal calcium sensor VILIP-1, and contributes to the development of bone cancer pain. Moreover, P2X3 receptor antagonist A-317491, which successfully reduces chronic inflammatory and neuropathic pain behavior in animals[58,59], also relieves cancer bone pain[60]. However, A-317491 effect was limited to attenuation of bone pain related behaviors in the early stage of tumor development, while had no effect as tumor progressed to an advanced point. Similarly, oral administration of AF-353, another potent and selective P2X3 and P2X2/3 receptor antagonist, attenuated bone cancer pain, but had no preventing effect on cancer-induced bone destruction[55]. P2X receptors blockade was found to be an efficacious pain easing strategy also in a model of melanoma developed in mice paw[61]. In this context, tumor cell inoculation caused a pain related behavior that was increased by intra-plantar injection of ATP, and reduced, in a dose-dependent fashion, by P2 receptor broad-spectrum antagonists. Interestingly, the authors reported a contemporary up-regulation of P2X3 receptors in DRG. Discussed evidence suggests that specific blockade of P2X3 or P2X2/3 receptors will not be sufficient to minimize cancer related pain. In fact, the role of P2X receptors in cancer pain is not restricted to P2X3 and neuronal cells. On the contrary, non-neuronal cells play a pivotal role in chronic pain states[51,57,62] and immune-cancer cells expressed P2X4 and P2X7 receptors are involved in inflammatory and neuropathic pain development[29,30,47,53,54,60]. P2X4 or P2X7 null mice show reduced nociceptive sensitivity and development of neuropathic pain states. Moreover, similar results can be obtained by *in vivo* administration of P2X7 antagonists[6] However, neither P2X7 genetic ablation nor receptors' antagonist administration was able to alleviate bone cancer pain[63]. The different efficacy of P2X7 antagonist in reducing inflammatory and neuropathic pain versus bone cancer pain can be ascribed to a diverse mechanism of generation of the painful stimulus, which is probably more complex in cancer than in other pathological situations.

P2X RECEPTORS AS PHARMACOLOGICAL TARGETS FOR CANCER PAIN

Pharmacological approaches to different kind of cancer pain are based on patient's status. There are three main classes of drugs for cancer pain management: opioids, non-opioids and adjuvant analgesics[64]. Normally, pharmacotherapy consist in an appropriate selection of drugs, belonging to the three categories mentioned, and it is structured to optimize positive outcomes and minimize side-effects[4]. Terminal oncological patients generally experience chronic pain that can be eased by palliative care improving their quality of life and helping them to face the prospect of death[1]. Suramin, a broad spectrum P2 receptor antagonist, showed clinically relevant antitumor activity in prostate cancer, as reflected by reduction of prostate specific antigen levels, pain relief, and a relatively long time to disease progression[65,66]. Moreover, suramin underwent phase Ⅲ clinical trials for refractory prostate cancer, giving encouraging results as palliative therapy and delaying disease progression[67]. However, the mechanism of action of suramin is essentially unknown as it binds to several proteins including growth factors and is not only a P2 receptor antagonist but also acts as agonist of ryanodine receptors. Nevertheless, pain-easing activity of suramin could be attributed, at least partially, to P2X3 or P2X2/3 blockade. Indeed, as mentioned before, different P2X3, P2X2/3 specific antagonists proved effective in reduce bone cancer pain perception in animal models[53,60,68] and refractory prostate cancer generally metastasize to bone. P2X2 and P2X2/3 receptors have also been involved in development of opioid treatment resistance in severe pain states associated to cancer metastasis[68]. Chizhmakov *et al*[69] demonstrated that opioids

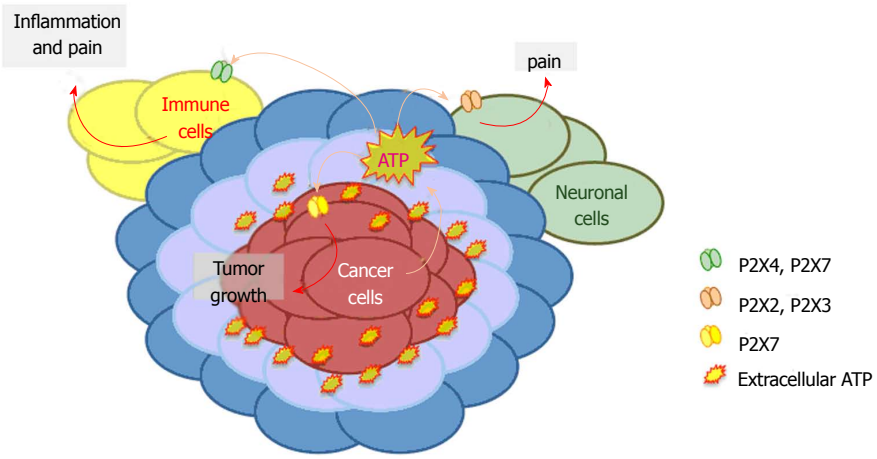

Figure 2 Schematic representation of solid tumor generated by cancer cells (red) surrounded by the normal tissue cells (light and dark blue) releasing extracellular adenosine triphosphate in surrounding environment (in yellow). Adenosine triphosphate (ATP) acts through three different ways: (1) binding P2X7 receptors, ATP facilitates tumor growth; (2) binding the P2X2, P2X3, P2X2/3 receptors expressed by neuronal cells (in green), ATP triggers pain perception; and (3) binding P2X4 and P2X7 receptors expressed by immune cells (in yellow), ATP causes inflammation and pain.

are able to inhibit P2X3 and P2X2/3 receptor responses in sensory neurons[69], but also that cancer cells, co-cultured with neurons, release diffusible factors able to alter opioid-dependent P2Xs inhibition, probably causing opioid resistance[69].

Another interesting target for the development of anti-cancer, pain-easing therapies is P2X7. At the moment different P2X7 antagonists are undergoing clinical trials for their efficacy in reducing neuropathic and inflammatory pain[70,71], and its role in pain pathways is confirmed by the involvement of P2X7 receptor, expressed by spinal microglia, in induction of morphine tolerance[72]. Moreover, P2X7 potent and selective antagonists A-740003 and A-438079 both showed anti-nociceptive activity in rodents[69,73]. Accordingly, P2X7 receptor antagonist brilliant blue G reduced pain perception in a rat model of bone cancer[74]. However, in a similar mice model A-438079 failed to alleviate bone cancer pain[63]. These discrepancies could be ascribed to the different animal models, cell players examined, and dissimilar specificity of the drugs administered. A strategy based on P2X7 blockade could not only be efficacious in reducing cancer pain but also tumor growth, vascularization and spreading. In fact, we recently demonstrated that P2X7 receptor affects cancer development, causing increased tumor engraftment, growth rate and angiogenesis[75]. Interestingly, both P2X7 antagonist AZ-10606120 and P2X7 inhibitor oxidized-ATP caused an evident tumor regression accompanied by reduced vascular endothelial growth factor secretion and consequently a decrease in tumor blood vessels[76]. Accordingly, different studies show a P2X7 blockade dependent reduction of melanoma and glioma growth[49]. However, data on glioma are debatable, in fact Fang and colleagues also reported Brillant Blue G dependent glioma growth acceleration[75].

A growing body of literature has also attributed a role to P2X7 in tumor dissemination[65,77-80] and drugs acting at the receptor have been proposed as anti-metastatic agents[8,80]. Interestingly, drugs as different as the traditional Chinese medicine compound Emodin, and the statin atrovastin both act at tumor cells inhibiting their migratory ability *via* P2X7 blockade[79,80].

Treatment of oncological patients with intravenous infusions of ATP at high doses was also proposed as strategy to fight tumor growth taking advantage of ATP cytotoxic properties. ATP administration intravenously proved to be effective in arresting tumor progression in rodent models of hormone-refractory prostate[81] and colon cancer[82]. However, the possible side effects due to pain development after intravenous administration of millimolar doses of ATP to patients have to be taken into account. In fact, it is known that application/injection of ATP causes pain perception associated with vascular changes[61,62] and this effect was also observed in clinical trials administering high doses of ATP to patients[83]. On the other hand, short infusion of low ATP concentrations did not caused pain when administered to terminal oncological patients, but improved their quality of life[84] and reduced cachexia[85]. However, administration of ATP to patients with less severe forms of cancer did not produce any positive effect on their quality of life or in tumor regression[84].

Interestingly, ATP has been suggested also as adjuvant chemotherapy facilitating the passage of anticancer drugs such as doxorubicin and mytomicin C[81,86,87]. However, chemotherapy itself causes pain, which could be enhanced by ATP administration. Summarizing, even if ATP administration to cancer patients is representing an interesting strategy to eradicate tumor cells, probably will be causing pain as major side effect and will be, then, inadequate for the treatment of tumors associated with severe pain sensation.

CONCLUSION

Cancer pain is one of the most serious problems in oncology, depending on the growing number of cancer patients and, luckily, of cancer survivors. For these reasons identification of adequate anti-pain therapies, improving the quality of life of oncological patients is attracting widespread interest. Due to the different cellular components involved in tumor, classically administered anti-pain pharmacological approaches not always satisfy cancer patient's needs. In this review, we underline a possible connection between pain perception and tumor growth in cancer mediated by P2X receptors (Figure 2). Described

evidence suggests that P2X receptors are good pharmacological targets for the treatment of cancer and associated pain. Reported literature supports the hypothesis that the ideal anti-cancer strategy will be obtained with a calibrated mixture or combination of P2X2, P2X3, P2X4 and P2X7 receptor antagonists, which will reduce/resolve at the same time tumor growth and relative pain.

REFERENCES

1 **Portenoy RK**, Lesage P. Management of cancer pain. *Lancet* 1999; **353**: 1695-1700 [PMID: 10335806 DOI: 10.1016/S0140-6736(99)01310-0]
2 **Marcus DA**. Epidemiology of cancer pain. *Curr Pain Headache Rep* 2011; **15**: 231-234 [PMID: 21556709 DOI: 10.1007/s11916-011-0208-0]
3 **van den Beuken-van Everdingen MH**, de Rijke JM, Kessels AG, Schouten HC, van Kleef M, Patijn J. Prevalence of pain in patients with cancer: a systematic review of the past 40 years. *Ann Oncol* 2007; **18**: 1437-1449 [PMID: 17355955 DOI: 10.1093/annonc/mdm056]
4 **Fallon MT**. Neuropathic pain in cancer. *Br J Anaesth* 2013; **111**: 105-111 [PMID: 23794652 DOI: 10.1093/bja/aet208]
5 **Burnstock G**. Purinergic mechanisms and pain--an update. *Eur J Pharmacol* 2013; **716**: 24-40 [PMID: 23524093 DOI: 10.1016/j.ejphar.2013.01.078]
6 **Volonté C**, Apolloni S, Skaper SD, Burnstock G. P2X7 receptors: channels, pores and more. *CNS Neurol Disord Drug Targets* 2012; **11**: 705-721 [PMID: 22963440 DOI: 10.2174/187152712803581137]
7 **Adinolfi E**, Raffaghello L, Giuliani AL, Cavazzini L, Capece M, Chiozzi P, Bianchi G, Kroemer G, Pistoia V, Di Virgilio F. Expression of P2X7 receptor increases in vivo tumor growth. *Cancer Res* 2012; **72**: 2957-2969 [PMID: 22505653 DOI: 10.1158/0008-5472.CAN-11-1947]
8 **Jelassi B**, Chantôme A, Alcaraz-Pérez F, Baroja-Mazo A, Cayuela ML, Pelegrin P, Surprenant A, Roger S. P2X(7) receptor activation enhances SK3 channels- and cystein cathepsin-dependent cancer cells invasiveness. *Oncogene* 2011; **30**: 2108-2122 [PMID: 21242969 DOI: 10.1038/onc.2010.593]
9 **North RA**, Jarvis MF. P2X receptors as drug targets. *Mol Pharmacol* 2013; **83**: 759-769 [PMID: 23253448 DOI: 10.1124/mol.112.083758]
10 **Kawate T**, Michel JC, Birdsong WT, Gouaux E. Crystal structure of the ATP-gated P2X(4) ion channel in the closed state. *Nature* 2009; **460**: 592-598 [PMID: 19641588 DOI: 10.1038/nature08198]
11 **Toulme E**, Tsuda M, Khakh BS, Inoue K. On the Role of ATP-Gated P2X Receptors in Acute, Inflammatory and Neuropathic Pain. In: Kruger L, Light AR. Translational Pain Research: From Mouse to Man. Boca Raton, FL: CRC Press, 2010 [PMID: 21882468 DOI: 10.1201/9781439812105-c10]
12 **Woolf CJ**. What is this thing called pain? *J Clin Invest* 2010; **120**: 3742-3744 [PMID: 21041955 DOI: 10.1172/JCI45178]
13 **Kidd BL**, Urban LA. Mechanisms of inflammatory pain. *Br J Anaesth* 2001; **87**: 3-11 [PMID: 11460811 DOI: 10.1093/bja/87.1.3]
14 **Moilanen E**. Two faces of inflammation: an immunopharmacological view. *Basic Clin Pharmacol Toxicol* 2014; **114**: 2-6 [PMID: 24286361 DOI: 10.1111/bcpt.12180]
15 **Vranken JH**. Elucidation of pathophysiology and treatment of neuropathic pain. *Cent Nerv Syst Agents Med Chem* 2012; **12**: 304-314 [PMID: 23033930 DOI: 10.2174/18715241280376064 5]
16 **Jay GW**, Barkin RL. Neuropathic pain: etiology, pathophysiology, mechanisms, and evaluations. *Dis Mon* 2014; **60**: 6-47 [PMID: 24507705 DOI: 10.1016/j.disamonth.2013.12.001]
17 **Jimenez Andrade JM**, Mantyh P. Cancer Pain: From the Development of Mouse Models to Human Clinical Trials. In: Kruger L, Light AR. Translational Pain Research: From Mouse to Man. Boca Raton, FL: CRC Press, 2010 [PMID: 21882467]
18 **Portenoy RK**. Treatment of cancer pain. *Lancet* 2011; **377**: 2236-2247 [PMID: 21704873 DOI: 10.1016/S0140-6736(11)60236-5]
19 **Cain DM**, Wacnik PW, Turner M, Wendelschafer-Crabb G, Kennedy WR, Wilcox GL, Simone DA. Functional interactions between tumor and peripheral nerve: changes in excitability and morphology of primary afferent fibers in a murine model of cancer pain. *J Neurosci* 2001; **21**: 9367-9376 [PMID: 11717370 DOI: 10.1046/j.1529-8027.2002.02011_5.x]
20 **Clohisy DR**, Mantyh PW. Bone cancer pain. *Cancer* 2003; **97**: 866-873 [PMID: 12548588 DOI: 10.1002/cncr.11144]
21 **Virk MS**, Lieberman JR. Tumor metastasis to bone. *Arthritis Res Ther* 2007; **9** Suppl 1: S5 [PMID: 17634144 DOI: 10.1186/ar2169]
22 **Falk S**, Uldall M, Heegaard AM. The role of purinergic receptors in cancer-induced bone pain. *J Osteoporos* 2012; **2012**: 758181 [PMID: 23091774 DOI: 10.1155/2012/758181]
23 **Foley KM**. Advances in cancer pain. *Arch Neurol* 1999; **56**: 413-417 [PMID: 10199328 DOI: 10.1001/archneur.56.4.413]
24 **Probstner D**, Thuler LC, Ishikawa NM, Alvarenga RM. Phantom limb phenomena in cancer amputees. *Pain Pract* 2010; **10**: 249-256 [PMID: 20070548 DOI: 10.1111/j.1533-2500.2009.00340.x]
25 **Vaso A**, Adahan HM, Gjika A, Zahaj S, Zhurda T, Vyshka G, Devor M. Peripheral nervous system origin of phantom limb pain. *Pain* 2014; **155**: 1384-1391 [PMID: 24769187 DOI: 10.1016/j.pain.2014.04.018]
26 **Christo PJ**, Mazloomdoost D. Cancer pain and analgesia. *Ann N Y Acad Sci* 2008; **1138**: 278-298 [PMID: 18837907 DOI: 10.1196/annals.1414.033]
27 **Geber C**, Breimhorst M, Burbach B, Egenolf C, Baier B, Fechir M, Koerber J, Treede RD, Vogt T, Birklein F. Pain in chemotherapy-induced neuropathy--more than neuropathic? *Pain* 2013; **154**: 2877-2887 [PMID: 23999056 DOI: 10.1016/j.pain.2013.08.028]
28 **Warwick RA**, Hanani M. The contribution of satellite glial cells to chemotherapy-induced neuropathic pain. *Eur J Pain* 2013; **17**: 571-580 [PMID: 23065831 DOI: 10.1002/j.1532-2149.2012.00219.x]
29 **Lundstedt D**, Gustafsson M, Steineck G, Malmström P, Alsadius D, Sundberg A, Wilderäng U, Holmberg E, Johansson KA, Karlsson P. Risk factors of developing long-lasting breast pain after breast cancer radiotherapy. *Int J Radiat Oncol Biol Phys* 2012; **83**: 71-78 [PMID: 22079722 DOI: 10.1016/j.ijrobp.2011.05.065]
30 **Di Franco R**, Falivene S, Ravo V, Mammucari M, Sarli E, Baffini S, De Palma G, Pepe A, Traettino M, Muto M, Cappabianca S, Muto P. Management of painful bone metastases: our experience according to scientific evidence on palliative radiotherapy. *Anticancer Res* 2014; **34**: 1011-1014 [PMID: 24511047]
31 **Turk DC**, Wilson HD, Cahana A. Treatment of chronic non-cancer pain. *Lancet* 2011; **377**: 2226-2235 [PMID: 21704872 DOI: 10.1016/S0140-6736(11)60402-9]
32 **Tsuda M**, Masuda T, Tozaki-Saitoh H, Inoue K. P2X4 receptors and neuropathic pain. *Front Cell Neurosci* 2013; **7**: 191 [PMID: 24191146 DOI: 10.3389/fncel.2013.00191]
33 **Wirkner K**, Sperlagh B, Illes P. P2X3 receptor involvement in pain states. *Mol Neurobiol* 2007; **36**: 165-183 [PMID: 17952660 DOI: 10.1007/s12035-007-0033-y]
34 **Burnstock G**, Di Virgilio F. Purinergic signalling and cancer. *Purinergic Signal* 2013; **9**: 491-540 [PMID: 23797685 DOI: 10.1007/s11302-013-9372-5]
35 **Tsuda M**, Tozaki-Saitoh H, Inoue K. Pain and purinergic signaling. *Brain Res Rev* 2010; **63**: 222-232 [PMID: 19931560 DOI: 10.1016/j.brainresrev.2009.11.003]
36 **Abbracchio MP**, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signalling in the nervous system: an overview. *Trends Neurosci* 2009; **32**: 19-29 [PMID: 19008000 DOI: 10.1016/j.tins.2008.10.001]
37 **Hamilton SG**, McMahon SB. ATP as a peripheral mediator of pain. *J Auton Nerv Syst* 2000; **81**: 187-194 [PMID: 10869719

DOI: 10.1016/S0165-1838(00)00137-5]
38 **Pellegatti P**, Raffaghello L, Bianchi G, Piccardi F, Pistoia V, Di Virgilio F. Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. *PLoS One* 2008; **3**: e2599 [PMID: 18612415 DOI: 10.1371/journal.pone.0002599]
39 **Berglund E**, Berglund D, Akcakaya P, Ghaderi M, Daré E, Berggren PO, Köhler M, Aspinwall CA, Lui WO, Zedenius J, Larsson C, Bränström R. Evidence for Ca(2+)-regulated ATP release in gastrointestinal stromal tumors. *Exp Cell Res* 2013; **319**: 1229-1238 [PMID: 23499741 DOI: 10.1016/j.yexcr.2013.03.001]
40 **Martins I**, Tesniere A, Kepp O, Michaud M, Schlemmer F, Senovilla L, Séror C, Métivier D, Perfettini JL, Zitvogel L, Kroemer G. Chemotherapy induces ATP release from tumor cells. *Cell Cycle* 2009; **8**: 3723-3728 [PMID: 19855167 DOI: 10.4161/cc.8.22.10026]
41 **Schumacher D**, Strilic B, Sivaraj KK, Wettschureck N, Offermanns S. Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. *Cancer Cell* 2013; **24**: 130-137 [PMID: 23810565 DOI: 10.1016/j.ccr.2013.05.008]
42 **Pellegatti P**, Falzoni S, Pinton P, Rizzuto R, Di Virgilio F. A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion. *Mol Biol Cell* 2005; **16**: 3659-3665 [PMID: 15944221 DOI: 10.1091/mbc.E05-03-0222]
43 **Adinolfi E**, Cirillo M, Woltersdorf R, Falzoni S, Chiozzi P, Pellegatti P, Callegari MG, Sandonà D, Markwardt F, Schmalzing G, Di Virgilio F. Trophic activity of a naturally occurring truncated isoform of the P2X7 receptor. *FASEB J* 2010; **24**: 3393-3404 [PMID: 20453110 DOI: 10.1096/fj.09-153601]
44 **Michaud M**, Martins I, Sukkurwala AQ, Adjemian S, Ma Y, Pellegatti P, Shen S, Kepp O, Scoazec M, Mignot G, Rello-Varona S, Tailler M, Menger L, Vacchelli E, Galluzzi L, Ghiringhelli F, di Virgilio F, Zitvogel L, Kroemer G. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. *Science* 2011; **334**: 1573-1577 [PMID: 22174255 DOI: 10.1126/science.1208347]
45 **Stagg J**, Beavis PA, Divisekera U, Liu MC, Möller A, Darcy PK, Smyth MJ. CD73-deficient mice are resistant to carcinogenesis. *Cancer Res* 2012; **72**: 2190-2196 [PMID: 22396496 DOI: 10.1158/0008-5472.CAN-12-0420]
46 **Baricordi OR**, Melchiorri L, Adinolfi E, Falzoni S, Chiozzi P, Buell G, Di Virgilio F. Increased proliferation rate of lymphoid cells transfected with the P2X(7) ATP receptor. *J Biol Chem* 1999; **274**: 33206-33208 [PMID: 10559192 DOI: 10.1074/jbc.274.47.33206]
47 **Adinolfi E**, Pizzirani C, Idzko M, Panther E, Norgauer J, Di Virgilio F, Ferrari D. P2X(7) receptor: Death or life? *Purinergic Signal* 2005; **1**: 219-227 [PMID: 18404507 DOI: 10.1007/s11302-005-6322-x]
48 **Amoroso F**, Falzoni S, Adinolfi E, Ferrari D, Di Virgilio F. The P2X7 receptor is a key modulator of aerobic glycolysis. *Cell Death Dis* 2012; **3**: e370 [PMID: 22898868 DOI: 10.1038/cddis.2012.105]
49 **Hattori F**, Ohshima Y, Seki S, Tsukimoto M, Sato M, Takenouchi T, Suzuki A, Takai E, Kitani H, Harada H, Kojima S. Feasibility study of B16 melanoma therapy using oxidized ATP to target purinergic receptor P2X7. *Eur J Pharmacol* 2012; **695**: 20-26 [PMID: 22981895 DOI: 10.1016/j.ejphar.2012.09.001]
50 **Surprenant A**, North RA. Signaling at purinergic P2X receptors. *Annu Rev Physiol* 2009; **71**: 333-359 [PMID: 18851707 DOI: 10.1146/annurev.physiol.70.113006.100630]
51 **Donnelly-Roberts D**, McGaraughty S, Shieh CC, Honore P, Jarvis MF. Painful purinergic receptors. *J Pharmacol Exp Ther* 2008; **324**: 409-415 [PMID: 18042830 DOI: 10.1124/jpet.106.105890]
52 **Liu M**, Yang H, Fang D, Yang JJ, Cai J, Wan Y, Chui DH, Han JS, Xing GG. Upregulation of P2X3 receptors by neuronal calcium sensor protein VILIP-1 in dorsal root ganglions contributes to the bone cancer pain in rats. *Pain* 2013; **154**: 1551-1568 [PMID: 23707265 DOI: 10.1016/j.pain.2013.04.022]
53 **Wu JX**, Xu MY, Miao XR, Lu ZJ, Yuan XM, Li XQ, Yu WF. Functional up-regulation of P2X3 receptors in dorsal root ganglion in a rat model of bone cancer pain. *Eur J Pain* 2012; **16**: 1378-1388 [PMID: 22528605 DOI: 10.1002/j.1532-2149.2012.00149.x]
54 **Jimenez-Andrade JM**, Mantyh WG, Bloom AP, Ferng AS, Geffre CP, Mantyh PW. Bone cancer pain. *Ann N Y Acad Sci* 2010; **1198**: 173-181 [PMID: 20536932 DOI: 10.1111/j.1749-6632.2009.05429.x]
55 **Kaan TK**, Yip PK, Patel S, Davies M, Marchand F, Cockayne DA, Nunn PA, Dickenson AH, Ford AP, Zhong Y, Malcangio M, McMahon SB. Systemic blockade of P2X3 and P2X2/3 receptors attenuates bone cancer pain behaviour in rats. *Brain* 2010; **133**: 2549-2564 [PMID: 20802203 DOI: 10.1093/brain/awq194]
56 **Jarvis MF**. Contributions of P2X3 homomeric and heteromeric channels to acute and chronic pain. *Expert Opin Ther Targets* 2003; **7**: 513-522 [PMID: 12885270 DOI: 10.1517/14728222.7.4.513]
57 **Jarvis MF**. The neural-glial purinergic receptor ensemble in chronic pain states. *Trends Neurosci* 2010; **33**: 48-57 [PMID: 19914722 DOI: 10.1016/j.tins.2009.10.003]
58 **Jarvis MF**, Burgard EC, McGaraughty S, Honore P, Lynch K, Brennan TJ, Subieta A, Van Biesen T, Cartmell J, Bianchi B, Niforatos W, Kage K, Yu H, Mikusa J, Wismer CT, Zhu CZ, Chu K, Lee CH, Stewart AO, Polakowski J, Cox BF, Kowaluk E, Williams M, Sullivan J, Faltynek C. A-317491, a novel potent and selective non-nucleotide antagonist of P2X3 and P2X2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat. *Proc Natl Acad Sci USA* 2002; **99**: 17179-17184 [PMID: 12482951 DOI: 10.1073/pnas.252537299]
59 **McGaraughty S**, Wismer CT, Zhu CZ, Mikusa J, Honore P, Chu KL, Lee CH, Faltynek CR, Jarvis MF. Effects of A-317491, a novel and selective P2X3/P2X2/3 receptor antagonist, on neuropathic, inflammatory and chemogenic nociception following intrathecal and intraplantar administration. *Br J Pharmacol* 2003; **140**: 1381-1388 [PMID: 14623769 DOI: 10.1038/sj.bjp.0705574]
60 **Hansen RR**, Nasser A, Falk S, Baldvinsson SB, Ohlsson PH, Bahl JM, Jarvis MF, Ding M, Heegaard AM. Chronic administration of the selective P2X3, P2X2/3 receptor antagonist, A-317491, transiently attenuates cancer-induced bone pain in mice. *Eur J Pharmacol* 2012; **688**: 27-34 [PMID: 22634164 DOI: 10.1016/j.ejphar.2012.05.008]
61 **Fujita M**, Andoh T, Sasaki A, Saiki I, Kuraishi Y. Involvement of peripheral adenosine 5′-triphosphate and P2X purinoceptor in pain-related behavior produced by orthotopic melanoma inoculation in mice. *Eur J Neurosci* 2010; **31**: 1629-1636 [PMID: 20525075 DOI: 10.1111/j.1460-9568.2010.07185.x]
62 **Khakpay R**, Polster D, Köles L, Skorinkin A, Szabo B, Wirkner K, Illes P. Potentiation of the glutamatergic synaptic input to rat locus coeruleus neurons by P2X7 receptors. *Purinergic Signal* 2010; **6**: 349-359 [PMID: 21103218 DOI: 10.1007/s11302-010-9198-3]
63 **Hansen RR**, Nielsen CK, Nasser A, Thomsen SI, Eghorn LF, Pham Y, Schulenburg C, Syberg S, Ding M, Stojilkovic SS, Jorgensen NR, Heegaard AM. P2X7 receptor-deficient mice are susceptible to bone cancer pain. *Pain* 2011; **152**: 1766-1776 [PMID: 21565445 DOI: 10.1016/j.pain.2011.03.024]
64 **Paice JA**, Ferrell B. The management of cancer pain. *CA Cancer J Clin* 2011; **61**: 157-182 [PMID: 21543825 DOI: 10.3322/caac.20112]
65 **Reyno LM**, Egorin MJ, Eisenberger MA, Sinibaldi VJ, Zuhowski EG, Sridhara R. Development and validation of a pharmacokinetically based fixed dosing scheme for suramin. *J Clin Oncol* 1995; **13**: 2187-2195 [PMID: 7666077]
66 **Kobayashi K**, Vokes EE, Vogelzang NJ, Janish L, Soliven B, Ratain MJ. Phase I study of suramin given by intermittent infusion without adaptive control in patients with advanced

cancer. *J Clin Oncol* 1995; **13**: 2196-2207 [PMID: 7666078]
67 **Small EJ**, Meyer M, Marshall ME, Reyno LM, Meyers FJ, Natale RB, Lenehan PF, Chen L, Slichenmyer WJ, Eisenberger M. Suramin therapy for patients with symptomatic hormone-refractory prostate cancer: results of a randomized phase III trial comparing suramin plus hydrocortisone to placebo plus hydrocortisone. *J Clin Oncol* 2000; **18**: 1440-1450 [PMID: 10735891]
68 **Chizhmakov I**, Yudin Y, Mamenko N, Prudnikov I, Tamarova Z, Krishtal O. Opioids inhibit purinergic nociceptors in the sensory neurons and fibres of rat via a G protein-dependent mechanism. *Neuropharmacology* 2005; **48**: 639-647 [PMID: 15814099 DOI: 10.1016/j.neuropharm.2004.12.009]
69 **Chizhmakov I**, Mamenko N, Volkova T, Khasabova I, Simone DA, Krishtal O. P2X receptors in sensory neurons co-cultured with cancer cells exhibit a decrease in opioid sensitivity. *Eur J Neurosci* 2009; **29**: 76-86 [PMID: 19077126 DOI: 10.1111/j.1460-9568.2008.06556.x]
70 **Alves LA**, Bezerra RJ, Faria RX, Ferreira LG, da Silva Frutuoso V. Physiological roles and potential therapeutic applications of the P2X7 receptor in inflammation and pain. *Molecules* 2013; **18**: 10953-10972 [PMID: 24013409 DOI: 10.3390/molecules180910953]
71 **King BF**. Novel P2X7 receptor antagonists ease the pain. *Br J Pharmacol* 2007; **151**: 565-567 [PMID: 17471176 DOI: 10.1038/sj.bjp.0707266]
72 **Chen ML**, Cao H, Chu YX, Cheng LZ, Liang LL, Zhang YQ, Zhao ZQ. Role of P2X7 receptor-mediated IL-18/IL-18R signaling in morphine tolerance: multiple glial-neuronal dialogues in the rat spinal cord. *J Pain* 2012; **13**: 945-958 [PMID: 22968128 DOI: 10.1016/j.jpain.2012.06.007]
73 **Honore P**, Donnelly-Roberts D, Namovic MT, Hsieh G, Zhu CZ, Mikusa JP, Hernandez G, Zhong C, Gauvin DM, Chandran P, Harris R, Medrano AP, Carroll W, Marsh K, Sullivan JP, Faltynek CR, Jarvis MF. A-740003 [N-(1-{[(cyanoimino)(5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide], a novel and selective P2X7 receptor antagonist, dose-dependently reduces neuropathic pain in the rat. *J Pharmacol Exp Ther* 2006; **319**: 1376-1385 [PMID: 16982702 DOI: 10.1124/jpet.106.111559]
74 **Huang ZX**, Lu ZJ, Ma WQ, Wu FX, Zhang YQ, Yu WF, Zhao ZQ. Involvement of RVM-expressed P2X7 receptor in bone cancer pain: mechanism of descending facilitation. *Pain* 2014; **155**: 783-791 [PMID: 24447511 DOI: 10.1016/j.pain.2014.01.011]
75 **Fang J**, Chen X, Zhang L, Chen J, Liang Y, Li X, Xiang J, Wang L, Guo G, Zhang B, Zhang W. P2X7R suppression promotes glioma growth through epidermal growth factor receptor signal pathway. *Int J Biochem Cell Biol* 2013; **45**: 1109-1120 [PMID: 23523696 DOI: 10.1016/j.biocel.2013.03.005]
76 **Takai E**, Tsukimoto M, Harada H, Sawada K, Moriyama Y, Kojima S. Autocrine regulation of TGF-β1-induced cell migration by exocytosis of ATP and activation of P2 receptors in human lung cancer cells. *J Cell Sci* 2012; **125**: 5051-5060 [PMID: 22946048 DOI: 10.1242/jcs.104976]
77 **Takai E**, Tsukimoto M, Harada H, Kojima S. Autocrine signaling via release of ATP and activation of P2X7 receptor influences motile activity of human lung cancer cells. *Purinergic Signal* 2014; **10**: 487-497 [PMID: 24627191 DOI: 10.1007/s11302-014-9411-x]
78 **Ren S**, Zhang Y, Wang Y, Lui Y, Wei W, Huang X, Mao W, Zuo Y. Targeting $P2X_7$ receptor inhibits the metastasis of murine P388D1 lymphoid neoplasm cells to lymph nodes. *Cell Biol Int* 2010; **34**: 1205-1211 [PMID: 20722629 DOI: 10.1042/CBI20090428]
79 **Jelassi B**, Anchelin M, Chamouton J, Cayuela ML, Clarysse L, Li J, Goré J, Jiang LH, Roger S. Anthraquinone emodin inhibits human cancer cell invasiveness by antagonizing P2X7 receptors. *Carcinogenesis* 2013; **34**: 1487-1496 [PMID: 23524196 DOI: 10.1093/carcin/bgt099]
80 **Ghalali A**, Wiklund F, Zheng H, Stenius U, Högberg J. Atorvastatin prevents ATP-driven invasiveness via P2X7 and EHBP1 signaling in PTEN-expressing prostate cancer cells. *Carcinogenesis* 2014; **35**: 1547-1555 [PMID: 24451147 DOI: 10.1093/carcin/bgu019]
81 **Shabbir M**, Thompson C, Jarmulowiczc M, Mikhailidis D, Burnstock G. Effect of extracellular ATP on the growth of hormone-refractory prostate cancer in vivo. *BJU Int* 2008; **102**: 108-112 [PMID: 18325054 DOI: 10.1111/j.1464-410X.2008.07578.x]
82 **Bian S**, Sun X, Bai A, Zhang C, Li L, Enjyoji K, Junger WG, Robson SC, Wu Y. P2X7 integrates PI3K/AKT and AMPK-PRAS40-mTOR signaling pathways to mediate tumor cell death. *PLoS One* 2013; **8**: e60184 [PMID: 23565201 DOI: 10.1371/journal.pone.0060184]
83 **White N**, Burnstock G. P2 receptors and cancer. *Trends Pharmacol Sci* 2006; **27**: 211-217 [PMID: 16530853 DOI: 10.1016/j.tips.2006.02.004]
84 **Beijer S**, Hupperets PS, van den Borne BE, Wijckmans NE, Spreeuwenberg C, van den Brandt PA, Dagnelie PC. Randomized clinical trial on the effects of adenosine 5′-triphosphate infusions on quality of life, functional status, and fatigue in preterminal cancer patients. *J Pain Symptom Manage* 2010; **40**: 520-530 [PMID: 20598849 DOI: 10.1016/j.jpainsymman.2010.01.023]
85 **Agteresch HJ**, Dagnelie PC, van der Gaast A, Stijnen T, Wilson JH. Randomized clinical trial of adenosine 5′-triphosphate in patients with advanced non-small-cell lung cancer. *J Natl Cancer Inst* 2000; **92**: 321-328 [PMID: 10675381 DOI: 10.1093/jnci/92.4.321]
86 **Munerati M**, Cortesi R, Ferrari D, Di Virgilio F, Nastruzzi C. Macrophages loaded with doxorubicin by ATP-mediated permeabilization: potential carriers for antitumor therapy. *Biochim Biophys Acta* 1994; **1224**: 269-276 [PMID: 7981242 DOI: 10.1016/0167-4889(94)90200-3]
87 **Mo R**, Jiang T, DiSanto R, Tai W, Gu Z. ATP-triggered anticancer drug delivery. *Nat Commun* 2014; **5**: 3364 [PMID: 24618921 DOI: 10.1038/ncomms4364]

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ORIGINAL ARTICLE

Identification of host miRNAs that may limit human rhinovirus replication

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Abstract

AIM: To test whether the replication of human rhinovirus (HRV) is regulated by microRNAs in human bronchial epithelial cells.

METHODS: For the present study, the human cell line BEAS-2B (derived from normal human bronchial epithelial cells) was adopted. DICER knock-down, by siRNA transfection in BEAS-2B cells, was performed in order to inhibit microRNA maturation globally. Alternatively, antisense oligonucleotides (anti-miRs) were transfected to inhibit the activity of specific microRNAs. Cells were infected with HRV-1B. Viral replication was assessed by measuring the genomic viral RNA by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Association between microRNA-induced-silencing-complex and viral RNA was detected by Ago2 co-immunoprecipitation followed by RT-qPCR. Targetscan v.6 was used to predict microRNA target sites on several HRV strains.

RESULTS: Here, we show that microRNAs affect replication of HRV-1B. DICER knock-down significantly reduced the expression of mature microRNAs in a bronchial epithelial cell line (BEAS-2B) and in turn, increased the synthesis of HRV-1B RNA. Additionally, HRV-1B RNA co-immunoprecipitated with argonaute 2 protein, an important effector for microRNA activity suggesting that microRNAs bind to viral RNA during infection. In order to identify specific microRNAs involved in this interaction, we employed bioinformatics analysis, and selected a group of microRNAs that have been reported to be under-expressed in asthmatic bronchial epithelial cells and were predicted to target different strains of rhinoviruses (HRV-1B, -16, -14, -27). Our results suggest that, out of this group of microRNAs, miR-128 and miR-155 contribute to the innate defense against HRV-1B: transfection of specific anti-miRs increased viral replication, as anticipated *in-silico*.

CONCLUSION: Taken together, our results suggest that pathological changes in microRNA expression, as already reported for asthma or chronic obstructive pulmonary disease have the potential to affect Rhinovirus replication and therefore may play a role in virus-induced exacerbations.

Key words: Human Rhinovirus; MicroRNAs; MiR-155; SiRNA; Lentiviral transduction; Antiviral innate immunity

Core tip: Our results show for the first time that: (1) DICER knock-down increases HRV-1B replication in hu-

man bronchial epithelial cells; (2) the genomic RNA of human rhinovirus (HRV)-1B interacts directly with the miRISC during infection; and (3) inhibition of two microRNAs predicted to target HRV-1B, *i.e.*, miR-128 and miR-155, favors viral replication. This supports a role for cellular microRNAs in the antiviral response to HRV-1B mounted by bronchial epithelial cells, and suggests that pathological microRNA dysregulation may contribute to the poor antiviral immunity in diseases such as asthma and chronic obstructive pulmonary disease.

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INTRODUCTION

MicroRNAs (or miRs) are small, non-coding RNAs that regulate gene expression at the post-transcriptional level. MicroRNAs are synthesized in the nucleus as long primary transcripts. These undergo a complex maturation process that includes cleavage by DICER in the cytoplasm, yielding the single-stranded mature form[1]. Mature microRNAs then associate with Argonaute (AGO) proteins[2]. These constitute the core of the effector complex often referred to as microRNA-induced silencing complex[3]. MicroRNAs modulate the rate of translation and the stability of target mRNAs by binding to partially complementary sites, within the 3' untranslated region (UTR) of their targets. Not requiring a perfect match, microRNAs of a given sequence can interact with many different mRNAs and therefore, the expression of several genes can be modulated by a single microRNA[4]. It is not straightforward to predict which genes can be regulated by a specific microRNA thus complex algorithms such as Targetscan[5] have been developed for this purpose.

Being part of the mechanisms that cells use to regulate and fine tune gene expression, it is not surprising that microRNAs play a role in many different biological processes such as development, cellular differentiation, maturation or apoptosis, highlighting their importance for normal cell function. As a consequence, their deregulation perturbs gene expression and can have pathological consequences, as evidenced by their involvement in cancer[6]. DICER is essential for mouse embryonic development[7] and later on for correct lung epithelium morphogenesis[8]. MicroRNA expression is highly regulated during lung development[9] in both mouse and human, suggesting an important role during this process[10]. Also in culture, the microRNA profile changes considerably during human bronchial epithelial cell differentiation, controlling the expression of genes involved in this process[11]. Several are the examples of microRNAs involved in the regulation of the immune response. For instance, miR-155 deficient mice showed impaired immune responses in addition to lungs with evident airway remodelling[12]. Importantly, the expression of microRNAs is not regulated only during development but also by a variety of extracellular stimuli such as cytokines, pathogen components and infection. For example, bacterial lipopolysaccharide (LPS) causes up-regulation of miR-155[13] but down-regulation of miR-125b in murine macrophages[14]. As expected by their opposite regulation, the two microRNAs have opposite effects on the expression of tumor necrosis factor α (TNF-α), which is secreted after LPS treatment. Often microRNAs are used by cells to fine tune or reinforce their responses, by targeting signalling molecules. For instance, in monocytes and macrophages, interleukin 1 β (IL-1β), TNF-α and LPS have been shown to up-regulate miR-146a/b. Perhaps in order to prevent excessive inflammation, the microRNA inhibits the expression of IL-1 receptor-associated kinase 1 and 2 and TNF receptor-associated factor 6 which are important signalling proteins of innate immunity pathways[15]. Also vesicular stomatitis virus (VSV) or Influenza A infection up-regulates miR-146a/b with the effect of promoting replication of the virus[16,17]. Respiratory syncytial virus can alter cellular microRNA expression[18,19]. In particular, in human bronchial epithelial cells the down-regulation of miR-221 favours viral replication by counteracting the induction of apoptosis in infected cells[20].

In addition to acting on host mRNAs, microRNAs can directly bind and regulate viral RNA and therefore hamper viral replication. Interestingly, such direct antiviral function has been conserved from plants to mammals[21]. In humans, antiviral microRNAs have been shown to act both independently and as important effectors of the interferon (IFN) system, which is paramount in innate antiviral immunity. For instance, Hepatits C Virus (HCV) exemplifies how microRNAs can influence viral tissue tropism and work either in favour or against viruses. In fact, miR-122, a liver-specific microRNA is able to facilitate HCV replication[22]. Conversely, IFN-β down-regulates miR-122 while up-regulating the expression of other microRNAs with sequence-specific antiviral activity[23]. Recently, IFN-β treatment of HeLa cells was shown to induce the expression of miR-23b[24] which in turn was able to limit HRV-1B replication, by reducing the levels of the very low-density lipoprotein receptor. DICER-deficient murine macrophages were also more permissive to VSV than wild type cells, with no difference in type I IFN production[25]. DICER and several microRNAs interfere also with the replication of Influenza A virus[26-28] and retroviruses such as Human Immune Deficiency Virus-1[29] and Primate Foamy Virus-1[30].

Considering the many examples of antiviral microRNAs reported, we hypothesized that Human Rhinoviruses (HRV) are targeted by microRNAs. While responsible for most of the common colds in healthy subjects, HRVs are a major trigger of chronic obstructive pulmonary disease (COPD) and asthma exacerbations[31-33] which represent a significant problem for disease management. HRVs predominantly infect epithelial cells of the upper and lower airways[34-37]. They are non-enveloped viruses of the

Picornaviridae family, and are subdivided in three species (A, B and C) based on sequence homology. HRV-A and -B comprise approximately 100 different strains which are also divided into two groups depending on receptor usage. Rhinoviruses of the major subgroup, such as HRV-16, use the intercellular adhesion molecule 1 for entry[38] while the low-density lipoprotein receptor family[39] is used by members of the minor subgroup, such as HRV-1B. Shortly after entry, the single stranded genomic RNA functions as mRNA, driving the expression of a single open reading frame that encodes all viral proteins. The double-stranded RNA, that forms during viral replication, is detected by cellular receptors such as Toll-like Receptor 3 and the RNA helicases *RIG-I* (retinoic acid inducible gene) and melanoma differentiation associated gene-5[40] leading to the induction of IFNs (and downstream IFN-regulated genes) and pro-inflammatory cytokines[41].

A bioinformatics study suggested that human microRNAs may effectively bind to the genome of Rhinoviruses[42] and artificial siRNAs have been shown to inhibit HRV-16 replication[43]. Here we show that microRNAs in general may be involved in the defence against Rhinovirus in human bronchial epithelial cells and that the inhibition of two microRNAs predicted to target HRV-1B, miR-128 and miR-155, increased the accumulation of intracellular viral RNA. To our knowledge, this is the first experimental study to provide evidence suggesting that constitutively expressed cellular microRNAs regulate HRV replication.

MATERIALS AND METHODS

Cell culture

BEAS-2B and THP-1 cells were grown in RPMI medium supplemented with Glutamax I (GIBCO) and 10% heat inactivated foetal bovine serum (FBS, GIBCO). All cells were cultured at 37 ℃ in a humidified 5% CO2 incubator. For the infection experiments, RPMI with Glutamax I supplemented with 2% FBS was used.

Propagation of HRV-1B and infection experiments

HRV-1B was a gift from Professor Sebastian L. Johnston (Imperial College, London). HRV-1B was grown on Ohio HeLa cells (obtained from the American Type Culture Collection) grown in Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented with 10% FBS, 50 IU/mL penicillin, 50 mg/mL streptomycin, 2 mmol/L L-glutamine. Briefly, HeLa cells were grown up to around 70%-80% confluency. Cells were washed once with Hank's Balanced Salt Solution (GIBCO) and then HRV was added. The cell culture vessel was then kept at room temperature on a plate rocker for 1 h. Subsequently, more infection medium was added and finally the cell culture vessel was incubated at 33 ℃ (in a humidified incubator, in air with 5% CO_2) for 16-24 h. After performing three cycles of freeze-thaw at -80 ℃, the supernatant (containing the virus) was recovered, cell debris were removed by centrifugation at 800 × g at 4 ℃ and used to inoculate more HeLa cells. Finally, the virus-containing medium was centrifuged at 800 × g at 4 ℃ and then filtered through a 0.2 mm syringe filter. All the infection experiments presented here were performed with the same viral stock. The latter was titrated to 5.5 × 10^6 viral particles/mL by 50% tissue culture infective dose (TCID50) as previously described[44].

For infection experiments, BEAS-2B cells were grown until about 80% confluent, detached by trypsin treatment and seeded at 0.75 × 10^5 cells/well in 24-well plates. Twenty four hours later, growth medium was replaced with infection medium for about 16 h before infection with HRV-1B. In each experiment, before infection, the cells present in two wells were counted using an improved Neubauer 0.1 mm haemocytometer. Cells were detached by trypsin treatment and re-suspended in 1 mL/well. The cell count obtained was used to calculate the volume of viral stock necessary for the desired multiplicity of infection (MOI) according to the following formula: mL of viral stock = (MOI × number of cells)/TCID50. Cells to be infected were first washed with 1 mL/well of Phosphate buffered saline (PBS) and then 0.2 mL/well of the viral suspension were added. Cells were then incubated at room temperature on a plate rocker for one hour. At this point, residual virus was removed and cells were washed as above. Then, either 0.5 mL/well of infection medium was added, or cells were harvested (0 h post-infection) using 0.5 mL/well of TRI-Reagent (Ambion). Plates were placed in the incubator for the indicated time, before collection in TRI-Reagent.

Transfections

Negative control siRNA and an anti-DICER siRNA were purchased from Ambion (Silencer® Select Validated siRNA). Anti-DICER (Ambion siRNA ID: s23754) sense strand sequence (5'-3' sequence): GAUCCUAUGUUCAAUCUAAtt. Antisense strand sequence (5'-3' sequence): UUAGAUUGAACAUAGGAUCga. Negative control #2 (Ambion siRNA ID: 4390846) sequence not provided.

BEAS-2B cells were seeded at 0.75 × 10^5 cells/well in 24-well plates. Twenty four hours later cells were transfected as follows. Two hundred μL/well of growth medium; for each well to be transfected, 1.5 μL of Oligofectamine (Invitrogen) were diluted in 10 μL of plain RPMI and mixed by pipetting. In a separate microcentrifuge tube, 0.15 μL of 50 μmol/L siRNA were diluted in 40 μL of plain RPMI medium and mixed by pipetting. The tubes were left at room temperature. After 5 min, the contents of the two tubes were mixed together by pipetting and left at room temperature. Twenty minutes later, the mixture was mixed again by pipetting and dispensed on the cells (50 μL/well giving a final siRNA concentration of 30 nmol/L). The following day, the medium present in the wells was replaced with fresh infection medium. BEAS-2B cells were transfected every 36 h (3 d) following the protocol described. This required that 48 h post-transfection cells were detached by trypsin treatment and seeded at the density specified earlier. One day after the last transfection, the medium was replaced with infection medium. Cells were then infected with HRV-1B 24 h

later (48 h after the third transfection).

All the anti-miRs (Anti-miR™ miRNA Inhibitors) were purchased from Ambion: anti-miR negative control#1 cat.n: AM17010; anti-hsa-miR-18a, cat.n: AM12973; anti-hsa-miR-19b, cat.n: AM10629; anti-hsa-miR-106b, cat.n: AM10067; anti-hsa-miR-128, cat.n: AM11746; anti-hsa-miR-155, cat.n: AM12601.

Anti-miR transfections were performed using INTERFERin, as recommended by the manufacturer's protocol. BEAS-2B cells were seeded at 0.75×10^5 cells/well in 24-well plates. Twenty four hours later, the culture medium was replaced with 500 μL/well of growth medium. For each well to be transfected, 1.2 μL of 50 μmol/L anti-miR were diluted in 100 μL of plain RPMI and mixed by pipetting. Immediately after, 4 μL of INTERFERin were added and mixed by pipetting. After 20 min incubation at room temperature, 100 μL/well were used, giving a final anti-miR concentration of 100 nmol/L. The following day, cells were infected with HRV-1B.

Lentivirally transduced BEAS-2B cell lines

The lentivirally transduced BEAS-2B cell lines were generated as previously described[45]. The genomic region encompassing miR-155 was amplified using the following primers, forward: AAGCTTTATGCCTCATCCTCTGAGTGC; reverse: CTCGAGACGAAGGTTGAACATCCCAGTGACC. The insert was cloned into pSUPER plasmid between the sites HindⅢ and XhoI, excised with EcoRI and MluI and hence subcloned in pLVTHM, generating the construct pLVTHM_BIC.

HEK293T cells were co-transfected with 5 μg of pLVTHM_BIC (containing miR-155) or pLVTHM (used as negative control) 3.75 μg of pPAX2 and 1.5 μg of pMD2G using Superfect (Qiagen) according to the manufacturer's protocol. Supernatants were used to transduce BEAS-2B cells. Four days after transduction, selection of positively transduced cells (GFP+) was achieved by cell sorting, performed on a BD FACSAria Ⅲ cell sorter.

Reverse transcription-quantitative polymerase chain reaction analysis

Total cellular RNA was extracted using TRI-Reagent (Ambion) according to the manufacturer's instructions. RNA samples were quantified using the spectrophotometer Nanodrop 1000 (Thermo Scientific). Reverse transcription (RT) reactions were performed using the same amount of RNA for all samples, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems part number: 4368813) with random hexamers as primers. MicroRNA reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed using Taqman microRNA assay kits from applied biosystems (part number: 4427975) that contain a specific RT primer and Taqman qPCR primers and probe necessary to quantify expression of a specific microRNA. In both cases, the cDNA obtained was used to perform qPCR using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems part number: 4364341) on a 7900HT Real-Time PCR machine (Applied Biosystems) with the standard Taqman thermal cycling conditions for assays using "Taqman probes" (95 ℃ for 10 min; 95 ℃ for 15 s; 60 ℃ for 1 min - data collection) or modified as follows for assays using "Perfect probe" (95 ℃ for 10 min; 95 ℃ for 15 s; 50 ℃ for 30 s - data collection; 72 ℃ for 15 s). Fold differences in gene expression were calculated using the comparative Ct method for relative quantification[46]. Unless otherwise stated, gene expression was calculated using GAPDH as reference gene, and the average delta Ct value of the negative control as calibrator. Gene expression assays from Applied Biosystems (part n: 4331182): AGO2 (EIF2C2 - Assay ID: Hs01085579_m1); COL1A1 (Assay ID: Hs00164004_m1); DICER1 (Assay ID: Hs00229023_m1); GAPDH (part n: 4352934); SMAD2 (Assay ID: Hs00183425_m1). Gene expression assays from PrimerDesign Ltd (Southampton, United Kingdom): HRV-1B (Taqman probe); and IFNB1 (Perfect probe); IL13RA1 (Taqman probe); PU.1 (Taqman probe). MicroRNA expression assays (part n: 4427975): hsa-miR-18a (Assay ID: 002422); hsa-miR-19b (Assay ID: 000396); hsa-miR-106b (Assay ID: 000442); hsa-miR-128 (Assay ID: 002216); hsa-miR-155 (Assay ID: 002623).

Co-immunoprecipitation protocol

The antibodies used were: anti-AGO2, clone 11A9 (rat IgG2a) available from Ascenion GmbH, Munich, Germany[47]; normal rat IgG (cat.n: sc-2026, Santa Cruz biotechnology, inc). Protein G-coated Sepharose beads (Amersham) were prepared for IP reactions as follows. Beads were collected by centrifugation in a bench top centrifuge, at 3000 rpm (= 800 × g) for 1 min at 4 ℃. Beads were washed 3 times with ice-cold lysis buffer (PBS 1X, NP-40 0.5%, EDTA pH8 2 mmol/L, Glycerol 20%). Beads were then blocked in ice-cold lysis buffer with a final concentration of 1 mg/mL of BSA and 1 mg/mL of sonicated salmon sperm DNA (sspDNA) rotating end over end, for at least 1 h at 4 ℃. After this incubation, beads were collected by centrifugation and washed three times as above and resuspended in an equal volume of ice-cold lysis buffer.

THP-1 cells (human monocyte cell line, used as an additional control) were collected by centrifugation at 300 × g for 5 min at 4 ℃. BEAS-2B cells were first trypsinised to detach them from the plastic. Trypsin was inhibited by adding growth medium (containing 10% FBS) and cells were then collected by centrifugation as above. In both cases, cells were then washed twice in sterile ice-cold PBS (GIBCO) and finally the cell pellet was resuspended in 50 μL/10^6 cells of ice-cold complete lysis buffer (composed as specified above and containing in addition 100 U/mL RNAse inhibitor - Applied Biosystems; 1X complete cocktail protease inhibitor - Roche; DTT 0.5 mmol/L, all added just before use) incubated on ice for 10 min. Cell lysates were kept on ice during the entire procedure. They were cleared of the residual cell debris by centrifugation, at top speed in a bench top microcentrifuge, at 4 ℃ for 15 min. Pellets were discarded while supernatants were collected and diluted ten times, so that 1 mL of lysate would correspond to 2×10^6 cells. One mL aliquots were dispensed in pre-chilled eppendorfs, while 10 μL were

Table 1 Sequence details of miR-18a, -19b, -106b, -128, -155

miR name in text	miRBase accession number	mature miR sequence
miR-18a	MIMAT0000072	UAAGGUGCAUCUAGUGCAGAUAG
miR-19b	MIMAT0000074	UGUGCAAAUCCAUGCAAAACUGA
miR-106b	MIMAT0000680	UAAAGUGCUGACAGUGCAGAU
miR-128	MIMAT0000424	UCACAGUGAACCGGUCUCUUU
miR-155	MIMAT0000646	UUAAUGCUAAUCGUGAUAGGGGU

placed into 0.5 mL of TRI-Reagent and constitute the input sample. The same amount of anti-AGO2 specific antibody or the corresponding IgG negative control isotype antibody was then added to each 1mL-aliquot of cell lysate at this stage and finally, the samples were left rotating end over end at 4 ℃ overnight. 50 μL of protein G-coated sepharose beads, prepared as described above, were added to each co-IP reaction, and incubated rotating end over end at 4 ℃ for 2 h. Beads were then collected by centrifugation and then washed five times as already described, using 1mL of ice-cold washing buffer. The latter was different for THP-1 (PBS 1X, NaCl 160 mM, NP-40 0.05%, Glycerol 10%) or BEAS-2B cells (PBS 1X, NaCl 860 mmol/L, NP-40 0.05%, Glycerol 10%). Beads were then washed once more with ice-cold PBS (1x) and resuspended in 100 μL of RNAse-free water. 2.5 μL of proteinase K (20 mg/mL) was added to each tube. Beads were incubated for 15 min at 37 ℃ in a rotomixer at 1000 rpm. Finally, 500 μL of TRI-Reagent (Ambion) were added to each tube. Beads were then vortexed briefly and incubated at room temperature for about 10 min. Eppendorfs were then spun at top speed in a bench-top centrifuge for 1 min. Supernatants were transferred into new eppendorfs while the collected beads were discarded. RNA isolation was then performed as usual. The isolated RNA, was then either used straightaway for RT-qPCR reactions, or stored at -80 ℃ for later use.

AGO2 co-IP calculations

Relative RNA levels were calculated using the following formula: $2^{-[Ct(IP) - Ct(Input)]}/2^{-[Ct(GAPDH\ IgG) - Ct(Input)]}$. Inclusion of the Ct values from the input normalises for mRNA abundance in the cell lysate.

Bioinformatics analysis

The genomic sequences of different HRVs were obtained from the National Center for Biotechnology Information website. HRV-1B version number D00239.1; HRV-16 version number L24917.1; HRV-14 version number K02121.1; HRV-27 version number FJ445186.1.

The microRNA sequences used with Targetscan[48] were obtained from[49]. The microRNA sequences used with miRanda[50] and probability of interaction by target accessibility (PITA)[51] were obtained from miRBase 21[52]. Mature microRNA sequences for miR-18a, -19b, -106b, -128, -155, were identical across the two datasets and are reported in Table 1. MicroRNA target site predictions on viral genomes were obtained by running the appropriate program, locally on a Linux Ubuntu 10.04 LTS system. TargetScan software version 6, miRanda 3.3a and PITA were run with default parameters.

Results were then sorted by context+ score for Targetscan, Energy for miRanda and ddG for PITA. No cut-off value is recommended for Targetscan; Energy threshold of -7 (for miRanda) and ddG threshold of -10 (for PITA[53]) are recommended, besides the default parameters.

In Targetscan, the context+ score is calculated based on the seed class (6-mer, 7-mer-1a, 7-mer-m8 or 8-mer), the extent of miR 3'-binding, the miR:target stability, the AU composition of the target site, its distance from the closest end of the target RNA, and the number of sites on the same target RNA.

MiRanda first identifies regions of complementarity between miR and target RNA and afterwards calculates the free energy of the RNA duplex.

In PITA, after the identification of possible RNA duplexes, the algorithm calculates the net free energy (ddG). The latter is the difference between the stability of the miR:target (energy gained, dGduplex) minus the energy necessary to unfold the region surrounding the target site (dGopen) which is necessary for microRNA binding.

The schematics showing the bioinformatics predictions were generated using BioPerl[54].

Statistical analysis

Statistical significance was calculated using the tools integrated in the software GraphPad Prism v.6. Unless otherwise stated, the unpaired t-test was used for pairwise comparisons. If multiple conditions were compared altogether, one-way analysis of variance with Bonferroni post-test correction was used.

RESULTS

DICER knock-down favours HRV-1B replication

In order to test if microRNAs have a role during HRV infection of bronchial epithelial cells, we disrupted the biogenesis of microRNAs by knocking-down DICER in BEAS-2B cells. The latter is a virally immortalized cell line derived from healthy human bronchial epithelial cells[55] that has often been employed as a convenient model for HRV infection of this cell type, *e.g.*,[56]. To establish if DICER knock-down would compromise substantially the expression of mature microRNAs, we performed several rounds of transfection with either siRNA against DICER, or negative control siRNA. Total cellular RNA was extracted 48 h after each transfection (Figure 1A) and the siRNA efficacy was verified measuring

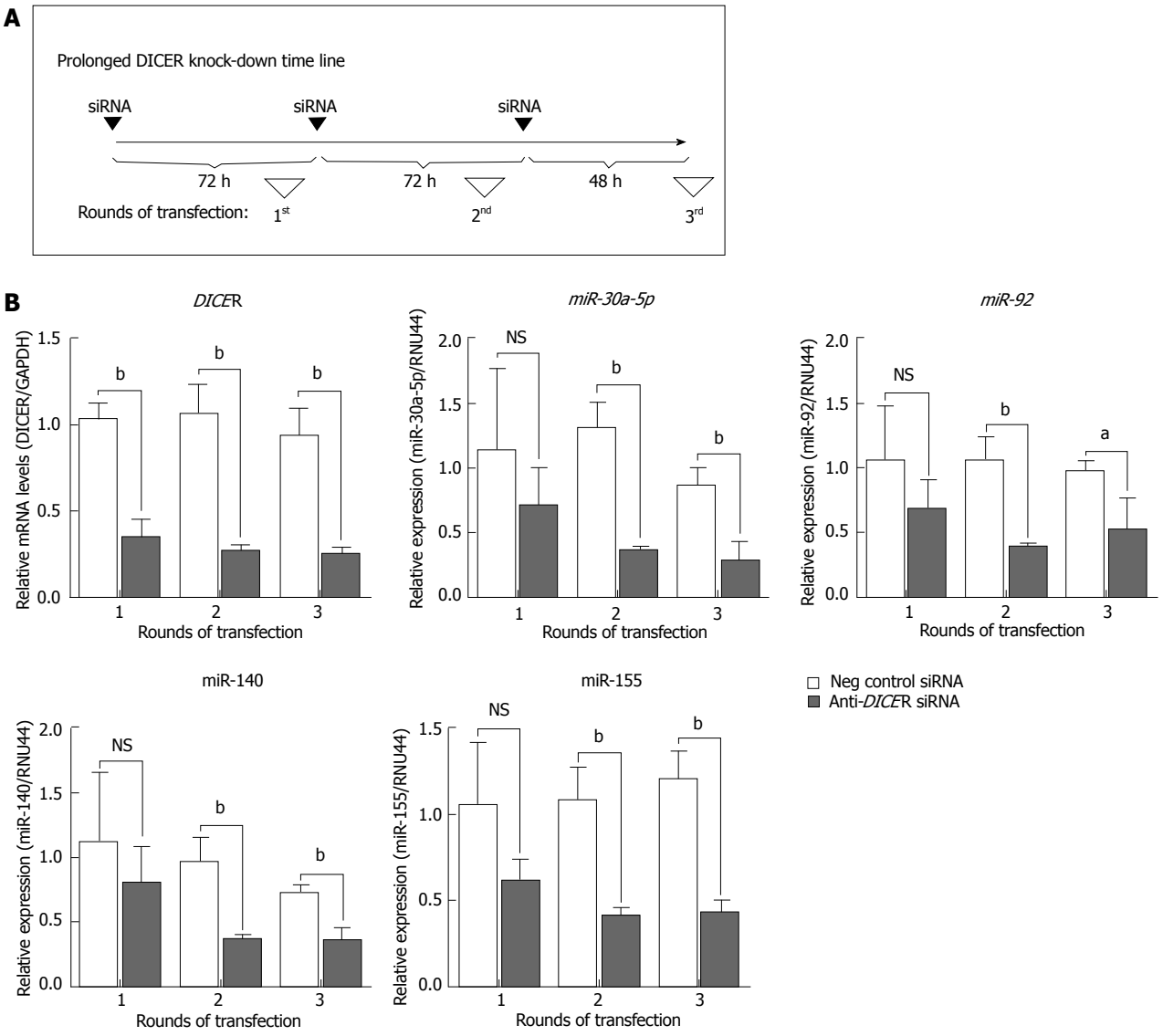

Figure 1 Prolonged knock-down of DICER is necessary in order to effectively lower the levels of mature microRNAs. Thirty nmol/L of either anti-DICER or negative control siRNA were used. A: Global timeline of the experiment; B: DICER mRNA or expression of the indicated microRNAs (miR-30a-5p, -92, -140, 155) was quantified by reverse transcription-quantitative polymerase chain reaction. The values plotted represent the mean ± SD, of three independent experiments. For all graphs, unpaired *t* test was used to calculate the *P* values for anti-DICER *vs* negative control siRNA samples. $^{a}P < 0.05$; $^{b}P < 0.01$. NS: Not significant.

DICER mRNA by RT-qPCR. In addition, we measured the expression of 4 miRs (miR-155, -140, -92, 30a-5p) to detect changes in the production of cellular microRNAs (Figure 1B). On the one hand, we found that DICER mRNA was significantly and consistently reduced by the anti-DICER siRNA since the first round of transfection. On the other hand, 48 h after the first transfection, the levels of mature microRNAs were only marginally affected compared to negative control. In fact, the microRNAs appeared to be reduced by less than 50% and such a difference was not statistically significant. After the second and third round of transfection instead, all four microRNAs were significantly reduced, with residual levels scoring below 50% of the respective negative controls. Therefore, we infected BEAS-2B cells with three different doses of HRV-1B, after three rounds of siRNA transfection (Figure 2). For each dose of virus used, DICER knock-down increased the intracellular levels of viral RNA, as measured 8 h post-infection. This experiment was repeated three more times using only the intermediate dose of virus (MOI = 0.01) confirming the previous results: cells lacking microRNAs (Figure 3A and B) had about 40% more viral RNA than negative control, both at 8 and 24 h post-infection (Figure 3C). Crucially, the levels of IFN-β mRNA were not different between cells transfected with either anti-DICER or negative control siRNA (Figure 3D). This suggested that DICER knock-down did not alter the expression of IFN-β neither before nor during viral infection. Therefore, higher levels of viral RNA were not due to altered IFN-β induction, suggesting that the disruption of microRNA maturation affected viral replication through a mechanism independent from the IFN-β pathway. Considering that there was no difference in the level of viral RNA that entered the cells (0 h post-infection) but differences were detected only at 8 and 24 h post-infection, the mechanism responsible for

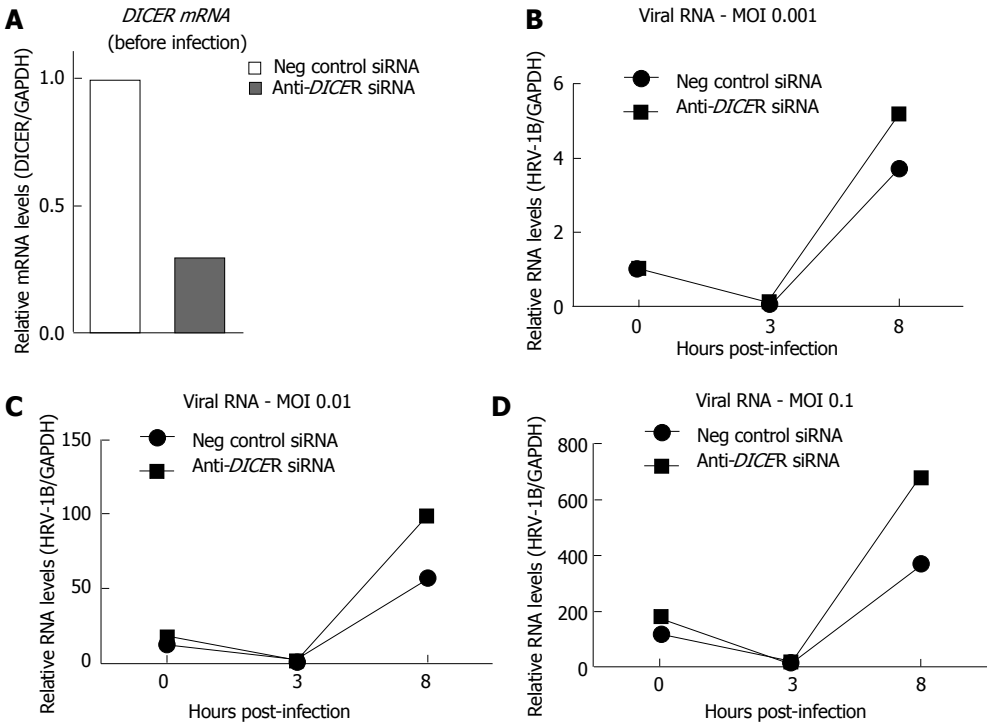

Figure 2 Prolonged DICER knock-down enhanced human rhinovirus-1B replication (preliminary experiments). BEAS-2B cells were transfected for three rounds with either a negative control siRNA or anti-DICER siRNA. Forty-eight hours after the third transfection, cells were infected with the indicated amount of HRV-1B, expressed as MOI (multiplicity of infection). Reverse transcription-quantitative polymerase chain reaction was used to quantify (A) DICER mRNA levels before infection or (B-D) HRV-1B RNA at 0, 3 or 8 h post-infection (HPI). The plotted values represent the average of qPCR duplicates from one experiment. The 0 HPI sample at MOI 0.001 was used as calibrator for all samples.

the higher accumulation of intracellular viral RNA was likely to involve intracellular events of viral replication. Given these considerations, we speculated that one possibility was that mature microRNAs interacted directly with the viral genome. Such interaction would hamper viral replication even if only mildly, as the results from our DICER knock-down experiment suggest.

AGO2 protein interacts with HRV-1B RNA

In order to test if microRNAs interacted directly with the RNA of HRV-1B, we performed RNA immunoprecipitations using an antibody specific for AGO2 (AGO2 co-IP thereafter). With this technique (Figure 4A), it is possible to identify the mRNA molecules that are bound by AGO2 protein and therefore, are targeted by microRNAs[47]. Although human cells express 4 Argonaute proteins (AGO1-4) several reports showed that they associate to largely overlapping pools of microRNAs and target mRNAs. Moreover, AGO2 co-IP has been shown to correctly identify direct targets of both endogenous and exogenously added microRNAs, *e.g.*,[57-61]. In addition, we validated the choice of AGO2 in our protocol by showing that microRNAs were strongly co-purified and that mRNAs, already known to be microRNA targets, could be readily detected in the immunoprecipitated fraction (Figure 5). BEAS-2B cells were infected with HRV-1B, and AGO2 co-IP was performed on cells harvested 6 h post-infection (Figure 4B). Under these conditions, AGO2 was as strongly associated with HRV-1B RNA as it was with SMAD2 mRNA. The latter constitutes a positive control, as it has already been shown to be a target of miR-155[62]. In contrast, GAPDH was not significantly co-precipitated with AGO2, as compared to when an irrelevant antibody was used. The interaction of AGO2 with the genomic RNA of HRV-1B suggested that the latter may be bound by microRNAs during infection of bronchial epithelial cells.

Antagonists of miR-155 or miR-128 enhance HRV-1B replication

To identify specific microRNAs that could directly target HRV-1B, we performed a bioinformatics analysis. We ran Targetscan[5] using a list of microRNAs that we found to be expressed in human primary bronchial epithelial cells (PBECs) as measured by Taqman low density microRNA arrays (Table 2). A number of those microRNAs were predicted to target HRV-1B with varying degrees of efficiency, as summarized by the "context+ score" where the lower the value the better. Among these, we focused on microRNAs that were also shown to be under-expressed in the asthmatic bronchial epithelium[63]. We selected one microRNA (miR-18a) predicted to be very efficient at targeting the virus, two microRNAs with average scores (miR-155 and miR-128) and two microRNAs predicted to be less likely to target the virus (miR-106b and miR-19b) (Table 3). In addition, the five microRNAs had putative sites also on other Rhinoviruses such as HRV-16, -14, -27. Interestingly, of the five microRNAs, miR-155 was predicted to target all the tested genomes in a very similar region at their 3' end (Table 3 and Figure 6).

In order to test experimentally the *in-silico* predictions,

Table 2 MicroRNAs expressed in healthy primary bronchial epithelial cells

Let-7a	miR-149	miR-218	miR-345	miR-501-5p	miR-93
Let-7b	miR-152	miR-221	miR-34a	miR-502-3p	miR-9
let-7c	miR-155	miR-222	miR-34c-5p	miR-502-5p	miR-95
let-7d	miR-15a	miR-223	miR-361-5p	miR-503	miR-96
let-7e	miR-15b	miR-22	miR-362-3p	miR-505	miR-98
let-7f	miR-16	miR-224	miR-362-5p	miR-519a	miR-99a
let-7g	miR-17	miR-23a	miR-365	miR-523	miR-99b
miR-100	miR-181a	miR-23b	miR-374a	miR-532-3p	
miR-101	miR-181c	miR-24	miR-374b	miR-532-5p	
miR-103	miR-182	miR-25	miR-375	miR-542-3p	
miR-106a	miR-183	miR-26a	miR-376a	miR-545	
miR-106b	miR-184	miR-26b	miR-376c	miR-548d-5p	
miR-107	miR-185	miR-27a	miR-379	miR-574-3p	
miR-10a	miR-186	miR-27b	miR-410	miR-576-3p	
miR-125a-3p	miR-18a	miR-28-3p	miR-411	miR-579	
miR-125a-5p	miR-18b	miR-28-5p	miR-422a	miR-582-3p	
miR-125b	miR-191	miR-296-5p	miR-423-5p	miR-582-5p	
miR-126	miR-192	miR-29a	miR-424	miR-589	
miR-127-3p	miR-193a-3p	miR-29b	miR-425	miR-590-5p	
miR-128	miR-193a-5p	miR-29c	miR-429	miR-597	
miR-129-3p	miR-193b	miR-301a	miR-449a	miR-598	
miR-130a	miR-194	miR-301b	miR-449b	miR-618	
miR-130b	miR-195	miR-30b	miR-450a	miR-625	
miR-132	miR-197	miR-30c	miR-450b-5p	miR-627	
miR-134	miR-198	miR-31	miR-452	miR-628-5p	
miR-135a	miR-19a	miR-320	miR-454	miR-629	
miR-135b	miR-19b	miR-323-3p	miR-455-3p	miR-636	
miR-136	miR-200a	miR-32	miR-455-5p	miR-642	
miR-138	miR-200b	miR-324-3p	miR-483-5p	miR-652	
miR-139-5p	miR-200c	miR-324-5p	miR-484	miR-655	
miR-140-3p	miR-202	miR-328	miR-485-3p	miR-660	
miR-140-5p	miR-203	miR-330-3p	miR-486-3p	miR-671-3p	
miR-141	miR-204	miR-331-3p	miR-486-5p	miR-708	
miR-142-3p	miR-205	miR-331-5p	miR-487a	miR-744	
miR-145	miR-20a	miR-335	miR-489	miR-758	
miR-146a	miR-20b	miR-339-3p	miR-491-5p	miR-885-5p	
miR-146b-5p	miR-210	miR-339-5p	miR-494	miR-886-3p	
miR-148a	miR-212	miR-340	miR-495	miR-886-5p	
miR-148b	miR-21	miR-342-3p	miR-500	miR-92a	

Here are reported only the miRs with Ct values lower than 40, in at least 2 (out of 4) samples of primary bronchial epithelial cells (PBECs) from healthy donors.

we transfected BEAS-2B cells with microRNA antagonists (anti-miRs). To test the efficiency of anti-miR transfection, we measured the expression of miR-18a, -106b, -128 and -155 by RT-qPCR, 24 h post-transfection (Figure 7) confirming reduction (from 70% to 90%) of the measured microRNAs. Twenty four hours after anti-miR transfection, BEAS-2B cells were infected with HRV-1B. Cells were collected 8 h post-infection and viral RNA was quantified by RT-qPCR (Figure 8). The results showed that, as expected given their positive "context + scores", miR-106b and miR-19b did not alter viral replication. The microRNA predicted to be the best match for HRV-1B, miR-18a, did not affect viral replication either. However, when miR-155 or miR-128 was silenced, we observed an increase in viral RNA of approximately 50% compared to a negative control anti-miR. Notably, the different constructs transfected did not alter the levels of GAPDH mRNA across all the samples of infected cells (Figure 9B). As an additional control we also noted that the total cellular RNA did not change among the samples transfected with specific anti-miRs (Figure 9A). In addition, the same analysis on independently transfected cells that were not subsequently infected with HRV-1B (Figure 9C and D) confirmed that these parameters were not affected by any of the anti-miRs used. These observations suggest that the measured increase of intracellular viral RNA was not influenced by the normalisation method adopted, and that in our experiments cell viability was not significantly altered by the different anti-miRs transfected.

Finally, we generated two lentivirally transduced BEAS-2B cell lines, in order to study the effect of miR-155 over-expression on the replication of HRV-1B (Figure 10). These experiments showed that from 40% to 50% less viral RNA accumulated in cells that over-expressed miR-155, compared to the relative negative control, thus reinforcing the results obtained for this microRNA in the anti-miR experiments.

DISCUSSION

Innate immunity plays a vital role in the antiviral response of human cells. In particular, the importance of the IFN

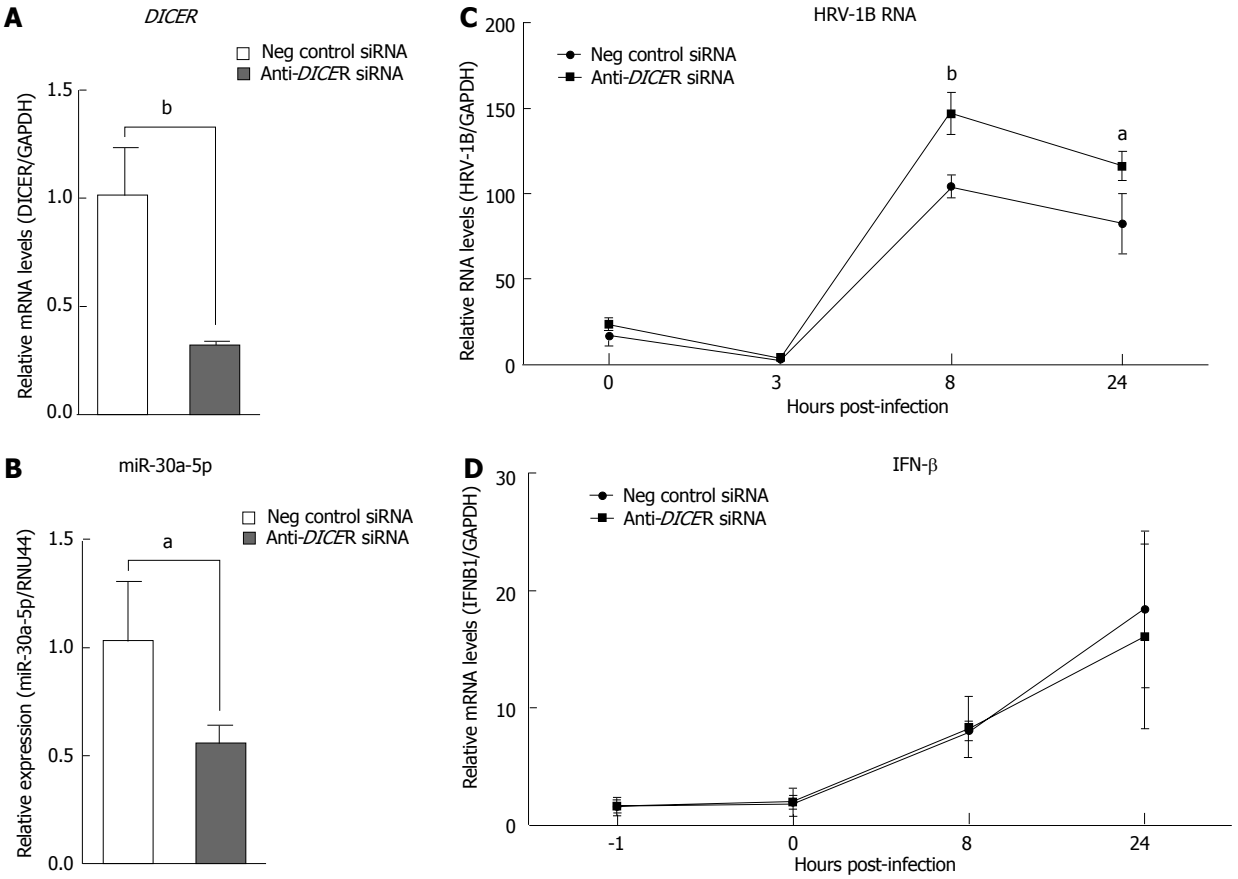

Figure 3 Prolonged DICER knock-down leads to higher levels of viral RNA. BEAS-2B cells were transfected for three rounds (using 30 nmol/L of siRNA) and then infected with human rhinovirus (HRV)-1B at MOI 0.01. Cells collected just before infection were used to measure DICER mRNA levels (A) and miR-30a-5p expression (B) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). HRV-1B RNA (C) and IFNB1 mRNA (D) levels in infected cells were measured by RT-qPCR at the indicated time points. In (D) the time point "-1" refers to uninfected cells, collected just before infection. In (C) all the samples were normalised as done for data in Figure 2 [0 h post-infection (HPI), MOI 0.001 sample as calibrator]. Plotted values represent the mean ± SD, of three independent experiments. For all panels, unpaired *t* test was used to calculate the *P* values for anti-DICER *vs* negative control siRNA samples.[a]*P* < 0.05; [b]*P* < 0.01. NS: Not significant; MOI: Multiplicity of infection.

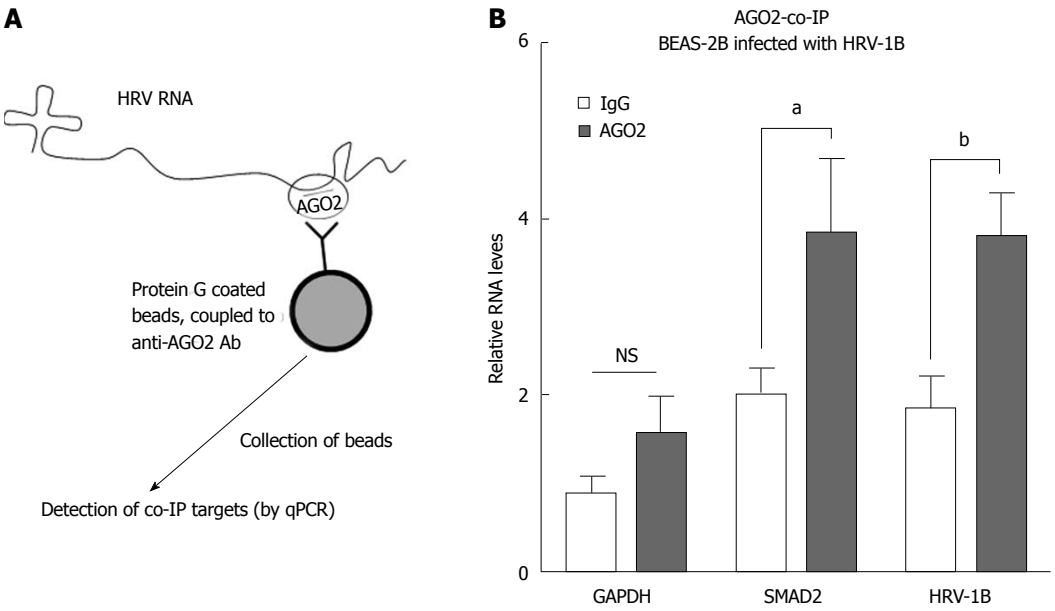

Figure 4 Human rhinovirus-1B RNA co-immunoprecipitates with AGO2 protein in BEAS-2B cells. A: Schematic representation of the rationale behind AGO2 co-IP experiments; B: BEAS-2B cells were infected with HRV-1B at MOI 0.01. Six hours post-infection cells were collected and AGO2 co-IP was performed. Plotted values represent the mean ± SD, of three independent experiments. The formula used takes into account the abundance of the RNAs before immunoprecipitation (see materials and methods). Unpaired *t* test was used to calculate the *P* values for AGO2 *vs* IgG control. NS: Not significant (*P* > 0.05); [a]*P* < 0.05; [b]*P* < 0.01. MOI: Multiplicity of infection; HRV: Human rhinovirus.

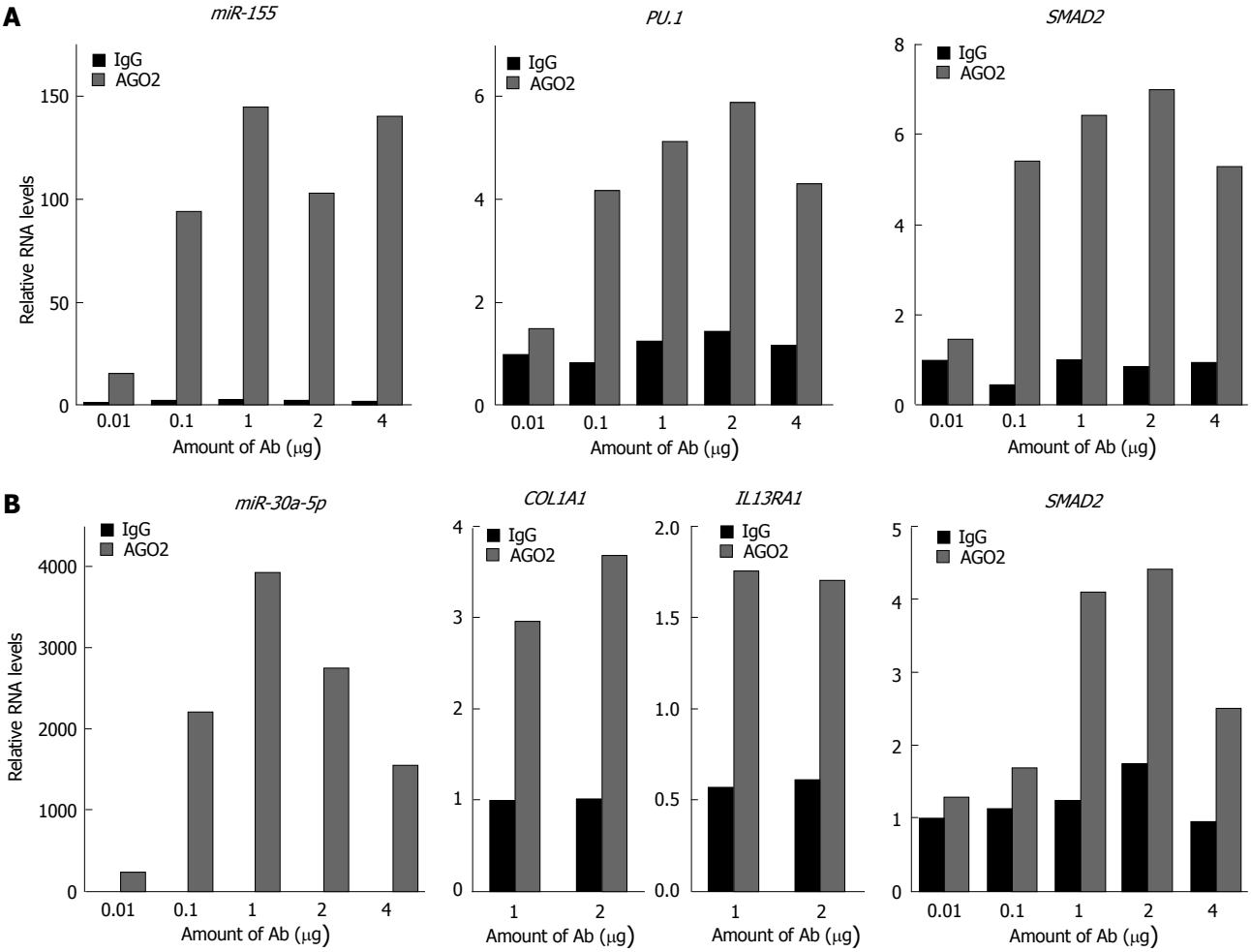

Figure 5 Antibody titration for AGO2 co-IP. These experiments were performed in order to determine the most convenient antibody concentration to use for (A) THP-1 (used as additional control) and (B) BEAS-2B cells: 1μg was chosen as it would give the highest enrichment of both microRNAs and target mRNAs. These results show that microRNAs are very tightly associated with AGO2 protein, and confirm that microRNA-regulated mRNAs are also considerably co-purified. Previous work from our group has shown that PU.1, SMAD2 and IL13RA1 are targeted by miR-155[45,62,89] while COL1A1 has been shown to be targeted by miR-29b elsewhere[90]. IgG refers to the negative control antibody, AGO2 refers to the anti-AGO2 antibody used. Plotted values represent the average of qPCR duplicates from one experiment.

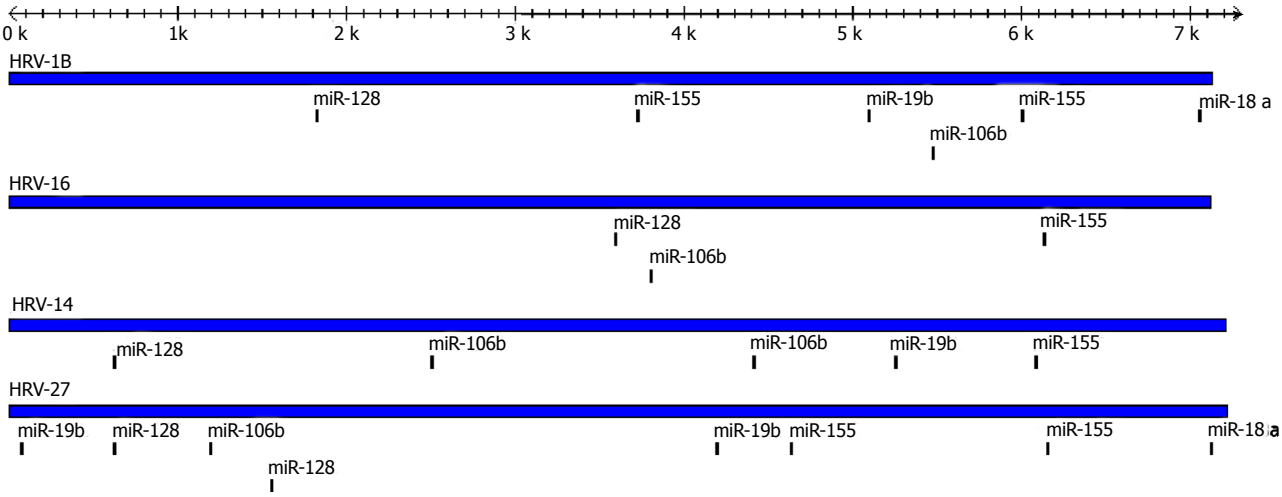

Figure 6 Schematic of targetscan predictions. Indicated are the target sites predicted for miR-18a, -19b, -106b, -128, -155 on two strains of HRV-A (HRV-1B and -16) and two strains of HRV-B (HRV-14 and -27). HRV: Human rhinovirus.

system in antiviral immunity has been long recognized and studied[64]. Early, innate immune responses are particularly well suited for protection from viruses that result in acute infections, such as Rhinoviruses. Although it is still

Table 3 Targetscan predictions on human rhinovirus -1B, -16, -14, -27

Virus	MiR	Site type	Site start	Site end	Context + score	Alignment
HRV-1B	18a	7mer-1a	7053	7059	0.163	site: GUGCACUGUAUAUCCCACCUUAU |||||| |||||| miR: GAUAGACGUGAUCUAC----- -GUGGAAU
HRV-1B	128	7mer-m8	1822	1828	-0.017	site: AAACAUUAGUAUGUACACUGUGC ||||||| miR: UUUCUCUGGCCAAGUGACACU
HRV-1B	155	7mer-m8	3716	3722	-0.014	site: AUAAAUCCAAUCAAU- - - - AGCAUUAG |||| ||||||| miR: UGGGGAUAGUGCUAAUCGUAAUU
HRV-1B	155	7mer-m8	5998	6004	-0.013	site: UGAUUCUAAACCAAUAGCAUUAG ||||||| miR: UGGGGAUAGUGCUAAUCGUAAUU
HRV-1B	19b	8mer-1a	5088	5095	0.021	Site: UAAUUUACAAAUUGUUUUGCACA ||||||| miR: AGUCAAAACGUACCUAAACGUGU
HRV-1B	106b	7mer-m8	5471	5477	0.025	Site: CCAGAAUGUAAUUUAGCACUUUC ||||||| miR: UAGACGUGACAGUCGUGAAAU
HRV-16	128	7mer-m8	3593	3599	-0.04	Site: GAUCUUAGACACUUUCACUGUGC |||| ||||||| miR: UUUCUCUGGCCAAGUGACACU
HRV-16	155	7mer-m8	6133	6139	-0.036	Site: UUCCAAACUUAAGCUAGCAUUAG ||||||| miR: UGGGGAUAGUGCUAAUCGUAAUU
HRV-16	106b	7mer-1a	3802	3808	0.107	Site: GCAAACAAUAAUUGCCACUUUAA |||||| miR: UAGACGUGACAGUCGUGAAAU
HRV-14	128	7mer-1a	620	626	-0.059	Site: CAUAUAUAUACAUAUACUGUGAU |||||| miR: UUUCUCUGGCCAAGUGACACU
HRV-14	155	7mer-m8	6082	6088	-0.056	Site: ACUUACACUAAAAGAAGCAUUAU ||||||| miR: UGGGGAUAGUGCUAAUCGUAAUU
HRV-14	19b	7mer-m8	5255	5261	-0.001	Site: GGACCAAACACAGAAUUUGCACU ||||||| miR: AGUCAAAACGUACCUAAACGUGU
HRV-14	106b	8mer-1a	2503	2510	0.047	Site: CAGAACUACCUACAUGCACUUUA ||||||| miR: UAGACGUGACAGUCGUGAAAU
HRV-14	106b	7mer-m8	4414	4420	0.057	Site: UCCACCAGAUCCCAAGCACUUUG ||||||| miR: UAGACGUGACAGUCGUGAAAU
HRV-27	18a	7mer-1a	7121	7127	-0.134	Site: AGGUUCUUGUGAUACCACCUUAC |||||| miR: GAUAGACGUGAUCUACGUGGAAU
HRV-27	19b	7mer-m8	66	72	-0.11	Site: CUGGUAUUUUGUACC- - - UUUGCACG ||||| ||||||| miR: AGUCAAAACGUACCUAAACGUGU
HRV-27	128	7mer-1a	619	625	-0.053	Site: AUAUAUAGCAUAUAUACUGUGAU |||||| miR: UUUCUCUGGCCAAGUGACACU
HRV-27	155	7mer-1a	6152	6158	0.018	Site: CUUAUGUUAGUUUGGGCAUUAAG |||||| miR: UGGGGAUAGUGCUAAUCGUAAUU
HRV-27	106b	8mer-1a	1193	1200	0.025	Site: CAAAAUUUCACAGUG--GCACUUUA ||| ||||||| miR: UAGACGUGACAGUCGUGAAAU
HRV-27	128	7mer-1a	1552	1558	0.049	Site: CCCAGUAUACCUGUU--ACUGUGAC ||| |||||| miR: UUUCUCUGGCCAAGUGACACU
HRV-27	19b	7mer-m8	4192	4198	0.058	Site: CAAAUGUCUCAGAAGUUUGCACC ||||||| miR: AGUCAAAACGUACCUAAACGUGU
HRV-27	155	7mer-m8	4632	4638	0.097	Site: UGUGUUGAACCCUGAAGCAUUAG ||||||| miR: UGGGGAUAGUGCUAAUCGUAAUU

HRV: Human rhinovirus.

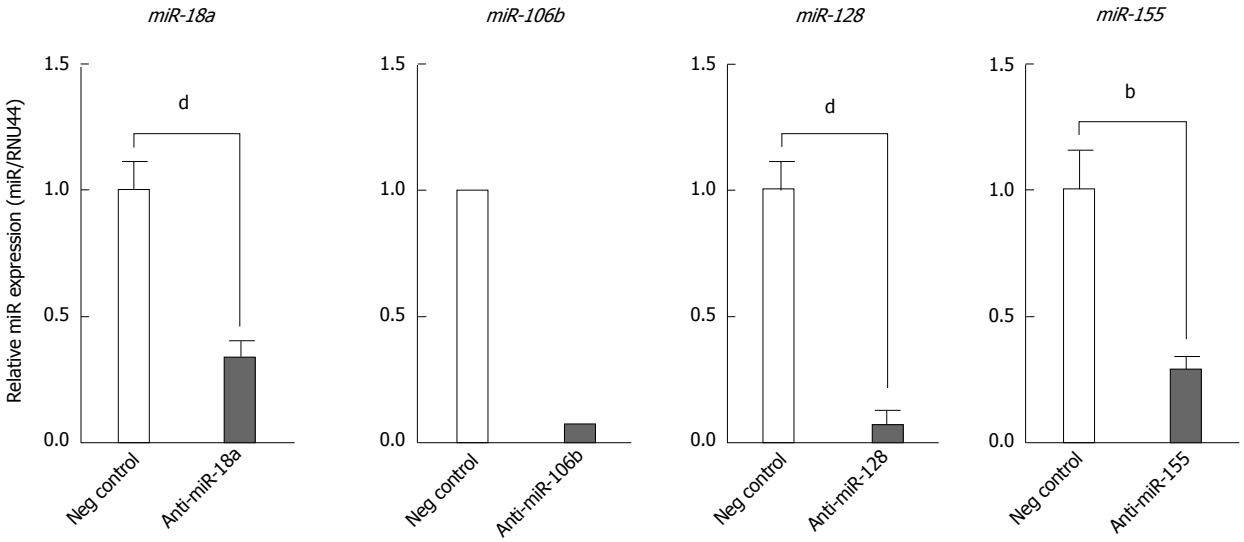

Figure 7 Effect of anti-miR transfection on microRNA expression in BEAS-2B cells. MicroRNA expression was quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) 24 h post-transfection with either a negative control anti-miR or the indicated specific anti-miR. Plotted values for miR-18a, -128 and -155 represent the mean ± SD of three independent experiments. Unpaired *t* test was used to calculate *P* values for cells transfected with the specific anti-miR *vs* negative control anti-miR. $^{b}P < 0.01$; $^{d}P < 0.01$. The values plotted for miR-106b represent the average of qPCR duplicates from one experiment.

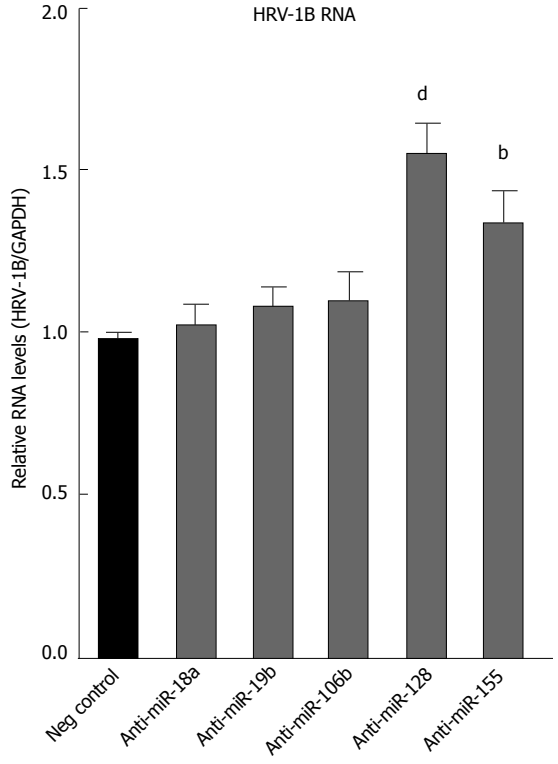

Figure 8 Antagonists of miR-155 and miR-128 enhance human rhinovirus-1B replication in BEAS-2B cells. BEAS-2B cells were transfected with 100 nmol/L of the indicated anti-miR. The following day, cells were infected with HRV-1B (multiplicity of infection of 0.01). HRV (human rhinovirus)-1B RNA was measured by RT-qPCR from samples collected at 8 h post-infection. Plotted values represent the mean ± SD, from 3 independent experiments. The *P* values were calculated for each specific anti-miR vs the negative control anti-miR, using one way ANOVA with Bonferroni correction. $^{b}P < 0.001$; $^{d}P < 0.0001$.NS: Not significant.

under debate whether virus-induced RNA interference (RNAi) is an antiviral mechanism actually conserved in human cells[65,66] it has become clear that microRNAs play an important role in human innate antiviral immunity.

We hypothesized that microRNAs inhibit the replication of human Rhinoviruses. This would be of interest in the fields of asthma and COPD, since Rhinoviruses constitute major exacerbation triggers of these conditions. In particular, it has been shown that Rhinoviruses replicate more in PBECs from asthmatics than in cells from healthy subjects and that the former express less IFN-β and IFN-gamma during infection[44,67]. Recently, it has been reported that the bronchial epithelium of asthmatics present a dysregulated microRNA profile[63] with prevalence of under-expressed microRNAs. Therefore, if microRNAs that are under-expressed in asthmatic PBECs were able to hold back Rhinovirus replication, it can be speculated that also their deficiency may contribute to the weaker antiviral immunity of asthmatic cells.

MicroRNA maturation involves the activity of two endonucleases that progressively shorten the initial transcript. DROSHA cleaves the primary microRNA (pri-miR) in the nucleus, while DICER is responsible for trimming the precursor microRNA (pre-miR) in the cytoplasm, and finally the mature microRNA assembles with AGO proteins in order to be functional[3,4]. Hence, impairing the activity of either DROSHA or DICER has been shown to lead to a reduction of mature microRNAs[68,69]. In order to test whether microRNAs affect the replication of Human Rhinovirus, we opted to target DICER in bronchial epithelial cells. A very specific protocol had to be followed to generate the desired knock down of microRNAs; transfecting BEAS-2B cells with anti-DICER siRNA for three consecutive times showed that microRNAs were reduced to about 40%-30% only after the second round of transfection (Figure 1) equivalent to five days of DICER silencing.

Our results are in agreement with other published work where, 48 h after transfection of anti-DICER siRNA, only a subset of microRNAs was affected, and in general their reduction was very rarely above 50%[68,70-72].

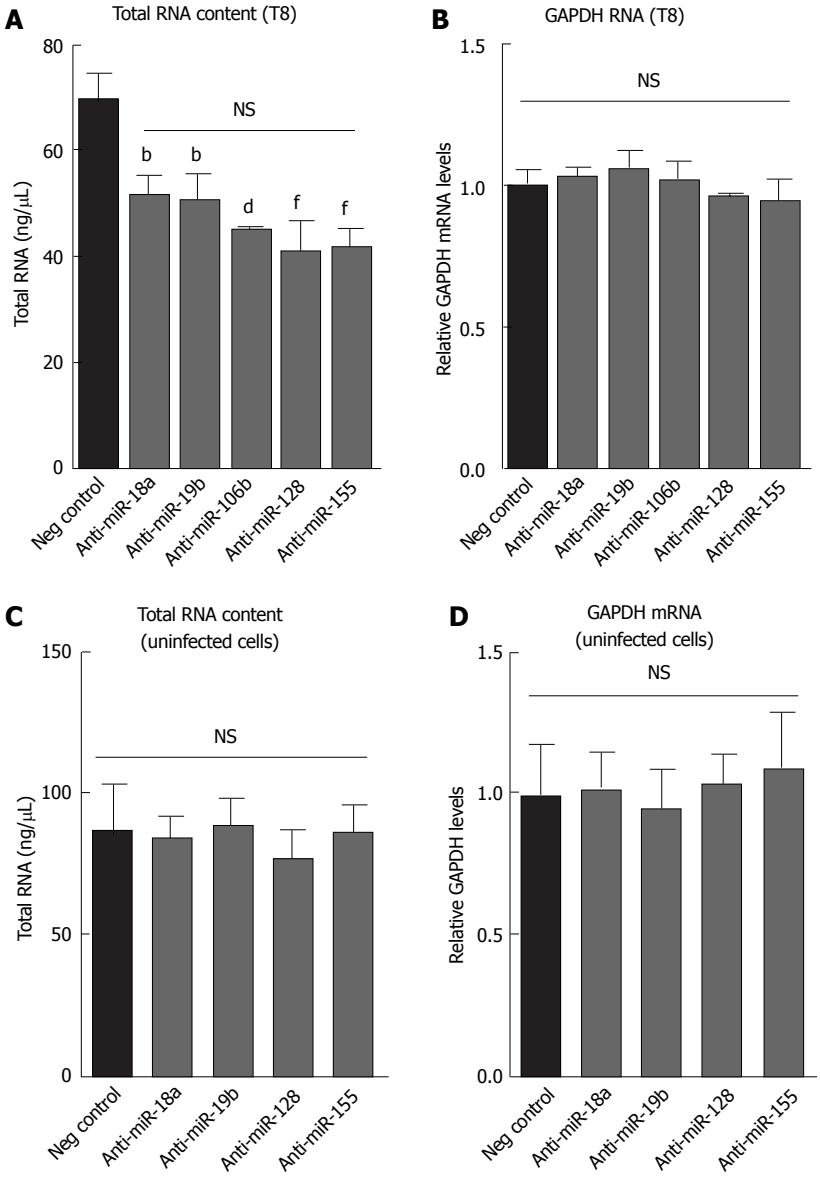

Figure 9 Effect of anti-miR transfection on total cell RNA and GAPDH expression in BEAS-2B cells. (A and B) Values obtained for the samples used for Figure 5 or (C and D) from cells transfected with the indicated anti-miRs but not infected with HRV-1B. The values plotted represent the mean ± SD of three independent experiments. The *P* values were calculated across all the anti-miRs, using one way ANOVA with Bonferroni correction. In A, each specific antimiR was significantly different *vs* negative control ($^{b}P < 0.01$; $^{d}P < 0.001$; $^{f}P < 0.0001$) but not significantly different to one another. NS: Not significant.

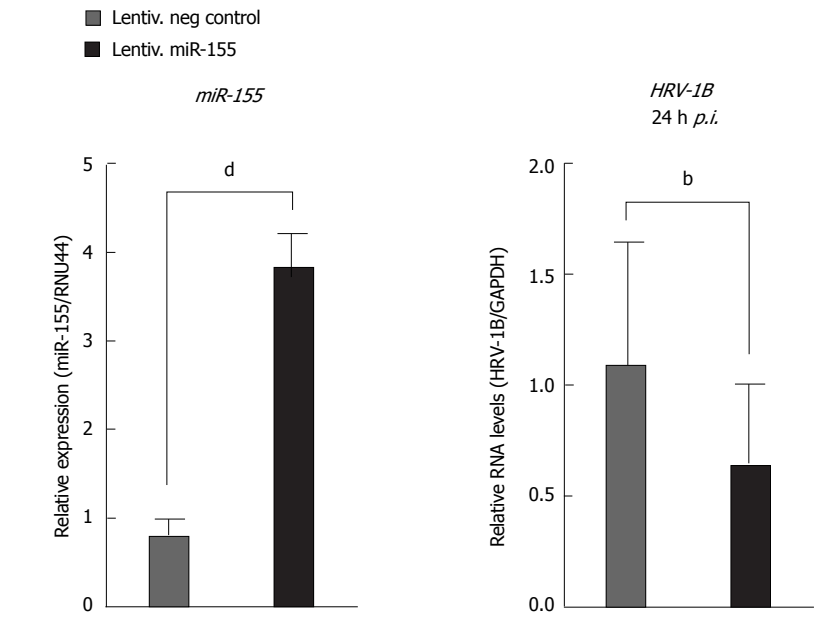

Figure 10 MiR-155 over-expression inhibits human rhinovirus-1B replication. Lentivirally transduced BEAS-2B cells were infected with human rhinovirus-1B (HRV-1B) at an MOI of 0.01. MiR-155 expression of uninfected cells or HRV-1B RNA (24 h post-infection) was measured by RT-qPCR from three independent experiments. *P* values for miR-155-overexpressing cells *vs* negative control cells were calculated using the unpaired *t* test for miR-155 expression, or the ratio paired *t* test for HRV-1B RNA; $^{b}P < 0.01$, $^{d}P < 0.001$.

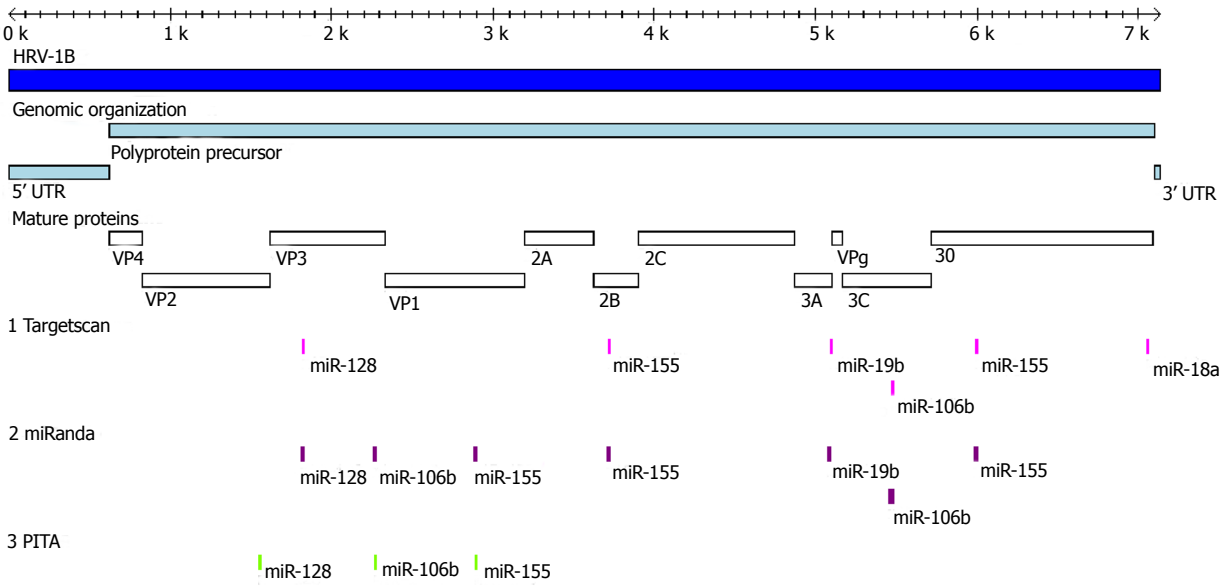

Figure 11 Schematic comparison of microRNA predictions on human rhinovirus-1B. Indicated are the target sites predicted for miR-18a, -19b, -106b, -128, -155 on HRV-1B by Targetscan, miRanda and PITA algorithms. In addition, the genomic organization and the boundaries of the viral mature proteins are shown.

Table 4 MiRanda predictions on human rhinovirus-1B

Virus	miR	Site start	Site end	Score	Energy (kcal/mol)		Alignment
HRV-1B	106b	2257	2276	141	-18.24	miR:	uaGACGUGACAGUCGUGAAAu
							||||| ||| || ||||
						site:	uuCUGCA-UGUAAGGACUUUu
HRV-1B	155	3704	3723	156	-17.59	miR:	ugGGGAUAGUGCUAAUCGUAAUu
							:|| |||| | ||||||||
						site:	aaUCCAAUCA-- --A-UAGCAUUAg
HRV-1B	106b	5459	5478	160	-14.6	miR:	uagACGUGACAGUCGUGAAAu
							||: | | |:||||||||
						site:	gaaUGUAAU--UUAGCACUUUc
HRV-1B	155	2878	2901	144	-14.2	miR:	ugGGGAUAGU--GCUAAUCGUAAUu
							|:|| || : ||||||||:
						site:	cuCUUUACCAUUUCUUAGCAUUGc
HRV-1B	155	5984	6005	159	-13.65	miR:	ugGGGAUAGUGCUAAUCGUAAUu
							:: ||| || ||||||||
						Site:	gaUUCUA--AACCAAUAGCAUUAg
HRV-1B	128	1809	1829	147	-13.12	miR:	uuucucuggcCAAGUGACACu
							|| ||||||
						Site:	acauuaguauGUACACUGUGc
HRV-1B	19b	5071	5095	153	-10.35	miR:	agucaAAACG- - - UACCUAAACGUGu
							||| | || | ||||||
						Site:	uguaaUUUACAAAUUGUUUUGCACa

HRV: Human rhinovirus.

Schmitter *et al*[69] showed that an anti-DICER shRNA had to be expressed for at least 3 to 7 d in HEK cells, in order to effectively abolish microRNA repression of a luciferase construct. In addition, Gantier *et al*[73] specifically studied microRNA stability, showing that different miRs have different turnover rates, and estimated that the average half-life in HEK cells is about 5 d.

DICER knock-down, and the subsequent reduction of microRNAs, allowed an increase of viral RNA following infection with HRV-1B (Figure 3) suggesting that the microRNA machinery affected the intracellular stages of Rhinovirus replication. In addition, considering that AGO proteins are responsible for the activity of microRNAs, our AGO2 co-IP results suggest that microRNAs bound the viral RNA during infection of bronchial epithelial cells (Figure 4). This encouraged us to use bioinformatics in order to identify possible antiviral microRNA candidates. From the candidates identified by Targetscan, we selected five microRNAs known to be deficient in asthmatic PBECs: miR-18a, -19b, -106b, -128 and -155 ([63] and our data, not shown). Using anti-miRs, we confirmed that, out of those microRNAs, miR-128 and miR-155 had antiviral activity against HRV-1B in BEAS-2B cells (Figure 8): inhibition of miR-128 or

Table 5 PITA predictions on human rhinovirus-1B

Virus	miR	Site start	Site end	dG duplex	ddG		Alignment
HRV-1B	155	2901	2893	-15.12	-11.87	Site:	UCUUUACCAUUUCUUAGCAUUGCG
							|| || |||||||:
						miR:	UGGGGAUAGU-GCUAAUCGUAAUU
HRV-1B	128	1562	1554	-16.4	-11.11	Site:	CUAACAUAAGACCAAUCACUGUAUCA
							|||||||
						miR:	UUUCUCUGGCCAAGUGACACU
HRV-1B	106b	2276	2268	-17.2	-10.9	Site:	GUUUCUGCAUGUAAGGACUUUUG
							|||||||||||
						miR:	UAGACGUGACAGUCGUGAAAU

The cut off was set at -10 (ddG). HRV: Human rhinovirus.

Table 6 Full target site list predicted by PITA on human rhinovirus-1B

Virus	microRNA	Start	End	dGduplex	dGopen	ddG
HRV-1B	miR-155	2901	2893	-15.12	-3.24	-11.87
HRV-1B	miR-128	1562	1554	-16.4	-5.28	-11.11
HRV-1B	miR-106b	2276	2268	-17.2	-6.29	-10.9
HRV-1B	miR-18a	2124	2116	-14.81	-4.9	-9.9
HRV-1B	miR-106b	4777	4769	-17.81	-7.96	-9.84
HRV-1B	miR-19b	6668	6660	-14.1	-5.07	-9.02
HRV-1B	miR-18a	7047	7039	-23.1	-15.01	-8.08
HRV-1B	miR-155	3186	3178	-14.3	-7.56	-6.73
HRV-1B[1]	miR-155	3723	3715	-14.9	-8.5	-6.39
HRV-1B	miR-19b	2016	2008	-12.11	-5.91	-6.19
HRV-1B	miR-18a	2570	2562	-16.8	-11.26	-5.53
HRV-1B	miR-18a	3108	3100	-13.5	-8.1	-5.39
HRV-1B	miR-128	3609	3601	-11.5	-6.32	-5.17
HRV-1B[1]	miR-19b	5095	5088	-12.53	-7.64	-4.88
HRV-1B[1]	miR-106b	5478	5470	-15.3	-10.46	-4.83
HRV-1B[1]	miR-155	6005	5997	-11.6	-7.01	-4.58
HRV-1B	miR-18a	4144	4138	-12.06	-7.91	-4.14
HRV-1B	miR-128	3513	3505	-14.3	-10.41	-3.88
HRV-1B	miR-19b	77	70	-10.42	-6.61	-3.8
HRV-1B	miR-106b	5542	5534	-10.6	-6.95	-3.64
HRV-1B	miR-155	5028	5020	-8.1	-4.94	-3.15
HRV-1B	miR-18a	4792	4784	-11.5	-8.37	-3.12
HRV-1B	miR-155	3552	3544	-12.6	-9.54	-3.05
HRV-1B	miR-106b	3585	3577	-12	-9.41	-2.58
HRV-1B	miR-18a	1686	1680	-14.19	-11.87	-2.31
HRV-1B	miR-106b	722	714	-4.36	-2.12	-2.23
HRV-1B	miR-128	2562	2554	-14.3	-12.1	-2.19
HRV-1B	miR-155	3975	3967	-13.8	-11.77	-2.02
HRV-1B	miR-128	442	434	-17.2	-15.21	-1.98
HRV-1B	miR-155	6621	6613	-6.5	-4.56	-1.93
HRV-1B	miR-155	4551	4543	-7.3	-5.68	-1.61
HRV-1B	miR-155	5978	5970	-7.9	-6.41	-1.48
HRV-1B	miR-18a	1431	1423	-6.6	-5.13	-1.46
HRV-1B	miR-19b	3248	3240	-8.71	-7.66	-1.04
HRV-1B	miR-106b	6598	6590	-11.8	-10.94	-0.85
HRV-1B[1]	miR-128	1829	1822	-12.9	-12.08	-0.81
HRV-1B	miR-155	6962	6954	-6	-5.38	-0.61
HRV-1B	miR-106b	569	563	-10.2	-9.77	-0.42
HRV-1B	miR-155	870	862	-9.57	-9.26	-0.3
HRV-1B	miR-18a	5478	5471	-10.7	-10.46	-0.23
HRV-1B	miR-155	3584	3576	-9.6	-9.41	-0.18
HRV-1B	miR-155	6258	6250	-9.17	-8.98	-0.18
HRV-1B	miR-19b	3550	3542	-10.4	-10.38	-0.019
HRV-1B	miR-106b	5269	5261	-10	-10	0.0096
HRV-1B	miR-106b	6533	6525	-11.4	-11.44	0.047
HRV-1B[1]	miR-18a	7059	7053	-18.7	-19.34	0.64
HRV-1B	miR-19b	2309	2301	-6.4	-7.08	0.68
HRV-1B	miR-19b	5076	5068	-9.2	-10.03	0.83
HRV-1B	miR-106b	177	169	-14.3	-15.51	1.21
HRV-1B	miR-155	624	616	-9.39	-11.23	1.84

HRV-1B	miR-106b	1105	1098	-9.2	-11.47	2.27
HRV-1B	miR-155	4265	4257	-6.91	-9.29	2.38
HRV-1B	miR-155	6641	6633	-5.8	-8.21	2.41
HRV-1B	miR-18a	4866	4858	-8.3	-11.02	2.72
HRV-1B	miR-18a	569	561	-7	-9.77	2.77
HRV-1B	miR-106b	6286	6278	-3.51	-6.53	3.02
HRV-1B	miR-128	6964	6956	-5.69	-8.77	3.08
HRV-1B	miR-155	2384	2376	-4.1	-7.44	3.34
HRV-1B	miR-155	234	226	-6.15	-9.76	3.61
HRV-1B	miR-19b	622	614	-7.4	-11.06	3.66
HRV-1B	miR-106b	441	435	-10.5	-15.27	4.77
HRV-1B	miR-155	6240	6232	-6	-12.02	6.02
HRV-1B	miR-106b	6184	6176	-7.9	-14.56	6.66
HRV-1B	miR-106b	549	541	-6.3	-15.77	9.47
HRV-1B	miR-106b	7007	6999	-7.61	-18.39	10.78

[1]Sites identified also by targetscan. HRV: Human rhinovirus.

miR-155 lead to a modest but significant increase of viral RNA, which was roughly similar to what obtained by the prolonged DICER silencing. Conversely, miR-155 over-expression inhibited viral replication (Figure 10).

MicroRNA-128 has been shown to play an important role in the control of apoptosis in glioblastoma[74-76]. MiR-128 has also been shown to regulate EGFR expression in non-small-cell lung cancer cell lines[77] and to be down-regulated in the airway epithelium of smokers[78]. It would certainly be of interest to investigate whether the lack of miR-128 in asthmatic PBECs contributes to their higher EGFR expression[79] or if this microRNA affects the virally induced apoptosis in bronchial epithelial cells[44]. However, considering the short time post-infection adopted (8 h), it seems unlikely that in our experiments the inhibition of miR-128 increased viral replication by affecting the induction of apoptosis.

MicroRNA-155 plays an important role in different processes[80] from physiological ones such as haematopoiesis, cellular differentiation and immune responses, to malignancies[13,81-85]. Importantly, miR-155 has been shown to be up-regulated by a series of microbial components such as LPS. Although the functions of miR-155 in non-hematopoietic cells have been much less studied, the antiviral activity we postulate for miR-155 in bronchial epithelial cells would fit with the large involvement of this microRNA in immunity.

It was surprising that the inhibition of miR-18a did not affect HRV-1B replication, despite the promising context+ score calculated by Targetscan. Therefore, we used two additional microRNA predictive programs on HRV-1B: miRanda and PITA (see Figure 11 and Tables 4 and 5). Despite large overlap between the sites identified by Targetscan and miRanda, the latter did not find a miR-18a target on HRV-1B. PITA identified favourable sites only for miR-155, -128 and -106b, in order of predicted efficiency. In combination with our experimental results (only miR-128 and -155 affected viral replication) this analysis highlights the importance to adopt multiple prediction algorithms. Moreover, the results from PITA suggest that the presence of secondary structures in the viral genome may prevent miR-18a from interacting with its target site. As a matter of fact, target site accessibility has been shown to be an important factor in determining microRNA efficacy[51]. Of the three algorithms, PITA is the only one to take into account this feature of microRNA biology (see materials and methods for more details). Notably, PITA could identify all the sites also predicted by Targetscan, but estimated their accessibility too low for the microRNA to have an effect on HRV-1B (Table 6).

The Rhinovirus genomic RNA is directly translated into a single polyprotein which further matures into separate proteins. Therefore, it is a single transcriptional unit *i.e.*, it is comparable to a single cellular mRNA. However, while miRs are often found to target the 3'UTR of cellular mRNAs, the putative miR sites for HRV-1B fall within the coding region (Figure 11). This is in agreement with what already shown for HCV[23] and Influenza virus[28] and represents a striking difference between microRNA targets on cellular mRNAs and those on viral RNA.

Further investigation is needed to understand the mechanistic details of what shown by our experiments. Our results cannot exclude the possibility that the observed effects were due to an indirect mechanism. It could be argued that the microRNA inhibitors could have affected the IFN-related antiviral machinery. For instance, miR-155 has been shown to target SOCS1, an inhibitor of type I IFN signalling[86]. So, it can be assumed that the inhibition of miR-155 would favour viral replication by increasing SOCS1. However, also miR-19b has been shown to target SOCS1[87] but, in our experiments, the inhibition of this microRNA did not have any effect on viral replication.

Despite these limitations, our data altogether suggest that HRV-1B may be directly targeted by microRNAs. In fact (1) prolonged DICER knock-down did not affect the basal levels of IFN-beta mRNA or its induction by infection; (2) AGO2 protein interacted with HRV-1B RNA during viral replication (6 h post-infection); (3) miR-155 and miR-128 have putative target sites on HRV-1B, as predicted by three different bioinformatics tools; and (4) inhibition of either miR led to a modest but significant increase of intracellular viral RNA, while over-expression of miR-155 had the opposite effect.

In conclusion, our study suggests that cellular microRNAs play a role in the innate immune response against

Rhinoviruses. We also showed that two microRNAs that are under-expressed in the asthmatic epithelium, can affect HRV-1B replication. Therefore, our work encourages speculating that the asthmatic microRNA deficiency recently uncovered[63,88] may contribute to the higher replication of Rhinoviruses in asthmatic PBECs.

COMMENTS

Background

Human Rhinovirus (HRV) is one of the major causes of asthma and chronic obstructive pulmonary disease (COPD) exacerbations, which can be life threatening and represent an unmet clinical need. MicroRNAs, non-coding RNA molecules that regulate gene expression, also participate in innate antiviral immunity.

Research frontiers

Bronchial epithelial cells from asthmatic patients have a defective innate immune response to HRV infection, notably by producing lower levels of interferons, compared to cells from healthy subjects. However, the role of microRNAs in such insufficient immune response has not been investigated, despite the recent finding that microRNA expression is dysregulated in asthmatic cells.

Innovations and breakthroughs

Several reports have highlighted the importance of microRNAs as part of the innate antiviral immunity. Often, microRNAs have been shown to inhibit viral replication by directly targeting viral RNA. While it has been demonstrated that miR-23b could limit HRV replication by an indirect mechanism, this is the first report showing evidence of direct interaction between the microRNA machinery and HRV RNA.

Applications

By uncovering a role for microRNAs in the antiviral response of bronchial epithelial cells to HRV, this study may inspire future strategies for therapeutic intervention in the management of HRV-induced exacerbations of asthma and COPD.

Terminology

MicroRNAs are short RNA molecules that generally inhibit gene expression by binding to partially complementary sequences in the 3' Un-Translated Region of cellular mRNAs. Such inhibition can involve both increased mRNA degradation and inhibition of translation.

Peer review

In the manuscript, some interesting results have been observed that both miR-128 and miR-155 target HRV-1B. Over-expression of miR-155 inhibits HRV-1B RNA accumulated in BEAS-2B cell line.

REFERENCES

1 **Bartel DP**. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; **116**: 281-297 [PMID: 14744438 DOI: 10.1016/S0092-8674(04)00045-5]

2 **Meister G**, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* 2004; **15**: 185-197 [PMID: 15260970 DOI: 10.1016/j.molcel.2004.07.007]

3 **Peters L**, Meister G. Argonaute proteins: mediators of RNA silencing. *Mol Cell* 2007; **26**: 611-623 [PMID: 17560368 DOI: 10.1016/j.molcel.2007.05.001]

4 **Filipowicz W**, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 2008; **9**: 102-114 [PMID: 18197166 DOI: 10.1038/nrg2290]

5 **Lewis BP**, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005; **120**: 15-20 [PMID: 15652477 DOI: 10.1016/j.cell.2004.12.035]

6 **Garzon R**, Calin GA, Croce CM. MicroRNAs in Cancer. *Annu Rev Med* 2009; **60**: 167-179 [PMID: 19630570 DOI: 10.1146/annurev.med.59.053006.104707]

7 **Bernstein E**, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. Dicer is essential for mouse development. *Nat Genet* 2003; **35**: 215-217 [PMID: 14528307 DOI: 10.1038/ng1253]

8 **Harris KS**, Zhang Z, McManus MT, Harfe BD, Sun X. Dicer function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci USA* 2006; **103**: 2208-2213 [PMID: 16452165 DOI: 10.1073/pnas.0510839103]

9 **Williams AE**, Moschos SA, Perry MM, Barnes PJ, Lindsay MA. Maternally imprinted microRNAs are differentially expressed during mouse and human lung development. *Dev Dyn* 2007; **236**: 572-580 [PMID: 17191223 DOI: 10.1002/dvdy.21047]

10 **Tomankova T**, Petrek M, Kriegova E. Involvement of microRNAs in physiological and pathological processes in the lung. *Respir Res* 2010; **11**: 159 [PMID: 21092244 DOI: 10.1186/1465-9921-11-159]

11 **Martinez-Anton A**, Sokolowska M, Kern S, Davis AS, Alsaaty S, Taubenberger JK, Sun J, Cai R, Danner RL, Eberlein M, Logun C, Shelhamer JH. Changes in microRNA and mRNA expression with differentiation of human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2013; **49**: 384-395 [PMID: 23590309 DOI: 10.1165/rcmb.2012-0368OC]

12 **Rodriguez A**, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, van Dongen S, Grocock RJ, Das PP, Miska EA, Vetrie D, Okkenhaug K, Enright AJ, Dougan G, Turner M, Bradley A. Requirement of bic/microRNA-155 for normal immune function. *Science* 2007; **316**: 608-611 [PMID: 17463290 DOI: 10.1126/science.1139253]

13 **O'Connell RM**, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci USA* 2007; **104**: 1604-1609 [PMID: 17242365 DOI: 10.1073/pnas.0610731104]

14 **Tili E**, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, Fabbri M, Alder H, Liu CG, Calin GA, Croce CM. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol* 2007; **179**: 5082-5089 [PMID: 17911593 DOI: 10.4049/jimmunol.179.8.5082]

15 **Taganov KD**, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci USA* 2006; **103**: 12481-12486 [PMID: 16885212 DOI: 10.1073/pnas.0605298103]

16 **Hou J**, Wang P, Lin L, Liu X, Ma F, An H, Wang Z, Cao X. MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. *J Immunol* 2009; **183**: 2150-2158 [PMID: 19596990 DOI: 10.4049/jimmunol.0900707]

17 **Buggele WA**, Johnson KE, Horvath CM. Influenza A virus infection of human respiratory cells induces primary microRNA expression. *J Biol Chem* 2012; **287**: 31027-31040 [PMID: 22822053 DOI: 10.1074/jbc.M112.387670]

18 **Thornburg NJ**, Hayward SL, Crowe JE. Respiratory syncytial virus regulates human microRNAs by using mechanisms involving beta interferon and NF-κB. *MBio* 2012; **3**: pii: e00220-12 [PMID: 23249809 DOI: 10.1128/mBio.00220-12]

19 **Bakre A**, Mitchell P, Coleman JK, Jones LP, Saavedra G, Teng M, Tompkins SM, Tripp RA. Respiratory syncytial virus modifies microRNAs regulating host genes that affect virus replication. *J Gen Virol* 2012; **93**: 2346-2356 [PMID: 22894925 DOI: 10.1099/vir.0.044255-0]

20 **Othumpangat S**, Walton C, Piedimonte G. MicroRNA-221 modulates RSV replication in human bronchial epithelium by targeting NGF expression. *PLoS One* 2012; **7**: e30030 [PMID: 22272270 DOI: 10.1371/journal.pone.0030030]

21 **Müller S**, Imler JL. Dicing with viruses: microRNAs as antiviral factors. *Immunity* 2007; **27**: 1-3 [PMID: 17663977 DOI: 10.1016/j.immuni.2007.07.003]

22 **Jopling CL**, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 2005; **309**: 1577-1581 [PMID: 16141076 DOI: 10.1126/science.1113329]

23 **Pedersen IM**, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, David M. Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature* 2007; **449**: 919-922 [PMID: 17943132 DOI: 10.1038/nature06205]

24 **Ouda R**, Onomoto K, Takahasi K, Edwards MR, Kato H, Yoneyama M, Fujita T. Retinoic acid-inducible gene I-inducible miR-23b inhibits infections by minor group rhinoviruses through down-regulation of the very low density lipoprotein receptor. *J Biol Chem* 2011; **286**: 26210-26219 [PMID: 21642441 DOI: 10.1074/jbc.M111.229856]

25 **Otsuka M**, Jing Q, Georgel P, New L, Chen J, Mols J, Kang YJ, Jiang Z, Du X, Cook R, Das SC, Pattnaik AK, Beutler B, Han J. Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. *Immunity* 2007; **27**: 123-134 [PMID: 17613256 DOI: 10.1016/j.immuni.2007.05.014]

26 **Matskevich AA**, Moelling K. Dicer is involved in protection against influenza A virus infection. *J Gen Virol* 2007; **88**: 2627-2635 [PMID: 17872512 DOI: 10.1099/vir.0.83103-0]

27 **Ma YJ**, Yang J, Fan XL, Zhao HB, Hu W, Li ZP, Yu GC, Ding XR, Wang JZ, Bo XC, Zheng XF, Zhou Z, Wang SQ. Cellular microRNA let-7c inhibits M1 protein expression of the H1N1 influenza A virus in infected human lung epithelial cells. *J Cell Mol Med* 2012; **16**: 2539-2546 [PMID: 22452878 DOI: 10.1111/j.1582-4934.2012.01572.x]

28 **Song L**, Liu H, Gao S, Jiang W, Huang W. Cellular microRNAs inhibit replication of the H1N1 influenza A virus in infected cells. *J Virol* 2010; **84**: 8849-8860 [PMID: 20554777 DOI: 10.1128/JVI.00456-10]

29 **Triboulet R**, Mari B, Lin YL, Chable-Bessia C, Bennasser Y, Lebrigand K, Cardinaud B, Maurin T, Barbry P, Baillat V, Reynes J, Corbeau P, Jeang KT, Benkirane M. Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* 2007; **315**: 1579-1582 [PMID: 17322031 DOI: 10.1126/science.1136319]

30 **Lecellier CH**, Dunoyer P, Arar K, Lehmann-Che J, Eyquem S, Himber C, Saïb A, Voinnet O. A cellular microRNA mediates antiviral defense in human cells. *Science* 2005; **308**: 557-560 [PMID: 15845854 DOI: 10.1126/science.1108784]

31 **Johnston SL**, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, Symington P, O'Toole S, Myint SH, Tyrrell DA. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 1995; **310**: 1225-1229 [PMID: 7767192 DOI: 10.1136/bmj.310.6989.1225]

32 **Johnston NW**, Johnston SL, Duncan JM, Greene JM, Kebadze T, Keith PK, Roy M, Waserman S, Sears MR. The September epidemic of asthma exacerbations in children: a search for etiology. *J Allergy Clin Immunol* 2005; **115**: 132-138 [PMID: 15637559 DOI: 10.1016/j.jaci.2004.09.025]

33 **Seemungal TA**, Harper-Owen R, Bhowmik A, Jeffries DJ, Wedzicha JA. Detection of rhinovirus in induced sputum at exacerbation of chronic obstructive pulmonary disease. *Eur Respir J* 2000; **16**: 677-683 [PMID: 11106212 DOI: 10.1034/j.1399-3003.2000.16d19.x]

34 **Gern JE**, Galagan DM, Jarjour NN, Dick EC, Busse WW. Detection of rhinovirus RNA in lower airway cells during experimentally induced infection. *Am J Respir Crit Care Med* 1997; **155**: 1159-1161 [PMID: 9117003 DOI: 10.1164/ajrccm.155.3.9117003]

35 **Friedlander SL**, Busse WW. The role of rhinovirus in asthma exacerbations. *J Allergy Clin Immunol* 2005; **116**: 267-273 [PMID: 16083778 DOI: 10.1016/j.jaci.2005.06.003]

36 **Hayden FG**. Rhinovirus and the lower respiratory tract. *Rev Med Virol* 2004; **14**: 17-31 [PMID: 14716689 DOI: 10.1002/rmv.406]

37 **Mosser AG**, Brockman-Schneider R, Amineva S, Burchell L, Sedgwick JB, Busse WW, Gern JE. Similar frequency of rhinovirus-infectible cells in upper and lower airway epithelium. *J Infect Dis* 2002; **185**: 734-743 [PMID: 11920291]

38 **Greve JM**, Davis G, Meyer AM, Forte CP, Yost SC, Marlor CW, Kamarck ME, McClelland A. The major human rhinovirus receptor is ICAM-1. *Cell* 1989; **56**: 839-847 [PMID: 2538243 DOI: 10.1016/0092-8674(89)90688-0]

39 **Hofer F**, Gruenberger M, Kowalski H, Machat H, Huettinger M, Kuechler E, Blaas D. Members of the low density lipoprotein receptor family mediate cell entry of a minor-group common cold virus. *Proc Natl Acad Sci USA* 1994; **91**: 1839-1842 [PMID: 8127891 DOI: 10.1073/pnas.91.5.1839]

40 **Wang Q**, Nagarkar DR, Bowman ER, Schneider D, Gosangi B, Lei J, Zhao Y, McHenry CL, Burgens RV, Miller DJ, Sajjan U, Hershenson MB. Role of double-stranded RNA pattern recognition receptors in rhinovirus-induced airway epithelial cell responses. *J Immunol* 2009; **183**: 6989-6997 [PMID: 19890046 DOI: 10.4049/jimmunol.0901386]

41 **Slater L**, Bartlett NW, Haas JJ, Zhu J, Message SD, Walton RP, Sykes A, Dahdaleh S, Clarke DL, Belvisi MG, Kon OM, Fujita T, Jeffery PK, Johnston SL, Edwards MR. Co-ordinated role of TLR3, RIG-I and MDA5 in the innate response to rhinovirus in bronchial epithelium. *PLoS Pathog* 2010; **6**: e1001178 [PMID: 21079690 DOI: 10.1371/journal.ppat.1001178]

42 **Watanabe Y**, Kishi A, Yachie N, Kanai A, Tomita M. Computational analysis of microRNA-mediated antiviral defense in humans. *FEBS Lett* 2007; **581**: 4603-4610 [PMID: 17825824 DOI: 10.1016/j.febslet.2007.08.049]

43 **Phipps KM**, Martinez A, Lu J, Heinz BA, Zhao G. Small interfering RNA molecules as potential anti-human rhinovirus agents: in vitro potency, specificity, and mechanism. *Antiviral Res* 2004; **61**: 49-55 [PMID: 14670593 DOI: 10.1016/j.antiviral.2003.08.005]

44 **Wark PA**, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, Holgate ST, Davies DE. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J Exp Med* 2005; **201**: 937-947 [PMID: 15781584 DOI: 10.1084/jem.20041901]

45 **Martinez-Nunez RT**, Louafi F, Friedmann PS, Sanchez-Elsner T. MicroRNA-155 modulates the pathogen binding ability of dendritic cells (DCs) by down-regulation of DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). *J Biol Chem* 2009; **284**: 16334-16342 [PMID: 19386588 DOI: 10.1074/jbc.M109.011601]

46 **Livak KJ**, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402-408 [PMID: 11846609 DOI: 10.1006/meth.2001.1262]

47 **Rüdel S**, Flatley A, Weinmann L, Kremmer E, Meister G. A multifunctional human Argonaute2-specific monoclonal antibody. *RNA* 2008; **14**: 1244-1253 [PMID: 18430891 DOI: 10.1261/rna.973808]

48 **Garcia DM**, Baek D, Shin C, Bell GW, Grimson A, Bartel DP. Weak seed-pairing stability and high target-site abundance decrease the proficiency of lsy-6 and other microRNAs. *Nat Struct Mol Biol* 2011; **18**: 1139-1146 [PMID: 21909094 DOI: 10.1038/nsmb.2115]

49 Targetscan miR families [Internet]. Available from: URL: http://www.targetscan.org/vert_61/vert_61_data_download/miR_Family_Info.txt.zip

50 **John B**, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. *PLoS Biol* 2004; **2**: e363 [PMID: 15502875 DOI: 10.1371/journal.pbio.0020363]

51 **Kertesz M**, Iovino N, Unnerstall U, Gaul U, Segal E. The role of site accessibility in microRNA target recognition. *Nat Genet* 2007; **39**: 1278-1284 [PMID: 17893677 DOI: 10.1038/ng2135]

52 **Kozomara A**, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res* 2014; **42**: D68-D73 [PMID: 24275495 DOI: 10.1093/

nar/gkt1181]
53 **PITA FAQs.** Segal lab of computational biology [Internet]. Available from: URL: http://genie.weizmann.ac.il/pubs/mir07/mir07_notes.html
54 **Stajich JE**, Block D, Boulez K, Brenner SE, Chervitz SA, Dagdigian C, Fuellen G, Gilbert JG, Korf I, Lapp H, Lehväslaiho H, Matsalla C, Mungall CJ, Osborne BI, Pocock MR, Schattner P, Senger M, Stein LD, Stupka E, Wilkinson MD, Birney E. The Bioperl toolkit: Perl modules for the life sciences. *Genome Res* 2002; **12**: 1611-1618 [PMID: 12368254 DOI: 10.1101/gr.361602]
55 **Reddel RR**, Ke Y, Gerwin BI, McMenamin MG, Lechner JF, Su RT, Brash DE, Park JB, Rhim JS, Harris CC. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res* 1988; **48**: 1904-1909 [PMID: 2450641]
56 **Papadopoulos NG**, Papi A, Meyer J, Stanciu LA, Salvi S, Holgate ST, Johnston SL. Rhinovirus infection up-regulates eotaxin and eotaxin-2 expression in bronchial epithelial cells. *Clin Exp Allergy* 2001; **31**: 1060-1066 [PMID: 11467997 DOI: 10.1046/j.1365-2222.2001.01112.x]
57 **Karginov FV**, Conaco C, Xuan Z, Schmidt BH, Parker JS, Mandel G, Hannon GJ. A biochemical approach to identifying microRNA targets. *Proc Natl Acad Sci USA* 2007; **104**: 19291-19296 [PMID: 18042700 DOI: 10.1073/pnas.0709971104]
58 **Hendrickson DG**, Hogan DJ, Herschlag D, Ferrell JE, Brown PO. Systematic identification of mRNAs recruited to argonaute 2 by specific microRNAs and corresponding changes in transcript abundance. *PLoS One* 2008; **3**: e2126 [PMID: 18461144 DOI: 10.1371/journal.pone.0002126]
59 **Landthaler M**, Gaidatzis D, Rothballer A, Chen PY, Soll SJ, Dinic L, Ojo T, Hafner M, Zavolan M, Tuschl T. Molecular characterization of human Argonaute-containing ribonucleoprotein complexes and their bound target mRNAs. *RNA* 2008; **14**: 2580-2596 [PMID: 18978028 DOI: 10.1261/rna.1351608]
60 **Su H**, Trombly MI, Chen J, Wang X. Essential and overlapping functions for mammalian Argonautes in microRNA silencing. *Genes Dev* 2009; **23**: 304-317 [PMID: 19174539 DOI: 10.1101/gad.1749809]
61 **Dueck A**, Ziegler C, Eichner A, Berezikov E, Meister G. microRNAs associated with the different human Argonaute proteins. *Nucleic Acids Res* 2012; **40**: 9850-9862 [PMID: 22844086 DOI: 10.1093/nar/gks705]
62 **Louafi F**, Martinez-Nunez RT, Sanchez-Elsner T. MicroRNA-155 targets SMAD2 and modulates the response of macrophages to transforming growth factor-{beta}. *J Biol Chem* 2010; **285**: 41328-41336 [PMID: 21036908 DOI: 10.1074/jbc.M110.146852]
63 **Solberg OD**, Ostrin EJ, Love MI, Peng JC, Bhakta NR, Hou L, Nguyen C, Solon M, Nguyen C, Barczak AJ, Zlock LT, Blagev DP, Finkbeiner WE, Ansel KM, Arron JR, Erle DJ, Woodruff PG. Airway epithelial miRNA expression is altered in asthma. *Am J Respir Crit Care Med* 2012; **186**: 965-974 [PMID: 22955319 DOI: 10.1164/rccm.201201-0027OC]
64 **Donnelly RP**, Kotenko SV. Interferon-lambda: a new addition to an old family. *J Interferon Cytokine Res* 2010; **30**: 555-564 [PMID: 20712453 DOI: 10.1089/jir.2010.0078]
65 **Parameswaran P**, Sklan E, Wilkins C, Burgon T, Samuel MA, Lu R, Ansel KM, Heissmeyer V, Einav S, Jackson W, Doukas T, Paranjape S, Polacek C, dos Santos FB, Jalili R, Babrzadeh F, Gharizadeh B, Grimm D, Kay M, Koike S, Sarnow P, Ronaghi M, Ding SW, Harris E, Chow M, Diamond MS, Kirkegaard K, Glenn JS, Fire AZ. Six RNA viruses and forty-one hosts: viral small RNAs and modulation of small RNA repertoires in vertebrate and invertebrate systems. *PLoS Pathog* 2010; **6**: e1000764 [PMID: 20169186 DOI: 10.1371/journal.ppat.1000764]
66 **Jeang KT**. RNAi in the regulation of mammalian viral infections. *BMC Biol* 2012; **10**: 58 [PMID: 22734679 DOI: 10.1186/1741-7007-10-58]
67 **Contoli M**, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, Kebadze T, Mallia P, Stanciu LA, Parker HL, Slater L, Lewis-Antes A, Kon OM, Holgate ST, Davies DE, Kotenko SV, Papi A, Johnston SL. Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006; **12**: 1023-1026 [PMID: 16906156 DOI: 10.1038/nm1462]
68 **Kuehbacher A**, Urbich C, Zeiher AM, Dimmeler S. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circ Res* 2007; **101**: 59-68 [PMID: 17540974 DOI: 10.1161/CIRCRESAHA.107.153916]
69 **Schmitter D**, Filkowski J, Sewer A, Pillai RS, Oakeley EJ, Zavolan M, Svoboda P, Filipowicz W. Effects of Dicer and Argonaute down-regulation on mRNA levels in human HEK293 cells. *Nucleic Acids Res* 2006; **34**: 4801-4815 [PMID: 16971455 DOI: 10.1093/nar/gkl646]
70 **Suárez Y**, Fernández-Hernando C, Pober JS, Sessa WC. Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. *Circ Res* 2007; **100**: 1164-1173 [PMID: 17379831 DOI: 10.1161/01.RES.0000265065.26744.17]
71 **Winter J**, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol* 2009; **11**: 228-234 [PMID: 19255566 DOI: 10.1038/ncb0309-228]
72 **Yang JS**, Lai EC. Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. *Mol Cell* 2011; **43**: 892-903 [PMID: 21925378 DOI: 10.1016/j.molcel.2011.07.024]
73 **Gantier MP**, McCoy CE, Rusinova I, Saulep D, Wang D, Xu D, Irving AT, Behlke MA, Hertzog PJ, Mackay F, Williams BR. Analysis of microRNA turnover in mammalian cells following Dicer1 ablation. *Nucleic Acids Res* 2011; **39**: 5692-5703 [PMID: 21447562 DOI: 10.1093/nar/gkr148]
74 **Adlakha YK**, Saini N. MicroRNA-128 downregulates Bax and induces apoptosis in human embryonic kidney cells. *Cell Mol Life Sci* 2011; **68**: 1415-1428 [PMID: 20924637 DOI: 10.1007/s00018-010-0528-y]
75 **Peruzzi P**, Bronisz A, Nowicki MO, Wang Y, Ogawa D, Price R, Nakano I, Kwon CH, Hayes J, Lawler SE, Ostrowski MC, Chiocca EA, Godlewski J. MicroRNA-128 coordinately targets Polycomb Repressor Complexes in glioma stem cells. *Neuro Oncol* 2013; **15**: 1212-1224 [PMID: 23733246 DOI: 10.1093/neuonc/not055]
76 **Zhang Y**, Chao T, Li R, Liu W, Chen Y, Yan X, Gong Y, Yin B, Liu W, Qiang B, Zhao J, Yuan J, Peng X. MicroRNA-128 inhibits glioma cells proliferation by targeting transcription factor E2F3a. *J Mol Med* (Berl) 2009; **87**: 43-51 [PMID: 18810376 DOI: 10.1007/s00109-008-0403-6]
77 **Weiss GJ**, Bemis LT, Nakajima E, Sugita M, Birks DK, Robinson WA, Varella-Garcia M, Bunn PA, Haney J, Helfrich BA, Kato H, Hirsch FR, Franklin WA. EGFR regulation by microRNA in lung cancer: correlation with clinical response and survival to gefitinib and EGFR expression in cell lines. *Ann Oncol* 2008; **19**: 1053-1059 [PMID: 18304967 DOI: 10.1093/annonc/mdn006]
78 **Schembri F**, Sridhar S, Perdomo C, Gustafson AM, Zhang X, Ergun A, Lu J, Liu G, Zhang X, Bowers J, Vaziri C, Ott K, Sensinger K, Collins JJ, Brody JS, Getts R, Lenburg ME, Spira A. MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. *Proc Natl Acad Sci USA* 2009; **106**: 2319-2324 [PMID: 19168627 DOI: 10.1073/pnas.0806383106]
79 **Puddicombe SM**, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, Davies DE. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J* 2000; **14**: 1362-1374 [PMID: 10877829 DOI: 10.1096/fj.14.10.1362]
80 **Faraoni I**, Antonetti FR, Cardone J, Bonmassar E. miR-155

gene: a typical multifunctional microRNA. *Biochim Biophys Acta* 2009; **1792**: 497-505 [PMID: 19268705 DOI: 10.1016/j.bbadis.2009.02.013]

81 **Higgs G**, Slack F. The multiple roles of microRNA-155 in oncogenesis. *J Clin Bioinforma* 2013; **3**: 17 [PMID: 24073882 DOI: 10.1186/2043-9113-3-17]

82 **O'Connell RM**, Rao DS, Chaudhuri AA, Boldin MP, Taganov KD, Nicoll J, Paquette RL, Baltimore D. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med* 2008; **205**: 585-594 [PMID: 18299402 DOI: 10.1084/jem.20072108]

83 **Dudda JC**, Salaun B, Ji Y, Palmer DC, Monnot GC, Merck E, Boudousquie C, Utzschneider DT, Escobar TM, Perret R, Muljo SA, Hebeisen M, Rufer N, Zehn D, Donda A, Restifo NP, Held W, Gattinoni L, Romero P. MicroRNA-155 is required for effector CD8+ T cell responses to virus infection and cancer. *Immunity* 2013; **38**: 742-753 [PMID: 23601686 DOI: 10.1016/j.immuni.2012.12.006]

84 **Vigorito E**, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, Das PP, Miska EA, Rodriguez A, Bradley A, Smith KG, Rada C, Enright AJ, Toellner KM, Maclennan IC, Turner M. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity* 2007; **27**: 847-859 [PMID: 18055230 DOI: 10.1016/j.immuni.2007.10.009]

85 **Ceppi M**, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, Santos MA, Pierre P. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proc Natl Acad Sci USA* 2009; **106**: 2735-2740 [PMID: 19193853 DOI: 10.1073/pnas.0811073106]

86 **Wang P**, Hou J, Lin L, Wang C, Liu X, Li D, Ma F, Wang Z, Cao X. Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. *J Immunol* 2010; **185**: 6226-6233 [PMID: 20937844 DOI: 10.4049/jimmunol.1000491]

87 **Pichiorri F**, Suh SS, Ladetto M, Kuehl M, Palumbo T, Drandi D, Taccioli C, Zanesi N, Alder H, Hagan JP, Munker R, Volinia S, Boccadoro M, Garzon R, Palumbo A, Aqeilan RI, Croce CM. MicroRNAs regulate critical genes associated with multiple myeloma pathogenesis. *Proc Natl Acad Sci USA* 2008; **105**: 12885-12890 [PMID: 18728182 DOI: 10.1073/pnas.0806202105]

88 **Jardim MJ**, Dailey L, Silbajoris R, Diaz-Sanchez D. Distinct microRNA expression in human airway cells of asthmatic donors identifies a novel asthma-associated gene. *Am J Respir Cell Mol Biol* 2012; **47**: 536-542 [PMID: 22679274 DOI: 10.1165/rcmb.2011-0160OC]

89 **Martinez-Nunez RT**, Louafi F, Sanchez-Elsner T. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). *J Biol Chem* 2011; **286**: 1786-1794 [PMID: 21097505 DOI: 10.1074/jbc.M110.169367]

90 **Ogawa T**, Iizuka M, Sekiya Y, Yoshizato K, Ikeda K, Kawada N. Suppression of type I collagen production by microRNA-29b in cultured human stellate cells. *Biochem Biophys Res Commun* 2010; **391**: 316-321 [PMID: 19913496 DOI: 10.1016/j.bbrc.2009.11.056]

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ORIGINAL ARTICLE

Short- and long-term effects of silver nanoparticles on human microvascular endothelial cells

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Abstract

AIM: To study the response to silver nanoparticles (Ag NP) of human microvascular endothelial cells, protagonists of angiogenesis.

METHODS: We cultured human microvascular endothelial cells and endothelial colony-forming cells in their corresponding growth medium. Stock solutions of Ag NP were prepared in culture medium and sonicated before use. They were added at different concentrations and for different times to culture media. The toxicity of Ag NP was investigated by measuring the reduction of yellow tetrazolium salt to dark purple formazan (MTT assay) at 575 nm. After staining with trypan blue, cell proliferation was assessed by counting viable cells. The lactate dehydrogenase leakage assay was performed on culture media by following the oxidation of NADH to NAD+ and monitoring the reaction kinetically at 340 nm. Reactive oxygen species production was quantified using 2'-7'-dichlorofluorescein diacetate. The alkaline comet assay was performed after mixing the cells with low melting-point agarose. Electrophoresis was then conducted and the samples were stained with ethidium bromide and analyzed with a fluorescence microscope.

RESULTS: Ag NP are cytotoxic in a dose and time dependent fashion for HMEC. At high concentrations, Ag NP determine loss of membrane integrity as demonstrated by the increased activity of lactate dehydrogenase in the culture medium. Ag NP rapidly stimulate the formation of free radicals. However, pre-incubation with Trolox, apocynin, or N-acetyl-L-cysteine, antioxidants which have different structure and act through different mechanisms, is not sufficient to prevent cytotoxicity. Ag NP also induce DNA damage dose-dependently, as shown by comet assay. When exposed to sublethal concentrations of Ag NP for long times, the cells remain viable but are growth retarded. Interestingly, removal of Ag NP partially rescues cell growth. Also genotoxicity is reversible upon removal of Ag NP from culture medium, suggesting that no permanent modifications occur. It is noteworthy that Ag NP are cytotoxic and genotoxic also for endothelial progenitors, in particular for endothelial colony-forming cells, which participate to angiogenesis.

CONCLUSION: Silver nanoparticles are cytotoxic and genotoxic for human microvascular endothelial cells and might become a useful tool to control excessive angiogenesis.

Key words: Endothelial cells; Nanoparticles; Genotoxicity; Cytotoxicity; Angiogenesis

Core tip: We studied the sensitivity to silver nanoparticles of microvascular endothelial cells, which are responsible for tissue homeostasis and fundamental in angiogenesis. Silver nanoparticles are cytotoxic and lead to membrane leakage. Cytotoxicity is not prevented by the antioxidants Trolox, N-acetyl-L-cysteine or apocynin. Silver nanoparticles also induce DNA damage as demonstrated by comet assay. When exposed to sublethal concentrations of silver nanoparticles for long times, the cells remain viable but are growth retarded.

Interestingly, removal of silver nanoparticle rescue cell growth, suggesting that no permanent modifications occur. Silver nanoparticles are cytotoxic and genotoxic also for endothelial progenitors, which contribute to angiogenesis.

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INTRODUCTION

Angiogenesis is a complex multistep process resulting in the formation of new capillaries from pre-existing vessels[1]. Protagonists in angiogenesis are the microvascular endothelial cells which are stimulated by angiogenic factors to proliferate, invade the surrounding tissues and undergo differentiation to form new vessels[1]. Therefore, impairing endothelial cell survival and growth can effectively block angiogenesis when excessive, as described in tumors, chronic inflammatory diseases, retinopathies, psoriasis among others[1]. Indeed, anti-angiogenic drugs are currently used in therapy to control angiogenesis in different pathological conditions[2].

Silver nanoparticles (Ag NP) are the most widely commercialized among all nanomaterials, because they show antiseptic properties. Consequently, they are used in the production of catheters or implantable devices as well as in cosmetics and household products[3]. Many studies have reported the cytotoxicity of Ag NP in several systems *in vitro* and *in vivo*[4]. Few studies focused on endothelial cells. Ag NP induce cell injury through the activation of the NFkB pathway in human endothelial cells from the umbilical vein (HUVEC)[5]. Exposure of rat brain endothelial cells to Ag NP leads to a marked cytotoxicity which is independent from the Ag ion release[6] and partially related to the induction of an inflammatory response[7]. Ag NP inhibit Vascular Endothelial Growth Factor (VEGF)-induced cell proliferation and migration in bovine retinal endothelial cells and promote apoptosis[8,9]. They also reduce VEGF and interleukin 1β-induced permeability in porcine retinal endothelial cells[10]. In contrast with these studies, Kang *et al*[11] showed that Ag NP stimulated the murine endothelial cell line SVEC4-10 to produce angiogenic factors and nitric oxide and to activate VEGF-receptor pathways. This study also reports that Ag NP increase angiogenesis in Matrigel plug assay.

To our knowledge, no data are available about the effects of Ag NP on human dermal microvascular endothelial cells (HMEC). It is noteworthy that endothelial cells show a high degree of phenotypic heterogeneity[12] and it is therefore relevant to analyze cells from different districts. Indeed, because of the diversity of hemodynamics and of signals from different tissue microenvironments, microvascular endothelial cells diverge from endothelial cells of the large vessels in gene expression profile[13].

We here show the short- and long-term effects of Ag NP on HMEC viability and growth, crucial events in angiogenesis. Interestingly, also endothelial colony-forming cells (ECFC), rare circulating endothelial cells which participate to angiogenesis[14], are sensitive to Ag NP.

MATERIALS AND METHODS

Reagents

Ag NP were purchased from NanoAmor (Houston, United States). According to the manufacturer, Ag NP chemical specifications are the following: purity, 99.5%; average particle size, 35 nm; specific surface area, 30-50 m^2/g; particle morphology, spherical; crystallographic structure, cubic. Stock solutions of these nanoparticles were prepared in MCDB131 and EBM-2 media without fetal bovine serum (FBS) at concentration of 1080 μg/mL and kept at 4 ℃ for 1 mo. Stock solutions were sonicated several times just before preparing appropriate dilutions in culture medium. Unless otherwise specified, all the reagents were from Sigma (Oakville, Canada).

Cell culture, MTT and lactate dehydrogenase assay

HMEC were obtained from Centers for Disease Control and Prevention (Atlanta, United States). They exhibit typical cobblestone morphology, express cell-surface molecules, including CD31 and CD36, secrete von Willebrand's Factor, and take up acteylated low-density lipoprotein[15]. HMEC were grown in MCDB131 (Invitrogen, Milan, Italy) containing Epidermal Growth Factor (EGF) (10 ng/mL), hydrocortisone (1 μg/mL) and 10% FBS on 2% gelatin-coated dishes. ECFC were from Lonza (Milano, Italy) and grown in EBM-2 medium supplemented with 10% FBS and hydrocortisone, human EGF, VEGF, human basic Fibroblast Growth Factor, ascorbic acid, heparin and gentamicin/amphotericin B, according to the manufacturer's instruction[16].

For MTT assay the cells were seeded in 96 well/plates. MTT is a sensitive quantitative colorimetric assay measuring the reduction of yellow tetrazolium salt MTT to dark purple formazan by succinate dehydrogenase, mainly in mitochondria, and it is now widely accepted as a reliable way to examine cytotoxicity[17]. Briefly, the medium was replaced with medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT, 0.5 mg/mL). At the end of the incubation, media were removed and formazan crystals generated by the cellular reduction activity were dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured at 575 nm. In long-term experiments, cells were trypsinized, stained with trypan blue (0.4%) and viable cells were counted[16].

To evaluate cell membrane integrity, the lactate dehydrogenase (LDH) leakage assay was performed. Culture medium was collected and centrifuged at 4000 rpm for 10 min. 50 μL of medium was resuspended in a solution containing phosphate buffer 0.1 mol/L pH 7.0, 25 μL so-

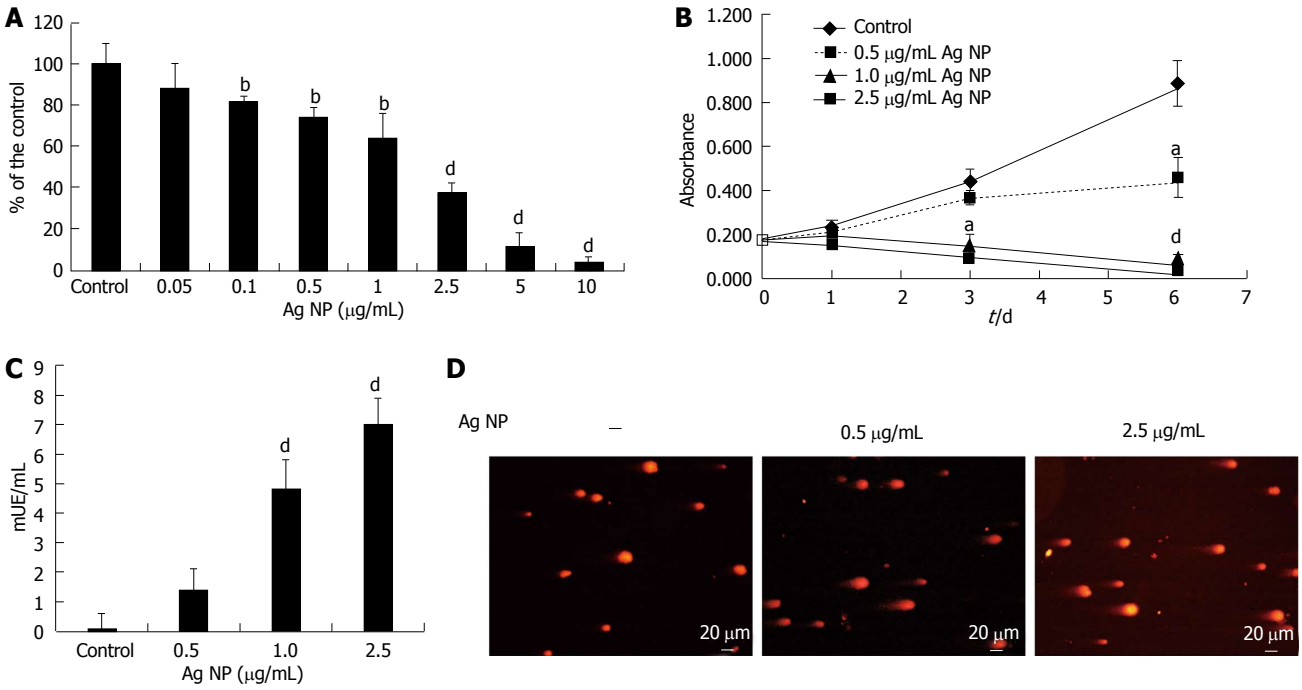

Figure 1 Silver nanoparticles are cytotoxic and genotoxic for human dermal microvascular endothelial cells. (A) Human dermal microvascular endothelial cells (HMEC) were exposed to various concentrations of silver nanoparticles (Ag NP) for 72 h or (B) were treated with different concentrations of Ag NP for different times. MTT assay was then performed. In (A) data are expressed as the percentage of control and in (B) as absorbance. Data represent the mean ± SD deviation of at least three separate experiments in triplicate. *P* value was calculated *vs* untreated cells: $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$. C: HMEC were treated with 0.5, 1.0 and 2.5 μg/mL Ag NP for 24 h and the LDH leakage assay was performed. Data are expressed as enzyme unit/mL culture medium and represent the mean ± SD of five separate experiments. *P* value was calculated *vs* untreated cells: $^{d}P < 0.001$; D: Comet assay was performed on HMEC treated with Ag NP for 24 h. After staining with ethidium bromide, the slides were analyzed with a fluorescence microscope.

dium pyruvate 23 mmol/L, 12.5 μL NADH 14 mmol/L. LDH catalyzes the reduction of pyruvate to lactate in the presence of reduced nicotinamide adenine dinucleotide (NADH) at pH 7.0. The reaction was monitored kinetically at 340 nm by the rate of decrease in absorbance resulting from the oxidation of NADH to NAD+ proportional to the activity of LDH in the sample. Data are expressed as enzyme unit/ml culture medium.

Reactive oxygen species production

Intracellular oxidative stress was quantified using 2'-7'-dichlorofluorescein diacetate (DCFH). Cells were seeded into black bottomed 96 plates (Greiner bio-one, Frickenhausen, Germany) and 24 h later exposed for 30 min to different concentrations of NP in a 20 μmol/L DCFH solution. Intracellular oxidative stress was measured by monitoring the emission at 529 nm of the DCFH dye using Promega Glomax Multi Detection System. H_2O_2 was used as a positive control. The results are the mean of three independent experiments performed in quadruplicate. Data are shown as the fold increase in reactive oxygen species (ROS) levels of Ag NP treated cells compared to control ± SD.

Single cell electrophoresis - comet assay

The alkaline comet assay was performed on HMEC and ECFC cells. Cells were seeded at 10000/cm^2 in 24 well-plates 24 h prior to treatment. After treatment, cells were trypsinized, mixed with low melting-point agarose and spread on pretreated slides which were allowed to dry. The slides were then immersed in ice cold lysis solution (Tris-HCl 0.01 mol/L pH 10, NaCl 2.5 mol/L, EDTA 0.1 mol/L, NaOH 0.3 mol/L, Triton 1%, DMSO 10%) and incubated at 4 ℃ for 60 min. Electrophoresis was conducted in ice cold running buffer (NaOH 0.3 mol/L, EDTA 0.001 mol/L) for 30 min at 300 mA. The slides were then rinsed, fixed in ice-cold methanol for 3 min and dried at room temperature. Cells were stained with ethidium bromide and analyzed with a fluorescence microscope.

Statistical analysis

Statistical significance was determined using the Student's *t* test and set at *P* values less than 0.05. In the figures $^{a}P < 0.05$; $^{b}P < 0.01$; $^{d}P < 0.001$.

RESULTS

High concentrations of Ag NP are cytotoxic

HMEC cells were exposed to various concentrations of Ag NP for 72 h. By MTT assay we found that Ag NP reduced the viability of HMEC cells dose dependently (Figure 1A). Cytotoxicity was statistically significant at a concentration 0.1 μg/mL ($P < 0.01$) with an Inhibitory Concentration 50 (IC_{50}) 1.86 ± 0.13 μg/mL. The kinetics of NP-induced cytotoxicity were then examined using 0.5, 1.0 and 2.5 μg/mL Ag NP (Figure 1B). 1.0 and 2.5 μg/mL Ag NP had dramatic effects on cell viability,

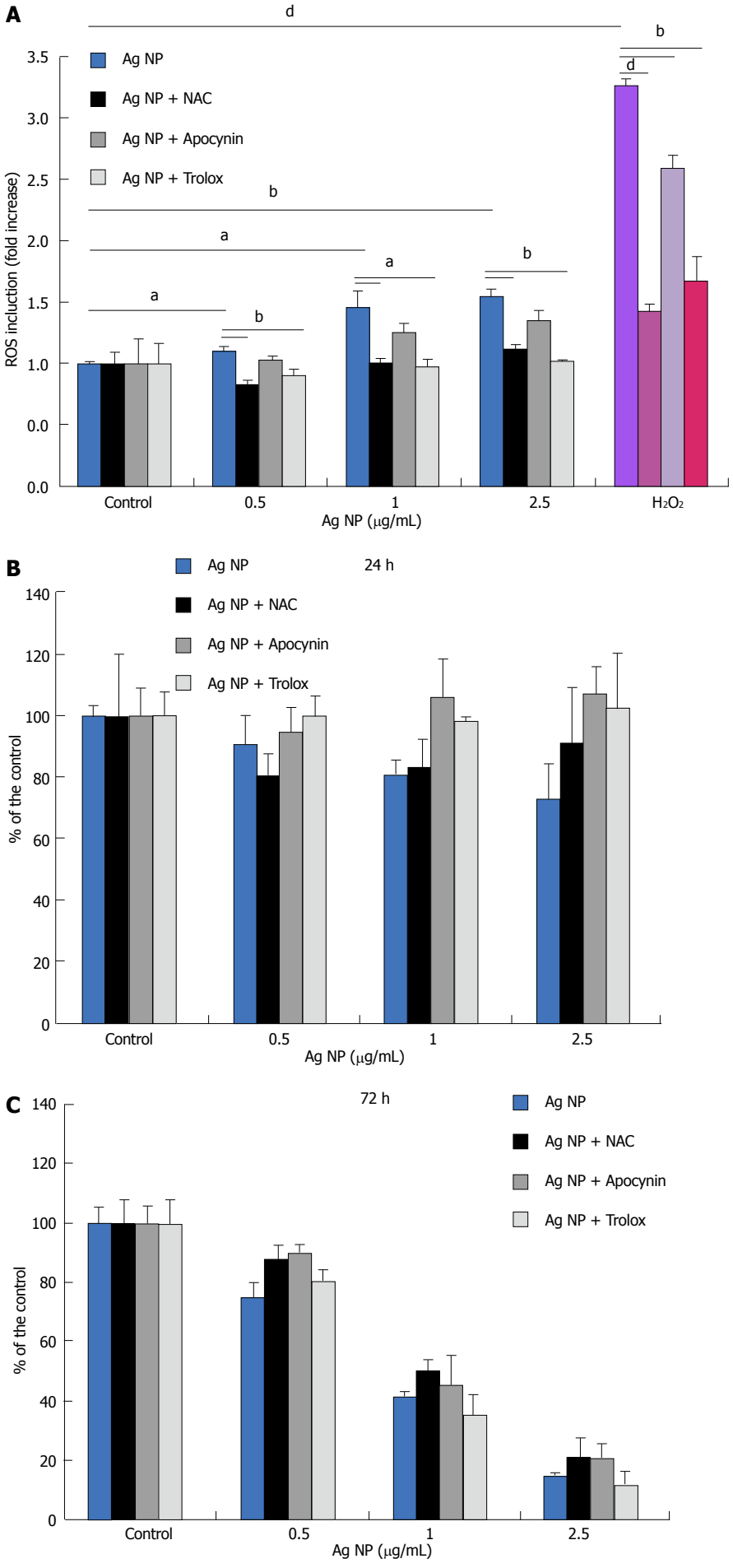

Figure 2 Silver nanoparticles induce the formation of reactive oxygen species, but antioxidants do not prevent silver nanoparticles' cytotoxicity. A: HMEC cells were pretreated with antioxidants and then exposed to various concentrations of Ag or H_2O_2 (100 μmol/L) for 30 min. ROS generation was then measured. Data are shown as the fold increase in ROS levels of treated cells compared to the corresponding control. Each bar is the mean of three separate experiments ± SD. P value was calculated *vs* untreated cells: $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$; B: HMEC were pre-incubated with Trolox (40 μmol/L), NAC (5 mmol/L) or apocynin (10 μmol/L) for 2 h before adding different concentrations of Ag NP. MTT assay was performed after 24 h (B) and 72 h (C). Data are expressed as the percentage of control and represent the mean ± SD of four separate experiments. HMEC: Human dermal microvascular endothelial cells.

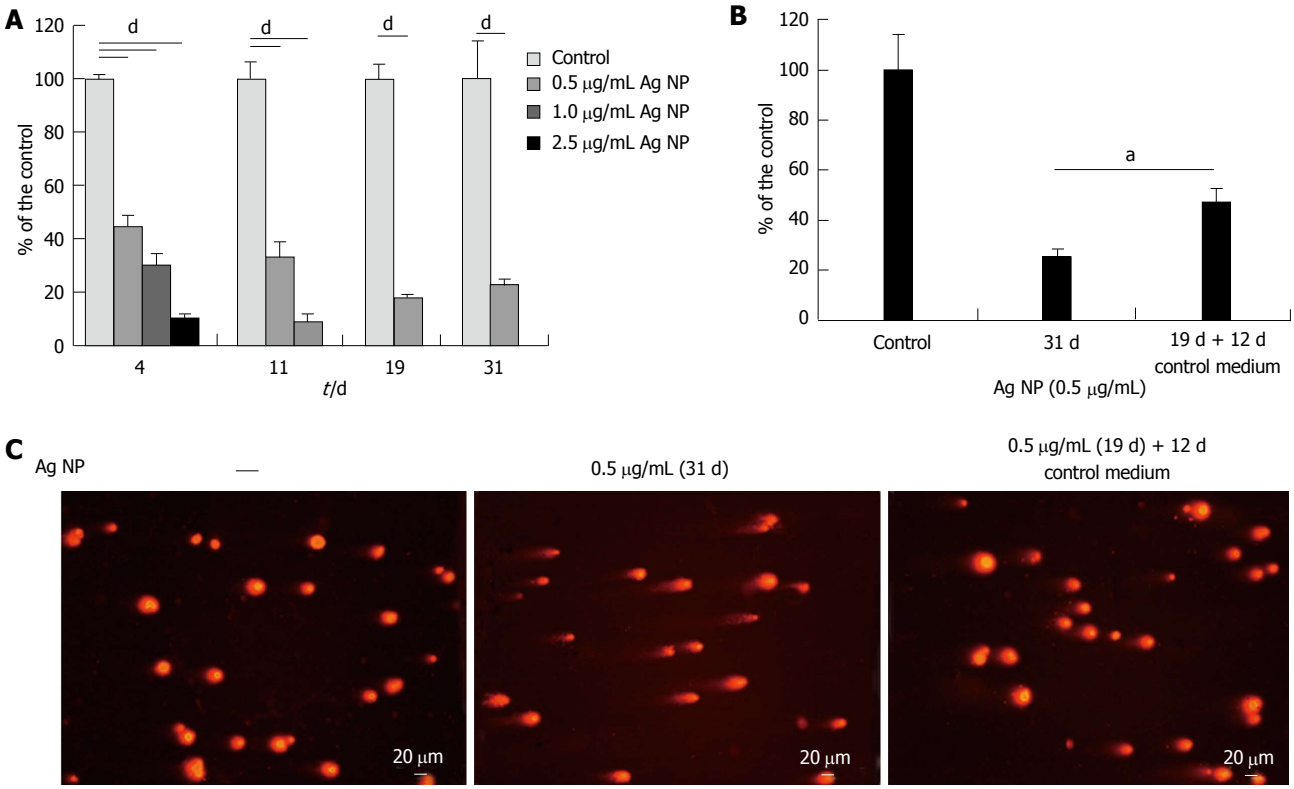

Figure 3 Low concentrations of silver nanoparticles reversibly retard human dermal microvascular endothelial cells growth. A: Human dermal microvascular endothelial cells (HMEC) were exposed to various concentrations of silver nanoparticles (Ag NP). All the samples were trypsinized and counted on the same day in which the controls reached confluence. After counting, the cells were re-seeded at the same density; B: On day 19, part of the samples were seeded in the absence of Ag NP and counted at day 31. In (A) and (B) data are expressed as the percentage of control. Data represent the means ± SD of four separate experiments. In (A) *P* value was calculated *vs* untreated cells, $^{d}P < 0.001$; and in (B) *P* value was calculated *vs* cells cultured for 31 d continuously with Ag NP, $^{a}P < 0.05$; C: Comet assay was performed as described on HMEC treated with Ag NP (0.5 μg/mL) for 31 d or after removal of the nanoparticles from the culture media on day 19.

while HMEC survived with 0.5 μg/mL Ag NP. This experiment was also performed by counting the cells. We found no viable cells with the high concentrations of Ag NP while we observed an increase of cell number in HMEC treated with 0.5 μg/mL Ag NP (data not shown). These results demonstrate that Ag NP are cytotoxic in a dose and time dependent fashion. Interestingly, HMEC displayed the same sensitivity to Ag NP coated with Poly-VinylPyrrolidone (data not shown), thus indicating that the toxic effect is not due to the release of Ag ions from their surface upon the contact with aqueous solutions.

LDH is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity. We measured LDH activity after treating HMEC for 24 h with Ag NP and observed a marked dose dependent increase of LDH activity in the medium after exposure to 1.0 and 2.5 μg/mL Ag NP (Figure 1C), while the leakage was very low in HMEC treated with 0.5 μg/mL of nanoparticles. These results indicate that high concentrations of Ag NP induce damage to the cellular membranes.

We then evaluated whether exposure to Ag NP for 24 h induced DNA strand breaks in HMEC cells by comet assay. As shown in Figure 1D, Ag NP induced a dose dependent level of genotoxic stress as demonstrated by the formation of comets.

Antioxidants do not prevent Ag NP cytotoxicity

Initially, we measured the generation of ROS in response to Ag NP for 30 min. In HMEC, we found a significant dose-dependent increase of ROS (Figure 2A). We also evaluated the capability of three antioxidants with different mechanisms of action to inhibit ROS formation in response to Ag NP in HMEC. To this purpose, we used Trolox, a synthetic cell-permeable analogue of α-tocopherol which scavenges peroxyl and alkoxyl radicals, and N-acetyl-L-cysteine (NAC), a thiol compound that is a precursor of reduced glutathione and increases the activity of superoxide dismutase[18]. We also utilized apocynin, an inhibitor of NADPH oxidase[19]. We pre-incubated the cells with Trolox (40 μmol/L), apocynin (10 μmol/L), or NAC (5 mmol/L) for 2 h before adding different concentrations of Ag NP or H_2O_2 as a positive control. Figure 2A shows that the three antioxidants reduced the formation of ROS induced by Ag NP and by H_2O_2. To evaluate the role of oxidative stress in Ag NP-mediated toxicity, we treated the cell with the three antioxidants before adding Ag NP for 24 and 72 h. As shown in Figure 2B, antioxidants did not exert any protective effect against Ag NP in HMEC. Accordingly, we detected DNA damage by comet assay also when HMEC were pre-incubated with antioxidants prior to the addition of Ag NP (data not shown).

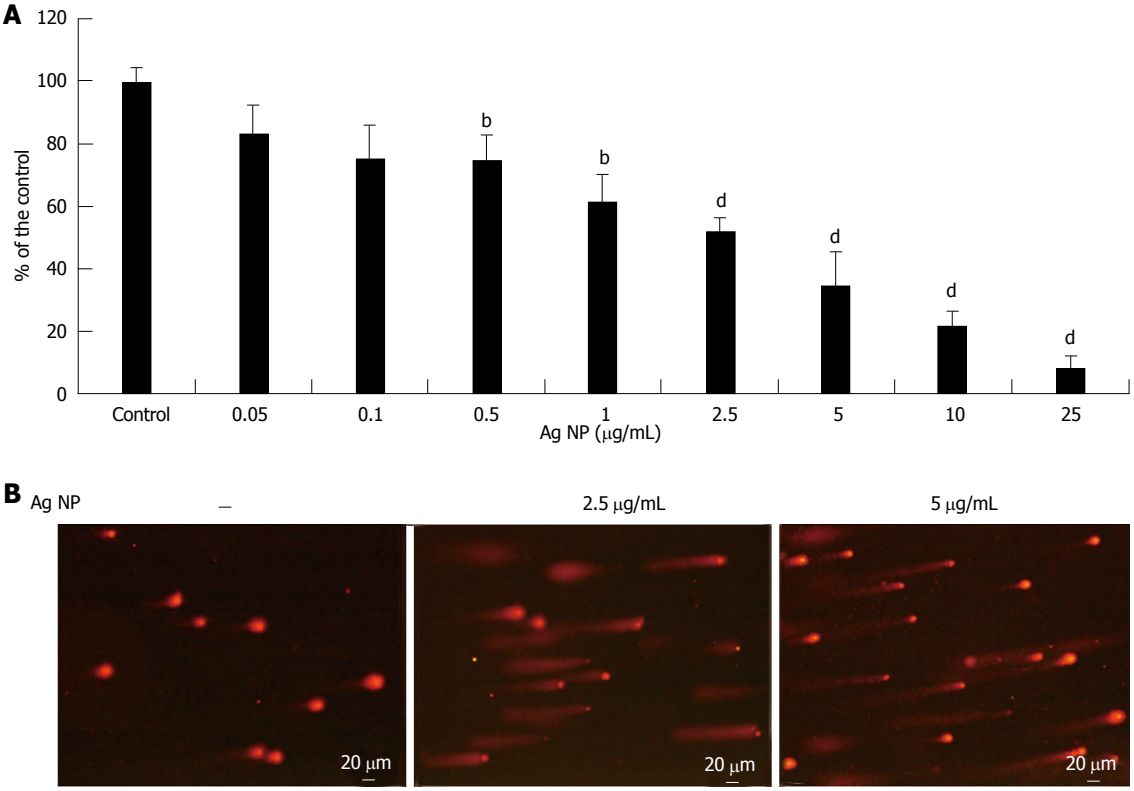

Figure 4 Silver nanoparticles are cytotoxic and genotoxic for endothelial colony-forming cells. A: Endothelial colony-forming cells (ECFC) were treated with various concentrations of silver nanoparticles (Ag NP) for 72 h. MTT assay was then performed and data are expressed as the percentage of control. Data represent the mean ± SD of at least three separate experiments. *P* value was calculated *vs* untreated cells: $^{b}P < 0.01$, $^{d}P < 0.001$. B: Comet assay was performed on ECFC treated with Ag NP (2.5 and 5 μg/mL) for 24 h as described.

Low concentrations of Ag NP induce a reversible growth inhibition

We then evaluated the effects of long term exposure to Ag NP. HMEC were treated with different concentrations of Ag NP and counted on the day in which the control untreated cells reached confluence. After counting, Ag NP-treated or untreated cells were re-seeded at the same density.

As shown in Figure 3A, 2.5 μg/mL Ag NP are highly cytotoxic and almost no viable cells are observed after 4 d treatment, while 11 d were necessary to have no viable cells when 1.0 μg/mL Ag NP was used. Only the 0.5 μg/mL Ag NP treated cells could be cultured for 31 d. At day 19, the cells were counted, re-seeded and propagated with or without 0.5 μg/mL Ag NP for additional 12 d. We show that HMEC partially recovered their proliferative potential after removing Ag NP from the medium (Figure 3B). In parallel, we performed comet assay and show that also genotoxicity was reversible upon removal of Ag NP from culture medium (Figure 3C).

Ag NP are cytotoxic for ECFC

We extended part of our studies to ECFC, which are known to contribute to new vessel formation[14]. We evaluated the effects of different concentrations of Ag NP and found that, similarly to HMEC, Ag NP were cytotoxic in a dose dependent fashion (Figure 4A). It is noteworthy that ECFC are less sensitive to Ag NP than HMEC, since the IC_{50} we observed was 2.40 ± 0.24 μg/mL. We also found that Ag NP induced a marked DNA damage as detected by comet assay (Figure 4B).

DISCUSSION

The possibility of exploiting nanoparticles as alternative candidates to treat different diseases is under intensive investigation and represents a major challenge for modern medicine. Indeed, nanoparticles could serve as active components or could be useful to deliver functional moieties into their targets.

In particular, Ag NP are cytotoxic and genotoxic in various cell types including cancer cell lines[9,20], and induce DNA damage in human peripheral blood cells[21]. Consequently, the possibility to exploit their cytotoxic activity to treat proliferative diseases is of interest. Little is known about the effects of Ag NP on endothelial cells[5-7] and, to our knowledge, no data are available on human microvascular endothelial cells, pivotal regulators of tissue homeostasis and fundamental players in angiogenesis.

We here show that Ag NP are cytotoxic and genotoxic for HMEC. These results suggest that Ag NP might represent a challenging tool to treat all those diseases characterized by exaggerated angiogenesis[1]. Considering that: (1) angiogenesis is one of the hallmark of cancer[22]; and (2) human cancer cells are susceptible to the cytotox-

ic effects of Ag NP, Ag NP might also be promising to control proliferative diseases since they could target both the vasculature and the neoplastic cells. To this purpose, it is worth underscoring that also endothelial progenitors, specifically ECFC, circulating endothelial cells with intrinsic *in vivo* vessel forming ability[14], are sensitive to Ag NP. Interestingly, ECFC are emerging as important players in angiogenesis and also as useful tools in regenerative medicine[14].

Several mechanisms have been proposed to explain how Ag NP exert their activity. Ag NP are cytotoxic through inducing oxidative stress[23], impairing mitochondrial function[24], damaging DNA[25] and promoting inflammation[5]. In HMEC we show that Ag NP damage DNA, and this might account for the decreased viability of Ag NP-treated cells.

The contribution of free radicals to Ag NP toxicity in HMEC is puzzling. Indeed, while the production of ROS rapidly increases upon exposure to Ag NP, the three antioxidants used, which have different structure and act through different mechanisms[18,19], do not prevent Ag NP cytotoxicity. On the contrary, the antioxidant N-acetyl cysteine inhibited Ag NP cytotoxicity in HUVEC[5]. This discrepancy might be due to the well known differences occurring between micro- and macro-vascular endothelial cells and to different culture conditions used[12].

The reduced viability observed in HMEC exposed to Ag NP correlates with increased leakage of LDH, a cytosolic enzyme detected out of the cells when membrane integrity is lost. Recently, based on the evidence that discrepancies exist between the results of LDH release assay and MTT assay, the interference of Ag NP with LDH assay has been investigated[26]. While it is possible that the presence of Ag NP underestimates LDH leakage, in our system the results from LDH assay match the data from MTT assay.

When treated with sub-lethal, low concentrations of Ag NP, HMEC can be propagated for a month and are growth retarded for the duration of the experiment. This growth inhibition is partially reversible. Indeed, after 19 d, part of the cells were not exposed to Ag NP anymore and began to grow at a faster rate than HMEC maintained in the presence of Ag NP, suggesting that no permanent modifications occur as a consequence of a long-term exposure to Ag NP.

Most reports indicate that Ag NP promote an anti-angiogenic phenotype in endothelial cells of various species and derived from different districts by impairing their viability and inhibiting their growth, migration and differentiation in response to angiogenic factors[5-10]. Moreover, Ag NP were anti-angiogenic when evaluated in chick chorioallantoic membranes and in matrigel plugs in mice[27]. At the moment, we are aware of a single report demonstrating that Ag NP induce angiogenesis[11]. These discordant findings might result from the use of a transformed endothelial cell line[11]. While cell lines are useful for some experiments, they appear to be less suited for studies focused on the regulation of cell survival, proliferation and apoptosis[28].

In conclusion, our results demonstrate that Ag NP are toxic for microvascular endothelial cells and for endothelial precursors, thus indicating that they could be exploited to contrast angiogenesis. Moreover, it should be recalled that any substance that enters the blood stream will be in direct contact with the endothelium. Consequently, in case Ag NP are used to deliver drugs, the possibility of their cytotoxic effect on the endothelium lining the microvasculature should be taken into account.

More studies are necessary to delineate the mechanisms involved in Ag NP toxicity on HMEC and to translate our findings in *in vivo* models.

COMMENTS

Background

Because of their strategic location at the interface between blood and vessels, endothelial cells are readily exposed to various molecules, some of which may promote maladaptive functional changes or direct injury. Recently, the therapeutic potential of nanoparticles as active component or as vehicle to deliver drugs has been investigated. The study of the effects of nanoparticles on endothelial cells is therefore of paramount interest. Little is known about the sensitivity to silver nanoparticles of microvascular endothelial cells, which are highly-specialized regulators of tissue homeostasis and are fundamental in angiogenesis, *i.e.*, the formation of new capillaries from pre-existing vessels.

Research frontiers

The possibility of exploiting nanoparticles as alternative candidates to treat different diseases is under intensive investigation and represents a major challenge for modern medicine. This work shows that Ag NP are cytotoxic and genotoxic for capillary endothelial cells. Therefore, these results suggest that Ag NP might represent a challenging tool to treat all those diseases characterized by exaggerated angiogenesis, *i.e.*, the formation of new capillaries from pre-existing vessels. Indeed, angiogenesis is a crucial pathogenic event in malignant tumors, chronic inflammatory diseases, retinopathies, and psoriasis.

Innovations and breakthroughs

This is the first study to report the short and long term effects of Ag NP on capillary endothelial cells.

Applications

By demonstrating the cytotoxic effect of Ag NP on capillary endothelial cells, this study may represent a future strategy for therapeutic intervention in all those diseases characterized by pathologic angiogenesis.

Terminology

Angiogenesis is a complex multistep process resulting in the formation of new capillaries from pre-existing vessels. Protagonists in angiogenesis are the capillary endothelial cells which are stimulated by angiogenic factors to proliferate, invade the surrounding tissues and undergo differentiation to form new vessels. Nanoparticles are microscopic particles with dimension less than 100 nm. In particular, silver nanoparticles (Ag NP) are the most widely commercialized among all nanomaterials, because of their antiseptic properties.

Peer review

This is a very well written and designed manuscript. The purpose of the study is clear. Method, results and discussion are well documented.

REFERENCES

1 **Carmeliet P**. Angiogenesis in life, disease and medicine. *Nature* 2005; **438**: 932-936 [PMID: 16355210]

2 **Gerger A**, LaBonte M, Lenz HJ. Molecular predictors of response to antiangiogenesis therapies. *Cancer J* 2011; **17**: 134-141 [PMID: 21427557]

3 **Chaloupka K**, Malam Y, Seifalian AM. Nanosilver as a new generation of nanoproduct in biomedical applications. *Trends Biotechnol* 2010; **28**: 580-588 [PMID: 20724010 DOI: 10.1016/j.tibtech.2010.07.006]

4 **de Lima R**, Seabra AB, Durán N. Silver nanoparticles: a brief review of cytotoxicity and genotoxicity of chemically and

biogenically synthesized nanoparticles. *J Appl Toxicol* 2012; **32**: 867-879 [PMID: 22696476 DOI: 10.1002/jat.2780]

5 **Shi J**, Sun X, Lin Y, Zou X, Li Z, Liao Y, Du M, Zhang H. Endothelial cell injury and dysfunction induced by silver nanoparticles through oxidative stress via IKK/NF-κB pathways. *Biomaterials* 2014; **35**: 6657-6666 [PMID: 24818879 DOI: 10.1016/j.biomaterials.2014.04.093]

6 **Grosse S**, Evje L, Syversen T. Silver nanoparticle-induced cytotoxicity in rat brain endothelial cell culture. *Toxicol In Vitro* 2013; **27**: 305-313 [PMID: 22954533 DOI: 10.1016/j.tiv.2012.08.024]

7 **Trickler WJ**, Lantz SM, Murdock RC, Schrand AM, Robinson BL, Newport GD, Schlager JJ, Oldenburg SJ, Paule MG, Slikker W, Hussain SM, Ali SF. Silver nanoparticle induced blood-brain barrier inflammation and increased permeability in primary rat brain microvessel endothelial cells. *Toxicol Sci* 2010; **118**: 160-170 [PMID: 20713472 DOI: 10.1093/toxsci/kfq244]

8 **Kalishwaralal K**, Banumathi E, Ram Kumar Pandian S, Deepak V, Muniyandi J, Eom SH, Gurunathan S. Silver nanoparticles inhibit VEGF induced cell proliferation and migration in bovine retinal endothelial cells. *Colloids Surf B Biointerfaces* 2009; **73**: 51-57 [PMID: 19481908 DOI: 10.1016/j.colsurfb.2009.04.025]

9 **Gurunathan S**, Lee KJ, Kalishwaralal K, Sheikpranbabu S, Vaidyanathan R, Eom SH. Antiangiogenic properties of silver nanoparticles. *Biomaterials* 2009; **30**: 6341-6350 [PMID: 19698986 DOI: 10.1016/j.biomaterials.2009.08.008]

10 **Sheikpranbabu S**, Kalishwaralal K, Venkataraman D, Eom SH, Park J, Gurunathan S. Silver nanoparticles inhibit VEGF- and IL-1beta-induced vascular permeability via Src dependent pathway in porcine retinal endothelial cells. *J Nanobiotechnology* 2009; **7**: 8 [PMID: 19878566 DOI: 10.1186/1477-3155-7-8]

11 **Kang K**, Lim DH, Choi IH, Kang T, Lee K, Moon EY, Yang Y, Lee MS, Lim JS. Vascular tube formation and angiogenesis induced by polyvinylpyrrolidone-coated silver nanoparticles. *Toxicol Lett* 2011; **205**: 227-234 [PMID: 21729742 DOI: 10.1016/j.toxlet.2011.05.1033]

12 **Danese S**, Dejana E, Fiocchi C. Immune regulation by microvascular endothelial cells: directing innate and adaptive immunity, coagulation, and inflammation. *J Immunol* 2007; **178**: 6017-6022 [PMID: 17475823]

13 **Chi JT**, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, Brown PO. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci USA* 2003; **100**: 10623-10628 [PMID: 12963823]

14 **Reinisch A**, Hofmann NA, Obenauf AC, Kashofer K, Rohde E, Schallmoser K, Flicker K, Lanzer G, Linkesch W, Speicher MR, Strunk D. Humanized large-scale expanded endothelial colony-forming cells function in vitro and in vivo. *Blood* 2009; **113**: 6716-6725 [PMID: 19321860 DOI: 10.1182/blood-2008-09-181362]

15 **Ades EW**, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol* 1992; **99**: 683-690 [PMID: 1361507]

16 **Baldoli E**, Maier JA. Silencing TRPM7 mimics the effects of magnesium deficiency in human microvascular endothelial cells. *Angiogenesis* 2012; **15**: 47-57 [PMID: 22183257 DOI: 10.1007/s10456-011-9242-0]

17 **Castiglioni S**, Casati S, Ottria R, Ciuffreda P, Maier JA. N6-isopentenyladenosine and its analogue N6-benzyladenosine induce cell cycle arrest and apoptosis in bladder carcinoma T24 cells. *Anticancer Agents Med Chem* 2013; **13**: 672-678 [PMID: 23094912]

18 **Monticone M**, Taherian R, Stigliani S, Carra E, Monteghirfo S, Longo L, Daga A, Dono M, Zupo S, Giaretti W, Castagnola P. NAC, tiron and trolox impair survival of cell cultures containing glioblastoma tumorigenic initiating cells by inhibition of cell cycle progression. *PLoS One* 2014; **9**: e90085 [PMID: 24587218 DOI: 10.1371/journal.pone.0090085]

19 **Petrônio MS**, Zeraik ML, Fonseca LM, Ximenes VF. Apocynin: chemical and biophysical properties of a NADPH oxidase inhibitor. *Molecules* 2013; **18**: 2821-2839 [PMID: 23455672 DOI: 10.3390/molecules18032821]

20 **Satapathy SR**, Mohapatra P, Preet R, Das D, Sarkar B, Choudhuri T, Wyatt MD, Kundu CN. Silver-based nanoparticles induce apoptosis in human colon cancer cells mediated through p53. *Nanomedicine* (Lond) 2013; **8**: 1307-1322 [PMID: 23514434 DOI: 10.2217/nnm.12.176]

21 **Flower NA**, Brabu B, Revathy M, Gopalakrishnan C, Raja SV, Murugan SS, Kumaravel TS. Characterization of synthesized silver nanoparticles and assessment of its genotoxicity potentials using the alkaline comet assay. *Mutat Res* 2012; **742**: 61-65 [PMID: 22178963 DOI: 10.1016/j.mrgentox.2011.12.003]

22 **Hanahan D**, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646-674 [PMID: 21376230 DOI: 10.1016/j.cell.2011.02.013]

23 **Foldbjerg R**, Olesen P, Hougaard M, Dang DA, Hoffmann HJ, Autrup H. PVP-coated silver nanoparticles and silver ions induce reactive oxygen species, apoptosis and necrosis in THP-1 monocytes. *Toxicol Lett* 2009; **190**: 156-162 [PMID: 19607894 DOI: 10.1016/j.toxlet.2009.07.009]

24 **Teodoro JS**, Simões AM, Duarte FV, Rolo AP, Murdoch RC, Hussain SM, Palmeira CM. Assessment of the toxicity of silver nanoparticles in vitro: a mitochondrial perspective. *Toxicol In Vitro* 2011; **25**: 664-670 [PMID: 21232593 DOI: 10.1016/j.tiv.2011.01.004]

25 **Hackenberg S**, Scherzed A, Kessler M, Hummel S, Technau A, Froelich K, Ginzkey C, Koehler C, Hagen R, Kleinsasser N. Silver nanoparticles: evaluation of DNA damage, toxicity and functional impairment in human mesenchymal stem cells. *Toxicol Lett* 2011; **201**: 27-33 [PMID: 21145381 DOI: 10.1016/j.toxlet.2010.12.001]

26 **Oh SJ**, Kim H, Liu Y, Han HK, Kwon K, Chang KH, Park K, Kim Y, Shim K, An SS, Lee MY. Incompatibility of silver nanoparticles with lactate dehydrogenase leakage assay for cellular viability test is attributed to protein binding and reactive oxygen species generation. *Toxicol Lett* 2014; **225**: 422-432 [PMID: 24463055 DOI: 10.1016/j.toxlet.2014.01.015]

27 **Kemp MM**, Kumar A, Mousa S, Dyskin E, Yalcin M, Ajayan P, Linhardt RJ, Mousa SA. Gold and silver nanoparticles conjugated with heparin derivative possess anti-angiogenesis properties. *Nanotechnology* 2009; **20**: 455104 [PMID: 19822927 DOI: 10.1088/0957-4484/20/45/455104]

28 **Boerma M**, Burton GR, Wang J, Fink LM, McGehee RE, Hauer-Jensen M. Comparative expression profiling in primary and immortalized endothelial cells: changes in gene expression in response to hydroxy methylglutaryl-coenzyme A reductase inhibition. *Blood Coagul Fibrinolysis* 2006; **17**: 173-180 [PMID: 16575254]

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