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REVIEW

Chronic alcohol consumption potentiates the development of diabetes through pancreatic β-cell dysfunction

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Abstract

Chronic ethanol consumption is well established as a major risk factor for type-2 diabetes (T2D), which is evidenced by impaired glucose metabolism and insulin resistance. However, the relationships between alcohol consumption and the development of T2D remain controversial. In particular, the direct effects of ethanol consumption on proliferation of pancreatic β-cell and the exact mechanisms associated with ethanol-mediated β-cell dysfunction and apoptosis remain elusive. Although alcoholism and alcohol consumption are prevalent and represent crucial public health problems worldwide, many people believe that low-to-moderate ethanol consumption may protect against T2D and cardiovascular diseases. However, the J- or U-shaped curves obtained from cross-sectional and large prospective studies have not fully explained the relationship between alcohol consumption and T2D. This review provides evidence for the harmful effects of chronic ethanol consumption on the progressive development of T2D, particularly with respect to pancreatic β-cell mass and function in association with insulin synthesis and secretion. This review also discusses a conceptual framework for how ethanol-produced peroxynitrite contributes to pancreatic β-cell dysfunction and metabolic syndrome.

Key words: Ethanol consumption; Type 2 diabetes; Pancreatic β-cells; Glucokinase; Metabolic syndrome

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Core tip: Chronic ethanol consumption induces Glucokinase (GCK) downregulation and inactivation through Tyr nitration, resulting in pancreatic β-cell apoptosis and dysfunction. Additionally, GCK proteins nitrated following ethanol treatment may be more susceptible to ubiquitination and consequent degradation than are native proteins of control cells. Peroxynitrite-mediated GCK downregulation or inactivation may perturb glucose metabolism and cellular antioxidant defense mechanisms, increasing susceptibility to insulin resistance and type-2 diabetes. Furthermore, peroxynitrite-generated activating transcription factor 3 may play as a major upstream regulator of GCK

downregulation in β-cell dysfunction and apoptosis.

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INTRODUCTION

Chronic alcohol consumption is associated with the increase of mortality, liver injuries, pancreatitis, hypertension, cardiomyopathy, and cerebrovascular diseases[1,2]. Although alcohol abuse is considered as a detrimental risk factor for human public health, there are evidences that low-to-moderate alcohol intake may exhibit certain beneficial effects, particularly regarding cardiovascular events, insulin resistance, and type-2 diabetes (T2D) through an augmentation of glucose-stimulated insulin secretion (GSIS) and insulin sensitivity[3,4]. Recently, some study also suggested that moderate drinking of wine drinking could decrease the incidence of nonalcoholic fatty liver disease, which is closely related to metabolic diseases including insulin resistance and obesity[5,6]. Most of these suggestions are based on epidemiological and controlled clinical data showing a J- or U-shaped relationship between alcohol consumption and the development of T2D[6,7], but they have still not fully explained the relationship between ethanol consumption and T2D. Furthermore, the mechanisms involved in the amelioration of insulin sensitivity by modest alcohol consumption ameliorates insulin sensitivity are not clearly understood. Recent studies demonstrated that drinking alcohol was associated with a loss of body weight and an increase of adiponectin secretion[8-10]. The enhancing effects of alcohol consumption on the release of plasma adiponectin may be very useful for the prevention of metabolic syndrome[6,11-13], since it was well known that adiponectin has a role to improve insulin sensitivity and fatty acid oxidation in various tissues. However, a recent study suggested that the administration of moderate amounts of ethanol to people or rodents with metabolic disorders, such as obesity or impaired glucose metabolism, could induce a higher risk of complications than was observed in healthy people[14].

Since alcohol is primarily metabolized by the enzyme alcohol dehydrogenase (ADH) in hepatocytes, the major functional cells that make up 70%-85% of the liver's cytoplasmic mass, the liver is the most susceptible organ for alcohol-induced damages[15-17]. A number of molecular mechanisms have been associated with the development and progression of alcohol-induced liver disease (ALD), which are determined by direct toxic effects of ethanol and indirect effects *via* various tissues and organs such as brain, heart, lung, and the neutrophils and macrophages of the immune system[15,18,19]. There is no question that ALD is considered as one of the major causing factors for increasing of morbidity and mortality *via* hepatic complications worldwide[20,21]. The "two-hit" hypothesis that is contributed to the progression from nonalcoholic fatty liver disease (NAFLD) to nonalcoholic steatohepatitis (NASH) can also be reflected to the development of alcoholic fatty liver disease (AFLD) to alcoholic steatohepatitis (ASH)[20,22,23]. An individual who has primary insult (fatty liver) by alcohol exposure may be predisposed to secondary insults such as steatohepatitis, liver inflammation and cirrhosis. A major stimulus for excessive cytokine production seen in the progression of AFLD to ASH is gut-derived endotoxin, lipopolysaccharide (LPS), a complex molecule found in the cell wall of many bacteria. Another stimulus contributing to ASH development is the generation of reactive oxygen species (ROS), as a by-product of alcohol metabolism in the liver[24]. Elevated levels of ROS are closely related to the development of ASH and NASH, which are regulated by the peroxidation of lipids, proteins, or DNA and mitochondrial damage. In general, the generation of ROS as a consequence of alcohol metabolism are caused by the alteration of mitochondrial oxidative chain *via* two major enzymes, cytochrome P450 2E1 (CYP2E1) and catalase that can metabolize alcohol to acetaldehyde[20,25,26]. ASH and NASH have a similar pathogenesis and histopathology but a distinct etiology and epidemiology[27]. Although ASH and NASH represent advanced stages of AFLD and NAFLD, respectively, the conditions responsible for the progression of uncomplicated liver steatosis to ASH or NASH are presently unknown. AFLD/ASH and NAFLD/NASH are increasingly relevant public health issues because of their close association with the worldwide epidemic of diabetes and obesity. These chronic liver diseases are common and are expected to substantially affect healthcare expenditures in future years.

In fact, pancreatic β-cells have a good compensatory response for increased insulin demand during the progression to insulin resistance and hyperglycemia[28]. However, if the cells were consistently overloaded, pancreatic β-cells fail to produce insulin, resulting in pancreatic β-cell dysfunction and subsequently, predisposes to the development of T2D[28]. Evidence from humans and rodents suggests that this condition may occur in part because of a decreased β-cell mass through its apoptosis[29,30]. Several reports also suggested that chronic ethanol consumption induced insulin resistance in insulin-sensitive tissues including liver and skeletal muscle by interfering with insulin signaling and causing

pancreatic β-cell apoptosis[31-33]. These effects are related to mitochondrial dysfunction and oxidative stress induced by acetaldehyde, the highly reactive metabolite of ethanol. Furthermore, it was well known that insulin-producing β-cells are very susceptible to changes in ER homeostasis[28,34,35]. ER stress excessively increased in pancreatic β-cells was associated with lipid accumulation and ROS production, lead to β-cell apoptosis and eventually, diabetes[36]. Therefore, pancreatic β-cells may be sensitive to ethanol-induced ER stress and oxidative stress, which represent the earliest events in glucose intolerance and are associated with mitochondrial dysfunction[37]. This reasoning suggests that the association of alcoholism with T2D is based on β-cell damage caused by ethanol consumption, but it remains controversial whether ethanol consumption inhibits insulin secretion by β-cells. In addition, the mechanisms underlying ethanol-mediated pancreatic β-cell dysfunction and apoptosis are not fully defined.

In the present review, we will also discuss the relationship between chronic ethanol consumption and metabolic disorders and suggest underlying mechanisms with a special focus on alcoholic fatty liver and pancreatic β-cell dysfunction and apoptosis *via* glucokinase nitration and its downregulation. As well, this review proposes potential therapeutic strategies to combat metabolic syndrome associated with pancreatic β-cell dysfunction and apoptosis induced by chronic exposure to ethanol.

ALCOHOL CONSUMPTION AND LIVER DISEASES

Alcohol consumption is recognized as leading life style risk factors that are associated with the development of metabolic syndrome including T2D as well as mental disability[38]. In particular, it has been known for many years that chronic alcohol consumption causes the development of hepatic steatosis, alcoholic hepatitis, and ultimately cirrhosis[39,40]. The pathogenesis of liver injury resulting from acute or chronic ethanol consumption may be different depends on various etiological characteristics[41]. Many other cell types, ranging from the innate immune cells to the liver parenchymal cells, *i.e.*, hepatocytes are involved in alcohol-induced liver injury[42]. Alcohol dehydrogenease (ADH) is the major pathway involved in oxidative ethanol metabolism in the liver and produces acetaldehyde, a highly reactive toxic molecule. Acetaldehyde produced from alcohol oxidation is typically metabolized rapidly to acetate, which is catalyzed by aldehyde dehydrogenase (ALDH)[43,44]. Alcohol oxidation induced by ADH and ALDH is accompanied by the conversion of NAD^+ to the reduced NADH, increasing the cellular NADH/NAD^+ ratio[15,45]. Additionally, ethanol-inducible CYP2E1, one of cytochrome P450 isozymes, is also predominantly involved in ethanol oxidation to acetaldehyde in the liver[15,46]. As like previously described, CYP2E1-medaited alcohol metabolism highly produces ROS and lipid peroxidation products, which lead to tissue damage. Another enzyme catalase located in peroxisomes can also oxidize ethanol by hydrogen peroxide (H_2O_2)-dependent pathway; however, this is considered as a minor pathway for alcohol metabolism[18]. It was well known that the oxidation of alcohol and produced acetaldehyde induced the changes of NADH/NAD^+ ratio in reduction-oxidative state of both the cytosol and mitochondria of hepatocytes[47]. NADH generated in mitochondria is conversely oxidized to NAD^+ by the electron transport chain. Similarly, in the condition of chronic alcohol consumption, the alterations in a variety of metabolic pathways observed may be caused by the change of NADH/NAD^+ ratio in the redox state. In particular, the increases of NADH/NAD^+ ratio by chronic ethanol consumption are correlated with a loss of mitochondrial membrane potential through mitochondrial permeability transition (MPT) opening[47]. Moreover, several studies also demonstrated that NADH is a key reducing cofactor for reduction of glutathione and an essential cofactor for pro-oxidative enzymes that generate reactive oxygen and nitrogen species (ROS and RNS, respectively),which could alleviate oxidative stress[48,49]. In general, superoxide ($O_2^{\bullet-}$) radical was produced at mitochondrial respiratory chain of various cell types including hepatocytes and pancreatic β-cells[50-52]. Despite of an important role of $O_2^{\bullet-}$ in alcohol consumption-mediated liver injury, oxidative damage induced by alcohol consumption is not sufficiently explained by $O_2^{\bullet-}$ alone[53]. Indeed, reactive radical involved in the oxidative damage caused by alcohol consumption is hydroxyl free radical ($^{\bullet}OH$), which is dependently formed by $O_2^{\bullet-}$production; its formation is associated with damaging enzymes, membrane lipids, and nucleic acids[53-55].Unless the generated free radical is quickly disposed of, the lifetime of $O_2^{\bullet-}$generated in mitochondria respiratory chain was prolonged and subsequently, the increased $O_2^{\bullet-}$ is sufficient to lead toxic effects *via* the formation of more potent oxidant H_2O_2through the reduction of $O_2^{\bullet-}$ by using superoxide dismutase (SOD)[56,57]. And also, superoxide is formed to peroxynitrite ($ONOO^-$), a strong oxidizing and nitrating species, by reacting with $NO^{\bullet}$[53,58].

Kupffer cells considered as hepatic macrophages is believed to play an important role in early liver injury event by exposure of alcohol *via* the recognition of endotoxin/LPS in the portal circulation[59,60]. Of the many factors associated with the development of alcoholic liver diseases, endotoxin/LPS, which is derived *via* the activation of gram-negative bacteria in gut, functions a major role in the progressive regulation of hepatic steatosis, inflammation, and

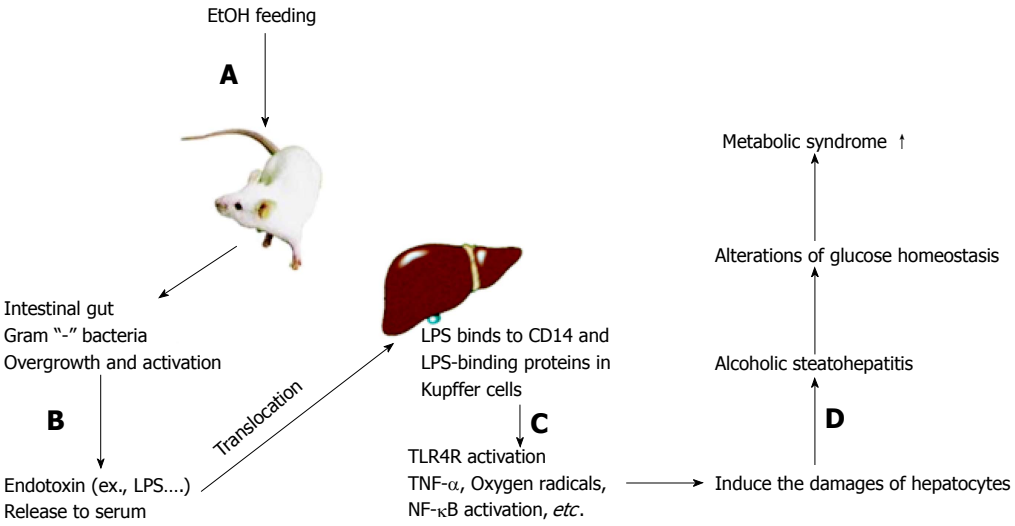

Figure 1 Proposed model by which ethanol feeding induces hepatocyte damage. A: Male 6-wk-old C57BL/6J mice (*n* = 8 in each group) were fed an ethanol diet for 8 wk. Individually caged mice were placed on a Lieber-Decarli regular liquid diet (Dyets; control diet: #710027, or ethanol diet: #710260). Mice were pair-fed with the control *vs* the 5% (v/v) ethanol diet for 8 wk; B: Alcohol intake induces the overgrowth and activation of intestinal gram-negative bacteria and subsequently increases the production of endotoxin (lipopolysaccharide, LPS) and its release into the blood; C: Released LPS activates toll-like receptor 4 (TLR4) on Kupffer cells, which are predominantly distributed in the lumen of hepatic sinusoids and exhibit endocytic activity against blood-borne materials entering the liver; D: Activated TLR4 drives Kupffer cells to produce inflammatory cytokines, chemokines, and reactive oxygen species *via* NF-kB activation, initiating an inflammatory cascade and hepatocyte damage.

fibrosis[61]. Recent experimental and clinical data also support that the stimulation of innate immune system is important to the progressive development of ALD[62]. The concept that the alter-regulation of innate immune response is a sufficient to induce ASH and NASH is supported by findings that alcohol consumption or high-fat diet intake increases the release of gut-derived endotoxin to the portal circulation, activating the hepatic immune Kupffer cells to produce pro-inflammatory cytokines, TNF-α and interleukin (IL)-6, and oxygen radicals[63-65].

There are several lines of evidence showing the role of gut-derived endotoxin on the development of both ASH and NASH (Figure 1). First, the intake of alcohol induces the damage the gastrointestinal barrier and subsequently diffuses the luminal bacterial products into the portal blood[66-68]. Second, acute and chronic alcohol feeding increased and/or activated intestinal gram-negative bacteria, subsequently elevating the production of endotoxin/LPS and its releasing into the blood. The production and release of endotoxin are observed in both human subjects and animal models of NAFLD/NASH. The serum levels of endotoxin released from alcohol-activated gram-negative bacteria were increased 5- to 20-fold in ASH patients, 3-fold in high-fat diet-fed healthy individuals, and 6- to 20-fold in NAFLD patients compared with normal subjects; the levels were also high compared with those of patients with non-alcoholic cirrhosis[63]. Third, LPS released from the gut microflora activates toll-like receptor 4 (TLR4) on Kupffer cells predominantly distributed in the lumen of hepatic sinusoids[69-71]. Fourth, activated TLR4 in Kupffer cells enhances the production of inflammatory cytokines, chemokines, and reactive oxygen species *via* NF-kB activation and increases the initiation of an inflammatory cascade and hepatocyte damage[72]. In general, oxidative stress induced by alcohol consumption causes the modification of hepatic proteins, which trigger immune reactions and consequent damage to hepatocytes. Actually, chronic ethanol consumption-mediated liver injury is concordantly associated with an increase in TNF-α, correlated with an increase in serum endotoxin levels[73]. In fact, macrophages release numerous cytokines when stimulated with endotoxin. The increased release of proinflammatory mediators (such as TNF-α, IL-1, IL-6, and ROS) and the infiltration of other inflammatory cells (such as neutrophils) eventually drive liver injury[66,74,75]. Accumulating evidence from experiments using knockout mice of these genes or their receptors suggests central roles for these inflammatory cytokines in the pathogenesis of ALD[76,77]. In fact, when monocytes and Kupffer cells are exposed to ethanol consumption, the expression and activities of endogenous signaling molecules and transcription factors are alternatively regulated[76,78]. Kupffer cells were activated by low concentrations of endotoxin (pg/mL to ng/mL), which is dependently regulated by CD14 receptor[66,79]. And also, when LPS binds to TLR4, the cooperation of LPS co-receptors, CD14and LPS-binding protein (LBP) are required to the interaction of LPS with TLR-4[59,79]. Since LBP, an acute phase reactant produced by hepatocytes, directly binds LPS and facilitates the association between LPS and CD14, low levels of endotoxin can be recognized by CD14 in the presence of LBP[59,80]. TLR4 deficiency prevented the development of both

ASH and NASH in association with the decrease inflammatory cytokines[63,81]. TLR4 can activate two distinct pathways, a MyD88-dependent and a MyD88-independent pathway[63,82]. Several studies using mouse models deficient in MyD88 or TLR4 supported that the pathogenic effect of TLR4 signaling in ASH is mediated *via* MyD88-independent pathway[63,82]. In a while, TLR4 signaling in the pathogenesis of NASH, including NF-kB activation and the upregulation of inflammatory cytokines is mediated by MyD88-dependent signaling[63,83-85]. In contrast to ASH, the development of NASH is closely associated with insulin resistance, which is correlated with endocrine interregulation between adipose tissue and the liver. Interestingly, the activation of TLR4 by MyD88-dependent pathway in macrophages was inhibited by adiponectin secreted from adipose tissue[63,82,86]. However, several studies show that there is no relationship between adiponectin and ASH. Despite of the differential regulation of adiponectin on both NAFLD/NASH and ASH, we cannot exclude the possibility that adiponectin may contribute to the pathogenesis of ASH and NASH by another signaling molecules or pathways.

To determine the responsiveness to endotoxins, including LPS, in ASH, we used LPS/D-galactosamine (GalN)- or ConA-induced hepatitis liver injury models showing the pathology similar to human liver disorders[87]. Liver histological evidences such as T cell infiltration, necrosis, and apoptosis, are rapidly induced within 8-24 h[88]. Despite of extensive studies, the exact molecular mechanisms involved in Con A- or LPS/GalN-induced hepatitis remain elusive. Several studies demonstrated that activated cytokines and their downstream signals, Janus kinase-signal transducer and activator of transcription factor (JAK-STAT), are important to T cell-mediated liver injury by regulating various cellular functions[89-93]. In general, binding of these cytokines enhanced the phosphorylation of the receptor-associated JAKs, which, in turn, activate the STATs, including STAT1, 2, 3, 4, 5, and 6[94]. Phosphorylated STATs form dimers and translocate into the nucleus to activate the transcription of many target genes, including the SOCS (suppressor of cytokine signaling) family of inhibitory proteins[95,96], which then turn off the JAK-STAT signaling pathway. Our previous data show that the infiltrating granulocytes, massive inflammation and necrosis observed in these hepatitis models are regulated by the IFN-γ-activated STAT1 pathway, which is correlated with T cell activation, as endotoxin-induced hepatitis liver injury is significantly abolished in STAT1$^{-/-}$ or IFN-γ$^{-/-}$ mice[97]. In addition, serum transaminase levels, hepatic inflammation, and necrosis increased in ethanol-fed mice are strongly potentiated by a low-dose injection of ConA[98].

ALD is induced by the direct toxic effects of alcohol and its metabolite, acetaldehyde, in various cell types in the liver[99]. In particular, alcoholic steatosis is considered as a key initial metabolic alteration in the progressive development of ALD. Lipid droplets in hepatocyte cytoplasm may inhibit cellular functions and/or make hepatocytes more susceptible to toxic or stress factors. The pathogenesis of alcoholic steatosis are caused by various mechanisms including the induction of fatty acid synthesis, inhibition of fatty acid oxidation, and reduction of very low-density lipoprotein (VLDL) secretion into the blood[100]. Furthermore, increasing evidence also suggests that adipose tissue dysfunction may impact hepatic lipid metabolism. Therefore, lipid homeostasis *via* interregulation of the liver-adipose tissue axis is important to regulate whole-body energy homeostasis[101,102]. White adipose tissue (WAT) is the major organ used to store excess energy for maintaining the status of energy homeostasis, whereas when the energy is required to other organs, WAT enhances the lipolysis and then produced free fatty acids (FFAs) is released to other organs[101-103]. When excess fatty acids produced by a fat storage function disordered in WAT are released and taken up by the liver, hepatic steatosis is easily developed[104]. Indeed, the mechanisms involved in hepatic fat accumulation *via* the influx of FFAs into the liver are well established in the "two-hit" model of non-alcoholic fatty liver disease (NAFLD) progression. Similarly, several clinical studies have demonstrated that alcoholic steatohepatitis (ASH) is also associated with the loss of fat mass and higher liver fat[105]. Ethanol consumption promotes the lipolysis of adipose tissues and the production of excess FFAs, followed by the accumulation of a large amount of fat droplets in the cytoplasm of hepatocytes, causing hepatic steatosis. These developments are also correlated with increases in lipogenesis and/or gluconeogenesis induced by elevated fatty acid synthase (FAS) and/or PEPCK expression, respectively.

Different from Western populations, Southeast Asian populations, including that of Korean, have many lean patients with T2D or metabolic disorders that may be caused by alcohol consumption[106]. Although ASH and NASH exhibits similar characteristics in histological pathology and progressive pattern to advanced liver disease[63], a number of comparable pathogenic mechanisms such as immune signaling and its pathological mechanisms in both ASH and NASH are still not clearly understood. Additional studies to divide the differences in the pathogenesis in ASH and NASH are required.

ALCOHOL CONSUMPTION AND PANCREATIC β-CELL DYSFUNCTION

Insulin resistance (IR) and pancreatic β-cell dysfunction are considered as two major independent-risk

factor for the onset of T2D[107,108], despite of some differences of these factors in each individual. The impaired glucose homeostasis and insulin insensitivity are generally used for the clinical diagnosis of T2D[109], but the pathophysiology underlying in peripheral organs such as muscle, liver, and adipose tissues are various in each individual. Therefore, to exactly identify which risk factors are involved in the development of each case of T2D patient is very important. Several previous reports have commonly suggested that IR was also closely associated with classic risk factors of T2D, including the enhancement of adiposity, hypertension, and hyperlipidemia[107,110]. A number of studies have also examined risk factors associated with β-cell dysfunction, but these studies rarely included multivariable adjustment. In the patients with T2D, IR increased in peripheral tissues with major metabolic functions, is accompanied with the reduction of insulin secretion induced by pancreatic β-cell dysfunction and apoptosis[111,112]. Furthermore, it is now well accepted that the failure of β-cells to compensate for insulin resistance is a prerequisite for T2D development. Although risk factors involved in the onset of T2D in individuals are differ according to various etiological phenomenons of IR and β-cell dysfunction[107,113], this discrepancy in IR and β-cell dysfunction may be caused by differing extents and durations of exposure to life style-related risk factors, such as high fat diet, alcohol consumption, smoking, and a lack of physical activity.

Indeed, previous studies suggested that chronic ethanol consumption is generally considered as a potent causing factor for chronic pancreatitis and is associated with 50%-70% of chronic pancreatitis patients[114]. However, chronic alcohol consumption alone does not cause chronic pancreatitis because a little heavy or chronic alcohol drinkers clinically develop to chronic pancreatitis[115,116]. The roles and involved mechanisms of alcohol consumption in the development of chronic pancreatitis are complex and poorly understood. Histological examination of pancreatic tissue from patients with chronic pancreatitis reveals inflammatory cells and extensive fibrosis. Although some studies have suggested that chronic alcoholics progress to chronic pancreatitis through the recruitment of inflammatory cells[117], present studies about the relationship between alcohol consumption and pancreatitis have not provide sufficiently evidence for explaining the roles and mechanisms involved in ethanol consumption-mediated pancreas damage, particularly, with respect to pancreatic β-cell dysfunction and apoptosis. The acute effects of ethanol consumption on insulin secretion by pancreatic β-cells remain controversial, but alcohol consumption is generally considered an independent risk factor for T2D[118,119]. Recently, chronic ethanol feeding in rodents was reported to cause pancreatic β-cell dysfunction and to decrease β-cell mass *via* apoptosis[34]. It is expected that the association of alcoholism with T2Dis based on this ethanol consumption-induced damage to β-cells, but the effects of ethanol consumption on β-cell dysfunction characterized by the inhibition of insulin secretion remain controversial, as both inhibition and enhancement of insulin secretion by ethanol consumption have been reported. Although acute ethanol consumption enhanced insulin secretion form pancreatic β-cells and thus increased insulin sensitivity in peripheral tissues, the exact regulatory mechanisms involved in these events are clearly unknown. These events may be an early defense mechanism to compensate for ethanol-inhibited basal insulin secretion. However, several studies demonstrated negative effects of ethanol on the function of β-cells, such as insulin synthesis and insulin secretion[120]. Excessive ROS production may be a mechanism involved in ethanol consumption-induced pancreatic β-cell dysfunction, thus triggering the development of T2D. Increased ROS may be one of the earliest events associated with mitochondrial dysfunction-mediated glucose intolerance, and may play an important sensitizer for the progressing of β-cell dysfunction and apoptosis[34]. Particularly, contrast to hepatocytes, pancreatic β-cells are characterized by their very low levels of the H_2O_2-inactivating enzymes catalase and glutathione peroxidase despite adequate expression of the superoxide ($O_2^{\bullet-}$)-inactivating SOD. Therefore, pancreatic β-cells have a high susceptibility to the toxicity of H_2O_2 and other produced ROS. As well, ROS produced *via* mitochondrial dysfunction are closely associated with increased endoplasmic reticulum (ER) stress. ER, localized in cellular cytoplasm, is responsible for the synthesis, packaging, and assembly of secretory and membrane proteins[34,121]. In particular, insulin-producing β-cells are very sensitive to ER stress *via* the changes of ER homeostasis since excessive ER stress remarkably inhibited insulin synthesis. ER stress is caused or characterized by the accumulation of unfolded response proteins and the increase of ER stress activators[122]. Excessive ER stress triggers lipid accumulation and ROS production in various cell types including β-cells and hepatocytes and, consequently, leads to diabetes mellitus *via* apoptotic cell death[34,123]. These conclusions have been supported by several intervention studies using genetic modifications or chemical chaperones[33]. The administration of moderate amounts of ethanol to rodents with metabolic disorders may increase the risk of complications in metabolic diseases or diabetes[124], as a result of the increased ER stress and ROS production with mitochondrial dysfunction. In addition, several studies demonstrated that the reduction in insulin content caused by ethanol was associated with reduced insulin synthesis, which has been attributed to ER stress. Despite convincing

evidence that chronic ethanol consumption acts as a risk factor for T2D[125,126], the direct effects of ethanol on the β-cell mass and its function and the exact mechanisms involved in β-cell dysfunction and apoptosis by chronic ethanol consumption are little known. Furthermore, as the pleiotropic effects of ethanol could be mediated by altered gene expression or epigenetic modifications[127,128], a comprehensive assessment about the impacts of ethanol on the expression or modification of genes related to the regulation of β-cell function should be considered. As well, we could not exclude the physiological roles of uncoupling protein 2 (UCP2), an isoform expressed in insulin-secreting cells, on the impairment of β-cell function (GSIS) and apoptosis by chronic ethanol consumption[129]. UCP2 exerts as a mitochondrial anion carrier protein and as antioxidant to reduce the generation of mitochondrial ROS by uncoupling of the proton motive force, possible tuning of redox homeostasis[130,131]. It has been reported that a β-cell-specific knock out of UCP2 was associated with increased mitochondrial membrane potential, ATP content and, subsequently, with elevated glucose-induced insulin secretion[132]. Conversely, UCP2 overepxression strongly attenuated GSIS, thereby indicating that uncoupling of the proton influx by UCP2 has a negative effect on insulin secretory responsiveness. As well, pancreatic β-cells isolated from UCP2 knock-out mice had chronically higher ROS compared to wild type mice[131,132]. Although the relationship between ethanol consumption and physiological roles of UCP2 did not clearly understood, several studies suggested that the impairment of redox homeostasis and energy metabolism induced by chronic ethanol consumption may be caused by the alteration of uncoupling mechanisms by UCP2, thereby results in pancreatic β-cell dysfunction and apoptosis[133].

CHRONIC ETHANOL CONSUMPTION AND THE DOWNREGULATION OF GLUCOKINASE

Glucokinase (GCK) plays an important role in the maintenance of blood glucose homeostasis, acting both as a metabolic sensor for GSIS in pancreatic β-cells and as the major regulator of glucose uptake in hepatocytes[134,135]. Previously, we demonstrated that chronic heperglycemia-induced β-cell dysfunction and apoptosis were closely related to GCK downregulation, which resulted in ROS production and AMPK hyperactivation[134,136]. However, the relevant upstream regulators of GCK downregulation remain elusive. One of major transcription factor essential to the maintenance of β-cell function is pancreatic duodenal homeobox-1 (PDX-1), which regulates the expression of genes such as insulin, islet amyloid polypeptide, GCK, and Glut2, which are[137]. Another transcription factor that regulates β-cell function is sterol regulatory element-binding protein-1c (SREBP-1c) and it binds directly to the SRE elements of GCK promoter, increasing GCK expression and enhancing the action of insulin on GCK transcription[137,138]. Recently, although the precise mechanisms remain unclear, we reported that lipotoxicity promoted the activity of activating transcription factor (ATF)-3, a member of the ATF/cAMP-responsive element binding protein subfamily, which was also associated with the inhibition of PDX-1-induced GCK promoter activity[139-141]. *ATF3*is an early-responsive gene to a variety of stresses and functions as a stress-inducible transcriptional repressor or activator[142,143]. Given its frequent induction by various cellular stressors, ectopic expression of ATF3 in heart, liver, and pancreatic β-cells causes cardiac enlargement, liver or pancreatic β-cell dysfunction and apoptosis, and impaired glucose metabolism and diabetes, and is associated with the induction of signals involved in pancreatic β-cell dysfunction, such as proinflammatory cytokines, nitric oxide, and high concentrations of glucose and free fatty acids[34,144]. Indeed, ethanol-induced oxidative stress may be associated with the induction of ATF3.

In addition, we suggested that ATF3 may play a critical role in ethanol-induced susceptibility to β-cell dysfunction and apoptosis, as well as hepatotoxicity, which can be triggered by an enhancement in peroxynitrite generation[141,145]. Peroxynitrite can stably react with cysteine and tyrosine residues on biological target proteins[146,147]. Similarly, NO generated by iNOS induction can directly induce the modification of target proteins through S-nitrosylation and nitration on cysteine and Tyr residues, respectively[148]. These modifications can also be associated with the changes of pathophysiological processes in organ tissues, and thus develops to diseases. A recent study demonstrated that insulin-induced neuronal nitric oxide synthase (nNOS) leads to S-nitrosylation of GCK, which helps to prevent the impaired glucose response and apoptosis of pancreatic β-cells[149]. However, other studies have demonstrated that peroxynitrite generated by cytokines or endotoxins can also induce impaired glucose homeostasis and pancreatic β-cell dysfunction and apoptosis[150,151].

Although the deleterious effects of chronic heavy alcohol consumption on the development of impaired fasting glucose or T2D are well known, the exact molecular mechanisms involved in chronic ethanol consumption-induced impaired glucose homeostasis and pancreatic β-cell dysfunction and apoptosis remain unclear. Recently, we demonstrated that chronic ethanol consumption-mediated pancreatic β-cell dysfunction and apoptosis are correlatively associated with peroxynitrite-mediated GCK down-

regulation and ATF3 induction[34]. As shown in Figure 2, pancreatic β-cell dysfunction and apoptosis were remarkably increased in chronic ethanol-fed mice. These processes were correlated with reductions in β-cell mass and insulin expression. Additionally, ethanol consumption inhibits the production of ATP and the synthesis or secretion of insulin in pancreatic β-cells. In contrast to previous studies showing that ethanol consumption is related to a decreased risk of T2D and cardiovascular disease, our data show that chronic ethanol consumption for 8 or 10 wk significantly impaired tolerance to both glucose and insulin, potentially as a result of reduced insulin synthesis or secretion *via* pancreatic β-cell dysfunction and apoptosis. However, we have also shown that the reduction in insulin synthesis detected in the β-cells isolated from ethanol-fed mice might be associated with the inhibition of insulin action and gluconeogenesis in the liver, correlated with hepatocellular damage caused by elevated plasma levels of alanine aminotransferase (ALT). These results suggest that the reduced insulin action upon glucose disposal in ethanol-fed mice was due to the reduction in insulin synthesis or secretion in pancreatic β-cells. Consistent with these results, Ser-307 phosphorylation of insulin receptor substrate 1 (IRS1) (inactive form) was strongly increased in the livers of ethanol-treated mice, whereas the activating Tyr-941 phosphorylation of IRS1 and Akt phosphorylation were significantly suppressed under the same conditions, suggesting reduced insulin action in the liver. Also, the increase of impaired glucose tolerance and the reduction of insulin sensitivity in peripheral tissues of ethanol-fed mice were strongly correlated with pancreatic β-cell apoptosis, characterized by increased levels of caspase-3 or PARP cleavage and the Bax/Bcl-2 ratio. The impaired glucose tolerance and pancreatic β-cell dysfunction of ethanol-fed mice are correlated with reductions in GCK protein expression and in those of glucose transporter GLUT2 and insulin. In contrast, the stress-inducible transcription factor ATF3 was significantly increased in pancreatic tissues and islet cells of ethanol-fed mice. These events were confirmed *in vitro* using the MIN6N8 pancreatic insulinoma cell line. In MIN6N8 cells, ethanol treatment strongly increased GCK expression at an early time point (6 h); expression remained high at 12 h but decreased thereafter, reaching baseline levels after 24 h, whereas ATF3 was significantly increased and predominantly localized in the nucleus. The early enhancement of GCK expression (6-12 h) following ethanol treatment might help to explain previous studies demonstrating that light-to-moderate ethanol consumption is associated with a lower risk of T2D. Indeed, several previous clinical studies demonstrated that low-to-moderate alcohol intake in obese participants or those with T2D was associated with low fasting glucose levels, which might subsequently lead to a diagnosis of non-T2D[6,8,10,151]. However, participants exposed to heavy alcohol consumption were more vulnerable to the development of impaired fasting glucose or T2D.

One explanation for these discrepancies might be associated with the amount and duration of ethanol exposure. Indeed, our studies show that chronic alcohol consumption for 8 or 10 wk significantly increased the development of impaired fasting glucose and β-cell apoptosis, followed by reductions in insulin and ATP production[34]. These results suggest that chronic ethanol consumption can completely abolish the early defense mechanism observed at an early time point of ethanol exposure in pancreatic β-cells and thus reduced the synthesis or secretion of insulin. Recently, we demonstrated that ATF3 inhibits the function of PDX-1 and decreases the synthesis of insulin in pancreatic β-cells, subsequently, leads to pancreatic β-cell dysfunction and apoptosis[152]. Similarly, our previous studies have also found that ATF3, especially, its C-terminal domain, is a potent upstream regulator for GCK downregulation and apoptosis induced by ethanol exposure in pancreatic β-cells[34]. Although the exact regulatory factors involved in the ethanol-induced susceptibility to pancreatic β-cell dysfunction and apoptosis remain clearly unknown, a number of evidences indicate that oxidative stress induced by ethanol exposure is a key cellular process to the development of organic toxicity, which can be triggered by enhancing peroxynitrite generation. Based on this knowledge, ethanol-induced ATF3 can be regulated by oxidative stress generated through CYP2E1 metabolism and may act as a critical regulator of the GCK downregulation induced by ethanol-generated oxidative stress in pancreatic β-cells. Several lines of evidence suggest that both ethanol-mediated GCK downregulation and ATF3 induction require ethanol metabolism (Figure 2). First, blocking ethanol metabolism with 4-MP attenuated the ethanol-induced reductions in GCK, insulin, and PDX-1 expression and the increase in ATF3. However, various effects of 4-MP on GCK downregulation were able to be mediated by ethanol-induced endogenous ATF3 or exogenously overexpressed ATF3 because 4-MP did not inhibit transfected ATF3 and/or ethanol-induced GCK downregulation. Second, acetaldehyde-induced GCK downregulation and β-cell apoptosis were inhibited by ATF3 depletion but not by 4-MP, suggesting that ATF3 may be a downstream regulator of ethanol metabolism and serve as an executive effecter of ethanol-mediated GCK downregulation. Our data also show that peroxynitrite-mediated GCK downregulation and apoptosis were attenuated by the peroxynitrite scavengers L-NMMA and uric acid (UA). In contrast to the potent effects of ATF3 on ethanol-induced GCK downregulation, ATF3 did not affect

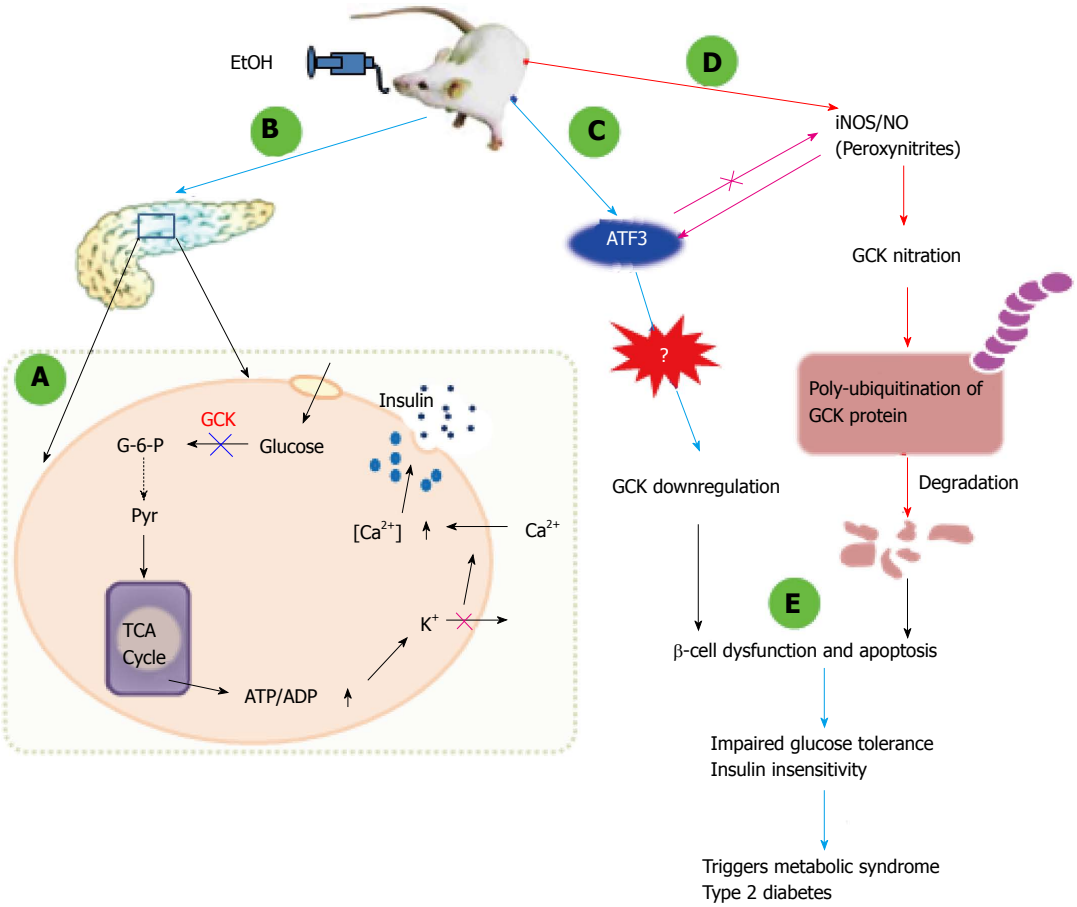

Figure 2 Proposed model by which peroxynitrite-mediated activating transcription factor 3 and glucokinase nitration in ethanol-fed mice trigger metabolic syndrome through pancreatic β-cell dysfunction and apoptosis. A: Under normal conditions, when glucose is taken up by pancreatic β-cells, it is converted to glucose-6-phosphate by glucokinase. Pyruvate transferred into the mitochondria activates the TCA cycle, which produces ATP. Increased ATP/ADP ratios prevent potassium efflux *via* inhibiting potassium channels and opening calcium channels. Increased intracellular calcium levels activate insulin maturation and its secretion; B: In mice fed with a 5% (v/v) ethanol diet for 8 wk, glucokinase (GCK) protein expression was significantly decreased in pancreatic tissues and isolated islet cells; C: Ethanol-fed mice showed high expression of activating transcription factor (ATF) 3. ATF3 is regulated by the ethanol metabolism pathway because ethanol-induced ATF3 was significantly decreased by 4-methylpyrazole, an inhibitor of cytochrome P450 2E1. ATF3 expression is regulated by the peroxynitrite-dependent pathway, but ATF3 did not affect the ethanol-mediated production of peroxynitrite. Despite this discrepancy, the C-terminal domain of ATF3 is involved in the downregulation of GCK. However, the role of ATF3 and the exact regulatory mechanisms involved in GCK downregulation are still not fully understood; D: Ethanol-produced peroxynitrite significantly increased GCK nitration on tyrosine residues; this effect was abolished by an iNOS inhibitor, L-NMMA, or the peroxynitrite scavengers, uric acid and deferoxamine. Interestingly, the nitrated GCK was more susceptible to ubiquitination than were the native proteins of control cells, making it vulnerable to degradation; E: Cells with downregulated GCK expression lack the ability to synthesize insulin and eventually progress to apoptosis. Mice lacking the ability to produce insulin may easily develop metabolic syndrome with impaired glucose tolerance and insulin insensitivity, resulting in type 2 diabetes.

ethanol-mediated iNOS or NO production, indicating that ATF3 may be a downstream regulator of the ethanol metabolism-mediated iNOS/NO pathway, which associates GCK downregulation with β-cell apoptosis. Mice lacking hepatic glucokinase show typical signs of maturity-onset type 2 diabetes of the young (MODY2), which results from impaired glucose tolerance caused by the failure of glucokinase activity to facilitate hepatic glucose utilization and glycogen synthesis[135,153]. We have demonstrated that ethanol-generated peroxynitrite can directly regulate GCK levels and activity. Similar to NO generated by iNOS, exogenously administrated NO can also directly change the structure of target proteins *via* nitration and S-nitrosylation on Tyr and cysteine residues, respectively, thereby altering pathophysiological processes[154-156]. Several previous studies about the regulation of GCK activity have been focused to the role of protein interactions with the GCK regulator (GCKR) or glucose[48,157]. However, the precise control mechanisms involved in the regulation of cellular GCK activity or its expression at the posttranslational or posttranscriptional level, especially in β-cells, still remain unclear. In pancreatic β-cells, insulin was recently shown to activate neuronal-type nitric oxide synthase (nNOS), which forms a complex with GCK on the surface of secretory granules, leading to the S-nitrosylation of GCK following the dissociation of GCK from granules[158]. Furthermore, several previous studies have demonstrated that NO and endothelial nitric oxide synthase (eNOS) generated by insulin-like growth factor-1 (IGF-1) and insulin prevent serum starvation-induced RINm5F β-cell apoptosis[159]. However, in contrast to the protective

effect of insulin or IGF-1-induced iNOS and NO on GCK S-nitrosylation, which prevents pancreatic β-cell dysfunction, peroxynitrite generated through various stresses, such as cytokines or endotoxins, also serves as a vital mechanism that causes impaired glucose metabolism and apoptosis in rat, mouse, and human pancreatic β-cells[160]. Similar to previous reports showing that peroxynitrite can directly damage proteins, DNA, and lipids[161], our study also shows that ethanol metabolism-generated peroxynitrite increases Tyr nitration of GCK, which is correlated with GCK downregulation. Similarly, recombinant GST-GCK was effectively nitrated on its Tyr residues after incubation with peroxynitrite. However, ATF3 did not directly affect GCK nitration, although ATF3 plays a critical role in regulating GCK expression. Ethanol-induced GCK nitration and iNOS induction were not altered by C-terminal domain-deleted ATF3(ΔC) or ATF3 siRNA. In contrast, ATF3 strongly inhibited GCK nitration in ethanol-cotreated cells even though ethanol-induced iNOS expression was not affected by ATF3, possibly due to the nearly complete reduction in GCK protein expression elicited by the combination of ethanol and ATF3. Previous studies have demonstrated that nitration modification is associated with the functional loss of target proteins due to the inhibition of Tyr residues important to the activity of protein[161,162]. The activity of GCK in converting glucose to glucose-6-phosphate was also inhibited by ethanol and SIN-1, but reversibly restored by the peroxynitrite scavengers L-NMMA, UA, and DFO. However, the GCK inactivation induced by ethanol or SIN-1 was not implicated in the S-nitrosylation of GCK because DTT, an S-nitrosylation inhibitor, had little effect on the inhibition of GCK activity induced by ethanol or peroxynitrite. In addition, the interaction of immunoprecipitated GCK with pBad and the GCK translocation into the mitochondrial fraction were determined by peroxynitrite-mediated GCK nitration.

Protein nitration can also induce the functional loss of target proteins *via* rapid degradation[48,161,162]. Our data also show that the ethanol-mediated nitrated GCK may be more susceptible to ubiquitination than are the native proteins of control cells, resulting in degradation[34]. The levels of GCK nitration and ubiquitination were concurrently increased in ethanol-treated cells and correlated with the reduced GCK protein levels, suggesting that nitrated proteins can be more easily ubiquitinated and degraded. Our data also show that GCK downregulation and the reduction of its activity by ethanol exposure are predominantly regulated by the modification *via* tyrosine nitration. However, the exact mechanism involved in the GCK modification on Tyr residues by ethanol-generated peroxynitrite remains unclear. Further studies involving ESI-MA are needed to confirm which Tyr residues are involved in the peroxynitrite-induced GCK downregulation and inactivation. In addition, because antioxidant enzymes are sensitive to the oxidative toxic effects of peroxynitrite[150], the inactivation of cellular antioxidant defense enzymes observed in ethanol-treated mice or cells is caused by excessive production of peroxynitrite, subsequently, potentiates the harmful cellular or organ defects. Indeed, our data showed that peroxynitrite-mediated GCK downregulation was reversely improved by the treatment of antioxidants such as NAC and L-NMMA. Additionally, excessive ROS is involved in the induction of protein damage by enhancing the proteolytic susceptibility to unfolded proteins and increasing the accessibility of proteases to the peptide bonds of proteins[162]. Previously, we have demonstrated that chronic exposure to high glucose significantly reduces GCK translocation to the mitochondrial membrane, accompanied with the reduction of GCK protein expression, and then conversely, increases the interaction of Bax with a voltage-dependent anion chain (VDAC) located in mitochondrial membrane, resulting in β-cell apoptosis[136], which were correlated with the induction of oxidative stress and mitochondrial dysfunction[134]. Thus, it is possible that GCK inactivation by peroxynitrite-mediated nitration is, partially associated with the inhibition of cellular reduction-oxidation status and the increase of oxidative damage observed during ethanol consumption. In addition, despite the lack of an effect of ATF3 on ethanol-mediated GCK nitration at the posttranslational level *via* the induction of iNOS and NO production, ATF3 may still play as a key regulator in GCK downregulation and pancreatic β-cell dysfunction and apoptosis. Therefore, we are now examining whether ATF3 plays a critical role in the transcriptional regulation of GCK. We have identified five potential binding sites for ATF/CREB in the GCK promoter using the TRANSFAC transcription factor binding database. Moreover, we have found that ATF3 can directly interact with specific binding sites in the 5′-flanking region of the GCK promoter, and then confirmed these interactions using mutated versions of the constructs.

CONCLUSION

Increasing evidence supports the idea that chronic alcohol consumption may trigger the progression or development of type 2 diabetes through impaired glucose metabolism and pancreatic β-cell dysfunction and apoptosis. Moreover, low-to-moderate alcohol consumption can also induce structural changes in the GCK protein *via* tyrosine nitration; reducing the activity of the nitrated enzyme. Here, we show for the first time that chronic ethanol consumption induces GCK downregulation and inactivation *via* the nitration at the Tyr residues of GCK, followed by pancreatic β-cell dysfunction and apoptosis. Our results strongly suggest that GCK proteins are nitrated following ethanol treatment, making

them more susceptible to ubiquitination (compared with the native proteins of control cells) and thus vulnerable to degradation. Peroxynitrite-mediated GCK downregulation or inactivation may also perturb glucose metabolism and cellular antioxidant defense mechanisms, increasing susceptibility to insulin resistance and T2D. Furthermore, peroxynitrite-generated ATF3 may play as a potent upstream regulator of GCK downregulation and β-cell dysfunction and apoptosis.

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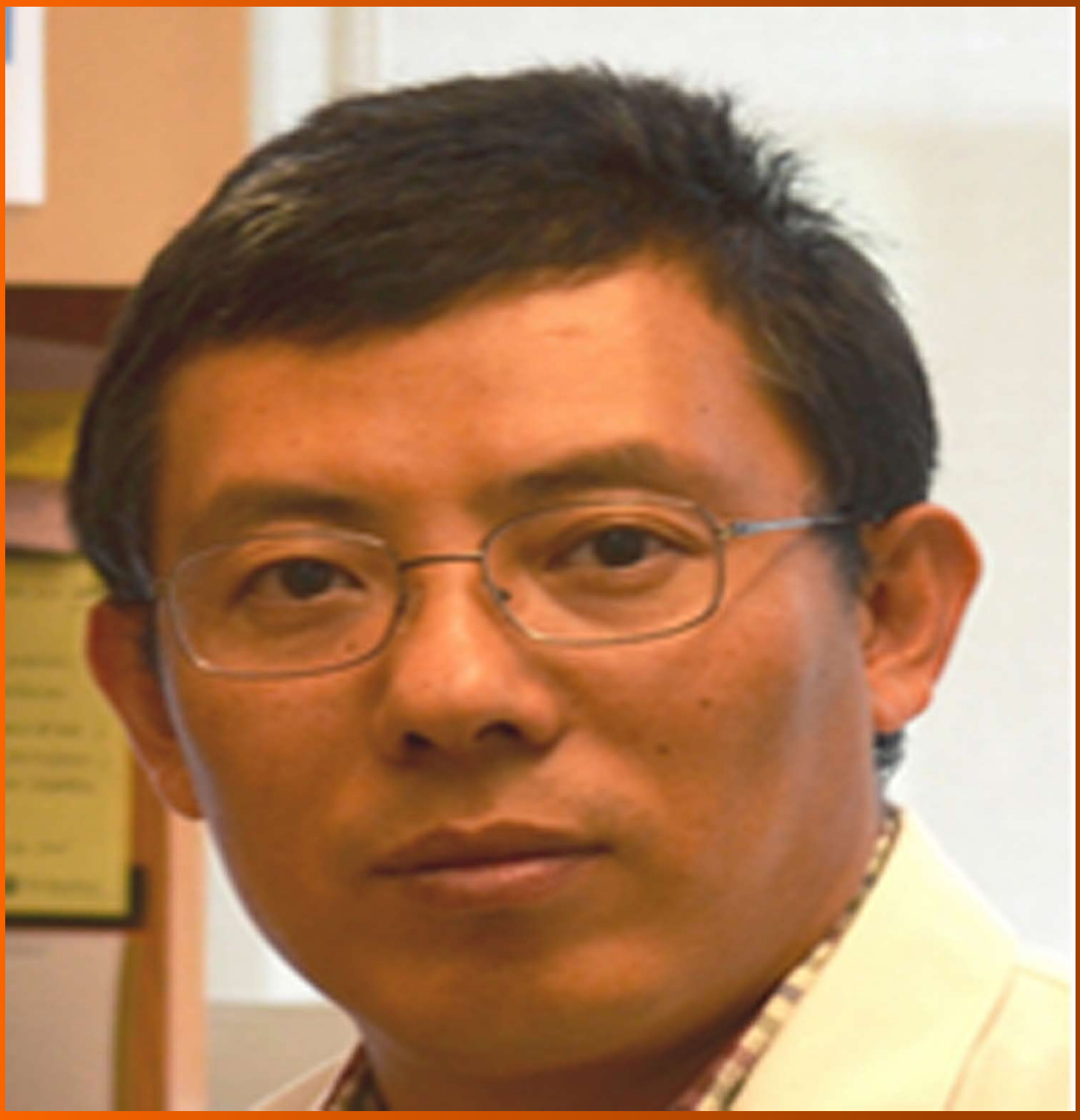

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EDITORIAL

Promise and challenges on the horizon of MET-targeted cancer therapeutics

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Abstract

MET (MNNG HOS transforming gene) is one of the receptor tyrosine kinases whose activities are frequently altered in human cancers, and it is a promising therapeutic target. MET is normally activated by its lone ligand, hepatocyte growth factor (HGF), eliciting its diverse biological activities that are crucial for development and physiology. Alteration of the HGF-MET axis results in inappropriate activation of a cascade of intracellular signaling pathways that contributes to hallmark cancer events including deregulated cell proliferation and survival, angiogenesis, invasion, and metastasis. Aberrant MET activation results from autocrine or paracrine mechanisms due to overexpression of HGF and/or MET or from a ligand-independent mechanism caused by activating mutations or amplification of MET. The literature provides compelling evidence for the role of MET signaling in cancer development and progression. The finding that cancer cells often use MET activation to escape therapies targeting other pathways strengthens the argument for MET-targeted therapeutics. Diverse strategies have been explored to deactivate MET signaling, and compounds and biologics targeting the MET pathway are in clinical development. Despite promising results from various clinical trials, we are still waiting for true MET-targeted therapeutics in the clinic. This review will explore recent progress and hurdles in the pursuit of MET-targeted cancer drugs and discuss the challenges in such development.

Key words: MET; Hepatocyte growth factor; Targeted therapy; Receptor tyrosine kinase; Cancer therapeutics

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Core tip: Aberrant activation of MET receptor tyrosine kinase signaling is frequently observed in many human cancers. Such activation not only affects cancer development and progression, but it also contributes to resistance against other cancer drugs. The inhibition of MET signaling is an attractive approach for cancer intervention, and pursuit of MET-targeted cancer therapeutics is underway. Even though promising results have been reported from various clinical trials, many challenges remain to be addressed before and even after the arrival of such drugs in the clinic.

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INTRODUCTION

It has been three decades since the discoveries of mesenchymal epithelial transition (MET) (MNNG HOS transforming gene) and its ligand, hepatocyte growth factor [HGF; also known as scatter factor (SF)][1-5]. MET, encoded by the proto-oncogene *MET* on chromosome 7 (7q31), is a receptor tyrosine kinase (RTK). Under physiological conditions, it is stimulated by HGF, mainly through a paracrine mechanism, which triggers a cascade of intracellular signaling networks. The signaling driven by this ligand-receptor pair is involved in mitogenesis, motility, and morphogenesis, and it is essential for many developmental and physiological processes[6-8]. Like that of many RTKs, MET signaling is tightly regulated, and its timely attenuation is crucial for proper regulation of its activities[9-11]. Inappropriate activation of this signaling cascade can cause hallmark cancer events that include deregulated cell proliferation, survival, transformation, angiogenesis, and invasion[12,13]. Several different mechanisms can lead to an aberrant MET signaling, including autocrine or paracrine activation resulting from overexpression of MET and or of HGF, and a ligand-independent mechanism caused by activating mutations or amplification of the *MET* gene[12].

Alteration of MET signaling has been reported in almost all types of human cancers and is often associated with poor prognosis[7,12] (http://www.vai.org/met/). The evidence provides a compelling rationale for targeting this pathway, and the rationale is strengthened by the fact that cancer cells often use the HGF-MET axis to escape therapies targeting other RTKs or signaling molecules[12,14-16]. Cancer treatment has been revolutionized by targeted therapy since the success of Gleevec (imatinib) for treating chronic myelogenous leukemia by inhibiting BCR-Abl tyrosine kinase activity[17]. Other targeted therapies include drugs targeting epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR)[11]. Targeted therapies are the trend in cancer treatment, even though not all such drugs have lived up to their promise, in part due to the complexity of the cancer genome[18-21]. Relative to how much we know about the molecular mechanisms of cancers and the numbers of suitable therapeutic targets that have been identified, the targeted therapies available in the clinic are quite limited. MET is one of the targetable molecules that are still lacking effective drugs for cancer treatment.

Over the years, diverse strategies have been explored to inhibit MET pathway activation, from blocking either ligand access or receptor dimerization to inhibiting MET kinase activity or preventing downstream signaling activation[12,22,23]. These efforts have led to the discovery of many MET inhibitors possessing distinct specificities and efficacies (Figure 1). There are hundreds of clinical trials aiming to bring MET inhibitors from the bench to bedside[12,23-25] (https://ccrod.cancer.gov/confluence/display/CCRHGF/Home). While many MET inhibitor trials have shown promising results, various challenges remain. For instance, which patient will benefit from MET-targeted therapeutics and what companion diagnostics will be needed for patient stratification? How can MET-targeted therapeutics be effectively used for tailoring a patient-oriented treatment plan?

MET SIGNAL TRANSDUCTION AND ACTIVITIES

MET is normally expressed in cells of epithelial or endothelial origin, while its ligand HGF is predominantly produced by mesenchymal cells[26]. This decoupling enables a tight regulation of MET signaling in tissues and cells where its activity is required, through the response of MET to gradients of HGF. When HGF binds, the MET receptor undergoes dimerization and a conformational change, leading to phosphorylation of its key tyrosine residues and recruitment of downstream signaling molecules. This recruitment triggers activation of important intracellular signaling pathways such as Ras-MAPK, PI3K-AKT, Src, STAT3, PLC-γ or Cdc42/Rac. Such activation is either mediated by scaffolding adaptors like Gab1 and Grb2 or by direct binding of signaling molecules to the multisubstrate-docking sites in the MET cytoplasmic region[27-30]. MET signaling is subject to timely attenuation that is regulated by several mechanisms, including phosphatase-mediated dephosphorylation, receptor turnover, and negative feedback inhibition[27,31-34]. Such regulation determines the duration and threshold of the MET signal output, how cells will respond, and what biological activities will be induced.

Diverse biological activities, from cell proliferation and survival to cell motility and invasion, can be induced by MET signaling, but it is still vague how the overall response to this signaling is produced. For instance, under what circumstances stimulated cells will proliferate rather than migrate or invade. Among many signaling pathways downstream of MET, activation of Ras-MAPK/ERK is crucial for cell proliferation; PI3K-Akt contributes more to cell survival; Cdc42/Rac activation induces cell motility; and STAT3 has been implicated in cell transformation and tubulogenesis[6-8,34]. Nonetheless, these intracellular pathways often have crossover activities, and they interplay to carry out many complicated biological roles driven by MET, such as invasion and branching morphogenesis[6-8,34]. The signal output and cellular response can be further complicated by crosstalk between the HGF-MET axis and many other cell-surface receptors or signaling pathways[12,34].

MET-mediated biological activities are part of many developmental and physiological processes. For example, the HGF-MET axis is essential for the developing placenta, embryonic liver, and limb muscles during embryogenesis[35-37], and it may be involved in the early development of the lungs, kidneys, and mammary glands[38-42]. HGF promotes formation of blood vessels and lymphatic vessels[43-45], and it plays a role in developing

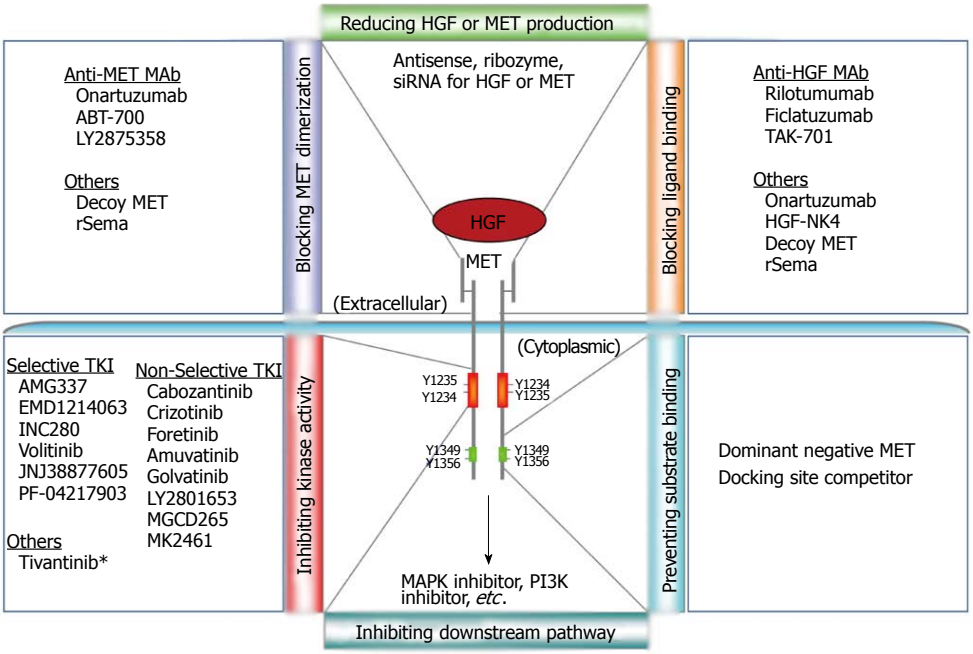

Figure 1 MET-targeted therapeutic strategies and inhibitors. Many strategies have been explored to inhibit MET signaling: blocking ligand binding or receptor dimerization; inhibiting MET kinase activity; preventing substrate binding; reducing hepatocyte growth factor (HGF) or MET production; and inhibiting downstream pathways. Shown in the diagram are representative inhibitors in each category, many of which are in clinical trials. Y1234 and Y1235 are two essential tyrosine residues in the TK-domain of MET, and Y1349 and Y1356 in its docking site are key tyrosine residues for substrate binding. Tivantinib is a non-ATP-competitive, selective MET inhibitor, but it also exhibits MET-independent antitumor activity by inhibiting microtubules. rSema: Recombinant MET Sema domain fragment.

neurons[46,47]. Physiologically, MET signaling is indispensable for liver regeneration and repair[48-50] and for skin wound healing[51]; it also contributes to insulin secretion and glucose metabolism[52,53]. A significant role of this signaling axis in the regulation of stem cell activity has also been found[54]. HGF can stimulate the migration and differentiation of mesenchymal stem cells (MSCs) while inhibiting their proliferation[55,56], and it can induce differentiation of bone marrow stem cells into hepatocytes[57,58].

MET SIGNALING IN CANCERS

The link of MET to cancer can be traced back thirty years. MET was originally cloned from a carcinogen-induced chromosome rearrangement in a human osteosarcoma cell line as part of the TPR-MET fusion[1,59], an oncogenic product which was also observed in human gastric carcinoma[60]. The most decisive evidence of MET signaling in cancers came from the identification of its germline and somatic mutations in papillary renal cell carcinomas (RCC)[61]. These mutations mainly locate in the tyrosine kinase (TK) domain of MET, resulting in constitutive activation of its signaling[62]. Such mutations have also been sporadically identified in childhood hepatocellular carcinoma (HCC) and head neck squamous cell carcinoma (HNSCC)[63,64]. Interestingly, MET mutations identified in other human solid cancers (such as lung and gastric cancers, and melanoma and thyroid carcinomas) are mostly in the extracellular semaphorin (Sema) domain and the juxtamembrane (JM) domain[65-69]. In lung adenocarcinoma, it is estimated that 4% of the tumors have exon 14 skipping in the MET mRNA due to splicing site mutations, and thus have JM-domain defect[20]. These non-TK-domain mutations likely affect ligand binding or CBL-mediated turnover of MET, thereby altering MET signal transduction. Besides genetic abnormalities, MET signaling is mostly altered through a paracrine or autocrine activation mechanism by inappropriate increases in MET and/or HGF expression. Evidence of such alterations has been documented in almost all types of human cancers, and high MET and HGF expressions are often correlated with invasive phenotype and poor prognosis[7,12]. Alternatively, aberrant MET activation in cancer cells can be the result of amplification of the *MET* gene, which is found in gastric, esophageal, lung, colorectal, and breast cancers[70-76].

Another important aspect of MET signaling in cancers emerged from studies of drug resistance. Activation of the HGF-MET axis has become one of the most crucial mechanisms that cancer cells adapt to bypass therapies targeting other oncogenic pathways. The first such evidence came from the analysis of non-small-cell lung cancer (NSCLC) patients treated with gefitinib (an EGFR inhibitor), which revealed amplification of *MET* as a mechanism for gefitinib resistance[14]. This mechanism accounts for acquired resistance to EGFR-targeted therapies (gefitinib and erlotinib) in about 5% of NSCLCs that harbor EGFR-activating mutations and are primarily sensitive to the treatment[77-80]. *MET* amplification is also associated with acquired resistance to cetuximab or panitumumab

(both EGFR-targeted monoclonal antibodies) in patients with metastatic colorectal cancer (mCRC)[81]. An alternative mechanism of resistance to EGFR-targeted therapies in lung cancer *via* MET signaling may be through up-regulation of HGF expression[82-84]. This mechanism could have a widespread impact on drug resistance to various anticancer kinase inhibitors[16]. For instance, stroma-derived HGF contributes to innate and acquired resistance to vemurafenib (RAF inhibitor) treatment of melanomas[15], and it may trigger resistance to ALK inhibitors in EML4-ALK lung cancer cells[85]. MET activation also confers tumor resistance to chemotherapy or radiotherapy.

Aberrant MET activation can elicit a multitude of biological consequences, ultimately leading to tumorigenesis and metastasis[6-8,12]. It causes oncogenic transformation and provides growth and survival signals to cancer cells by overactivating numerous downstream pathways (RAS-MAPK, PI3K-AKT, and STAT3, to name a few). In animal models, overexpression of MET in the liver results in HCC[86], while targeted expression of mutant MET in mammary epithelium leads to the development of breast cancers[87,88]. In parallel, MET causes invasive behavior of cancer cells, leading to metastasis; this is achieved by its abilities to up-regulate multiple extracellular matrix-degrading proteases, inducing the epithelial-to-mesenchymal transition and activating cell-mobilizing machinery[6-8,12]. The HGF-MET axis has also been implicated in the regulation of cancer stem cell activities in colon cancer and glioblastoma[89-91]. Besides direct contribution to the pathogenesis of cancer cells, MET signaling can enhance angiogenesis to strengthen tumor-supporting circuitry for promoting growth and survival[43,92,93].

DEVELOPMENT OF MET-TARGETED THERAPEUTICS: THE PROMISE

The indisputable role of MET signaling in cancer has made it a promising target for cancer intervention. Many approaches have been taken to try to effectively inhibit MET signaling activation in cancer cells[12,22-23]. Resulting from these efforts is a spectrum of targeted inhibitors having diverse biochemical and biological properties, including neutralizing antibodies to MET or HGF, small-molecule tyrosine kinase inhibitors (TKIs) of MET, and others (Figure 1). To date, more than two dozen MET-targeted inhibitors are in clinical development, with hundreds of trials conducted or underway, either as a single agent or in combination with other cancer drugs[12,23-25].

Ficlatuzumab (AV-299), rilotumumab (AMG102), and TAK-701 are humanized anti-HGF monoclonal antibodies that block HGF-dependent paracrine/autocrine MET activation. Ficlatuzumab, as a single agent for patients with advanced solid tumors, showed a partial benefit of stable disease in phase Ⅰ trials. Favorable responses were observed in a subgroup of NSCLC patients who had low MET expression when ficlatuzumab was used in combination with an EGFR inhibitor in a phase Ⅱ study[94-97]. Patients with refractory advanced solid tumors had a response of stable disease when treated with rilotumumab alone[98]. Recent phase Ⅱ clinical trials of rilotumumab in combination with chemotherapy extended progression-free survival (PFS) in patients with gastric cancer[99]. A benefit of combining rilotumumab with panitumumab was reported in a randomized phase Ⅱ trial of patients with mCRC who carry wild-type KRAS[100]. TAK-701, which has been tested in a phase Ⅰ trial, inhibits HGF-mediated resistance to gefitinib in an NSCLC tumor model[101,102].

Unlike HGF blockers, onartuzumab (MetMAb, a humanized monovalent antibody to MET) neutralizes MET by inhibiting HGF binding and receptor dimerization[103,104]. In a phase Ⅰ dose-escalation study, onartuzumab, as a single agent and in combination with bevacizumab, was well tolerated in patients with advanced solid tumors[105], while in a preclinical model it enhanced the antitumor efficacy of anti-VEGF biologics[106]. Several phase Ⅱ/Ⅲ trials of onartuzumab in combination with bevacizumab and or chemotherapeutic agents have been initiated for treating cancers such as mCRC, glioblastoma, NSCLC, breast cancer, and gastric cancer. In a randomized phase Ⅱ trial, onartuzumab and erlotinib in combination had a favorable outcome in MET-positive NSCLC patients, with MET expression of 2+ or 3+ scores based on immunohistochemical (IHC) staining[107]. This group of patients had a significant improvement of PFS (median 2.9 mo *vs* 1.5 mo) and overall survival (OS; median 12.6 mo *vs* 3.8 mo) relative to the placebo plus erlotinib controls. However in the MET-negative group, a worse OS was observed relative to the control group[107]. This result has led to a phase Ⅲ trial of onartuzumab plus erlotinib in MET-positive advanced NSCLC patients[108]. ABT-700 and LY2875358, two other antagonist antibodies against MET, are also being evaluated in early-phase trials[109,110].

While the anti-HGF and anti-MET biologics provide unique target specificity and long-lasting efficacy, the majority of potent MET inhibitors in clinical development are small-molecule TKIs. These are either selective or non-selective inhibitors of MET, and they mostly compete for the ATP-binding pocket in the TK-domain. Examples of selective inhibitors include AMG337, EMD1214063 (MSC2156119J), INC280 (INCB028060), and volitinib (HMPL-504); no significant safety concerns have been reported from early-phase studies of these oral inhibitors[111-114]. EMD1214063 is potent in suppressing the activities of both wild-type MET and its activating mutants in preclinical models[112,115,116], and ongoing phase Ⅰ/Ⅱ trials will evaluate its safety/efficacy in NSCLC and HCC. Several phase Ⅰ/Ⅱ trials of INC280 are recruiting patients with cancers including advanced cases of HCC, NSCLC, glioblastoma, or melanoma. A global phase Ⅱ study of volitinib has been initiated for papillary RCC[114]. PF-04217903, another potent selective TKI of MET, has been discontinued from trials due to a strategic

development decision[117].

Non-selective MET TKIs represent a different class of blockers; among them are crizotinib, cabozantinib, and foretinib. Crizotinib (Xalkori, PF-02341066), which was primarily designed for MET inhibition and displayed antitumor effects in MET-amplified NSCLC, is a multikinase inhibitor of MET, ALK, and ROS1[118-120]. Crizotinib is FDA-approved for treatment of ALK-fusion NSCLC patients, and it has shown antitumor activity in advanced ROS1-rearranged NSCLC[120]. A durable response to crizotinib was reported in an NSCLC patient with MET amplification but no ALK rearrangement[121]. Patients with MET-amplified esophagogastric adenocarcinoma or recurrent glioblastoma have also shown clinical responses to crizotinib treatment[122,123]. A cross-tumoral phase Ⅱ trial of crizotinib has been launched in patients with locally advanced and/or metastatic tumors that carry ALK and/or MET alteration. Ongoing trials also include crizotinib in combination with erlotinib or other inhibitors.

Cabozantinib (XL184) inhibits multiple molecular targets, including MET, VEGFR2, RET, AXL KIT, and FLT3, and it suppresses tumor growth, angiogenesis and metastasis[124]. Cabozantinib (Cometriq) was approved by the FDA as an orphan product for treating medullary thyroid cancer (MTC) in late 2012. A phase III trial for this rare human cancer showed a response rate of 28% for cabozantinib versus none for placebo (median PFS of 11.2 mo *vs* 4.0 mo), most likely due to its inhibitory activity on RET; it showed significant but manageable toxicity[125]. Cabozantinib phase Ⅱ trials displayed clinical activity in metastatic CRPC, resulting in improvement in PFS, bone scans, and pain, and a reduction of soft tissue lesions[126,127]. Clinical trials of cabozantinib are under way for several other cancer types, including NSCLC, glioblastoma, breast, ovarian, and urothelial cancers. Its combination with erlotinib has been tested in advanced NSCLC in an early-phase trial[128]; many other combination studies involving cabozantinib are under way.

Foretinib (XL880, EXEL-2880, and GSK1363089) is a multikinase inhibitor targeting MET, VEGFR2, RON, TIE-2, PDGFRβ, KIT, FLT3, and AXL. It has shown antitumor activity in xenograft tumors and in a phase Ⅰ trial of metastatic or unresectable solid tumors[129,130]. With a manageable toxicity profile, it demonstrated in a phase Ⅱ trial a high response rate in advanced papillary RCC patients who had germline MET mutations[131]. Phase Ⅱ trials are studying foretinib in metastatic gastric cancer, recurrent/metastatic HNSCC, and triple-negative breast cancer. Phase Ⅰ/Ⅱ trials also test combinations of foretinib with erlotinib against locally advanced/metastatic NSCLC or with lapatinib against HER2-overexpressing metastatic breast cancer. Other non-selective TKIs of MET include amuvatinib (MP470), golvatinib (E7050), LY2801653, MGCD265, and MK-2461, all of these inhibit both MET and other targets[132-136].

Tivantinib (ARQ197), unlike other MET TKIs, is a non-ATP-competitive small-molecule inhibitor of MET that has a broad spectrum of antitumor activity[137]. It selectively binds to inactive/unphosphorylated MET and inhibits its autophosphorylation[138]. Clinical development of tivantinib is being actively pursued. Examples from completed phase Ⅱ trials include tivantinib as a second-line treatment for advanced HCC, as well as its combination with erlotinib for previously treated NSCLC[139,140]. These results have led to the launches of phase Ⅲ trials of tivantinib either as a monotherapy or in combination, among many other ongoing clinical studies.

CHALLENGES OF MET-TARGETED THERAPEUTICS

Despite promising results, there have also been setbacks to MET-targeted therapeutics development, and numerous challenges remain to be addressed. For instance, rilotumumab in several phase Ⅱ trials showed no or limited efficacy in patients with metastatic RCC, castration-resistant prostate cancer (CRPC), or recurrent glioblastoma or ovarian cancer[141-144]. Foretinib as a single agent lacked efficacy in unselected patients with metastatic gastric cancer in a phase Ⅱ study[145]. The COMET-1 phase Ⅲ trial of cabozantinib in men with metastatic CRPC failed to meet its primary endpoint of demonstrating a statistically significant improvement of overall survival relative to prednisone treatment[146]. Further, the MARQUEE phase Ⅲ trial of tivantinib plus erlotinib and the MetLung phase Ⅲ trial of onartuzumab plus erlotinib in patients with advanced NSCLC were terminated following independent review board examination. Both trials failed to demonstrate any meaningful efficacy of combination compared with erlotinib alone[25,108,147]. Also, recent studies have demonstrated that tivantinib has cytotoxic activity independent of MET[148,149], raising the question of whether the clinical antitumor efficacy of this selective MET inhibitor was solely due to MET inhibition.

Such setbacks lead to the question of whether those clinical trials targeted the right groups of cancer patients for MET inhibition. One major challenge in patient selection is a lack of reliable biomarkers for companion diagnosis. IHC staining of MET has been widely used for assessing its protein status in tumors; patients with MET-IHC-positive gastric cancer displayed the greatest survival benefit from rilotumumab in combination with chemotherapy[99]. However, such staining unexpectedly failed in selecting patients for the MetLung phase Ⅲ trial, even though this biomarker was able to identify responders (MET-IHC score 2+ or 3+) for onartuzumab plus erlotinib treatment in a phase Ⅱ study[25,107,108,150]. It remains to be seen why MET-IHC biomarker failed in the latter trial and whether improvements in sensitivity and specificity would make it more reliable for patient stratification.

Other potential biomarkers include *MET* mutations

and *MET* amplification (gains in gene copy number or chromosome 7 polysomy). Germline *MET* TK-domain mutations are highly predictive for papillary RCC patients' response to foretinib treatment[131]. Nonetheless, *MET* mutation as a biomarker in general has its limitations, because a majority that are found in broader cancers are non-TK-domain mutations[20,65-69] whose value in predicting response to MET inhibitors remains undetermined. Fluorescence *in-situ* hybridization (FISH) of *MET* has been evaluated for a link between *MET* amplification and patient response in MET inhibitor trials[99,111,122,131,145,150]. Even though the sample sizes were mostly small for interpretation, patients with *MET*-amplified tumors usually have better response, indicating the value of *MET*-FISH as a predictive biomarker. The power of this biomarker will be further defined when results from several ongoing trials enrolling *MET*-amplified cancer patients become available. In addition, serum/plasma HGF and soluble MET concentrations have also been used in exploratory biomarker analyses[95,99,150].

While improved biomarkers are vital for better design and success of MET inhibitor trials, developing the proper therapeutic strategy to maximize drug efficacy in the clinic is equally important. The latter can be challenging, because alterations of MET signaling found in the majority of human cancers are not activating mutations or amplifications of *MET*, and the alterations are often accompanied by the activation of other pathways[7,12]. The use of MET-targeted drugs as a monotherapy has its own merit if MET alteration is the oncogenic driver; however, it might not perform well when different oncogenic pathway(s) is co-activated. The existence of crosstalk between MET and other pathways such as EGFR and VEGFR also complicates the outcome of MET-targeted monotherapy, providing further rationale for drug combination[12,34]. Clinical trials of various combination therapies have aimed to simultaneously inhibit MET and other molecular targets. However, determining what drug combination is best suited to which patient in the clinic demands a comprehensive platform of multiplexed molecular diagnoses. On the other hand, several targeted therapies, including those of EGFR and B-RAF, may theoretically benefit from combination with MET inhibitors to prevent MET signaling-mediated drug resistance[14-16,77-84]. Such combination strategies, nevertheless, are practically challenging because this resistance mechanism only accounts for a fraction of the resistant cases.

CONCLUSION

MET signaling is highly important in cancer malignancy, making it a promising molecular target for cancer intervention. The development of MET-targeted drugs is in full gear at multiple fronts, but numerous challenges remain. The failure of several late-stage clinical trials has put such development under scrutiny, but encouraging results are coming from other trials and new developments. Future trials should also put the activity of MET inhibitors on cancer invasion and metastasis in perspective. Inevitably, drug resistance will be expected for MET-targeted therapeutics, and such events have been observed in *in vivo* studies[151-154]. To enhance the performance of MET inhibition and to prevent cross-drug resistance, engineering bi-specific or multi-specific antibodies against MET and other cell surface receptors may provide a solution[155-157]. Many clinical benefits can be expected from the eventual approval of MET-targeted drugs.

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EDITORIAL

Animal venom studies: Current benefits and future developments

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Abstract

Poisonous organisms are represented in many taxa, including kingdom Animalia. During evolution, animals have developed special organs for production and injection of venoms. Animal venoms are complex mixtures, compositions of which depend on species producing venom. The most known and studied poisonous terrestrial animals are snakes, scorpions and spiders. Among marine animals, these are jellyfishes, anemones and cone snails. The toxic substances in the venom of these animals are mainly of protein and peptide origin. Recent studies have indicated that the single venom may contain up to several hundred different components producing diverse physiological effects. Bites or stings by certain poisonous species result in severe envenomations leading in some cases to death. This raises the problem of bite treatment. The most effective treatment so far is the application of antivenoms. To enhance the effectiveness of such treatments, the knowledge of venom composition is needed. On the other hand, venoms contain substances with unique biological properties, which can be used both in basic science and in clinical applications. The best example of toxin application in basic science is α-bungarotoxin the discovery of which made a big impact on the studies of nicotinic acetylcholine receptor. Today compositions of venom from many species have already been examined. Based on these data, one can conclude that venoms contain a large number of individual components belonging to a limited number of structural types. Often minor changes in the amino acid sequence give rise to new biological properties. Change in the living conditions of poisonous animals lead to alterations in the composition of venoms resulting in appearance of new toxins. At the same time introduction of new methods of proteomics and genomics lead to discoveries of new compounds, which may serve as research tools or as templates for the development of novel drugs. The application of these sensitive and comprehensive methods allows studying either of venoms available in tiny amounts or of low abundant components in already known venoms.

Key words: Venom; Poison; Snake; Scorpion; Spider; Toxin

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Core tip: Animal venoms are complex mixtures mostly of peptides and proteins. Recent studies have indicated that the single venom may contain up to several hundred different components producing diverse physiological

effects. The knowledge of venom composition as well as structure and properties of its components on the one hand may give the clue for better treatment of bites and stings on the other hand it may lead to the discovery of new medicines. Recent developments in research methods gave a great impulse to animal venom studies, which may result in the entry of new drugs to the market.

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INTRODUCTION

Poisonous organisms are wide spread throughout the globe and represented in many biological taxa. A large number of poisonous species belongs to the kingdom Animalia. Both vertebrates (fishes, amphibians, reptiles, birds, and mammals) and invertebrates (coelenterates, worms, arthropods, molluscs, echinoderms) may be poisonous. Venomous animals permanently or periodically contain in their organisms substances, which are toxic to other species. Even small doses of such compounds in the body of another animal cause painful disorders and sometimes death. Some venomous animals have special venom glands producing venom; others contain toxic substances in the various tissues of the body. A number of animals have a specialized wounding apparatus (organ) for the introduction of venom into the body of a predator or a prey. In the coelenterates (hydras, sea anemones, jellyfishes) these are stinging cells, in arthropods (scorpions, bees, wasps) - multicellular glands connected with the sting, and in fishes - similar glands connected with spines on the fins (*e.g.,* scorpionfish) and on gill covers (sea dragon). In many animals (millipedes, spiders, and snakes), venom glands are associated with mouthparts and venom is injected into the prey's body at the bite or puncture. In venomous animals, which have venom glands, but do not have a special apparatus for the venom injection, such as amphibians (salamanders, newts, frogs and others), glands are located in various skin sites. Of venomous terrestrial animals the most studied are snakes, scorpions and spiders. Among the marine animals, these are jellyfishes, anemones and cone snails. Animal venoms are complex mixtures, compositions of which depend on species producing venom.

SHORT HISTORY OF VENOM STUDIES

THE HISTORY OF the venom study traces back more than two millennia. The earliest survived detailed synopsis of venomous animals and their bites belongs to Aristotle (384-322 BC. Aristotle "Historia Animalium"). Modern scientific understanding of venomous snakes and venoms began to take shape thanks to the Italian scientists Francesco Redi and Felice Fontana, who worked in the city of Pisa in the 17 and 18 centuries, respectively. Physician, biologist, linguist and poet Francesco Redi[1] (1626-1697) published in 1664 a treatise on poisonous snakes "Osservazioni intorno alle vipere". He found that snake bile is not toxic as it was accepted at that time, and toxicity is in the venom released from the teeth at the bite. Redi is considered as one of the founders of toxinology, a specialist area of science dealing with animal, microbial, and plant venoms, poisons and toxins. A hundred years later, in the 18 century, another Italian, Felice Fontana, discovered snake venom glands and obtained snake venom, which he used for a variety of experiments with animals. Fontana corrected Redi: venom acts on the animals not by getting in stomach as Redi believed, but in the blood.

Intensive modern biochemical studies of venoms began in the middle of the last century, although some proteins in the venoms of snakes have been discovered much earlier. For example, one of the earliest reports on snake venom phosphodiesterase activity refers to 1932[2]. However, only since the late 50's early 60's of the last century, a broader study of snake venom proteins began. For example, in 1959 snake venom protease was isolated by the Japanese group[3], and in 1963 Chang and Lee[4] published a paper, which described isolation of α-bungarotoxin. The latter work has become a classic. To date, it is the most cited in the field of venom studies, and α-bungarotoxin is still one of the most selective markers of certain subtypes of nicotinic acetylcholine receptor. According to the PubMed database, more than 1000 articles devoted to the study of venoms are published annually, and the total number of publications is more than 40000. About half of these publications deal with snake venoms.

HEALING POWER OF ANIMAL VENOMS

The exact historical moment, when people have learned to benefit from venoms and to use them for medical purposes, is very difficult to trace. However, in ancient Rome animal venoms were used to produce drug for the treatment of smallpox, leprosy, and fever, as well as for wound healing. Many doctors and philosophers of ancient Greece wrote about the action of snake venom. The Greek physician Nicander of Colophon described the action of snake venom and the composition of antidotes. Particularly, snake venom was widespread as a remedy in the Middle Ages and until the 19 century it was part of many antidotes. Back in the 1st century AD, there was a mixture containing snake venom - the so-called theriac. It was manufactured in pharmacies in Europe until the 18 century. But the real use of snake venom began in the late 19 century, when French

scientist Albert Calmette found that if animals were injected with venom in small doses, their blood serum became a strong antidote[5]. First snake venoms were used only for antivenom production, but then their use has expanded. It was reported that in patients with epilepsy seizures were ceased after they being bitten by a rattlesnake, and in 1934 it was found that cobra venom in small doses has a potent analgesic activity - many times greater than morphine, with the difference that the venom does not cause drug addiction. Since that time venom was part of the drug against asthma, hypertension and even leprosy. So far, ointments and cremes (Cobroxin, Viprosal, Cobratoxan, *etc.*) based on venoms are produced in many countries. However, such use of venoms is hardly effective as their content in the preparations is extremely low, and the effect is achieved due to nonspecific action. Nevertheless, there is a certain demand for these products.

COMPOSITION OF ANIMAL VENOMS

ANIMAL VENOMS are extremely rich and complex natural sources of biologically active molecules that have a variety of molecular targets and functions. Venoms are classified by origin - venoms of snakes, scorpion venoms, spider venoms, *etc.*, or by their effects on the body - neurotoxic, hemotoxic venoms, *etc*. Animal venoms usually are aqueous solutions containing a significant number of components, most of the peptide and protein nature[6]. Several hundred different substances - toxins, may be present in single venom. Since one of the main objectives of the venom is killing or immobilization of the prey, its components are highly toxic. Animal toxins cause significant dysfunction of the nervous, cardiovascular and muscular systems. Poisoning by animal venoms cause serious illness in humans and can lead to death. For the treatment of such poisoning, antisera obtained against crude venom are generally used[7].

The venoms usually consist of enzymes (phospholipases, proteases, oxidases, *etc.*), proteins without enzymatic activity (neurotoxins, disintegrin, *etc.*), and peptides. Such a complex composition of the venom results in the combined effects on vital systems of the body and severe poisoning. Peptide toxins acting on ion channels (*e.g.,* ion channel blockers) are widely represented in venoms. Generally, they affect nervous system and are the principal active components of spider, scorpion, Cone snail and Elapidae snake venoms[8]. Spider and scorpion neurotoxins act mostly on voltage-gated ion channels, while snake neurotoxins - on ligand-gated channels, in particular on nicotinic acetylcholine receptors. Diverse conotoxins from Cone snails block both voltage gated channels and acetylcholine receptors.

In venoms, two classes - oxidases and hydrolases, represent enzymes. The highest enzyme content is in the snake venoms. Only single oxidase, L-amino acid oxidase, is found in venoms so far, while hydrolases are very abundant and include phospholipases A2, proteinases/peptidases, acetylcholinesterases, and hyaluronidases. Non-enzymatic proteins are more diversified and, for example, in snakes include so called three finger toxins, disintegrins, proteinase inhibitors, nerve growth factor, *etc*[9]. Peptides and polypeptides are the principal constituents of spider, scorpion and cone snail venoms and comprise various neurotoxins, antimicrobial peptides, immuno-modulating peptides, *etc.*

Such a wide range of peptides and proteins with different biological functions makes animal venoms valuable source of new compounds both for use in basic research and for the development of new medicines.

STATUS QUO IN THE USE OF ANIMAL VENOMS

DESPITE THE long history of venom handling, only few toxins found real application. Several toxins are widely used in basic research as biochemical tools. α-Bungarotoxin mentioned above is the best example. Discovered more than fifty years ago[4] it is still unsurpassed as selective and efficient pharmacological tool for the study of α7 and muscle type nicotinic acetylcholine receptors. α-Conotoxins, peptide neurotoxins, are more versatile tools for this receptor studies as they distinguish various subtypes and even different binding sites within one receptor molecule[10]. Some scorpion and spider toxins show very high selectivity toward particular types of voltage-gated ion-channels and are used in investigations on these channels[8]. Venom proteins affecting hemostasis also represent useful tools for exploring different processes within this sophisticated system.

Several venom components especially those from snake venoms have found extensive applications in the diagnosis of haemostatic disorders. Some diagnostics marketed by Pentapharm Ltd. (Switzerland) can be mentioned as examples. Thus, prothrombin activator Ecarin from *Echis carinatus* venom is used for the detection of abnormal type of prothrombin, serine protease Protac® from *Agkistrodon contortrix*-for determination of protein C and protein S, and metalloprotease RVV-X from *Daboia russelli*-for determination of FX and screening assay for lupus anticoagulants[11].

A new fibrin adhesive (sealant) was developed by a group of researchers from the Center for the Study of Venoms and Venomous Animals, in Sao Paulo State, Brazil. This sealant is a biological and biodegradable product produced without using human blood. The components of this novel sealant were extracted from large animals and a serine proteinase isolated from *Crotalus durissus terrificus* snake venom. A new fibrin adhesive was tested in periodontal surgery and found to be a good alternative to traditional sutures[12].

The application of snake venom proteinase in

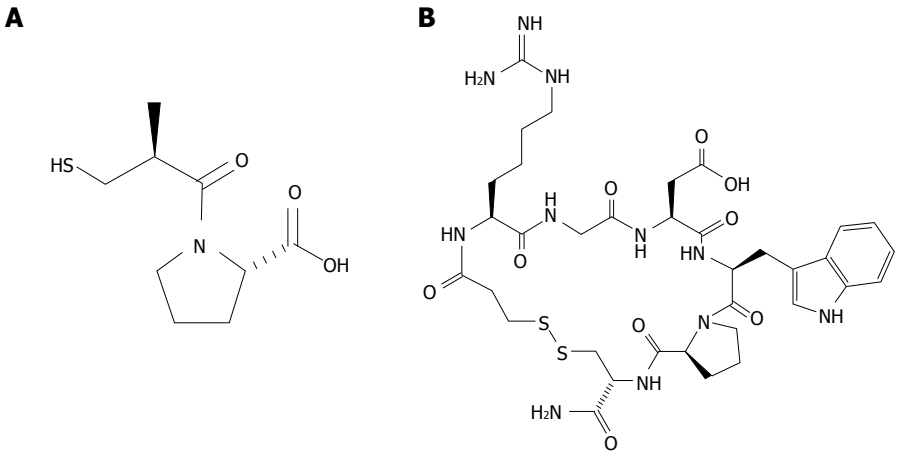

Figure 1 Chemical structures of captopril (A) and eptifibatide (B).

biotechnology was proposed by Australian company Venom Supplies Pty Ltd. The serine proteinase notanarin, which is a structural homologue of blood coagulation factor Xa, was positioned as an alternative to this factor in molecular biology for recombinant protein cleavage (http://www.venomsupplies.com/cleavage-compound/).

It should be noted that despite the great promises, only few medications are developed on basis of animal venom ingredients. The barriers on this difficult road are perfectly considered in a recent review[13]. Yet, several drugs based on the venom components have been successfully elaborated. In this row, Captopril® or Enalpril® (Figure 1A), a drug that treats hypertension, should be mentioned first. It was developed in the late 1970s and early 1980s based on bradykinin potentiating peptide, a peptide from the pit viper *Bothrops jararaca* venom. Captopril is an angiotensin-converting enzyme inhibitor and its development became a paradigm for "rational drug design". The year production of captopril is about 1000 tons.

Two more drugs based on animal toxins, anti-coagulants Integrilin® (Eptifibatide, Figure 1B) and Aggrastat® (Triofiban), are used now to prevent heart attacks or the formation of blood clots. Eptifibatide is a cyclic heptapeptide derived from a protein found in the venom of the south eastern pygmy rattlesnake *Sistrurus miliarius barbouri*. It belongs to the class of the so-called arginine-glycine-aspartate (RGD) - mimetics, reversibly binds to platelets and selectively blocks the platelet glycoprotein Ⅱb/Ⅲa receptor. Eptifibatide is marketed by Millennium Pharmaceuticals, Inc. and Schering Corporation and distributed by Schering Corporation. Tirofiban is a peptidomimetic based on disintegrin echistatin from the saw-scaled viper *Echis carinatus.* It also mimics the RGD sequence and is an antiplatelet drug[14]. Tirofiban is administered to reduce the rate of thrombotic cardiovascular events.

Recently a real neurotoxic peptide was turned into a medicine. This is Prialt® (Ziconotide), an analgesic from cone snail venom[15]. Ziconotide is a synthetic form of ω-conotoxin M-VIIA, peptide from the venom of *Conus magus* marine snail; it acts as a specific blocker of neuronal N-type calcium channels and is used to treat a variety of chronic pain syndromes.

The above examples show that the new drugs can be developed based on proteins and peptides from animal venoms. The number of new compounds found in venoms is growing steadily and some of them can be used for this purpose as well.

PROSPECTS AND DIRECTIONS OF DEVELOPMENT IN THE FIELD OF ANIMAL VENOMS

In relation to subjects discussed above it is reasonable to assume that one of the areas in the future venom research will be the search for new compounds, which may serve as a basis for the development of new medicines or used as new molecular tools. Since all available venoms have already been well studied, rare or very small animals will be used as research objects. Also previously unexplored compounds present in the venoms in extremely small quantities may be included in studies. In this direction, progress in high-throughput "omics" methodologies (proteomics, transcriptomics and genomics) make a great contribution to the identification and characterization of new scaffolds[16,17]. In particular, in the last decade, the utilization of these protocols for deciphering the toxin composition of venoms in great details becomes a common practice. Even the new term "venomics", which is the proteomic characterization of venom proteomes, has emerged. For example, the venom profiling of the rear-fanged snake *Thamnodynastes strigatus* using venomics has revealed new types of biologically important molecules including a new kind of matrix metalloproteinase that is unrelated to the classical snake venom metalloproteinases[18].

The search for prodrugs of medications acting on central nervous system among animal venom components is especially promising. The data available show that some of venom peptides appear to be capable of go through the blood brain barrier (BBB). Thus, bee venom apamin, a peptide interacting with certain types of potassium channels, and its nontoxic

analogue can penetrate BBB[19] that makes these peptides promising candidates for BBB shuttles. Other peptide chlorotoxin from scorpion venom was shown also penetrate BBB and its fluorescently labelled analogue was used for detecting cancer foci and metastases noninvasively[20]. Chlorotoxin-linked dye Tumor Paint BLZ-100 that "light ups" cancer cells so that surgeons can precisely target brain tumors is entering phase 1b study in human patients. Some venom proteins also possess capability to cross BBB (for review see[21]). These findings open up the new possibilities for emergence of medicines for the central nervous system treatment.

It should be noted that the genome of king cobra *Ophiophagus hannah* has been sequenced recently[22]. However, no new types of venom proteins or peptides revealed by this study have been reported so far.

Another way for the progress in the area of animal venoms is the development of new drugs based on known toxins. However even well known toxins may have unexpected or unwanted effects *in vivo*, as their targets may be expressed differently in the body. This problem requires a careful study in each particular case before a compound can be considered as a drug lead. Currently, several companies are moving in this direction. Thus, Celtic Biotech Iowa Inc. develops novel therapeutic products for the treatment of solid cancers and pain, which are based on crotoxin from pit-viper *Crotalus durissus terrificus* and cardiotoxin from cobra *Naja atra.* ReceptoPharm, United States-based biotechnology company, has completed phase I trials with modified versions of toxins from cobra venoms. The company plans to conduct further clinical trials in multiple sclerosis, motor neuron disease, adrenomyeloneuropathy and viral infections. The United States biotechnology company Kineta begins randomized placebo controlled trials in psoriatic arthritis and psoriasis of drug candidate ShK-186, which is a synthetic peptide that blocks Kv1.3 potassium ion channels. ShK-186 was derived from ShK neurotoxin of Caribbean anemone *Stichodactyla helianthus*. It should be noted that the work in this sector is very risky and competitive. A number of companies, which have begun work on the development of new toxin-based drugs, were forced to stop operation or ceased to exist.

A few words about antivenom therapy should be said here as well. It is currently the only available effective treatment for envenomation. "Omics" technologies have also a strong impact in this field. Transcriptomic analyses can identify regional variations in venom composition, which should be taken into account at development of specific and efficacious antivenoms. Utilizing transcriptomic analyses cDNA sequences encoding main toxic proteins can be easily determined. Large amount of particular cDNA can be prepared and directly used for immunization. For example, cDNA encoding a novel P-Ⅱ type metalloproteinase from *Bothrops asper* venom glands was cloned, sequenced and used for DNA immunization of animals[23]. The equine antibodies induced by immunization with plasmid encoding the metalloproteinase completely neutralized the hemorrhagic activity of the whole Bothrops asper venom. This technique opens a new way to the treatment of envenomation.

Despite the great progress in animal venom studies, some challenges remain yet in this area. Thus, the development of effective, cheap and safe antivenoms is in great demand. To address this problem, the composition of animal venoms specific to the area and its relation to the known one should be established. Until now, even the venoms of some very dangerous poisonous animals are not fully characterized. This concerns, for example, the Australian jellyfishes. The box jellyfish *Chironex fleckeri*, causing so-called Irukandji syndrome, produces extremely potent and rapid-acting venom. Envenomed victims can show symptoms ranging from headaches, severe pain, nausea and vomiting to pulmonary oedema, cardiac failure and severe hypertension resulting in death. However, the exact venom composition is not known and no well established treatment exists. The same is true for some other venomous animals (*e.g.,* scorpions and spiders) inhabiting other part of globe. The problem is complicated in rural areas where even existing antivenoms are not always available.

In conclusion, it is still largely expected that introduction of new "omics" methodologies in venom studies will greatly enhance the discovery of new toxins which may be used as templates for drug design. These methodologies provide a number of advantages for the development of new tools for basic research, clinical diagnostics, and novel medicines.

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EDITORIAL

MicroRNAs as mediators of cardiovascular disease: Targets to be manipulated

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Abstract

Cardiovascular disease has been the leading cause of death worldwide for the last few decades. Even with the rapid progression of the biomedical field, conquering/managing cardiovascular disease is not an easy task because it is multifactorial disease. One of the key players of the development and progression of numerous diseases is microRNA (miRNA). These small, non-coding RNAs bind to target mRNAs to inhibit translations of and/or degrade the target mRNAs, thus acting as negative regulators of gene expressions. Accumulating evidence indicates that non-physiological expressions of miRNAs contribute to both development and progression of cardiovascular diseases. Since even a single miRNA can have multiple targets, dysregulation of miRNAs can lead to catastrophic changes of proteins that may be important for maintaining physiologic conditions of cells, tissues, and organs. Current knowledge on the role of miRNAs in cardiovascular disease is mostly based on the observational data such as microarray of miRNAs in animal disease models, thus relatively lacking insight of how such dysregulation of miRNAs is initiated and regulated. Consequently, future research should aim to elucidate the more comprehensive mechanisms of miRNA dysregulation during pathogenesis of the cardiovascular system so that appropriate counter-measures to prevent/manage cardiovascular disease can be developed.

Key words: Cardiovascular diseases; MicroRNA; Heart; Endothelial cells; Smooth muscle cells

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Core tip: Accumulating evidence indicates that microRNAs (miRNAs) play important roles in the development and progression of cardiovascular diseases. To date, observational studies such as miRNA-profiling in diseased animals and/or patients have provided valuable information regarding their roles in cardiovascular diseases. For example, dysregulated miRNAs under pathologic conditions have been identified, and their possible targets, whose down-regulation may have contributed to the development of corresponding disease, have been examined. Nevertheless, future studies should

be more focused on identifying key mechanisms of miRNA dysregulation during pathogenesis of the cardiovascular system so that optimized counter-measures to prevent/manage cardiovascular disease can be designed and developed.

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INTRODUCTION

Despite enhanced understanding of the pathogenesis of cardiovascular system, it still is challenging to manage/treat cardiovascular disease, making the cardiovascular disease leading cause of death worldwide. Cardiovascular disease is a multifactorial disease having number of risk factors such as obesity, hypertension, dyslipidemia, and diabetes. Development and progression of cardiovascular disease has been associated with non-coding RNA-mediated change of gene expressions that are critical for the maintenance of cardiovascular system[1]. Over the last few decades, small, non-coding microRNAs (miRNAs) have emerged as critical players in controlling physiological and pathological processes, and accumulating evidence indicates that the development and progression of cardiovascular disease are also regulated by miRNAs[2-4]. Since the field of miRNA-dependent physiologic/pathologic regulation of cardiac cells, or the heart, evolves rapidly, it is always high time to overview the field and to re-adjust the strategies for the future studies.

CARDIOVASCULAR DISEASE AND MICRORNA

MicroRNAs are single-stranded RNAs that bind to the complementary sequences present on the 3'UTR (untranslated region) of target mRNAs, subsequently suppressing target protein expressions[5]. Since an individual miRNA targets multiple mRNAs, the manipulation of miRNAs can have a significant impact on intracellular networks. Such concept of miRNA-dependent regulation of cellular signaling has been empirically proved in various diseases including cardiovascular disease[4,6,7]. For example, pertaining to the role of miRNAs in coronary artery disease, miR-21 has been reported to be up-regulated in atherosclerotic plaques, and knockdown of miR-21 using anti-sense oligonucleotide reduced neointima formation in balloon injured animals[8]. For the case of remodeling process after myocardial infarction, miR-29 family has been reported to be down-regulated in the region of fibrous tissue formation and extracellular matrix deposition, increasing expressions of its target genes such as fibrillin and various isotypes of collagens[9]. As to the vascular inflammation and miRNA, endothelial cell enriched miR-126 inhibited vascular adhesion molecule 1 (VCAM-1) expression and decreased leucocyte binding to activated endothelial cells[10]. Furthermore, miR-195 significantly down-regulated production of inflammatory cytokines such as interleukin 1 beta (IL-1β), IL-6, and IL-8 in vascular smooth muscle cells[11]. In some cases, miRNAs have impact on more than one aspects of cardiovascular system to develop pathologic conditions. For example, patients with hypertension are at an increased risk of cardiovascular disease[12], and the etiology of hypertension encompasses abnormally increased vascular tone, endothelial dysfunction, and cardiac hypertrophy, and miRNA-dependent regulation has been implicated in all of these conditions[13]. These examples clearly demonstrate that miRNAs play important roles in modulating pathophysiologic function of cardiovascular system.

TYPICAL PATTERN OF STUDY ON CARDIOVASCULAR DISEASE AND MICRORNA

As described above, numerous studies have elucidated the role of miRNAs in cardiovascular disease and provided valuable information for further research. For most of studies focusing on the role of miRNAs in disease, (1) identification of miRNA in a specific disease, (2) target identification of the miRNA, and (3) functional validation of the targets using gain and/or loss of function approaches are the reoccurring theme of the studies. As an example, a previous study examined the role of miRNA in cardiac hypertrophy[14]. In that particular study, (1) pro-hypertrophic miRNAs (miR-212/132) were identified; (2) their functional role was evaluated by either overexpressing or knockout them; and (3) FoxO3 was identified as their target. However, there are relatively few studies provided information on (1) what kind of stimulus induce or repress expression of a specific miRNA; and (2) how that stimulus operates remains insufficient regarding the role of miRNAs in cardiovascular disease. It is important that future studies also focus on this type of information to establish an effective miRNA-based therapeutic strategy.

INVESTIGATING REGULATION OF SPECIFIC MIRNAS UNDER PARTICULAR PATHOLOGIC CONDITIONS

Although there are studies focused on bona fide transcriptional regulation of miRNAs[15,16], what may be also useful to develop therapies and/or therapeutic strategies for cardiovascular disease is to elucidate

how specific miRNAs are regulated under a particular pathologic condition. For example, miR-1, one of the most enriched miRNAs in heart, has been associated with different types of heart diseases. The expression of miR-1 has been reported to be decreased in cardiac hypertrophy and atrial fibrillation[17,18], while increased expression of miR-1 was observed in heart failure[19]. Although these findings clearly state that miR-1 play crucial roles in modulating multiple cardiovascular diseases, they do not provide information on how such bi-directional regulation of miR-1 expression is achieved. The importance of elucidating the mechanisms of particular miRNAs under specific circumstance comes from the uncertainty, at least for now, of using miRNAs as therapeutic tools. One of the most recent clinical trials utilizing miRNA-based therapeutic approach is the use of miravirsen, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide designed to neutralizing miR-122, for the treatment of hepatitis C virus (HCV)[20]. Although miravirsen reduced the level of HCV RNA in a dose-dependent fashion without viral resistance, there remain some issues related to the role of miR-122 in other physiologic and/or pathologic conditions. For example, low expression level of miR-122 in hepatocellular carcinomas (HCCs) has been associated with a poor prognosis[21], and deletion of miR-122 in mouse resulted in hepatosteatosis, hepatitis, and HCC-like tumor development[22]. These studies indicate that the potential benefits and drawbacks of using miravirsen must be carefully weighed during further clinical development. Furthermore, optimized means of effective miRNA delivery to target tissues or organs are yet to be developed. Although a number of different approaches to facilitate effective miRNA delivery, such as nanotechnology-based[23], lipid-based[24], and virus-based miRNA delivery systems[25], have been designed, there still remains issue of toxicity which often led to ultimate death of animals and target specific delivery[26,27]. Thus, as contingency strategy, it may be necessary to start to find means of regulating specific miRNAs *in situ* other than delivering exogenous miRNAs to effectively utilize miRNAs as potent therapeutic target for treating diseases.

ALTERNATIVE APPROACHES TO MODULATE EXPRESSIONS OF MIRNAS

One of the alternative approaches to modulate *in vivo* miRNA expression is using small molecules to regulate expressions of miRNAs[28]. The very first evidence was demonstrated by the study of Gumireddy *et al*[29]. In that particular study, the authors identified 2 small molecules as selective and effective inhibitors of miR-21 expression[29]. Few years later, small molecule-induced up-regulation of miRNA, miR-122, was also demonstrated[30]. Recently, a compound called Rubone has been reported to induce miR-34a expression, suppressing growth of HCC[31]. Another strategy for inhibiting the production of mature miRNAs involves inhibition of Dicer, miRNA processing nucleases. It has been reported that streptomycin prevented the processing of pre-miR-21 by binding to the Dicer processing site[32]. Additionally, the processing of pre-miR-372 and 373 by Dicer was also significantly inhibited by small molecule inhibitors[33]. The structural characteristics of miRNA, such as stem loops in pre-miRNAs and the bulges in miRNAs, are suspected to enable them to be targeted by small molecules[34,35]. In conjunction with such effort to find small molecules that modulate miRNA expressions, computer-aided approaches are getting attentions in RNA-targeting lead compound (small molecule) identification[36,37]. These *in silico* approaches are expected to improve the pipelines in a cost-effective way compared to the traditional approaches that are usually expensive and time-consuming.

CONCLUSION

Diverse roles of miRNAs in physiologic and/or path-ophysiologic conditions make them a very attractive modality to manage/treat multifactorial diseases such as cardiovascular disease. Nevertheless, using miRNAs as a therapeutic drug faces a major obstacle of developing efficient delivery methods. Consequently, finding means of regulating specific miRNAs is important to effectively utilize miRNAs as potent therapeutic target for treating diseases. Elucidating detailed mechanisms by which miRNAs are regulated under physiologic and/or pathologic conditions is imperative to design novel and potent miRNA-based therapeutic strategy. Especially for the case of using small molecules to modulate miRNA expressions, more structural and thermodynamic information on the interaction of those two molecules are required. Given the importance of miRNAs in pathogenesis of cardiovascular diseases and the promise they hold as viable therapeutic modality, miRNA-based therapeutics are expected to revolutionize the way of treating cardiovascular diseases in near future.

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MINIREVIEWS

Adipocyte activation of cancer stem cell signaling in breast cancer

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Abstract

Signaling within the tumor microenvironment has a critical role in cancer initiation and progression. Adipocytes, one of the major components of the breast microenvironment, have been shown to provide pro-tumorigenic signals that promote cancer cell proliferation and invasiveness *in vitro* and tumorigenicity *in vivo*. Adipocyte secreted factors such as leptin and interleukin-6 (IL-6) have a paracrine effect on breast cancer cells. In adipocyte-adjacent breast cancer cells, the leptin and IL-6 signaling pathways activate janus kinase 2/signal transducer and activator of transcription 5, promoting the epithelial-mesenchymal transition, and upregulating stemness regulators such as Notch, Wnt and the Sex determining region Y-box 2/octamer binding transcription factor 4/Nanog signaling axis. In this review we will summarize the major signaling pathways that regulate cancer stem cells in breast cancer and describe the effects that adipocyte secreted IL-6 and leptin have on breast cancer stem cell signaling. Finally we will introduce a new potential treatment paradigm of inhibiting the adipocyte-breast cancer cell signaling *via* targeting the IL-6 or leptin pathways.

Key words: Breast cancer; Interleukin-6; Cancer stem cells; Adipocyte; Microenvironment; Epithelial-mesenchymal transition; Leptin

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Core tip: We discuss the relationship between adipocytes in the microenvironment and breast cancer cells. We emphasize the role of adipocyte-secreted leptin and interleukin-6 in inducing breast cancer cell epithelial-mesenchymal transition and activating stemness pathways. Finally we summarize possible microenvironmental therapeutic targets and the potential role of non-coding RNAs in adipocyte-breast cancer interactions.

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INTRODUCTION

Breast cancer occurs through the accumulation of genetic and epigenetic changes, activating oncogenes and silencing tumor suppressors in the mammary ductal or lobular tissues. Ductal carcinomas make up

approximately 85% of diagnosed breast cancers, and are the focus of this review[1]. Ductal tumors progress linearly beginning as atypical hyperplasia that grow into ductal carcinoma *in situ* (DCIS) lesions which later acquire malignant phenotypes and become invasive ductal carcinomas[2]. The final stage of breast cancer involves dissemination of primary tumor cells and colonization of distant tissues by metastatic tumor cells.

Gene expression profiling has been used to identify molecular subtypes of breast cancer with different prognosis and treatment responses. Luminal A and B both express estrogen (ER) and progesterone receptors (PR), differing primarily in their proliferation kinetics. Luminal subtype breast cancers comprise the majority of tumors and are among the best prognosis, in part due to availability of antiestrogen therapies. Human epidermal growth receptor breast cancers overexpress or amplify HER2 and respond to targeted anti-HER2 therapies. Basal-like breast cancers are frequently triple-negative ($ER^-/PR^-/HER2^-$), often harbor *P53* mutations, and are aggressive with poor prognosis. A newly described molecular subtype, claudin-low breast cancers, also do not express ER and PR, but are identified through their characteristic lack of cell-cell adhesion molecules (claudins) and basal cytokeratins.

White adipose tissues account for approximately 80% of the volume of the adult breast, and are composed of a heterogeneous collection of cells including adipocytes, fibroblasts, capillaries, immune cells, and extracellular matrix. It was long believed the primary function of adipose tissue was energy storage; in fact stromal adipose is a complicated endocrine organ. Adipose tissues produce a wide variety of adipokines and signaling molecules that play numerous roles in breast tumor formation and progression[3]. This relationship is cemented by a well-established link between obesity and breast cancer. Obesity is a major risk factor for breast cancer development and patient survival, with a 33% increase of cancer mortality in obese patients[4]. The majority of the mammary microenvironment consists of adipocytes and adipocyte precursors. Mesenchymal stem cells differentiate into adipocytes through the two stages of adipogenesis, driven by transcription factors peroxisome proliferator-activated receptor γ and the C/EBP family. Initially mesenchymal stem cells commit to the adipocyte lineage forming preadipocytes, which become mature adipocytes through terminal differentiation[5]. Both preadipocytes[6] and mature adipocytes increase breast cancer growth, with marked effects on migration and the colony forming ability of breast cancer cells. Moreover, cancer associated adipocytes undergo phenotypic changes, forming a more supportive tumor niche[7]. Identifying the mechanisms of this relationship could lead to novel targets for prevention and treatment of breast cancer.

The standard of care for breast cancer is a combination of surgery, radiation and chemotherapy. Treatment success varies depending on molecular subtype of the tumor, and additional adjuvant and targeted therapies are available. While adjuvant hormonal therapies such as Tamoxifen are effective for ER^+ patients, and targeted therapies such as the monoclonal antibody Trastuzumab are effective for $HER2^+$ patients, there are no targeted treatments available for patients with basal-like or claudin-low breast cancer[8]. Additionally, drug resistance is a major factor in the treatment failure of all molecular subtypes. One suspected culprit of resistance is cancer stem cells. The cancer stem cell model describes an intratumoral subpopulation of cancer cells that have unregulated stem cell properties, primarily self-renewal and multipotent differentiation, which drive tumorigenesis and tumor heterogeneity[9]. First isolated from AML cell populations by using flow cytometry to sort cells based on the molecular markers $CD34^+$ $CD38^-$[10], cancer stem cells have been identified in breast cancer as the $CD44^+$ $CD24^{-/low}$ $ALDH1^+$ cell population[11,12]. Cancer stem cells are resistant to traditional cancer therapies due to their quiescence, DNA repair capabilities and overexpression of drug efflux pumps[13].

In part through the activation of cancer stem cell signaling, the tumor microenvironment plays a critical role in the development and progression of breast tumors. Targeting the microenvironment has the potential to inhibit cancer stem cells, preventing drug resistance and relapse across all molecular subtypes. This is an attractive therapeutic option due to the relative genetic stability and the reduced risk of resistance of the microenvironment[14]. Therapies targeting the microenvironment have been successful in multiple myeloma through targeting multiple myeloma cell-bone marrow interactions using bisphosphonates[15] and bortezomib[16]. Aromatase inhibitors, a recent success story in breast cancer, target post-menopausal estrogen produced by extragonadal aromatization in stromal cells as well as breast tissues and tumors[17].

In this review we will focus on the relationship between breast cancer cells and mature adipocytes, with emphasis on two of the best studied adipocyte secreted signaling molecules, leptin and interleukin-6 (IL-6). These molecules promote breast cancer progression through activation of the epithelial-mesenchymal transition and cancer stem cell signaling in breast cancer cells, and are potential novel microenvironmental targets.

BREAST CANCER STEM CELL SIGNALING

A number of signaling pathways that have fundamental roles in the regulation of self-renewal and differentiation of adult and embryonic stem cells have been linked to breast cancer stem cells. Adipocyte secreted leptin and IL-6 can activate many of these pathways, dysregulating self-renewal and differentiation within breast cancer cells. Targeting these signaling pathways within the microenvironment may be an important method of

targeting breast cancer stem cells. While numerous stemness pathways have been identified, we will focus on three of the best characterized: the Notch, Wnt and octamer binding transcription factor 4 (OCT-4)/ Sex determining region Y-box 2/Nanog pathways.

Notch

The Notch receptor is an important developmental mediator of self-renewal and regulator of cell fate decisions in many cell types, including within the mammary gland[18]. When ligand is bound, ADAM and γ-secretase proteases cleave the Notch receptor. The cleavage product is transported to the nucleus, where it activates gene transcription. In both basal like and ER^+/PR^+ breast cancer cell models Notch activates histone deacetylase SIRT2, which deacetylates and activates ALDH1A1, and increases mammosphere formation[19]. Inhibition of Notch signaling using a neutralizing antibody is sufficient to significantly reduce mammosphere formation of DCIS cells *in vitro*, indicating a crucial function in breast cancer stem cell signaling[20]. Constitutive Notch activation is a common feature of early stage breast cancer[20], and high levels of Notch are correlated with poor breast cancer prognosis[21].

Wnt

The Wnt signaling pathway is crucial for embryonic development, and is involved in cell fate determination, proliferation and cell migration. When Wnt ligand is present, it binds the Frizzled receptor, allowing β-catenin to be transported to the nucleus and activate gene transcription[22]. In the absence of Wnt, β-catenin is targeted for proteasomal degradation. Wnt pathway target genes such as LEF1 and AXIN2 are upregulated in breast cancer cells, especially in breast cancer stem cell populations[23]. Wnt signaling is important for breast cancer stem cell self renewal, when treated with Wnt inhibitor DKK1, ER^+ and ER^- breast cancer cells had reduced mammosphere formation[23]. Unregulated activation of the Wnt pathway can occur *via* mutations in downstream Wnt target genes, β-catenin and over-expression of the Wnt ligand. Secretion of Wnt ligand from cells in the microenvironment has a paracrine effect on cells on the invasive edge of tumors, increasing their proliferative and invasive abilities[24].

Oct-4/SOX2/Nanog signaling axis

Oct-4 is a member of the POU transcription factor family, and is critical for self-renewal of undifferentiated cells. It is normally only expressed in embryonic stem and embryonic germ cells, and is used as a marker for undifferentiated cells. Oct-4 is necessary to maintain stem cells in a pluripotent state[25]. Through interaction with HMG domain protein SOX2, Oct-4 activates transcription of several genes in pluripotent cells, including *Fgf4*, *Utf1*, *Fbx15,* and the genes encoding themselves, *Sox2* and *Pou5f.* SOX2 and Oct-4 synergy also activates transcription of key pluripotent embryonic regulator Nanog[26], a homeodomain protein that maintains the primitive ectoderm in the embryo[27]. Nanog is expressed in cells that are able to form pluripotent stem cell lines, and plays a key role in inhibition of differentiation in these cells, as well as activation of self-renewal. Nanog also activates Oct-4 transcription, although it is not necessary for Oct-4 expression[28].

These key pluripotency transcription factors share numerous targets, and they are essential to the transcriptional pathways that regulate embryonic stem cell identity[29]. Therefore it is not surprising that all three are frequently activated in breast cancer stem cells[30-32]. Overexpression of Nanog enhances ER^+/PR^+ breast cancer cell ALDH1 expression and drug resistance[30], as well as invasiveness and mammosphere formation[33]. Nanog overexpression also increases tumor formation *in vivo*[33]. SOX2 is highly expressed in early stage breast tumors, and knockdown prevents mammosphere formation as well as delaying tumor formation *in vivo*[32]. OCT-4 overexpression in healthy primary breast tissue cultures generated cells capable of tumor initiation[34].

EPITHELIAL-MESENCHYMAL TRANSITION

Through the epithelial-mesenchymal transition (EMT), epithelial cells lose cell polarity, cell-cell adhesion and undergo cytoskeletal reorganization gaining a motile, invasive phenotype. In healthy cells, EMT plays a critical role in development, embryogenesis and wound healing through the reorganization of tissues and germ layers[35]. The classic markers for EMT are loss of E-cadherin, a protein necessary for cell adhesion, and increase in N-cadherin. Additional mesenchymal proteins include smooth-muscle actin, vimentin and fibronectin. Change in expression of these markers is used to characterize EMT *in vitro*[36].

The tumor microenvironment produces EMT signaling molecules, promoting the mesenchymal phenotype necessary for cancer progression and metastasis. Cells in the inflamed microenvironment secrete transforming growth factor-β (TGF-β), stimulating Snail and Slug which transcriptionally repress E-cadherin[37]. Hypoxia in the microenvironment activates HIF-1, stimulating transcription of EMT activating protein Twist. In ER^+ breast cancer cells, Twist is activated by locally produced IL-6 through signal transducer and activator of transcription 3 (STAT3) signaling[38]. EMT is also tightly regulated by microRNA signaling, most notably through repression of E-cadherin activators by the miR-200[39] and miR-34[40] families. miR-200 is significantly downregulated in breast cancer, a possible mechanism of EMT activation[41]. Loss of E-cadherin also increases the tumorigenicity of cancer cells, and is correlated with increased cancer grade[37].

Cancer stem cells and cells that have gone through EMT share many common characteristics. Breast cancer stem cells have protein expression consistent with EMT, decreased E-cadherin and increased N-cadherin and Slug expression[42]. Furthermore, non-tumorigenic immortalized

human mammary cells that have undergone EMT are enriched for cancer stem cell markers such as $CD44^+$/$CD24^-$ and signaling proteins SOX2, Nanog and octamer binding transcription factor 4 (OCT4)[42]. When mammary epithelial cells transformed through HER2 overexpression undergo EMT *in vitro*, the proportion of cancer stem cells is significantly increased. Human mammary epithelial cells transformed with a V12H-Ras oncogene overexpressing EMT proteins Twist or Snail were able to form tumors *in vivo* from significantly fewer cells than control transformed cells[42]. EMT is an important process for cancer progression and metastasis, generating invasive cells with a cancer stem cell-like phenotype, however the exact relationship between EMT and cancer stem remains to be determined.

ADIPOSE SIGNALING

Adipose tissue is an amalgamation of adipocytes, fibroblasts, stromal precursors and immune cells. Adipose signaling regulates fatty acid metabolism, homeostasis, and insulin signaling *via* adipocyte-secreted factors such as adiponectin and leptin. Adipose tissue has significant immune and inflammatory signaling functions involving adipokines such as IL-6 and tumor necrosis factor-α (TNF-α). While IL-6 and TNF-α are classically produced by the non-adipocyte members of the adipose tissue, it has been demonstrated that cancer associated adipocytes secrete IL-6, one of the primary cytokines involved in adipocyte-tumor cell interaction.

The stromal microenvironment plays a critical role in breast cancer formation and progression, however the pro-tumorigenic abilities of mature adipocytes have only been recognized in the past 10 years[7]. In order to isolate the effects of cytokines from those of adipocyte produced estrogen, it is important to demonstrate the effects in both ER^+ and ER^- model systems. Using conditioned media from adipocyte culture plates, both ER^+ and ER^- breast cancer cells had significant increases in proliferation and survival[43]. Crosstalk between adipocytes and breast cancer cells leads to change in phenotype of the adipocyte cells in addition to the changes seen in breast cancer cells. Mature adipocytes co-cultured with breast cancer cells exhibit delipidation, loss of terminal differentiation markers and overexpression of inflammatory cytokines and adipokines. The expression of these signaling molecules plays a critical role in the tumor supporting functions of adipocytes[7] (Figure 1). Two of the best-characterized signaling molecules in the breast cancer-adipocyte relationship are leptin and IL-6.

Leptin in the tumor microenvironment

Leptin is a 16 kd protein encoded by the *ob* gene, first discovered in *ob/ob* mutant mice that exhibit an obese phenotype due to the lack of satiety. The classical function of leptin is appetite control. When fat stores reach a certain level, leptin is secreted, and activates leptin receptors (OB-R) in the brain to indicate satiety. While OB-R is found at highest concentrations in the hypothalamus, it has been identified in almost all tissues. Levels of circulating leptin directly correlate with total fat mass, as larger fat cells produce more. Leptin signaling is dysregulated in obese subjects, with up to seven times higher leptin secretion compared to lean[44].

Leptin receptor OB-R is a member of the class I cytokine receptor family. Different isoforms of the OB-R protein are able to activate several classical cytokine-signaling pathways, including the JAK/STAT, PI3K and MAPK pathways. Through these pathways leptin likely has significant, diverse effects on physiology and disease, many of which are not fully understood[45].

Leptin signaling is a significant factor in the adipocyte-tumor signaling relationship. OB-R mRNA expression is highly upregulated in patient tumors of all breast cancer subtypes, with little to no expression seen in normal epithelial tissue[46]. Leptin signaling enhances tumor formation, proliferation and invasion by activation of cancer stem cell signaling pathways Notch and Wnt, and by activating numerous oncogenic pathways, including HER2, AKT, STAT3 and NFkB. Both leptin and OB-R are significantly upregulated in breast cancer stem cells, which exhibit increased sensitivity to leptin signaling due to higher receptor expression[47]. In ER^+/PR^+ breast cancer cells, STAT3 activation *via* leptin signaling also increases expression of chaperone binding protein Hsp90, resulting in upregulation of the HER2 oncogene. shRNA knockdown of STAT3 inhibited leptin induced HER2 upregulation[48].

Silencing of OB-R using shRNA in triple negative breast cancer cells inhibited expression of Nanog, SOX2 and OCT4. These cells had reduced cell proliferation and mammosphere formation, indicative of a reduction in self-renewal. Additionally, OB-R silenced cells went through mesenchymal to epithelial transition, with increased E-Cadherin and decreased vimentin expression[49].

Through the use of two mouse models, leptin-deficient *ob/ob* mice and OB-R deficient *db/db*, the role of leptin in tumorigenesis was confirmed *in vivo*. Both models form early onset obesity with nearly identical characteristics, however *ob/*ob mice have no circulating leptin, whereas *db/db* have high levels of leptin. Using tumors resected from MMTV-WNT-1 transgenic mice, a single cell suspension was injected into *db/db*, *ob/ob* or wild type mice. Tumor growth was detected earlier in *db/db* compared to wild type, and tumor volume was up to 8 times that of the WT tumors. *Ob/ob* mice formed tumors at a significantly lower rate, with volumes similar in size to the wild type tumors. Through the use of a limiting dilution assay, the authors demonstrated that injection tumor cells from wild type mice resulted in 100% secondary tumor growth formation, whereas the same number of cells from leptin deficient tumors were unable to form secondary tumors[50]. This provides *in vivo* evidence

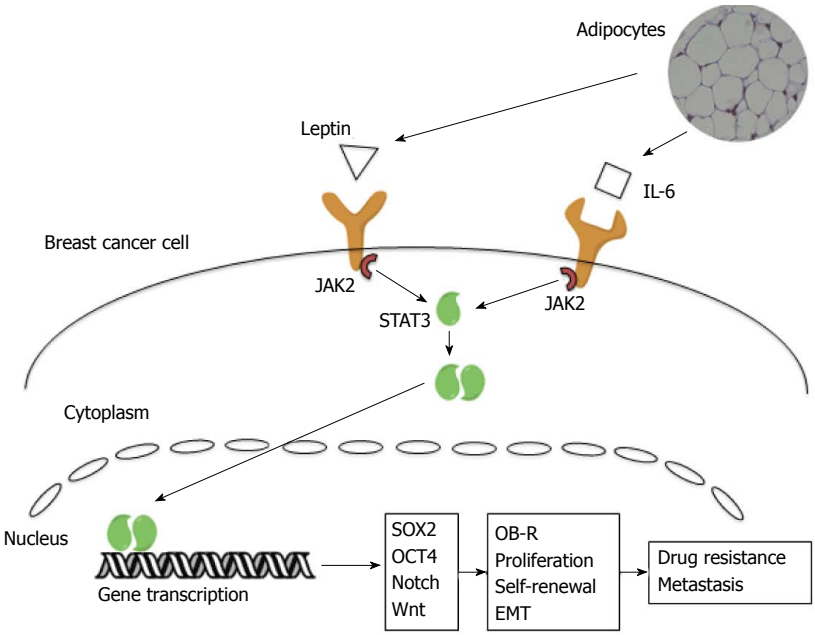

Figure 1 Adipocyte secreted leptin and interleukin-6 has a paracrine effect on nearby breast cancer cells. Through activation of the JAK2/STAT3 pathway, stemness factors SOX2 and OCT4 as well as EMT factors Notch and Wnt are transcribed. This leads to increased cancer cell proliferation and self-renewal entry into EMT and increased expression of leptin receptor OB-R. IL-6: Interleukin-6; OB-R: Leptin receptor; JAK2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; SOX2: Sex determining region Y-box 2; OCT4: Octamer binding transcription factor 4; EMT: Epithelial mesenchymal transition.

of the necessity of leptin for breast cancer initiation and progression as well as implicating its role in breast cancer stem cell self-renewal. In triple-negative breast cancer cell cultures, OB-R activation increases levels of stem cell regulator Notch[21]. Inhibition of leptin signaling decrease expression of both Wnt and Notch in carcinogen induced mouse mammary tumors[51].

It is clear that leptin plays a significant role in activation of breast cancer stem cell signaling. Leptin activates several stemness pathways including the OCT-4/SOX2/Nanog axis, Notch signaling and Wnt/β-catenin signaling. Through these pathways leptin increases self-renewal, tumor initiation and ALDH1 expression, indicating an important role in adipocyte mediated pro-tumor signaling.

IL-6 in the tumor microenvironment

Adipose tissue is a significant source of IL-6, producing approximately one third of IL-6 found in the plasma. In healthy adipose tissue, non-adipocyte members produce the majority of adipose IL-6[44]. There are seven members of the IL-6 family; IL-6, oncostatin M (OSM), IL-11, leukemia inhibitory factor (LIF), cardiotrophin-like cytokine (CLC), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1). There are 3 plasma membrane receptors, gp130, LIFR and OSMR, which activate the JAK/STAT, MAPK and PI3K pathways. Through these pathways IL-6 cytokines activate genes involved in inflammation, differentiation, survival, apoptosis and proliferation[52]. Within the adipose tissue, IL-6 stimulates glucose uptake, and activates glucose and fatty acid oxidation as well as insulin release[53].

Serum level of IL-6 is a negative prognostic marker in breast cancer patients[51,54]. While adipocyte secretion of IL-6 is low, proximity with tumor cells upregulates IL-6 expression. As mature adipocytes are the most common cells in tumor stroma, the combined amount of adipocyte IL-6 may have a significant impact on tumor cells[7]. IL-6 stimulates invasion in both ER^+ and ER^- breast cancer cells, similar to the phenotype observed in adipocyte/breast cancer cell co-culture[7]. When ER^+/ER^- breast cancer cells were treated with adipocyte-conditioned media, addition of an IL-6 blocking antibody significantly inhibited the pro-invasive effect[7]. However, depletion of IL-6 does not completely eliminate the invasive effects, supporting the model that multiple secreted molecules are important in the adipocyte-breast cancer cell interaction[55].

IL-6 activates transcription of OCT4 though the janus kinase 2 (JAK2)/STAT3 pathway, inducing EMT[38]. In triple negative breast cancer cells, there is higher IL-6/JAK2/STAT3 pathway activity in $CD44^+CD24^-$ cancer stem cells compared to the differentiated $CD44^+CD24^+$ population[56]. Addition of IL-6 to culture media increased the proportion of cancer stem cells in triple negative breast cancer cell lines as well as in primary cells isolated from triple negative tumors[57]. Both breast cancer stem cells and mesenchymal breast cancer cells secrete up to 1000-fold more IL-6 than non-stem epithelial breast cancer cells, indicating the presence of an autocrine positive feedback loop[58].

FUTURE WORK AND THERAPEUTIC IMPLICATIONS

There is significant evidence that both adipocyte secreted IL-6 and leptin have pro-EMT activity, as well as promoting self-renewal and cancer stem cell signaling, however, these are just two of many signaling factors produced by adipocytes. Complete characterization of the adipocyte secretome in the breast cancer microenvironment is an important next step in the investigation of the adipocyte-breast cancer signaling relationship. Dirat *et al*[7] have demonstrated

the phenotypic plasticity of adipocytes in culture with breast cancer cells, further description of the exact mechanisms and consequences of cancer-associated adipocytes will contribute significantly to our knowledge of the tumor microenvironment. While the breast cancer microenvironment is heterogeneous, adipocytes are the primary constituent. Blocking adipocyte-cancer cell interactions has the potential to inhibit cancer stem cell activity and prevent tumor initiation/progression.

Conditioned media from adipocytes treated with Genistein and Sulforaphane has been shown to inhibit mammosphere formation of breast cancer cells[59,60]. Furthermore, there is already a clinically available anti-IL-6 antibody, Tocilizumab. Developed as a treatment for inflammatory rheumatic diseases, Tocilizumab may be useful for inhibiting adipocyte/breast cancer cell IL-6 signaling[61]. There also is significant interest in targeting leptin signaling for treatment of breast cancer. Leptin-signaling inhibition has anti-tumor effects in both ER^+, ER^- and triple negative *in vitro* and *in vivo* models of breast cancer[62,63]. OB-R inhibitors, including leptin muteins[64], leptin peptide modulators[65], antibodies[66] and nanobodies[67] are under development, but have yet to enter clinical trials.

Future studies will reveal if other adipocyte-derived factors contribute to tumorigenesis. There are many adipocyte-secreted cytokines, with only a few currently investigated. It is likely that non-coding RNAs such as miRNAs and long non-coding RNAs (lncRNAs) are also involved in stromal-tumor cell signaling. Microvesicles such as exosomes mediate paracrine and endocrine transfer of miRNA and lncRNA, as well as proteins including TGF-β[68]. Targeting exosome mediated signaling may provide unique methods of inhibiting the adipocyte-breast cancer relationship.

miRNAs dysregulation is seen in almost all cancers[69] affecting nearly every hallmark of cancer[70]. Depending on the protein targeted, miRNAs act as either oncogenes or tumor suppressors, and miRNAs have a direct role in breast cancer stemness and progression[71,72]. Recently miRNAs with specific roles in adipogenesis and obesity have been identified[73]. There is differential miRNA expression in the adipocytes of obese mice compared to lean, including downregulation of miR-200 family microRNAs[74]. The miR-200 family inhibits epithelial mesenchymal transition through targeting of key EMT regulators such as ZEB1, SIP1[75] and SIRT1[41]. miR-200c expression is suppressed by IL-6, another potential mechanism for adipocyte-mediated increase in EMT signaling[76]. MiRNAs have important functions in both adipocytes and breast cancer cells, and may serve as signaling molecules or modify cancer cell interactions in the tumor microenvironment.

Current research on lncRNAs is rapidly changing the current paradigm of cell signaling. Initially found to have important regulatory roles during development and stem cell biology, lncRNAs may be involved in dysregulated signaling associated with transformation. lncRNA have diverse roles, and function during nearly all levels of gene expression. Differential lncRNAs expression may be used to predict patient outcomes or targeted to disrupt cell signaling[77]. Through profiling of preadipocyte and adipocyte transcriptomes, lncRNAs that control adipogenesis were identified[78]. Dissection of lncRNA's specific roles in adipocytes and breast cancer cells may provide new avenues of treatment.

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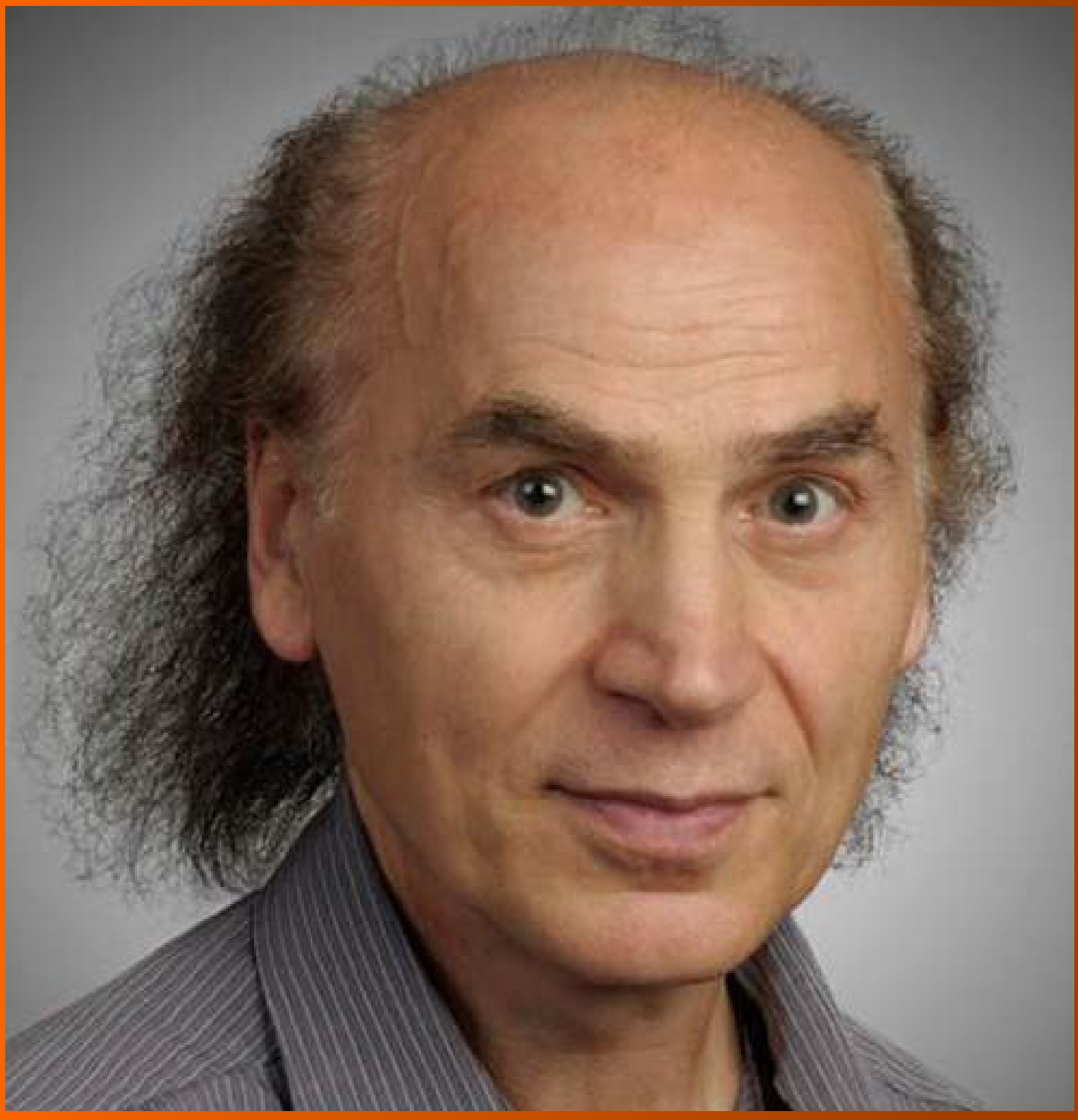

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EDITORIAL

Mechanism of DNA damage tolerance

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Abstract

DNA damage may compromise genome integrity and lead to cell death. Cells have evolved a variety of processes to respond to DNA damage including damage repair and tolerance mechanisms, as well as damage checkpoints. The DNA damage tolerance (DDT) pathway promotes the bypass of single-stranded DNA lesions encountered by DNA polymerases during DNA replication. This prevents the stalling of DNA replication. Two mechanistically distinct DDT branches have been characterized. One is translesion synthesis (TLS) in which a replicative DNA polymerase is temporarily replaced by a specialized TLS polymerase that has the ability to replicate across DNA lesions. TLS is mechanistically simple and straightforward, but it is intrinsically error-prone. The other is the error-free template switching (TS) mechanism in which the stalled nascent strand switches from the damaged template to the undamaged newly synthesized sister strand for extension past the lesion. Error-free TS is a complex but preferable process for bypassing DNA lesions. However, our current understanding of this pathway is sketchy. An increasing number of factors are being found to participate or regulate this important mechanism, which is the focus of this editorial.

Key words: DNA damage tolerance; Template switching; DNA damage bypass; DNA replication; Replicative stress; Translesion synthesis; Ubiquitination; Sumoylation

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Core tip: DNA damage may compromise genome integrity and lead to cell death. Cells have evolved a variety of processes to respond to DNA damage including damage repair and tolerance mechanisms. The DNA damage tolerance (DDT) pathway promotes the bypass of single-stranded DNA lesions encountered by DNA polymerases during DNA replication. This prevents the stalling of DNA replication. Two mechanistically distinct DDT branches, translesion synthesis and template switching have been characterized. However, our current understanding of DDT is far from complete and that of template switching is especially sketchy. This editorial focuses on recently identified components and regulators of DDT.

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DNA DAMAGE TOLERANCE MECHANISMS

The genomic DNA of a living organism is subject to damage by both internal and external chemical and physical agents, leading to various types of lesions. Cells employ DNA damage repair and tolerance/bypass mechanisms as well as damage checkpoints to respond to these lesions[1]. DNA double-stranded breaks (DSBs) can be repaired by homologous recombination (HR) or non-homologous end joining, while single-stranded DNA (ssDNA) lesions are subject to repair by nucleotide excision repair, base excision repair, or DNA mismatch repair[2-6]. DNA damage such as base modification, if left unrepaired in S phase of the cell cycle, stalls the progression of DNA replication, because the replicative polymerases are not able to recognize the modified bases and use them as template for nucleotide incorporation. The DNA damage tolerance (DDT) mechanism (also known as DNA damage bypass or post-replication repair) enables DNA replication to circumvent the lesions, thereby allowing the completion of DNA replication, and leaving the damages to be repaired later[7,8].

Recent studies indicate that DNA damage makes DNA synthesis on both the leading and lagging strands discontinuous as a result of the uncoupling of DNA polymerase and DNA helicase or the reinitiation of DNA synthesis at a distance from the lesion[9-11]. This creates a ssDNA gap behind the replication fork with the DNA lesion at the 3' end of the gap (Figure 1A). This ssDNA gap can be filled by two distinct DDT mechanisms. One is translesion synthesis (TLS) in which the replicative DNA polymerase is temporarily replaced by a special TLS polymerase pol ζ or η that can replicate across DNA lesions[12] (Figure 1B). As TLS polymerases lack proofreading activity and have flexible active sites that can recognize modified nucleotides, they may incorporate the wrong nucleotides (Figure 1B). As such, TLS synthesis is potentially mutagenic and error-prone. In fact, TLS is a major source of cellular mutagenesis. The other DDT process is template switching (TS) in which the stalled nascent strand switches temporarily to the newly synthesized undamaged sister strand for replication over the lesion (Figure 1C and D). Therefore, TS is an error-free process. Pairing between the two newly synthesized strands in TS is promoted by strand invasion (Figure 1C). The resulting structure is then turned into a sister chromatid junction (SCJ) after gap filling using the damage-free sister strand as a template (Figure 1D). SCJ is later resolved to yield two duplex DNA strands, completing the damage bypass process (Figure 1E). Note that both TLS and TS take place behind the replication fork, and may happen during or after DNA replication. There is evidence suggesting that TS starts early in S phase, whereas TLS doesn't start until late S phase[13-15].

The sliding DNA clamp PCNA plays a central role in DDT regulation as its post-translational modifications act as a molecular switch to control the choice of DDT pathways[16]. PCNA is a ring-shaped homotrimer that is loaded to primed DNA template by the clamp loader RFC. It encircles DNA to act as a sliding platform for recruiting many factors involved in DNA replication and DNA damage response. For example, PCNA interacts with replicative DNA polymerases (ε and δ) to serve as their processivity factor. DNA damage such as base modification encountered by the replication fork induces the ubiquitination of PCNA[17] (Figure 2). PCNA is first mono-ubiquitinated at lysine 164 (K164) by the E2-E3 ubiquitinase Rad6/Rad18[17], and can be further poly-ubiquitinated *via* the formation of K63-linked ubiquitin chains by another E2-E3 ubiquitinase Rad5/Ubc13/Mms2[17] (Figure 2). Mono-ubiquitinated PCNA specifically promotes TLS as it preferentially binds TLS polymerase ζ or η, which is believed to facilitate the replacement of replicative polymerases with TLS polymerases[16,18] (Figure 2). On the other hand, poly-ubiquitinated PCNA activates the Rad5-dependent error-free TS pathway (Figure 2), but the underlying mechanism has yet to be elucidated[16].

COMPONENTS OF THE DDT MACHINERY AND CROSS-TALK BETWEEN DDT AND OTHER DNA DAMAGE RESPONSE MECHANISMS

More and more factors are being implicated in DDT, especially the error-free TS branch of DDT (Table 1). The fact that many of these factors are known to also function in other DNA damage repair and checkpoint mechanisms suggests an extensive crosstalk between DDT and these pathways.

MRX complex has 3'-5' exonuclease activity and ssDNA endonuclease activity, and is known to act in DSB recognition and processing[19]. Recent epistasis analyses place MRX in both the TLS and TS branches of DDT[20]. Moreover, there is evidence for a physical interaction between MRX and Rad18[20]. These findings suggest that MRX acts early in DDT, likely to process the 3' end of the ssDNA gap at stalled replication fork and help recruit Rad18[20].

Ino80 chromatin remodeling complex is known to associate with replication forks and aid in fork progression and recovery of stalled forks[21-23]. Ino80 was also found to facilitate Rad18 recruitment to stalled forks, PCNA poly-ubiquitination, Rad51 recruitment and formation of SCJ intermediate of error-free DDT[23]. Therefore, Ino80 plays a role in error-free DDT during DNA replication in S phase. Whether it also

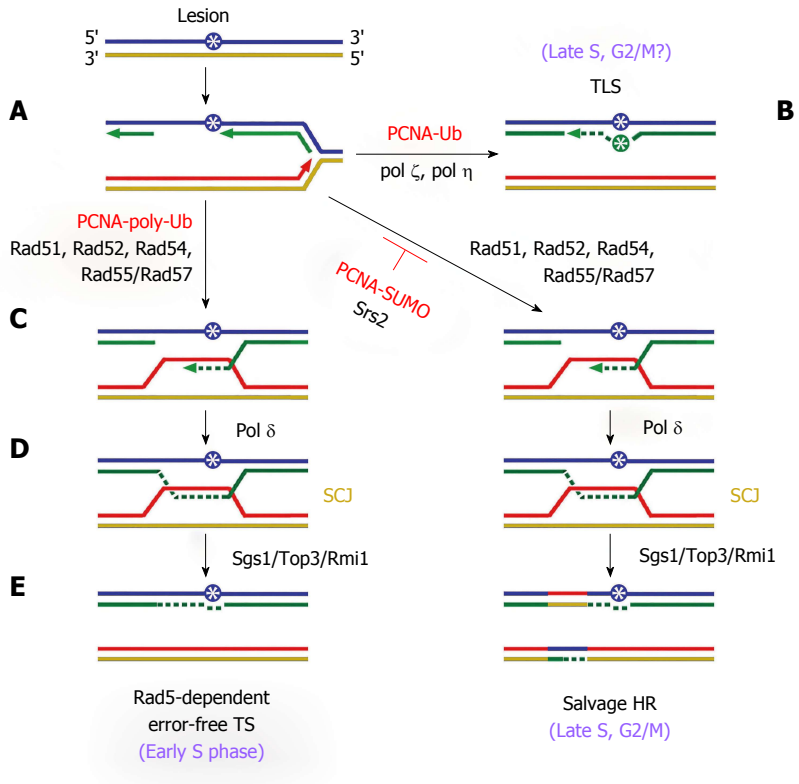

Figure 1 DNA damage tolerance mechanisms. DNA damage (asterisk in blue circle) stalls DNA replication. A: Reinitiation of DNA synthesis results in the formation of a ssDNA gap. Shown is a ssDNA gap on the lagging strand; B: PCNA mono-ubiquitination induces a switch from replicative polymerase to a TLS polymerase Pol ζ or Pol η, resulting in gap filling *via* TLS, which may incorporate the wrong nucleotide (asterisk in green circle); C-E: PCNA poly-ubiquitination activates the Rad5-dependent error-free TS pathway. Gap filling is achieved by strand invasion mediated by Rad51, Rad52, Rad54, and Rad55/Rad57 and repair synthesis carried out by Pol δ (C), followed by the formation of SCJ (D), and resolution of the SCJ by Sgs1/Top3/Rmi1 (E). DDT can also proceed through the salvage HR pathway that also produces the SCJ intermediate. The salvage pathway is hyper-recombinogenic and prone to crossover as indicated. It is normally inhibited by sumoylated PCNA and Srs2. G2: Gap 2; M: Mitotic; PCNA: Proliferating cell nuclear antigen; S: Synthesis; SUMO: Small ubiquitin-like modifier; TLS: Translesion synthesis; SCJ: Sister chromatid junction; Ub: Ubiquitin.

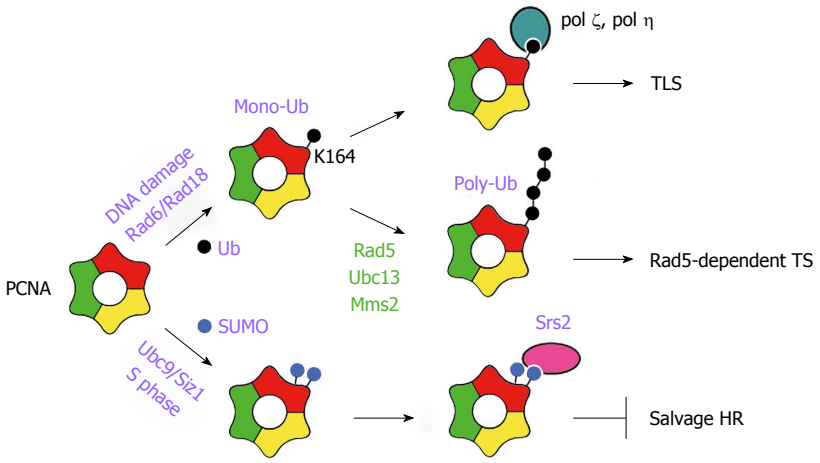

Figure 2 Regulation of DNA damage tolerance by proliferating cell nuclear antigen modification. PCNA is a homotrimer that can be modified at K164 by either ubiquitin or SUMO. It can also be sumoylated at K127. PCNA mono-ubiquitination (mono-Ub) by Rad6/Rad18 facilitates the recruitment of TLS polymerases thereby promoting TLS. Extension of mono-Ub with a K63-linked Ub chain (poly-Ub) by Rad5/Ubc13/Mms2 promotes Rad5-dependent error-free TS pathway. PCNA sumoylation recruits Srs2 that inhibits the salvage HR pathway. HR: Homologous recombination; K: Lysine; PCNA: Proliferating cell nuclear antigen; S: Synthesis; SUMO: Small ubiquitin-like modifier; TLS: Translesion synthesis; TS: Template switching; Ub: Ubiquitin.

plays a role in TLS has not been examined.

Exo1 is a major 5'-3' nuclease responsible for the resection of dsDNA break ends, and functions in DNA damage repair, recombination, replication, and telomere integrity[24,25]. There is increasing genetic evidence implicating Exo1 and its nuclease activity in error-free TS pathway[20,26-28]. Moreover, Exo1 was found to be required for the formation of SCJ

Table 1 Components and regulators of DNA damage tolerance[1]

Factor/complex	Functions/properties
Rad6/Rad18	E2 ubiquitin conjugase/E3 ubiquitin ligase complex; promotes mono-ubiquitination of PCNA at lysine 164 (PCNA-K164)
Rad5/Mms2/Ubc13	E3 ubiquitin ligase/E2 ubiquitin conjugase complex; promotes poly-ubiquitination of PCNA-K164; has ATPase activity
Pol30	Forms a homotrimer (PCNA); its ubiquitination and sumoylation regulates the choice of DDT mechanism
Ubc9/Siz1	E2 SUMO conjugase/E3 SUMO ligase complex; promotes PCNA sumoylation
DNA polymerase ζ	TLS polymerase; consists of subunits Rev3, Rev7, Pol31 and Pol32 with Rev3 being the catalytic subunit
Rev1	dCMP transferase; required for TLS by Pol ζ
DNA polymerase η (Rad30)	TLS polymerase
DNA polymerase δ (Pol3/Pol31/Pol32)	Lagging strand DNA polymerase; Pol3 is the catalytic subunit; promotes repair synthesis in error-free DDT; Pol32 is not essential for DNA replication but is required for repair synthesis
Rad51	Components of HR responsible for homology search and strand invasion; Rad51 molecules bind ssDNA to form a presynaptic
Rad52	filament important for subsequent strand invasion; both Rad55 and Rad57 are paralogs of Rad51; Rad55/Rad57 stabilizes Rad51
Rad54	nucleoprotein filament and inhibits Srs2 helicase activity
Rad55/Rad57	
Srs2	3′-5′ helicase and translocase; disrupts Rad51 nucleoprotein filaments; inhibits salvage HR pathway thereby channeling DNA lesions to Rad5 pathway
Sgs1/Top3/Rmi1	Resolves SCJ intermediates generated in error-free DDT
RFA (replication factor A) (Rfa1/Rfa2/Rfa3)	ssDNA binding complex; is involved in DNA replication, repair and recombination; RFA-ssDNA nucleofilament helps recruit Rad18
Exo1	5′-3′ exonuclease; promotes resection of dsDNA break ends; functions in DNA damage repair, recombination, replication, and telomere integrity; is required for SCJ formation in error-free DDT
9-1-1 complex (Ddc1/Mec3/Rad17)	Ring shaped DNA clamp; is involved in DNA check point signaling; facilitates Pol ζ recruitment; is required for SCJ formation in error-free DDT; interacts with Exo1
MRX complex (Mre11-Rad50-Xrs2)	3′-5′ exonuclease and ssDNA endonuclease; is potentially involved in both TLS and TS branches of DDT; interacts with Rad18
Shu complex (Shu1/Shu2/ Csm2/Psy3)	Is involved in error-free DDT; interacts with Rad55/Rad57
Ino80	Chromatin remodeler; associates with replication forks and promotes fork progression; required for Rad18 and Rad51 recruitment to replications forks, PCNA poly-ubiquitination and SCJ formation
Elg1	Unloads PCNA from chromatin during DNA replication; regulates choice of DDT pathway
Hmo1	HMGB homolog; binds ssDNA and DNA with an altered conformation; bends DNA; interacts with Elg1; may help channel DNA lesions to Rad5 pathway; required for SCJ formation in S phase
Fun30	Chromatin remodeler; facilitates long-range resection of dsDNA ends; potentially regulates choice of DDT pathway

[1]Listed are DDT factors in *S. cerevisiae*. ATPase: Adenosine triphosphatase; dCMP: Deoxycytidine monophosphate; DDT: DNA damage tolerance; dsDNA: Double stranded DNA; HMGB: High-mobility group protein B; HR: Homologous recombination; K: Lysine; PCNA: Proliferating cell nuclear antigen; RFA: Replication factor A; S: Synthesis; SCJ: Sister chromatid junction; ssDNA: Single stranded DNA; SUMO: Small ubiquitin-like modifier; TLS: Translesion synthesis; TS: Template switching; Ub: Ubiquitin.

intermediate of error-free DDT[27] .

9-1-1 complex resembles PCNA in assuming a ring structure that can encircle DNA[29]. 9-1-1 has been long established as a DNA damage sensor and a component of checkpoint signaling. It also plays a role in TLS by facilitating the recruitment of TLS polymerase pol ζ[30]. Recent genetic evidence also implicates 9-1-1 in error-free DDT[28]. 9-1-1 is required for SCJ formation[28]. 9-1-1's function in error-free DDT is separate from its canonical role in G1/S and S phase checkpoint signaling. 9-1-1 is loaded by Rad24 to 5′ end of ssDNA region/gap and is known to associate with many checkpoint and repair proteins[29]. Recently, 9-1-1 was found to also bind Exo1 and stimulate its end resection activity[28,31]. This raises the possibility that 9-1-1 recruits Exo1 to the 5′ end of the ssDNA gap behind stalled replication fork to extend the gap[28].

Error-free DDT is intimately linked to HR as a subset of HR factors has been shown to play roles in it. These include Rad51, Rad52, Rad54, Rad55 and Rad57 that are believed to carry out homology search, strand invasion and pairing of newly synthesized strands from the two sister chromatids[27,32,33]. Rad51 molecules bind the stalled nascent DNA strand to form a presynaptic filament that is later involved in strand invasion[34], and the Rad51 paralogs Rad55 and Rad57 form a complex to facilitate the formation or maintenance of Rad51 filament[35,36].

DNA polymerase δ carries out DNA synthesis for gap filling after the stalled strand switches template during error-free DDT[27]. This results in the formation of a SCJ (Figure 1D) that is resolved by Sgs1/Top3/Rmi1 complex, completing the error-free DDT process[37-39].

SALVAGE HOMOLOGOUS RECOMBINATION PATHWAY OF DDT

Besides the Rad5-dependent error-free DDT pathway, there is an alternative HR-mediated DDT mechanism referred to as the salvage HR pathway that does not depend on PCNA ubiquitination[27,28,40-42]. This pathway, if allowed to act during DNA replication, would generate

hyper-recombinogenic intermediates that may lead to chromosome rearrangement and genome instability. The existence of the salvage HR pathway was suggested by the finding that *RAD52* is a high copy suppressor of replicative stress-sensitivity of a mutant in which PCNA cannot be ubiquitinated[40]. Consistently, deletion of the "antirecombinase" Srs2 suppresses the sensitivity of cells lacking Rad6 to DNA damage[43]. This suppression is dependent on the HR repair pathway since it is blocked when *RAD51, RAD52, RAD54, RAD55* or *RAD57* is absent[43]. Moreover, this suppression was found to be specific for Rad5-dependent DDT[40,44-46]. These findings are consistent with the notion that the salvage HR pathway functions independently of the Rad5-dependent error-free TS pathway to bypass DNA lesions, and is normally hindered by Srs2.

Srs2 is a DNA helicase that has the ability to disrupt Rad51-ssDNA nucleofilament, and is thus called an "antirecombinase"[47,48]. The role of Srs2 in inhibiting recombination during replication is closely linked to the sumoylation of PCNA at K164 or K127 by Ubc9/Siz1[40,45]. Unlike PCNA ubiquitination that is induced by DNA damage, PCNA sumoylation is independent of DNA damage[17]. Sumoylation of PCNA is cell cycle dependent as it only occurs in S phase. Srs2 was found to preferentially interact with sumoylated PCNA, leading to the notion that sumoylated PCNA recruits Srs2 to replicating DNA to prevent unwanted recombination during normal DNA replication, and blocks salvage HR pathway in the presence of replicative stress[40,45].

The Rad5-dependent pathway and salvage HR pathway both employ template switching to bypass DNA lesions encountered by the replication fork. As such, it is not surprising that these two pathways share many components such as HR factors Rad51, Rad52, Rad54, Rad55 and Rad57. Both pathways generate SCJ intermediates that are resolved by Sgs1/Top3/Rmi1[27,49]. It was found recently that the 9-1-1 complex is required for SCJ formation in both the Rad5-dependent and salvage HR pathways[28], suggesting that the putative 9-1-1 mediated processing of the ssDNA gap is needed for each pathway.

Despite their mechanistic similarity, the Rad5 and salvage HR pathways clearly differ in that the former is dependent on Rad5/Ubc13/Mms2 mediated PCNA poly-ubiquitination and the latter is not. In addition, they are distinguishable regarding their timing of action in the cell cycle. There is evidence indicating that the Rad5 pathway operates in early S phase, whereas salvage pathway acts in late S or G2/M phase[28,41,49]. This is presumably because the salvage HR pathway is normally kept in check in S phase by sumoylated PCNA and Srs2, and only becomes derepressed when the level of PCNA sumoylation starts to decline in late S phase[28,49,50]. Moreover, the salvage pathway is hyper-recombinogenic and thus has a high propensity to yield chromosomal rearrangement, but the Rad5 pathway is not. This might, at least in part, stem from the fact that salvage pathway acts in the absence of Srs2 while the Rad5 pathway operates in the presence of Srs2. Since Srs2 is known to suppress crossover in HR and promote noncrossover HR[51-53], it is likely that the salvage pathway is prone to crossover and the Rad5 pathway is free of crossover. Lastly, Srs2 has recently been found to also limit D-loop extension, or repair synthesis, during HR and template switching[54], which may also contribute to the prevention of DNA rearrangement in Rad5 dependent error-free DDT.

REGULATION OF THE CHOICE OF DDT PATHWAY

The choice of DDT pathway greatly impacts genome integrity as mutagenesis by TLS and hyper-recombination by salvage HR pathway can lead to the accumulation of harmful mutations and chromosomal rearrangements[55,56]. There is increasing evidence for the existence of multiple factors/mechanisms that ensure the preferential employment of the Rad5-dependent error-free pathway for DNA damage bypass. First, TLS, Rad5 pathway and salvage HR pathway seem to operate within distinct time windows in relation to DNA replication, with Rad5 pathway acting in early S phase, and TLS and salvage HR pathway acting in late S or G2/M phase[13,14,28,41]. Thus Rad5 pathway is the earliest DDT mechanism available to deal with DNA lesions encountered by replication forks (Table 1).

Secondly, Srs2 is believed to channel DNA lesions to Rad5 pathway by inhibiting salvage HR pathway. This is based on the finding that Srs2 deletion (or blocking PCNA sumoylation) suppresses *rad5*Δ hypersensitivity to DNA damage in a Rad51-dependent fashion[40,44,45]. Since the salvage HR pathway is normally kept in check in S phase by PCNA sumoylation and Srs2, lack of Srs2 function would allow the salvage HR pathway to work ectopically in S phase. Alternatively, or in addition, lack of Ssr2 function increases the "scheduled" function of salvage HR pathway in late S and G2/M phase. Regardless of its possible timing, the derepression of the salvage HR pathway allows *rad5*Δ cells to effectively bypass DNA lesions and stay viable, albeit at a cost of genome stability.

As Srs2 has a general antirecombinase activity of disrupting Rad51-ssDNA nucleofilament, it should in theory also inhibit the Rad5 pathway as it inhibits the salvage HR pathway. However, Rad5 pathway is apparently not subject to inhibition by Srs2. What then prevents Srs2 from blocking Rad5 pathway? Recent discoveries on potential functions of the Shu complex and Rad55/Rad57 in error-free DDT provided a possible answer to this question. The Shu complex consists of Shu1, Shu2, Psy3 and Csm2, and genetic evidence has implicated it in error-free DNA damage repair and DDT[57-59]. The Csm2 subunit of Shu complex associates with the auxiliary HR factor Rad55/Rad57. Importantly, Rad55/Rad57 promotes the formation of Rad51 nucleofilament and may counter

the anti-recombination function of Srs2[35,36,60-62]. It is possible that Shu complex contributes to error-free DDT by recruiting Rad55/Rad57[63]. Shu complex itself may be recruited to stalled DNA replication fork directly or indirectly by PCNA poly-ubiquitination. In short, Shu complex may help prevent Srs2 from inhibiting Rad5-dependent pathway. The notion that Srs2 specifically inhibits the salvage HR pathway is in full accordance with *srs2*Δ mediated suppression of the hypersensitivity to replicative stress of cells lacking Rad5 pathway[40,44,45]. An alternative explanation for why Rad5 pathway is not subject to inhibition by Srs2 is that ubiquitination of K164 of PCNA by Rad5/Ubc13/Mms2 competes with PCNA-K164 sumoylation that is necessary for Srs2 recruitment[18].

Interestingly, similar to Srs2 deletion, deletion of Elg1, Hmo1 or Fun30 also suppresses hypersensitivity to replicative stress of cells lacking Rad5 pathway, suggesting that these three functionally diverse proteins also regulate the choice of DDT pathway[49,64,65]. Elg1 is homologous to Rfc1, the largest subunit of PCNA loader RFC[16]. Elg1 associates with the other subunits of RFC (Rfc2 thorough Rfc5) to form a RFC like complex (Elg1-RLC) that is known to play a role in genome stability[66]. Elg1 deletion causes an abnormal accumulation of sumoylated and unmodified PCNA on replicating chromatin[64,67]. Elg1 physically interacts with PCNA with a preference for sumoylated PCNA[64]. Elg1-RLC can unload unmodified and sumoylated PCNA from chromatin during DNA replication[64,67]. Given that sumoylated PCNA recruits Srs2, it is not surprising that Srs2 also accumulates on chromatin in the absence of Elg1[64]. Although these findings suggest a role of Elg1 in regulating the error-free DDT pathway by controlling the level of chromatin-associated PCNA, they do not readily provide an explanation of *elg1*Δ mediated suppression of *rad5*Δ hypersensitivity to replicative stress.

Hmo1 is a chromatin architecture protein homologous to HMGB (High mobility group box) protein[68]. It preferentially binds ssDNA or DNA with altered conformations, and has the ability to bend DNA[69,70]. Hmo1 was found to physically interact with Elg1[49]. However, genetic evidence suggests Hmo1 to act in parallel with Elg1 (and Srs2) in regulating DDT pathway choice[49]. Hmo1 is suggested to facilitate the channeling of DNA lesions to the Rad5-dependent pathway and prevent the salvage HR pathway and TLS. There is evidence suggesting that the regulatory function of Hmo1 in DDT is dependent on its DNA bending activity[49]. In addition to its potential role in regulating DDT pathway choice, Hmo1 was suggested to function specifically in the Rad5-dependent pathway. This was based on the finding that Hmo1 is required for SCJ formation in S phase, which coincides with the time window of Rad5 pathway, but is not required for SCJ formation in late S or G2/M phase of the cell cycle. It was thought that Hmo1 facilitates SCJ formation by binding and stabilizing sister chromatid bridges and hemicatenanes formed at replication forks with ssDNA gaps[49]. Note that unlike Hmo1, Elg1 and Srs2 are not required for SCJ formation, and are therefore unlikely to be participants of the error-free DDT process[49].

Fun30 is a chromatin remodeler that has been shown to facilitate long-range resection of dsDNA ends that proceeds HR[71-73]. This is believed to underlie its function in promoting DSB repair[71-74]. We obtained evidence that implicates Fun30 in the regulation of DDT[65]. Fun30 deletion suppresses *rad5*Δ hypersensitivity to replicative stress, which is unlikely due to a reduction in DNA end resection[65]. This suppression is dependent on Rad51 but not TLS or DNA damage repair pathways including nucleotide excision repair, base excision repair and non-homologous end joining[65]. It is possible that Fun30 negatively regulates the salvage HR pathway as Srs2 does.

WORKING MODEL FOR DDT MACHINERY

We incorporate recent findings on DDT described above in a working model as follows. When a DNA lesion stalls the replication fork in S phase, a ssDNA gap forms behind the fork (Figure 1A). RFA then binds ssDNA to form RFA-ssDNA nucleofilament that recruits Rad18, thereby starting the DDT process[11,75]. RFA-ssDNA filament also activates the replication checkpoint[76]. Ino80 chromatin remodeler that normally associates with replication forks to promote fork progression remodels chromatin at stalled replication fork, resulting in a chromatin environment that facilitates Rad18 recruitment. MRX complex may process the ssDNA gap by resecting its 3' end, and also by helping to recruit Rad18 *via* physical interaction.

Rad18 recruited to the ssDNA gap then binds Rad6 to form a E2-E3 ubiquitinase that mono-ubiquitinates K164 on PCNA that is associated with the 3' end of the gap. At this point, mono-ubiquitinated PCNA can lead to TLS or error free TS branches of DDT. On the one hand, mono-ubiquitinated PCNA can promote TLS by facilitating the replacement of replicative polymerases bound to PCNA with TLS polymerases (Figure 1B). 9-1-1 also plays a role in TLS by facilitating the recruitment of TLS polymerase pol ζ. On the other hand, Rad6/Rad18 complex may recruit Rad5/Ubc13/Mms2 E2-E3 ubiquitinase *via* physical interaction between Rad18 and Rad5[77]. Rad5/Ubc13/Mms2 further ubiquitinates mono-ubiquitinated PCNA. Poly-ubiquitinated PCNA then signals for error-free TS. 9-1-1 loaded to the 5' end of the ssDNA gap facilitates error-free TS by recruiting Exo1 for gap expansion, which is presumably important for subsequent events in error-free DDT.

Rad51 binds to the stalled nascent DNA strand to form a Rad51-ssDNA presynaptic filament. The extent and stability of this filament is determined by a balance between the opposing actions of Srs2 and Rad55/Rad57. Shu complex may help recruit Rad55/Rad57 and Rad51. With the help of Rad52 and Rad54, the Rad51 presynaptic filament then searches for

homology and invades the DNA duplex of the sister chromatid, and pairs with the nascent complementary sister strand (Figure 1C). Hmo1 may facilitate strand invasion by binding and stabilizing sister chromatid bridges and hemicatenanes.

After annealing with its sister strand, the nascent strand from the damaged DNA duplex is extended by replicative DNA polymerase pol δ over the DNA lesion (Figure 1C). This D-loop extension or repair synthesis is limited and transient, as it is hindered by Srs2. After the completion of repair synthesis, the 3′ end of the newly synthesized sequence switches back to the ssDNA gap on the damaged DNA. This effectively fills the ssDNA gap and results in the formation of an SCJ structure (Figure 1D). SCJ is then resolved by Sgs1/Top3/Rmi1 complex to form two duplex DNA strands (Figure 1E), concluding the error-free TS process.

CONCLUSION

More than four decades of studies of DNA damage tolerance/bypass have yielded significant advances in our knowledge of this important process including the characterization of the TLS polymerases and the discovery of PCNA ubiquitination as a molecular switch for TLS and TS pathways. However, the current understanding of DDT especially error-free DDT is far from complete, and many questions remain unanswered: (1) What determines the choice between TLS and Rad5-dependent TS? Specifically, what triggers poly-ubiquitination of mono-ubiquitinated PCNA? What promotes the interaction between Rad5 and Rad18? (2) How PCNA poly-ubiquitination activates downstream TS events? Does it directly recruit a component(s) involved in error-free DDT? (3) Are the three subunits of PCNA subject to independent or coordinated ubiquitination and sumoylation? (4) Does stalling DNA replication induce changes in local chromatin structure that are necessary for error-free DDT? (5) How do Elg1, Hmo1 and Fun30 regulate the choice of DDT pathway? (6) Are recently identified DDT components MRX complex, 9-1-1, Shu complex and Hmo1 directly or indirectly involved in DDT? (7) Does ssDNA gap at stalled replication fork have to be expanded by MRX complex and/or Exo1 for DDT to occur? (8) What determines the putative time windows in the cell cycle in which TLS, Rad5 pathway and salvage HR pathway operate? (9) What prevents the Rad5 pathway from being hyper-recombinogenic? and (10) Does 9-1-1 complex associate with the 5′ end of the ssDNA gap at stalled replication fork and help recruit DDT components such as Exo1? Addressing these questions would help yield a comprehensive understanding of the molecular mechanism and regulation of DDT.

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EDITORIAL

DNA damage responses in cancer stem cells: Implications for cancer therapeutic strategies

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Abstract

The identification of cancer stem cells (CSCs) that are responsible for tumor initiation, growth, metastasis, and therapeutic resistance might lead to a new thinking on cancer treatments. Similar to stem cells, CSCs also display high resistance to radiotherapy and chemotherapy with genotoxic agents. Thus, conventional therapy may shrink the tumor volume but cannot eliminate cancer. Eradiation of CSCs represents a novel therapeutic strategy. CSCs possess a highly efficient DNA damage response (DDR) system, which is considered as a contributor to the resistance of these cells from exposures to DNA damaging agents. Targeting of enhanced DDR in CSCs is thus proposed to facilitate the eradication of CSCs by conventional therapeutics. To achieve this aim, a better understanding of the cellular responses to DNA damage in CSCs is needed. In addition to the protein kinases and enzymes that are involved in DDR, other processes that affect the DDR including chromatin remodeling should also be explored.

Key words: Cancer stem cell; DNA damage response; DNA repair; Cancer therapy

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Core tip: Cancer stem cells (CSCs) are thought to be at the root of cancer recurrence and therapeutic resistance. Eradication of CSCs could be a key to successful cancer therapeutics. Studies have shown that CSCs possess highly efficient ability to process DNA damage. This elevated DNA damage response (DDR) is believed to be responsible for the resistance to DNA damaging agents. Thus, targeting enhanced DDR in CSCs may be a promising strategy to facilitate elimination of these highly tumorigenic cells by conventional therapies.

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INTRODUCTION

Many tumors contain phenotypically and functionally heterogeneous cancer cells. Among them, a minority of cell subpopulation, termed cancer stem cell (CSC), which possesses the self-renewal capacity and is able to generate the heterogeneous lineage of cancer cells, has been believed to be responsible for the initiation and maintenance of the tumors. Besides their self-renewal and differentiation properties shared with stem cells, CSCs are characterized with high tumorigenic potential and are also referred to as tumor-initiating cells[1]. Recent data suggest that CSCs are more resistant to chemotherapy and radiotherapy than non-stem cells[2]. Relatively successful cancer treatments shrink the bulk of tumor but often fail to eliminate the CSCs, resulting in the recurrence of tumors. Combined with their high tumorigenic potential, CSCs are believed to be responsible for tumor therapy resistance, tumor relapse, and metastasis.

Many chemotherapeutic drugs, such as platinum-based agents as well as radiotherapy, kill cancer cells by inducing DNA damage. Therefore, cells that efficiently repair DNA damage can potentially survive therapeutic agents. Indeed, prompt activation of DNA damage responses (DDR) and enhanced DNA repair capacity have been implicated as contributors to increased resistance to therapy in the CSC population in gliomas and breast cancer[3,4].

DDR

Cells incur a large number of DNA lesions from endogenous processes. Many lesions can block transcription, DNA replication, and chromosome segregation, resulting in cell death or gene mutations. Fortunately, cells have evolved an intricate machinery to deal with these lesions. This machinery, known as the DDR, can recognize these DNA lesions and repair them. Meanwhile, cells with genome injury can be arrested at specific checkpoints to allow repair of lesions before they are converted into permanent mutations. When damage is too significant, a cell may undergo apoptosis to avoid the passing of damage to their progeny. Although DDR is critical to the maintenance of genome stability, highly efficient DNA repair machinery can reduce the efficacy of DNA damaging agents in treating cancers. DNA damaging agents used for cancer therapy can induce various DNA lesions, such as covalent crosslinks between DNA bases (cisplatin, carboplatin, oxaliplatin and mitomycin C), base alkylation (methyl methanesulphonate and temozolomide), DNA single-strand breaks (SSB) and double-strand breaks (DSB) (camptothecin and etoposide). The DDR pathways in cancer cells can greatly affect the consequences of cancer treatment with DNA-damaging agents (Figure 1).

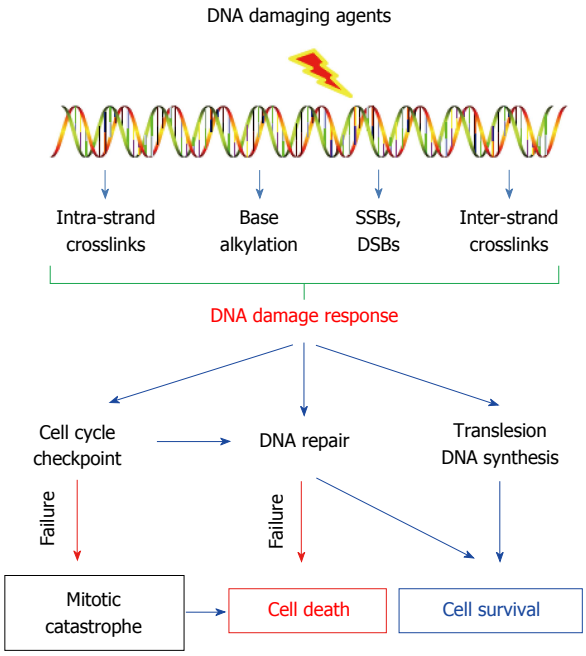

Figure 1 DNA damage responses and consequences in cancer treatment with DNA-damaging agents. DNA-damaging agent-induced DNA lesions can (1) activate cell cycle checkpoint to arrest the cell cycle progression to allow time for repair before the damage is passed on to daughter cells; (2) activate specific DNA repair pathways to remove the lesions; (3) or enables continuous functioning of replication by bypassing the DNA lesions through translesion DNA synthesis. Thus, highly efficient DDR can promote the survival of cancer cells upon DNA-damaging agent treatment.

Repair of intra-strand crosslinks

Cisplatin induces primarily 1, 2-intra-strand crosslinks between adjoining purines in DNA, *e.g.*, *cis*-Pt(NH_3)$_2$d(GpG) (Pt-GG), with Pt bound to two adjacent guanines, and *cis*-Pt(NH_3)$_2$d(ApG) (Pt-AG), with Pt bound to an adenine and an adjacent guanine. These damages contribute 90% of the total damage introduced by cisplatin[5-8]. The intra-strand crosslinks induced by platinum-based therapeutic agents are mainly processed by nucleotide excision repair (NER), which is carried out by a minimal set of proteins including XPA, XPC-RAD23B, XPG, RPA, ERCC1-XPF, TFIIH, PCNA, DNA polymerase δ or ε, and DNA ligase I, in human cells[9,10]. These proteins are sequentially assembled at the site of DNA lesions in chromatin[11-13], where they carry out the dual incision of the damage-containing DNA. Approximately 24-32 nucleotides are then removed, and a new fragment is synthesized to restore the DNA structure[14-16].

Repair of base alkylation and SSB

Many alkylating agents such as cyclophosphamide and temozolomide induce DNA damage by attaching an alkyl group to adenine or guanine, leading to replication fork stalling and subsequent apoptosis[17]. These DNA lesions are mainly repaired by base excision repair (BER). In BER, the alkylated bases

are first excised from the DNA strand by the lesion specific DNA repair enzymes known as glycosylases. The resulting abasic sites or apyrimidinic/apurinic (AP) sites are further recognized and excised by an AP endonuclease, APE1. The resulted SSBs can be bound by poly(ADP-ribose) polymerase 1 (PARP1), which synthesizes PAR polymers attached to itself and other repair factors[18,19]. Afterwards, BER proteins such as DNA polymerase β and XRCC1-DNA ligase α are recruited to sites of DNA damage for DNA synthesis and gap filling. If the AP site is not repaired, the SSB will persist at the site of damage, cause replication fork collapse during the S-phase, and subsequently is converted to DSB.

Repair of DSBs

DSBs arise from ionizing radiation, free radicals, topoisomerase inhibitors, and replication of a SSB. Homologous recombination (HR) and nonhomologous end joining (NHEJ) are the two major DSB repair pathways that have been studied extensively. In NHEJ, the DSB ends are simply reconnected without the use of a repair template. This process requires Ku70, Ku80, DNA-dependent protein kinases (DNA-PKs), XRCC4, and DNA ligase IV[20]. The Ku70/Ku80 heterodimer first binds to DNA ends and recruits DNA-PKs, which function to keep the broken DNA ends in close proximity and to recruit end-processing factors, such as Artemis, polynucleotide kinase/phosphatase (PNKP), APE1, and tyrosyl-DNA phosphodiesterase 1 (TDP1), to prepare the DNA ends for ligation by XRCC4-DNA ligase IV[21].

NHEJ is active throughout the cell cycle. In contrast, HR-directed DSB repair is confined to the late S and G2 phases of proliferating cells when sister chromatids are available as repair templates. HR repair is initiated by the generation of 3′ single-stranded DNA (ssDNA) by resection of the 5′ end, and the binding of replication protein A (RPA). This initial processing of the DNA ends involves the activity of several nucleases, *e.g.*, the MRN (MRE11-RAD50-NBS1) complex, Bloom's syndrome helicase (BLM), CtBP-interacting protein (CtIP), exonuclease 1 (EXO1), and DNA replication ATP-dependent helicase (DNA2)[22]. A recombinase, *e.g.*, RAD51 in eukaryotes, is loaded onto these specifically processed DNA ends to displace RPA to form a RAD51-ssDNA nucleofilament with the assist of breast cancer 1 (BRCA1), BRCA2, and several RAD51 paralogues[20]. The RAD51-ssDNA nucleofilament can invade homologous duplex DNA to search for and couple with a homologous sequence elsewhere in the genome to form a displacement D-loop in which DNA synthesis is initiated to replace the DNA surrounding the former break site.

Repair of inter-strand crosslinks

Inter-strand crosslinks (ICLs) are able to inhibit transcription and replication by blocking DNA strand separation. Among the chemotherapeutic agents, cisplatin, cyclophosphamide, melphalan, and mitomycin C (MMC) are considered inducers of ICLs[23]. The repair of ICLs is complex and involves a coordination of several DNA repair pathways such as NER, HR, Translesion synthesis (TLS), and Fanconi anemia (FA) pathway[23]. ICLs can be recognized by NER machinery and incised by ERCC1-XPF endonucleases, resulting in unhooking of the lesion, which is bypassed by TLS. When ICLs encounter a replication fork during S phase, the collapse of the replication fork can lead to formation of DSB, which can be further processed by HR[24]. However, the collapse of the replication fork can be avoided by FA complementation group M (FANCM)-mediated fork regression or stabilization.

Translesion DNA synthesis

TLS is a general DNA damage tolerance mechanism, which allows the cell to replicate over DNA lesions. DNA lesions that block replication can be bypassed by specialized low-fidelity DNA polymerases, which are capable of replicating across DNA damage. The most abundant class of these DNA polymerases belongs to the Y-family, including Pol η, Pol κ, Pol ι, and REV1[25]. TLS is facilitated by PCNA mono-ubiquitylation, which is regulated by Rad6-Rad18 E2-E3 ubiquitylating enzymes and USP1 deubiquitylating enzyme at sites of DNA damage[26-28]. During TLS, the first polymerase inserts a nucleotide opposite the lesion and the second polymerase, usually DNA polymerase ζ, extends beyond the lesion. TLS plays an important role in the development of cisplatin resistance and is critical in the repair of ICLs[29].

Activation of cell cycle checkpoint machinery

Cell cycle checkpoints can be activated to arrest cell cycle progression when DNA damage or replication stalls are sensed, so that cells will have sufficient time to repair the damage before it is passed on to daughter cells. The checkpoint pathways in the mammalian cells can be regulated by the ATM (Ataxia Telangiectasia mutated) and ATR (ATM and Rad3-related) protein kinases. ATM and ATR phosphorylate a number of substrates such as Chk1 and Chk2. The activated Chk1 and Chk2 further phosphorylate p53 to stabilize the protein, enhancing the effects of p53 in response to DNA damage. Cdc25A is also phosphorylated by Chk1 and Chk2, leading to a rapid degradation of G1/S transition activators, and enforcing the G1-arrested state[30]. Cdc25C phosphorylation by Chk1 and Chk2 triggers G2 checkpoints to make sure DNA is fully replicated and undamaged before cells undergo chromosome condensation and nuclear division. Targeting cell cycle checkpoints has been proposed as a novel anticancer strategy. ATM inhibitors (such as KU55933) have been shown to effectively increase the sensitivity of cancer cells to DNA damaging agents[31]. The inhibitors of Chk1, Chk2, and Cdc25 have also

been suggested to enhance the cytotoxicity of various antitumor agents[32].

DDR IN CSCS

Stem cells persist and function throughout the entire life of an organism to regulate tissue homeostasis and regeneration. The functional importunity and the long life of stem cells suggest that they must be armed to maintain genomic integrity in unique ways different from those of somatic cells[2]. Indeed, human embryonic stem (ES) cells have been shown to display high levels of DNA repair capacity[33]. While reduced DNA repair capacity and loss of genomic integrity have been linked to differentiation or impaired function of stem cells[34]. However, contradictory results also showed that human ES cells are deficient in DNA repair capacity and cell cycle arrest but use high propensity to apoptosis as a strategy to ensure genomic integrity[35].

Given that CSCs share common properties with stem cells and are resistant to the treatment with various DNA damaging agents, CSCs were also proposed to possess the elevated DDR to process DNA damage more efficiently than bulk cancer cells. It has been reported that CD133+ glioma stem cells (GSCs) contribute to glioma radio-resistance and tumor regeneration through enhanced cell cycle checkpoint response and DNA repair. This is further supported by the finding that the radio-resistance of CD133+ GSCs can be overcome with a specific inhibitor of the Chk1 and Chk2 checkpoint kinases[3]. Further investigation demonstrated that L1CAM (CD171), which is preferentially expressed and is induced by DNA damage in GSCs, is responsible for the enhanced activation of cell cycle checkpoint and radio-resistance of GSCs by upregulating NBS1 expression[36]. Glioblastoma-initiating cells (GICs) also exhibit enhanced basal activation of SSB repair due to enhanced activation of the key SSB repair player PARP1. The growth, self-renewal, and DNA damage repair capacity of GICs can be inhibited by PARP inhibition, leading to an enhanced sensitization of GICs to radiation[37]. In addition, GICs exhibit hypermethylation in the promoter of O^6-methylguanine DNA methyltransferase (*MGMT*) gene. The epigenetically silenced MGMT might be responsible for the resistance of GICs to temozolmide[38] due to MGMT's ability to reverse alkylation at the O^6 positon of guanine and neutralize the cytotoxicity of alkylating agents such as temozolmide[39].

In addition to glioma stem cells, enhanced DNA repair capacity was also found in other CSCs. For example, enhanced repair of DNA DSBs and upregulated expression of DSB repair genes were found in CD133+ CSCs isolated from the A549 human lung carcinoma cell line[40]. Similarly, DDR and the expression of various repair proteins are also found to be highly up-regulated in $Lin^-CD29^HCD24^H$ tumor-initiating cells isolated from mouse mammary gland tumors, indicating an elevated DDR in these CSCs[41]. Significant increase in the expression of DNA repair-related genes, such as BRCA1 and RAD51, have also been observed in pancreatic putative CSCs compared with bulk cells. These spheroid cells also repair DNA breaks more efficiently than bulk cells after treatment with gemcitabine[42]. Furthermore, a strong activation of Chk1 and cell cycle arrest were observed in patient-derived non-small-cell lung cancer (NSCLC) stem cells compared to their corresponding differentiated cells after exposure to DNA damaging agents. Treatment with Chk1 inhibitors in combination with chemotherapy dramatically reduced the NSCLC stem cell population *in vitro* by promoting premature cell cycle progression and inducing mitotic catastrophe[43].

Although considerable reports suggest that competent DNA repair machinery is utilized by CSCs to survive DNA damaging agent treatment, some controversial observations also exist. The DSB repair capacity was found to be significantly reduced in CD133+ glioma stem cells compared with the glioma cell lines. In addition, the intra-S-phase checkpoint was absent in these CD133+ cells, albeit G2 checkpoint activation was intact. Consistent with reduced DNA repair capacity, CD133+ glioma stem cells are more sensitive to radiation than the established glioma cell lines[44]. In another report, although CD133+ glioma stem cells were shown to display elongated cell cycle and enhanced basal activation of Chk1 and Chk2, neither BER nor SSB repair was found to be increased in CD133+ compared to CD133- glioma cells[45]. Interestingly, Lim *et al*[46] reported a more efficient HR repair in GICs, but neither a extended cell cycle nor augmented activation of checkpoint proteins was observed in these cells. The reduced DDR was also reported in other CSCs. For example, esophageal CSCs exhibited compromised induction of p53 and inhibited G1 checkpoint arrest, as well as attenuated DNA repair capacity upon exposure to DNA damaging agents, as compared to non-CSCs[47]. NSCLC TICs showed an impaired DDR due to deficient activation of various checkpoint factors, *e.g.*, ATM, DNA-PKcs, Krűppel-associated protein 1 (KAP1), and FA complementation group D2 (FANCD2), leading to compromised cell cycle checkpoints[48]. We have recently determined the NER efficiency of ovarian CSCs, defined by either CD44+CD117+ or spheroid growing in serum-free culture, after treatment with cisplatin. Surprisingly, although ovarian CSCs exhibited resistance to cisplatin treatment compared to their corresponding bulk cancer cells, no increased capability to remove cisplatin-induced DNA lesions was found in these CSCs[49]. In contrast, we revealed an elevated expression of DNA polymerase η and constitutively high levels of mono-ubiquitylated PCNA in various ovarian CSCs, indicating that enhanced TLS could be responsible for cisplatin resistance in these cells[49].

The controversial observations of the DDR in CSCs indicate that enhanced DDR can be a contributor in

the resistance of CSCs to DNA damaging agents, but this may not be applied to all CSCs. Due to the genetic heterogeneity in cancer, CSCs isolated from different cell lines and patients may have different responses to DNA damage. Furthermore, the heterogeneity in CSCs and uncertainty in CSC markers may also result in different subpopulations of CSCs from the same cell line or patient[50], which may also exhibit different DDR. Another possible explanation for the conflicting results is that the different culture conditions of CSCs may affect sensitivity and responses of CSCs to DNA damaging agents as well.

CHROMATIN REMODELING IN STEM CELLS

Chromatin creates a natural barrier against access to DNA in many nuclear functions. It is now clear that chromatin modifications have an integral role in DNA damage repair. Following DNA damage, the chromatin needs to be in an "open" state in order to allow the repair factors to access the DNA molecule. Increasing evidence indicates that DDR can be extensively impacted by the modulations to chromatin structure. Chromatin structure in stem cells is different from differentiated cells. Many stem and progenitor cells have been classically described as having a typical open chromatin conformation that is mostly devoid of heterochromatin[51]. Electron microscopy examination indicated that heterochromatin is predominant in differentiated cells but less common in ES cells[52]. Analysis of global chromatin compaction using nuclease also indicated that chromatin is more accessible, and thus more sensitive to nuclease digestion in ES cells than differentiated cells.

In addition to the histological evidence, the idea of "open chromatin" is also supported by other analyses. For example, immunoblotting and immunofluorescence analyses have demonstrated a reduced amount of histone H3 tri-methylation on Lys9 (H3K9me3), a marker of heterochromatin, suggesting that ES cells possess considerably less heterochromatin than differentiated cells. ChIP-seq analyses further showed that heterochromatic marks H3K9me3 and H3K27me3 cover 16% and 12% of the genome of differentiated IMR90 cells, respectively, but both of them cover only 4% in human ES cells[53]. On the other hand, it appears that ES cells have higher global level of histone acetylation, a general mark of open chromatin, as compared to differentiated cells. In addition, some indirect evidence also support "open chromatin" in ES cells. A fraction of loosely bound architectural chromatin proteins was found in ES cells by using Fluorescence recovery after photobleaching (FRAP) assay, while this fraction is absent in differentiated cells. Collectively, these findings indicate that chromatin in ES cells is globally decondensed and hyperdynamic compared with differentiated cells.

Given that stem cells use distinct characteristic of chromatin structure for regulation of self-renewal and differentiation, it is likely that histone modifications and chromatin remodeling upon DNA damage may be different between stem cells and differentiated cells. Among the limited data regarding DNA damage-induced chromatin modifications in stem cells, γH2AX was mostly investigated. It has been reported that γH2AX foci can be observed under microscope in an open chromatin structure even in the absence of exogenous DNA damage in pluripotent mouse ES (mES)[54]. Pluripotent mES cells can contain more than 100 large γH2AX foci, which is equivalent to the number of foci that are produced in differentiated cells in response to about 4 Gy X-rays. In comparison, intact differentiated mammalian cells normally exhibit fewer than 5 large γH2AX foci per cell. It was proposed that the decondensed chromatin structure of pluripotent mES cells, mainly maintained by enhanced global histone acetylation and reduced histone methylation, as well as abundant chromatin remodeling complexes, contributes to the development of larger γH2AX foci[54]. Further relaxation of ES cell chromatin by lessening the level of the linker histone H1 causes ES cells hyper-resistant to DNA damage, as the DDR is enhanced in the context of open chromatin[55]. Although DNA damage induces γH2AX foci in both differentiated and stem cells, the distribution of γH2AX in the nucleus of stem cells is distinct. Momcilovic *et al*[56] used a 405 nm laser to induce DNA damage in a defined subnuclear area of several ES cells and found that unlike in mouse embryonic fibroblasts (MEF), γH2AX staining was not confined to the affected nuclear region in ES cells. Instead, γH2AX specific signal was uniformly distributed through the entire nuclear area, perhaps due to the high dynamics of chromatin in pluripotent stem cells[56].

It is noteworthy that not all stem cells have unique pattern of γH2AX in response to DNA damage. Like differentiated cells, unirradiated hematopoietic stem cells displayed extremely low levels of γH2AX foci, while these cells showed an immediate induction of γH2AX foci after exposure to 2 Gy IR[57]. Similarly, the percentage of cells with nuclear foci of γH2AX after IR was not significantly different in stem glioma (CD133+) and non-stem glioma (CD133-) cells at both 1 and 24 h after treatment[45]. Thus, the distinct performance of histone H2AX phosphorylation upon DNA damage in various kinds of stem cells reflects various DNA damage responses in different stem cells.

TARGETING THE DDR TO FACILITATE ERADICATION OF CSCS

Genotoxic agents including ionizing radiation (IR) and many chemotherapeutic drugs that cause DNA damage have been used for cancer therapy for many years. As discussed above, the DDR is a determinant

factor in cell sensitivity to DNA damaging agents. Thus, the protein kinases and enzymes that are involved in the DDR, such as ATM, ATR, Chk1, Chk2, and PARP1, have been identified as promising targets for cancer therapeutics[58]. Due to the enhanced DDR in most types of CSCs, modulation of the DDR in CSCs became a new therapy strategy in the treatment with DNA damaging agents. For example, a specific inhibitor of the checkpoint kinases debromohymenialdisine (DBH) has been demonstrated to reverse the radio-resistance of GSCs defined as CD133+[3]. PARP inhibitor (olaparib) was also able to sensitize GICs to radiation[37]. In addition, NSCLC-stem cell survival can be dramatically reduced by Chk1 inhibitors (SB218078 and AZD7762) in combination with chemotherapy[43]. Recently, the work in our lab revealed depleted miR-93 expression in ovarian CSCs, which is, at least partially, responsible for the upregulated DNA polymerase η expression. Transfection of miR-93 into ovarian CSCs increased their sensitivity to cisplatin treatment, probably through downregulation of DNA polymerase η and thus inhibition of TLS of CSCs[49]. These findings provide a new avenue to facilitate the eradication of CSCs by platinum-based chemotherapeutics. However, given that adult stem cells also possess enhanced DDR, the DDR inhibitors aimed at eliminating CSCs could also sensitize the adult stem cells to DNA damaging agent, resulting in severe side effects. Therefore, identifying the differences in the DDR between adult stem cells and CSCs, and finding the Achilles' heel in the mechanisms protecting CSCs is critical to the development of potential drugs for facilitating eradication of CSCs by DNA damaging agents.

CONCLUSION

Dysregulated DDR is observed in many cancers and is responsible for the genomic instability that related to tumorigenesis and tumor progression[59]. The DDR defects provide an Achilles's heel that can be exploited for cancer therapy[60-63]. The enhanced DDR found in CSCs is considered a protector from the insult of DNA damaging agents. Thus, reducing DDR in CSCs would be a promising therapy strategy for achieving elimination of CSCs with the conventional radio-/chemo-therapy.

Currently, most studies are focusing on the protein kinases and enzymes that are involved in the DDR. As discussed above, the DDR in CSCs can be affected by many factors, among which chromatin remodeling plays an important role in facilitating the DDR in cells following DNA damage. Although stem cells display an open chromatin conformation, there is lack of information on the chromatin structure in CSCs. Understanding the chromatin structure and chromatin remodeling in CSCs after the treatment with DNA damaging agents would provide a novel avenue to interfere with the DDR in this cells. Another question we need to address is how CSCs respond to DNA damaging agents *in vivo*. Most of the current studies were conducted using in vitro cultured cells. However, CSCs dwell in a specialized microenvironment denoted as "stem cell niche", which contains diverse stromal cells, vascular networks, soluble factors, and extracellular matrix component. These components may affect the DDR in CSCs. In addition, the CSC niche can protect CSCs by sheltering them from diverse genotoxic insults, leading to an enhanced therapeutic resistance[64]. Thus, the future design of therapy to eliminate CSCs needs consideration of the tumor microenvironment. Finally, the phenotypic heterogeneity of CSCs in different tumors, and even in the same tumor, should be considered[65]. Identifying the dysregulated DDR components in different CSC subpopulations is critical to selecting targets that can be used to efficiently shut down their specific DDR.

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EDITORIAL

Topographic patterns of vascular disease: HOX proteins as determining factors?

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Abstract

Steadily increasing evidence supports the idea that genetic diversities in the vascular bed are, in addition to hemodynamic influences, a major contributing factor in determining region-specific cardiovascular disease susceptibility. Members of the phylogenetically highly conserved *Hox* gene family of developmental regulators have to be viewed as prime candidates for determining these regional genetic differences in the vasculature. During embryonic patterning, the regionally distinct and precisely choreographed expression patterns of HOX transcription factors are essential for the correct specification of positional identities. Apparently, these topographic patterns are to some degree retained in certain adult tissues, including the circulatory system. While an understanding of the functional significance of these localized *Hox* activities in adult blood vessels is only beginning to emerge, an argument can be made for a role of *Hox* genes in the maintenance of vessel wall homeostasis and functional integrity on the one hand, and in regulating the development and progression of regionally restricted vascular pathologies, on the other. Initial functional studies in animal models, as well as data from clinical studies provide some level of support for this view. The data suggest that putative genetic regulatory networks of *Hox*-dependent cardiovascular disease processes include genes of diverse functional categories (extracellular matrix remodeling, transmembrane signaling, cell cycle control, inflammatory response, transcriptional control, *etc*.), as potential targets in both vascular smooth muscle and endothelial cells, as well as cell populations residing in the adventitia.

Key words: *Hox*; Blood vessel; Cardiovascular disease; Positional identity; Endothelial cell; Vascular smooth muscle cell

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Core tip: Accumulating evidence indicates regionally restricted HOX expression patterns in the adult arterial tree that may reflect a topographic vascular HOX code for specifying positional identities in various cell types of the circulatory system. We propose that this positional information is critical for maintaining local vessel wall homeostasis and that its disruption plays an important role in the development of vascular diseases with distinct topographic preferences. This editorial discusses emerging molecular data in support of this novel concept.

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INTRODUCTION

According to epidemiological data from the World Health Organization (WHO), diseases of the circulatory system, collectively known as cardiovascular diseases (CVDs) are the leading cause of death world-wide (http://www.who.int/mediacentre/factsheets/fs317/en), and a great number of resources have been committed to study the pathophysiology of CVDs with the goal of improving therapeutic interventions. The most common forms of CVD include atherosclerosis of various segments of the arterial tree (coronary, carotid, and cerebral arteries, aortic arch, abdominal artery), aneurysms of the thoracic and abdominal aorta, as well as cerebral arterial aneurysms, peripheral arterial disease (PAD), and venous insufficiency.

Although certain types of CVDs share some of the same pathogenic characteristics (*e.g*., neointima and atherosclerotic plaque formation as a result of chronic inflammation in atherosclerosis, medial degeneration in aortic aneurysms), they usually develop at distinct anatomic locations. As the most prevalent CVD risk factors including hypertension, diabetes, smoking, alcohol, lack of physical activity, obesity, high blood cholesterol and triglycerides, and stress are all systemic, regional genetic differences in the vascular bed are likely to be critical for these topographic disease patterns, as they cannot be attributed to hemodynamic differences alone. This view is underscored by aortic homograft transplant experiments in canines, in which atherosclerosis- susceptible segments of the abdominal aorta were transplanted into the atherosclerosis-resistent thoracic aorta and *vice versa*[1]. Examination of transplanted vessel segments subsequent to keeping the animals on an atherogenic diet revealed that the abdominal segments readily developed atherosclerotic lesions in their thoracic host environment, whereas the thoracic aortic segments continued to be free from lesion formation in the abdominal aorta. These results are consistent with data from a large-scale clinical study involving approximately 12000 patients that were treated surgically for atherosclerotic occlusive disease with a follow-up of over 25 years[2]. In this study the arterial bed was divided into four anatomical categories for analysis including the coronary arterial bed, the major branches of the aortic arch, the visceral branches of the abdominal aorta and the terminal abdominal aorta with its major branches. The data showed that the response to systemic risk factors is distinctly different in each of the four categories[2].

A search for potential determinants underlying these inherent differences in the response to systemic pathogenic triggers will have to consider the circumstance that vascular smooth muscle cells (VSMCs) residing in distinct segments of the arterial tree originate from different sources depending on their anatomic location. Lineage-mapping involving chick-quail chimera identified at least seven different sources including proepicardium, secondary heart field, neural crest, mesangioblasts, somites, splanchnic mesoderm and mesothelium that contribute to defined arterial segments, in addition to various types of progenitor cells residing primarily in either the media or adventitia with a more universal distribution[3]. Overall, this fate map reveals a segmented pattern of the arterial tree that seems to reflect the metameric patterning of the vertebrate embryo. As segment identities, and therefore diversities, are specified by members of the phylogenetically highly conserved family of *Hox* transcriptional regulators, these genes have to be viewed as prime candidates for determining different positional identities in the vascular bed that reflect regional differences in CVD susceptibility.

HOX-SPECIFIED POSITIONAL IDENTITIES IN THE CIRCULATORY SYSTEM

The conserved *Hox* gene family constitutes a genetic system of unique properties that is utilized initially during embryonic patterning for specifying positional identities along the anterior-posterior (A-P) axis[4,5]. The mouse and human genome harbor 39 *Hox* genes that are organized into four separate clusters designated *Hoxa, -b, -c,* and *-d*. Alignment of these transcriptionally unipolar clusters based on sequence similarities reveals 13 paralogous groups of *Hox* genes that are activated sequentially in distinct A-P embryonic domains such that genes of groups 1 and 2 are expressed first in the most anterior regions, whereas group 13 *Hox* genes are activated last by following the A-P morphogenetic progression. This modus of activation generates unique domains of combinatorial *Hox* activities at any given location of the embryo that has been referred to as the *Hox* code in analogy to the postal zip code for specifying positional identities[6]. Data obtained by large-scale gene expression profiling

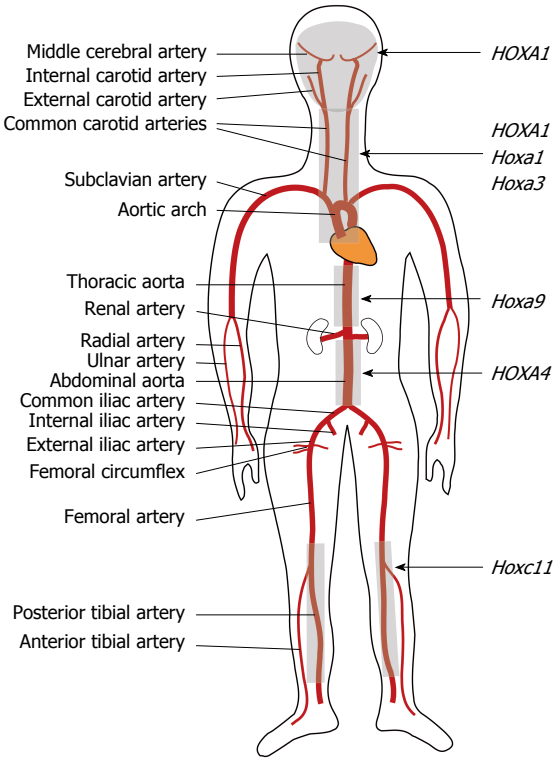

Figure 1 Map of *Hox* functional domains in the arterial tree. The schematic shows a rough outline of the main human arterial segments. Localization of vascular defects associated either with mutated human *HOX* or mouse *Hox* alleles as indicated at the right were used to generate a cursory map of *HOX*/*Hox* activity domains (grey shading) assuming that the defects observed in the mouse map to roughly equivalent anatomic positions in humans. The demarcation of the *HOXA4* activity domain is based on down-regulation of *HOXA4* expression associated with AAA. The *Hoxa9* domain in the thoracic aorta signifies an atherosclerosis-resistant segment.

of adult fibroblasts derived from different anatomic regions in humans suggest that this embryonically established topographic *Hox* code is, at least to some degree, retained in the adult[7], where it is believed to be critical for maintaining positional identities by regulating local differentiation and signaling events.

Initial evidence for the existence of a topographic *Hox* code in the circulatory system came from *LacZ* reporter gene studies in transgenic mice that revealed remarkable regionally restricted expression patterns for *Hoxc10, Hoxc11,* and *Hoxa3* in subpopulations of VSMCs of the media, as well as in endothelial cells (ECs) within distinct segments of the vascular bed of young adult (6 wk), as well as 1 year old mice[8]. Apparently, this presumptive vascular *Hox* code is instrumental in maintaining vessel wall integrity and homeostasis as indicated by the region-specific vascular remodeling events upon its interruption. Specifically, this was demonstrated by inducing changes in the vascular *Hoxc11* expression pattern that is normally restricted to the distal limb vasculature of adult mice (Figure 1). By utilizing an integrated tetracycline regulatory system and *Transgelin* (*Tagln*) promoter elements that drive expression universally in VSMCs of TetOn*(Tagln-Hoxc11)* transgenic mice upon doxycycline (dox) induction, these mice developed severe vessel wall defects (medial thinning, elastic laminae fragmentation, intimal lesion formation) in arterial segments where *Hoxc11* is normally not expressed (carotid artery, aortic arch, thoracic aorta), whereas overexpression of *Hoxc11* in its natural vascular domain of activity, including the lower femoral artery, resulted in a drastic increase in vessel diameter but without the structural defects observed upon ectopic expression[9]. Furthermore, human *HOX* transcriptome analysis of vascular ECs derived from different anatomic locations revealed specific *HOX* expression signatures that are believed to determine positional identities and regulate endothelial differentiation[10].

Compared to gene expression profiling, an alternative approach to consider for mapping *Hox* activities in the circulatory system is to determine *Hox* functional domains by mutational analysis in mice and by linking human congenital vascular defects to mutant *HOX* alleles. The *Hoxa3*^{tm1Mrc} mutant is perhaps the first case in which disruption of a *Hox* gene has been linked to severe cardiovascular defects in mice that include absence of the right carotid artery and stenosis of the aortic valves, in addition to abnormalities of the cardiac chambers, as well as other developmental defects[11]. In humans and mice, a homozygous *HOXA1/Hoxa1* mutation was linked to a complex syndrome that includes malformations of the cerebral vasculature, the internal carotid arteries, and the cardiac outflow tract in addition to neuronal defects[12-14]. Mice homozygous for the *Hoxc11*^{tm1Mrc} targeted allele developed greatly enlarged femoral arteries that were phenotypically very similar to the enlarged vessels seen upon induced overexpression of *Hoxc11* in the distal hindlimb vasculature[9]. Furthermore, and of potential clinical relevance is the downregulation of *HOXA4* expression associated with abdominal aortic aneurysms[15]. Interestingly, *HOXA4* exhibits an A-P gradient in expression levels along the aorta (ibid) that might overlap with a gradient for *Hoxa9* expression, for which highest levels were found in the thoracic aorta of mice[16] as discussed below.

HOX-REGULATED GENETIC PATHWAYS IN THE VASCULATURE - POTENTIAL CVD RELEVANCE

The vascular functions of *Hox* genes have perhaps been studied most extensively in cells of endothelial lineage within the context of angiogenesis as observed in normal physiological scenarios (*e.g.*, wound healing) and under pathological conditions (*e.g.*, tumorigenesis). This work has yielded useful insight into *Hox*-dependent pathways regulating EC phenotypic properties. Accordingly, all *HOX* genes of paralogous group 3 were found to stimulate angiogenesis albeit through different pathways[17]. *HOXD3* promotes expression of *integrin* $\alpha 5$ and $\beta 3$ subunit genes in response to FGF2[18-20];

heterodimers of these subunits with other subunits generate fibronectin receptors $\alpha5\beta1$ and $\alpha v\beta3$ that facilitate adhesion and migration through fibronectin-rich granulation tissue during wound healing[17]. Consistent with this is the *HOXD3*-regulated increase in expression of the urokinase plasminogen activator (*uPA*) gene[18]. The human *HOXD3* paralogs *HOXA3* and *HOXB3* also promote angiogenesis although through different pathways that involve increased EC migration and expression of *Mmp14* and *uPAR* (*HOXA3*) and enhanced expression of the angiogenic ligand Ephrin A1 (*HOXB3*)[17]. Increased EC migration in tissue culture was also observed for *HOXA9* through direct transcriptional activation of the *Ephrin receptor B4* (*EPHB4*) gene[21]. *HOXD10*, on the other hand, was found to be inactive during angiogenesis but expressed in quiescent vascular endothelium, and pro-angiogenic factors ($\beta4$ and $\alpha3$ integrins, *MMP14, uPAR* and *cyclin D1*) were downregulated in *HOXD10*-transfected ECs[22]. Likewise, *HOXA5* is reportedly inactive in angiogenic endothelium but expressed in quiescent ECs where *VEGFR2*, *ephrin A1*, *HIF1*α, and *COX2* were downregulated upon transfection with *HOXA5*[23].

As most of these *Hox* expression data and functional correlations have been obtained in cell culture systems, some of these results may not reflect the vascular patterns and functions observed *in vivo.* For example, *Hoxa3* expression is induced together with *Hoxd3* during cutaneous healing of excisional wounds in mouse[24]. However, the same gene was found to be abundantly expressed in VSMCs of major blood vessels in addition to ECs but in a topographically restricted manner[8].

As pointed out above, induction of ectopic *Hoxc11* expression in VSMCs of TetOn *(Tagln-Hoxc11)* transgenic mice on an FVB/NTac background resulted in severe vascular defects. Consequently, these mice offer an opportunity to start defining *Hoxc11*-regulated pathways of pathological vessel wall remodeling. Preliminary results showed induction of the apoptotic marker CASP3, as well as matrix protein metalloproteinase MMP2 and MMP9 expression in medial VSMCs of the thoracic aorta following doxycycline (dox)-induced HOXC11 expression[9]. Both MMP2 and MMP9 are involved in extracellular matrix (ECM) breakdown and restructuring of the vessel wall, as well as breakdown of the basement membrane and cell migration[25,26], *i.e.*, events that are consistent with the formation of the type of lesion formation observed in the thoracic aorta of these mice. Interestingly, in the lower femoral artery, where *Hoxc11* overexpression resulted in an enlargement of an otherwise structurally normal vessel, only MMP2 was induced upon dox-treatment. Accordingly, the differential histological changes observed in response to *Hoxc11* ectopic *vs* overexpression were mirrored by distinct differences in molecular responses, in this case MMP9 expression, that is usually associated only with injury and inflammatory responses[25]. Furthermore, data from chromatin immune-precipitation (ChIP) and transient co-transfection assays suggested that *Mmp2* and *Mmp9* are transcriptionally regulated by HOXC11, thus linking *Hoxc11* directly to the regulation of vascular remodeling pathways involving these MMPs.

In a separate study, a search for factors that contribute to the commonly greater susceptibility to atherosclerosis in the aortic arch *vs* the thoracic aorta found that members of *Hox* paralogous groups 6-10 exhibit higher expression levels in the former across various mammalian species including mouse, rat, and porcine[16]. Remarkably, essentially the same differential *Hox* expression profiles were observed in human embryonic stem cells that were differentiated *in vitro* either along the neuroectoderm pathway into aortic arch-like VSMCs or along the paraxial mesoderm pathway into thoracic aorta-like VSMCs. These data suggest that *Hox*-specified positional identities of VSMCs established during development are retained in adulthood. Apparently this positional diversity is reflected by differential responses to inflammatory stimuli that are critical for the progression of vascular diseases such as atherosclerosis. This was illustrated by the greater activity and binding to DNA of the pro-inflammatory transcription factor NF-κB in aortic arch *vs* thoracic aorta VSMCs. Moreover, this NF-κB pattern apparently is, at least in part, due to a reciprocal regulatory relationship with Hoxa9 such that the higher levels of HOXA9 expression in the thoracic aorta repress *NF-*κ*B* transcriptional activity, whereas the high levels of NF-κB actitivity in the arch repress *Hoxa9* expression[16,27].

As mentioned before, downregulation of already low *HOXA4* expression levels in the abdominal aorta was associated with abdominal aortic aneurysms (AAAs)[15] that are much more prevalent than thoracic aortic aneurysms (TAAs), which themselves occur primarily in the ascending aorta, *i.e.,* the proximal region of the aortic arch[28]. While there exist many differences in the etiology, histology, and genetics of the two diseases, it is perhaps noteworthy that both develop predominantly in vessel segments that are situated at the low ends of *Hox* expression gradients with a greater pro-inflammatory predisposition in both cases, even though the inflammatory component in TAAs is lower than in AAAs[28]. This raises the intriguing question whether high levels of *Hoxa9* and *Hoxa4* expression may protect against the development of both aneurysms and atherosclerosis in the descending aorta, aka thoracic aorta. In that case, the further reduced *HOXA4* levels clinically associated with AAA are likely the consequence of inflammatory stimuli, a view supported by *in vitro* data showing reduced *HOXA4* expression in cultured human VSMCs and ECs treated with inflammatory cytokine IFN-γ[15].

Recent *in vitro* silencing experiments of *HOX* activities (*HOXB7, HOXC6, HOXC8*) in multipotent stem cells isolated from the adventitia of the human

thoracic aorta provided evidence for *Hox*-dependent differentiation into VSMCs involving epigenetic mechanisms[29]. This raises the interesting possibility that *Hox* genes may be critical regulators of vascular cell populations that drive phenotype switching between homeostatic/regenerative *vs* pathological remodeling in vascular tissues in a region-specific manner.

CONCLUSION

Their persistent topographic expression patterns in post-natal vascular tissues suggest that *Hox* genes play a critical role in maintaining vessel wall homeostasis in a region-specific manner. Consequently, the idea that *Hox*-linked genetic polymorphisms affecting either their functional quality or expression levels may underlie CVD susceptibility in discrete portions of the vascular tree is a valid proposition. Albeit limited, the currently available *in vivo* data derived from analyses of *Hox* knockout and conditional *Hox* gain-of-function mutant mice, as well as from clinical studies support that view. Initial efforts of defining the genetic regulatory networks of *Hox*-dependent CVD processes implicate genes of diverse functional categories (ECM remodeling, transmembrane signaling, cell cycle control, inflammatory response, transcriptional control, *etc.*), as potential targets. The finding of region-specific reciprocal interactions of a *Hox* gene with a pro-inflammatory factor (NF-κB) is intriguing and might be of universal relevance in the search for therapeutic approaches. Within this context, defining the epigenetic mechanisms controlling *Hox* activities in blood vessels will be critical as indicated by data that associate hypomethylation of CpG islands within the *HOXC11/HOXC9* genomic interval with the ectopic activation of *HOXC10* and *-9* in atherosclerotic coronary arteries[30].

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EDITORIAL

Lack of new antiinfective agents: Passing into the pre-antibiotic age?

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Abstract

The lack of newly developed antibiotics, together with the increase in multi-resistance of relevant pathogenic bacteria in the last decades, represents an alarming signal for human health care worldwide. The number of severely infected persons increases not only in developing but also in highly industrialized countries. This relates in first line to the most severe form of a bacterial infection, sepsis and the septic shock syndrome, with high mortality on critical care units. No particular anti-sepsis drug is available, and the therapy with conventional antibiotics more and more fails to provide a survival benefit. Due to the fact that the pharmaceutical industry has withdrawn to a high degree from the development of anti-infectious agents, a huge challenge for health care is approaching in the 21st century. In this article, these problems are outlined and possible alternatives are presented which may be helpful to solve the problem.

Key words: Antimicrobial resistance; Antibiotics; Sepsis; Antimicrobial peptides; Lipoproteins; Inflammation; Cytokines; Endotoxins

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Core tip: The dramatic increase in multiresistance of relevant pathogenic bacteria can be assumed to be one of the main global health care problems of the 21st century. At the same time, the development of new antibiotics decreases considerably from which a significant lack in the health supply for combating infections can be scheduled. In this paper, alternative strategies are discussed which are focused mainly on the development and application of antimicrobial peptides. These can easily be synthesized in high amounts under moderate costs, have usually a high therapeutic index, and do not have a tendency for resistance emergence. Recent developments are presented from which it may be deduced that the most serious form of a bacterial infection, sepsis and septic shock, may be treatable in near future.

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DESCRIPTION OF THE PROBLEM

The assessment of infections has changed completely in the last 30 years. Up to the 1980's it was believed that all bacterial infections were controllable with antibiotics: "It's time to close the book of infectious diseases"[1]. But already at the end of the 1990's, the general view changed: "Bacterial infections are a worldwide threatening of human health, in developed states they are the third frequent, in countries of the Third World "even the most frequent cause of death"[2].

These new assessments can be summarized in the statement: "Bacterial infections are the largest health care challenges of the 21st century".

The reasons for this paradigm change are complex. One of the main reasons is certainly the drastic increase in bacterial resistance: The occurrence of bacterial drug resistance, which is - among others - caused by wrong antibiotic medication and the excessive use of antibiotics in animal husbandry, represents an increasing threatening for human health. This situation is communicated also more and more in the media, for example in a contribution by the well-renowned British BBC[3]. The uncontrolled emission of antibiotics in mass animal husbandry, leading to a contamination of the soils and the water, represents a selection pressure on bacteria from the environment such as *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) with subsequent increase in resistance. As examples for the increase in resistance, it should be stated that more than 90% of the *S. aureus* strains are resistant to all penicillins and other related antibiotics, and many, *i.e.,* around 30% nowadays with increasing tendency, are resistant even to the newer methycillin-related drugs (MRSA). Further relevant resistant germs with high infection potential are beside MRSA, vancomycin-resistant *Enterococci*, fluorquinon-resistant *P. aeruginosa*, and ESBL (extended spectrum beta-lactamase)-resistant *Escherichia coli* and *Klebsiella pneumonia*. Infections induced by multiresistant strains frequently lead to heavy infections, for example to wound, urinary, and respiratory infections which finally may lead to the life-threatening sepsis syndrome with multi-organ failure.

More than 100000 patients suffer annually from MRSA infections in Germany, with a mortality rate of up to 10% by sepsis or pneumonia ("Deutsche-Sepsisgesellschaft")[4]. Very important germs with high infection and mortality potential are also the MDR (multi-drug resistant). *Mycobacterium tuberculosis* strains (MDRMt). These are in particular in Eastern Europa a huge threatening of human health, which does not lead to short-time infections as described above but to a long-term infirmity of infected people with a high death-rate. For these bacteria, in contrast to those described above, even the bioactive compounds from their cell wall have not been characterized so far. Another reason for the paradigm change mentioned above is that in the last decades pharmaceutical companies have brought to market only two new classes of antibiotics, which moreover have only effects against particular bacteria. This situation is expressed by the statement: "We are facing a huge crisis worldwide not having an antibiotics pipeline". (Dr. Janet Woodcock, Director of CDER at FDA)[5]. The lack in the development of new antibiotics is illustrated in Figure 1 showing a dramatic decrease of newly approved compounds.

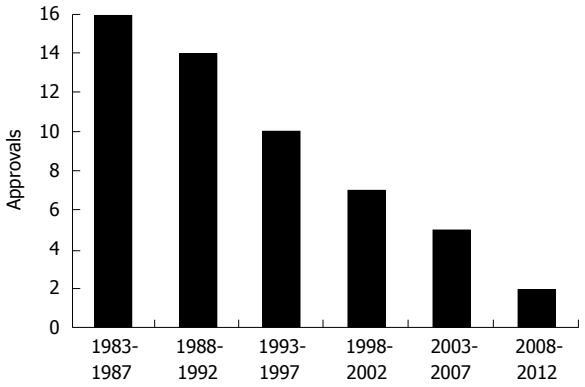

Figure 1 New systemic antibacterial agents approved by the United States Food and Drug Administration per 5-year period, through 2012. From Boucher *et al*[6], with permission by Oxford Press.

There is no evidence that this situation will change. In the pipeline of the pharmaceutical industry no broad-band developments are reported. This fact leads to ironic or even sarcastic formulations from experts such as the following: Who Will Mourn the Passing of the Pharmaceutical Industry[7]?

Details of "Medicines in Development" against a variety of bacterial and viral infections, mostly by small Biotech companies, is found in the 2013 report[8].

The failure of the pharmaceutical industry to develop new antibiotics has of course a rational background: The development of anti-infective drugs is a very laborious, long-lasting, and expensive procedure, and is frequently "crowned" by failure[9]: Thus, for example, the development of an effective anti-sepsis medicament (see below) has been described as "Money graveyard" for pharmaceutical companies, because in more than 20 clinical phases Ⅲ there was no survival benefit for patients, but the companies had invested overall some billion $. Thus, drug development is targeted to fulfill primarily the promise of the return of investment rather than creating new anti-infective drugs.

MECHANISMS OF SEPSIS INDUCTION

Bacterial infections may give rise to a variety of different

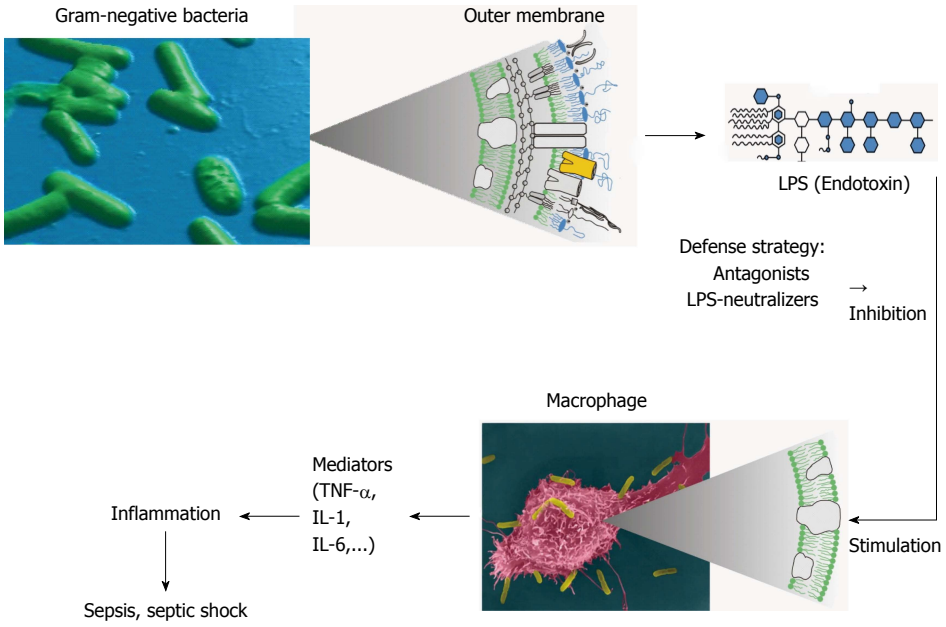

Figure 2 Mechanisms of lipopolysaccharide-induced cytokine (mediator) secretion with subsequent induction of inflammation and sepsis. Without therapeutical intervention, the interaction of the toxins leads to cell activation with subsequent release of inflammation-inducing compounds eventually resulting in sepsis. Intervention strategies may be performed by *e.g.*, AMP and LPS/LP-neutralizers. LPS: Lipopolysaccharide; IL: Interleukin; TNF: Tumor necrosis factor.

diseases, from harmless local infections affording no treatment over more severe local infections such as skin and skin-related infections up to the most severe form, the sepsis. In contrast to other infections such as respiratory or urogenital tract diseases, sepsis is a systemic disease, *i.e.,* it is spreading over the blood stream. For this, no particular drug is available since the withdrawal of Drotrecogin-α (recombinant protein C, Xigris®) in October 2012, which anyway was only suitable for a small number of patients. The numbers known from the USA may give a hint regarding the high relevance of the septic syndrome in health care, when compared with the data from other diseases: annual death cases United States: Sepsis 215000; Myocardial Infarction 193000, Lung cancer 153000.

In selected video presentations, the dramatic kind of occurrence and frequency of sepsis are presented most impressive[10,11]. It is well known that septic shock may occur for patients, whose bodies are weakened due to other traumata (heavy burns, accidents, surgery, *etc.*), who have a priori a weak immune system, or who are infected due to unfortunate reasons (even an inaccurate treatment of a tooth may lead to sepsis because bacteria from the mouth may infiltrate the bloodstream). It is important to note that not the bacteria and their toxins, but rather the overshooting reaction of the body to these stimuli is responsible for the induction of the sepsis syndrome. Whereas the presence of the inflammation-inducing bacterial "pathogenicity factors" (PFs) such as endotoxin [lipopolysaccharide (LPS)] for Gram-negative and lipoprotein (LP) from Gram-positive source at low concentrations is beneficial for the immune system in particular in the early childhood, the negative consequences at high concentrations lead to the well-known cytokine storm (Figure 2)[12].

The lack in progress in the fight against sepsis has not changed during the last decades, however, the occurrence of drug-resistant strains has severely worsened the situation. Whereas for the drug-susceptible bacteria an elaborate therapy by applying suitable antibiotics and "adjunctive" and "volume replacement" procedures in critical care units may lead to some therapeutic success, bacteria such as MRSA and ESBL - resistant strains from *Escherichia coli* and *Klebsiella pneumonia* cannot be treated adequately. Even if the immune system is potent enough to combat the bacterial invasion, the release of the bacterial toxins LPS and LP from the cell envelopes of bacteria induces the immunity to go haywire. These toxins react with cells of the immune system in a heavy way by producing so-called cytokines (mediators), which leads to a self-poisoning of the body. As shown in Figure 2, the liberation of LPS - which may take place by the action of the immune system or only by cell division of the bacteria - from the bacterial outer membrane is responsible for the heavy inflammation reaction by interacting with cell receptors, as shown here on macrophages (for LPS: Toll-like receptor 4, for LP: Toll-like receptor 2), with subsequent cell signaling.

In Figure 2 also possible intervention strategies are depicted, like antibacterial drugs, LPS neutralizers, and protein antagonists such as antibodies (see below).

Even if suitable antibiotics would be available, this frequently does not necessarily improve the health of the patients, but in contrary may worsen their state, because due to bacterial killing the bacterial toxins are now released from the bacterial cell envelopes with a

Table 1 Nomenclature of terms used in severe systemic infections

Term	Definition
Endotoxemia	Occurrence of Lipopolysaccharide (Endotoxin) in Blood
Bacteremia	Occurrence of living bacteria in blood
SIRS	Generalized hyperinflammatory reaction with different causes, Infections, Burns, Traumata
Sepsis	SIRS induced by an infection
Severe Sepsis	Sepsis with organ dysfunction
Septic Shock	Sepsis with shock

SIRS: Systemic inflammatory response syndrome.

severe increase in the inflammation reaction. "There are more and more results from animal models and from clinical studies that the antibiotic-induced release of biologically active LPS leads to a worsening of the critical state of septic patients"[13].

A detailed characterization of the terms and symptoms, which may occur in humans due to a systemic inflammation is given in the Table 1.

REASONS FOR FAILURE OF PREVIOUS ANTI-SEPSIS THERAPIES

Regarding all previous efforts for the development of anti-septic compounds, the corresponding experimental therapies have failed to improve the clinical outcome of severe sepsis and septic shock, and sometimes even increased mortality. The targets of current and past anti-septic strategies can be divided into five categories[9]: (1) Cell-signaling; (2) Coagulation pathway; (3) Endotoxin; (4) Immunomodulation; and (5) Specific mediators.

From the analysis of published data it can be deduced that seven agents yielded at least some improvement in clinical trials: a high-dose corticosteroid, two anti-endo-toxin therapies, a human recombinant IL-1 receptor antagonist, an intensive insulin therapy, an intravenous immunoglobulin, and a recombinant activated protein C10.

High dose corticosteroids, have been used until 1989. At the beginning, the compounds actually showed some survival benefit, but further analyses revealed that the survival rate in patients with septic shock was even lowered[14]. IL-1 receptor antagonists were used until 1997 and again showed a survival benefit at first, which could not be confirmed in follow-up trials. The same applies to monoclonal anti-TNFα antibodies (afemilomab, Abbott), which showed some positive effects, but no overall increase in survival. The use of anti-IFNγ antibodies (INNO-202, Innogenetics) showed some benefit in monkeys (reported in 2003), but for unknown reasons was not further pursued through clinical studies. Intensive insulin therapy (IIT) is not recommended at this time because the first encouraging results could not be transferred to other ICU-populations. Furthermore, the application of IIT resulted in significant harm to patients by severe hypoglycemia.

Details of the numerous attempts for combating sepsis and the reasons of failure are described by Fink and Shaw Warren[15].

The common problem of the strategies mentioned above is that all concepts (with the exception of compounds that are targeting the bacteria's endotoxins) do only tackle some aspects of severe sepsis rather than addressing the body's whole inflammatory state, triggered by bacteria or rather bacterial PFs. Accordingly, these strategies had been - at best - only be partially successful.

An alternative approach relates to immunomodulation mediated by short peptides to bolster host immunity, rather than reacting directly with bacteria or their PFs. Representative compounds are defensins, cathelicidins, dermicines and derivatives thereof, which are able to stimulate the expression of cytokines and chemokines. Some data indicate that these peptides may modulate the inflammatory cascade by targeting multiple inflammatory mediators, which may lead, among others, to an alteration of cell membrane function connected with the inhibition of TLR4 signaling induced by LPS. However, no data are available that would indicate their clinical usefulness in sepsis.

The latest failed clinical trials concern two in preclinical investigations very promising compounds, talactoferrin, a recombinant lactoferrin produced by Agennix, and the LPS-antagonist Eritoran by EISAI corporation. Both compounds did not succeed in phase Ⅲ studies, an explanation may be with the former compound that talactoferrin is focused mainly on Gram-negative bacteria, but may not have enough potential to neutralize endotoxins (see, *e.g.*[16]), and with the latter that LPS-antagonists of course cannot act against Gram-positive bacteria and their toxins.

Presently, only two anti-septic compounds are under development. The approach by Medinox (United States) is in phase Ⅰ/Ⅱ and exploits the compound NOX100 (Irish Pharm. Healthcare Assoc., 2010), which is believed to neutralize excessive NO produced during inflammation. Again, only one side-aspect of bacterial sepsis is addressed, and accordingly, it is unlikely that the compound will be broadly efficacious and of benefit for sepsis patients. Similar applies to compound cyto-FAB (anti-TNF, AstraZeneca), which has entered clinical phase Ⅱ trials (Irish Pharm. Healthcare Assoc., 2010). The current therapeutic situation is best described by the following statement: "Mostly old therapeutic concepts in a 'new design' are currently pursued, although it has been reported that 395 medicines and vaccines are under clinical evaluation, with 88 of them denoted as antibiotics/antibacterials"[8]. As can be concluded from the above, an efficacious and safe anti-septic drug is yet to be developed.

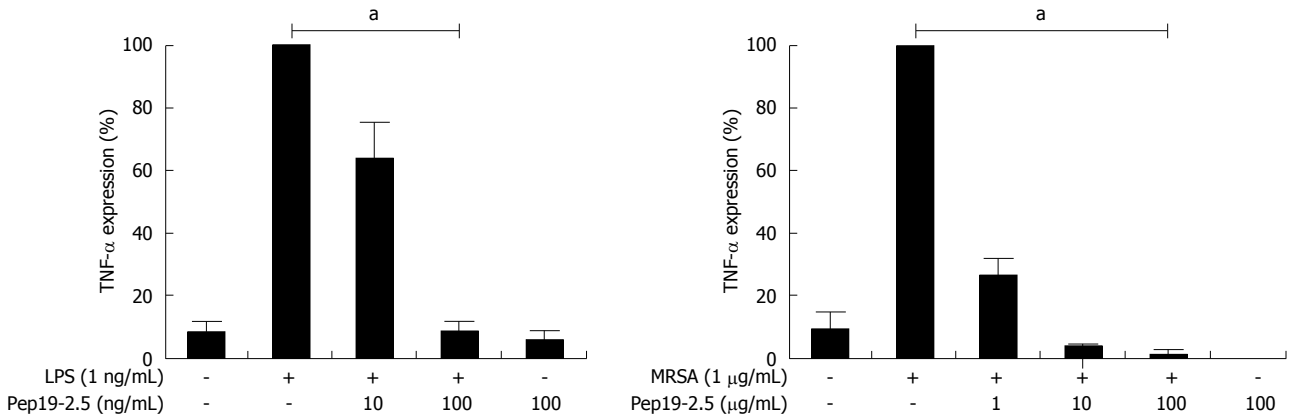

Figure 3 Determination of tumor necrosis factor-α expression levels from human lung tissue with lipopolysaccharide from *Salmonella minnesota* R60 and heat-killed MRSA combined with different concentrations of Pep19-2.5 (Aspidasept®). Depicted is the mean ± SEM of 5 independent experiments. Assuming parametric distribution data were log-transformed and groups were compared for statistical significant differences by use of One-Way Repeated Measure ANOVA ($^aP < 0.05$). From[19] with permission of American Society for Microbiology. LPS: Lipopolysaccharide; TNF: Tumor necrosis factor.

NEW APPROACH WITH ANTIMICROBIALS. HOPE FOR THE 21st CENTURY?

At the beginning of the 1990's, more and more investigators focused on the design and synthesis of antimicrobial peptides (AMP) in the fight against bacteria and their toxins, mainly based on the use of endogenous (body-own) AMPs such as cathelicidins, defensins, and dermicins. Up to now, these activities have not led to a newly approved drug, except for daptomycin, a lipopeptide which acts only against Gram-positive bacteria. The reasons for the inefficacy are manifold: AMPs are efficacious mucosal factors, but naturally never exposed to the systemic environment, rendering them inherently toxic at concentrations required to bind the bacteria and their toxins when used systemically. A second point is the fact that the bacterial surface structures, which are the first line of attack for the AMPs, show so many variations that not a single compound is able to act as broad-band drug. Nearly all of these approaches were directed against bacteria.

We have chosen another approach: Our rational was to engineer simpler, smaller synthetic polypeptides, which are essentially non-toxic, but still capable of binding PFs. Originally based on comprehensive biophysical studies of natural occurring antimicrobial proteins (*e.g.,* cyclic peptides from anti-*Limulus* factor, shortened peptide variants from human lactoferrin, porcine NK-lysine, human granulysin), we have developed a new class of synthetic polypeptides (lead compound: Aspidasept®), which possess potent antibacterial activities. These were originally scheduled to bind to and neutralize the lipid A part of Gram-negative LPS, but in the meantime could be shown also to neutralize Gram-positive lipopeptides/proteins[17-19] (a neutralization of peptidoglycan and lipoteichoic acid could also be observed, but it can be highly doubted that these are relevant PFs, as still proposed[20]).

The neutralization of the PFs from nearly all bacteria prevents the over-shooting inflammation reactions induced by these during sepsis. A variety of *in vitro*, *in vivo* and *ex vivo* data is indicative of high inflammation-decreasing activity and efficient protection (*e.g.,* strong weakening of infections, increased survival). As example for the inhibition of the inflammation reaction, in Figure 3 data from an *ex vivo* model of the human lung are presented. A schematic view on the processes taking place in human blood in the absence (up) and presence (down) of Aspidasept® are shown in Figure 4.

Aspidasept's scheduled therapeutic concentrations in sepsis patients are far below the concentrations at which side effects ("no observed adverse effect level", NOAEL) do occur. In fact, experiments in rats by a CRO (contract research organization) which have been conducted in accordance to ICH guideline M3, revealed that at a concentration of 0.5 mg/kg per day with infusions for 14 d no toxic effects were found. Therefore, this concentration is within the NOAEL. At 3.2 mg/kg per day, slight toxic effect occurred, indicating that this belongs to the MTD (maximum tolerated dose). Both concentrations are more than 200-fold higher than the scheduled concentration in septic patients. Therefore, these data provide for the first time the opportunity to develop an equally safe and efficacious medication against sepsis.

CONCLUSION

It cannot be expected that in the next decades new effective broad-band antibiotics will enter the market. Since the increase in multi-resistance and the demographic development - with a growing proportion of elder people - will continue, a huge burden to the health care system can be supposed. Therefore, to solve the urgent problems concomitant with lack of new medicaments and increasing resistance, new

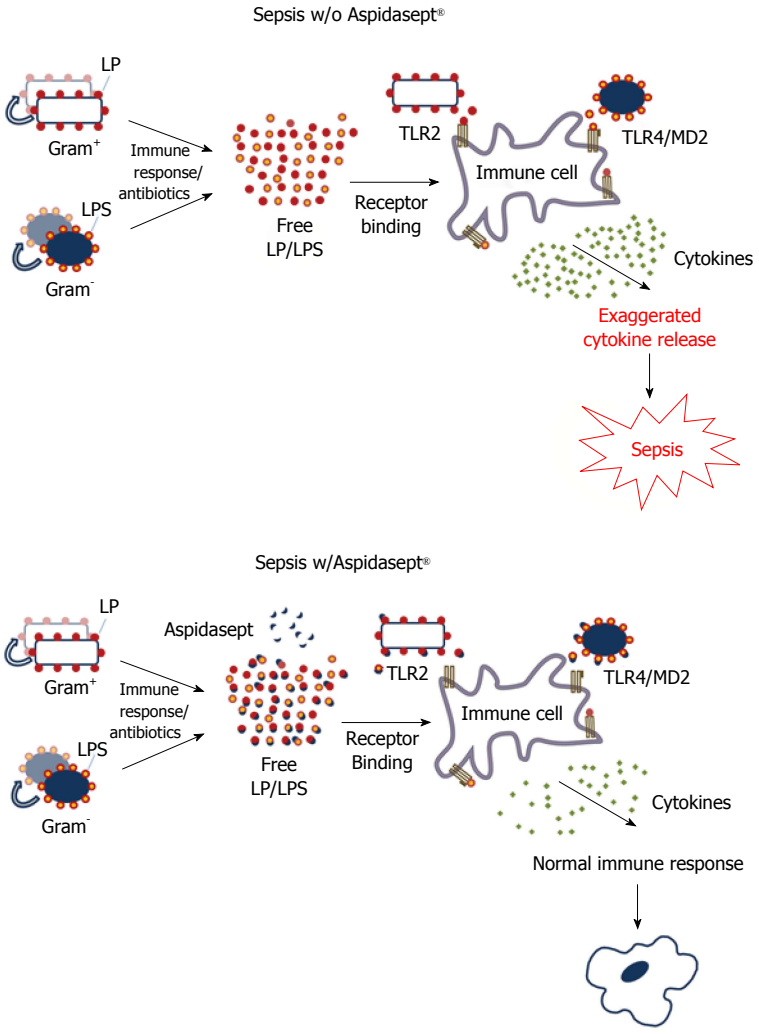

Figure 4 Compound Aspidasept® (Amino acid sequence GCKKYRRFRWKFKGKFWFWG; EU, United States, and Japan patent filed) is able to inhibit very efficiently the cytokine production induced by the pathogenicity factors endotoxin/lipoproteins or by the bacteria *in vitro*, *in vivo* (various mouse models), and *ex vivo* (human lung, see Figure 3). These inhibition properties of Aspidasept® comprise various relevant bacterial strains from Gram-negative and Gram-positive origin, as well as Mycobacteria, in particular *M. tuberculosis*. Aspidasept® has low toxicity (the NOAEL, no observed adverse effect level' is 200 to 500 times higher than the planned therapeutical doses). Beside its inflammation-inhibiting activity, it is able to bind the multi-resistant bacteria thus limiting their spreading. LPS: Lipopolysaccharide.

approaches must be developed. The presented data of antimicrobial peptides with high efficiency to neutralize the bacterial toxins may be one chance for the future. Whether this development will be the only promising approach, is unclear. However, one of the main arguments for the development of AMP is the rather simple and cheap synthesis up to very large scales (kg). In upcoming clinical trials it should turn out whether the highly successful preclinical data *in vitro*, *in vivo*, and *ex vivo* can be confirmed successfully also for septic patients as significant decrease in lethality. This would be the first therapeutical approach with antimicrobial peptides in clinical practice. A success would be also insofar important, since development of resistance against these AMPs is less probable.

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EDITORIAL

Is erythroferrone finally the long sought-after systemic erythroid regulator of iron?

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Abstract

Iron metabolism is regulated on the cellular and the systemic level. Over the last decade, the liver peptide "hepcidin" has emerged as the body's key irons store regulator. The long postulated "erythroid regulator of iron", however, remained elusive. Last year, evidence was provided, that a previously described myokine "myonectin" may also function as the long sought erythroid regulator of iron. Myonectin was therefore renamed "erythroferrone". This editorial provides a brief discussion on the two functions of erythroferrone and also briefly considers the emerging potential role of transferrin receptor 2 in erythropoiesis.

Key words: Erythroid regulator of iron; Hepcidin; Iron metabolism; Myonectin; Systemic iron regulation

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Core tip: Iron metabolism is regulated on the cellular and the systemic level. Over the last decade, the small, 25-amino-acid liver peptide "hepcidin" has emerged as the body's key irons store regulator. The long postulated "erythroid regulator of iron", however, remained elusive. Several candidates have been suggested in the literature, however all had to be dismissed. Last year, evidence was provided, that erythroferrone, a previously described myokine, may also function as the long sought erythroid regulator of iron. While erythroferrone appears to be an important iron regulator for erythropoiesis, transferrin receptor 2 also emerges as a player in this regulation.

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IRON AND LIFE

Iron is a very important metal for all cells; iron deficiency results in cell death, but iron overload can be similarly detrimental. Adult humans have about 3 to 5 g of iron in the body, the vast majority of which is bound to haemoglobin[1]. The major iron storage in the body occurs in the liver, or more specifically, the

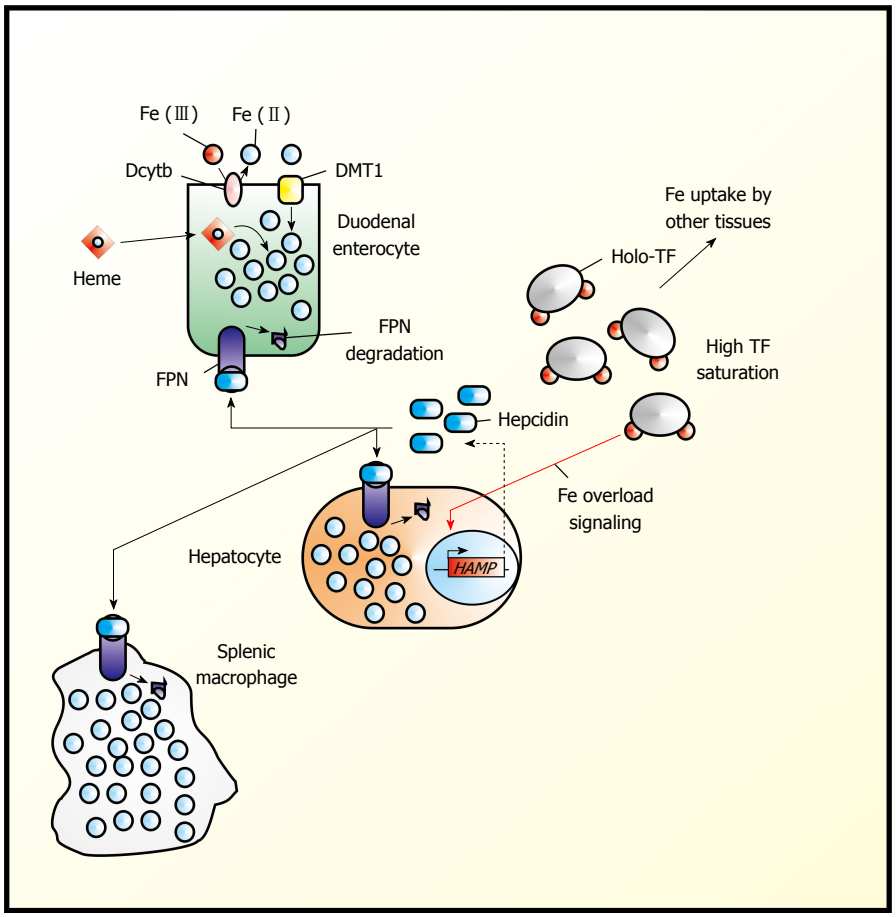

Figure 1 Hepcidin in the regulation of systemic iron homeostasis. Systemic iron (Fe) homeostasis is primarily regulated by the hepcidin-ferroportin (FPN1) axis. Duodenal enterocytes import dietary Fe from several sources including heme Fe and non-heme Fe, which typically must be reduced at the level of the apical membrane by chemical reductants such as ascorbate[26,27] or by plasma membrane oxidoreductases (*e.g.*, Dcytb), The reduced Fe then enters a common intracellular pool of Fe. "Sensing" the level of transferrin (TF) saturation and levels of Fe stores under conditions of Fe overload, hepatocytes up-regulate the expression of hepcidin, which is then released into the plasma where it can then bind to ferroportin (FPN1), thereby triggering FPN1 internalization and proteosomal degradation[1].

hepatocytes, where most iron is bound to ferritin[2]. As iron is such a double-edged sword, its metabolism has to be tightly regulated and for a long time, both, a store regulator and a erythroid regulator have been hypothesised to regulate systemic iron needs[3]. It was also shown early on that both, iron deficiency and increased erythropoiesis were able to up-regulate iron absorption[4,5].

HEPCIDIN AS THE IRON STORE REGULATOR

In 2000/2001, a small (25-amino-acid) hepatic antimicrobial peptide, LEAP-1 or hepcidin, was first described[6,7]. It was soon to be connected to iron metabolism and now is widely accepted to be the "store regulator"[5]. Murine hepcidin was found to be over-expressed during iron overload[8]. It is synthesized in response to high iron load and secreted by the liver into the blood stream, where the majority may bind unspecifically to albumin, while a small fraction (the active fraction?) will bind specifically to β_2-macroglobulin, from which it can be delivered to ferroportin[9]. Once bound to ferroportin, the only known mammalian cellular iron exporter, it will trigger proteosomal ferroportin degradation and thereby lower iron release into the circulation (Figure 1)[1].

PROPOSED ERYTHROID REGULATORS OF IRON

The nature of the erythroid regulator on the other hand remained elusive. In order to be considered the erythroid regulator, the molecule should be independent of the total body iron stores, but up-regulated in anaemia; it should be up-regulated by erythropoietin and it should trigger the down-regulation of hepcidin. It also should be down-regulated after blood transfusion. Two candidates have been earlier identified, Growth Differentiation Factor 15 (GDF15) and Twisted Gastrulation (TWSG1)[10,11]. The former was identified as being up-regulated in thalassaemic serum by a transcriptional profiling approach during erythropoiesis and as being able to *in vitro* suppress hepcidin expression[10]. By transcriptome analyses of erythropoiesis, TWSG1 was identified as

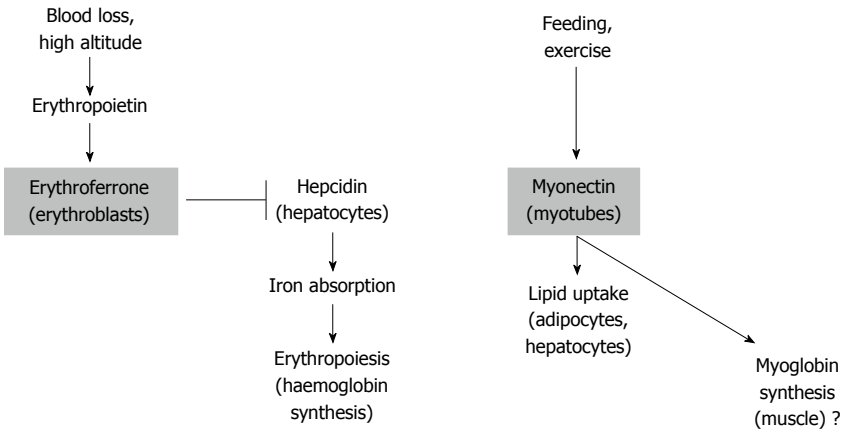

Figure 2 Two potential actions of erythroferrone. After blood loss, stress erythropoiesis is triggered by erythropoietin. This results in erythroferrone release from erythroblasts and in turn down-regulation of hepcidin. As a result, iron absorption and hemoglobin synthesis and erythropoiesis are increased (left). In response to feeding and exercise, myonectin (erythroferrone) produced by myotubes triggers fatty acid uptake by adipocytes and hepatocytes (right). Considering the function of erythroferrone in erythropoiesis, regulation of myoglobin is suggested to be a likely consequence of myonectin release (far right).

being up-regulated in early stages and again being able to (indirectly) suppress hepcidin expression[11]. For both these factors, however, evidence that they would constitute the erythroid regulator remained weak. Such, GDF15 levels are not correlated to hepcidin levels[12]. Even soluble transferrin receptor 1 was proposed to play the role of the erythroid regulator as it increases in iron deficiency and during increased erythropoiesis[13,14], however, this proposal also had to be dismissed, as it has no effect on iron absorption or hepcidin expression[14].

ERYTHROFERRONE - THE ERYTHROID REGULATOR?

Earlier this year erythroferrone, 340-amino acid soluble protein, was suggested for this role[15]. In response to erythropoietin, erythroblasts produce erythroferrone, which in turn suppresses hepcidin expression, resulting in increased release of iron from cellular iron stores[15]. Knockout mice for erythroferrone fail to rapidly suppress hepcidin expression in response to haemorrhage[15]. They also show a haemoglobin deficit, indicative of an impediment of erythropoiesis[15]. Erythroferrone expression in spleen and bone marrow increases during anaemia of inflammation and this contributes to iron mobilisation and recovery from anaemia[16].

MYONECTIN - A MYOKINE

These data provide strong evidence for erythroferrone finally being the (or at least an) erythroid regulator of iron metabolism. However, erythroferrone had earlier been described as "myonectin" or "C1q tumor necrosis factor α-related protein isoform 15" (CTRP15)[17]. [Unfortunately the same name "myonectin" was used in the same year for the related, but not identical "C1q tumor necrosis factor α-related protein isoform 5 (C1QTNF5)"[18] by Lim *et al*[19] for a myokine, the level of which is increased in insulin-resistant rats and mice and in myocytes depleted of mitochondrial DNA. This 243-amino acid soluble protein induces the phosphorylation on AMP-dependent protein kinase and acetyl-CoA carboxylase[20]]. CTRP15/myokine's circulating level was described to be tightly regulated by the metabolic state (suppressed during fasting and up-regulated after re-feeding) and predominantly expressed in skeletal muscle[17].

A MYOKINE AS ERYTHROID REGULATOR OF IRON?

These authors did not include bone marrow or spleen in their expression analysis (the two tissues where erythropoietin significantly stimulated erythroferrone expression[15]) and neither analysed a knockout mouse of myonectin for a muscle phenotype (which was not reported for the erythroferrone knockout mouse[15]). Thus, it appears at first glance difficult to reconcile the two proposed functions of erythroferrone/myokine as those of a factor released into the circulation. Exercise, unless performed at high altitude, is not usually considered to stimulate erythropoiesis[21], neither is feeding. Thus, how can the apparent conundrum be solved that a myokine, that produced during exercise and feeding, enhancing fatty acid uptake by myocytes and hepatocytes[17] should be a master regulator of iron metabolism in erythropoiesis[15]? There appears to be discussion on whether continuous exercise (on sea level) can raise the erythropoietin levels and therefore erythropoiesis[22], thus there may be a way of reconciling the two functions of erythroferrone now described in the literature. Furthermore, a peptide stimulating haemoglobin formation in erythropoiesis may also have a role in myosin formation in the muscle (Figure 2).

REGULATION OF ERYTHROFERRONE BY TRANSFERRIN RECEPTOR 2

While preparing this short editorial, several papers were published that provided evidence for a role of transferrin receptor 2 in the regulation of erythropoiesis. Wallace *et al*[23] reported that mice lacking matriptase-2 and transferrin receptor 2 are characterised by severe anaemia. These mice have a significantly increased erythroferrone expression when compared to their wild type litter mates during stress erythropoiesis. Similar results were presented by Nai *et al*[24] showing that mice lacking both matriptase-2 and transferrin receptor 2 have increased erythrocyte counts when compared to littermates only lacking matriptase-2. When analysing mice, lacking transferrin receptor 2 in the bone marrow only, Nai *et al*[25] reported that these mice exhibit increased numbers of nucleated erythroid cells in the bone marrow and increased erythroferrone levels in the spleen and in erythroid precursors.

CONCLUSION

C1q tumour necrosis factor α-related protein isoform 15, originally called myonectin and now referred to as erythroferrone, appears to play two different regulatory roles; that of a myokine and that of an erythroid regulator of iron. On the whole, evidence for its function as erythroid regulator of iron appears to be stronger than that of its function as myonectin. Transferrin receptor 2 also plays a regulatory role in erythropoiesis, down-regulating erythroferrone and thereby erythropoiesis. Transferrin receptor 2 may be involved in the iron sensing that results in the regulation of red blood cell formation.

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REVIEW

Multifunctional facets of retrovirus integrase

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Abstract

The retrovirus integrase (IN) is responsible for integration of the reverse transcribed linear cDNA into the host DNA genome. First, IN cleaves a dinucleotide from the 3′ OH blunt ends of the viral DNA exposing the highly conserved CA sequence in the recessed ends. IN utilizes the 3′ OH ends to catalyze the concerted integration of the two ends into opposite strands of the cellular DNA producing 4 to 6 bp staggered insertions, depending on the retrovirus species. The staggered ends are repaired by host cell machinery that results in a permanent copy of the viral DNA in the cellular genome. Besides integration, IN performs other functions in the replication cycle of several studied retroviruses. The proper organization of IN within the viral internal core is essential for the correct maturation of the virus. IN plays a major role in reverse transcription by interacting directly with the reverse transcriptase and by binding to the viral capsid protein and a cellular protein. Recruitment of several other host proteins into the viral particle are also promoted by IN. IN assists with the nuclear transport of the preintegration complex across the nuclear membrane. With several retroviruses, IN specifically interacts with different host protein factors that guide the preintegration complex to preferentially integrate the viral genome into specific regions of the host chromosomal target. Human gene therapy using retrovirus vectors is directly affected by the interactions of IN with these host factors. Inhibitors directed against the human immunodeficiency virus (HIV) IN bind within the active site of IN containing viral DNA ends thus preventing integration and subsequent HIV/AIDS.

Key words: Retrovirus integrase; Integration; Host factors; Atomic structure; Human immunodeficiency virus integrase inhibitors

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Core tip: This review examines the multifunctional properties of retrovirus integrase (IN) besides its key function of integrating the viral DNA into host chromosomes. IN has a major role in the maturation of the virus, reverse transcription and nuclear transport of the preintegration complex. IN binds to cellular cofactors

for uncoating of the core and to other cellular proteins that guide the preintegration complex to preferred regions on the host genome for integration. Understanding these IN functions has resulted in the production of clinical IN strand transfer inhibitors to prevent human immunodeficiency virus (HIV/AIDS) and development of retrovirus vectors for human gene therapy.

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INTRODUCTION

Retroviruses are ubiquitous in nature and are found in yeast to insects and to the animal and plant kingdoms. In retroviruses, the single stranded viral RNA genome is reverse transcribed by the viral reverse transcriptase into a double strand DNA copy that is subsequently integrated into the host genome. The integration process that inserts the viral DNA into the host DNA is mediated by the viral encoded integrase (IN). Retroviruses are mobile elements that distinguish themselves from other viruses because their DNA genomes become an integral part of the host genome, an essential requirement in their replication cycle. There are slight variations in this integration process in the above mentioned biological systems, some of which are called Ty1/copia and Ty3/gypsy elements or LTR retrotransposons. This review will focus on IN found in alpharetroviruses, gammaretroviruses, lentiviruses which includes human immunodeficiency virus (HIV), and spumaviruses. The purpose of the review will be to briefly highlight the most current salient features of the many functional facets of the retrovirus IN and identify critical papers and reviews that provide in-depth information for a specific subject.

HIV is the most studied retrovirus with regards to its biology, biochemistry and pathogenesis. A general retrovirus replication cycle is depicted in Figure 1 along with location of steps for FDA approved HIV drug therapies. Each step from the binding of the virus particle to a cellular receptor to the release of a new virus particle is described in the legend. In Figure 1, IN plays a role in step 3: uncoating of the internalized viral core, step 4: reverse transcription of the viral RNA, step 5: nuclear transport of the preintegration complex (PIC), step 6: integration of viral DNA into the host genome, and step 13: maturation.

IN DOMAIN ORGRANIZATION

The retrovirus IN is located at the C-terminal end of the Pol protein that is part of a larger precursor polyprotein (Gag-Pol) encapsulated within the virus particle. The retrovirus IN was first identified and purified from an alpharetrovirus[1] and genetically shown to be necessary for integration[2-5]. The avian retrovirus or Rous sarcoma virus (RSV) and HIV IN proteins are 286 and 288 residues in length, respectively, while the prototype foamy virus (PFV) IN is 392 residues[6] (Figure 2). RSV IN possesses three structural domains comprising an N-terminal domain (NTD) (residues 1-44) that contains a zinc-binding HHCC motif, a catalytic core domain (CCD) (residues 58-213) and a C-terminal domain (CTD) (residues 222-268). HIV IN has similar size domains and linkers as RSV IN. Both PFV and murine leukemia virus (MLV) IN (408 residues) possess an additional N-terminal extension domain (NED) (approximately 50 residues) attached adjacent to the NTD of IN.

BIOLOGICAL FUNCTIONS OF IN

Role of IN in viral maturation

The retrovirus structural Gag proteins are approximately 80% of the total mass of the viral proteins. The reverse transcriptase (RT) and IN are derived from a separate Gag-Pol precursor polyprotein produced by translational frameshifts. This precursor polyprotein accounts for approximately 2% to 3% of the total viral protein mass (Figure 3). Despite this lower level of IN in virus particles, studies using site-directed mutagenesis of HIV IN highly suggest that deletion and missense mutations affect HIV assembly and release[7]. The mechanisms associated with these defects are not well understood but may include activation of the viral protease located within Gag-Pol and dimerization of this precursor polyprotein.

The recent discovery of inhibitors that do not interact within the catalytic active site of HIV IN has provided unforeseen information on a structural role for IN in virus particle maturation. These inhibitors called LEDGINS[8] or ALLINIS (allosteric IN inhibitors)[9] prevent interactions of IN with a cellular chromatin binding protein lens epithelium-derived growth factor (LEDGF/p75), a transcriptional cofactor that guides the HIV PIC (Figure 1) to active transcription sites for integration (see host factors). In addition to inhibiting integration, significant structural defects are caused by these inhibitors resulting in abnormal formation of electron dense HIV virus cores thus producing non-infectious particles upon egress[10-12] (Figure 1). Other studies have shown that these inhibitors significantly induce HIV IN monomers to produce inactive oligomers *in vitro*[10,13,14]. These results highly suggest that IN plays a critical role in virus maturation (Figure 1).

Reverse transcription requires IN functions

RT and IN are linked together covalently at the C-terminal end of Gag-Pol precursor polyprotein (Figure 3). The processing of the precursor protein to RT and IN by the viral protease in the virus particle is different between RSV and HIV. For RSV, the Pol product from the polyprotein is a 95 kDa polypeptide termed β that forms

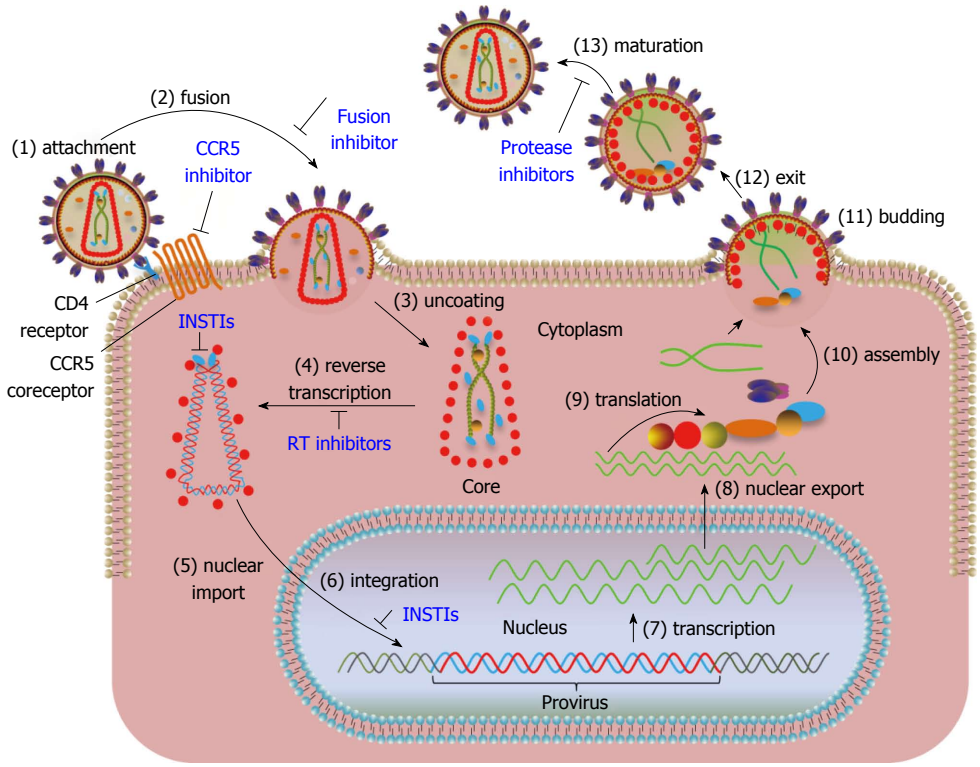

Figure 1 Overview of human immunodeficiency virus type 1 replication cycle. Steps in the viral replication cycle are numbered. The steps where FDA approved drugs exist are marked in blue. (1) The trimeric envelope glycoprotein (gp120/gp41) of human immunodeficiency virus (HIV) binds to the CD4 receptor and CCR5 co-receptors present on host cell surface. Maraviroc, a CCR5 receptor antagonist blocks the binding of viral gp120 to CCR5 and prevents the entry of virus into cells; (2) Viral membrane fuses with cellular membrane allowing the entry of the virus into the cytoplasm. Enfuvirtide, a fusion inhibitor binds to gp41 and prevents the formation of an entry pore for the capsid of the virus, thus blocking the entry of virus into the cytoplasm; (3) The virus undergoes uncoating of the core; (4) HIV RNA is reverse-transcribed by reverse transcriptase into double stranded DNA within the core. Reverse-transcriptase inhibitors block the synthesis of viral DNA; (5) Integrase (IN) cleaves two nucleotides adjacent to conserved CA nucleotides from both 3'-ends of viral DNA (referred as 3'-processing in text) and forms the preintegration complex (PIC). The PIC is transported into the nucleus through a cellular nuclear import pathway. IN strand transfer inhibitors (INSTIs) bind to the IN-DNA complex after 3'-processing rendering the cytoplasmic PIC defective for integration but not nuclear transport; (6) IN facilitates the joining of viral DNA to the host DNA (termed strand transfer in text). Integrated viral DNA is referred to as "provirus". A cellular protein, lens-epithelium derived growth factor (LEDGF/p75), preferentially targets the integration of viral DNA within transcriptionally active regions. INSTIs block the joining of processed viral DNA ends into cellular DNA; (7) The viral DNA is transcribed by cellular RNA pol II into viral RNAs; (8) Full-length and alternatively spliced RNAs are transported out of nucleus; (9) Viral proteins are synthesized from the RNA template; (10) Full-length viral RNA and newly synthesized proteins begin the assembly of new virus particles at the inner surface of cell membrane; (11) Immature viral particles bud out of the membrane and released (12); and (13) Proteolytic processing of viral proteins results in the maturation of viral particle. Protease inhibitors block the formation of mature proteins by inhibiting the proteolytic processing of the viral polyproteins.

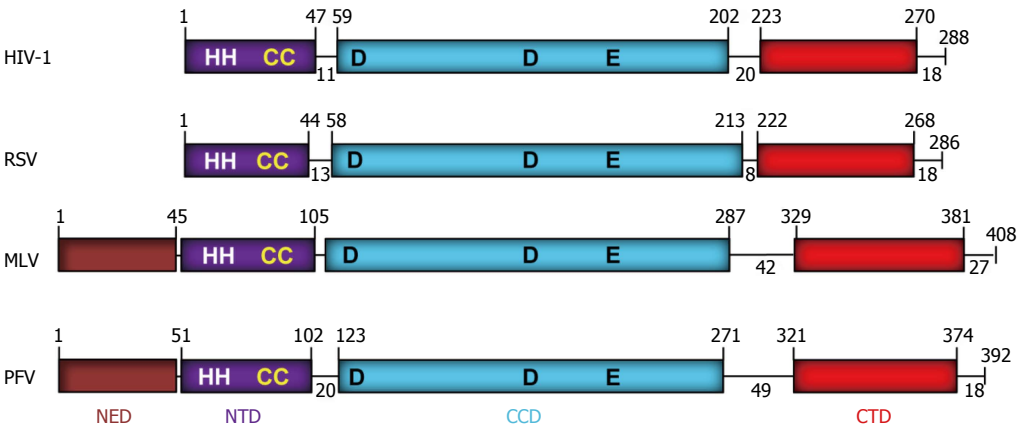

Figure 2 Domain organization of retroviral integrases. The domains (NED: N-terminal extension domain; NTD: N-terminal domain; CCD: Catalytic core domain; and CTD: C-terminal domain) are depicted with different colors. The number of residues in each domain are indicated. The domains are separated by protein linkers of various sizes. NTD contains the zinc binding HH-CC motif and the D-D-35-E motif in CCD binds Mg^{2+} which constitutes the active site. The CTD shows a topology of SH3-like domain, a characteristic feature associated with protein-protein and protein-DNA interactions. The exact MLV domain sizes have not been determined experimentally except for the CTD[99]. A short tail of disordered amino acids is located on the C-terminal end of each IN. IN: Integrase; HIV: Human immunodeficiency virus; RSV: Rous sarcoma virus; MLV: Murine leukemia; PFV: Prototype foamy virus.

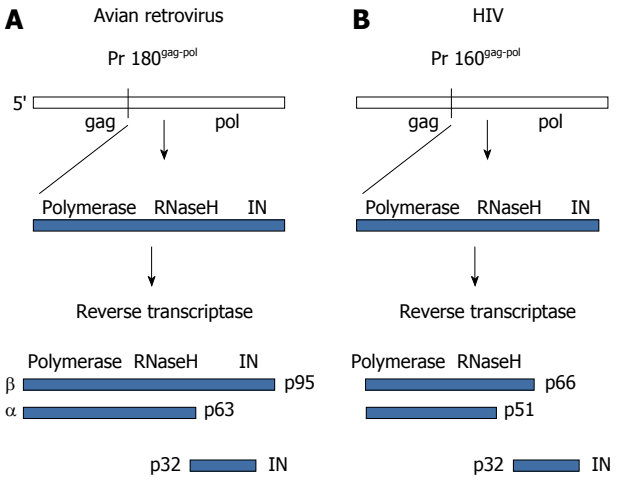

Figure 3 Structural organization and proteolytic processing of reverse transcriptase and integrase. A: The precursor Gag-Pol protein is processed by the viral protease into an intermediate polyprotein. The avian polyprotein exists as a dimer of the β subunit that is processed into the active αβ subunits (RT) and integrase (IN). The α subunit contains the active polymerase and RNaseH sites while β retains IN residues. RT does not possess integration capabilities; B: Cleavage of the human immunodeficiency virus (HIV) polyprotein produces the active dimeric RT and IN. The HIV p66 subunit possesses the polymerase and RNaseH active sites. RT: Reverse transcriptase.

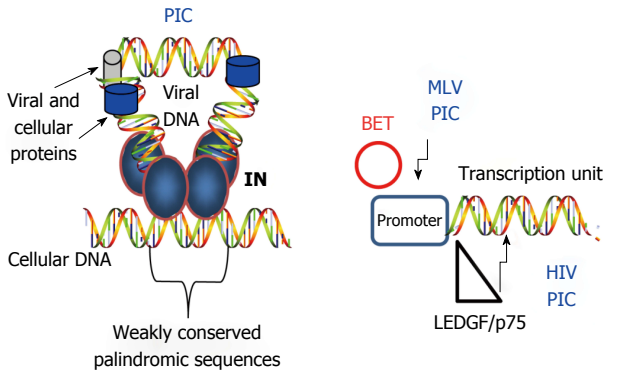

Figure 4 Selection of cellular DNA target sites for integration. Left: IN within the PIC is guided to host sites by several mechanisms including recognition of weak palindromic sequences by IN, nuclear topography of the cellular host DNA site, chromatin structure, and several kinds of associated histone tails. Only IN (labeled), RT (blue circle) and LEDGF/p75 (gray cylinder) are identified in the figure; Right: Cell proteins (BET and LEDGF/p75) influence the PIC to preferentially insert the viral DNA into specific regions of the host genome. IN: Integrase; PIC: Preintegration complex; HIV: Human immunodeficiency virus; MLV: Murine leukemia virus.

dimers (Figure 3). The viral protease cleaves the dimers into active RT, termed αβ, which causes the release and activation of the 32 kDa IN. The αβ polypeptide still contains a single copy of IN residues that facilitates a processive mechanism for reverse transcription and its associated RNaseH activity. In contrast, HIV IN is released entirely from the polyprotein producing RT (p66/p51) (Figure 3), similarly to MLV IN (not shown).

Site-specific mutations introduced into the cleaved HIV IN result in reverse transcription defects but not integration[15]. The exact mechanisms associated with these RT defects are mostly unknown. HIV IN (Figure 3) appears to have a physical interaction with RT (p66/p51) that is necessary for normal viral DNA synthesis[16-19]. In normal HIV infected cells, IN interacts with the major structural viral capsid protein (CA) whose interactions affect the normal uncoating process of the core and thus reverse transcription[20]. Following the above theme that viral core uncoating affects reverse transcription, recent results suggest that the cellular protein DYNLL1 is required for early viral DNA synthesis and uncoating *via* a physical interaction of DYNLL1 with HIV IN[21]. As these authors suggested, IN-DYNLL1 interactions may be a potential target for future inhibitors.

Role of IN in the preintegration complex, its nuclear transport and in integration

Although IN has many roles in the retrovirus replication cycle, IN is the catalytic component necessary for concerted integration of the viral DNA ends into the host genome (Figures 4 and 5). A pivotal study clearly demonstrated that linear MLV DNA acts as the substrate for integration *in vivo*[22]. The high molecular weight PIC containing a copy of the linear viral DNA was isolated from MLV infected cells (Figures 1 and 4). This complex is capable of integrating the viral ends in a concerted fashion into a target substrate *in vitro*. DNA sequence analysis of the integrated viral DNA demonstrated that IN inserted the 3′ OH ends into opposite strands of the target DNA producing the 4 bp host site duplication found associated with the integrated MLV provirus *in vivo* or a 6 bp duplication with RSV (Figure 5). The host site duplication for HIV is 5 bp.

There are numerous publications (many not indicated here) describing the integration capabilities of the PIC for MLV[22,23] and later for HIV[24-27] at the biochemical, genetic and inhibition of strand transfer by IN inhibitors (Figure 1). The PIC is composed of linear DNA, viral proteins IN, RT, CA, matrix (MA), viral protein R (Vpr) and several cellular associated proteins (Figure 4). In the cytoplasm, IN cleaves a dinucleotide from the 3′ OH ends of blunt DNA before transport of the PIC into the nucleus (Figure 5). HIV IN also appears to play a role in the nuclear transport of the PIC along with other viral and cellular proteins[28,29]. After transport, IN inserts the 3′ OH recessed DNA ends into the cellular DNA genome. As described later, the HIV IN strand transfer inhibitors (STIs) interact within the active site of IN and the 3′ processed LTR ends in the cytoplasmic PIC, thus inactivating the complex preventing integration into cellular DNA[27]. The retrovirus PIC has provided major insights into retrovirus integration but has a severe limitation for providing sufficient quantities necessary for biochemical studies.

IN interactions with host factors and cellular target site selection

IN is the dominant factor for selection of weakly conserved palindromic sequences at the target site *in vivo*[30-32] (Figure 4). Whole genomic sequencing of DNA target sites demonstrates that HIV IN prefers

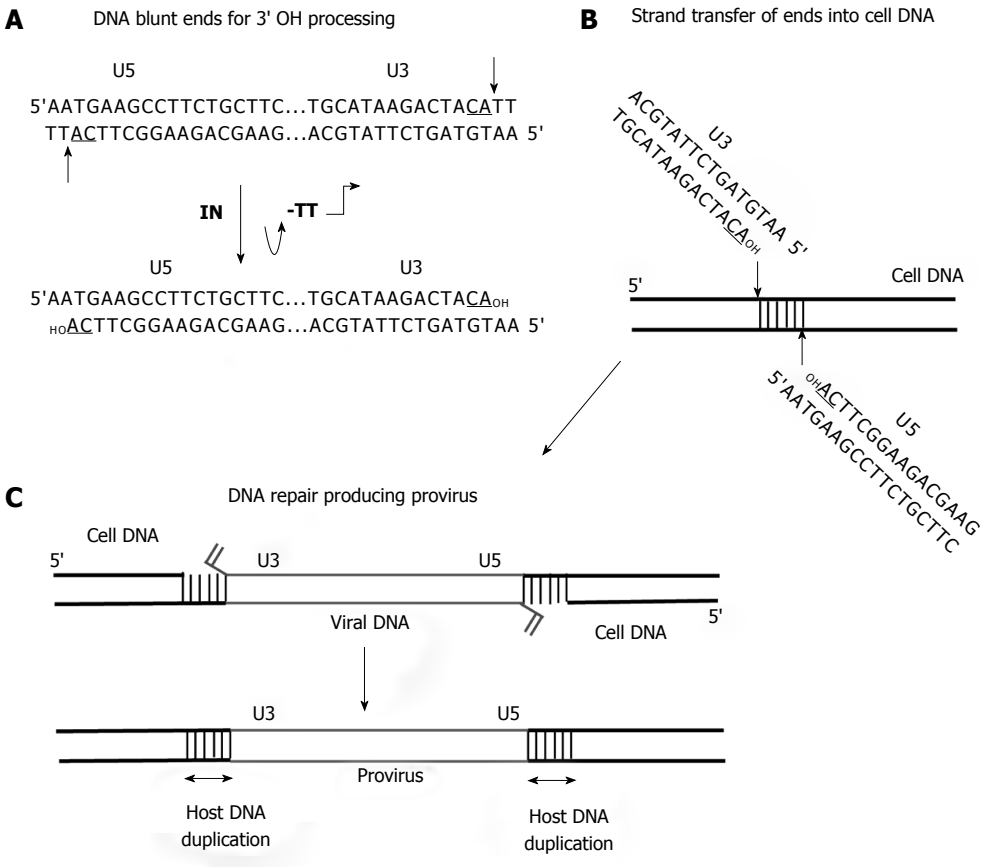

Figure 5 Requirement for 3' OH processing and strand transfer activities to facilitate concerted integration of viral DNA by integrase into target DNA. A: The linear approximately 10 kb DNA with only the ends is shown. The U3 and U5 RSV DNA blunt ends with the underlined conserved CA dinucleotide on the catalytic strand are indicated. The thin arrows indicate 3' OH processing of two nucleotides by IN producing the recessed substrate for strand transfer activity; B: Concerted integration of the viral linear DNA ends by integrase (IN) into the target DNA is by a transesterification mechanism; C: The insertion of viral DNA into the target produces a 6 bp stagger cut by RSV IN that is repaired by cellular proteins producing the integrated provirus. RSV: Rous sarcoma virus.

to integrate its viral DNA into active transcriptional units[30] while MLV IN favors integration sites near the promoter region of genes and not into transcriptional units[33]. Further studies demonstrated that RSV IN essentially integrates its DNA into the host genome at random sites[34,35]. These results suggested that HIV and MLV IN interact with specific host factors that direct IN to preferred sites in the host genome.

Major studies have centered on the ability of LEDGF/p75, a transcriptional co-activator, to guide the HIV PIC to integrate its DNA into active transcriptional sites[36-41] (Figure 4). Besides LEDGF/p75, cellular Nup153 is indispensable for the integration of HIV DNA into the peripheral edge of the nuclear DNA suggesting that nuclear topography is also an important for HIV integration site selection[42]. For MLV IN, recent studies demonstrated that IN interacts with the chromatin BET (bromo- and extra-terminal domain) proteins (BRD2, BRD3 and BRD4) directing it to the promoter region of genes[39,43-45] (Figure 4). To date, there are no reports that have identified cellular proteins involved in host site selection by avian retroviruses. In summary, IN is capable of integrating its viral DNA into numerous segments of the host genome but is preferentially directed to specific regions by host factors.

BIOCHEMICAL AND STRUCTURAL PROPERTIES OF IN

Solution properties of IN

Biochemical and biophysical studies of retrovirus IN has been extensive over the last 37 years. Most studies have used recombinant RSV, HIV, MLV and PFV IN. Only the avian retrovirus IN has been purified from virus particles[1,46]. As stated above, IN cleaves a dinucleotide from both 3' OH blunt ends of the viral DNA and subsequently inserts the recessed ends into a target DNA by a transesterification reaction (Figure 5). The biochemistry of these reactions and other structural properties of IN were recently reviewed[47].

The subunit structure of IN is varied and highly dependent on the purification process and different salt requirements to maintain solubility for each expressed recombinant IN. PFV IN is soluble (10-15 mg/mL) in 0.2 mol/L NaCl[48,49] while RSV IN is highly soluble (30 mg/mL) in 0.2 mol/L $(NH_4)_2SO_4$[50]. PFV IN is monomeric[49] while RSV IN is a dimer in solution[50-52]. Recombinant HIV IN has been purified as a monomer, dimer and tetramer[47,53,54]. Recent studies suggest that the active form of HIV IN is a monomer that is responsible for assembly of the synaptic complex (SC) or intasome

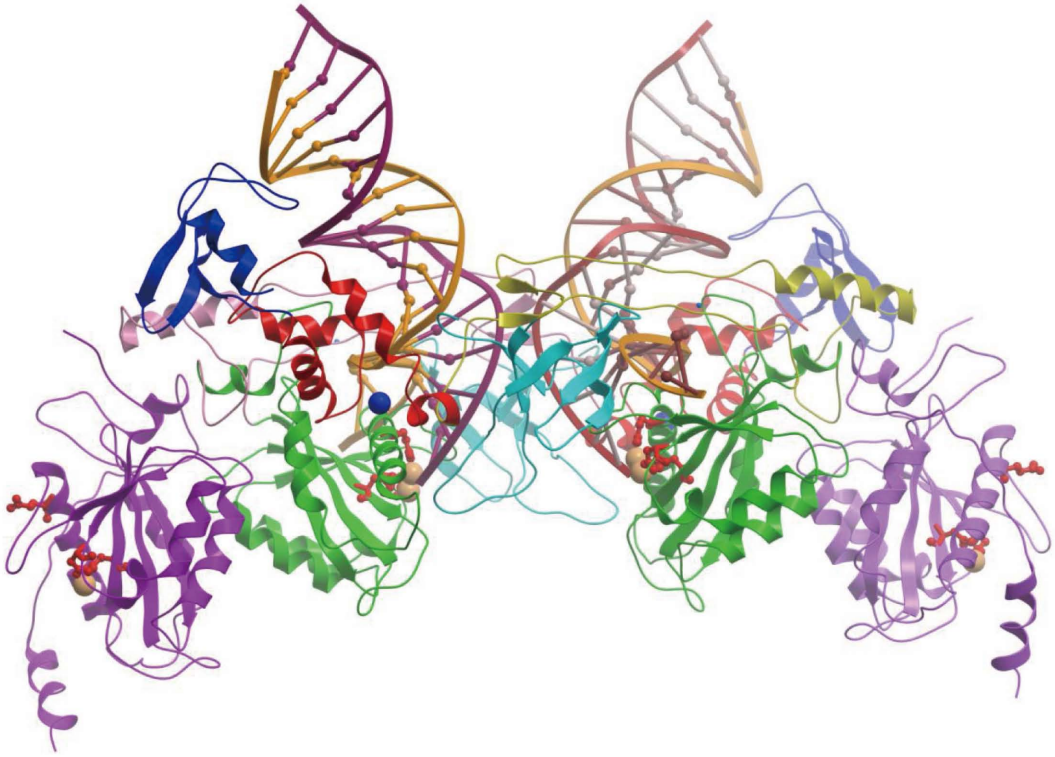

Figure 6 Prototype foamy virus intasome structure. The intasome consist of an integrase (IN) tetramer with two viral DNA ends. The 17/19 bp DNA interacts extensively with the two inner monomers of IN. Among the inner monomers, the N-terminal extension domain, N-terminal domain, catalytic core domain (CCD) and C-terminal domains are shown in blue, red, green and cyan, respectively. The DDE residues are identified as red amino acids. Among the outer protomers, only the CCD (shown in purple) was resolved. The cartoon was redrawn from the PDB ID 3OY9 in Molsoft ICM-Browser. The Zn^{2+} and Mg^{2+} metal ions are shown with blue and orange spheres, respectively.

capable of efficient concerted integration of two oligonucleotides into a target substrate *in vitro*[53]. The HIV IN monomer purified in the presence of EDTA is converted into a tetramer in the presence of Zn^{2+} without the formation of a dimer intermediate[53,54]. Studies have demonstrated that a tetramer of IN is associated with HIV, PFV and RSV SC capable of concerted integration[50,55-57].

The oligomeric state of retrovirus IN play key roles in virus maturation upon release of the virus particle, enzymatic activities for 3′ OH processing and concerted integration of viral DNA and possibly for reverse transcription in the cytoplasmic PIC.

Retroviral IN superfamily and PFV intasome structure

The retrovirus IN superfamily comprises proteins that are involved in a variety of activities like replication and repair of DNA, V(D)J recombination, and DNA transposition. The DDE domain of retrovirus IN (Figure 2) is highly conserved in proteins capable of similar catalytic reactions including MuA transposase, human RAG1 recombinase, the RNaseH domain on the retrovirus RT (Figure 3) and other bacterial and human endonucleases[58,59]. Structures of many of the above protein have been resolved at the atomic level in complex with DNA. However, among the retrovirus INs only the four-domain PFV intasome (PFV IN-DNA complex) has been resolved (Figure 6).

The crystallographic study of the full-length PFV IN with bound viral DNA revealed, for the first time, the architecture of retrovirus intasome assembly and atomic details of the IN-DNA interactions[57,60] (Figure 6). The PFV intasome contains a tetramer of IN, which consists of a dimer of the "inner" subunits that make extensive protein-protein and protein-DNA interactions, and a pair of the "outer" subunits that appear to play structural roles. All four domains of the IN subunits and the inter-domain linkers interact extensively with DNA to bridge between the two viral DNA ends and capture the target DNA. The engagement of the viral DNA sequence and catalysis *in trans* is a feature shared with related transposase systems[59,61].

The determination of the atomic structure of the PFV intasome greatly enhanced our understanding of IN interactions with 3′ OH recessed ends in the active site and showing how a tetramer of IN catalyzes the concerted integration reaction[57]. Other structural studies have shown how PFV IN is bound to the viral DNA in its ground state prior to 3′ OH processing or strand transfer[62]. Lastly, the PFV intasome in complex with target DNA after insertion of the 3′ OH ends (strand transfer complex) demonstrated how severely the target DNA is bent upon insertion of the viral DNA ends[60,63].

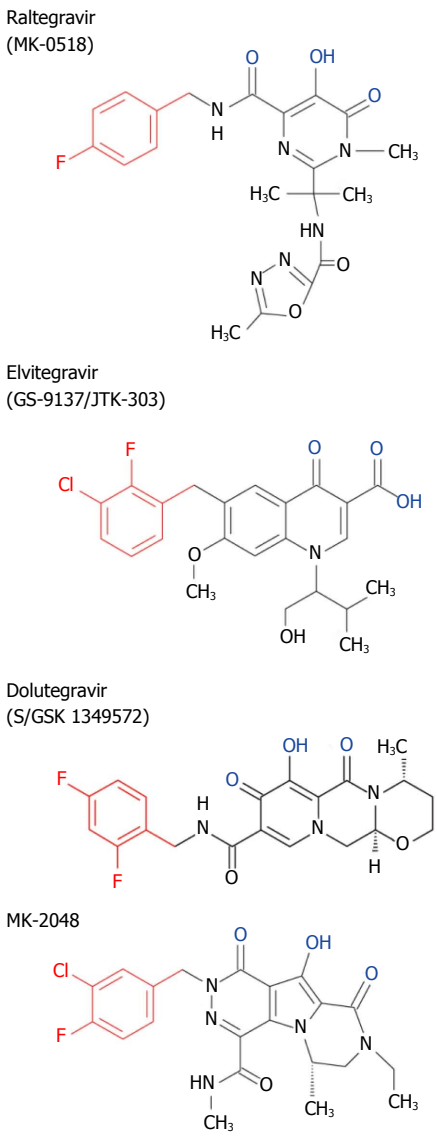

Figure 7 Chemical structure of strand transfer inhibitors. Conserved metal chelating moiety similar to diketo acid is shown in blue and a conserved halogenated benzyl group that interacts with the penultimate nucleotide of DNA is shown in red.

HIV IN INHIBITORS

Strand transfer inhibitors and their interactions within the active site of HIV IN

The requirement for integration of the viral DNA into the host genome for replication renders IN as a natural target for development of inhibitors. Numerous studies in the 1990s to identify relevant HIV IN inhibitors culminated with a study demonstrating that IN STI prevent the integration of the viral DNA into the cell genome resulting in inhibition of HIV replication in cells[27]. Further studies established that the STI competed with target DNA binding to IN suggesting a unique conformation of IN for the strand transfer reaction[64]. The diketo acid pharmacophore of the STI also bound to the divalent metal ion in the active site of IN[65].

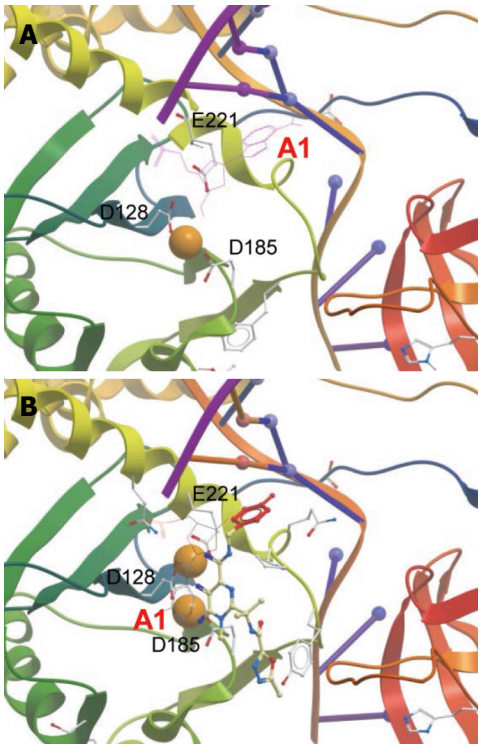

Figure 8 Active site within prototype foamy virus intasome in apo (A) and Raltegravir-bound state (B). Orange spheres indicate Mg^{2+}. Active sites residues are labeled. Only the terminal 3' adenosine (marked A1) is displayed in its chemical form to show the displacement upon RAL binding. The diketo acid group of Raltegravir (RAL) interacts with the metal ions. Adenine is π-stacked against the RAL metal-chelating scaffold. The halobenzoyl group of RAL is marked red. The figures were constructed from PDB entries 3L2R and 3OYA, respectively, in Molsoft ICM-Browser.

Crystallization studies of the four-domain PFV IN intasome without (Figure 6) and with STIs demonstrated the interactions of these inhibitors within the active site of IN[57,62,66,67] (see Figures 7 and 8 for brief description of STIs and their mechanism). There are currently three clinical STIs approved by the FDA for treatment of HIV/AIDS (Figure 7). They are Raltegravir (RAL), Elvitegravir (EVG) and Dolutegravir (DTG). The HIV IN STIs are interfacial inhibitors whose mechanisms are similar to other compounds that target bacterial DNA gyrase and topoisomerase IV as well as anticancer drugs targeting human topoisomerases[68,69]. Interfacial inhibitors, like the HIV STIs, bind to a site produced at the interface of two (or more) molecules bound together resulting in a functional complex. In the case of STIs, the interfaces are formed by the viral terminal CA nucleotides and specific IN residues in the PFV intasome[57,67] (Figure 8). A recent book[70] and several reviews of HIV IN inhibitors provide an extensive overview of the discovery of STIs and their mechanisms[47,71-73].

HIV and RSV synaptic complexes are kinetically stabilized by STIs

The atomic structures of the three-domain HIV and RSV IN (Figure 2) in complex with viral DNA are unknown. Crystallization of these INs in complex with

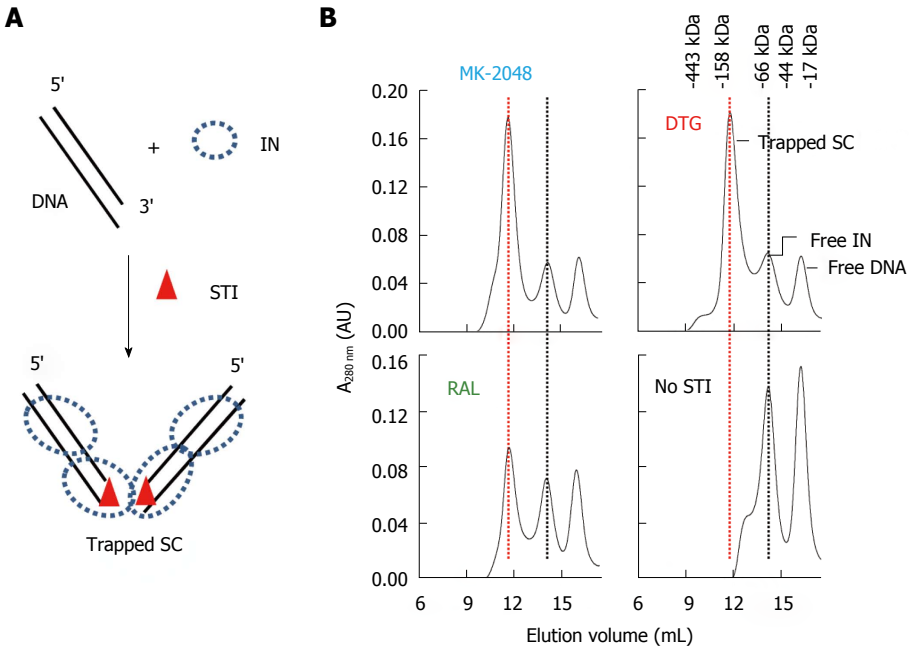

Figure 9 Identification of strand transfer inhibitor-trapped Rous sarcoma virus synaptic complex by size-exclusion chromatography. A: Schematic of Rous sarcoma virus (RSV) IN, 3' OH recessed ODN, and strand transfer inhibitor (STI) used to assemble STI-trapped synaptic complex (SC); B: MK-2048 and Dolutegravir (DTG) efficiently produce trapped SC while Raltegravir had moderate efficiency. No trapped SC is produced in the absence of STI. Elution positions of STI-trapped SC (red line), free IN and DNA are marked. Molecular weight markers are indicated.

their viral DNA substrates at high IN concentrations has not been successful for numerous biochemical reasons including aggregation of HIV IN and limited stability of the complexes.

The HIV SC at low nM concentrations can be physically trapped in the presence of HIV IN STIs as analyzed by native agarose gel electrophoresis[74,75]. There are strong structural similarities in the CCD between HIV, PFV and RSV IN (Figure 2)[47,51,52,57,67,76,77] suggesting that under appropriate conditions the highly soluble RSV IN could also be trapped in the presence of the appropriate oligonucleotide (ODN) substrate and STIs. Assembly of a trapped RSV SC at high IN concentrations (1.5 mg/mL; 45 μmol/L) in solution required the presence of 3' OH recessed viral ODN ranging in sizes (16/18R to 18/20R) and DTG, RAL or MK-2048[50] (Figure 9). DTG and MK-2048 (an investigational inhibitor) possess a significantly longer dissociative half-life than RAL within an HIV IN-DNA complex allowing these STIs to capture and stabilize the IN-DNA complex[78,79]. Without the STI presence, the transient RSV SC produced in the concerted integration pathway could not be isolated highly suggesting the STI-trapped SC is kinetically stabilized. Efforts are ongoing to resolve the atomic structure of this kinetically stabilized STI-trapped SC and the RSV strand transfer complex.

Clinical results using HIV IN STIs and drug resistance

The previously described three clinical STIs (Figure 7) are the newest class of inhibitors to treat HIV/AIDS that must be used in combinational drug therapy with reverse transcriptase, protease and entry inhibitors. RAL and EVG were FDA approved for clinical use in 2007 and 2012, respectively, while DTG was approved in 2013. IN STIs are superior to other regimens in first-line therapies[80,81]. All three IN STIs have a high safety record and display strong efficacy[81].

HIV has a high mutational rate, approximately 3 × 10^{-5} mutations/base pair per cycle[82]. Thus, like with RT and protease inhibitors, the use of IN STIs has resulted in the emergence of drug-resistant viral strains that causes a loss of drug effectiveness. Both RAL and EVG possess a low genetic barrier giving rise to drug-resistant IN mutants (G140S/Q148H, N155S, Q148K, F121Y, T66I/S153Y) in tissue culture as well as in treated patients[72,83]. In contrast, DTG has a high genetic barrier and to date significant resistant IN mutants have been nearly absent in treatment-naïve patients[81,84,85]. The higher genetic barrier of DTG in patients may be due to its longer dissociative half-life from the HIV IN-DNA complex suggesting that DTG extended binding may be a significant factor for prevention of drug-resistance[86]; also shown to be a factor in producing kinetically stabilized RSV SC (Figure 9). HIV carrying the rare signature IN R263K mutation (others are H51Y and E138K) observed with DTG apparently cannot co-exist in combination with many of the above classical IN resistance mutations[87]. HIV is apparently unable to compensate for these mutations which induce a fitness cost that prevents the virus from evading inhibitor pressure.

Taken these demonstrated properties of DTG, investigators have slightly modified its structure to

formulate a long-acting injectable nanoparticle (200 nm) suitable for clinical administration on a quarterly basis[88]. In these preclinical studies, GSK744 provided high-level protection against repeated simian/human immunodeficiency virus challenge in rhesus macaques. This study suggests that GSK744 in combination with a similar injectable RT inhibitor could decrease adherence problems associated with pre-exposure prophylaxis.

RETROVIRAL VECTORS

Human gene therapy

Retroviruses are natural vectors for transfer of genetic information between cells. For example, the insertion of an oncogene derived from one cell by a retrovirus into another cell occurs naturally[82]. Numerous commercial and investigative retroviral vectors have been developed to accomplish a wide variety of experimental protocols as well as the study of human diseases[89]. This permanent transfer of genetic information by retroviruses has led to an explosion of ideas for human gene therapies using gammaretroviral (MLV) and lentiviral (HIV) vectors.

The selection of DNA target sites by IN in cells is highly influenced by the interactions of IN with host cofactors and nuclear import properties of the PIC (Figure 4, see above). The selective property of MLV IN to preferentially integrate its viral DNA in the promoter regions of genes had placed a hold on human gene therapy using MLV vectors to treat SCID-X1 (x-linked severe combined immunodeficiency) in boys, which was successful[90-92]. Insertional mutagenesis lead to T-cell acute lymphoblastic leukemia in 5 of the 20 patients caused by transactivation of LMO2 or CCND2 proto-oncogenes[93,94]. Recently, a highly modified self-inactivating (SIN) MLV vector with deleted viral U3 enhancer sequences provided the same efficacy as the original MLV vectors and with no leukemogenesis produced after 33 mo[95]. Future observations of these patients using this modified MLV vector will determine further use of this kind of SIN vector. SIN HIV vectors have been used in human trials and have the advantage that they can effectively transfect resting cells due to their nuclear import properties[89,96]. Finally, SIN avian retrovirus vectors have been developed but have not been used in human gene therapy[97]. Consistent with the observation that avian retroviruses integrate randomly into cellular DNA[34,35], genome-wide analysis of integration sites by a SIN avian retrovirus vector in human hematopoietic stem/progenitor cells demonstrated that integration is random and not near hotspots containing proto-oncogenes like LMO2[98].

CONCLUSION

This review has outlined the multiple functions that the retrovirus IN has in the life cycle of retroviruses besides the key function of DNA integration. The development of the three clinical STIs (Figure 7) directed against HIV IN has provided a strong premise for supporting basic scientific research[73]. The future development of advanced STIs for pre-exposure prophylaxis, the search for new IN inhibitors and the discovery other IN functions are currently underway. The potential utilization of retrovirus vectors for human gene therapy only adds to this future spectrum for scientific investigation of the retrovirus IN.

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REVIEW

Role of ZAC1 in transient neonatal diabetes mellitus and glucose metabolism

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Abstract

Transient neonatal diabetes mellitus 1 (TNDM1) is a rare genetic disorder representing with severe neonatal hyperglycaemia followed by remission within one and a half year and adolescent relapse with type 2 diabetes in half of the patients. Genetic defects in TNDM1 comprise uniparental isodisomy of chromosome 6, duplication of the minimal TNDM1 locus at 6q24, or relaxation of genomically imprinted *ZAC1*/*HYMAI*. Whereas the function of HYMAI, a non-coding mRNA, is still unidentified, biochemical and molecular studies show that zinc finger protein 1 regulating apoptosis and cell cycle arrest (ZAC1) behaves as a factor with versatile transcriptional functions dependent on binding to specific GC-rich DNA motives and interconnected regulation of recruited coactivator activities. Genome-wide expression profiling enabled the isolation of a number of Zac1 target genes known to regulate different aspects of β-cell function and peripheral insulin sensitivity. Among these, upregulation of *Pparγ* and *Tcf4* impairs insulin-secretion and β-cell proliferation. Similarly, Zac1-mediated upregulation of *Socs3* may attenuate β-cell proliferation and survival by inhibition of growth factor signalling. Additionally, Zac1 directly represses *Pac1* and *Rasgrf1* with roles in insulin secretion and β-cell proliferation. Collectively, concerted dysregulation of these target genes could contribute to the onset and course of TNDM1. Interestingly, Zac1 overexpression in β-cells spares the effects of stimulatory G-protein signaling on insulin secretion and raises the prospect for tailored treatments in relapsed TNDM1 patients. Overall, these results suggest that progress on the molecular and cellular foundations of monogenetic forms of diabetes can advance personalized therapy in addition to deepening the understanding of insulin and glucose metabolism in general.

Key words: Diabetes mellitus; *ZAC1*; Transient neonatal diabetes mellitus 1; Genomic imprinting; Insulin; Glucose; Target genes; Tailored therapy

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Core tip: Accidents of nature leading to rare genetic diseases can provide important insights into the molecular and cellular foundations of related common diseases. Various genetic anomalies at chromosome 6q24 manifest with life-threatening transient neonatal diabetes mellitus (TNDM1). All of these genetic defects share overexpression of the maternally imprinted transcriptional regulator *ZAC1*. Genome-wide expression profiling identified a number of downstream target genes sharing a critical role in insulin secretion, β-cell proliferation, and survival. Importantly, Zac1 overexpression in β-cells spares the effects of G-protein signaling on insulin secretion opening the prospect for tailored therapy in TNDM1 patients.

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INTRODUCTION

The individual, societal, and economic burden of the diabetes epidemic has raised steadily over the last decades despite continuous refinements in dietary and pharmacological therapies[1]. On current trends, at least one in 10 people alive today are at risk to suffer from diabetes at some stage during their lifespan. At the same time, the total number of individuals being at risk to develop diabetes is predicted to double in a generation from 150 million in 2000 to 300 million by 2025. Existing medical treatments frequently ameliorate diabetic conditions without reinstating normal metabolism and thus leave many patients exposed to debilitating, or even life-threatening, impairments[2]. In conjunction with genetic predisposition (particularly in some ethnicities) the epidemic derives mainly from type 2 diabetes (T2D) and associated conditions termed "diabesity" and "metabolic syndrome". These states encompass various risk factors such as obesity, overly rich nutrition, and a sedentary lifestyle[3]. Although lifestyle modifications (balanced diet, more exercise, and weight reduction) are well-recognized to delay, or even prevent, T2D in persons at risk, such cautionary measures are notoriously hard to sustain[4].

A large number of epidemiological and experimental studies have aimed to detect the genetic and environmental factors underlying the onset, progression, and treatment response of diabetes over the last decades. Contrary to the well-established damage of insulin-producing β-cells by autoimmune processes in type 1 diabetes (T1D), the pathophysiological basis of T2D is still incompletely understood and involves multiple cellular and physiological systems influencing each other's set points, thresholds, and metabolic functions[5]. Insulin resistance typically evolves during the course of T2D as result of a diminished sensitivity to insulin's metabolic regulatory functions in several peripheral tissues (fat, muscle, and liver) aggravated by reduced exercise, aging, and obesity. T2D represents a relative, rather than an absolute insulin deficiency, as a result of a progressive malfunction of β-cells to secrete sufficient amounts of insulin for sustaining efficient carbohydrate and lipid homeostasis[6]. Conclusively, refined insight into the pathways that underlie β-cell dysfunction at a physiological and molecular scale remains desirable to advance personalized treatment of T2D.

How can we examine human β-cells when these are barely accessible? Single gene defects encoding severe β-cell dysfunction with manifest diabetes provide unique inroads to disease mechanisms if the causative gene can be isolated. In this regard, positional cloning enabled the identification of heterozygous mutations in the hepatic transcription factors HNF1A and HNF4A as the cause of early-onset diabetes[7,8]. This important discovery drove forth the subsequent discovery of a transcription factor network underpinning normal β-cell development[9] as well as susceptibility to T2D[10,11]. Hence, monogenetic diseases can guide biological studies aimed to unlock the cellular and molecular basis of the specific condition under study, but may have also important implications for related multifactorial diseases.

Based on this perspective, we will discuss in this review recent progress in the molecular genetics of transient neonatal diabetes mellitus (TNDM) and how these new findings advance insight into β-cell development and physiology. Specifically, we will focus on the transcriptional regulator ZAC1 (*z*inc-finger protein 1 regulating *a*poptosis and cell *c*ycle arrest), which is encoded by a genomically imprinted gene at 6q24 in human. TNDM is characterized by a unique clinical course manifesting prenatally with T1D and an increased risk for T2D in adolescence. Importantly, the identification of various target genes over the last years eventually enabled insight into ZAC1's role in pancreatic β-cell development and maturation and potential pharmacological treatments of newborns suffering from TNDM.

NEONATAL DIABETES MELLITUS

Neonatal diabetes mellitus (NDM), also recognized as "early-onset" diabetes mellitus, manifests unregulated hyperglycemia during the first 6 mo of life and is a rare disorder developing uniformly in all races and ethnic groups. Typically, NDM presents with intrauterine growth retardation (IUGR), reduced subcutaneous fat, failure to thrive, and low or undetectable C-peptide levels (a peptide of 31 amino acids connecting the A-chain and the B-chain of proinsulin)[12]. NDM commonly arises from mutations in genes that play a

crucial role in pancreas development, β-cell apoptosis, insulin processing, and the regulation of insulin release[13]. NDM has an incidence of 1 in 300000 to 500000 live births, whereby current data indicate that approximately over half (57%) of the cases are transient (TNDM), depend on initial insulin application, and resolve spontaneously in less than one and a half year, only to relapse in adolescence.

Different genetic defects have been identified as the source of TNDM; these include mutations in the potassium channel genes *ABCC8* (TNDM2; OMIM 310374) and *KCNJ11* (TNDM3; OMIM 610582)[14], *insulin*[15], and occasionally *HNF1B*[16]. About two-third of all cases of TNDM derive from genetic mutations or loss of genomic imprinting at a locus on 6q24 (TNDM1; OMIM 603044)[13] encoding *ZAC1* (alias *PLAGL1*), which is in the focus of the remainder of this review, and *HYMAI* (hydatiform mole *a*ssociated and *i*mprinted transcript) whose biological function remains unknown so far.

TNDM1

Clinical picture and course

TNDM1 was firstly described as a clinical entity in 1926[17]. Similar to NDM, newborns suffering from TNDM1 represent with perinatal hyperglycemia, undetectable or very low insulin levels, and severe dehydration. Altogether, this life threatening condition requires immediate insulin therapy. IUGR develops in > 95% of TNDM1 cases during the third trimester and concurs with severely reduced subcutaneous fat formation in neonates[18]. Consistent with insulin's hypothesized role in growth stimulation, postnatal substitution initiates a fast catch-up growth and normalizes TNDM1 patients' height and weight by the age of 2 years.

Additionally, TNDM1 patients can display a number of symptoms varying in penetrance and unrelated to diabetes such as macroglossia, umbilical hernia, cardiac anomalies, brain malformation, and mental impairments in lately diagnosed newborns. These features are shared among TNDM1 infants irrespective of the underlying genetic anomalies[18].

It is important to note, that insulin dependency declines within a few months postnatally (median treatment duration 3 mo) and rarely extends for up to one and a half year. Following on, TNDM1 remits and patients represent as normoglycemic, free of glycemic compromise, and sustain normal growth[19]. This reversible course seems compatible with a temporary rather than a persistent defect in the control of insulin synthesis and/or secretion. Yet, approximately one half of all TNDM1 patients develop in adolescence or early adulthood signs of T2D[20] and require dietetic as well as pharmacological treatments (sulfonylurea and insulin) to ameliorate hyperglycemia. Puberty-related hormonal changes and resumption of somatic growth are known to impose an increased demand on insulin's availability, may compromise pancreatic functions, and ultimately, lead to relapse with T2D in persons at risk.

Alternatively, the same genetic defect underlying impaired insulin secretion, and consequently IUGR, may predispose to relapse in later life[21]. Epigenetic mechanisms, particularly DNA-methylation, are thought to mediate epigenetic programming in response to early life conditions[22,23] and are increasingly recognized to interact with genetic risk factors.

Collectively, TNDM1 represents with neonatal hyperglycemia, infant remission, and adolescent relapse. IUGR and hyperglycemia suggest a decline in available insulin due to a deficit in β-cell number and/or compromised insulin secretion.

Molecular and clinical genetics of TNDM1

Paternal uniparental disomy of chromosome 6 (UPD6) was firstly discovered in two TNDM1 patients in 1995[24]. The same report presciently suggested that TNDM1 is a genomic imprinting disorder[25] - a hypothesis that has been confirmed as early as five years later[26,27]. Expression at the TNDM1 locus on chromosome 6q24 is confined to the paternally derived allele with the maternal allele being silenced. This process, termed genomic imprinting, is initiated in the respective parental germ cells and involves DNA methylation as a central mechanism[28]. As a result, the expressed paternal *ZAC1* allele is unmethylated at a confined region of 1 kb in size, the so called differentially methylated region (DMR), while the silenced maternal allele is methylated. The DMR straddles the shared promoter of *ZAC1* and *HYMAI* establishing monoallelic expression in healthy individuals. Contrarily, a defect in DMR methylation results in relaxation of *ZAC1/HYMAI* imprinting with biallelic expression in TNDM1[29]. Although it has not been formally proven whether one or both of these genes underlie TNDM1, it appears decent to assign a critical role to *ZAC1* based on its molecular and cellular properties (see below). In contrast, the non-coding RNA *HYMAI* is transcribed from the same imprinted promoter and in the same orientation as *ZAC1* and is ubiquitously expressed throughout development and maturation although its function remains still unresolved[27,30]. Notwithstanding the limited information on *HYMAI*, further studies are looked for with respect to the critical role of non-coding RNAs in the control of imprinted gene expression[28].

In addition to the proximal promoter, *ZAC1* contains a second promoter some 50 kb upstream, which is devoid of genomic imprinting, drives biallelic *ZAC1* expression, and predominates in blood cells[31]. Although biallelic expression may prevail in some tissues or across defined periods, monoallelic expression from the imprinted promoter is preserved during mouse development and seems to fulfil a particular role in tissues regulating glucose metabolism and/or showing dynamic changes in perinatal Zac1 expression (see below). Still, the possibility of postnatal switches in

promoter usage[32] during postnatal remission and/or relapse with T2D should not dismissed and requires further analysis.

Although half of the TNDM1 patients relapse with T2D in early adulthood, *ZAC1* seems not to encode a T2D susceptibility locus. Whereas the chromosomal region 6q22-25 has been initially captured in some genome-wide association studies, particularly in the Pima Indian population[33], this finding was refuted in a recent large case-control study in which not a single *ZAC1* nucleotide polymorphism (SNP) associated with T2D[34]. This study lacked however sufficient power to address a parental impact on SNP effects - an important issue since investigations in the Iceland population evidenced several imprinted loci among regions with a disease association dependent on parental origin[35].

In addition to the original discovery of UPD6, duplication of the TNDM1 region, and maternal hypomethylation at the DMR have been identified subsequently as a genetic cause of TNDM1[36].

Children suffering from UPD6 (40%) can display additional symptoms unmasked by uniparental disomy (*e.g.*, recessive mutations). Since UPD6 is the result of a stochastic reproductive error, it is non-hereditary. In contrast, duplication of 6q in TNDM1 patients (32%) comprises a critical region of approximately 160 kb and can be passed to offspring with manifestation in males[37]. The majority of these duplications are submicroscopic, differ in breakpoints, but share a similar phenotype[36].

Hypomethylation at the DMR in TNDM1 patients (28%) leads to erasure of all maternal methylated CpG residues and suggests that normal *ZAC1* gene dosage has to be severely disrupted to manifest TNDM1. It is important to note, that approximately 50% of TNDM1 patients with *ZAC1* DMR hypomethylation suffer from hypomethylation at other imprinted loci as well[38]. This raises the possibility that defects in the imprinting machinery (*e.g.*, loss of ZFP57, a zinc-finger transcription factor containing a KRAB domain[39]) or imprinted gene networks (IGN) can give rise to TNDM1[32].

In sum, TNDM1 patients show genetic defects leading to overexpression of ZAC1 from the imprinted region of chromosome 6q24. The underlying chromosomal anomalies comprise paternal UPD6, paternally derived duplication of 6q24, and maternal methylation defects at the DMR[20,29].

TRANSGENIC MOUSE MODELS OF TNDM1

ZAC1 overexpression in TNDM1 stimulated the creation of suitable mouse models to analyze the underlying pathology. For this purpose, a P1-derived artificial chromosome (PAC) of 175 kb comprising the entire *ZAC1* and *HYMAI* loci was integrated with multiple copies into the mouse genome[40]. In accord with the inheritance in human, hyperglycemia in neonate mice was confined to offspring derived from paternal transmission of the overexpressed PAC.

Interestingly, the effects from *ZAC1* overexpression in mice largely recapitulated human TNDM1 with neonate hyperglycemia, juvenile remission, and adult glucose intolerance[40]. Yet, neonate growth was not retarded at birth nor were aged mice overt diabetic. Histological examination of pancreata from TNDM1 mice suggested a reduction in the development and existence of all endocrine cell types including β-cells concomitantly with a downregulation of the important developmental transcription factors Ngn3, Pax6, and Pdx1. Puzzlingly, in late pregnancy and postnatally transgenic β-cells assumed a rapid catch-up growth as a result of increased proliferation and/or decreased apoptosis and surpassed in numbers their normal counterparts. Irrespective of this increase in neonatal β-cell numbers, insulin content and secretion were diminished and resulted in postnatal hyperglycemia. Still, an ongoing increase in β-cell numbers enabled their duplication and reinstatement of normal glucose tolerance in juvenile mice. This compensatory increase was, however, not sustained in aged mice, which suffered from an accelerated decline in β-cell numbers and compromised insulin secretory capacity when compared to controls.

Together, these findings in mice corroborate *ZAC1/HYMAI's* maternal imprinting status and evidence the impact of these genes on pancreatic β-cell proliferation, differentiation, and insulin secretion from late gestation through adulthood. Consistent with these data, ZAC1 overexpression in rodent β-cell lines[41,42] reduces glucose-stimulated insulin secretion (GSIS) as will be discussed in more detail in the section on target genes.

In contrast to ZAC1 overexpression in pancreatic β-cells, results from Zac1 deficient mice ($Zac1^{+/-pat}$) evidenced no obvious effects on β-cell proliferation or insulin secretion[43]. $Zac1^{+/-pat}$ mice represented with IUGR, altered bone formation, impaired pulmonary respiration, hydrocephaly, disturbed neural cell numbers, and neonatal lethality[43,44]. Notably the same authors[43] assigned to *Zac1* a critical role in the regulation of an IGN, which on its own may affect β-cell function and confound *Zac1* defects.

All in all, these studies support a role of *ZAC1* overexpression in TNDM1 and prompt the question through which molecular and cellular mechanisms *ZAC1* contributes to the clinical picture and course of TNDM1.

TRANSCRIPTIONAL REGULATOR ZAC1

The zinc-finger protein Zac1 was originally isolated from clonal corticotroph tumor cells derived from mice[45] and shortly afterwards from malignantly transformed ovary surface epithelial cells[46]. Zac1 overexpression studies together with appropriate knock-down experiments

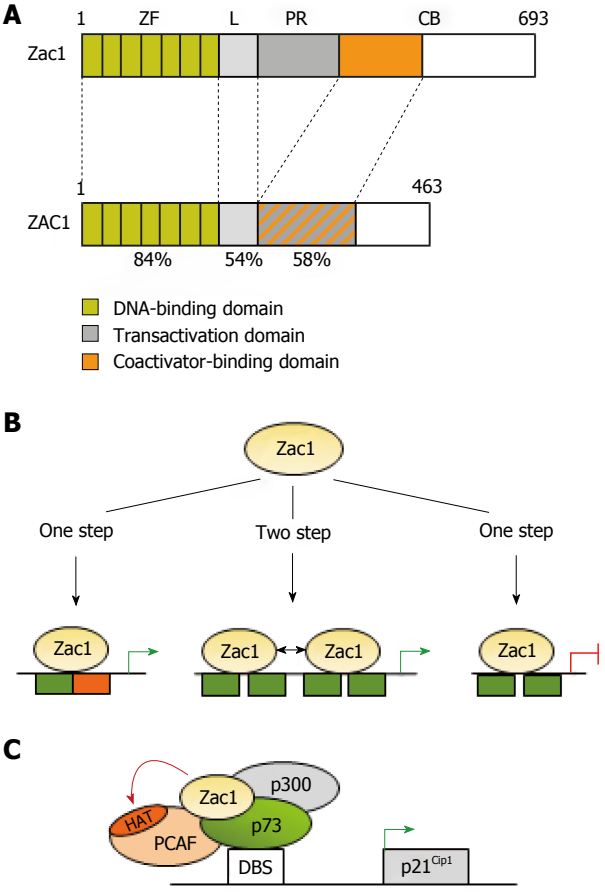

Figure 1 Transcriptional activities of Zac1. A: Schematic drawing of Zac1 proteins. Domains are boxed and numbers refer to amino acids and percentage of homology between mouse and human. The N-terminal DNA binding domain is highly conserved between human and mice and comprises seven canonical C2H2-type zinc fingers (ZF). The linker domain (L) in conjunction with the proline-rich region (PR) confers transactivation in mice, which is further enhanced through the C-terminus's coactivator binding (CB) domain. Contrarily, transactivation and coactivator recruitment jointly localize to ZAC1's C-terminus; B: Zac1 recognizes different DNA-elements to confer transcriptional regulation. Monomeric Zac1 binding to GC-rich palindromes (left) or dimerization at G/C rich direct repeat elements (middle) results in transactivation. On the other hand, monomeric Zac1 binding to G/C rich half sites causes repression (right); C: Zac1 coactivation of p73. Following recognition of its DNA-binding site (DBS), the transcription factor p73 recruits Zac1 together with the general coactivator p300 and PCAF to the *p21*^{Cip1} promoter during early neural differentiation. Due to its scaffolding function, Zac1's zinc fingers stabilize this interaction and enhance additionally PCAF's histone acetyltransferase (HAT) activity. This event enhances histone acetylation at the *p21*^{Cip1} promoter and subsequent transcription.

evidenced Zac1's role in the regulation of apoptosis and cell cycle arrest and stimulated its name-giving[45,47,48]. In agreement with these observations, human *ZAC1*[48], undergoes methylation-sensitive silencing in various tumors including ovary and mammary cancer[46,49].

Human and mouse ZAC1 proteins consist of 463 and 693 amino acids, respectively, and share a highly conserved amino-terminal DNA-binding domain enclosing seven zinc fingers conforming with the classical C2H2-type (Figure 1A). Apart from that, a central and C-terminal domain enriched in proline and glutamic acid residues, respectively, exists solely in mice[48]. The region adjacent to the DNA-binding domain, termed linker-region, together with the central proline-rich region confers transactivation in mice, which is enhanced by the C-terminus' interaction with the general coactivators p300/CBP[50]. On the other side, transactivation and coactivator recruitment are merged in ZAC1's C-terminus and illustrate how different protein motives are used species-specifically in transactivation (Figure 1A).

Monomeric Zac1 can bind to palindromic GC-rich DNA elements to confer transactivation (Figure 1B). Similarly, Zac1 dimerization at direct and reverse G/C-rich repeat DNA elements confers transactivation whereas monomeric binding to single half sites leads to repression[51] (Figure 1B). In addition to DNA binding, zinc finger 6 and 7 interact with p300 to increase (palindromes and repeats) or decrease (half sites) its catalytic activities[50]. This interrelationship between Zac1's DNA address code and enzymatic regulation of transcription is thought to support efficient regulation of target genes.

Moreover, Zac1 can coregulate the activity of various members of the nuclear receptor and p53 families without binding on its own to DNA due to its scaffolding function and concomitant coactivator regulation[52-54] (Figure 1C). The *p53* family comprises the eponym *p53* and the relatives *p63* and *p73*, all of which share critical roles in cell renewal and proliferation, cell fate commitment and differentiation, and ultimately apoptosis[55]. As an example, the transcription factor p73 tethers the coactivators PCAF/p300 and Zac1 jointly to the *p21*^{Cip1} (*Cdkn1a*) promoter in a model of early neuronal differentiation. Consistent with its scaffolding function Zac1 strengthens *via* its zinc fingers the interaction between PCAF and p300 and enhances at the same time PCAF's catalytic activity (Figure 1C)[52]. This finding evidences an authentic Zac1 coactivator function and suggests that similar mechanisms apply to Zac1-dependent nuclear receptor coregulation[53].

In summary, Zac1 transcription factor activities are determined by sequence-specific DNA binding and interconnected regulation of coactivator activities. Additionally, Zac1 can act as coregulator for unrelated transcription factors by stabilizing complex formation with recruited coactivators and enhancement of their catalytic functions.

ZAC1 TARGET GENES IN THE PANCREAS

Zac1's role as transcription factor and coregulator offers the opportunity to gain insight into its function in TNDM1 *via* the identification of downstream target genes. In the context of this review we will pay particular attention to those targets, which are expressed in pancreatic β-cell islets and are likely to share a role in insulin and glucose metabolism. Readers interested in further Zac1 functions are referred to recent reviews[32,56].

In support of *ZAC1's* potential role in TNDM1, strong immunoreactive signals were detected in the nuclei of insulin positive cells, whereas the exocrine pancreas was only sparsely stained in adult human[42]. Refined immunohistochemistry in mice evidenced strong Zac1 signals at prenatal and neonatal stages [(embryonic day 18 (E18) and postnatal day 1 (P1)] in the endocrine pancreas of both insulin-expressing and non-expressing cells. Moreover, sparse immunoreactivity was spread throughout the exocrine pancreas. Postnatally, Zac1 immunoreactivity rapidly declined in either pancreas compartment (P4 to P12) in accord with previous reports[40,57]. Subsequently, Zac1 expression was maintained at lower levels in insulin-positive cells in early adult mice (P42) but was largely absent in the parenchyma consistent with the findings in human[42].

Together, these findings corroborate ZAC1 expression in insulin-positive cells in human and mice and reveal dynamic changes in perinatal expression levels.

PAC1

PAC1 (*alias ADCYAP1R1*) encodes a G-protein coupled receptor (GPCR) for the neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) and was the first Zac1 target gene to be identified[45,58,59]. Zac1 regulates *Pac1* gene expression and related promoter reporter constructs in a cell type-specific fashion as evidenced by activation in cerebellar granule cells, epithelial kidney cells, and osteosarcoma cells *vs* inhibition in pituitary gonadotroph cells and pancreatic β-cells[58,60,61] (Hoffmann A, Spengler D. Unpublished findings. 2012). The neuropeptide PACAP was firstly purified from sheep hypothalamus[62] and has multiple functions on the neuroendocrine, endocrine, and nervous system[63]. Pancreatic islets are richly innervated *via* pancreatic ganglia that derive from the vagus dorsal motor nucleus. Both ganglia and islets contain PACAP as well as the related vasointestinal peptide (VIP) among other neurotransmitters. This distribution suggests that PACAP can act at the same time as neurotransmitter and islet endocrine peptide[64].

The VIP/PACAP receptor family comprises PAC1 (specific for PACAP), VPAC1, and VPAC2 (both with equal affinity for VIP and PACAP)[65]. Pancreatic islets express all of these receptors, which couple to stimulation of adenylate cyclase[66,67]. Increases in intracellular levels of cAMP activate protein kinase A (PKA) and guanine nucleotide exchange factors of the Epac family that enhance insulin secretion through distinct pathways involving K_{ATP} channels together with the exocytotic machinery[68].

Intracellular glucose metabolism presents the major stimulus for GSIS. This process commences with the uptake of glucose into β-cells *via* the glucose transporter protein 2 (Glut-2). Breakdown of glucose leads to ATP production and an enhanced ATP/ADP ratio followed by closing of K_{ATP} channels. Subsequently, plasma membrane depolarization and Ca^{2+} influx stimulate the exocytosis of insulin.

PACAP and VIP are thought to potentiate glucose-stimulated, but barely resting, insulin secretion by increasing intracellular Ca^{2+} concentrations[64]. In accord with this hypothesis, Pac1 null mice develop apparently normal and represent with inconspicuous resting plasma glucose and insulin levels[69]. On the other side, the response to PACAP on GSIS was reduced by half in isolated islets from Pac1 null mice. Moreover, these mice showed reduced GSIS following intravenous or oral glucose administration. Hence, *Pac1* seems to control the insulin-secretory response to insulin itself in addition to its potentiation. Consistent with this possibility, Pac1 null islets showed a diminished insulin response to glucose indicating Pac1's critical role for optimal GSIS during food intake[69]. A possible explanation for this finding is that intracellular PACAP, independently of neural release, triggers PKA activation necessary for optimal calcium influx in the context of GSIS.

In contrast to PACAP's role for insulin secretion, its effects on long-term β-cell mass have been less studied. Islets commonly decline during the course of T2D and aggravate the increased demand for insulin. Interestingly, PACAP overexpression in β-cells ameliorated the effects of streptozotocin-induced cell death as evidenced by lower plasma glucose levels in transgenic mice when compared to controls[70]. Morphometric analysis showed a tendency towards increased β-cell mass suggestive of a role for PACAP in the regulation of islet cell proliferation or differentiation. Compatible with this view, PACAP enhances the growth of pancreatic cancer cells through cAMP/PKA-dependent stimulation of the MAP kinase pathway together with the upregulation of nuclear oncogenes[71]. Relatedly, other studies suggest that intracellular PACAP protects β-cell mass by preventing harmful exogenous insults on the β-cell[72,73].

All in all, these studies raise the possibility that altered *ZAC1* dosage in TNDM1 reduces *PAC1* expression in β-cells, GSIS, the insulin-secretory response itself, and β-cell proliferation or survival on the long term.

PPARγ

Genome-wide expression analysis[74] led to the identification of *Pparγ* (peroxisome proliferator-activated receptor) as a Zac1 target gene. PPARγ belongs to the nuclear hormone receptor family with an important function in adipogenesis, lipid and glucose metabolism, differentiation, antiproliferation, apoptosis, and inflammation[75]. The 5'end of *PPARγ* is subject to alternative splicing that produces two protein isoforms differing in 30 amino acids present in the PPARγ2, but absent in the PPARγ1, amino terminus[76]. PPARγ2 expression prevails in adipose tissue, whereas PPARγ1 is more widely expressed comprising heart and skeletal muscle, kidney, liver, spleen, colon, pituitary, brain, and pancreas. ZAC1 binds *in vitro* and *in vivo* to the

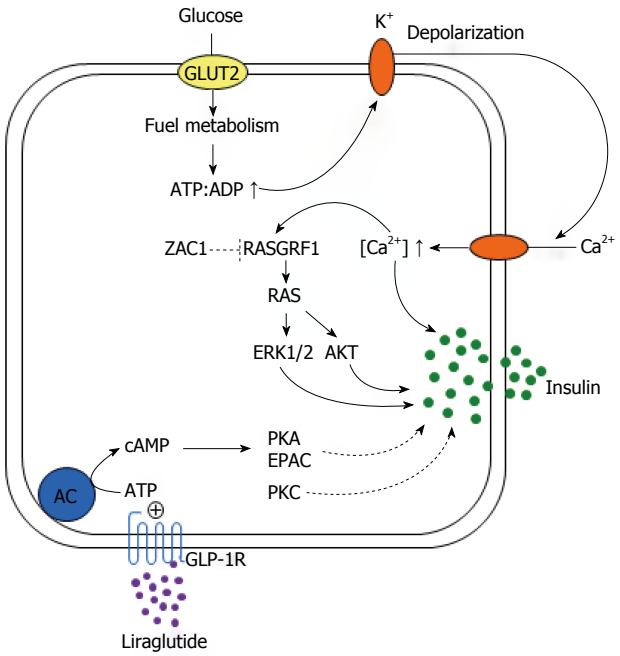

Figure 2 Roles for RAS protein-specific guanine nucleotide-releasing factor 1 in insulin secretion and transient neonatal diabetes mellitus type 1. Glucose is the main stimulus for insulin secretion. Once transported into pancreatic β-cells by glucose transporter 2 (GLUT2), metabolisation of glucose results in the production of ATP. A subsequent rise in the ATP:ADP ratio drives closure of ATP-regulated K+ channels and accumulation of intracellular K^+. Heightened K^+ concentrations lead to depolarization of the plasma membrane and promote opening of voltage-dependent Ca^{2+} channels with subsequent influx of Ca^{2+} and increases in free cytoplasmatic Ca^{2+}. This event stimulates exocytosis of insulin from the insulin-storing secretory granules through different routes including activation of the Ca^{2+}-calmodulin activated RAS protein-specific guanine nucleotide-releasing factor 1 (*RASGRF1*), a direct ZAC1 target gene. In its activated state the small G-protein RAS couples to ERK1/2 and PI3K/AKT signaling among other downstream effectors, which jointly enhance the exocytotic process of insulin secretion. ZAC1 overexpression in transient neonatal diabetes mellitus type 1 (TNDM1) is predicted to directly repress *RASGRF1* and consequently GSIS. Contrarily, PKA signaling is undisturbed in TNDM1 as well as the potentiating effects of the GLP-1R agonist liraglutide on GSIS.

proximal promoter region of *PPARγ1* in various cell types (neural cells, pituitary cells, and colon cells among others) derived from rodent or men[74]. Functionally, ZAC1 antiproliferation partly depends on PPARγ1 expression and associates with the upregulation of PPARγ target genes contributing to differentiation (*KER20* and *TSC22*), apoptosis (*POX*) or growth inhibition (*PTEN*).

Notably, PPARγ overexpression has been suggested to attenuate glucose- and potassium-induced stimulated insulin secretion[77,78]. This effect is thought to relate to PPARγ's upregulation of uncoupling protein-2 (UCP-2), which reduces the availability of ATP needed for the energy-consuming exocytotic process of insulin secretion.

On the contrary, β-cell specific deletion of *Pparγ* causes islet hyperplasia with unaltered glucose homeostasis[75]. A plausible Pparγ target gene contributing to these effects is *Pten* (phosphatase and tensin homologue deleted on chromosome 10), which diminishes intracellular levels of phosphatidylinositol *via* inhibition of phosphatidylinositol-3-kinase (PI3K). As a result, deletion of *Pten* enhances cell proliferation and survival without disrupting differentiation[79].

Interestingly, *Pten's* effects on β-cell proliferation extend to models of T2D and deteriorated insulin secretion in aged mice. In this regard, continuous β-cell specific deletion of *Pten* protects against T2D ensuing from high-fat diet or deficient leptin-signaling due to a gain in β-cell mass under basal and diabetic circumstances[80]. Similarly, a knockout of *Pten* at adult age manifests an enhanced β-cell mass and proliferation as result of a sustained response to mitogenic stimulation during aging[81].

In summary, these findings support that enhanced ZAC1 expression may impair insulin secretion and β-cell proliferation *via* induction of *PPARγ* and its downstream targets *UCP-2* and *PTEN*. Because this pathway has not formally been proven to exist in pancreatic β-cells, further studies on this transcriptional network are looked for.

Rasgrf1

The small G-protein Ras integrates multiple upstream signaling pathways and connects them to different downstream effectors comprising the Raf-MEK-Erk kinase cascade, the PI3K-Akt pathway, and RalGDS family members among others[82]. Ras alternates between an active GTP-bound and inactive GDP-bound state. This balance is tightly regulated by GTPase-activating proteins (GAPs) and guanine nucleotide-exchange factors (GEFs), respectively. Various GEF proteins have been isolated; among these RAS protein-specific guanine nucleotide-releasing factor 1 (RASGRF1) and RASGRF2 are stimulated by the G-protein subunits β and γ or by calmodulin-mediated Ca^{2+} influxes (Figure 2).

Genome-wide expression profiling for Zac1 target genes in neural cells evidenced downregulation of *Rasgrf1*[74]. Originally thought to be confined to postnatal brain, Rasgrf1 expression has been also detected in adult mice islets[83]. Consistent with this report, cytoplasmatic RASGRF1 expression colocalizes with nuclear ZAC1 expression in adult human insulin-positive cells with weak expression in the parenchyma[42]. A similar expression pattern exists in mice with Rasgrf1 and Zac1 mRNA and protein expression correlating inversely with each other pre- and postnatally. Accordingly, Rasgrf1 was weakly expressed at E18 when Zac1 expression is high and rapidly increased postnatally when Zac1 expression declined. Expression of both Zac1 and Rasgrf1 in two mouse β-cell models (Min6 and R7T1) was similar to P4 neonate islets from mice with a strong decrease of Rasgrf1 expression following Zac1 transfection. Chromatin-immunoprecipitation (ChIP) analysis evidenced that Zac1 bound at multiple predicted DNA-binding sites at the *Rasgrf1* promoter

and associated with the presence of repressive histone marks[42].

R7T1 β-cells were stably transfected to express double amounts of Zac1 as an *in vitro* model to simulate the effects of biallelic ZAC1 expression in TNDM1. Zac1 overexpressing β-cells showed moderately delayed differentiation with unaltered cell proliferation. Importantly, Zac1 overexpression impaired glucose-, insulin-, and potassium-induced depolarization and the subsequent activation of MAPK and PI3K pathways driving insulin secretion (Figure 2). Consistent with a crucial function of Rasgrf1 in Zac1-dependent insulin secretion, reinstatement of Rasgrf1 expression in Zac1 overexpressing R7T1 β-cells normalized insulin secretion[42]. Moreover, subcutaneous transplantation of parent, but not of Zac1 overexpressing R7T1 β-cells ameliorated hyperglycemia in streptozotocin-treated diabetic mice.

Notably, activated Ras may additionally switch on the GEF function of RalGEF and Tiam1. Hereby, Tiam1 facilitates the action of Rac1 with a regulatory role in GSIS[84] and RalGEF enhances the activity of the small G-protein Ral with a regulatory role in different steps of insulin exocytosis[85,86]. Hence, Zac1-dependent Rasgrf1 repression may integrate additional pathways beyond canonical MAPK and PI3K signaling, which overall result in an impaired GSIS.

Activation of GPCRs leading to an increase in intracellular levels of cAMP (see above), are known to optimize the insulinotropic effects of glucose. In this regard, the glucagon-like peptide 1 receptor (GLP-1R) stimulates insulin secretion independent of Ras pathways. Indeed, liraglutide, a clinically used GLP-1 analog, undistinguishably stimulated MAPK and PI3K pathways and downstream insulin secretion in stable mock- and Zac1-tranfected R7T1 β-cells and strongly reduced hyperglycemia following their subcutaneous transplantation in experimental diabetic mice.

Interestingly, the oral or intravenous glucose tolerance tests display a subnormal insulin response in adolescent TNDM1 patients whilst glucagon-stimulated insulin secretion is preserved[87]. Hence, β-cells in TNDM1 patients seem to sustain the capability to secrete insulin in response to stimulatory GPCR pathways, but not to glucose stimulation.

In summary, this work evidences a critical role for the regulatory Ras/Rasgrf1 dyad in GSIS. Zac1-dependent repression of Rasgrf1 diminishes the activation of Ras' downstream effectors comprising canonical and non-canonical pathways in insulin secretion. On the contrary, GPCR's capability to potentiate GSIS seems to be spared from Zac1 overexpression in accord with findings from adolescent TNDM1 patients (Figure 2).

Socs3

The neural stem cell line (C17.2) has been used for additional genome-wide expression profiling and led to the identification of *Socs3* (suppressor of cytokine signaling) as potential Zac1 target gene[88]. SOCS proteins comprise a group of eight members (SOCS1 through SOCS7 and CIS) all of which encompass a COOH-terminal SOCS box and a characteristic central Src homology-2 (SH2) domain. Cytokine stimulation leads to the activation and nuclear translocation of the signal transducer and activator of transcription (STAT) proteins that in turn upregulate the expression of *SOCS* genes among others. These transcription factors recognize their DNA address code at regulatory regions of the *SOCS* genes, and once induced, CIS, SOCS1, SOCS2, and SOCS3 form a canonical negative feedback loop that serves to attenuate further cytokine signaling[89]. Similarly, SOCS family members might also inhibit tyrosine kinase signaling, particularly by the insulin-like growth factor-1 (IGF-1) and insulin receptors (see below).

The *Socs3* gene contains multiple predicted Zac1 DNA-binding sites clustered at the regulatory upstream region and the neighboring first intron. Complementary experiments including different *Socs3* promoter reporter assays in conjunction with various Zac1 expression constructs as well as ChIP studies evidenced that Zac1 confers transactivation in an additive manner following binding to the *Socs3* promoter and first intron. Similarly, ZAC1 potently induced *SOCS3* in human neuroblastoma cells indicating that regulation of this target gene is preserved in human cells[88]. Functionally, Zac1 transiently induces Socs3 in NSCs during the course of astroglial, but not of neural, differentiation. Zac1-dependent Socs3 expression peaks at the astroglial transition to maturation and has been suggested to prevent precocious astroglial differentiation by inhibition of related signaling pathways independent of Zac1's cell cycle arrest function. In accord with this hypothesis, Zac1 knock-down advanced astroglial differentiation. Conversely, knock-down of Socs3 in Zac1 overexpressing cells reinstated timely astroglial differentiation irrespective of a Zac1-driven sustained G1 arrest.

In sum, Zac1 transactivates in a lineage-specific manner *Socs3* in NSCs and thus fine tunes astroglial differentiation independent of Zac1's cell cycle arrest function[88]. Moreover, these findings raise the possibility of a Zac1 role for Socs3 regulation in TNDM1, particularly in pancreatic islets. Although there are no experimental data available yet, we will discuss in the following part the rationale for future studies to explore this topic.

Originally viewed as negative regulators of cytokine signaling, SOCS proteins have been more recently also suggested to attenuate insulin receptor signaling[90]. Contrarily to cytokine receptors, insulin and IGF-1 receptors contain tyrosine kinase activity on their own. Once bound by its receptor, insulin triggers autophosphorylation and catalytic activation leading to the phosphorylation of various intracellular targets, including insulin receptor substrate (IRS) proteins. In

their phosphorylated state, IRSs associate with multiple SH2 domain-containing proteins and enable that multiple signaling proteins are subsequently recruited to the plasma membrane where they regulate various signal transduction pathways controlling translocation of glucose transporters from intracellular vesicles to the plasma membrane, protein and glycogen synthesis, and ultimately cell proliferation and survival[90].

Insulin resistance denotes an impaired tissue response to insulin and presents an important pathogenic determinant in the manifestation of T2D. Heightened concentrations of proinflammatory cytokines comprising tumor necrosis factor-α (TNFα), interleukins (IL-1β and IL-6), and growth hormone (GH) during injury, obesity, or infection, are considered to advance the onset of insulin resistance in peripheral tissues[6]. In this context it is important to note that SOCS1 and SOCS3 proteins have been shown to disrupt signaling complex formation at insulin or IGF-1 receptors as a result of an enhanced degradation of the adaptor proteins IRS-1 and IRS-2. Consequently, insulin signaling declines in disease-relevant organs such as liver and muscle as well as in adipose tissue[90]. Because both ZAC1 and SOCS3 are expressed in these tissues, ZAC1 overexpression in TNDM1 patients may contribute to relapse with T2D in adults *via* sustained upregulation of SOCS3 and subsequent impairments in insulin signaling.

In addition to an effect on peripheral insulin signaling, SOCS3 appears also to affect pancreatic β-cells themselves[91]. Tyrosine phosphorylation of the insulin receptor and associated IRS proteins in response to insulin binding is inhibited by IL-1β *via* upregulation of SOCS3 expression and the formation of SOCS3/insulin receptor complexes. Together, these events result in reduced PI3K signaling and impaired long term survival of β-cells[91]. Relatedly, SOCS3 has been reported to attenuate growth factor signaling important to β-cell proliferation and cell mass[92]. In this respect, overexpression of SOCS3 exclusively in β-cells caused a reduction in β-cell volume by one third in transgenic mice and reduced STAT5 phosphorylation upon GH treatment of transgenic islets when compared to wild-type ones. Similar results were obtained for adenovirus mediated SOCS3 overexpression in primary islets. Notably, SOCS3 overexpression spared the effects of GLP-1, mediated by PKA, on cell proliferation. Collectively, these results suggest that SOCS3 inhibits β-cell proliferation by counteracting growth factor receptor signaling.

On the other side, a number of reports evidenced for SOCS3 a role in β-cell survival following exposure to cytotoxic factors. As far back as 2001, Socs3 has been suggested to protect against interferon-γ and IL-1β-mediated β-cell damage by reduced transcription of inducible nitric oxide synthase (iNOS)[93]. Similarly, TNFα-induced apoptosis with a possible role in T1D is prevented by Socs3 overexpression in rat INS β-cells[94]. In further support of these findings, ciliary neurotrophic factor signaling (CNTF) increased STAT3 (cytoplasmatic and nuclear phosphorylated forms) as well as Socs3 expression and protected against cytokine- or streptozotocin-induced apoptosis[95,96]. At the same time, increased Socs3 expression impaired GSIS in isolated rat neonatal islets.

Collectively, these results suggest that the effects from SOCS3 expression are context-dependent involving growth inhibition as well as improved survival following cytotoxic insults.

Overall, ZAC1-dependent *SOCS3* upregulation may contribute to different aspects in TNDM1 by: (1) facilitating development of peripheral insulin resistance in adults; (2) inhibiting prenatal β-cell proliferation; and (3) impairing GSIS. Future experimental studies are looked for to address these topics.

p57^{Kip2}

The cyclin-dependent kinase inhibitor (CKI) p57^{KIP2} fulfils a critical task in cell cycle progression and differentiation[97]. Cyclin-dependent kinases (CDK) represent an evolutionary conserved group of serine/threonine kinases whose catalytic activities are tightly controlled through the association with various cyclins. The expression levels of cyclins oscillate periodically across the cell cycle and stimulate the sequential formation of different CDK-cyclin complexes that drive the coordinate progression through the cell cycle.

Application of the neuropeptide PACAP (see above) rapidly increases p57^{Kip2} expression in embryonic cortical precursors and reduces the activity of CDK2, entry into S-phase, and the synthesis of DNA. At the same time PACAP enhanced the association of p57^{Kip2}, but not of p21^{Cip1} or p27^{Kip1}, with the kinase complex in accord with PACAP's antimitogenic function[98]. Hence, Zac1-dependent induction of *Pac1* in neural cells, but not in β-cells where *Pac1* is repressed, may contribute *via* enhanced p57^{Kip2} expression to antiproliferation.

Relatedly, Zac1 can induce *p57^{Kip2} via* an alternative route involving the basic helix-loop-helix (bHLH) protein Tcf4. The family of bHLH proteins coordinate cell lineage decisions with proliferation and differentiation and the subsequent migration of progenitors during neurogenesis among other functions[99]. These proteins share a bHLH domain enabling homo- or heterodimerization upon DNA-binding to E-box (CANNTG) motives. Two major groups can be distinguished: the spatiotemporally expressed specification factors (*e.g.*, NeuroD, neurogenin, Mash, and Math) and the ubiquitously expressed dimerization factors known as E-proteins. The latter group comprises the two splice variants of *E2A*, *E12* and *E47*, *HEB*, and *TCF4* (also called *E2-2*)[100]. Among these genes, *Tcf4* has been recently identified as direct Zac1 target gene during early neurogenesis[101]. Zac1 recognizes multiple GC-rich DNA-elements at both the promoter and first intron of *Tcf4* to confer synergistic transactivation as shown by reporter assays with various *Tcf4* promoter reporter plasmids and Zac1 constructs.

Moreover, sequential ChIP assays corroborated that Zac1 binding associates with the presence of active histone marks at *Tcf4*.

Interestingly, E-proteins share with Zac1 a cell cycle arrest function, which has been attributed to their induction of $p57^{Kip2}$ in neuroblastoma cells[102]. Compatible with these findings several predicted E-box motifs exist in the distal $p57^{Kip2}$ promoter and confer Zac1 transactivation *via* Tcf4 upregulation[101].

Collectively, these data suggest that $p57^{Kip2}$ is an indirect Zac1 target gene *via* transactivation of *Tcf4*. In this regard, a further investigation has pointed to a critical function of $p57^{Kip2}$ in β-cell replication[103]. Hyperinsulinism of infancy presents a complex syndrome in which β-cells fail to suppress the secretion of insulin under hypoglycemic conditions[104]. The majority of patients suffer from mutations of either in *KCNJ11* (encoding two subunits of the β-cell ATP-sensitive K^+ channel)[105] or *ABCC8* (encoding a sulfonylurea receptor)[106], both of which regulate the secretion of insulin.

Hyperinsulinism of infancy can be histologically distinguished into a diffuse form affecting all β-cells and a focal one in which a single cell population undergoes clonal expansion. Genetically, focal β-cell hyperplasia results from a duplication of the p terminus of paternal chromosome 11 at the cost of the maternal allele. This recombination event results in two mutated alleles at the *ABCC8/KCNJ11* loci and UPD for all genes telomeric to this site[107]. The duplicated segment harbors several paternally silenced imprinted genes including $p57^{KIP2}$ whose expression is extinguished in mutated β-cell descendants[108]. To evidence a role in β-cell proliferation, shRNA was used to silence $p57^{KIP2}$ in islets obtained from deceased donors free of pancreatic dysfunctions[103]. Interestingly, such $p57^{KIP2}$ knock-down β-cells reassumed replication when transplanted under the kidney capsule of immunodeficient hyperglycemic mice and showed a normal glucose-induced calcium influx in support of an intact β-cell function.

In sum, this study identifies biallelic silencing of $p57^{Kip2}$ as a critical pathogenic factor for focal hyperinsulinism. Functionally, $p57^{Kip2}$ seems to act as a gatekeeper of mature β-cell replication. In this respect, ZAC1 overexpression in TNDM1 may upregulate $p57^{KIP2}$ *via TCF4* at crucial stages of prenatal and postnatal development. Transgenic *ZAC1* mice provide a valuable model to explore this hypothesis in future studies.

CONCLUSION

The steady rise of the diabetic epidemic makes further progress on the genetic, cellular, and physiological basis of this multifactorial disease mandatory. Accidents of nature leading to well-defined genetic defects can provide valuable insights into β-cell function and glucose metabolism in general. TNDM1, a rare monogenetic disease, appears of particular interest in this respect given its variable clinical course representing postnatally with severe hyperglycemia and insulin deficiency, remission within one and a half year, and frequent relapse with T2D in adolescence. Identification of the genetic defects at chromosome 6q24 (UPD6, duplication of the TNDM1 region, relaxation of genomic imprinting) point to altered dosage of monoallelically expressed *ZAC1* as important pathogenic factor in TNDM1. Consistent with this view, *ZAC1* overexpression in transgenic mice simulated major aspects of the human disease. A set of biochemical and molecular studies showed that Zac1 represents a zinc-finger protein with versatile regulatory functions dependent on DNA-binding to specific GC-rich motives and interconnected regulation of recruited coactivator activities. Additionally, Zac1 can serve as coactivator for the p53 and nuclear receptor families even so this function is presently largely unexplored with respect to TNDM1. Contrarily, the role as canonical transcription factor allowed gaining insight into ZAC1's role in TNDM1 through the identification of downstream target genes. Genome-wide expression profiling enabled the isolation of a set of direct target genes known to regulate various aspects of β-cell function and peripheral insulin sensitivity. Among these, Zac1-dependent upregulation of the transcription factors Pparγ and Tcf4 may translate into an altered expression of downstream targets sharing a role in insulin secretion (*Ucp-2*) and β-cell proliferation (*Pten* and $p57^{Kip2}$) (Figure 3, middle). Similarly, Zac1-mediated transactivation of Socs3 may inhibit β-cell proliferation and survival by attenuating growth factor signaling. Moreover, altered ZAC1 dosage in TNDM1 may also extend to SOCS3 upregulation in peripheral tissues and underlie impaired insulin signaling in relapsed patients with T2D (Figure 3, right).

Additionally, Zac1-dependent repression of downstream target genes may as well contribute to reduced insulin secretion and β-cell proliferation. In this regard, *Pac1* and *Rasgrf1* were repressed by Zac1 in β-cells, but not in neural cells, indicating that Zac1's transcriptional or coregulatory activities are tissue-specific[42,53,59,61]. Pac1 enhances the insulin-secretory response to insulin, GSIS, and β-cell proliferation (Figure 3, left). Similarly, Rasgrf1 repression attenuated Ras-mediated activation of canonical and non-canonical pathways in GSIS. Even so, Zac1 overexpression in β-cells spared the effects of GPCR on GSIS. In any case, the concerted regulation of these target genes in β-cells and peripheral tissues may partly explain the clinical course of TNMD1 ranging from fatal neonatal hyperglycemia, subsequent remission, to adolescent relapse with T2D.

Interestingly, relapsed TNDM1 patients show in response to both the oral and intravenous glucose tolerance test a subnormal insulin secretion, whilst glucagon-stimulated insulin secretion is preserved[87]. This raises the possibility that insulin secretion *via*

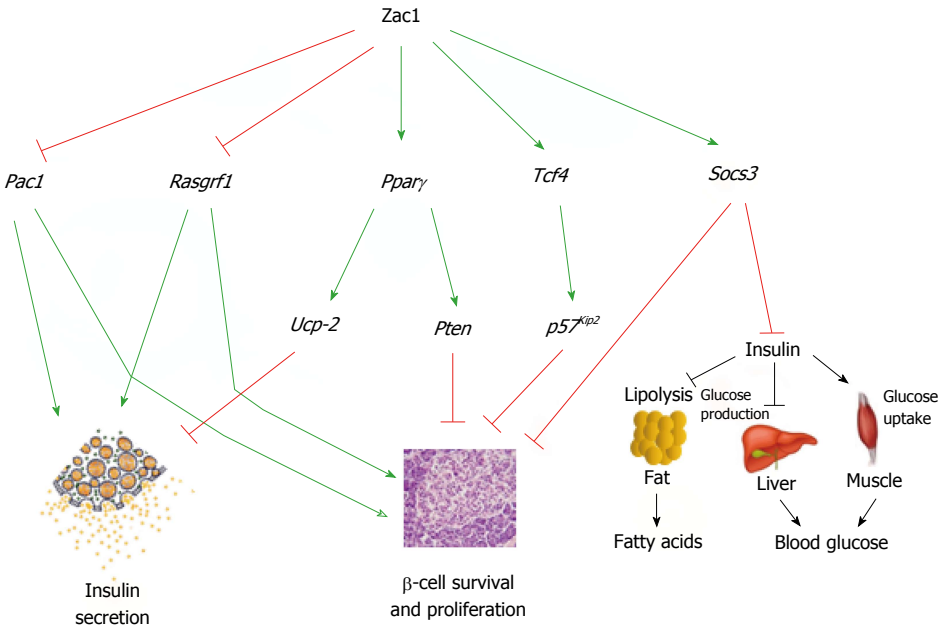

Figure 3 Integrated model of Zac1's role in β-cell function and glucose metabolism. Zac1 DNA-binding at downstream target genes confers transcriptional activation or repression in a partly tissue-specific manner in mice. In this respect Zac1 binding represses *Pac1* and *Rasgrf1* in pancreatic β-cells leading to an impaired insulin-secretory response and GSIS (left). Additionally, Zac1 upregulation of the transcription factors *Pparγ* and *Tcf4* may translate in enhanced expression of their downstream targets *Ucp-2*, *Pten*, and *p57*^{Kip2} with an inhibitory role in GSIS and β-cell proliferation (middle). Moreover, Zac1-dependent transactivation of *Socs3* may attenuate growth factor and insulin signaling in β-cells and peripheral tissues, respectively (right).

stimulatory G-protein signaling is maintained in TNDM1 and thus partly restores β-cell function while being compromised for GSIS. Indeed, treatments targeting the GLP-1 pathway have been lately reported as an interesting therapeutic option instead of insulin substitution in relapsed TNDM1 patients[109]. This finding corroborates previous work in experimental diabetic mice, where liraglutide treatment ameliorated hyperglycemia following subcutaneous transplantation of either parent or Zac1 overexpressing β-cells[42].

Overall, these results demonstrate that elucidation of the molecular and cellular foundations of rare monogenetic diabetic diseases can substantially advance our understanding of β-cell function and glucose metabolism in general. The advent of precision medicine[110] may not only offer access to personalized information we look for to stay healthy but may also lead to tailored treatments as suggested here for TNDM1 following ZAC1 overexpression.

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REVIEW

Biology of hyaluronan: Insights from genetic disorders of hyaluronan metabolism

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Abstract

Hyaluronan is a rapidly turned over component of the vertebrate extracellular matrix. Its levels are determined, in part, by the hyaluronan synthases, HAS1, HAS2, and HAS3, and three hyaluronidases, HYAL1, HYAL2 and HYAL3. Hyaluronan binding proteins also regulate hyaluronan levels although their involvement is less well understood. To date, two genetic disorders of hyaluronan metabolism have been reported in humans: HYAL1 deficiency (Mucopolysaccharidosis IX) in four individuals with joint pathology as the predominant phenotypic finding and HAS2 deficiency in a single person having cardiac pathology. However, inherited disorders and induced mutations affecting hyaluronan metabolism have been characterized in other species. Overproduction of hyaluronan by HAS2 results in skin folding and thickening in shar-pei dogs and the naked mole rat, whereas a complete deficiency of HAS2 causes embryonic lethality in mice due to cardiac defects. Deficiencies of murine HAS1 and HAS3 result in a predisposition to seizures. Like humans, mice with HYAL1 deficiency exhibit joint pathology. Mice lacking HYAL2 have variably penetrant developmental defects, including skeletal and cardiac anomalies. Thus, based on mutant animal models, a partial deficiency of HAS2 or HYAL2 might be compatible with survival in humans, while complete deficiencies of HAS1, HAS3, and HYAL3 may yet be recognized.

Key words: Hyaluronidase; Hyaluronan; Hyaluronidase 1; Mucopolysaccharidosis; Hyaluronidase 2; Hyaluronan synthase 2

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Core tip: This manuscript summarizes the phenotypes that have been associated with alterations in hyaluronan synthesis or degradation. It should serve as a reference

for those who are considering an alteration in hyaluronan metabolism as the cause of a genetic condition.

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INTRODUCTION

Hyaluronan (HA) is a straight chain polymer comprised of repeating disaccharide units of glucuronic acid and N-acetylglucosamine (→4]β-D-GlcA-[1→3]-β-D-GlcNAc-[1→)$_n$, and has substantial size heterogeneity in different tissues, although in most tissues it is greater than 10^6 daltons[1]. Although the presence of both uronic acid and an amino sugar define HA as a glycosaminoglycan (previously known as mucopolysaccharides), it differs from the other members of this family of compounds in that it is not sulfated or protein-linked, and rather than being synthesized in the Golgi, it is synthesized at the cell membrane and extruded into the extracellular space[2]. HA can assemble with tissue-specific binding proteins known as hyaladherins to form large complexes. Within the extracellular matrix, many structural and biological functions have been attributed to HA of different sizes. Examination of the phenotypes associated with naturally-occurring and laboratory-induced alterations in HA metabolism has facilitated knowledge of HA metabolism and function.

DISTRIBUTION AND FUNCTION OF HA

It is estimated that HA accounts for 15 g of the "average" 70 kg human[3]. Studies of adults have revealed the highest HA concentrations in soft connective tissues such as skin, synovial fluid, Wharton's jelly of the umbilical cord, and vitreous body[4]. The concentration of HA is also very high during embryogenesis and wound healing[5]. Low concentrations of HA are present in serum, where there is an average of 20-40 μg per liter[6,7], although there are many pathological states that are associated with and may cause increased levels of serum HA[4,8]. Based on whole body studies in the rat and other species, it is estimated that 50% of the HA is in the skin, 25% in the skeleton and joints, and most of the remaining HA is in brain, kidney, lung and muscles[4,9].

HA is critical to the assembly and structure of the extracellular matrix at both tissue and cellular levels. The large size of HA provides rigidity to some types of matrix and serves a scaffolding function for several constituents of the matrix. The hydrophilic groups of HA attract water to hydrate tissues, lubricate joints, and fill space. A HA-enriched, hydrated matrix facilitates cellular movement and proliferation that is critical for cellular migrations during early developmental processes and in the regeneration and remodeling of tissues such as during wound healing. HA has also been shown to play a role in inflammation and ovulation[10,11]. The functions of HA depend on its size; high molecular mass and low molecular mass HA typically have opposing effects on epithelial to mesenchymal transition, inflammation, and angiogenesis[12,13].

HA TURNOVER

Among extracellular matrix molecules, HA has one of the fastest rates of turnover[1,14,15], with an estimated one-third of the 15 g of HA in an average adult human turned over daily[8]. The half-life of HA was initially estimated to be 2-4 d[15], but it is now clear that the half-life varies among and within tissues because of differences in the rates of degradation or other forms of turnover. For example, HA has a half-life of about 2-5 min in blood[16,17], whereas it is estimated to be 2.5 d in the skin[18], and up to 18 d when bound to aggrecan in cartilage[19]. Approximately 30% of HA degradation occurs locally, within the tissue in which it is synthesized[18]. The remaining about 70% enters the lymphatic drainage where 90% is removed by the lymph nodes[20] and the remainder is taken up from the blood by the liver[16] and, to a lesser extent, the kidney and spleen[21]. Sinusoidal endothelial cells are responsible for uptake by the liver[22-24] *via* receptors with high affinity for HA and chondroitin sulfate[25]. Only about 1% of blood HA is eliminated each day in the urine[26], and this is limited to lower molecular mass HA (< 12000 Da) that can pass through the glomerular filtration barrier.

The internalization of HA is essential for its complete breakdown in cells, but identifying the receptor(s) responsible for uptake in each tissue has been challenging. Three receptors, HA receptor for endocytosis (HARE, also known as stabilin-2), cluster of differentiation antigen 44 (CD44), and lymphatic vessel endothelial receptor-1 (LYVE-1), are able to internalize HA for degradation *in vitro*, but their relevance *in vivo* is less clear. HARE localizes to endothelial cells of organs that internalize circulating HA including liver, lymph nodes and spleen, as well as to endothelial cells of the oviduct, corneal and lens epithelium, mesenchymal cells of the heart valve, ependymal cells of the brain, macrophages, and epithelial cells of the renal papillae[27]. HA uptake *via* clathrin-coated pits of liver endothelial cells[28] is inhibited by a HARE blocking antibody[29,30], and an anti-cluster of differentiation associated antigen 44 (CD44) blocking antibody inhibits both endocytosis and cleavage of HA[31]. The LYVE-1 receptor binds and internalizes HA in transfected human embryonic kidney cells[32]; much remains to be learned about

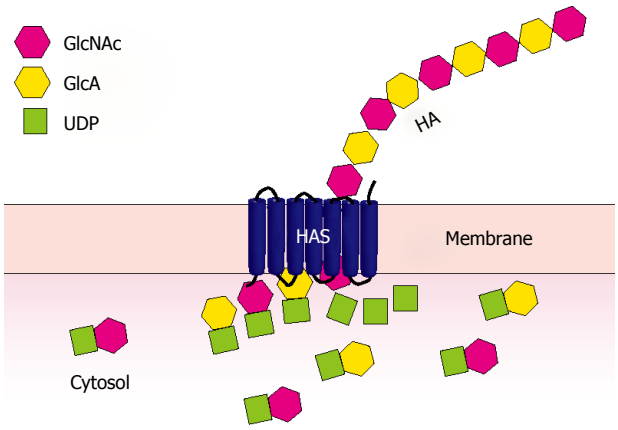

Figure 1 Hyaluronan synthesis. Uridine diphosphate (UDP)-linked sugar substrates, glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc), are sequentially added to the reducing end of the growing HA chain at the cytoplasmic aspect of the cell membrane through the action of hyaluronan synthase (HAS). The resulting sugar polymer is extruded into the extracellular matrix surrounding the cell.

its regulation[33]. It seems likely that each of these receptors can internalize HA under specific circumstances, but that HARE appears to be most important in the internalization of circulating HA. Only HARE knock-out mice have elevated circulating HA[30]; an increased concentration of serum HA was not found in mice lacking both the CD44 and LYVE-1 receptors[34]. CD44 appears to function primarily in the uptake of HA during pathological processes such as inflammation[35], although it may also have a role in local degradation of HA such as in cartilage[36] or developing heart[37].

Internalization of HA is likely preceded by its depolymerization. This concept was first proposed because the size of HA was found to be reduced after transit through lymph nodes[38]. In cartilage explants, a decrease in the size of steady-state HA compared to newly synthesized HA, also suggested that extracellular depolymerization of HA was an initial step in HA degradation[19]. A model whereby a cell surface hyaluronidase, putatively HYAL2 (described below), initiates the cleavage of HA before receptor-mediated internalization for intracellular degradation has been proposed[39]. Other modes of HA depolymerization, such as by oxygen-derived radicals[40], have also been suggested. The studies in cartilage explants also suggested that HA is internalized and degraded in conjunction with the binding domain of aggrecan[19], a finding that has not been explored in the context of current degradation models.

Among the HA binding proteins that have been identified, the recently described KIAA1199 is unique because its presence facilitates the degradation of HA[41]. Unlike CD44 which is concentrated in calveolin-rich lipid rafts, KIAA1199 appears to engage HA *via* a clathrin-coated pit pathway[41]. KIAA1199 facilitates the degradation of HA in some tissues, although it does not have homology to the active sites of mammalian hyaluronidases and it is unclear how it directly or indirectly depolymerizes HA. Variants of KIAA1199 that are associated with reduced *in vitro* HA degradative activity are described that have been found in several persons with non-syndromic hearing deficit[41,42]. The mouse orthologue of KIAA1199 also has HA degradative activity[43]. While the mode of action toward HA and the physiological role of KIAA1199 are not yet known, it has been proposed that KIAA1199 has a key role in HA catabolism in dermis and, possibly, brain[41].

The rapid rate of HA turnover in many tissues requires high rates of both synthesis and degradation. Taken together with the important functional roles of HA, one might expect that defects in either HA synthesis or degradation could cause clinical phenotypes. However, for these same reasons, it has been speculated that defects in HA metabolism may be lethal. To date, only two genetic disorders of HA metabolism have been described in humans and studies in the mouse provide evidence to support the earlier contentions that complete deficiencies in at least some HA metabolizing enzymes may be lethal during embryonic or fetal development.

HA SYNTHESIS

HA synthesis at the inner aspect of the plasma membrane takes place through the sequential transfer of uridine diphosphate (UDP)-GlcA and UDP-GlcNAc to the reducing terminus of the growing polysaccharide chain[44] (Figure 1). The first mammalian HA synthase to be identified, HAS1, was identified from cDNA libraries by functional expression; HAS2 and HAS3 were identified soon after by homology-based cloning[2]. Genes encoding these three enzymes, HAS1, HAS2, and HAS3, were mapped to human chromosomes 19q13.3, 8q24.12, and 16q22.1, respectively[45]. All of these enzymes are capable of HA synthesis, but of different average sizes and at different rates[46,47]. The three HAS enzymes are expressed in both embryonic and adult tissues; HAS2 is most broadly expressed during development, while HAS3 appears to be most broadly expressed in adult tissues[48,49]. The *in vivo* functions of the individual enzymes are becoming clearer through the characterization of mouse knockouts of these genes.

DEFICIENCIES IN HA SYNTHESIS

HAS1 is the least active of the synthases, requiring a very high concentration of UDP-GlcNAc[46,47]. No abnormalities were detected in initial studies of mice with a targeted disruption of *Has1*[50], although subsequent studies that focused on the brain revealed a mild increase in seizure activity[51]. Accelerated wound closure and decreased HA synthesis was recently demonstrated in *Has1*$^{-/-}$ *Has3*$^{-/-}$ mice, but the involvement of HAS1 appears minimal in comparison to HAS3[52]. Altered splicing of *HAS1* mRNA has

been reported in cases of malignant myeloma and Waldenström's macroglobulinemia[53,54], and inherited and acquired *HAS1* mutations are found in increased frequency in these conditions[55,56]. Despite this strong association, the relationship between this finding and the function of HAS1 remains to be determined.

HAS2 is recognized as the synthase responsible for the majority of HA synthesis during development[50]. *Has2*$^{-/-}$ mouse embryos die at embryonic day (E) 9.5 due to a failure to form the HA-rich cardiac cushion and initiate the epithelial to mesenchymal transition (EMT) required to form the valves and septa[57,58]. Exogenous HA could rescue the EMT defect in *ex vivo* explants from the developing cardiac cushion of *Has2*$^{-/-}$ embryos[58], providing evidence that HA regulates signal transduction pathways during development. The severity of the cardiac defect in *Has2*$^{-/-}$ embryos required that conditional deletions of *Has2* be used to study its role in other aspects of development. A deletion of *Has2* limited to the limb bud mesoderm of mice demonstrated that HA synthesis is also essential for growth and patterning of the limb, maturation of developing chondrocytes, and joint formation[59]. A nestin-cre driven deletion of *Has2* did not severely impact the developing brain, although the mice had increased seizure activity[51]. The conditional deletion of *Has2* in other tissues will be necessary to determine its full role in development; given the high HA levels in many developing tissues[60], other critical roles for HAS2 in development will likely be forthcoming.

Alterations in HA synthesis might be expected to affect heart development in humans and this led investigators to screen the DNA from 100 children with a ventricular septal defect for *HAS2* mutations[61]. In a single patient, a c.A1496T (p.Glu499Val) mutation was identified that reduced the activity of transiently expressed HAS2 by 40%. This suggests that partial HAS2 deficiency could be the cause of congenital heart defects in other patients. These results have not yet been replicated in independent cohorts.

The HAS3 enzyme is the most active of the HA synthases and produces HA of lower molecular mass[46]. Initial studies of *Has3*$^{-/-}$ mice showed no obvious phenotype although they were more resistant to ventilator-induced lung injury[62]. However, in recent studies focused on the brain, *Has3*$^{-/-}$ mice were found to have reduced HA, crowding of neurons and reduced extracellular space in specific regions of the brain[51]. These mice exhibited a significant increase in abnormal electrical activity and a substantial predisposition to seizure activity. The reduced extracellular space in the affected brain regions reduced the diffusion of molecules and provided evidence for a physiological role of HA in the regulation of brain extracellular space. Studies of the role of *HAS3* in human epilepsy have not yet been reported and there are only limited data regarding other clinical phenotypes[51].

INCREASED HA SYNTHESIS

The first disorder of increased HA synthesis to be described in humans concerned an individual with thickened skin and excessive skin folding[63]. The skin folding was particularly prominent in the face and limbs; skin of the trunk was thickened but not folded. That individual did not have other significant medical issues and the skin folds decreased with age. Histological studies demonstrated excessive extracellular HA in the skin. The concentration of serum HA was markedly increased, up to 1000 fold greater than normal. Although an underlying genetic cause was not reported, the authors speculated that a similar disorder affects the shar-pei dog.

The shar-pei dog was selectively bred to increase the presence and prominence of skin folds, resulting in the meat-mouth shar-pei, which has an extreme presentation of the trait and a condition termed hereditary cutaneous mucinosis. Demonstration that the material accumulating in the skin and serum is HA[64] and that these dogs have elevated levels of *HAS2* mRNA[65], led the condition to be renamed as hereditary cutaneous hyaluronosis[66]. Mapping studies linked the severe HA accumulation to duplications upstream of *HAS2* and to a familial fever syndrome that is found in shar-pei dogs[67]. However, the linkage between this duplication and the familial fever syndrome has recently been refuted[68].

Another naturally occurring animal model with increased levels of HA in the skin is the naked mole rat, a rodent recognized for its long life span (about 30 years) and resistance to cancer. Investigators seeking to identify the basis of the resistance to cancer discovered that fibroblasts derived from the naked mole rat synthesize very large quantities of high molecular mass HA[69]. The naked mole rat was found to express a reduced level of the HA-degrading enzyme, HYAL2, as well as a unique form of HAS2. HAS2 from the naked mole rat contains substitutions of Ser for Asn at two different and highly conserved regions of the enzyme. Together, these changes in HA metabolism result in increased HA of higher molecular mass. To demonstrate the relationship between the excessive high molecular mass HA and resistance to cancer, the authors used transient expression approaches to alter the levels of HAS2 or HYAL2. Either a reduction in HAS2 by siRNA, or an increase in HYAL2 by transient overexpression, resulted in a decrease in the extracellular HA and susceptibility to tumorigenesis[69].

Thus, in contrast to the severe developmental abnormalities identified in mice with HAS2 deficiency, an overexpression of HAS2 results in a much milder phenotype in rodents and dogs. It is even possible that the excessive synthesis of HA has some protective roles although the studies in the naked mole rat

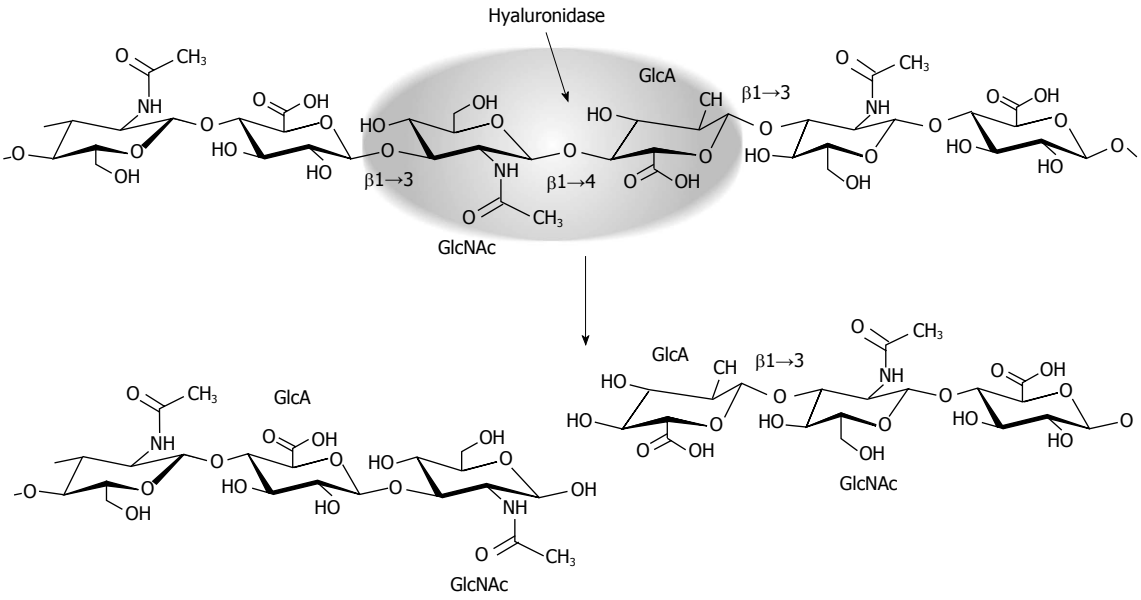

Figure 2 Hyaluronidase function. Mammalian hyaluronidases are endoglycosidases that hydrolyze the β1→4 linkage between the GlcNAc and GlcA disaccharide units that make up HA. This results in non-reducing and reducing termini; the non-reducing sugar becomes a substrate for the exoglycosidase β-glucuronidase.

indicated that other factors work in conjunction with HA to mediate the cancer resistance phenotype[69]. In humans, a better understanding of the full range of phenotypes that might be associated with alterations in HAS2 expression awaits the identification of additional patients.

DEGRADATION OF HA

Within the cell, the complete degradation of HA to monosaccharides requires the concerted action of an endoglycosidase, hyaluronidase, and the exoglycosidases β-N-acetyl-D-hexosaminidase and β-D-glucuronidase[70]. As an endoglycosidase, hyaluronidase cleaves the hyaluronan polymer internally at β1→4 linkages between β-N-acetylglucosaminine and β-D-glucuronic acid (Figure 2). The resulting oligosaccharides become substrates for the exoglycosidase β-D-glucuronidase which hydrolyzes the terminal non-reducing glucuronic acid, leaving a non-reducing β-N-acetylglucosaminine that can then be hydrolyzed by β-N-acetyl-D-hexosaminidase[71].

Two forms of hyaluronidase were originally characterized in human tissues, one with a neutral pH optimum that was rich in testes, and a second with an acid pH optimum that was found in most solid tissues and serum[72]. A glycosylphosphatidylinositol (GPI)-linked sperm protein important for fertilization, PH-20, was later found to have sequence similarity to bee venom hyaluronidase[73], and was soon recognized as the neutrally active hyaluronidase[74]; its gene, *SPAM1*, was mapped to human 7q31 (and mouse chromosome 6A2)[75]. The acid-active hyaluronidase in human plasma, HYAL1, was isolated[76], and mapped to human chromosome 3p21.3 (mouse chromosome 9F1-F2), a region previously characterized as having a tumor suppressor locus[77]. The human genome sequence allowed additional putative hyaluronidases to be identified, including HYAL2 which encodes an enzyme with weak activity toward high molecular mass HA[78] and mapped in close proximity to HYAL1[79]. Bioinformatic approaches revealed two tandemly repeated groups of three genes, which mapped to human chromosome 3p21.3 (centromere-*HYAL2-HYAL1-HYAL3-telomere*)[80] and 7q31.3 (centromere-*SPAM1-HYAL4-HYALP1-telomere*)[81,82]. An additional gene, *Hyal5*, is found downstream of *Spam1* in mice[83]. These genes were identified as putative hyaluronidase homologues based on sequence conservation but further studies demonstrated that *HYALP1* is an expressed pseudogene in humans[81], HYAL4 encodes a chondroitinase[84,85], and HYAL3 has no detectable *in vitro* activity[86,87]. Additional expression studies using human and mouse tissues showed that *HYAL1/Hyal1*, *HYAL2/Hyal2*, and *HYAL3/Hyal3* are broadly expressed in somatic tissues[81,88], while the expression of *SPAM1* is limited primarily to the testes[75] and HYAL4 to skeletal muscle and placenta[81]. The recently described HA binding/degradative protein, KIAA1199, is expressed in many tissues including brain, lung, pancreas, testis and ovary but is not expressed in liver, kidney or spleen[89].

HYALURONIDASE DEFICIENCIES

The first individual to be identified with a hyaluronidase deficiency was a young girl who had mild short stature, mild facial dysmorphism including a flattened nasal bridge, a bifid uvula and a submucosal cleft palate, and a presenting complaint of multiple periarticular

soft tissue masses involving both large and small joints; there was no evidence of visceral or neurological involvement. Histological and ultrastructural analyses of biopsies showed the periarticular masses to have a synovium that was massively expanded with macrophages containing numerous lysosomes containing fibrillar storage material; the skin showed similar pathology with fibroblasts containing increased numbers of lysosomes with excess macromolecular substrate. This work suggested some type of lysosomal storage disorder. Early biochemical investigations disclosed markedly elevated levels of serum HA and a deficiency of acid pH optimum hyaluronidase (and chondroitinase) and the condition, a new lysosomal storage disorder, was termed mucopolysaccharidosis IX[90]. This patient was subsequently found to have a 1361del37ins14 mutation in *HYAL1* that was predicted to result in a premature stop codon on one allele, and a c.G1412A (p.Glu268Lys) on the other allele; no HYAL1 activity was detectable[91]. Three additional patients with HYAL1 deficiency have since been identified in a consanguineous family of Saudi Arabian origin[92]. The proband in this family was initially characterized with idiopathic juvenile arthritis that did not respond to treatment with non-steroidal anti-inflammatory drugs. Two siblings of the proband were subsequently found to have joint abnormalities including joint effusions, synovial proliferation, and cysts. No serum HYAL1 activity was detected in any of the patients and all of them were homozygous for a c.104delT (p.Val35AlafsX25) mutation. The phenotypes of all 4 individuals with HYAL1 deficiency are less severe than that predicted for human hyaluronidase deficiency based on the broad distribution of HA in the human body and the significance of HA in many developmental and remodeling processes. Taken together, this information suggests that other enzymes (partially) compensate for the deficiency of HYAL1.

Consistent with the phenotype of persons with mucopolysaccharidosis IX, a *Hyal1*$^{-/-}$ mouse model did not show a generalized accumulation of HA[93]. The only detectable phenotype was a progressive loss of articular cartilage proteoglycan in the knee joint, starting as early as 3 mo of age. The mild phenotype in the mouse model is likely due, at least in part, to compensation by certain exoglycosidase activities, as mice deficient in both HYAL1 and β-hexosaminidases A and B (*Hyal1*$^{-/-}$*Hexa*$^{-/-}$*Hexb*$^{-/-}$) showed global HA accumulation that was significantly higher than that in either *Hyal1*$^{-/-}$ or *Hexa*$^{-/-}$*Hexb*$^{-/-}$ mice[94]. It seems likely that except in the joints the levels of β-hexosaminidase and β-glucuronidase are adequate to compensate for HYAL1 deficiency in mice, and by extrapolation, in humans as well. Interestingly, female *Hyal1*$^{-/-}$ mice had prolonged fertility; the basis for this is currently unclear[95].

HYAL2 is a broadly expressed glycosylphosphatidylinositol-linked cell surface protein whose localization and activity have been controversial. Its activity is weak, but has been successfully demonstrated toward high molecular mass HA which it appears to cleave to 20 kDa fragments[86,96]. HYAL2 interacts with CD44 and NHE1 proteins[97,98], which has led to the model shown in Figure 3, proposing that HYAL2 initiates the breakdown of extracellular HA, in partnership with the Na/H exchanger isoform 1 (NHE1) which acidifies the local environment[39]. That model further proposes that fragments generated by HYAL2 are internalized by receptor-mediated endocytosis. The generation of a *Hyal2*$^{-/-}$ mouse which exhibited mild anemia, craniofacial abnormalities, and evidence of pre-weaning lethality, did not completely support this model, as generalized storage of HA was not detected[99]. However, further studies demonstrated substantial (2/3) pre-weaning lethality in *Hyal2*$^{-/-}$ mice and severe cardiopulmonary dysfunction leading to death in 54% of *Hyal2*$^{-/-}$ mice by an average of 3.2 mo of age[100]. Accumulating extracellular HA in the heart, lung, and serum of these mice was of higher than average molecular mass, consistent with the model that HYAL2 may be important in initiating the depolymerization of HA in the extracellular matrix. The pre-weaning lethality in the mice suggests that a complete deficiency of HYAL2 may cause a severe, if not lethal, condition in humans, although the genetic background may provide important modifying effects that might either mitigate or exacerbate any of the above mentioned monogenic biosynthetic or catabolic enzyme disorders.

HYAL3 is broadly but weakly expressed in somatic cells. Although it is homologous to the other hyaluronidases, no activity has been clearly associated with the enzyme[86,87]. *Hyal3*$^{-/-}$ mice did not accumulate HA, and the only detectable phenotype was a minor change in the histopathology of the lungs[101]. However, the overexpression of HYAL3 in cultured baby hamster kidney cells resulted in increased HYAL1 activity, suggesting a role for HYAL3 in HA metabolism[87].

PH-20 (SPAM1) plays a role in both penetration of the cumulus barrier and binding of the zona pellucida during fertilization[102,103]. When it was initially mapped to human chromosome 7q31 and shown to be specifically expressed in the testes, it was thought that mutations in the gene might be identified as a cause of infertility[75], but no reports of mutations have been made. Studies in *Spam1*$^{-/-}$ mice have not provided insights regarding phenotype(s) that might be associated with human SPAM1 mutations because HYAL5 in mice can compensate for SPAM1 deficiency[104-106].

CONCLUSION

It is clear that the range of conditions that result from abnormalities in HA metabolizing enzymes is only partially understood. Monogenic dysfunction of enzymes involved in HA synthesis and HA catabolism can have marked phenotypic effects that can involve many

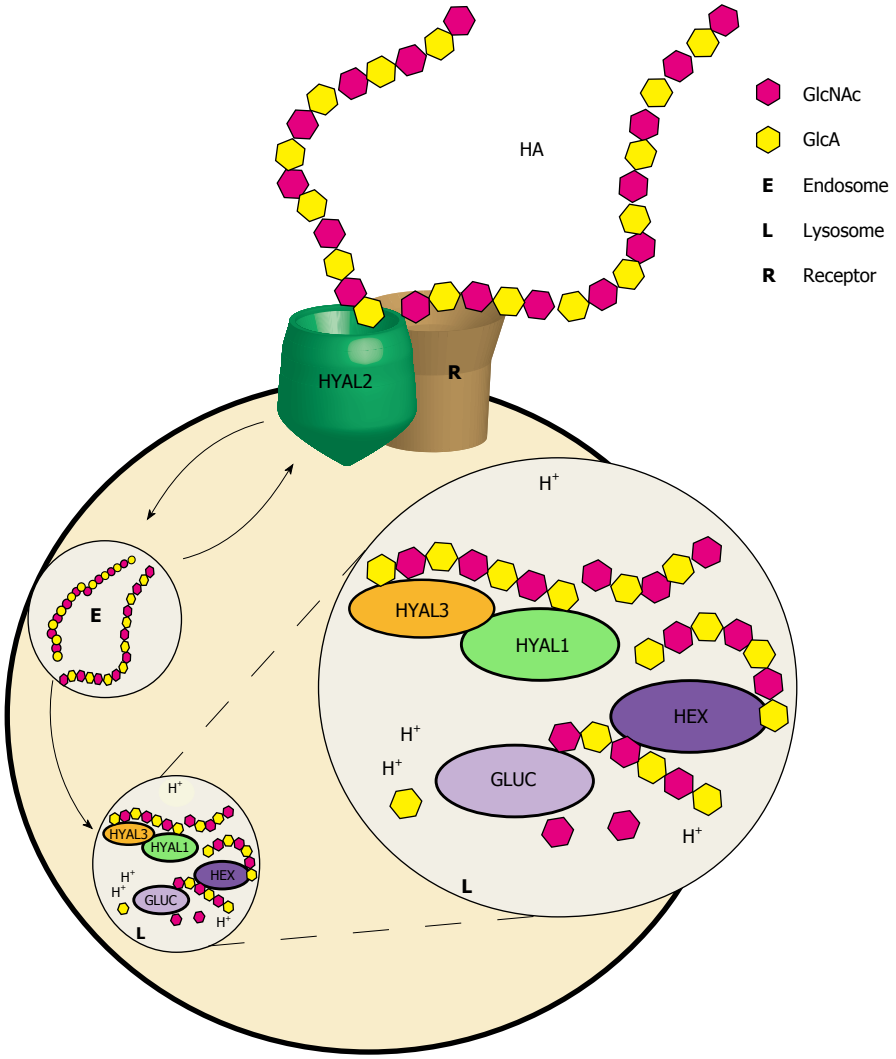

Figure 3 Proposed model for hyaluronan breakdown. Hyaluronan (HA) bound by a cell- or matrix-associated receptor such as CD44, HARE, or LYVE-1 is proposed to be hydrolyzed to intermediate-sized fragments by the GPI-linked HYAL2. The resulting fragments are then internalized by receptor-mediated endocytosis and transported to lysosomes. Once inside the lysosome, further degradation takes place through the action of the acid-active HYAL1. HYAL1 cleaves the intermediate-sized HA fragments to smaller fragments such that they become substrates for the sequential action of the exoglycosidases, β-glucuronidase (Gluc) and β-N-acetylhexosaminidase (Hex) which hydrolyze terminal GlcA and GlcNAc, respectively. The role of HYAL3 is unclear although its overexpression increases HYAL1 activity in cell culture based studies.

tissues and organ systems. It is likely that the ongoing broad application of next generation sequencing to identify the causes of unexplained phenotypes in humans will identify novel human conditions that result from variations in the HYAL and HAS enzymes and broaden the spectrum of already described conditions, furthering our understanding of their *in vivo* functions.

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REVIEW

Molecular basis of cleft palates in mice

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Abstract

Cleft palate, including complete or incomplete cleft palates, soft palate clefts, and submucosal cleft palates, is the most frequent congenital craniofacial anomaly in humans. Multifactorial conditions, including genetic and environmental factors, induce the formation of cleft palates. The process of palatogenesis is temporospatially regulated by transcription factors, growth factors, extracellular matrix proteins, and membranous molecules; a single ablation of these molecules can result in a cleft palate *in vivo*. Studies on knockout mice were reviewed in order to identify genetic errors that lead to cleft palates. In this review, we systematically describe these mutant mice and discuss the molecular mechanisms of palatogenesis.

Key words: Tbx1; Submucosal cleft palate; Incomplete cleft palate; Palatal shelf; Palatogenesis; Knockout mice

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Core tip: Cleft lip and/or palate is one of the most frequent congenital craniofacial anomalies observed. Multifactorial conditions, including genetic and environmental factors, induce the formation of cleft palates. We screened knockout mice with cleft palate phenotypes and observed approximately 180 mice with the anomaly. In order to understand the molecular regulatory mechanisms of palatogenesis and to identify genetic errors that lead to cleft palates, we aimed to review studies performed using knockout mice with cleft palates.

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INTRODUCTION

Cleft lip and/or palate (CL/P) is the most frequent congenital craniofacial anomaly observed in humans, with an incidence of 1 per 700 births worldwide[1]. Furthermore, 55% of the patients with CL/P are reported to have a multiple malformation syndrome[2]. CL/P involves a multifactorial etiology, both genetic and environmental. Teratogens that cause CL/P in humans include common environmental exposures, such as alcohol, smoking, infections, dioxin, estrogen, retinoic acid, and altitude (reviewed by Murray[1]). The offspring of parents with CL/P present a higher incidence of CL/P than those without a family history[1]. Gene-environment interactions for non-syndromic CL/P have also been reported[1]. Cleft palate (CP) cases include complete CP, incomplete CP, and soft palate clefts. The mildest form of cleft palates is the soft palate cleft or bifid uvula because the initial palatal fusion occurs in the anterior region of secondary palatal shelves. Incomplete CP and soft palate clefts can manifest together with submucosal CP. This review focuses on studies performed using knockout mice with CP, aiming to clarify the molecular regulatory mechanisms of palatogenesis and to identify genetic errors underlying mammalian cleft palates.

MAMMALIAN PALATOGENESIS

The palate is formed with the primary and secondary palate. The primary palate is derived from the frontonasal prominence and becomes a small anterior part of the adult hard palate. The secondary palatal shelves extend bilaterally from the internal aspects of the maxillary prominences and will become the adult hard and soft palates. The process of palatogenesis consists of several stages: palatal shelf formation, elevation, and midline fusion of the palatal shelves (Figure 1). The secondary palatal shelves develop between embryonic day (E) 11.5 and 12.5 in the mouse embryo (Figure 1A). At E13.5, the palatal shelves grow downward on each side of the tongue (Figure 1B). As the jaws develop, the tongue descends and the palatal shelves elevate to a horizontal position above the dorsum of the tongue (E14). Continuing their growth, the bilateral palatal shelves meet at the midline and fuse between E14.5 and E15.5 (Figure 1C).

The palatal shelves are composed of the neural crest-derived mesenchyme and ectoderm-derived epithelia, which cover the palatal mesenchyme (Figure 1D). Both elevation and fusion of the secondary palatal shelves occur in the midline from anterior to posterior. The secondary palatal shelves also fuse with the primary palate, separating the oral and nasal cavities. The anterior two-thirds of the palate forms the hard palate with neural crest-derived palatal bones (Figure 2A). The posterior one-third of the palate forms the bone-free soft palate and is involved in the palatopharyngeal sealing. Disruption at any stage of the formation, elevation, growth, or fusion of the secondary palatal shelves results in CP[3].

MOUSE MODELS FOR STUDYING THE MOLECULAR MECHANISMS OF PALATAL DEVELOPMENT

Major advances have been achieved regarding the molecular mechanisms that regulate palatal development using genetically engineered mice. Deletions in many genes of mice result in CP and the most frequent phenotype seen is complete CP (Figure 2B). Uniquely, *Tbx1*$^{-/-}$ mice present various phenotypes of CP[4], including complete CP (Figure 2B), incomplete CP (Figure 2C), and anterior CP (Figure 2D). Bone staining showed that some mice potentially had a submucosal CP (Figure 2G). These observations are in agreement with various CP phenotypes in humans.

In order to elucidate the molecular pathogenesis of CL/P, we conducted a literature search on PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and the Mouse Genome Informatics (MGI) from the Jackson Laboratory (http://www.informatics.jax.org). The search was limited to knockout mice with CP and excluded the teratogen-induced CP (Table 1). We also investigated diseases/syndromes using the Online Mendelian Inheritance in Man (OMIM) (http://omim.org). Not all the molecules involved in cleft palates in mice are correlated to CL/P in human (Table 1). When genes in Table 1 were analyzed by biological function using BioCarta (http://www.biocarta.com) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (http://www.genome.jp/kegg/pathway.html), the transforming growth factor (TGF), hedgehog, Wnt, fibroblast growth factor (FGF), and mitogen-activated protein kinase (MAPK) signaling pathways were found to be critical in palatogenesis (Table 2). When genes were analyzed by molecular function using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) database (http://pantherdb.org)[5], the most significantly enriched molecular function was the "transcription factor", especially the "homeobox transcription factors" (Table 2 and Figure 3).

To analyze mutant mice with cleft palates, the defects in palatal shelf development were divided into the following six categories (Table 3), which were modified from a previously published classification[3]. The first category is the failure of the palatal shelf formation. The gene mutations affect the initial development of the palatal shelf. The second one is the abnormal fusion of the palatal shelves and the mandible or tongue. Oral fusions between the palatal shelves and the tongue or mandible are rare. In *Tbx1* (T-box 1) knockout mice, the posterior part of the palatal shelves fuse to the mandible, inhibiting the

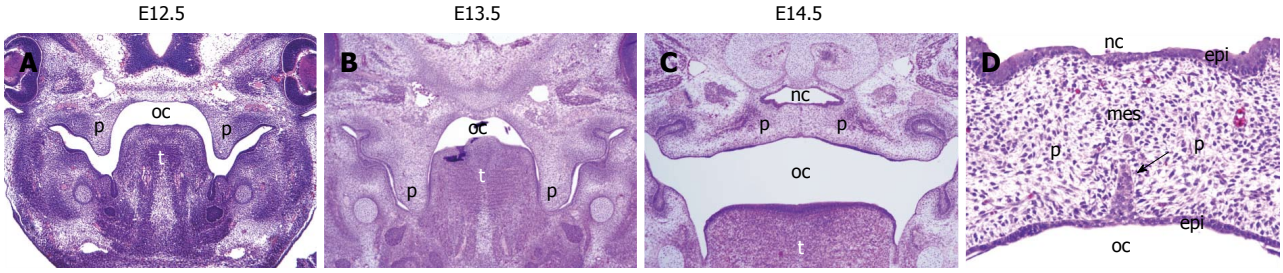

Figure 1 Palatogenesis in mice. Hematoxylin and eosin staining of coronal sections of the head of a wild-type mouse at embryonic day (E) 12.5 (A), E13.5 (B), and E14.5 (C, D). A: Mouse palatal shelves (p) develop from the maxillary prominences; B: By E13.5, the palatal shelves grow downward on each side of the tongue (t); C and D: At E14.5, the palatal shelves face each other along the midline above the tongue and fuse, separating the oral cavity (oc) from the nasal cavity (nc). The arrow in (D) indicates the medial edge epithelial (MEE) cells that constitute the midline epithelial seam. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Southwestern Medical Center and Tokyo Medical and Dental University. mes: Mesenchyme; epi: Epithelium.

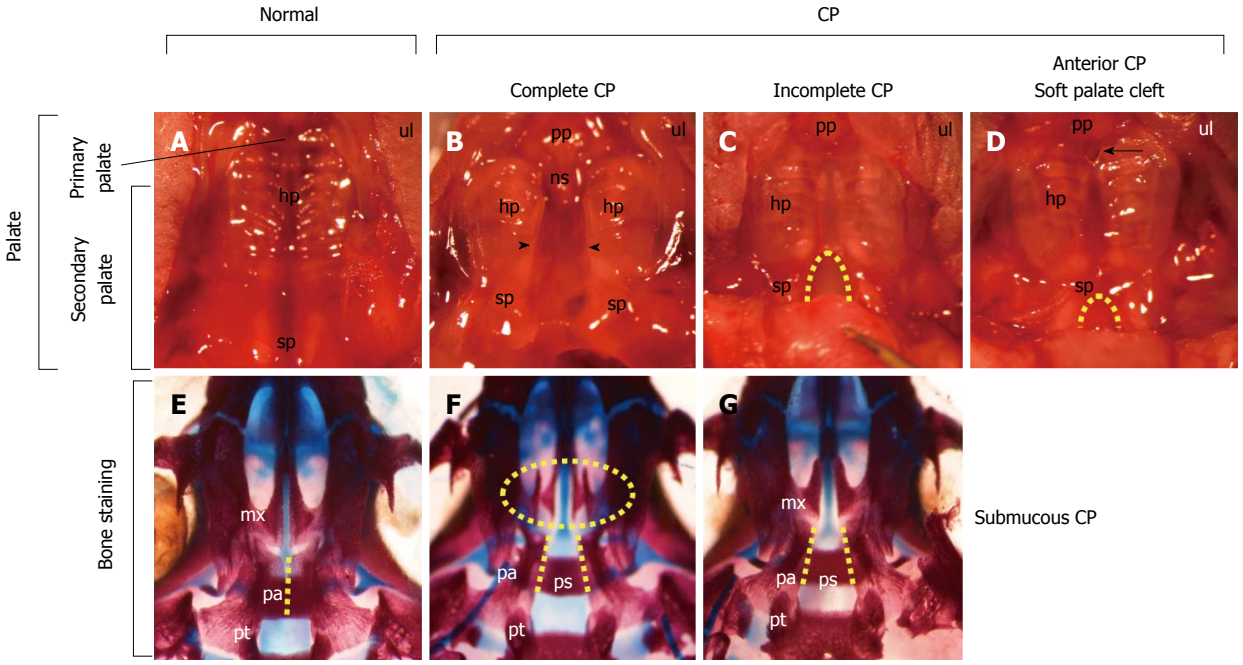

Figure 2 View of the palate from wild-type and *Tbx1*$^{-/-}$ mice with cleft palates. A-D: Ventral view of the maxilla of newborn wild-type (A) and *Tbx1*$^{-/-}$ mice with cleft palates (B-D). The palate consists of the primary palate (pp) and the secondary palate (sp), which consists of a hard palate (hp) and a soft palate (sp) (A). *Tbx1*$^{-/-}$ mice show complete cleft palate (CP) (arrowheads in B), incomplete CP (dashed line in C), and soft palate clefts associated with anterior CP (dashed line in D). An anterior CP (an arrow in D) is present at the junction between the primary palate and secondary palate, while the posterior palate remains fused; E-G: Ventral view of the cranial base of newborn wild-type (E) and *Tbx1*$^{-/-}$ mice (F, G) stained with alizarin red for mineralized bone and alcian blue for cartilage. Fusion of the bilateral palatal bones (pa) observed in the wild-type (dashed line in E) is absent in *Tbx1*$^{-/-}$ mice (dashed lines in F, G). The palatal shelves in the maxilla (mx) of *Tbx1*$^{-/-}$ mice with complete CP (oval dashed line in F) failed to grow toward the midline. Note the visible presphenoid bone (ps) associated with CP (F, G). Modified and used with permission from Funato *et al*[4]. ns: Nasal septum; pt: Pterygoid bone.

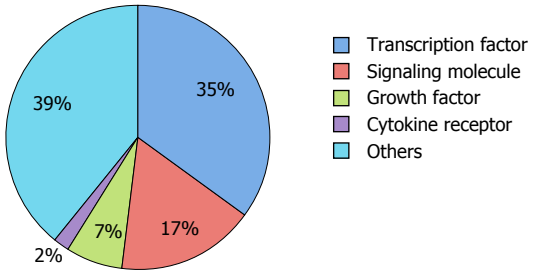

Figure 3 Gene ontology analysis of genes associated with cleft palate in mice. Gene ontology analysis of genes associated with cleft palate in mice was performed using the PANTHER classification system (http://pantherdb.org). The most significantly enriched molecular function was the "transcription factor" (P = 1.2 × 10^{-12}). A P value less than 0.05 was considered statistically significant.

elevation of the palatal shelves[4]. The third category is the failed or delayed palatal shelf elevation. Ablation of *Pax9* (paired box gene 9), *Pitx1* (paired-like homeodomain 1), *Gli2* (GLI family zinc finger 2), or *Osr2* (Odd-skipped related transcription factor 2) in the palatal mesenchyme results in the failed palatal shelf elevation[6-9], suggesting crucial roles for these transcription factors in controlling the mesenchymal cells during palatal shelf elevation. The fourth one is the failure of the palatal shelf development after elevation. The loss of *Msx1* (msh homeobox 1) and *Lhx8* (LIM homeobox gene 8) and the conditional ablation of *Tgfbr2* (transforming growth factor,

Table 1 Molecules involved in cleft palate in mice

Knockout mice with cleft palates			Humans		
Gene/category	**Protein**	**Ref.**	**OMIM**	**Syndrome**	**CL/P**
Growth factors, antagonist, and receptors					
Acvr1/Alk2 (*Wnt1-Cre*-mediated ablation)	Activin A receptor, type Ⅰ	[33]	[1]102576	Fibrodysplasia ossificans progressiva	nr
Acvr2a	Activin A receptor, type ⅡA	[34]	[1]102581	nr	nr
Bmp4	Bone morphogenetic protein 4	[35]	[1]112262	Microphthalmia, syndromic 6 Orofacial cleft 11	r
Bmp7	Bone morphogenetic protein 7	[36]	[1]112267	nr	nr
Bmpr1a/Alk3 (*Nestin-Cre*-mediated ablation)	Bone morphogenetic protein receptor, type ⅠA	[35]	[1]601299	Juvenile polyposis syndrome, Polyposis syndrome	nr
Chrd	Chordin	[37]	[1]603475	nr	nr
Ctgf	Connective tissue growth factor	[38]	[1]121009	nr	nr
Edn1	Endothelin 1	[39]	[2]131240	Auriculocondylar syndrome 3	r
Egfr	Epidermal growth factor receptor	[17]	[1]131550	nr	nr
Fgf9	Fibroblast growth factor 9	[40]	[1]600921	uc	nr
Fgf10	Fibroblast growth factor 10	[13,41]	[1]602115	Aplasia of lacrimal and salivary glands LADD syndrome	nr
Fgf18	Fibroblast growth factor 18	[42,43]	[1]603726	nr	nr
Fgfr1	Fibroblast growth factor receptor 1	[44]	[1]136350	Nonsyndromic cleft lip/palate Hartsfield syndrome Hypogonadotropic hypogonadism 2 Pfeiffer syndrome	r
Fgfr2 (knockout) (*Krt14-Cre*-mediated ablation)	Fibroblast growth factor receptor 2	[13,45]	[1]176943	Apert syndrome Crouzon syndrome Pfeiffer syndrome Saethre-Chotzen syndrome	r
Fst	Follistatin	[46]	[1]136470	nr	nr
Gabrb3	Gamma-aminobutyric acid A receptor, beta 3	[47]	[1]137192	Epilepsy, childhood absence, susceptibility to, 5	r
Gdf11/Bmp11	Growth differentiation factor 11	[48]	[1]603936	nr	nr
Gpr124	G protein-coupled receptor 124	[49]	[1]606823	nr	nr
Inhba	Inhibin, beta A/activin A	[50]	[1]147290	nr	nr
Pdgfc	Platelet-derived growth factor C	[51]	[1]608452	nr	r [52]
Pdgfra (knockout) (*Wnt1-Cre*-mediated ablation)	Platelet-derived growth factor receptor, alpha polypeptide	[53,54]	[1]173490	Gastrointestinal stromal tumor, somatic Hypereosinophilic syndrome, idiopathic, resistant to imatinib	r
Tgfb2	Transforming growth factor, beta 2	[55]	[1]190220	Loeys-Dietz syndrome, type 4	r
Tgfb3	Transforming growth factor, beta 3	[15,16,18]	[1]190230	Arrhythmogenic right ventricular dysplasia 1	r
Tgfbr1/Alk5 (*Wnt1-Cre*-, and *Nestin-Cre*-mediated ablation)	Transforming growth factor, beta receptor Ⅰ	[56,57]	[1]190181	Loeys-Dietz syndrome, type 1	r
Tgfbr2 (*Wnt1-Cre*-, and *KRT14-Cre*-mediated ablation)	Transforming growth factor, beta receptor Ⅱ	[12,58]	[1]190182	Loeys-Dietz syndrome, type 2	r
Vegfa	Vascular endothelial growth factor A	[59]	[2]192240	nr	nr
Membrane proteins					
Ceacam1	Carcinoembryonic antigen-related cell adhesion molecule 1	[60]	[1]109770	nr	nr
Efna5	Ephrin A5	[61]	[1]601535	nr	nr
Efnb1	Ephrin B1	[62]	[1]300035	Craniofrontonasal dysplasia	r
Efnb2	Ephrin B2	[63]	[1]600527	nr	nr
Fzd2	Frizzled class receptor 2	[64]	[1]600667	nr	nr
Itga5 (knockout) (*Mesp1-Cre*-mediated ablation)	Integrin alpha 5	[65,66]	[1]135620	nr	nr
Itgb1 (*Col2a1-Cre*-mediated ablation)	Integrin beta 1	[67]	[1]135630	nr	nr
Itgb8	Integrin beta 8	[68]	[1]604160	nr	nr
Jag1 (*Wnt1-Cre*-mediated ablation)	Jagged1	[69]	[2]601920	Alagille syndrome	nr
Jag2	Jagged2	[70]	[1]602570	nr	nr
Kcnj2	Potassium inwardly-rectifying channel, subfamily J, member 2	[71]	[1]600681	Andersen syndrome Atrial fibrillation, familial, 9 Short QT syndrome 3	r

Lrp6	Low density lipoprotein receptor-related protein 6	[72]	[1]603507	nr	nr
Ror2	Receptor tyrosine kinase-like orphan receptor 2	[73]	[1]602337	Robinow syndrome, autosomal recessive Brachydactyly, type B1	r
Ryk	Receptor-like tyrosine kinase	[74]	[1]600524	nr	nr
Ryr1	Ryanodine receptor 1, skeletal muscle	[75]	[1]180901	Central core disease King-Denborough syndrome Minicore myopathy with external ophthalmoplegia	nr
Sc5d/Sc5dl	Sterol-C5-desaturase (fungal ERG3, delta-5-desaturase) homolog (S. cerevisae)	[76]	[1]602286	Lathosterolosis	nr
Shh (*KRT14-Cre*-, and *Sox2-Cre*-mediated ablation)	Sonic hedgehog	[13,77]	[1]600725	Holoprosencephaly-3 Microphthalmia with coloboma 5 Single median maxillary central incisor	r
Smo/Smoh (*Wnt1-Cre*-mediated ablation)	Smoothened, frizzled class receptor	[78]	[1]601500	Basal cell carcinoma, somatic	nr
Tctn2	Tectonic family member 2	[79]	[1]613846	Meckel syndrome 8	r
Wls/Gpr177 (*Wnt1-Cre*-mediated ablation)	Wntless homolog (Drosophila)	[80]	[1]611514	nr	nr
Wnt5a	Wingless-type MMTV integration site family, member 5A	[81]	[1]164975	Robinow syndrome, autosomal dominant	r
Wnt9b (knockout) (*Foxg1-Cre*-mediated ablation)	Wingless-type MMTV integration site family, member 9B	[82,83]	[1]602864	nr	nr
Transcription and nucleolar factors					
Alx1	Aristaless-like homeobox 1	[84]	[1]601527	Frontonasal dysplasia 3	r
Alx3	Aristaless-like homeobox 3	[85]	[1]606014	Frontonasal dysplasia 1	r
Alx4	Aristaless-like homeobox 4	[85]	[1]605420	Frontonasal dysplasia 2 Parietal foramina 2 Craniosynostosis 5	Cleft alae nasi
Anp32b	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B	[86]	nr	nr	nr
Arid5	AT-rich interaction domain-containing protein 5A	[87]	[1]611583	nr	nr
Asxl1	Additional sex combs like 1	[88]	[1]612990	Bohring-Opitz syndrome Myelodysplastic syndrome, somatic	r
Barx1	BarH-like homeobox 1	[89]	[1]603260	nr	nr
Cdc42 (*Prrx1-Cre*-mediated ablation)	Cell division cycle 42	[90]	[1]116952	nr	nr
Chd7 (heterozygotes) (*Wnt1-Cre*-mediated ablation)	Chromodomain helicase DNA binding protein 7	[91,92]	[1]608892	CHARGE syndrome Hypogonadotropic hypogonadism 5 with or without anosmia	r
Cited2	CBP/p300-interacting transactivator, with Glu/Asp-rich C-terminal domain, 2	[93]	[1]602937	Atrial septal defect 8 Ventricular septal defect 2	nr
Crebbp/Cbp	CREB binding protein	[94]	[1]600140	Rubinstein-Taybi syndrome	nr
Dlx1	Distal-less homeobox 1	[95]	[1]600029	nr	nr
Dlx2	Distal-less homeobox 2	[95]	[1]126255	nr	nr
Dlx5	Distal-less homeobox 5	[96,97]	[1]600028	Split-hand/foot malformation 1 with sensorineural hearing loss	r
Dph1/Ovca1	DPH1 homolog (*S. cerevisiae*)	[98]	[1]603527	nr	nr
Eya1	Eyes absent 1 homolog (Drosophila)	[99]	[1]601653	Branchiootic syndrome 1 Branchiootorenal syndrome 1, with or without cataracts Anterior segment anomalies with or without cataract	r
Foxc2/Mfh1	Forkhead box C2	[100]	[1]602402	Lymphedema-distichiasis syndrome	r
Foxd3 (*Wnt1-Cre*-mediated ablation)	Forkhead box D3	[101]	[1]611539	uc	nr
Foxe1/Titf2/Fkhl15	Forkhead box E1	[102]	[1]602617	Bamforth-Lazarus syndrome Nonsyndromic orofacial clefting	r
Foxf2	Forkhead box F2	[103]	[1]603250	nr	nr
Gbx2	Gastrulation brain homeobox 2	[104]	[1]601135	nr	nr
Gli2	GLI family zinc finger 2	[8]	[1]165230	Culler-Jones syndrome Holoprosencephaly-9	r
Gli3	GLI family zinc finger 3	[105]	[1]165240	Greig cephalopolysyndactyly syndrome Pallister-Hall syndrome	r

Gsc	Goosecoid homeobox	[106]	[1]138890	Short stature, auditory canal atresia, mandibular hypoplasia, skeletal abnormalities	nr
Hand2/dHand	Heart and neural crest derivatives expressed 2	[107]	[1]602407	nr	nr
Hic1	Hypermethylated in cancer 1	[108]	[1]603825	nr	nr
Hoxa2	Homeobox A2	[19]	[1]604685	Microtia with or without hearing impairment (AD) Microtia, hearing impairment, and cleft palate (AR)	r
Irf6	Interferon regulatory factor 6	[109,110]	[1]607199	van der Woude syndrome Orofacial cleft 6 Popliteal pterygium syndrome 1	r
Jmjd6/Ptdsr	Jumonji domain containing 6	[111]	[1]604914	nr	nr
Kat6a/Moz/Myst3	K (lysine) Acetyltransferase 6A	[112]	[1]601408	nr	nr
Lhx7	LIM homeobox gene 7	[113]	nr	nr	nr
Lhx8	LIM homeobox gene 8	[11]	[1]604425	nr	r
Luzp1	Leucine zipper protein 1	[114]	[1]601422	nr	nr
Mef2c (*Wnt1-Cre*-mediated ablation)	MADS box transcription enhancer factor 2	[115]	[1]600662	Chromosome 5q14.3 deletion syndrome Mental retardation, stereotypic movements, epilepsy, and/or cerebral malformations	nr
Meox2	Mesenchyme homeobox 2	[116]	[1]600535	nr	nr
Mn1	Meningioma 1	[117]	[1]156100	Meningioma	nr
Mnt	Max binding protein	[118]	[1]603039	nr	nr
Msx1	Msh homeobox 1	[10,23]	[1]142983	Ectodermal dysplasia 3, Witkop type Orofacial cleft 5 Tooth agenesis, selective, 1, with or without orofacial cleft	r
Msx2 (missense mutation)	Msh homeobox 2	[119]	[1]123101	Craniosynostosis, type 2 Parietal foramina 1 Parietal foramina with cleidocranial dysplasia	r
Nabp2/Obfc2b/hSSB1	Nucleic acid binding protein 2	[120,121]	[1]612104	nr	nr
Osr2	Odd-skipped related transcription factor 2	[9]	[1]611297	nr	r
Pak1ip1	PAK1 interacting protein 1	[122]	[1]607811	nr	nr
Pax9	Paired box gene 9	[6]	[1]167416	Tooth agenesis, selective, 3	nr
Pbx1	Pre B cell leukemia homeobox 1	[83]	[1]176310	Leukemia, acute pre-B-cell	nr
Pds5a	PDS5, regulator of cohesion maintenance, homolog A (*S. cerevisiae*)	[123]	[1]613200	nr	nr
Phc1/Rae28	Polyhomeotic homolog 1	[124]	[1]602978	uc	nr
Pitx1	Paired-like homeodomain 1	[7,125]	[1]602149	Clubfoot, congenital, with or without deficiency of long bones and/or mirror-image polydactyly Liebenberg syndrome	r
Pitx2	Paired-like homeodomain 2	[126]	[1]601542	Axenfeld-Rieger syndrome, type 1 Iridogoniodysgenesis, type 2 Peters anomaly	nr
Pnn	Pinin	[127]	[1]603154	nr	nr
Prdm16	PR domain containing 16	[128]	[1]605557	Cardiomyopathy, dilated, 1LL Left ventricular noncompaction 8	nr
Prrx1/Prx1/Mhox	Paired related homeobox 1	[129]	[1]167420	Agnathia-otocephaly complex	r
Ptch1/Ptc1 (*Wnt1-Cre*-mediated ablation)	Patched 1	[130]	[1]601309	Basal cell nevus syndrome (Gorlin syndrome) Holoprosencephaly type 7	r
Pygo2 (*CMV-Cre*-mediated ablation)	Pygopus 2	[131]	[1]606903	nr	nr
Rax	Retina and anterior neural fold homeobox	[132]	[1]601881	Microphthalmia, isolated 3	nr
Recql4	RecQ protein-like 4	[133]	[1]603780	Baller-Gerold syndrome RAPADILINO syndrome Rothmund-Thomson syndrome	r
Runx2	Runt-related transcription factor 2	[134]	[1]600211	Cleidocranial dysplasia	r
Sall3	Spalt-like transcription factor 3	[24]	[1]605079	nr	nr
Satb2	SATB homeobox 2	[135,136]	[1]608148	Glass syndrome	r
Shox2	Short stature homeobox 2	[22]	[1]602504	nr	nr
Sim2	Single-minded family bHLH transcription factor 2	[137]	[1]600892	nr	nr

Smad4 (*Osr2-Cre*-mediated ablation)	SMAD family member 4	[138]	[1]600993	Juvenile polyposis/hereditary hemorrhagic telangiectasia syndrome Myhre syndrome	nr
Smad7	SMAD family member 7	[139]	[1]602932	uc	nr
Snai2	Snail family zinc finger 2	[140]	[1]602150	Piebaldism Waardenburg syndrome, type 2D	nr
Sox5	SRY (sex determining region Y)-box 5	[141]	[1]604975	nr	nr
Sox9 (heterozygous) (*Wnt1-Cre*-mediated ablation)	SRY (sex determining region Y)-box 9	[142,143]	[1]608160	Acampomelic campomelic dysplasia	r
Sox11	SRY (sex determining region Y)-box 11	[144]	[1]600898	Mental retardation, autosomal dominant, 27	nr
Sp8	Sp8 transcription factor	[145]	[1]608306	nr	nr
Tshz1	Teashirt zinc finger family member 1	[146]	[1]614427	Aural atresia, congenital	nr
Tbx1 (knockout) (*KRT14-Cre*-mediated ablation)	T-box 1	[4,147]	[1]602054	DiGeorge syndrome Velocardiofacial syndrome Conotruncal anomaly face syndrome Tetralogy of Fallot	r
Tbx2	T-box 2	[148]	[1]600747	nr	nr
Tbx22	T-box 22	[149]	[1]300307	Cleft palate with ankyloglossia submucous cleft palate (SMCP)	r
Tcof1 (heterozygous)	Treacher Collins-Franceschetti syndrome 1	[150]	[1]606847	Treacher-Collins syndrome	r
Tfap2A (*Wnt1-Cre*-mediated ablation)	Transcription factor AP-2 alpha	[151]	[1]107580	Branchio-oculo-facial syndrome	r
Trp63/Tp63	Transformation related protein p63	[152]	[1]603273	Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome 3 Orofacial cleft 8 Hay-Wells syndrome Limb-mammary syndrome	r
Vax1	Ventral anterior homeobox 1	[153]	[1]604294	Microphthalmia, syndromic 11	r
Whsc1	Wolf-Hirschhorn syndrome candidate 1	[154]	[1]602952	nr	nr
Zeb1	Zinc finger E-box binding homeobox 1	[155]	[1]189909	Corneal dystrophy	nr
Zfp640/Mzf6d	Zinc finger protein 640	[87]	nr	nr	nr
Zic3	Zinc finger protein of the cerebellum 3	[156]	[1]300265	Congenital heart defects, nonsyndromic Heterotaxy, visceral, 1 VACTERL association	r
Cytoplasmic proteins					
Akap8/Akap95	A kinase (PRKA) anchor protein 8	[157]	[1]604692	nr	nr
Apaf1	Apoptotic peptidase activating factor 1	[158]	[1]602233	nr	nr
B9d1	B9 protein domain 1	[159]	[1]614144	Meckel syndrome 9	nr
Cask	Calcium/calmodulin-dependent serine protein kinase	[160]	[1]300172	FG syndrome 4 Mental retardation and microcephaly with pontine and cerebellar hypoplasia	r
Cdkn1c/p57kip2	Cyclin-dependent kinase inhibitor 1C	[161,162]	[1]600856	Beckwith-Wiedemann syndrome IMAGe syndrome	r
Chuk/Ikk1/Tcf16	Conserved helix-loop-helix ubiquitous kinase	[163]	[1]600664	Cocoon syndrome	nr
Crk	v-crk sarcoma virus CT10 oncogene homolog	[164]	[1]164762	nr	nr
Ctnnb1 (*KRT14-Cre*-mediated ablation)	Catenin (cadherin-associated protein), beta 1,	[165,166]	[1]116806	Mental retardation, autosomal dominant 19	nr
Cyp26B1	Cytochrome P450, family 26, subfamily b, polypeptide 1	[167]	[1]605207	Craniosynostosis with radiohumeral fusions and other skeletal and craniofacial anomalies	nr
Cyp51	Cytochrome P450, family 51	[168]	[1]601637	nr	nr
Dhcr7	7-dehydrocholesterol reductase	[169,170]	[1]602858	Smith-Lemli-Opitz syndrome	r
Dhrs3	Dehydrogenase/reductase (SDR family) member 3	[171,172]	[1]612830	nr	nr
Dicer1 (*Pax2-Cre*-mediated ablation)	Dicer 1, ribonuclease type Ⅲ	[29]	[1]606241	Rhabdomyosarcoma, embryonal, 2 Goiter, multinodular 1 Pleuropulmonary blastoma	nr
Dlg1/Dlgh/Sap97	Discs large 1	[173]	[1]601014	nr	nr
Fuz	Fuzzy planar cell polarity protein	[174]	[1]610622	Neural tube defects	nr
Gab1	Growth factor receptor bound protein 2-associated protein 1	[175]	[1]604439	nr	nr

Gad1/Gad67	Glutamate decarboxylase 1	[176,177]	[1]605363	Cerebral palsy, spastic quadriplegic, 1	r
Glce	Glucuronyl C5-epimerase	[178]	[1]612134	nr	nr
Glg1	Golgi apparatus protein 1	[179]	[1]600753	nr	nr
Grb2	Growth factor receptor bound protein 2	[180]	[1]108355	nr	nr
Gsk3b	Glycogen synthase kinase 3 beta	[181]	[1]605004	nr	nr
Hs2st1	Heparan sulfate 2-O-sulfotransferase 1	[182]	[1]604844	nr	nr
Hspb11/Ift25	Heat shock protein family B (small), member 11	[183]	[1]604844	nr	nr
Ilk (*Col2a1-Cre*-mediated ablation)	Integrin linked kinase	[184]	[1]602366	nr	nr
Impad1/Jaws	Inositol monophosphatase domain containing 1	[185]	[1]614010	Chondrodysplasia with joint dislocations, GRAPP type	r
Inpp5e	Inositol polyphosphate-5-phosphatase E	[186]	[1]613037	Joubert syndrome 1 Mental retardation, truncal obesity, retinal dystrophy, and micropenis	nr
Kif3a (*Wnt1-Cre*-mediated ablation)	Kinesin family member 3A	[187]	[1]604683	nr	nr
Map3k7/Tak1 (*Wnt1-Cre*-mediated ablation)	Mitogen-activated protein kinase kinase kinase 7	[188,189]	[1]602614	nr	nr
Nprl3	Nitrogen permease regulator-like 3	[190]	[1]600928	nr	nr
Ofd1 (*CAG-Cre*-mediated ablation)	Oral-facial-digital syndrome 1 gene homolog (human)	[191]	[1]300170	Joubert syndrome 10 Orofaciodigital syndrome Ⅰ Simpson-Golabi-Behmel syndrome, type 2	r
Pdss2 (*Pax2-Cre*-mediated ablation)	Prenyl (solanesyl) diphosphate synthase, subunit 2	[192]	[1]610564	Coenzyme Q10 deficiency, primary, 3	nr
Piga (*EIIa-Cre*-mediated ablation)	Phosphatidylinositol glycan anchor biosynthesis, class A	[193]	[1]311770	Multiple congenital anomalies-hypotonia-seizures syndrome 2 Paroxysmal nocturnal hemoglobinuria, somatic	nr
Pkdcc/Vlk (*Sox2-Cre*-mediated ablation)	Protein kinase domain containing, cytoplasmic	[194,195]	[1]614150	nr	nr
Prickle1	Prickle homolog 1	[196]	[1]608500	Epilepsy, progressive myoclonic 1B	nr
Rad23b	RAD23b homolog (*S. cerevisiae*)	[197]	[1]600062	nr	nr
Rspo2	R-spondin 2 homolog (Xenopus laevis)	[198,199]	[1]610575	nr	nr
Schip1	Schwannomin interacting protein 1	[87]	nr	nr	nr
Sdccag8	Serologically defined colon cancer antigen 8	[200]	[1]613524	Bardet-Biedl syndrome 16 Senior-Loken syndrome 7	nr
Slc32a1/Viaat	Solute carrier family 32, member 1	[201,202]	nr	nr	nr
Spry1 (*Wnt1-Cre*-mediated ablation)	Sprouty homolog 1	[203]	[1]602465	nr	nr
Spry2	Sprouty homolog 2	[204]	[1]602466	nr	nr
Sumo1 (heterozygous)	SMT3 suppressor of mif two 3 homolog 1 (yeast)	[205]	[1]601912	Orofacial cleft 10	r
Ugdh (*Wnt1-Cre*-mediated ablation)	UDP-glucose dehydrogenase	[206]	[1]603370	nr	nr
Wdpcp	WD repeat containing planar cell polarity effector	[207]	[1]613580	uc	nr
Extracellular proteins					
Col2a1	Collagen, type Ⅱ, alpha 1	[208]	[2]120140	Achondrogenesis, type Ⅱ Stickler syndrome, type Ⅰ Kniest dysplasia	r
Hspg2	Heparan sulfate proteoglycan 2, perlecan	[209,210]	[1]142461	Dyssegmental dysplasia Schwartz-Jampel syndrome, type 1	nr
Serpinh1/Hsp47 (*Col2a1-Cre*-mediated ablation)	Serpine peptidase inhibitor, clade H, member 1	[211]	[1]600943	Osteogenesis imperfecta, type X	nr
Smoc1	SPARC related modular calcium binding 1	[212]	[1]608488	Microphthalmia with limb anomalies	r

[1]Before an OMIM entry number indicates a gene; [2]Before an OMIM entry number indicates that the entry includes a description of a gene of known sequence and a phenotype. OMIM: Online Mendelian Inheritance in Man (http://omim.org); CL/P: Cleft lip and/or palate; r: Reported; nr: Not reported; uc: Unclarified.

beta receptor Ⅱ) in the neural crest or *Shh* (sonic hedgehog) in the epithelium result in failure of the palatal shelf development[10-13]. The fifth category is the persistence of medial edge epithelial (MEE) cells. The palatal epithelia are regionally divided into three parts: oral, nasal, and MEE. The MEE cells are removed from

Table 2 Classification of genes associated with cleft palate in mice

	Genes
Signaling pathway	
TGF-beta signaling pathway	*Acvr1/Alk2, Acvr2a, Bmp4*[1], *Bmp7, Bmpr1a/Alk3, Cdc42, Chrd, Crebbp/Cbp, Cited2, Foxc2/Mfh1*[1], *Foxd3, Foxe1/Titf2/Fkhl15*[1], *Foxf2, Fst, Inhba, Gdf11/Bmp11, Map3k7/Tak1, Pitx2, Smad4, Smad7, Tgfb2*[1], *Tgfb3*[1], *Tgfbr1/Alk5*[1], *Tgfbr2*[1]
Hedgehog signaling pathway	*Bmp4*[1], *Bmp7, Crebbp/Cbp, Gli2*[1], *Gli3*[1], *Gsk3b, Ptch1/Ptc1*[1], *Shh*[1], *Smo/Smoh, Wnt5a*[1], *Wnt9b*
Wnt signaling pathway	*Acvr1/Alk2, Ctnnb1, Crebbp/Cbp, Edn1*[1], *Fzd2, Gsk3b, Lrp6, Map3k7/Tak1, Prickle1, Smad4, Smo/Smoh, Wnt5a*[1], *Wnt9b*
FGF signaling pathway	*Fgf10, Fgf18, Fgf9, Fgfr1*[1], *Fgfr2*[1], *Grb2, Spry1, Spry2*
MAPK signaling pathway	*Cdc42, Chuk/Ikk1/Tcf16, Egfr, Fgf10, Fgf18, Fgf9, Fgfr1*[1], *Fgfr2*[1], *Grb2, Map3k7/Tak1, Pdgfra*[1], *Tgfb2*[1], *Tgfb3*[1], *Tgfbr1/Alk5*[1], *Tgfbr2*[1], *Crk, Itgb1*
Cytokine-cytokine receptor interaction	*Acvr1/Alk2, Acvr2a, Bmp7, Bmpr1a/Alk3, Egfr, Inhba, Pdgfra*[1], *Pdgfc*[1], *Tgfb2*[1], *Tgfb3*[1], *Tgfbr1/Alk5*[1], *Tgfbr2*[1], *Vegfa*
CBL mediated ligand-induced downregulation of EGF receptors	*Egfr, Grb2, Pdgfra*[1]
Sprouty regulation of tyrosine kinase signals	*Egfr, Grb2, Spry2, Spry1*
NFkB activation	*Crebbp/Cbp, Chuk/Ikk1/Tcf16, Map3k7/Tak1, Smad4, Tgfbr1/Alk5*[1], *Tgfbr2*[1]
Adherens junction	*Crebbp/Cbp, Ctnnb1, Cdc42, Egfr, Fgfr1*[1], *Map3k7/ Tak1, Smad4, Snai2, Tgfbr1/Alk5*[1], *Tgfbr2*[1]
Focal adhesion	*Ctnnb1, Cdc42, Col2a1*[1], *Crk, Egfr, Gsk3b, Grb2, Itga5, Itgb1, Itgb8, Ilk, Pdgfra*[1], *Pdgfc*[1], *Vegfa*
Steroid biosynthesis	*Cyp51, Dhcr7*[1], *Sc5d/Sc5dl*
Cell cycle	*Crebbp/Cbp, Cdkn1c/p57kip2*[1], *Gsk3b, Smad4, Tgfb2*[1], *Tgfb3*[1]
Regulation of actin cytoskeleton	*Cdc42, Crk, Egfr, Fgf9, Fgf10, Fgf18, Fgfr1*[1], *Fgfr2*[1], *Itga5, Itgb1, Itgb8, Pdgfra*[1], *Pdgfc*[1]
Axon guidance	*Cdc42, Efna5, Efnb1*[1], *Efnb2, Gsk3b, Itgb1*
Endocytosis	*Cdc42, Egfr, Fgfr2*[1], *Pdgfra*[1], *Tgfbr1/Alk5*[1], *Tgfbr2*[1]
Angiogenesis	*Ctnnb1, Crk, Efnb1*[1], *Efnb2, Fgfr1*[1], *Fgfr2*[1], *Fzd2, Gsk3b, Grb2, Jag1, Jag2, Pdgfra*[1], *Pdgfc*[1], *Vegfa, Wnt5a*[1]
Family	
Homeobox protein	*Alx1*[1], *Barx1, Alx3*[1], *Alx4*[1], *Dlx1, Dlx2, Dlx5*[1], *Gbx2, Gsc, Hoxa2*[1], *Msx1*[1], *Msx2*[1], *Pax9, Prrx1*[1], *Pitx1*[1], *Pitx2, Rax, Shox2, Vax1*[1]
Tgf-beta receptor type Ⅰ and Ⅱ	*Acvr1/Alk2, Acvr2a, Bmpr1a, Tgfbr1/Alk5*[1], *Tgfbr2*[1]
Tgf-beta family	*Bmp4*[1], *Bmp7, Gdf11, Inhba, Tgfb2*[1], *Tgfb3*[1]
Tyrosine protein kinase	*Egfr, Fgfr1*[1], *Fgfr2*[1], *Pdgfra*[1], *Ror2*[1], *Ryk*
Ephrin	*Efna5, Efnb1*[1], *Efnb2*
Zinc finger protein	*Gli2*[1], *Gli3*[1], *Zic3*[1], *Hic1, Snai2*
Forkhead protein	*Foxc2*[1], *Foxd3, Foxe1*[1], *Foxf2*
T-box protein	*Tbx1*[1], *Tbx2, Tbx22*[1]
Sox transcription factor	*Sox5, Sox9*[1], *Sox11*
Heparin-binding growth factor family member/FGF	*Fgf9, Fgf10, Fgf18*
Sprouty	*Spry1, Spry2*
Smad	*Smad4, Smad7*
Integrin beta subunit	*Itgb1, Itgb8*
Frizzled	*Fzd2, Smo*
Wnt related	*Wnt5a*[1], *Wnt9b*
Serine-threonine protein kinase	*Ilk, Mpa3k7/Tak1*
LIM domain containing protein	*Lhx8*[1], *Lhx7, Prickle1*
EGF-like domain protein	*Jag1, Jag2*

[1]Genes involved in human cleft lip and/or palate. TGF: Transforming growth factor; FGF: Fibroblast growth factor; MAPK: Mitogen-activated protein kinase; CBL: Casitas B-lineage lymphoma; EGF: Epidermal growth factor.

the fusion line by epithelial cell migration, apoptosis, and epithelial-mesenchymal transdifferentiation[14]. *Tgfb3* (transforming growth factor, beta 3) or *Egfr* (epidermal growth factor receptor) knockout mice lack the adhesive interactions between the palatal shelves because the fate of MEE cells is altered[15-18]. In the last category, the cleft palate arises as a secondary defect, due to tongue or bone anomalies during development. For example, *Hoxa2* (homeobox A2) knockout mice exhibit CP, because depression of the tongue is inhibited by the abnormal attachment of the hyoglossus muscle to the greater horn of the hyoid[19,20].

There is molecular heterogeneity along the medial-lateral and anterior-posterior axes of palatal shelves. Regionally restricted expression of molecules provides distinct regulatory mechanisms for the development of palatal shelves. For instance, *Msx1, Shox2* (short stature homeobox 2), *Fgf10* (fibroblast growth factor 10), *Bmp2* (bone morphogenetic protein 2), and *Bmp4* (bone

Table 3 Six categories of defects that result in cleft palate in mutant mice

	Defects	Knockout mice
(1)	Failure of the palatal shelf formation (small palatal shelves)	*Acvr2a*[34,50], [1]*Fgfr2*[13], [1]*Lhx8*[11], *Pitx2*[126], *Itga5*[65], *Fst*[46]
(2)	Abnormal fusion of palatal shelves and tongue or the mandible	*Jag2*[70], [1]*Irf6*[109,110], [1]*Tbx1*[4], *Fgf10*[41]
(3)	Failure or delayed palatal shelf elevation	*Pax9*[6], [1]*Pitx1*[7], [1]*Osr2*[9], [1]*Gli2*[8], [1]*Tgfb2*[55], [1]*Pdgfc*[51], *Dhrs3*[172]
(4)	Failure of the palatal shelf development after the elevation	[1]*Msx1*[10], [1]*Lhx8*[11], [1]*Tgfbr2* (*Wnt1-Cre*-mediated ablation)[12]
(5)	Persistence of medial edge epithelial cells	*Apaf1*[158], [1]*Tgfb3*[18], *Egfr*[17], *Ctnnb1* (*K14-Cre*-mediated ablation)[166]
(6)	Secondary defect	[1]*Hoxa2*[19,20], [1]*Satb2*[135], *Acvr1/Alk2* (*Wnt1-Cre*-mediated ablation)[33]

[1]Genes involved in human cleft lip and/or palate.

morphogenetic protein 4) are exclusively expressed in the anterior region of the palatal shelves[4,13,21,22]. The ablation of *Msx1* in mice results in cell proliferation alterations in the anterior palatal mesenchyme and cleft palate[23]. *Shox2* shows restricted expression patterns in the anterior palatal mesenchyme and the ablation of *Shox2* in mice results in anterior cleft palates[22]. *Fgf10* is also expressed in the anterior palatal mesenchymal cells and induces *Shh* expression through its receptor Fgfr2 (fibroblast growth factor receptor 2) in the palatal epithelium[13]. On the other hand, *Pax9* is expressed in the posterior palatal shelves. Ablation of *Pax9* results in cleft palates because of a palatal shelf development defect[6,21]. Even though it is known that Tbx1 induces the expression of *Pax9* in the posterior part of palatal shelves[4], the mechanism of Tbx1-induced *Pax9* expression during palatogenesis remains unknown. There is also molecular heterogeneity along the medial-lateral axis of the palatal shelf. For instance, *Osr2* expression in the palatal shelf is characterized by a medial-lateral gradient. Loss of *Osr2* results in the failure of palatal shelf elevation because of the delayed development of the medial part of palatal shelf[9].

MOLECULAR PATHOGENESIS OF CLEFT PALATES

Since most of the studies in mice focus on complete CP, the pathogenesis of other CP phenotypes is not well understood. *Tbx1* is expressed in the developing palatal shelves in mice[4], highlighting the crucial function of *Tbx1* in regulating palatal development. Loss of *Tbx1* results in the abnormal fusion of the oral epithelia, which induces CP by preventing the elevation of palatal shelves[4]. The phenotypic variation in the *Tbx1*$^{-/-}$ palates strongly suggests that Tbx1 is involved in modifier genes and/or stochastic factors. *Tgfb3*$^{-/-}$ mice also exhibit either incomplete or complete CP[15,16]. Ablation of *Shox2* results in anterior cleft palates[22]. Knockout mice of *Sall3* (spalt-like transcription factor 3), which is expressed in the palatal mesenchyme, show hypoplasia of the soft palate and epiglottis[24]. These mice are unique models for studying the etiopathogenesis underlying the variety of CP phenotypes in humans.

A comprehensive list of molecules associated with CL/P in mice and their classification should provide insights into the genetic etiology of CL/P; however, the phenotype of knockout mice does not always recapitulate the phenotype in humans (Table 1). Since Table 1 includes the genes associated with tissue-specific conditional knockout mice, mutations of these genes may induce the phenotype of embryonic lethality in humans. Haploinsufficiency mutations of the *TBX1* mutation are associated with CP[25]; however, heterozygous mice with *Tbx1* are phenotypically normal, and *Tbx1*$^{-/-}$ mice have CP phenotypes[4], thereby suggesting a species-specific requirement for *Tbx1* dosage. Mutations of the *PVRL1* (poliovirus receptor-related 1 or Nectin 1) cause CL/P-ectodermal dysplasia syndrome and nonsyndromic CL/P (OMIM #225060), whereas *Pvrl1*$^{-/-}$ mice do not develop CP[26]. Lack of palatal phenotypes in mice may be a consequence of functional redundancy of *Pvrl* genes. Interestingly, *Smad4*, *Smad7*, *Fgf9*, *Fgf10*, and *Fgf18* are involved in CP in mice (Table 1), whereas *SMAD3* (OMIM *613795), *FGF8* (OMIM *600483), and *FGF17* (OMIM *603725) are involved in CP in humans.

Candidate genes for nonsyndromic CP in human must show a relevant spatio-temporal gene expression pattern in the developing palatal shelves, and induce a specific cleft palate phenotype when deleted[1]. Disease genes responsible for Mendelian forms of syndromic CP are also important in the etiology of nonsyndromic CP[27]. *TBX1* mutations have been found in patients with incomplete CP without clinical diagnosis of del22q11.2 syndrome[25]. *TBX1* is also one of the disease genes of conotruncal anomaly face syndrome (OMIM #217095), which is often associated with cleft palates, particularly submucosal CP, and bifid uvula. *Tbx1*$^{-/-}$ palatal phenotype in mice makes *Tbx1* a potential candidate gene for nonsyndromic CP, especially submucosal CP and incomplete CP in humans.

RECENT ADVANCES IN PALATOGENESIS

Even though many genes associated with CP have been identified, little is known about how the environment influences gene expression in palatogenesis, and palatal phenotype. Epigenetics, such as DNA methylation and chromatin remodeling, and the microRNA (miRNA) regulation could change gene expression profiles and phenotypes. Hundreds of miRNAs, small non-

coding RNAs that modulate gene expression at the post-transcriptional level are expressed in murine embryonic craniofacial tissue[28]. Conditional knockout mice of *Dicer1* (dicer 1, ribonuclease type Ⅲ), which regulates the generation of miRNA, resulted in disrupted palatogenesis[29], suggesting that the miRNA function may be important in mammalian palatogenesis. miR-140, which modulates BMP signaling, regulates palatogenesis in mice[30] and miR-17-92 modulates Tbx1 and Tbx3 (T-box 3) activity, resulting in orofacial clefting[31]. Interestingly, transcription of *Dicer1* is regulated by TP63 (transformation related protein p63)[32], whose mutations are associated with cleft palate phenotypes (Table 1). Since genes involved in miRNA generation and individual CP genes can both be modulated by several miRNAs, it is conceivable that complex gene-miRNA interactions exist during palatogenesis. Genetically engineered mice with miRNAs, which modulate CP genes, may provide new information on the gene interactions underlying the palatogenesis. Further studies on miRNA and methylated genes involved in palatogenesis are necessary to understand the environmental factors contributing to CP.

CONCLUSION

Studies with genetically engineered mice with CP reveal the importance of regulated molecular functions in palatogenesis and provide the opportunity to discover new genes implicated in palatogenesis. However, there is still much to learn about transcriptional regulation and molecular networks in palatogenesis. The interactions between environmental/stochastic factors and genes in the etiopathogenesis of CL/P require further studies. Teratogenic effects of dioxins and retinoic acid have been reported in mice[1]. Mutant mice with CP can also be used as models to assess environmental effects or gene-environment interactions. Epithelial abnormal fusion could be one of the stochastic causes that induce a variety of CP phenotypes in mice. Understanding the palatal epithelial functions during palatogenesis may also lead to the discovery of novel therapeutic methods for CL/P.

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REVIEW

SGF29 and Sry pathway in hepatocarcinogenesis

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Abstract

Deregulated c-Myc expression is a hallmark of many human cancers. We have recently identified a role of mammalian homolog of yeast SPT-ADA-GCN5-acetyltransferas (SAGA) complex component, SAGA-associated factor 29 (SGF29), in regulating the c-Myc overexpression. Here, we discuss the molecular nature of SFG29 in SPT3-TAF9-GCN5-acetyltransferase complex, a counterpart of yeast SAGA complex, and the mechanism through which the elevated SGF29 expression contribute to oncogenic potential of c-Myc in hepatocellularcarcinoma (HCC). We propose that the upstream regulation of SGF29 elicited by sex-determining region Y (Sry) is also augmented in HCC. We hypothesize that c-Myc elevation driven by the deregulated Sry and SGF29 pathway is implicated in the male specific acquisition of human HCCs.

Key words: c-Myc; SPT3-TAF9-GCN5-acetyltransferase complex; SGF29; Sry; Hepatocarcinogenesis

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Core tip: Deregulated c-Myc expression is a hallmark of many human cancers. We have recently identified a role of mammalian homolog of yeast SPT-ADA-GCN5-acetyltransferas (SAGA) complex component, SAGA-associated factor 29 (SGF29), in regulating the c-Myc overexpression. We propose that the upstream regulation of SGF29 elicited by sex-determining region Y (Sry) is also augmented in hepatocellularcarcinoma (HCC). We hypothesize that c-Myc elevation driven by the deregulated Sry and SGF29 pathway is implicated in the male specific acquisition of human HCCs.

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C-MYC AND HEPATOCARCINOGENESIS

c-*myc* is a protooncogene of the viral homolog of v-*myc* which causes myelocytomatosis[1-4]. The expression of c-Myc is tightly regulated by the many ligand-stimulated receptor signaling under normal condition[5]. The deregulated expression level of c-Myc contributes many aspects of cancer development including proliferation, growth, DNA replication, protein synthesis, metabolism, cell adhesion, angiogenesis, metastasis and immune responses by regulating the transcription of its target genes[6-11]. Only two-fold increase in the expression level of c-Myc can affect cell cycle progression[12]. In over 50% of human cancers, c-Myc is deregulated and/or activated[13-18]. In Burkitt lymphoma, the translocation of c-*myc* gene to the immunoglobulin gene causes the overexpression of c-Myc[19,20]. c-Myc is also highly expressed by the gene amplification in many human and rodent cancers including hepatocellularcacinoma (HCC)[21-23]. The 8q22-24 region including c-*myc* locus is involved in the early onset of HCC and represent the frequent amplification in early HCC[24,25]. In mice models, the expression level of c-Myc is also associated with the development of HCC[26-28]. In colon cancer and T cell leukemia, WNT and Notch signaling pathways are involved in the upregulation of c-Myc, respectively[29-32]. Conversely, suppression of c-Myc expression can cause tumor regression by inducing cell cycle arrest, differentiation, or senescence depending on the cell contexts in mice models. The effect of c-Myc downregulation on tumor regression is permanent in some tumors such as lymphoma and osteosarcoma, whereas in hepatocellular or breast cancers this effect is reversible[33-37]. Thus, inhibiting the c-Myc expression could be a promising therapeutic strategy[38-44].

TRANSCRIPTIONAL REGULATION OF C-MYC *VIA* SPT3-TAF9-GCN5-ACETYLTRANSFERASE

c-Myc has N-terminal transcriptional regulatory domain consisting of Myc boxes (Mb) Ⅰ, Ⅱ, Ⅲ, Ⅳ and nuclear localization signal. The basic Helix-Loop-Helix leucine-Zipper (bHLHZ) domains encompasses C-terminal region of c-Myc[45-50]. In the nucleus, c-Myc heterodimerizes with Max through their bHLHZ domains to bind to the E-box sequence (5'-CACGTG-3') in the gene regulatory region[51-54]. The E-box sequences mainly exist in the promoter or intron 1 of the target genes. Although E-boxes are occupied with other E-box-binding transcription factors including ChREBP, SREBP, HIF-1, NRF1, USF, TFE3, Clock, and Bmal to regulate cellular homeostasis in quiescent state, c-Myc is replaced with these factors upon its induction by ligand stimulation[11]. Genome-wide screening for c-Myc-bound target genes using chromatin immunoprecipitation (ChIP)-sequencing reveal that around 6000 genes can be bound with c-Myc in human genome[55]. Of these genes, a set of 300 genes are actually inducible by c-Myc and are involved in nucleotide metabolism, ribosome biogenesis, RNA processing, and DNA replication[56]. These genes are also suggested to be induced in c-Myc-overexpressing transformed cells. c-Myc can recruit some cofactor complexes including histone acetyltransferases, ubiquitin ligases, and kinases, and the N-terminal region of MbI and MbⅡ domains are required for the transcriptional activation and malignant transformation activities[57-59]. These domains can recruit three classes of histone acetyltransferase (HAT) complexes including SPT3-TAF9-GCN5-acetyltransferase (STAGA) complex[60], p300/CBP-associated factor (PCAF) complex[61,62] and the TIP60-containing complex[63] to activate the adjacent gene transcription. Of these three classes of HAT complexes, STAGA complex is thought to be responsible for the transactivation of c-Myc, because the only direct interaction between STAGA and c-Myc is demonstrated[64]. The *Saccharomyces cerevisiae* SPT-ADA-GCN5-acetyltransferase (SAGA) complex is the 1.8 MDa transcriptional coactivator that is highly conserved counterpart of STAGA complex consisting of 18 to 20 proteins[65-71]. The detailed information about these complexes is derived from the studies of yeast SAGA complex. SAGA is essential for about 10% of whole gene transcription[65,66,70,72]. The SAGA complex subunits are classified into four functional groups. The first group includes GCN5 acetyltransferase, ADA2B, ADA3 and SAGA-associated factor 29 (SGF29) regulating the acetylation of multiple lysine residues of histone H3 including H3K9, H3K14, H3K18 and H3K23[73-76]. The acetylation of histones is significantly implicated in the activation state of transcription[77-79]. Moreover, GCN5 can bind to the acetylation mark of histone H3 by its bromo domain[80]. The second group constitutes the ubiquitin-specific protease Ubp8, SGF11, SGF73 and SUS1 to perform the deubiquitinaion of H2B[81,82]. Deubiquitination of Lys123 of histone H2B eventually induce the phosphorylation of the C-terminal domain of RNA polymerase Ⅱ (Pol Ⅱ) to facilitate release of Pol Ⅱ into transcription elongation[83,84]. The third group contains TATA-binding protein (TBP)-associated factor (TAF) proteins that are also incorporated in the general transcription factor TFIID[67,68]. Since a complex consisting of the histone-fold domains of TAF6, TAF9, TAF12 and ADA1 have structural resemblance to the histone octamer, TAF proteins make the structural backbone of the SAGA complex[85]. The last group consists of Tra1 and SPT proteins to tether the SAGA complex to the specific transcriptional activators[67,86-91]. SPT proteins are also reported to be required for the recruitment of TBP to the target gene promoters[69,92-94]. These subunits cooperatively recruit the Pol Ⅱ and assemble the preinitiation complex to induce the target

gene transcription. Of these components, Tra1 and TAF proteins are essential for cellular viability in yeast[95,96]. Most of these components are highly conserved between yeast and human. The human orthologue of Tra1, Transactivation/transformation-associated protein (TRRAP), can interact with the Mb Ⅰ and Mb Ⅱ domains of c-Myc, which is essential for malignant transformation and for the hyperacetylation of histone H3 and H4[64,97-102]. In addition, TRRAP is implicated in the transactivation and transformation activity of E2F1, E2F4, p53, and E1A[98,103-105]. The acetyltransferase activity of human GCN5 is also reported to be involved in the malignant transformation potential of c-Myc[99]. Thus, STAGA complex has significant role in the c-Myc-mediated onset of many human malignancies.

INVOLVEMENT OF SGF29 IN HEPATOCARCINOGENESIS

The critical factor in transformation activity of c-Myc other than TRRAP and GCN5 is SGF29 which is also conserved between yeast and mammals. SGF29 is originally identified using the mass spectrometric analysis of yeast SAGA[81]. By directly interacting with ADA3, SGF29 is incorporated into the STAGA complex[106]. The structural domains of SGF29 are N-terminal coiled-coil domain and C-terminal double Tudor domains[107,108]. Tudor domain is originally cloned in Drosophila, and to date, about 30 Tudor-containing proteins have been identified in mammals such as SGF29, 53BP1, Spindlin1 and UHRF1 most of which can bind to a methylated lysine[75,109-115]. The Tudor domain-containing proteins belong to so-called histone reader proteins that can recognize the various sites for post-translational histone modifications including methylation, acetylation, ubiquitination, and phosphorylation[116]. The histone readers recruit specific protein complexes at the site of histone modifications. These modifications are referred to as histone code to regulate chromatin organization and gene transcription. Many human disorders are associated with the misreading of histone codes[117-119]. The Tudor domains of SGF29 specifically recognize the histone H3K4me3/2 sites in a manner of preference to tri-methylation in yeast and human cells[75,110]. Because tri-methylation of histone H3K4 is prerequisite for subsequent acetylation of histone H3[120] and no other subunits of SAGA complex harbor the Tudor domain, only SGF29 mediates a direct connection between histone H3K4me3/2 and acetylation of histone H3. Knocking down the yeast SGF29 or its mutated form of it decrease the acetylation levels of histone H3 without disturbing the integrity of SAGA complex because of the loss of its ability to bind histone H3K4me3/2 and to load SAGA complex at target gene promoters[67,75,121]. The histone H3K4me3/2 is frequently observed in the active promoter region of genes, and the histone H3 in the E-box sequences are also reported to be highly methylated[122-126]. These methylation of histone H3K4 in E-box occur prior to the induction of acetylation of H3 by c-Myc expression[127]. Because c-Myc is not thought to directly recognize these histone markers, SGF29 in the STAGA complex may help to recruit c-Myc to the E-box in cooperation with TRRAP[106]. The loaded c-Myc induces the local histone acetylation at the site of E-box leading to histone unwinding and significant induction of downstream target gene transcription[128]. In normal organs, SGF29 is robustly expressed in testis and modestly in thymus, spleen and lung. In rat hepatomas, three out of five cell lines overexpress SGF29 together with c-Myc[106]. In the presence of c-Myc, SGF29 enhance the transcriptional activating activity of the promoter of a c-Myc target gene, ornithine decarboxylase (ODC)[129,130], *in vitro*. Conversely, the deletion of SGF29 expression causes the decrease in this promoter activity in rat hepatoma K2 cells. The downregulation of SGF29 also abrogates the colony forming ability of K2 cells in soft agar and tumorigenicity in nude mice concomitant with the decreased expression of c-Myc target genes such as ODC, lactate dehydrogenase-A (LDH-A)[131,132] and metastasis-associated protein 1 (MTA1)[133], whereas growth rate in monolayer culture is not affected. Moreover, the lowered expression level of SGF29 suppresses the metastatic ability of K2 cells to lung[106]. Taken together, the increased SGF29 expression is closely associated with the oncogenic potential of c-Myc by controlling its target gene expression.

MALE SPECIFIC HEPATOCARCINOGENESIS AND SRY

How could the factor(s) cause the deregulated expression of SGF29? The *in silico* search for the transcription factor binding site in the promoter region of rat SGF29 gene predicts that ten transcriptional factors could bind this region[134]. These factors contain c-Myc, sex-determining region Y (Sry), AML-1a and GATA-1. Given that there are seven high-mobility group (HMG)-boxes (5'-AACAAAG-3'), Sry-binding DNA element, and high-expression level of SGF29 in testis, Sry may be the most potent regulator of SGF29 overexpression. Indeed, SGF29 is shown to be a novel Sry target gene because Sry can directly bind the proximal promoter region of rat *SGF29* gene and increase *SGF29* transcript[134]. Sry is originally identified as the male sex-determining gene and is located in the male-specific region of the Y chromosome[135-137]. Induction of Sry expression in the genital ridges drives the testis differentiation and causes the activation of Sox9 to induce the downstream target genes required for male development[138-140]. Sry is not so highly conserved in its N-terminal region among mammals, but these proteins harbor the same ability to control the sex determination in developmental process in a spatiotemporally regulated manner[141-143]. The highly conserved HMG DNA-binding domain of Sry elicits the crucial function in developmental process[139,144,145]. In general, transcription factor have

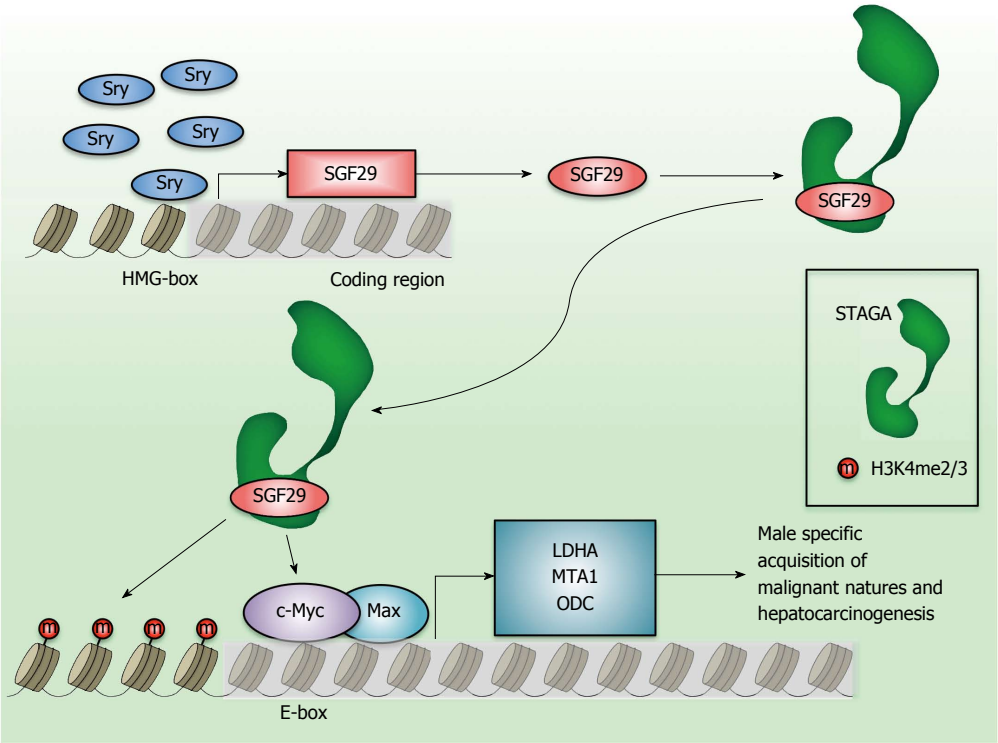

Figure 1 SAGA-associated factor 29 and Sry pathway in hepatocarcinogenesis. Deregulated c-Myc expression is a hallmark of many human cancers. The transcriptionally active marker of histone H3, H3K4me3/2 (red small circles depicted in left bottom of the figure), is frequently observed in the active promoter region of the E-box sequences in the c-Myc target genes. SGF29, a component of STAGA complex, which can interact with H3K4me2/3 induce the acetylation of histone at the sites of c-Myc loaded E-box leading to histone unwinding and significant induction of downstream target gene transcription. The upstream regulation of SGF29 elicited by male specific transcription factor, Sry, is also deregulated in HCC. Eventually, c-Myc target genes such as LDHA, ODC and MTA1 expression are induced, which could account for a molecular insight into the male specific acquisition of human HCC driven by the deregulated Sry and SGF29 pathway. SGF29: SAGA-associated factor 29; STAGA: SPT3-TAF9-GCN5-acetyltransferase; Sry: Sex-determining region Y; HCC: Hepatocellular carcinoma; LDH-A: Lactate dehydrogenase-A; ODC: Ornithine decarboxylase; MTA1: Metastasis-associated protein 1.

the DNA-binding and transactivation domains, but most of Sry proteins from different species except for mouse and rat do not have an obvious transactivation domain[145]. Although many Sry-binding partner proteins are reported to bind to the non-conserved region of Sry[146-149], physiological relevance between these interactions and testis differentiation remains obscure[150,151]. Although Sry expression is limited in brain, thymus and testis in adulthood, some HCC cell lines such as K2 cells overexpress this gene[134]. Moreover, the copy number gain or amplification of this gene locus occur in 11.8% (8/68) of human male HCC cases, which may reflect the fact that Sry is deregulated in some human HCC[152]. The deletion of Sry expression causes the lowered SGF29 expression in K2 cells together with the decreased level of LDHA and MTA1[134]. Like SGF29, the reduced Sry expression leads to the diminished colony forming ability in soft agar and tumorigenicity *in vivo*. The Sry expression solely does not bypath the malignant phenotype when SGF29 is deleted, which reveal that the induction of SGF29 brought by Sry is necessary for the acquisition of c-Myc-dependent transformation activity. Taken together, these findings suggest a hypothetical model of Sry and SGF29 pathway in male specific malignancy of HCC (Figure 1). The aberrant expression of Sry causes the elevation of SGF29, which is integrated into STAGA complex to augment the c-Myc target genes' expression. Because Sry is expressed only in male, this scheme may be the explanation of male-specific acquisition of malignancy and hepatocarcinogenesis.

CONCLUSION

STAGA is one of the histone acetyltransferase complex and crucial for malignant transformation activity of c-Myc. STAGA complex can bind to c-Myc, and the deletion of key components of STAGA suppress the c-Myc target genes' transcription. Thus, uncovering the regulatory mechanism of STAGA complex can sheds light on the oncogenesis driven by c-Myc. As a component of STAGA, SGF29 physically interact with transcriptionally active histone marker H3K4me2/3. Whether SGF29 simultaneously associates with both STAGA complex and histone H3K4me2/3 is unclear, SGF29 might facilitate the efficient recruitment of Pol Ⅱ to the promoter region of c-Myc target genes. We have shown that SGF29 expression is deregulated in some rodent HCCs and is important for tumorigenic activity of c-Myc. Furthermore, because Sry is shown to be directly upregulate the SGF29 transcription and the amplification of *Sry* gene is observed in human

male HCCs, Sry may be an attractive target for c-Myc-involved HCCs as well as SGF29. Given that c-Myc is deregulated in many human cancer types, the Sry-SGF29-c-Myc axis might be implicated in the onset of these cancers. However, not having any information about the expression level of SGF29 in cancers other than HCC, more detailed survey for this point will be required. On the other hand, in addition to Sry, c-Myc, AML-1a and GATA-1 may also bind the promoter region of *SGF29* gene in *in silico* data analysis. Whether these factors will be the real upstream regulator(s) of *SGF29* gene or not should be revealed. Although there are several protein complexes which are recruited to c-Myc, implications of these complexes in malignant transformations are unclear. Moreover, because the relationships between c-Myc and STAGA components other than SGF29, GCN5, and TRRAP are unknown, deubiquitination or TBP recruiting activities of STAGA toward c-Myc should be also clarified.

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REVIEW

Metabolic interplay between glycolysis and mitochondrial oxidation: The reverse Warburg effect and its therapeutic implication

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Abstract

Aerobic glycolysis, *i.e.*, the Warburg effect, may contribute to the aggressive phenotype of hepatocellular carcinoma. However, increasing evidence highlights the limitations of the Warburg effect, such as high mitochondrial respiration and low glycolysis rates in cancer cells. To explain such contradictory phenomena with regard to the Warburg effect, a metabolic interplay between glycolytic and oxidative cells was proposed, *i.e.*, the "reverse Warburg effect". Aerobic glycolysis may also occur in the stromal compartment that surrounds the tumor; thus, the stromal cells feed the cancer cells with lactate and this interaction prevents the creation of an acidic condition in the tumor microenvironment. This concept provides great heterogeneity in tumors, which makes the disease difficult to cure using a single agent. Understanding metabolic flexibility by lactate shuttles offers new perspectives to develop treatments that target the hypoxic tumor microenvironment and overcome the limitations of glycolytic inhibitors.

Key words: Hepatocellular carcinoma; Oxidative stress; Metabolic interventions; Aerobic glycolysis; Lactate

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Core tip: The Warburg effect plays a vital role in cancer cell proliferation and survival, and contributes to the initiation of tumor metastasis. To adapt to rapidly changing microenvironment for survival such as from normoxia to hypoxia, cancer cells vary in metabolic phenotype; "metabolic flexibility". Even in a hypoxic condition, oxidative cancer cells and/or stromal cells should theoretically exist to support the metabolic fuel for glycolytic cancer cells and handle lactate *via*

the dynamic shuttle; "the reverse Warburg effect". Treatments against tumor metabolism may aim to target two distinct metabolic pathways of glycolysis and mitochondrial oxidative phosphorylation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the seventh most common cancer and the third cause of cancer-related mortality all over the world[1]. Despite the recent development of various types of targeted agents, minimal improvements have been identified in the survival of patients with advanced HCC since the introduction of sorafenib 10 years ago[2]. To overcome the limitations of current anticancer agents, new strategies must be developed.

Most previous studies have strongly suggested the metabolic reprogramming of cancer cells into aerobic glycolysis, *i.e.,* the Warburg effect, in the process of carcinogenesis and adjustment for the hypoxic tumor microenvironment. Based on this concept, the development of anticancer agents that target the enzymes involved in glycolysis appears to be promising. 3-bromopyruvate (3-BP), is a potent anticancer agent that inhibits the glycolytic pathway primarily leads to a depletion of energy reserves. Previous studies have demonstrated that 3-BP could have strong anticancer effects in various cancer types. However, in a case study performed in Egypt, the killing effect of 3-BP was not as potent as expected in the treatment of a 28-year-old man who presented with stage Ⅳ metastatic melanoma. The patient died of cancer progression even though he had received 3-BP treatment[3].

Based on the cancer progression despite 3-BP treatment in this case, mechanisms beyond the Warburg effect may contribute to cancer cell survival, *i.e.,* evading the glycolytic pathway or attenuating the 3-BP anticancer effect. One potential explanation of the mechanism is that cancer cells might have a preference to produce energy reserves *via* mitochondrial oxidative phosphorylation (OXPHOS) rather than high glycolysis according to their surrounding conditions. The cells use lactate from tumor stromal cells, which is the end product of glycolysis and can fuel mitochondrial OXPHOS after conversion to pyruvate. This phenomenon has been referred to as the "reverse Warburg effect", which indicates increased aerobic glycolysis of stromal cells adjacent to tumor cells[4]. Another potential reason for 3-BP resistance might be the capability of tumor cells to balance the redox potentials, which play a key role in drug detoxification and cellular protection from oxidative injury by free radicals and peroxides, *i.e.,* "chemoresistance" to 3-BP[5].

In this review, we describe the clinical implication of the Warburg effect with a high redox potential, and the roles of mitochondrial OXPHOS with a focus on lactate shuttles beyond the Warburg effect. Given the importance as modulators of tumor cell metabolism, these approaches may represent promising therapeutic targets to potentiate the anticancer effect of 3-BP, which has been well-known as the strongest inhibitor of glycolysis in cancer.

WARBURG EFFECT: CURRENT CLINICAL IMPLICATIONS IN CANCER

Warburg first reported an anomalous characteristic of energy metabolism in cancer cells[6,7], even in the presence of oxygen, cancer cells can accelerate glycolysis rather than mitochondrial OXPHOS; "aerobic glycolysis". Aerobic glycolysis is seemingly contradictory phenomena because cancer cells must compensate for the 18-fold lower efficiency of ATP production afforded by glycolysis as compared to mitochondrial OXPHOS. To compensate this lower efficiency, the cells upregulate glucose transporters such as GLUT1[8-10]. This inefficient energy metabolism provides cancer cells with several advantages: (1) balancing the redox potential inner cell; and (2) increased biosynthesis of intermediate macromolecules, anti-apoptosis, and efficient signaling through metabolites as compared with mitochondrial OXPHOS[9].

In hypoxic tumor cells, the overexpression of glucose transporters and glycolytic enzymes such as hexokinase Ⅱ (HK Ⅱ), phosphofructokinase (PFK), phosphoglycerate kinase, and lactate dehydrogenase (LDH), has been investigated[11]. It was reported that high serum levels of glucose transporter (GLUT) 1, GLUT 3, aldolase-B, and HK Ⅱ have been significantly associated with poor prognosis in various types of malignancies[12]. Among them, HK Ⅱ, involved in the first rate-limiting step of glycolysis, has been related to anti-apoptosis. A predominant fraction of HK Ⅱ was bound to the voltage-dependent anion channel (VDAC) at the outer membrane of the mitochondria. Among the proposed mechanism of chemoresistance in cancer cells was the decreased availability of free VDAC sites that interact with pro-apoptotic proteins (Bax). HK Ⅱ binds to the VDAC site and this interaction prevents the activation of Bax[13]. In other words, HK Ⅱ play a key role for prevention of chemotherapy-induced, mitochondria-mediated, tumor cell apoptosis. In addition, this glycolysis pathway can be further accentuated under the hypoxic conditions in many tumors: the hypoxic condition can upregulate glucose transporters and induce multiple enzymes of the

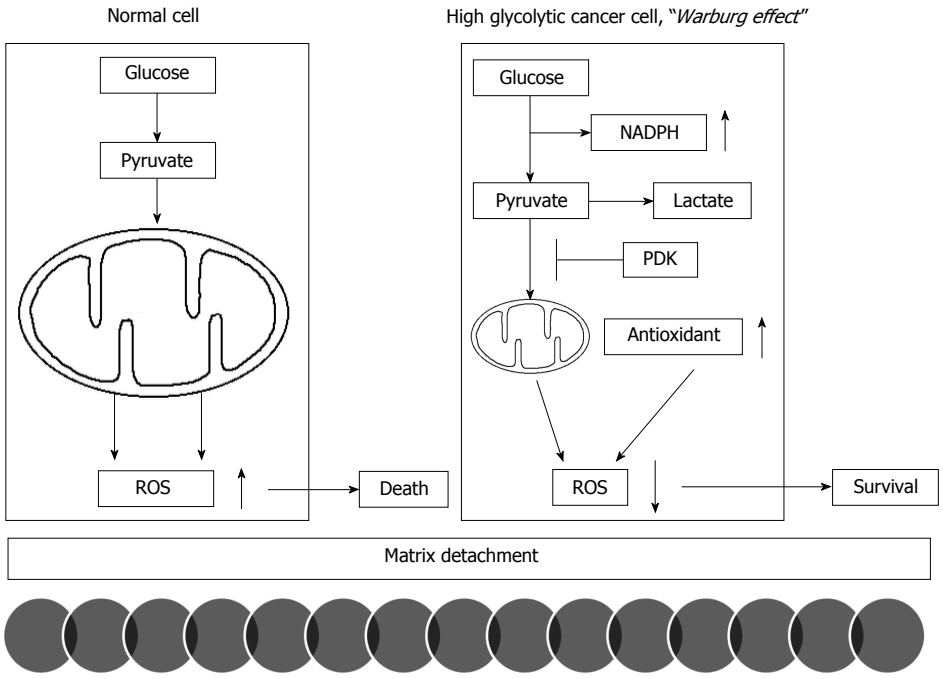

Figure 1 The Warburg effect contributes to the initiation of tumor metastasis by lowering intracellular reactive oxygen species levels. After matrix detachment, cancer cells decrease intracellular ROS levels through inducing enzymes involved in the glycolysis and antioxidant systems for their survival. The Warburg effect is strongly linked with modulation of ROS system. PDK: Pyruvate dehydrogenase kinase; ROS: Reactive oxygen species.

glycolytic pathway[10,14,15]. For example, both Ras oncoprotein and hypoxia can independently increase HIF1a and HIF2a transcription factor levels which upregulate glycolysis[14,16,17]. Our group demonstrated that hypoxia stimulates HCC cellular growth through hexokinase Ⅱ induction, which thereby may participate in HCC progression, and the blockage of this enzyme may be therapeutically efficacious in human HCCs[18,19]. Although tumor cells can redirect energy metabolism of "aerobic glycolysis", it has become apparent that oxygenation, which ranges from normoxia to hypoxia, is not always static in tumor microenvironment, but instead dynamically fluctuates as a result of the dynamic changes of the tumor-associated neovasculature[20].

WARBURG EFFECT WITH ROS: INITIATION OF METASTASIS

The acceleration of glycolysis in cancer cell energy metabolism could reduce the production of reactive oxygen species (ROS) by less reliance on mitochondrial OXPHOS and simultaneously enhance the redox potential *via* an increase in NADPH in the pentose phosphate pathway as byproducts for biosynthetic pathways of proliferation. This contribution of the Warburg effect to the balance of redox potential plays a pivotal role in the initiation of metastasis; matrix detachment. While normal cells attenuate mitochondrial OXPHOS in response to matrix detachment for their survival, many cancer cells already limit mitochondrial OXPHOS before detachment because of the Warburg effect. Normal cells activate PDK4 to inhibit PDH following detachment to upregulate glycolytic pathway. However, cancer cells already express high levels of PDK1 under attached conditions[21]. PDK1 and PDK3 expression in various cancers significantly correlates with patients' prognosis: tumor histological grades and disease-free survival[22,23]. PDK inhibition or PDH activation in cancer cells stimulates mitochondrial OXPHOS and thereby increase ROS production. Excess production of intracellular ROS levels increase their susceptibility to cell death after matrix detachment, which leads to a decreased metastatic potential[21]. Therefore, the Warburg effect allows cancer cells to evade cellular oxidative stress that would be produced by mitochondrial OXPHOS for glucose metabolism[24]. Thus, the reduction of ROS levels, which promotes metastasis, may represent an advantage given by the Warburg effect (Figure 1). Increased glucose consumption diverts more glucose carbon into the oxidative branch of the pentose phosphate pathway, which represents a major source to generate NADPH[25]. NADPH is a critical cofactor for the replenishment of reduced glutathione (GSH) in a cell. Cancer cells can further enhance this antioxidant generation pathway *via* PKM inhibition when oxidative stress increases[26].

In addition to the Warburg effect, cancer cells also potentiate antioxidant systems to cope with increased oxidative stress[25]. For example, while MnSOD is induced following matrix detachment in normal cells[27], MnSOD is constitutively overexpressed in cancer cells. Furthermore, increased MnSOD expression in cancer is significantly associated with poor prognosis[27-29]. An enhanced antioxidant capacity allows cancer cells to

better survive detachment-induced oxidative stress and initiate to metastasize. In a lung cancer mouse model, antioxidant treatments have consistently reduced oxidative stress and accelerated lung cancer progression[30].

LIMITATIONS OF THE WARBURG EFFECT AND GLYCOLYTIC INHIBITORS

To adapt to rapidly changing microenvironment for their survival such as from normoxia to hypoxia[31,32], each cancer cells may vary in metabolic phenotype even in a single tumor mass; "metabolic flexibility"[33]. Because total ATP production *via* the glycolytic pathway does not generally exceed 50%-60%[34], mitochondrial OXPHOS still, to a certain extent, contributes to ATP generation in cancer cells, *i.e.,* a mixture of glycolysis and mitochondrial OXPHOS[35]. The ratio of contribution to ATP production in cancer cells can be rapidly changed to maintain pace with the alteration of the tumor microenvironment. Herst *et al*[36] reported that energy production for tumor cell growth was altered from mitochondrial OXPHOS to accelerated glycolysis according to changes from normoxia to hypoxia: mitochondrial OXPHOS contributes to total ATP production, which was 91% in normoxia and reduced to 36% in hypoxia.

Given this flexibility, all cancer cells did not completely depend on accelerated glycolysis. Previous studies reported that mitochondrial OXPHOS in many cancers can be well-functioned to produce ATPs[9,37-40]. These authors have concluded that the Warburg effect is a result of accelerated glycolysis which suppressed mitochondrial OXPHOS rather than the initial impairments in mitochondrial OXPHOS. If glycolysis is suppressed in cancer cells, the mitochondrial OXPHOS function can be recovered[35,39,41,42]. In a noticeable investigation, Fantin *et al*[39] demonstrated that when glycolysis was inhibited by suppression of LDH-A in cancer cells, mitochondrial OXPHOS could be restored to compensate for energy production. This study reported that both LDH-A and mitochondrial function were modulated by the metabolite level such as pyruvate and the NADH/NAD+ ratio. This result indicates that cancer cells have the capacity of regulating ATP production by mitochondrial OXPHOS to adapt to the rapidly changing microenvironment.

According to a proposal by Smolková *et al*[41], metabolic phenotypes in cancer cells can be simply divided into two subgroups with each condition of the tumor microenvironment: (1) enhanced glycolysis and suppressed mitochondrial OXPHOS with a hypoxic condition; and (2) relatively suppressed glycolysis and restoration of mitochondrial OXPHOS with nutrient shortage because of high proliferation rates. This proposal explained that the Warburg phenotype is not a universal finding, and mitochondrial respiration impairment is not a fixed feature of cancer cells[41].

Although the glycolytic inhibitors targeting the Warburg effect have been investigated in various cancer types, the glycolytic inhibitors with the exception of 3-BP (a lactate analog)[18,19,43-70], 3-BrOP (a 3-bromopyruvate derivative)[71-74], and dichloroacetate (DCA)[75-98] have demonstrated low efficacy in arresting tumor growth when used alone[99]; these inhibitors include 2-deoxy-D-glucose (a glucose analog)[70,100-112], lonidamine (a derivative of indazole-3-carboxylic acid)[113-132], methyl jasmonate on HK[133-161], 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one on PFK[162-166], and iodoacetate on glyceraldehydes-3-phosphate dehydrogenase (GAPDH)[167-170].

Among various glycolytic inhibitors, 3-BP could only target two energy pathways of glycolysis and mitochondrial OXPHOS. The mechanism for 3-BP action suggested that mitochondrial HK Ⅱ is essential for the high glycolytic capacity *via* the utilization of mitochondrial ATP rather than cytosolic ATP, and the lowering of mitochondrial OXPHOS capacity by limiting Pi and ADP delivery to the mitochondria[171,172]. In contrast, DCA would affect only in cancer cells having impaired mitochondria function by PDK inhibition: otherwise alternative energy sources could compensate for the inhibited glycolysis through the competent mitochondria[173]. No phase Ⅲ randomized clinical trial of glycolytic inhibitors has exhibited satisfactory clinical outcomes[174].

Of note, the previously described studies that have reported the anticancer effects of these glycolytic inhibitors, with the exception of 3-BP, have exhibited common characteristics: (1) a low efficacy for anticancer effects when used alone; (2) capable of sensitizing cancer cells to conventional chemotherapy drugs, such as 5-fluorouracil, cisplatin, doxorubicin, and sorafenib; (3) the role of a sensitizer to make cancer cells vulnerable to radiotherapy and photodynamic therapy; and (4) an increase in the intracellular ROS levels as an important mechanism to induce their apoptosis.

PARADIGM SHIFT FROM THE WARBURG EFFECT TO THE REVERSE WARBURG EFFECT BASED ON LACTATE SHUTTLE

To explain metabolic flexibility, *i.e.,* mitochondrial OXPHOS, the reverse Warburg effect was suggested[175]; cancer cells educate carcinoma-associated fibroblasts (CAFs) to enhance aerobic glycolysis, and CAFs thereby produce lactate, which was converted to pyruvate and utilized for mitochondrial OXPHOS in cancer cells[175,176]. Tumor cells and CAFs influence each other in energy metabolites for co-evolution in cancer progression. A growing body of evidence indicates that lactate as an end product of glycolysis in hypoxic cancer cells and/or CAFs is not a waste product. Lactate can be used as

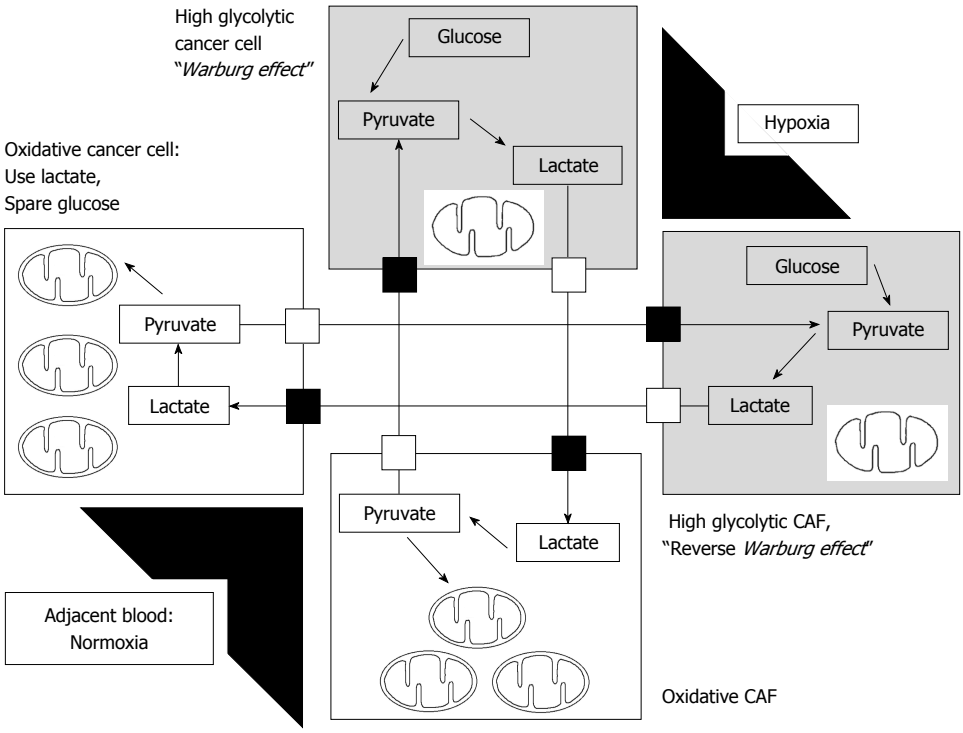

Figure 2 Metabolic interplay between high glycolytic cells and mitochondrial OXPHOS cells *via* lactate shuttle: the black box indicates monocarboxylate transporter 1, and the white box indicates monocarboxylate transporter 4: The gray zone means the hypoxic condition. Through MCT1 and 4, energy metabolites such pyruvate and lactate go in and out between glycolytic and oxidative cells: two roles of energy fuel and maintenance for acid-base balance in tumor microenvironment. MCT: Monocarboxylate transporters; CAF: Carcinoma associated fibroblast.

energy fuel for oxygenated tumor cells and/or oxidative CAFs as shown in Figure 2. Lactate is converted to pyruvate by LDH-B, which can enter the mitochondria to produce ATP in the cells with restored mitochondrial OXPHOS[31,40,176-181]. Particularly, oxidative tumor cells in normoxic microenvironment use mitochondrial OXPHOS to spare glucose, which can be utilized by glycolytic tumor cells located in hypoxic microenvironment.

During communication between glycolytic and oxidative cells, MCT1 and MCT4 are key players in this metabolic cross-talk. Influx of lactate by oxidative cancer cells occurs through MCT1, whereas lactate is released through MCT4[177]. MCT1 inhibition can make a metabolic shift from mitochondrial oxidation to glycolysis, which thereby induce glucose consumption. MCT4 inhibition can directly induce cell death *via* accumulation of intracellular lactic acid in hypoxic tumor cells[177]. Clinical phase I trials investigating MCT1 inhibitors are ongoing (http://clinicaltrials.gov/show/NCT01791595).

In addition to metabolic fuel by lactate shuttle, this phenomenon can maintain an acid-base balance *via* the prevention of the development of a fatal acidic environment in cancer cells[181,182]. Koukourakis *et al*[181,182] reported that increased expression of MCT1, LDH, and PDH in CAFs metabolically utilize lactate produced by tumor cells. In the other way, previous studies reported that some CAFs can undergo aerobic glycolysis and provide nearby oxidative cancer cells with the released lactate[176,182,183] (Figure 2).

Furthermore, accumulation of lactic acid, which represents high glycolysis rates in hypoxic conditions, reflects poor vascularity caused by rapid tumor growth. Previous studies demonstrated that lactate released from glycolytic tumor cells through MCT4 can stimulate angiogenesis and tumor growth *via* IL-8 dependent pathway[184]. Taken together, these findings establish important roles for lactate shuttles in tumors: (1) it acts as both a metabolic fuel; and (2) maintains an acid-base balance in cancer cells; and (3) signals for angiogenesis in hypoxic microenvironment. Finally, cancer cells can adapt to rapid changes in the tumor microenvironment through the metabolic interplay between oxidative and glycolytic cells, such as glycolytic and oxidative tumor cells and glycolytic and oxidative stromal cells, through lactate shuttle: "tumor heterogeneity and metabolic flexibility" (Figure 2).

THERAPEUTIC IMPLICATIONS OF TARGETING THE METABOLIC INTERACTION BETWEEN HIGH GLYCOLYTIC CELLS AND MITOCHONDRIAL OXPHOS CELLS

Although increasing evidence has recently indicated that mitochondrial OXPHOS and lactate shuttle may

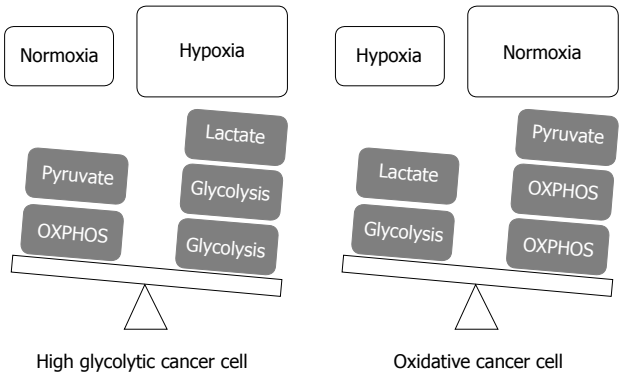

Figure 3 Metabolic flexibility of cancer cells depends on the tumor microenvironment: metabolic changes according to oxygen gradients. In hypoxic condition, glycolysis mainly contributes to produce ATPs with lowering reactive oxygen species production, thereby closely links with initiation of metastasis. In normoxic condition, mitochondrial oxidation more contributes for energy production than glycolysis. For survival, cancer cells adjust to dynamic changes between hypoxia and normoxia in tumor microenvironment. OXPHOS: Oxidative phosphorylation.

contribute to cancer cell survival and progression, the Warburg effect still plays a pivotal role in cancer cell metabolism and the initiation of metastasis in hypoxic conditions as previously discussed. Therefore, therapeutic strategies should focus on both targets, glycolysis and mitochondrial OXPHOS. Even though some studies reported that mitochondrial OXPHOS is also one of 3-BP targets[185], 3-BP has been mainly demonstrated to be the most potent glycolytic inhibitor among various types of inhibitors. However, including our studies, the results of *in vivo* studies that have used human HCC cell lines did not exhibit complete remission, but showed only partial remission after 3-BP treatment[18,19,43-70]. One reason why 3-BP did not completely suppress tumor growth might be the low efficiency to suppress mitochondrial OXPHOS, the lactate shuttle, and high redox potential in cancer cells. To overcome the weakness of glycolytic inhibitors, the inhibitors to target mitochondrial OXPHOS and other involved mechanisms such as the suppression of ROS production, might be effective when used simultaneously with glycolytic inhibitors as a combination treatment.

The metabolic interplay (lactate shuttle) between glycolytic and oxidative cells in tumors is modulated by two simple compartments for ATP production; glycolytic pathway and mitochondrial OXPHOS. Therefore, a combined treatment targeting both glycolysis and mitochondrial OXPHOS, could potentially be effective in suppression of tumor growth. Although various agents have been introduced to suppress mitochondrial metabolism, two agents, including metformin and glutamate dehydrogenase 1, were noticeable regarding their safety and potency. The metformin is widely used for Type Ⅱ diabetes in practical fields and has anti-cancer effects in animal models. Previous studies demonstrated that metformin inhibits complex Ⅰ *via* the inhibition of ubiquinone reduction and independently stimulates ROS production by the complex Ⅰ flavin[186-188]. It might be more clinically meaningful in terms of tolerable safety for a long period as demonstrated in diabetic patients.

Another promising agent that targets mitochondrial metabolism is the mitochondrial enzyme glutamate dehydrogenase 1 (GDH1). Previous reports demonstrated that glutamine may be utilized as the energy fuel for mitochondrial OXPHOS in cancer cells[189-192]. GDH1 is upregulated in human cancers and important for redox homeostasis by controlling the intracellular levels of its product alpha-ketoglutarate and subsequent metabolite fumarate, which subsequently activates glutathione peroxidase 1, *i.e.,* the antioxidant system. Targeting GDH1 by a small molecule inhibitor, the purpurin analog R162, resulted in an imbalanced redox homeostasis, which led to suppress tumor growth[193].

As described above, the common characteristics of glycolytic inhibitors were to increase ROS levels in cancer cells. Thus, a further increase in ROS stress using exogenous ROS enhancers combined with glycolytic inhibitors might effectively increase ROS levels above the threshold stimulating cell death pathways. The vulnerability for ROS stress in normal and cancer cells is quite different. Normal cells have tolerability for a certain level of exogenous ROS stress because of their high antioxidant capacity for lowering the ROS level and thereby prevent to reach the cell-death threshold[194,195]. In cancer cells, the increased ROS production from metabolic disarrangement and rapid proliferation may induce an upregulation of antioxidant capacity, *i.e.,* vulnerable redox equilibrium with high ROS production and elimination to maintain the ROS levels below the threshold for cell death[196,197]. Thus, cancer cells would be more vulnerable to increased oxidative stress induced by exogenous ROS enhancers that directly or indirectly suppress the antioxidant system[198-201]. These characteristics may provide a biochemical basis for a combination treatment of glycolytic inhibitors and ROS enhancers.

CONCLUSION

There have been a lot of advances to understand the importance of the Warburg effect and the metabolic interplay between glycolytic and oxidative cells in terms of lactate shuttle in previous decades. As previously discussed, the Warburg effect plays a vital role in cancer cell proliferation and survival in hypoxia, and also contributes to the initiation of tumor metastasis as matrix detachment. This effect is also highly linked to lowering the ROS levels to remain away from the cell death threshold. However, to survive under hypoxic conditions, a small portion of cancer cells and/or stromal cells should potentiate their mitochondrial OXPHOS rather than glycolysis to prevent a hostile acidic environment of lactate accumulation, which is the end product of the gly-

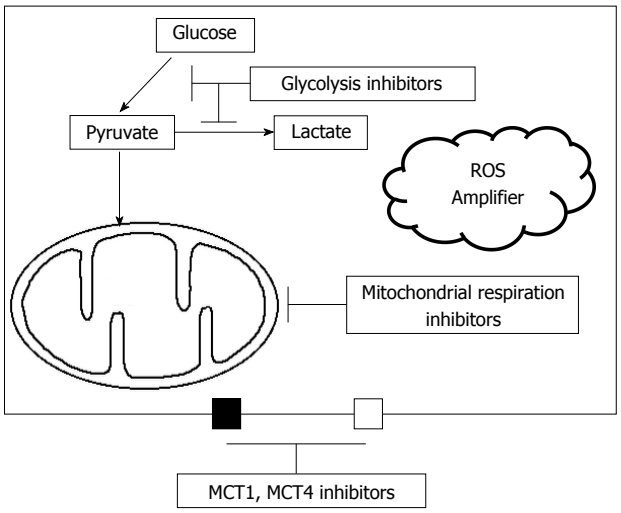

Figure 4 Potential therapeutic targets in metabolic interactions are suggested. A combination therapy might be promising; two targets that block the glycolysis and mitochondrial oxidative phosphorylation or block the glycolysis pathway and amplify ROS levels. Combination treatments targeting both the glycolysis and mitochondrial oxidation, or antioxidant systems can efficiently suppress energy production, or induce ROS-mediated apoptosis. ROS: Reactive oxygen species; MCT: Monocarboxylate transporters.

colytic pathway in hypoxic cancer cells. Thus, even in a hypoxic condition, oxidative cancer cells and/or stromal cells should theoretically exist to support the metabolic fuel for glycolytic cancer cells and handle their waste of lactate *via* the dynamic shuttle of lactate. Tumor heterogeneity exists in tumor mass under hypoxia, and metabolic flexibility is one adaptation mechanism to oxygen gradients (Figure 3).

Treatments against tumor metabolism may aim to target two distinct metabolic pathways of glycolysis and mitochondrial OXPHOS as a combination treatment (Figure 4). Although targeting one specific element of the tumor metabolism could often be ineffective because of the dynamic changes between glycolysis and mitochondrial OXPHOS, targeted treatments against glycolytic-oxidative cell interactions through the inhibition of glycolysis and mitochondrial metabolism might become a promising treatment for advanced stage HCC in clinical practice.

Given the unsatisfactory results of tyrosine kinase inhibitors in HCC treatment, a better understanding of the dynamic interactions between intracellular interactions, such as glycolysis and sensitivity of ROS amplification, and the intercellular interplay, such as glycolytic and mitochondrial OXPHOS cells in cancers, is critical to elucidate the heterogeneous biological features of HCC and identify effective strategies.

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REVIEW

Roles of the canonical myomiRs miR-1, -133 and -206 in cell development and disease

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Abstract

MicroRNAs are small non-coding RNAs that participate in different biological processes, providing subtle combinational regulation of cellular pathways, often by regulating components of signalling pathways. Aberrant expression of miRNAs is an important factor in the development and progression of disease. The canonical myomiRs (miR-1, -133 and -206) are central to the development and health of mammalian skeletal and cardiac muscles, but new findings show they have regulatory roles in the development of other mammalian non-muscle tissues, including nerve, brain structures, adipose and some specialised immunological cells. Moreover, the deregulation of myomiR expression is associated with a variety of different cancers, where typically they have tumor suppressor functions, although examples of an oncogenic role illustrate their diverse function in different cell environments. This review examines the involvement of the related myomiRs at the crossroads between cell development/tissue regeneration/tissue inflammation responses, and cancer development.

Key words: Muscle microRNAs; MiR-1; MiR-206; MiR-133a; MiR-133b; Cell development; Cancer

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Core tip: The roles of the canonical muscle-associated microRNAs are reviewed, including microRNA families miR-1 and miR-133, and single miR-206, which are collectively known as the "myomiRs". The myomiRs act at the crossroads of the molecular regulation of muscle cells, linking between pathways for cell differentiation, development and maintenance, but also potentiate aberrant cell growth in numerous non-muscle cancers. Typically myomiRs are downregulated in cancers, but some myomiRs are upregulated in a few cancers, yet each dysregulation event advances tumor progression. The review examines normal and disease-linked molecular changes associated with the myomiRs, and collates the extensive literature into accessible tables.

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INTRODUCTION

MicroRNAs (miRs) are short single strand RNA molecules (typically 22 nt) which interact in a semi-complementary manner with numerous target gene mRNAs, directed by a short "seed sequence", destining the targeted mRNA for degradation or for translational inhibition, and thus an miR can downregulate the functional expression of the target gene. In this manner a single miR can influence the abundance of numerous independent gene targets, and aid in the co-ordinate regulation of members of diverse cell signalling pathways, as well as metabolic pathways and basic cell proliferation or developmental processes. Three miR families, miR-1, miR-133 and miR-206 constitute the original (canonical) myomiRs and were considered muscle specific because of their prevalence in skeletal and cardiac muscle[1-5] and for their central roles in the regulation of myogenesis, muscle development and muscle remodelling[6-8]. Although other muscle enriched miRs such as miR-499 and -208, and others with key roles in cardiac muscle development have been identified, and although the term "myomiR" is now often used to denote several miRs encoded within myosin genes, for brevity this review is restricted to discussion of the three canonical myomiRs.

In man the genes encoding the canonical myomiR are organized into three cistrons encoding partners (miR-1-2, miR-133a-1), (miR-1-1, miR-133a-2) and (miR-133b, miR-206) and are located on chromosomes 18q11.2, 20q13.33 and 6p12.2, respectively. In this review we examine the roles of the myomiRs in normal tissue development and their emerging functions in various non-muscle tissues and their influence on the progression of cancers. The dysregulation of expression of the myomiRs in cancers is often related to a significant worsening patient prognosis, *via* the deregulation of a variety of validated gene targets.

The two mature miR-1 isomers have identical sequence, as have the two miR-133a isomers. The mature miR-133 isomers are also highly similar, differing only at the 3'-terminal base, with miR-133a$_{1/2}$ terminating G-3' and miR-133b with A-3', respectively. Independent upstream enhancers have been identified for the cistronic *miR-1-2 -133a-1* genes, as well as for the cistronic *miR-1-1/-133a-2* genes which are intronic to the *C20orf166* gene[9]. These independent enhancers allow the different isomer genes to be independently expressed under cell specific regulation.

DIFFERENT ROLES OF MYOMIRS IN MUSCLE

MicroRNA-1 and -133 were initially identified during the development and differentiation of skeletal muscle[7] and cardiac muscle[2,6]. Both *miR-1/-133a* gene cistrons are canonically expressed in skeletal and cardiac muscle[5,9], whilst the *miR-133b/-206* gene cluster is expressed in developing skeletal muscle[5] but not (significantly) in cardiac muscle, defining seminal roles of miR-1 and miR-133a in muscle biogenesis, and specifically in cardiac biogenesis[2,6]. A cartoon illustrating some of the major effects of myomiRs during differentiation of embryonic tissue and during tissue regeneration is shown in Figure 1.

MiR-133a has a regulatory role from the earliest differentiation of myogenic stem cells into myoblasts[7,10] continuing throughout the growth of structurally complex muscle tissues[7,11], and has homeostatic functions for muscle maintenance and protection in mature muscle, or in muscle regeneration from muscle progenitor cells after skeletal muscle stress or injury[5]. Key studies show miR-1, -133b and -206 acting during early development of skeletal myocytes through to the homeostatic maintenance of skeletal muscle[3,4,8], with miR-133b/-206 also having functions in neuromuscular synapse development and maintenance[12], as detailed in Tables 1 and 2.

Others have noted that the canonical myomiRs act as balanced regulators, often specifying broadly opposing functions. The miRs-1 and -206 are semi-homologous with closely similar mature sequences (and identical seed sequences), and target some genes in common, as well as independent targets. The identical mature seed sequences of miRs-133a and -133b implies they would share many targets in common, yet each of these miRs appear to have distinct cellular functions, with miR-133a expression common to all muscle and miR-133b abundant in all muscle types, except cardiac muscle. Loosely, the cell signalling pathways targeted by miR-1/-206 tend to have opposing functions to the regulatory pathways targeted by miR-133a/-133b. Both miR-1/ -206 act to promote myogenic differentiation, while the miR-133 isomers maintain the undifferentiated state and promote cell growth; hence co-expression of the myomiRs likely aids maintenance of homeostasis under normal cellular conditions.

This difference in expression of the related myomiR members in cardiac muscle compared to skeletal muscle may be associated with the physiological specialization of cardiac muscle, or its greater constancy of fibre type and function. In contrast, skeletal muscles constitute a variety of differentiated fibre types and are more plastic, capable of undergoing marked changes in myofibre content and physiology related to the level of use and workload[1,3]. As understanding of the molecular

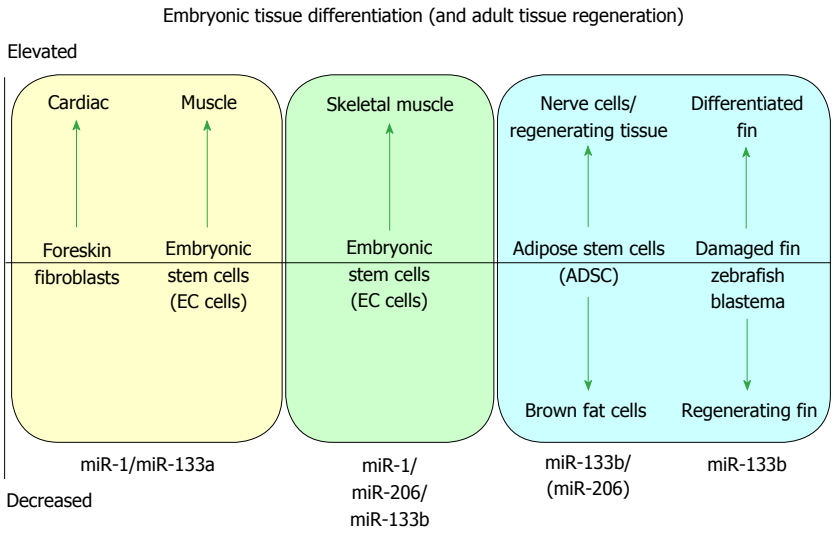


Figure 1 The roles of the myomiRs during embryonic tissue differentiation and adult tissue regeneration. Elevated levels of miR-1 and miR-133a are essential for differentiation of cardiac muscle[10,15], whilst miR-1, miR-206 and miR-133b are required for skeletal muscle differentiation[7,13]. Elevation of miR-133b levels in adipose stem cells leads to differentiation to a nerve-cell like fate[71], whilst reduction in miR-133b leads to brown fat cell differentiation[74]. Strong depletion of miR-133b and elevated Fgf allows regeneration of damaged zebrafish appendages[67,69].

regulation of muscle types have deepened, it is clear that the physiological and functional specializations are also reflected in the functions of the myomiRs.

CARDIAC MYOGENESIS

Studies with mammalian stem cells reveal broad functions for the myomiRs in the definition of primary differentiation pathways. Both miR-133 and miR-1 have roles in early cell programs leading to differentiation of muscle[2,10,13]. Pluripotent mammalian embryonic stem (ES) cells undertake cell fate decisions controlled by activation and repression of lineage-specific gene sets. These decisions are dictated by signalling networks which progressively narrow and specify the potential of ES cells as differentiation progresses. Muscle specific miR-133(a) and miR-1 both promote mesoderm formation from ES cells and suppress ectoderm and endoderm fates[2], but later during further differentiation into cardiac muscle progenitors, these miRs appear to have opposing regulatory functions[11,13]. Many non-muscle cell genes are repressed by miR-1 and miR-133 during this early ES cell differentiation program, suggesting that these two miRNAs may have general roles to regulate early ES cell-fate decisions from pluripotent cells[13], with miR-1 specifically targeting the translational repression of Dll-1 and Cdk9[10].

In vivo, the deletion of both *miR-133a1/2* genes causes lethal cardiac (ventricular-septal) abnormalities in about half of the mouse embryos or neonates, while mice deficient in only one of either miR-133a-1 or -133a-2 have phenotypically normal hearts[14]. Skeletal muscles are normal in both double and single mutant miR-133a mice (dead and surviving), implying that miR-133b can replace the absent miR-133a species in skeletal muscle and continue the regulation of normal development. In double mutant mice lacking all miR-133a, smooth muscle gene expression was activated 2-4 × and cardiomyocytes (but not cardio-fibroblasts) proliferated 2.5 × faster than normal, accompanied by increased expression of miR-133a targets, including PTBP2, CDC42, cell cycle control factors and cyclins D1, D2 and B1[14]. Recently, both adult and neonatal human foreskin fibroblasts were found capable of being reprogrammed towards cardiac muscle by exogenous expression of only several factors, myocardin, HAND2, T-box-5, GATA4, and miR-1, miR-133a and miR-499[15]. These stimulated cells expressed cardiac specific proteins and showed spontaneous contractility, emphasizing the role of these miRs in the control of specific cell development programs *via* the modulation of specific factor targets. Further, both human and mouse fibroblasts can be reprogrammed to form cardiomyocyte-like cells by overexpression of cardiac transcription factors (Gata4, Mef2c, and Tbx5 (GMT) or GMT plus Mesp1 and Myocd) along with miR-133a, which directly represses Snai1 which normally regulates EMT processes[16]. Interestingly, exogenous miR-133b can also downregulate Snai1 expression, suppressing fibroblast genes and upregulate the expression of a number of characteristic cardiac cell genes *in vitro*, yet it cannot replace miR-133a during normal heart development *in vivo*.

CARDIAC MUSCLE INJURY

Heart contractility and heart rate are stimulated during chronic pressure overload by activation of the sympathetic nervous system causing catecholamine release. The catecholamines activate β-adrenergic receptors and overstimulation is a component of heart

disease. MiR-133 directly targets adenylate cyclase VI and the catalytic subunit of PKA, both elements of the β1AR signal transduction cascade, reducing signalling[17]. Similarly carvedilol, an *in vivo* β-adrenergic blocker, improves the cardiac function in infarcted rats by restoring miR-133 expression, resulting in reduced cardiomyocyte apoptosis[18]. *In vitro* overexpression of miR-133a in cardiac cells has similar effects to carvedilol by downregulating caspase-9 and caspase-3 expression in the presence of H_2O_2. Overexpression of miR-133a also reduces ROS and malondialdehyde content, and increases SOD activity and GPx levels, protecting cardiomyocytes from apoptosis. Studies in mouse by Caré *et al*[6] also demonstrated that downregulation of both miR-133 and miR-1 are involved in cardiac hypertrophy. Specific targets of miR-133 such as RhoA, Cdc42 and Nelf-A/ WHSC2 can accumulate and contribute to the hypertrophy of cardiac myocytes during infarction.

SKELETAL MUSCLE MYOGENESIS

Liu *et al*[2,9] (2007, 2010) also established a fundamental model of differential expression of cistronic *miR-1* and *miR-133a* genes during myogenesis and differentiation of skeletal muscle, smooth muscle and cardiac muscle. The factor MEF2 controls expression of the miR-1-2/-133a-1 cistron *via* at least two MEF2 enhancer loci: one MEF2 enhancer located 3' upstream of the *miR-1-2* gene, a second intragenic MEF2 enhancer located upstream of the *miR-133a-1* gene and a third (less defined) locus far upstream that requires MyoD for expression[19]. Transcripts of *pri-miR-1-2/-133-a-1* (bi-cistron), *pri-miR-1* and *pri-miR-133a-1* genes indicate that both proximal enhancers are functional[1]. Others have emphasized the distribution of these regulatory enhancers[2] drawing attention to the differential expression of these cistronic miRs which the regulatory factor binding sites provide. The regulation of expression of the cistronic miRs by these key muscle development regulatory factors, which are themselves targets of repression by these self-same miRs, also emphasizes the precise inter-regulatory control of each of the various developmental program factors.

Notably, the 2,6-disubstituted purine reversine can induce differentiation reversal (de-differentiation) of C2C12 murine myoblast cells back into multipotent progenitor cells[20]. This occurs by inhibition of Aurora A and B protein kinases, reducing histone H3 phosphorylation, which in turn induces chromatin remodelling and restores cell multipotentency. Reversine treatment also stimulates expression of polycomb genes, Phc1 and Ezh2, leading to inhibition of expression of the muscle-specific transcription factors, myogenin, MyoD, and Myf5[21]. Concomitantly, reversine strongly inhibits miR-133a expression in C2C12 cells through the reduced expression of SRF transcription factor and by reduction of its binding to the miR-133a enhancer and by reduced epigenetic histone modifications on the miR-133a promoter, including reduced trimethylation, phosphorylation, and acetylation[22]. The co-overexpression of a miR-133a mimic along with reversine treatment prevents C2C12 myoblast de-differentiation, indicating the central role of the inhibition of miR-133a expression to the de-differentiation process. Significantly, reversine induced de-differentiation of committed cells is not limited to myoblasts, and reversine treatment can transform primary murine dermal fibroblasts into myogenic-competent cells within regenerating muscle *in vivo*[23].

Skeletal muscle myogenesis also involves the IGF signalling pathway[24], which activates muscle proliferation and differentiation *via* the PI3K/AKT pathway. The IGF pathway is regulated by miR-133-a1 which directly inhibits translation of IGF-1R protein, resulting in repression of PI3K/AKT pathway activity. IGF-1, which increases and activates IGF-1R during myogenesis by binding and inducing its phosphorylation, also indirectly activates myogenin, which in turn activates miR-133 activity. Thus miR-133 provides a negative regulation loop to monitor and control PI3K/AKT pathway activity. Similarly, miR-1 targets and reduces the activity of IGF-1 in differentiating C2C12 skeletal muscle cells and in heart muscle during cardiac failure states[25], meanwhile active IGF-1 signalling pathway downregulates miR-1 *via* repression of FoxO3a transcription factor. Thus, miR-1 also mediates the activity of the IGF-1 signal pathway and is itself feed-back regulated by the IGF-1 signal transduction cascade. Significantly, IGF-1 signalling (IGF-1 and IGF-1R) has key roles in the growth and development of many tissues[26], and also in the progression of many cancers (see later).

SKELETAL MUSCLE PLASTICITY

Skeletal muscles are plastic tissues in which the ratios of muscle fibre type (slow or fast twitch, smooth muscle, *etc*.) are to some degree responsive to remodelling through environmental input and nerve control. The muscle fibre type is maintained by the type of nerve signals received by the muscle, and transition between fast and slow twitch fibres can occur over time if nerve signals are changed from slow to fast type, and *vice versa*[27]. Similarly, prolonged workload or exercise can alter muscle fibre type and its metabolism to allow it to better respond before exhaustion. Muscle development programs regulated by miR-1 and miR-133a play important roles in muscle remodelling[4], and in hypertrophic skeletal muscle miR-1 and -133 levels are decreased[28], indicating that functional overload of muscle induces regulatory alterations which are in part influenced *via* altered miR activities.

REGULATORS OF MYOMIR EXPRESSION

Myostatin is a repressor of myogenesis, and its down-regulation allows increase of miR-1, -133a, -133b and -206 expression, activating muscle cell proliferation[29]. In contrast, myogenic factors myogenin and MyoD are well known positive regulators of myomiR expression in muscle that bind upstream of *miR-1/-133a* genes at defined enhancer regions[2,11]. SRF, MyoD and MEF2 are also direct transcriptional activators of myogenesis-related miR-1 expression in cardiac muscle[2,30]. Since downregulation of myostatin permits expression of miR-133b/-206, and MyoD and myogenin also binds the miR-206 promoter[11], suggesting that miR-206/-133b expression in muscle may also be in part controlled by MyoD/ myogenin.

The ERK1/2 signalling pathway also regulates expression of miR-133 during myogenesis in the C2C12 cell model[31], and its activity is also feedback influenced by miR-133. During myogenesis both miR-133a and -133b are upregulated, and both FGFR1 and PP2AC which function in the ERK1/2 pathway signal transduction are negatively regulated post-transcriptionally by both miRs. Inhibition of ERK1/2 pathway signalling inhibits C2C12 cell proliferation, stimulating initiation of differentiation and forming small truncated myotubes. Importantly, ERK1/2 signalling pathway activity negatively regulates expression of miR-133, providing a feedback loop between miR-133 levels and ERK1/2 signalling activity, forming an additional reciprocal mechanism for regulating myogenesis.

Recently other cellular factors have been identified that influence post-transcriptional maturation or bio-availability of myomiRs in muscle. mTOR regulates miR-1 indirectly in regenerating mouse skeletal muscle and differentiating myoblasts[32]. mTOR most likely affects MyoD protein stability, which then alters miR-1 expression through the availability of MyoD to bind its upstream enhancer. A pathway downstream of mTOR also operates in which miR-1 suppression of HDAC4 results in production of follistatin, which subsequently activates myocyte fusion. This suggests that an mTOR-miR-1-HDAC4-follistatin pathway regulates myocyte fusion during myoblast differentiation and in regenerating skeletal muscle.

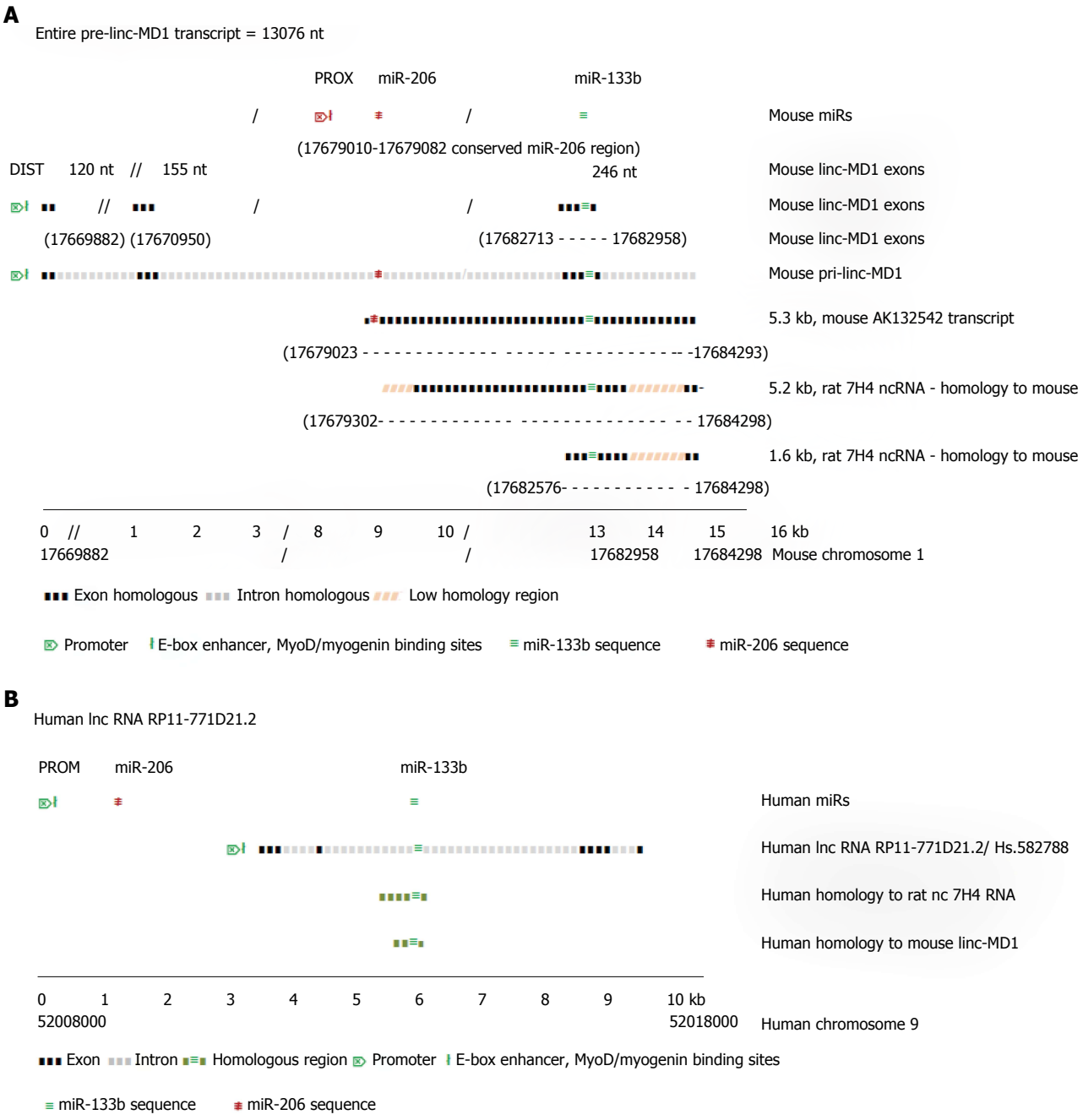
King *et al*[33] (2014) demonstrated that the RNA-binding TDP-43 protein interacts with miR-1/-206 family (but not the miR-133 family) in skeletal myoblast cells, limiting their bioavailability by preventing interaction with the RISC silencing complex, noting this is the first observation of a mechanism differentiating between mature bicistronically encoded miRs, which selectively modulates the bioactivity of downstream targets of the sequestered miRs. TDP-43 accumulates in motor neurons during ALS, a neuromuscular wasting disease. Two miR-1/-206 targets, IGF-1 and HDAC-4 are elevated in both ALS-model transgenic mouse muscle and in cells modified to overexpress TDP-43. The authors suggest the decreased miR-1 (-206) activity in ALS affected muscle could alter retrograde signalling at the NMJ through the dysregulation of both HDAC-4 and MEF-2, whereby miR-1 refines synaptic function by coupling changes in muscle activity to changes in presynaptic function[34].

Factors KSRP[35], MBNL1 and RNA binding protein LIN28[36] also positively and negatively regulate miR-1 biogenesis respectively. Additionally, miR-206 binds to 3'-UTR sites of KSRP transcript to inhibit KSRP expression in skeletal muscle[37]. Independently, miR-206 and KSRP are negative regulators of utrophin A, but unexpectedly, overexpression of miR-206 in both normal and dystrophic muscle cells promotes upregulation of utrophin A, *via* the downregulation of KSRP. Thus, miR-206 appears capable of switching between direct repression of utrophin A expression and the activation of its expression through decreased KSRP, the two molecular mechanisms providing close counter-regulation of utrophin A expression.

CISTRON MIR-206 AND MIR-133B

Although the microRNA-206 and -133b are thought typical of muscle specific miRs, little is known explicitly of their functions in skeletal muscle. Cesana *et al*[38] (2011) showed that *miR-133b* gene transcript in mouse is located within the precursor of the long (spliced) non-coding RNA linc-MD1 which is expressed under the control of an upstream distal (DIST) cistronic promoter[12] (Figure 2A), while the *miR-206* gene, which is located within the intron of linc-MD1, is transcribed autonomously under control of its own proximal (PROX) promoter. In proliferating myoblasts, only primary miR-206 transcript is expressed strongly, initiated from the PROX promoter. During mouse muscle differentiation, long distance interactions bring the DIST promoter into conjunction with PROX and the polyA addition regions of linc-MD1, facilitating the co-expression of linc-MD1 RNA, as well as the primary miR-206 transcript, with the PROX promoter activated by both MyoD and myogenin binding[11]. Notably, mature linc-MD1 RNA contains binding sites for miR-133 (and miR-135) acting as a binding "sponge" to downregulate their free abundance, in turn contributing to the expression regulation of the targets of these miRs, which include key muscle transcription factors[38]. In mouse muscle, the expression of mature linc-MD1 RNA mutually excludes the expression of miR-133b, which must be excised from the linc-MD1 pre-transcript. In the rat genome, a distinct ncRNA 7H4, covering less than half of the mouse linc-MD1 precursor transcript, but closely similar to a mouse RNA AK132542 transcript, also encodes the *miR-133b* gene, suggesting a similar function to linc-MD1 may occur in rat[39].

Figure 2A uses information from Cesana *et al*[38]


A
Entire pre-linc-MD1 transcript = 13076 nt
PROX
miR-206
miR-133b
Mouse miRs
(17679010-17679082 conserved miR-206 region)
DIST
120 nt
155 nt
246 nt
Mouse linc-MD1 exons
Mouse linc-MD1 exons
(17669882) (17670950)
(17682713 - - - - - 17682958)
Mouse linc-MD1 exons
Mouse pri-linc-MD1
5.3 kb, mouse AK132542 transcript
(17679023 - - - - -

(2011) and Rosenberg *et al*[40] (2006) to illustrate the aligned transcript regions of the mouse pri-linc-MD-1, the 5.3 kb random cloned mouse transcript cDNA (GenBank sequence) AK132542 and the 5.2 kb rat 7H4 ncRNA transcript. The independent pre-miR-206 transcript overlaps almost completely with the AK132542 transcript which is likely the mouse homolog of the expressed rat ncRNA 7H4. The *7H4 RNA* gene is almost fully conserved in the mouse genome, with the 7H4 RNA overlapping the 3' exon of *linc-MD1* gene (containing *miR-133b* gene) almost to the 3' terminus of the *miR-206* gene. Velleca *et al*[39] (1994) found two major transcripts of 7H4 RNA, a long 5.2 kb molecule, and a short 1.6 kb molecule coincident with the 3'-terminal region of the 5.2 kb transcript. The 1.6 kb fragment is much more abundant than the full length molecule, suggesting it is a product from the excision of miR-133b from the full length primary transcript. Notably, both long and short ncRNA 7H4 transcripts contain the entire *miR-133b* gene locus, suggesting they may function in rat similar to mature linc-MD1 in mouse, to bind complementary miRNAs, including miR-133. Similarly in man, ncRNA RP11-771D21.2 may represent the functional human homologue of mouse linc-MD1 RNA (Figure 2B).

Recently Legnini *et al*[41] (2014) reported that the mutually alternative synthesis of linc-MD1 and miR-133b is controlled by the pleiotropic mRNA regulator protein, HuR. In developing skeletal muscle, HuR favors accumulation of mature linc-MD1 by binding to it and repressing cleavage that would release pre-miR-133b. The level of HuR protein expression is also under the repressive negative control by miR-133 targetting, yet the sponging-up of miR-133 by the linc-MD1 helps consolidate HuR expression by forward positive control. Muscle developmental progression to later differentiation stages may involve overcoming this HuR-linMD1 repression of miR-133b expression by the independent miR-133a1/2 isomers which could downregulate HuR expression, allowing miR-133b excision, permitting developing muscle to exit from the control circuit. The level of linc-MD1 correlates inversely with the level of miR-135/-133b, which in turn control the expression of transcription factors MAML1 and MEF2C which are necessary for specific muscle gene expression. Thus, linc-MD1 activity provides another mechanism for pleiotropic regulation, slowing or activating muscle differentiation. Other evidence suggests the influence of HuR on many gene mRNA transcripts depends on the interplay of HuR with particular regulatory miRs that target and control the expression of the self-same mRNAs.

In sum, because the myomiRs target components of key signalling pathways and processes that control muscle cell development and maintenance, expression of the myomiRs is tightly regulated, often *via* feedback and feedforward circuits that provide both tight regulatory control and the ability to amplify myomiR expression. In muscle the myomiRs display high interconnectedness in terms of the regulation of their expression and the complementary processes that their regulatory functions influence. *MyomiR* genes are apparently cistronically encoded, yet expression of each of *myomiR* genes can be individually controlled by various transcription regulatory factors and other interactions such as RNA-RNA binding, such that expression of particular *myomiR* genes can be selectively enhanced under cellular conditions in which particular transcriptional regulatory factors are available.

ESTABLISHMENT OF NEUROMUSCULAR JUNCTIONS

MicroRNA miR-206 is expressed (virtually) exclusively in (developing) skeletal muscle[8], contributing to muscle differentiation programs through repression of Idl-3 protein expression, a downregulator of MyoD activity, as well as repressing the p180 subunit of DNA polymerase alpha, essential for DNA synthesis which occurs during differentiation[42]. MyoD itself promotes the expression of the miR-133 cistrons[11]. Further, in fast twitch muscle of mouse[12] and rat[43] miR-206 has been found to promote formation of new neuromuscular junctions following peripheral nerve denervation (scission). The expression of miR-206 and miR-133b are both upregulated strongly in muscle after denervation (as is 7H4 ncRNA), whereas miR-1 and miR-133a are downregulated. Four months after nerve scission, the re-innervated muscle was predominantly type II glycolytic fibres, suggesting that miR-206 may aid the determination of fibre type *via* down-regulation of MEF2 transcription factor activity[43]. Valdez *et al*[44] (2014) also examined the role of miR-133b and miR-206 on neuromuscular junction repair in injured mice. In miR-206 null mice, re-innervation was impaired following nerve injury, and in mice null for *-133b* and *-206* genes the same impaired neuromuscular repair was seen as in single gene *miR-206* null mice, whilst in single gene *miR-133b* null mice development and re-innervation proceeds normally following nerve injury. Together, these findings imply that miR-206 is the major regulator of nerve repair and reconnection to muscle following injury. In support, in miR-133b null mice Pitx3 levels were normal and impairment of locomotion was not detectable, controversially implying that miR-133b has no significant roles in neuron development, neuron maintenance and function *in vivo*[45]. In contrast, other studies with miR-206 null mice show no obvious phenotypic effects, muscles develop normally and mouse physiology appears normal, suggesting that other factors (including miR-133b) can replace miR-206 during development[46]. However, if the miR-206 null mice are then denervated, about 90% of both wt and miR-206 null mice recover and re-innervate after about

8 wk. This strongly suggests that other factors (including miR-133b) can provide redundant functions for the absent miR-206, including promoting compensatory peripheral nerve regeneration. Furthermore, miR-133b directly stimulates neurite outgrowth following nerve damage in rat brain after treatment with multipotent MSC cells[47], suggesting that elevation of levels of muscle miR-133b after muscle denervation is related to nerve regeneration, and that miR-133b may suffice in miR-206 null mice to replace absent functions. These various observations imply the likelihood that both miR-206 and -133b have functions in the recovery and maintenance of nerve-muscle signalling.

RETROGRADE SIGNALLING BETWEEN MUSCLE AND NERVE

Additionally, miR-206 targets BDNF which promotes efficient skeletal muscle regeneration following damage[48]. BDNF also controls the initiation and maintenance of the differentiated state of muscle cells, potentially *via* the regulation of retrograde signalling at the neuromuscular junction. The loss of neural input to muscle also causes HDAC4 to accumulate, reducing MEF2-regulated gene expression. Importantly, miR-206 targets HDAC4 and fibroblast growth factor signalling pathways in muscle. HDAC4 regulates neuromuscular-related gene expression and acts in the regulation of muscle remodelling, influencing the formation of appropriate nerve types which connect to the muscle[49]. Significantly, it has been shown that expression of miR-1/miR-133a is also regulated by an intragenic MEF2-enhancer[9], and miR-1 also regulates a MEF-2 dependent retrograde signal at the neuromuscular junction, suggesting that members of both myomiR cistons act to maintain neuromuscular homeostasis[34].

In mouse, members of the MyoD muscle transcription factor family, myf-4 and myogenin, are progressively downregulated during maturation from embryonic day 15 to the first postnatal weeks (weeks 1-3), coinciding with induction of muscle innervation[50,51]. In contrast, muscle denervation results in strong expression of MyoD and myogenin, preceding the accumulation of nAChR, α-subunit[39]. Additionally during myogenic differentiation, acetylcholinesterase transcript levels increase dramatically (5 ×), principally due to its stabilization by binding with HuR protein[52], consistent with a regulatory role of HuR in neuron excitability. Normally the expression of MyoD and myogenin is suppressed by activated nerve signal pathways, including by electrical conduction *per se*, and sets of muscle genes regulated by the MyoD family and myogenin are downregulated by increasing electrical activity and other nerve-derived signals. Thus again, a pronounced neuromuscular maintenance function for miR-206/-133b can be implied from interplay of signalling control between skeletal muscle and nerve. Both myogenin and MyoD induce the expression of miR-133b and -206, while repression of these factors inhibits their expression. On balance it appears that cistronic miR-206 and -133b and linc-MD1 homologues may contribute to programs of regulatory developmental gene expression in growing muscle and peripheral nerve, facilitating programs to interregulate the developing nerve connections with muscle, and speculatively aid in coordinating appropriate nerve and muscle gene expression programs, establishing interactions between skeletal muscles and their appropriate innervating nerves to maintain muscle fibre type and their correct neuromuscular junction associations.

MUSCLE MITOCHONDRIAL FUNCTION

Zhang *et al*[53] (2014) reported that miR-1 enters skeletal muscle mitochondria efficiently during muscle development whereby it stimulates the translation of specific mitochondrial genome-encoded transcripts, contributing positive regulation to muscle development. This stimulation of translation requires specific base-pairing between miR and its target mtRNA as well as interactions with mt-located Ago2 protein. These observations contrast earlier findings of Das *et al*[54] (2012) who showed that the mature miR-181c translocates into rat cardiac muscle mitochondria, reducing mitochondrial cytochome oxygenase 1 (mt-COX1) compared to mt-COX2 and mt-COX3 proteins. The reduced mt-COX1 causes mitochondrial complex IV remodelling, resulting in increased mt respiration and increased ROS generation. Recently, Das *et al*[55] (2014) used cationic nanoparticles to deliver miR-181c into rat cardiac mitochondria *in vivo,* causing cardiac dysfunction and a tendency to develop heart failure. Taken together, these studies reveal important new miR-mediated regulatory pathways in muscle mitochondria involving direct manipulation of mitochondrial gene expression by cytosolic miRNAs, including by a myomiR.

Importantly in both cardiac and skeletal muscle, mitochondrial UPC2/UCP3 uncoupling proteins regulate energy homeostasis and the rate of development and differentiation, with UPC2 repressing differentiation and promoting cell proliferation. However, MyoD activates miR-133a expression which in turn directly downregulates UCP2 mRNA to alleviate the developmental repression, suggesting a feedback network involving MyoD-miR-133a-UCP2[56]. Additionally, over-expression of myogenin and MyoD in mouse C2C12 myoblasts[57] increase expression strongly from the UCP3 promoter, but act weakly at the UCP2 promoter. Together these observations suggest UCPs helps maintain balance between muscle differentiation and proliferation during myogenesis, regulated by a MyoD-miR-133a-UCP2 feedback network and by differential responsiveness of UCP2 and UCP3 promoters to activation by myogenin and MyoD.

Furthermore, a downregulation of mitochondrial function is associated with skeletal muscle injury, including increased ROS and reduced cellular ATP generation. However, the recovery and regeneration of post-injury skeletal muscle involves the activation and proliferation of resident stem cells, including satellite cells and endothelial precursor cells followed by their differentiation into myocytes. Jash *et al*[58] (2014) showed during recovery from muscle injury that the AMPK-CRTC2-CREB and Raptor-mTORC-4EBP1 pathways are activated in satellite cells. mTORC1 positively regulated Ccnd1 translation, yet destabilized Ccnd1 mRNA. These opposing effects of mTORC1 were mediated by two miRs which target the 3'-UTR of Ccnd1 mRNA: one being miR-1 which in mTORC-knockdown muscle was downregulated, allowing Ccnd1 mRNA to accumulate. The authors suggest that mTORC may act to coordinate satellite cell proliferation during the activation of myogenesis.

MUSCLE DEGENERATION AND INFLAMMATION

During conditions of skeletal muscle atrophy and wasting, the cytokine TWEAK and its binding receptor Fn14 are elevated, activating catabolic and pro-inflammatory processes[59]. TWEAK inhibits expression of myoD, MEF2C and myogenin which in turn inhibits expression of miR-1, -133, and -206, suppressing differentiation of progenitor cells into myocytes. HMOX1, another factor associated with inflammation[60], also inhibits myoblast differentiation and myotube formation, by inhibition of expression of each of the myomiRs, again by limiting their transcription factors, MyoD and myogenin. Thus, both HMOX1 and TWEAK may be potentially involved in the regulation of broadly common inflammation associated pathways in skeletal muscle.

Interestingly, TWEAK[61] has a role in stimulating the proliferation of normal neonatal rat cardiomyocytes, increasing cell numbers accompanied by expression of cell proliferation markers Cyclin D2 and Ki67, and other cell cycle factors. In contrast, adult rat cardiomyocytes cannot be stimulated by TWEAK because of the developmental downregulation of its receptor Fn14 in adult cells, coincident with the loss of proliferation capacity in mammalian cardiomycetes several weeks after birth. Fn14 is present in neonatal cardiomyocytes, interacting with TWEAK to activate downstream signalling *via* ERK and PI3K signalling pathways, as well as *via* inhibiting glycogen synthase kinase-3β.

FAPs are quiescent progenitor cells resident in normal muscle that can facilitate myofibre regeneration after muscle damage by providing factors which stimulate proliferating myogenic progenitor cells. In dystrophic muscle disease, FAPs typically proliferate and give rise to their differentiated progeny, fibroblasts and adipocytes which replace muscle tissue. However, Saccone *et al*[62] (2014) found in early-stage disease dystrophic (mdx) mouse muscles that HDAC inhibitors can activate and commit FAPs themselves towards regeneration of muscle tissue, by derepressing a "latent" myogenic program. The inhibition of HDAC induces two core components of the myogenic transcriptional machinery, MyoD and BAF60C, which upregulate expression of miR-1-2, miR-133, and miR-206. The structural subunits of the BAF chromatin remodelling complex (BAF60a, BAF60b and BAF60c) bind to Brg1 (the core complex ATPase) and provide functional specificity[63]. BAF60c is a specific member of the complex during myogenesis, and is essential for the myogenesis process. Interestingly, BAF60a and BAF60b are targets for downregulation by miR-133 and miR-1/206, suggesting that such negative regulation increases the availability of (non-target) BAF60c to join the muscle remodelling complex. Furthermore, a recent study of inflammatory myopathies[64] including dermatomyositis, polymyositis, and inclusion body myositis found increased expression of the inflammatory cytokine TNFα was associated with decreased expression of miR-1, miR-133a, and miR-133b in all subtypes, plus the decreased expression of miR-206 in dermatomyositis. TNFα inhibited the expression of myogenic miRNAs in cells in an NF-κB-dependent manner, while the overexpression of miR-1, miR-206, or miR-133a/b could relieve the TNFα blockage of myogenic cell differentiation. Overall, the dysregulation of myomiR expression in muscle degenerative diseases was demonstrated to be intrinsic to the disease progression.

In diseased cardiac tissues, pre-inflammatory reactions involve upregulation of *CNN* genes, and *in vitro* the downregulation of CNN2 blocks multiple proinflammatory and profibrotic pathways in mouse activated primary cardiac fibroblasts (PCFBs)[65]. Immune cell chemotaxis towards CCN2-depleted PCFBs is also reduced strongly. CCN2 is a direct regulation target of miR-133b, and silencing of CCN2 expression by siRNA strongly decreases the expression of stretch-induced chemokines, matrix metalloproteinases, extracellular matrix and a cell-to-cell contact protein, indicating that CCN2 is involved in control of multiple signal pathways for muscle regeneration. Exogenous factors also influence muscle recovery after injury. PRP plasma is an enriched source of autologous platelet α-granule-derived growth factors and cytokines which can stimulate tissue healing[66]. When PRP is applied to injured rat soleus muscle, the recruitment, proliferation and differentiation of cells for muscle recovery is stimulated. Molecular analysis showed that 5 d after PRP treatment the expression of proinflammatory cytokines IL-1β, and TGF-β1 was increased strongly, which in turn induced expression of myogenic factors MyoD1, Myf5 and Pax7, and muscle IGF-1Eb isoform, and muscle recovery was strongly accelerated. Concomitantly miR-133a and miR-1 were downregulated (miR-133a markedly), while SRF was

upregulated, phosphoryled αB-cristallin was increased, as were apoptotic factors (NF-κB-p65 and caspase 3) which together indicate enhanced cell survival. Overall, PRP contributes to repair of injured skeletal muscle by *via* the control of secondary pathways (regulated by myomiRs and heat shock proteins) that modulate both inflammatory and myogenic pathways, with each contributing to the regulated tissue regeneration.

ROLES OF THE MYOMIRS IN OTHER TISSUES

Limb regeneration in lower vertebrates

Whilst miR-133 plays a central role is the repair of damaged muscle and nerves in mammals, in lower vertebrates such as amphibians and teleost fish which retain the capacity for regeneration of entire limbs after damage or loss, here the downregulation of miR-133 plays a central role in organizing the reactivation of cells for the repair of complex tissues. Yin and Poss[67] (2008) found that miR-133 controls complex biological processes involving formation of the regeneration blastema, a proliferative mesenchymal cell mass which is the progenitor for regeneration of the lost structures, ultimately developing into organized organs, including connective tissues, muscle, blood vessels and nerve tissues[67]. When zebrafish fins are excised, the depletion of miR-133 is controlled by increased Fgf signalling (Tables 1 and 2). In normal developed fins, high levels of miR-133 are maintained, accompanied by a cessation of Fgf signalling, indicating that high miR-133 levels normally suppresses tissue proliferation factors and signalling pathways, maintaining developed tissue homeostasis[68]. Increased miR-133b also influences spinal cord regeneration in adult zebrafish, reducing the level of RhoA protein, an inhibitor of axonal growth, and stimulating spinal cord regeneration of axons from neurons in particular brain structures[69].

MAMMALS

Corneal repair

In mouse cornea after laser ablation injury[70], miR-133b is the most strongly reduced miR (amongst others) during wound recovery, allowing increased expression of its targets which include CTGF growth factor, SMA, and COL1A1. Transforming growth factor β1-treated rabbit corneal fibroblasts also produced significant decrease in miR-133b, associated with significantly increased expression of CTGF, SMA, and COL1A1, and helped to minimise scar development during corneal recovery. See Table 2 for other tissues.

NERVE CELL DIFFERENTIATION

The miR-133b has roles in early stem cell differentiation leading to nerve development. ADSC stem cells can be induced to differentiate into neuron-like cells by IGF-Ⅰ signalling, which increases miR-133b expression *via* the downregulation of beta-Ⅲ-tubulin, Pitx3 and IGF-IR by translational repression of their proteins[71]. Neural differentiation from ADSC involves a feedback control mechanism in which IGF-I upregulates miR-133b, while miR-133b in turn downregulates the signal receptor activity, IGF-IR. Xin *et al*[47,72] found multipotent MSC cells regulate the growth of neurites *via* direct exosomal transfer of miR-133b to neural cells. Middle cerebral artery occluded rat brains elevate miR-133b in MSC exosomes, which stimulates neural regeneration. Exosomes transfer to adjacent astrocytes and neurons, reducing the expression of selected miR-133b targets, including CTGF and RhoA[72]. The first identified molecular role for miR-133b was in neural tissue[73] where it regulated the maturation of mammalian midbrain dopaminergic neurons (DNs), along with the paired-like homeodomain transcription factor Pitx3, which itself then regulated the transcription of miR-133b in a feedback control loop.

ADIPOCYTE DIFFERENTIATION

Several studies found that miR-133 a/b isomers play key roles in the differentiation of brown fat tissues from precursor cells after cold exposure[74-76]. Strong reduction of the transcription regulator MEF2 caused reduction of miR-133a, allowing an increase in the adipocyte progenitor specific Prdm16 (a miR-133 target), which promotes the differentiation of both myogenic precursor cells and white fat precursor cells into brown adipocytes. Adult mouse skeletal muscle satellite cells can differentiate into brown adipose *via* miRNA-133 targeting of Prdm16, leading researchers to suggest that the presence of these miRs may indicate energy dissipating cell lineages (muscle, nerve), compared to energy storing cells, such as white adipose tissue[76]. Mice with knockdown of *miR-133a1/a2* genes respond to cold exposure more strongly than wild-type animals and have increased insulin sensitivity and glucose tolerance associated with activation of the brown fat and thermogenic gene programs in subcutaneous white adipose tissue.

CHONDROCYTE DIFFERENTIATION

MiR-1 is highly expressed in the hypertrophic zone of growth plate cartilage, some 8-fold higher than in the proliferation zone[77]. MiR-1 strongly promotes chondrocyte proliferation and differentiation, including induction of the expression of chondrocyte markers Indian hedgehog and Col X, and acts by targetting HDAC4. Additionally HDAC4 negatively regulates chondrocyte hypertrophy by inhibiting Runx2, a critical transcription factor for chondrocyte hypertrophy. In contrast, miR-1 is repressed strongly during hypertrophic

Table 1 Roles and targets of the myomiRs, miR-1, -206, -133a, -133b

Factor(s)	Regulation	Regulator	Tissue/cell	Ref.
Fish and lower vertebrates: Development and regeneration				
Ttk protein kinase (mps1)	Upregulated mps1: a target of miR-133	Downregulation of miR-133 by Fgf	Regeneration of Zebrafish caudal fin (appendage)	[68]
RhoA	Downregulation of RhoA mRNA	Upregulation of miR-133b expression	Regenerating adult zebrafish spinal cord, axon outgrowth	[69]
RhoA	Downregulation of RhoA protein	Upregulation of miR-1 and miR-133 expression	Zebrafish muscle gene expression and regulation of sarcomeric actin organization	[166]
Cell cycle factors mps1, cdc37 and PA2G4, and cell junction components cx43 and cldn5	Upregulated mps1, cdc37, PA2G4, cx43, cldn5	Downregulated miR-133(a1) stimulates cardiac cell regeneration	Regenerating zebrafish cardiac muscle	[167]
miR-133b	MiR-133b found in developing somites, little in CNS tissues		Whole zebrafish embryos - normal development	[168]
SRF activates muscle specific genes and miRs; HDAC4 represses muscle gene expression	MiR-1 targets HDAC4, promoting myogenesis	In contrast, miR-133a represses SRF, enhancing myoblast proliferation	*X. laevis* embryos: skeletal muscle proliferation and differentiation in cultured myoblasts *in vitro* and in embryos *in vivo*	[7]
nAChR subunits UNC-29, UCR-63; MEF2	Subunits UNC-29, UCR-63, and MEF2 downregulated	miR-1 upregulated	*C. elegans* muscle at the neuromuscular junction	[34]
Mammalian pluripotent cells				
Muscle-specific microRNAs: miR-1 and miR-133a	MiR-1 and miR-133a have opposing functions during differentiation of progenitor cardiac muscles	Muscle-specific microRNAs, miR-1 and miR-133(a) upregulated	Promotion of mesoderm formation from mouse ES cells	[13]
Notch signalling, promotes neural differentiation and inhibits muscle differentiation; opposes miR-1 effects	Dll-1 translationally repressed	miR-1 upregulation, promotes cardiomycete differentiation	Mouse and human ES cell differentiation into muscle	[13]
$SRF^{-/-}$ EBs reflecting the loss of hematopoietic lineages in the absence of SRF	Early endoderm markers, Afp and Hnf4α: strongly down regulated	Increased miR-1 and miR-133a relieve the block on mesodermal differentiation	Mouse endoderm	[13]
Blood cell -specific genes, such as Cd53, CxCl4, and Thbs1, dramatically down regulated	Cd53, CxCl4, and Thbs1 expression was reinitiated by reintroduction of miR-1 or miR-133			
mES(miR-1)- and mES(miR-133a)-EBs compared to in control EBs	Nodal stimulated expression of endoderm markers Afp and Hnf4α in control EBs. Dramatically lower levels in mES(miR-1)- and mES(miR-133a)- EBs	miR-1 or miR-133 can each function as potent repressors of endoderm gene expression	mES cells, that lack either miR-1 or miR-133(a) during differentiation into EBs	[13]
IGF-1 IGF-1R	IGF-1 signalling and miR-133 co-regulate myoblast differentiation *via* a feedback loop	IGF-1 upregulates miR-133; miR-133 downregulates IGF-1R	Myogenic differentiation of C2C12 myoblasts; Mouse during development from embryonic to mature skeletal muscle	[24]
IGF-1	IGF-1 signalling and miR-1 coregulate differentiation of myoblasts *via* a feedback loop	IGF-1 signalling downregulates miR-1 by repression of FoxO3a; miR-1 down-regulates IGF-1	Differentiating C2C12 myoblasts	[25]
Reversine [2-(4-morpholinoanilino)-N6-cyclohexyladenine]	Decrease in active histone modifications; including trimethylation of histone H3K4/H3K36, phosphorylation of H3S10; Stimulates expression of polycomb genes Phc1 and Ezh2	miR-133a expression strongly inhibited by reversine; reduced acetylation of H3K14 at miR-133a promoter Reduced expression of myogenin, MyoD, Myf5 and Aurora A and B kinases	Reversine dedifferentiates murine C2C12 myoblasts back into multipotent progenitor cells, *via* extensive epigenetic modification of histones resulting in chromatin remodelling, and altered gene expression	[20-23]
FZD7 and FRS2	miR-1 promotes cardiac differentiation; miR-1 targets FZD7 and FRS2	Activitation of WNT and signalling cause MCPs differentiation into cardiomyocytes	Mouse and human ES cells	[169]
miR-206/133b cluster	*PAX7* gene expression unchanged; miR-206/133b cluster is not required for development, and survival of skeletal muscle cells	miR-206/133b cistron knock-out mice cells	Muscle satellite cell differentiation *in vitro*	[170]
Differentiating skeletal muscle				
DNA polymerase alpha	Repression of Idl-3 protein expression Repression of p180 subunit of DNA polymerase alpha	miR-206 up-regulated	Mouse skeletal muscle differentiation	[42]
MEF2 transcription factor	MEF2 activates of miR-1-2 and 133a-1 transcription; binds muscle-specific enhancer	Bicistronic primary transcript of miR-1-2 and 133a-1	Development of mammalian skeletal muscle	[9]

MRFs, Myf5, MyoD, Myogenin and MRF4	Myf5 essential for miR-1 and miR-206 expression during skeletal muscle myogenesis	Forced expression of MRFs in neural tube induces miR-1 and miR-206 expression	Chicken and mouse embryonic muscle	[171]
PTB and neuronal homolog nPTB, exon splicing factors	Downregulation of PTB protein by miR-133 (and miR-206)	Concurrent upregulation of miR-133 and induction of splicing of several PTB-repressed exons	During myoblast differentiation, microRNAs control a developmental exon splicing program	[172]
BDNF	BDNF downregulated	miR-206 upregulated	Differentiation of C2C12 myoblasts into myotubes	[48]
Fstl1 and Utrn	Fstl1 and Utrn downregulated	miR-206 upregulated	Skeletal muscle differentiation	[40]
Utrophin A (muscle)	Utrophin A down-regulated by both miRs	Upregulated miR-133b, miR-206	C2C12 mouse myoblasts, mouse soleus muscle	[173]
CNN3 gene	Negative correlation between miR-1 expression and CNN3 mRNA expression	Normal skeletal muscle	Tongcheng (Chinese) and Landrace (Danish) pigs	[174]
FGFR1 and PP2AC, members of ERK1/2 signalling pathway	miR-133 (a and b) activities increase during myogenesis	miR-133 directly downregulates expression of FGFR1 and PP2AC	Mouse C2C12 myoblast cells	[31]
ERK1/2 signalling pathway activity	ERK1/2 signalling activity suppresses miR-133 expression	Downregulation of expression of miR-133	A reciprocal mechanism for regulating myogenesis	
BAF chromatin remodelling complex (BAF60a, BAF60b and BAF60c)	Positive inclusion of BAF60c in the BAF chromatin remodeling complex	Expression of miR-133 and miR-1/206	Progression of developing somites in chick embryos	[63]
BAF chromatin remodelling complex	Negative regulation of BAF60a and BAF60b; exclusion from BAF chromatin remodelling complex	Expression of miR-133	Progression of developing somites in chick embryos	[63]
BAF chromatin remodelling complex	Exogenous upregulation of BAF60a and BAF60b		Delay in developing somites in chick embryos	[63]
Mitochondrial UCP2 and UCP3	MyoD activates miR-133a expression which in turn directly downregulates UCP2 mRNA	Feedback network involving MyoD-miR-133a-UCP2	Mouse skeletal and cardiac muscles; UCP2 imposes developmental repression	[56]
Mitochondrial UCP2 and UCP3	Exogenous overexpression of myogenin and MyoD transcription factors	Strong increase in UCP3 promoter, expression, weak effect at the UCP2 promoter	Mouse C2C12 myoblasts	[57]
Proliferating myogenic skeletal muscle cells				
MiR-206/133b cluster	MiR-206/133b cluster is not required for survival and regeneration of skeletal muscle	Muscle regeneration proceeds in Mdx mice *in vivo*	miR-206/133b cistron knock-out mice	[170]
Enhanced translation of specific mitochondrial genome-encoded transcripts	miR-1 enters muscle mitochondria and binds mtRNA targets along with Ago factor	Increased expression of mtRNA targets	Proliferating myogenic skeletal muscle cells after muscle injury	[53]
mTOR (serine/threonine kinase)	MyoD stability regulated by mTOR	Regulates miR-1 expression *via* MyoD availability	Regenerating mouse skeletal muscle and differentiating myoblast cells	[32]
AMPK-CRTC2-CREB and Raptor-mTORC-4EBP1 pathways	mTORC regulates timing of satellite cell proliferation during myogenesis	Knockdown of mTORC reduces miR-1 expression	Myogenenic satellite SCs proliferating and differentiating into myogenic precursors following rat skeletal muscle injury	[58]
HDAC4 regulates Pax7-dependent muscle regeneration	Pax7 stimulates SCs differentiation toward the muscle lineage, and limits adipogenic differentiation	HDAC4 upregulated in SCs differentiating into muscle cells	Myogenenic satellite SCs	[175]
pcRNA encoded by the H strand of the rat mitochondrial genome	Introduction of mt pcRNAs into injured muscle restoring mitochondrial mRNA levels; Intramuscular ATP levels were elevated after pcRNA treatment of injured muscle	Enhanced organellar translation and respiration; similarly reactive oxygen species were reduced; Resulted in accelerated rate of wound resolution	Injured rat skeletal muscle is associated with general downregulation of mitochondrial function; reduced ATP, and increased ROS	[176]
Cardiac muscle precursor cells				
GATA binding protein 4, Hand2, T-box5, myocardin, and microRNAs miR-1 and miR-133	Reprogrammed human fibroblasts show sarcomere-like structures and calcium transients; Some cells have spontaneous contractility	Forced over-expression of GATA binding protein 4, Hand2, T-box5, myocardin, and microRNAs miR-1 and miR-133	Human embryonic and adult fibroblasts activated to express cardiac markers	[15]
SRF, MyoD and Mef2 transcription factors	miR-1-1 and miR-1-2 During cardiogenesis miR-1 genes titrate critical cardiac regulatory proteins, control ratio of differentiation to proliferation	miR-1 genes upregulated; Elevated miR-1 targets downregulation of Hand2	Cardiac muscle precursor cells	[30]
Histone deacetylase inhibitor, trichostatin A forces differentiation, yet reduced miR-1 and miR-133a	miR-1 and miR-133a reduce cardiac specific Nkx2.5 protein and Cdk9	miR-1 and miR-133a increase during spontaneous differentiation of cardiac myoblasts	Mouse cardiac stem cells (ES cells)	[10]

Specific inhibition of HDAC4 modulates CSCs to facilitate myocardial repair	Positively proliferative myocytes increased in MI hearts receiving HDAC4 downregulated CSCs	CSCs with downregulated HDAC4 expression improved ventricular function, attenuated ventricular remodeling, promoted regeneration and neovascularization in MI hearts	Mouse CSCs transplanted into MI mouse hearts	[177]
Snai1	Overexpression of miR-133a (miR-133), Gata4, Mef2c, and Tbx5 (GMT) or GMT plus Mesp1 and MyocD improved cardiac cell reprogramming from mouse or human fibroblasts	miR-133a directly represses Snai1 expression, which silences fibroblast signatures; a key molecular process during cardiac reprogramming	Mouse/human fibroblasts more efficiently reprogrammed into cardiomycete-like cells	[16]
β1AR signal transduction cascade	Adenylate cyclase Ⅵ and the catalytic subunit of the cAMP-dependent PKA are components of β1AR transduction cascade	miR-133 directly targets β1AR, Adenylate cyclase Ⅵ and PKA	TetON-miR-133 inducible transgenic mice, subjected to transaortic constriction, maintained cardiac performance with attenuated apoptosis and reduced fibrosis *via* elevated miR-133 expression	[17]
ROS, MDA, SOD and GPx	miR-133 produced a reduction of ROS and MDA levels, and an increase in SOD activity and GPx levels	Overexpression of miR-133, a recognized anti-apoptotic miRNA	*In vitro* rat cardiomyocytes	[18]
Caspase-9	miR-133 directly suppresses caspase-9 expression resulting in downregulation of downstream apoptotic pathways	Overexpression of miR-133	*In vitro* rat cardiomyocytes	[18]
Spred1	miR-1 directly targets Spred1	miR-1 is upregulated in hCMPCs during angiogenic differentiation	hCMPCs	[178]
miRNA-1 and miRNA-133a	miRNA-1 and miRNA-133a have antagonistic roles in the regulation of cardiac differentiation	Forced overexpression of miR-1 alone enhanced cardiac differentiation, in contrast overexpression of miR-133a reduced cardiac differentiation, compared to control cells	Pluripotent P19.CL6 stem cells Overexpression of both miRNAs promoted mesodermal commitment and decreased expression of neural differentiation markers	[179]
Cardiac muscle				
Induction of GATA6, Irx4/5, and Hand2	Cardiac myocytes show defective heart development, altered cardiac morphogenesis, channel activity, and cell cycling	miR-1-2$^{-/-}$ gene knockout	Cardiac myocytes with knockout of both miR-1-2 genes	[180]
mt-COX1 mRNA	3′-UTR of mt-COX1 mRNA bound by miR-181c and Ago1 factor	Overexpression of miR-181c significantly decreased mt-COX1 protein, but not mt-COX1 mRNA level	Overexpression of miR-181c increased mitochondrial respiration and reactive oxygen species in neonatal rat ventricular myocytes	[54]
mt-COX1 mRNA	*In vivo* elevation of miR-181c in rat heart, reduces levels of mt-COX1 protein	Results in reduced capacity for strenuous exercise and evidence of heart failure	Rat cardiac muscle	[55]
Carvedilol, a β-adrenergic blocker	Induces upregulation of miR-133	Cytoprotective effects against cardiomyocyte apoptosis	Rat cardiac tissue, *in vivo*	[18]
GLUT4, and SRF	Both miRs downregulate SRF and KLF15	Both miR-133a and miR-133b target KLF15	Mouse cardiac myocytes	[181]
GLUT4 expression	Both basal and insulin-stimulated glucose uptake are increased	KLF15	Mouse muscle cell lines	[182]
MEF2 transcription factor	MEF2 directly activates transcription of miR-1-2 and 133a-1 binding muscle-specific enhancer between the genes	Bicistronic primary transcript of miR-1-2 and 133a-1	Development of mammalian cardiac muscle	[9]
Myocardium tissue	Enriched in miR-1, miR-133b, miR-133a		Heart structures of rat, Beagle dog and cynomolgus monkey	[183]
Gelsolin	One common miR-133a isomiR targets gelsolin gene more efficiently than standard isomer; New second rat miR-1 gene	Many isomiRs were detected by deep sequencing at higher frequency than the canonical sequence in miRBase	miRNA/isomiR expression profiles in the left ventricular wall of rat heart	[184]
CTGF	CTGF downregulated by both miRs	Exogenous upregulation of miR-133b (and miR-30c)	Cultured cardiomyocytes and ventricular fibroblasts	[185]
MT1-MMP	miR-133a upregulated	miR-133a targets MT1-MMP	Human left ventricular fibroblasts	[186]

Injured and regenerating cardiac muscle				
SERCA2a	Akt/FoxO3A-dependent pathway Activated SERC2a reduces phosphorylation of FoxO3a, allowing entry to nucleus and activation of miR-1 expression	Downregulation of miR-1 expression in failing heart muscle	Failing mouse heart muscle	[187]
IGF-1	IGF-1 signalling and miR-1 co-regulate differentiation of myoblasts *via* a feedback loop	IGF-1 signalling down-regulates miR-1 by repression of FoxO3a; miR-1 down-regulates IGF-1	Mouse heart muscle during cardiac failure states	[25]
Bim and Bmf	Only miR-133a expression enhanced under *in vitro* oxidative stress	miR-133a targets proapoptotic genes Bim and Bmf	Rat adult CPCs miR-1 favors differentiation of CPCs, whereas	[188]
Bim and Bmf	CPCs overexpressing miR-133a improved cardiac function by reducing Bim and Bmf	CPCs overexpressing miR-133a improved cardiac function, increasing vascularization and cardiomyocyte proliferation, reduced fibrosis and hypertrophy	CPCs overexpressing miR-133a in rat myocardial infarction model	[188]
MT1-MMP activity increased in both. Ischemia and reperfusion regions	Interstitial miR-133a decreased with ischemia *in vitro* and *in vivo*; reperfusion returned to steady-state	Phosphorylated Smad2 increased within the ischemia-reperfusion region	Ischemia-reperfusion Yorkshire pigs (90 min ischemia/120 min reperfusion)	[186]
Cardiovascular disease				
CNN2	Strong upregulation of CNN2 expression	miR-133b downregulated; miR-133b directly targets CNN2	Pre-inflammatory events in diseased cardiac tissues	[65]
Circulating platelet derived microparticles	Elevated miR-133		Patients with stable and unstable coronary artery disease	[189]
Acute MI causes upregulation of circulating serum miRs	miR-1, -133a, -133b, and -499-5p were about 15- to 140-fold elevated over control		Acute STEMI patients and experimental mouse MI model	[190]
Circulating miRNAs in serum of cardiovascular disease patients	Released miR-1 and miR-133a are localized in exosomes, and are released by Ca(2+) stimulation	Levels of miR-1, miR-133a, reduced in infarcted mouse myocardium model heart	miR release indicates myocardial damage	[191]
LVM after valve replacement in aortic stenosis	microRNA-133a is a significant positive predictor of LVM normalisation	miR-133 is a key element of the reverse remodelling process	Patients following valve replacement	[192]
Circulating levels of miR-133a	Elevated miR-133a (11-fold)		Troponin-positive acute coronary syndrome patients	[193]
Circulating levels of miR-133a	Elevated miR-133a	Improved potential regression of Left Ventricular Hypertrophy after valve replacement	Patients with aortic stenosis surgery	[194]
Apelin treatment reduces elevated circulating miRs	Elevated miR-133a, miR-208 and miR-1 reduced	High-fat diet elevated miRs and increased left ventricular diastolic and systolic diameters, and wall thickness	Obesity-associated cardiac dysfunction in mouse model	[195]
NAC treatment	Expressed miR-1, miR-499, miR-133a, and miR-133b were strongly depressed in the diabetic cardiomyocytes	NAC restored expression of miR-499, miR-1, miR-133a, and miR-133b significantly in the myocardium	Diabetic rat hearts	[196]
Myocardial junctin elevated	miR-1 targets junctin	NAC reduces junction levels	Development of diabetic cardiomyopathy in rat hearts	[196]
CAD associated ischemic heart failure	miR-133 expression decreased with increased severity of heart failure		Patients with CAD	[197]
Runx2	miR-133a targets Runx2		Transition of VSMCs to osteoblast-like cells	[198]
Increased alkaline phosphatase activity, osteocalcin secretion and Runx2 expression	miR-133a was decreased during osteogenic differentiation		Transition of VSMCs to osteoblast-like cells	[198]
Circulating miR-133a and 208a levels	Cardiac muscle-enriched microRNAs (miR-133a, miR-208a) elevated		Patients with coronary artery disease	[199]
Hypertrophic cardiac muscle				
Cx43 increased	miR-1 targets Cx43	Downregulation of miR-1 mediates induction of pathologic cardiac hypertrophy	Hypertrophic rat cardiomyocytes *in vitro* and *in vivo*	[200]
Cx43 downregulated	miR-1 targets Cx43	Cx43 protein downregulated in miR-1 Tg mice compared to WT mice	Cardiac-specific miR-1 transgenic (Tg) mouse model	[201]

Twf1 upregulated	miR-1 targets Twf1	Strong downregulation of miR-1 in pathologic hypertrophic cardiac cells compared to normal, induces Twf1 expression	*In vivo* in hypertrophic mouse left ventricle; and *in vitro* in phenylephrine-induced hypertrophic cardiomyocytes	[202]
RhoA, Cdc42, Nelf-A/WHSC2	Increased levels of RhoA, Cdc42, Nelf-A/WHSC2	Reduction miR-133a	Hypertrophic cardiac muscle	[6]
Calcineurin, agonist of cardiac hypertrophy	Increased Calcineurin activity; Cyclosporin A inhibits calcineurin	Reduced miR-133a; Prevents miR-133 down-regulation	Hypertrophic cardiac muscle; Cardiac hypertrophy reduced	[203]
NFATc4	NFAFc4 targetted by miR-133a	miR-133a	Cardiomyocyte hypertrophic repression	[204]
Interdependent Calcineurin-NFAT and MEK1-ERK1/2 signalling pathways in cardiomyocytes	MEK1-ERK1/2 signalling augments NFAT and NFAF gene expression; Activated calcineurin activates NFAT, inducing cardiac hypertrophy	MEK1 is part of mitogen-activated protein kinase (MAPK) cascade; MEK1 activates ERK directly	Hypertrophic growth response of mouse cardiomyocytes	[205]
Innervating skeletal muscle				
Innervated skeletal muscle	MyoD, Myf5, Mrt4, nAChRα Each is strongly repressed	Myogenin expression	Mouse skeletal muscle	[50,51]
Denervated muscle (unstimulated)		Myogenin expression up-regulated MyoD, Myf5, Mrt4, nAChRα All strongly stimulated	Mouse skeletal muscle	[51]
Electrically stimulated - Denervated muscle	Myogenin, MyoD, Myf5, Mrt4, partly stimulated; nAChRα inhibited		Mouse skeletal muscle	[51]
HDAC4 SRF	miR-1 promotes myogenesis by targetting HDAC4	miR-133 enhances myoblast proliferation by targetting SRF	Skeletal muscle proliferation and differentiation in myoblast cultures	[7]
Neural activity effect on muscle (HDAC4 - MEF2 Axis)	Loss of neural input leads to concomitant nuclear accumulation of HDAC4	HDAC4 inhibits activation of muscle transcription factor MEF2; results in progressive muscle dysfunction	MEF-2 activity strongly inhibited in denervated mouse skeletal muscle and in ALS muscle	[49]
Innervation and formation of airway smooth muscle	Sonic hedgehog (Shh) /miR-206/ BDNF	Shh signalling blocks miR-206 expression, which in turn increases BDNF protein	Shh coordinates innervation and formation of airway smooth muscle	[206]
nAChR subunits (UNC-29 and UNC-63); retrograde signalling	Subunits UNC-29, UCR-63 and MEF2 downregulated	miR-1 upregulated	*C. elegans* muscle at the neuromuscular junction	[34]
MEF2	Hnrpu, Lsamp, MGC108776, MEF2, Npy, and Ppfibp2 downregulated	miR-206 upregulated	Rat skeletal muscle/re-innervating muscle	[43]
HDAC4	HDAC4 (miR-206 target, prospective miR-133b target) downregulated	miR-206/-133b upregulated (and miR-1/-133a downregulated)	Mouse fast twitch skeletal muscle/re-innervating muscle	[12]
Regenerating injured muscle				
Hnrpu and Npy downregulated Ptprd downregulated	miR-1 upregulated miR-133a upregulated	miR-1, -133a, downregulated 1 mo after denervation, then increased 2 × at 4 mo after re-innervation	Rat skeletal soleus muscle after sciatic nerve injury and subsequent re-innervation	[43]
Hnrpu, Lsamp, MGC108776, MEF2, Npy, and Ppfibp2 downregulated	3 × increase in miR-206 1 mo later, after reinnervation; elevated at least 4 mo	Predominant type Ⅱ fiber at 4 mo, after nerve re-innervation	Rat skeletal soleus muscle after sciatic nerve injury and subsequent re-innervation	[43]
PP2A B56a	PP2A B56a downregulated	133a upregulated	Canine heart failure model: myocytes	[207]
CaMKII-dependent hyperphosphorylation of RyR2	VF myocytes had increased reactive oxygen species and increased RyR oxidation	miR-1 upregulated	Canine post-myocardial infarction model	[208]
Collagen upregulated	TGF-b1 and TGFbRII: upregulated	miR-133a or miR-590: downregulated	Canine model of acute nicotine exposure. Atrial fibrosis *in vivo*; cultured canine atrial fibroblasts *in vitro*	[209]
miR-208 upregulated	miR-1 and miR-133a downregulated		Human MI compared to healthy adult hearts	[210]
Myogenic proteins, MyoD1, myogenin and Pax7	Induced expression of MyoD1, myogenin and Pax7 several days after miR injection	Exogenous injection of miR-1, -133 and -206 promotes myotube differentiation	Regenerating injured mouse skeletal muscle	[211]
Cyclin D1/ Sp1	Cyclin D1/ Sp1 downregulated	miR-1/133 upregulated	Regenerating rat skeletal muscle	[212]
PRP, source of pro-inflammatory cytokines	Stong upregulation of the mRNA of pro-inflammatory cytokines IL-1β and TGF-β1; stimulation of both inflammatory and myogenic pathways; elevated heat shock proteins and increased phosphorylation of αB-cristallin	Stimulated tissue recovery *via* increased myogenic regulators MyoD1, Myf5, Pax7, and IGF-1Eb (muscle isoform) together with SRF; acts *via* increased expression of miR-133a with reduced levels of apoptotic factors (NF-κB-p65 and caspase 3)	Regenerating flexor sublimis muscle of rats, 5 d after injury and treated with PRP	[66]

Muscle degeneration				
Pro-inflammatory cytokine TWEAK	TWEAK upregulated	miR-1-1, miR-1-2, miR-133a, miR-133b and miR-206 downregulated	Degenerating/wasting mouse skeletal muscle	[59]
HMOX1 mediated by codependent inhibition of c/EBPδ binding to myoD promoter	HMOX1 inhibits differentiation of myoblasts and modulates miRNA processing HMOX1 effects partially reversed by enforced expression of miR-133b and miR-206	Downregulation of miR-1, miR-133a, miR-133b, and miR-206. Downregulation of MyoD, myogenin and myosin, and disturbed formation of myotubes. Upregulation of SDF-1 and miR-146a	Degenerating/wasting mouse skeletal muscle	[60]
Dystrophic muscular disease				
Circulating serum microRNAs	miR-1, miR-133a, and miR-206 highly abundant in Mdx serum	miR-1, miR-133a, and miR-206 downregulated or modestly upregulated in muscle	Muscle tissue from patients with Duchenne muscular dystrophy (Mdx)	[213]
Laminin α2 chain deficiency	miR-1, miR-133a, and miR-206 are deregulated in laminin α2 chain-deficient muscle	Laminin α2 chain-deficient mouse	Congenital muscular dystrophy type 1A tissue	[214]
Dystrophic process advances from prominent inflammation with necrosis and regeneration to prominent fibrosis	Deficiency in calpain leads initially to accelerated myofiber formation followed by depletion of satellite cells	Pax7-positive SCs highest in the fibrotic patient group; correlated with down-regulation of miR-1, miR-133a, and miR-206	Muscle from Limb-girdle muscular dystrophy 2 type Ⅰ patients	[215]
Transgenic overexpression of miR-133a1 (in dystrophin point mutation Mdx mice)	Extensive overexpression in skeletal muscle, lesser increase in heart	Normal skeletal muscle and heart development	Mdx mice (model for human muscular dystrophy), extensor digitorum longus muscle	[216]
miR-206 located in nuclear in both normal and DM1 tissues by *in situ* hybridization	Only miR-206 showed an over-expression in majority of DM1 patients	No change in expression of profiled miRs, miR-1, miR-133 (miR-133a/-133b), miR-181 (miR-181a/-181b/-181c)	Skeletal muscle (vastus lateralis) of from patients with myotonic dystrophy type 1 (DM1)	[217]
FAPs facilitate myofiber regeneration	HDAC inhibitors can activate FAPs towards muscle regeneration	Inhibition of HDAC induces MyoD and BAF60C expression, which causes up-regulation of miR-1-2, miR-133, and miR-206 expression	Early stage disease dystrophic mouse muscles, regeneration of myofibres	[62]
TDP-43	TDP-43 interacts with miR-1/-206 isomers, but not miR-133 isomers	Depleted miR-1/-206 allow targets IGF-1 and HDAC4 to accumulate in ALS muscle	Mouse ALS model injured motor neurons and muscle	[33]
Inflammation response in muscle				
Inflammatory myopathies	Increased expression of TNFα	Associated with decreased expression of miR-1, miR-133a, and miR-133b	Inflammatory myopathies including dermatomyositis, polymyositis, and inclusion body myositis	[64]
hBSMCs sensitized with IL-13	Increased muscle RhoA	Reduction of muscle miR-133a	Sensitized human bronchial smooth muscle cells (hBSMCs)	[218]

IGF: Insulin-like growth factor; IGF-1R: IGF-1 receptor; MRFs: Muscle regulatory factors; PTB: Polypyrimidine tract-binding protein; Fstl1: Follistatin-like 1; Utrn: Utrophin; mTOR: Mechanistic target of rapamycin; pcRNA: Polycistronic RNAs; SRF: Serum response factor; Snai1: Snail family zinc finger-1; β1AR: β-adrenergic receptor; PKA: Protein kinase A; MDA: Malondialdehyde; Spred1: Sprouty-related EVH1 domain containing protein 1; mt-COX1: Mitochondrial cytochrome c oxidase subunit 1; SERCA2a: Sarcoplasmic reticulum calcium ATPase 2a; LVM: Left ventricular mass; NAC: N-acetylcysteine; CAD: Coronary artery disease; StAR: Steroidogenic acute regulatory protein; mES: Mouse embryonic stem; EBs: Endodermal bodies; SCs: Stem cells; CSCs: Cardiac stem cells; MI: Myocardial infarcted; hCMPCs: Human-derived cardiomyocyte progenitor cells; VSMCs: Vascular smooth muscle cells; CPCs: Cardiac progenitor cells; STEMI: ST-segment elevation MI.

(late-stage) differentiation of chondrocytes in growth cartilage[78]. This differentiation could be reversed by transfection and overexpression of miR-1 which repressed the expression of aggrecan, the major cartilaginous proteoglycan gene in human chondrocytic HCS-2/8 cells and normal chicken chondrocytes. Thus miR-1 is a major effector in early growth and cell proliferation, and its repression at late differentiation stages is important for maintaining cartilage integrity.

MYOMIRS AND IMMUNOLOGICAL RESPONSES

Several reports of myomiR involvement in specialized immunological processes have recently emerged. Stumpova *et al*[79] (2014) found that several miRNAs, including miR-133b, were strongly expressed during *in vitro* maturation of human tolerogenic dendritic cells induced by exogenous IL-10 and TGF-β in comparison to miRs expressed in IL-4-induced and IFN-γ activated dendritic cells. MiR-133b and -206 have been previously reported to be expressed during differentiation of immunocompetent mouse Th17 cells, with miR-133b/-206 cistron transcription occurring along with expression at the nearby *Il17a/f* gene locus[80]. This feature of T cell differentiation towards an IL-17-producing phenotype is shared between αβ and γδ T cells, where the specific co-regulation of miR-133b and miR-206 with the Il17a/f locus extends to human Th17 cells, suggesting presence of miR-133b/-206 in T cells may be biomarkers for Th17-type immuno-reactions.

Table 2 Roles and targets of the myomiRs, miR-1, -206, -133a, -133b in other precursor cells and tissues

Factor(s)	Regulation	Regulator	Tissue/cell	Ref.
Nerve tissues				
Pitx3	Pitx3 downregulated	miR-133b	Mammalian midbrain DNs	[73]
Exosome-mediated transfer of miR-133b from MSC to brain astrocytes	miR-133b transfer from multipotent mesenchymal stromal cells to neural cells	miR-133b upregulated	Mouse MSCs to neural cells	[47]
Ctgf and RhoA	Ctgf and RhoA downregulated	miR-133b upregulated	Multipotent MSCs/Rat brain parenchymal cells	[72]
miR-133b null mice: Striatum dopamine levels unchanged, Pitx3 expression unaffected; motor coordination unaltered	miR-133b has no significant role on mDA neuron development and maintenance *in vivo*	Normal numbers of mDA neurons during development and aging of miR-133b null mice	Mouse mDA neuron development in $^{-/-}$miR-133b mutant mice	[45]
Acute or chronic morphine administration, or morphine withdrawal	miR-133b levels not affected		Rat VTA/ nucleus accumbens shell	[219]
GPM6A, a neuronal glycoprotein	microRNA-133b upregulation	Reduction in gmp6a at mRNA and protein level. Cell filopodium density was reduced	Hippocampus and prefrontal cortex of neonatal male rats stressed when in utero	[220]
Tac1 gene (neurotransmitter substance P)	Tac1 downregulated	miR-206 upregulated	MSCs-derived neural cells	[221]
Ketamine (antidepressive) administration	BDNF, a direct target gene of miR-206, was upregulated	miR-206 was downregulated by ketamine	Rat hippocampus tissue	[222]
Adipogenic tissues				
IGF-1 and IGF-1R	IGF-1 signalling and miR-133b co-regulate ADSC differentiation *via* a feedback loop	miR-133b downregulation of Pitx3; IGF-1 upregulates miR-133b; miR-133b downregulates IGF-1R	Adipose tissue-derived stem cell differentiation into neuron-like cells	[71]
Pdrm16	miR-133a directly targets Prdm16.	Downregulation of miR-133 resultsin differentiation of pre-adipocyte precursors into BAT	Mouse adipocyte differentiation to BAT	[74]
Pdrm16	miR-133 directly targets Prdm16	Downregulation of miR-133 resulted in differentiation of pre-adipocyte precursors into BAT	Mouse primary brown adipocyte (and myogenic) progenitor cells - differentiate into BAT or SAT	[75]
Pdrm16	miR-133 targets Prdm16 controlling brown adipose determination in skeletal muscle satellite cells	miR-133 downregulates Prdm16	Adult mouse skeletal muscle stem cells (satellite cells) differentiate into BAT	[76]
HDAC4 downregulation directs SCs towards adipocyte differentiation	Brown adipose master regulator Prdm16 is upregulated, while its inhibitor miR-133 is also downregulated	HDAC4 downregulated in SCs differentiating into adipocyte progenitor cells	Myogenic satellite SCs	[175]
GLUT4 expression	Both basal and insulin-stimulated glucose uptake are increased	KLF15	Mouse 3T3-L1 preadipocytes differentiating into adipocytes	[182]
Intrinsic insulin resistance	Elevated miR-133b	Undefined role	Adipose tissue of women with PCOS	[223]
Upregulation of LIM homeobox 8 and Zic family member 1 and downregulation of Homeobox C8 and Homeobox C9	Undefined relation of upregulated miR-206, miR-133b	Undefined relation with parallel upregulation of brite/beige markers, TBX1 and TMEM26	Human BAT from the supraclavicular region	[224]
Obesity development	Downregulation of miR-133b, miR-1	Undefined role	Adipose tissue from obese male C57BLJ6 mice	[225]
LXRα regulation of lipogenic genes	miR-1/miR-206 represses LXRα expression at both mRNA and protein levels	miR-1/miR-206-induces a decrease in lipogenic gene levels and lipid droplet accumulation	Mouse hepatocytes	[226]
Osteogenic tissues				
Development of bone on organic or inorganic substrates		miR-133 differentially expressed in osteoblasts grown on different substrates	Osteoblast	[227]
Runx2	miR-133 directly down-regulates Runx2	miR-133 up-regulated	Osteogenic differentiation from C2C12 mesenchymal cells	[228]
HDAC4	HDAC4 downregulates Runx2	miR-1 targets HDAC4, increasing Runx2 activity	Chondrocyte proliferation in cartilage growth plate	[77]
Aggrecan	miR-1 promotes late-stage differentiation of growing cartilage cells	miR-1 targets Aggrecan gene expression	Chicken chondrocytes and human HCS-2/8 cells	[78]
Alveolar cells				
VAMP2/ lung surfactant secretion	miR-206 targets VAMP-2	miR-206 overexpression decreased lung surfactant secretion	Lung alveolar type Ⅱ cells	[229]

Hormonal regulation				
L-thyroxine	miR-206/miR-133b downregulated	L-thyroxine treatment	L-thyroxine treated hypothroidic skeletal muscle from thyroidectomized patients	[230]
	miR-206/miR-133b upregulated	-	Hypothroidic human skeletal muscle	
Thyroid hormone/TEAD1	Thyroid hormone inhibits the slow muscle phenotype by upregulation of miR-133a1 which downregulates TEAD1	miR-133a1 is enriched in fast-twitch muscle and regulates slow-to-fast muscle fiber type conversion	Mouse muscle	[231]
Thyroid hormone/miR-133a1 TEAD1	myosin heavy chain I expression downregulated	TH indirectly downregulates myosin heavy chain I *via* miR-133a/TEAD1	Mouse muscle	[232]
L-thyroxine	pre-miR-206 and pre-mir-133b downregulated	L-thyroxine	L-thyroxine treated hypothyroidic mouse liver;	[232]
	50-500x increase expression of miR-1/-133a and miR-206/-133b	-	Hypothyroidic mouse liver	
Reduced insulin-mediated glucose uptake in cardiomycetes	Downregulation KLF15, which downregulates GLUT4	Forced overexpression of miR-133a and miR-133b	Rat cardiac myocytes	[181]
Cardiac myocyte glucose metabolism	Upregulation KLF15, which upregulates GLUT4	Silencing endogenous miR-133	Rat cardiac myocytes	[181]
Metabolic control of glucose uptake by GLUT4 transporter	Downregulates KLF15, which results in downregulation of GLUT4 levels	Chronic heart failure has depressed miR-133a and -133b levels	Rat cardiac myocytes during chronic heart failure and cardiac hyperthrophy	[181]
Atrial natriuretic factor expression upregulation	Enhanced at LVH and dramatically increased at CHF stage	Both miR-133a and miR-133b downregulated at CHF stage	LVH and CHF in salt-sensitive Dahl rats	[181]
Estrogen	Estrogen replacement strongly decreased IGF-1 protein level in muscles at 1 wk		Ovariectomized rat skeletal muscle	[233]
Multiple targets	miR-133a upregulated in BTBR mice		Pancreatic islets, adipose tissue, and liver from diabetes-resistant (B6) and diabetes-susceptible (BTBR) mice	[234]
Augmentation of adipocyte differentiation by norepinephrine does not alter myomiR levels	miRNAs miR-1, miR-133a and miR-206 specifically expressed both in brown pre- and mature adipocytes	miRNAs miR-1, miR-133a and miR-206 were absent from white adipocytes	Mouse brown adipocytes	[235]
Foxl2	miR-133b targets Foxl2; miR-133b inhibits Foxl2 binding to StAR and CYP19A1 promoter sequences	Foxl2 regulates StAR and CYP19A1 transcriptionally	Estradiol production in ovarian granulosa cells	[236]
Exosome release and cell to cell transfer				
Exosome-mediated transfer of miR-133b from MSCs to brain astrocytes	miR-133b transfer from multipotent mesenchymal stromal cells to neural cells	miR-133b upregulated	Mouse multipotent MSCs to neural cells	[47]
Cell to cell transfer of exosome-enriched extracellular particles	mir-133b promotes neural plasticity and recovery of function after stroke induced damage	miR-133b upregulated	Rat multipotent MSCs *via* transfer of exosome-enriched extracellular particles	[72]
Transplanted stem cells				
MSCs expressing miR-1	Upregulated miR-1	Increased rate of recovery, enhanced survival of transplanted MSCs and cardiomyogenic differentiation	Experimental ligation of the mouse left coronary artery to model myocardial infarction	[237]
Knockdown of Hes-1, member of Notch pathway	Upregulated miR-1 promotes the differentiation of MSCs into cardiac lineage	Role in survival of transplanted MSCs and cardiomyogenic differentiation	Mouse MSCs	[238]
Notch signalling and cardiomyocyte markers, Nkx2.5, GATA-4, cTnT, and Cx43	MSCs expressing exogenous miR-1		Mouse MSCs	[238]
Tissue inflammation				
Selective release of miRs during inflammation into serum	miR-133 selectively released		Review	[239]
Inflammation and cancer	MicroRNA, free radical, cytokine and p53 pathways		Review	[240]
Immunological switch which shapes tissue responses	TWEAK/Fn14 pathway		Review	[241]
Tumor biology	HMOX1		Review	[242]
GM-CSF	Direct supression of GM-CSF expression by miR-133	Elevated expression of miR-133a/-133b during oxidative stress	Mouse alveolar epithelial cells during oxidative stress	[82]

PI3K/Akt and IGF-1 pathways	Activation of PI3K/Akt and IGF-1 pathway activities	Downregulation of miR-133a (and other miRs) by AOM/DSS induced chronic inflammation	Mouse model: AOM/DSS-induced colitis-associated gastro-intestinal cancer	[83]
CTGF, SMA, and COL1A1	Increased expression of CTGF, SMA and COL1A1, which are miR-133b targets	Strong downregulation of miR-133b (and other miRs)	TGF-β treated rabbit corneal fibroblasts; Recovering mouse cornea after laser ablation,	[70]
IL-10 and TGF-β	Exogenous IL-10 and TGF-β induces miR-133b expression	Upregulation of miR-133b	Human tolerogenic dendritic cells during maturation	[79]
IL-17-producing T-cells	Upregulation of Il17a/f gene expression	miR-133b/-206 cistron transcription occurs along with nearby Il17a/f gene expression	Immunocompetent mouse Th17 cells	[80]
NLRP3 inflammasome which processes IL-1β by caspase-1 cleavage	miR-133a-1 suppresses activation of inflammasomes *via* suppression of expression of mitochondrial UCP2	miR-133a-1 overexpression in cells increases caspase-1 p10 and IL-1β p17 cleavage,	Differentiated mouse THP1 cells	[81]
Concanavalin A-induced fulminant hepatitis		miR-133a is the most strongly differentially upregulated miR	Mouse liver following ConA injection	[243]
Infection/immune response to influenza virus (H1N2)	miR-206 expression		Experimental influenza infection in pig lung	[244]
HIF-1α, and its regulator Four-and-a-half LIM (Lin-11, Isl-1 and Mec-3) domain 1 (Fhl-1)	Downregulation of miR-206 and upregulated HIF-1α and Fhl-1 in hypoxic lung tissue and PASMCs	miR-206 targets HIF-1α directly. Hypoxia-induced down-regulation of miR-206 promotes PH in PASMCs	Hypoxia-induced PH in hypoxic rat model in cultured hypoxic PASMCs	[245]
miR-206/NR4A2/NFKB1; Indirectly: inflammatory cytokines (IL6, IL1B, CCL5)	NFKB1 stimulates inflammatory cytokines (IL6, IL1B, CCL5)	Liposaccharides induce miR-206 expression which targets NR4A2 downregulation, which in turn allows upregulation of NFKB1 activity	Astrocyte-associated inflammation during recovery from chronic central nervous system injury	[246]
Cellular factors influencing myomir expression/activity				
miR-1/miR-133a				
Skeletal muscle				
Positive regulator	**Negative regulator**	**Regulated target miR**	**Tissue/cell**	**Ref.**
Myogenin, MyoD		Upregulates miR-1-1 and miR-133a-2 Upregulates miR-1-2 and miR-133a-1	Primary human myoblasts; C2C12 cells	[11]
SRF, MyoD and MEF2		Upregulates miR-1-2	Muscle somites	[30]
MEF2		Upregulates miR-1 and miR-133a	Skeletal muscle	[9]
KSRP (part of Drosha and Dicer complexes)	miR-206 binds 3'-UTR of KSRP and inhibits its expression	KSRP upregulates miR-1 expression	Skeletal muscle	[35,37]
RNA-binding protein LIN28		LIN28 upregulates miR-1 expression; LIN28 promotes pre-miR-1 uridylation by ZCCHC11 (TUT4)	Cardiac muscle of patients with muscular dystrophy	[36]
	MBNL1	MBNL1 downregulates miR-1 expression; MBNL1 binds to UGC motif in the loop of pre-miR-1 and competes for the binding of LIN28; MBNL1 blocks DICER processing of pre-miR-1	Cardiac muscle of patients with muscular dystrophy	[36]
CX43 and CACNA1C calcium channel	CX43 and CACNA1C both increased in both DM1-/DM2-affected hearts, contributing to the cardiac dysfunctions	CX43 and CACNA1C are direct targets of miR-1 repression	Cardiac muscle of patients with muscular dystrophy; CACNA1C and CX43 encode the main calcium- and gap-junction channels in heart	[36]
Utrophin A	miR-206 and KSRP are negative regulators of utrophin A	Overexpression of miR-206 promotes the upregulation of utrophin A, *via* the downregulation of KSRP	Normal and dystrophic muscle cells; miR-206 can switch between (1) direct repression of utrophin A expression, and (2) activation of its expression by decreasing KSRP, allowing close regulation	[37]
	Myostatin	Downregulates miR-1, miR-133a, miR-133b, miR-206	Mouse (35 d) pectoralis skeletal muscle	[29]
	SRF	Downregulates miRs-133a	Skeletal muscle	[1,3]
Prmt5 and Prmt4		Upregulates myomiR expression during differentiation	Mouse skeletal muscle	[247]
Smooth muscle				
Sp-1 transcription factor	pERK1/2	Upregulates miR-133(a)	VSMCs	[248]

Brg1		Upregulates miR-133 (ChIP complex with SRF)	Smooth muscle	[249]
Cardiac muscle				
GATA4, Nkx2.5, Myocardin, SRF		Upregulates miR-1 and miR-133a	Differentiating cardiac muscle	[5]
SRF plus Myocardin		Upregulates miR-1-1 and miR-1-2	Cardiomycetes	[30]
	Calcineurin	Downregulates miR-133a	Hypertrophic cardiac muscle	[203]
miR-206/ miR-133b				
Skeletal muscle				
Mrf5		Upregulates miR-1, miR-206	Skeletal muscle	[171]
Myogenin, MyoD		Upregulates miR-206	Primary human myoblasts; C2C12 cells	[11]
MyoD		Upregulates linc MD1 (encodes miR-133b)	Differentiating myoblasts	[11, 38]
		Binds to (E-box) enhancer of miR-206, miR-133b	skeletal muscle (mouse)	[12,40]
		Upregulates miR-206/miR-133b	Differentiated human foetal skeletal muscle cells	[250]
	FGF2 allows upregulation of Sp1/Cyclin D1	Downregulates p38-mediated miR-1/133 expression	Regenerating rat skeletal muscle	[212]
	Myostatin	Downregulates miR-133a, mir-133b, miR-1, and miR-206	Mouse (35 d) pectoralis skeletal muscle	[29]
	TWEAK downregulates myoD and MEF2c	Downregulates miR-1-1 and miR-133	Degenerating/wasting skeletal muscle	[59]
	HMOX1 downregulates MyoD and myogenin	Downregulates all myomiRs	Inflamed skeletal muscle	[60]
L-Thyroxine treatment		Downregulation of pri-miR-206 and pri-miR-133b No effect on miR-1/miR-133a pairs	Human skeletal muscle	[230]
Smooth muscle				
p-ERK	Activated extracellular signal-regulated kinase p-ERK inversely correlated with VSMC growth	Downregulates miR-133 expression	VSMCs	[248]
Other tissues				
Myogenin		Binds miR-206 enhancer (ChIP)	Fibroblast cell line:	[40]
IGF-I signalling		Upregulates miR-133b	Mouse Adipose derived stem cells	[71]
L-Thyroxine deficiency	Upregulated Col5a3	Strong upregulation of miR-133a and -133b	Hypothyroid mouse liver	[232]
	Downregulated Slc17a8, Gp2, Phlda1, Klk1d3, Klk1 and Dmbt1 Upregulated Vldlr and Akr1c19, and downregulated Upp2, Gdp2, Mup1, Nrp1, and Serpini2	Strong upregulation of miRs -1, -206		
L-Thyroxine treatment	Pre-miR-206 and Pre-miR-133b down-regulated	Upregulation of Gdp2 andMup1	Hypothyroid mouse liver *in vivo*, and in vitro mouse hepatocyte AML12 cells	[232]
PA2G4, mps1, cdc37, cx43, cldn5; cx43 is a miR-133 target	Upregulation of cell cycle factors mps1, cdc37, and PA2G4, and cell junction components cx43 and cldn5	Suppression of miR-133a1 stimulates cardiac cell proliferation	Regeneration of damaged Zebrafish cardiac muscle, associated with reduced miR-133a1	[167]
Fgf	Upregulated Fgf	Downregulates miR-133	Zebrafish regenerating fin blastema	[67]
SHP (nuclear receptor)	Downregulation of miR-206 in nuclear receptor SHP(-/-) mice		SHP(-/-) mice strain, mouse liver	[251]
AP1 transcription factor complex	AP1 induced miR-206 promoter transactivity and expression; this is repressed by YY1	ChIP analysis shows physical association of AP1 (c-Jun) and YY1 with miR-206 promoter	SHP(-/-) nuclear receptor mice strain, mouse liver	[251]
NR3B3	YY1 promoter transactivated by ERRgamma; this inhibited by SHP (NROB2)	Nuclear receptor ERRgamma (NR3B3) binding site on the YY1 promoter	Mouse liver	[251]
Novel cascade "dual inhibitory" mechanism governing miR-206 gene transcription by SHP	(1) SHP inhibition of ERRgamma leads to decreased YY1 expression	(2) Derepression of YY1 on AP1 activity, leads to activation of miR-206	Mouse liver	[251]
Il17a/f locus	miR-133b and miR-206 expression	Coregulated with IL-17 production	αβ and γδ T cells	[80]

IGF: Insulin-like growth factor; MRFs: Muscle regulatory factors; PTB: Polypyrimidine tract-binding protein; Fstl1: Follistatin-like 1; Utrn: Utrophin; mTOR: Mechanistic target of rapamycin; pcRNA: Polycistronic RNAs; SRF: Serum response factor; Snai1: Snail family zinc finger-1; β1AR: β-adrenergic receptor; PKA: Protein kinase A; MDA: Malondialdehyde; Spred1: Sprouty-related EVH1 domain containing protein 1; mt-COX1: Mitochondrial cytochrome C oxidase subunit 1; SERCA2a: Sarcoplasmic reticulum calcium ATPase 2a; LVM: Left ventricular mass; NAC: N-acetylcysteine; CAD: Coronary artery disease; StAR: Steroidogenic acute regulatory protein; VTA: Ventral tegmental area; BAT: Brown adipose tissue; SCs: Stem cells; PCOS: Polycystic ovary syndrome; LVH: Left cardiac ventricular hypertrophy; CHF: Chronic heart failure; DNs: Dopaminergic neurons; mDA: Midbrain dopaminergic; AOM: Azoxymethane; DSS: Dextran sulfate sodium; PH: Pulmonary hypertension; PASMCs: Pulmonary artery smooth muscle cells.

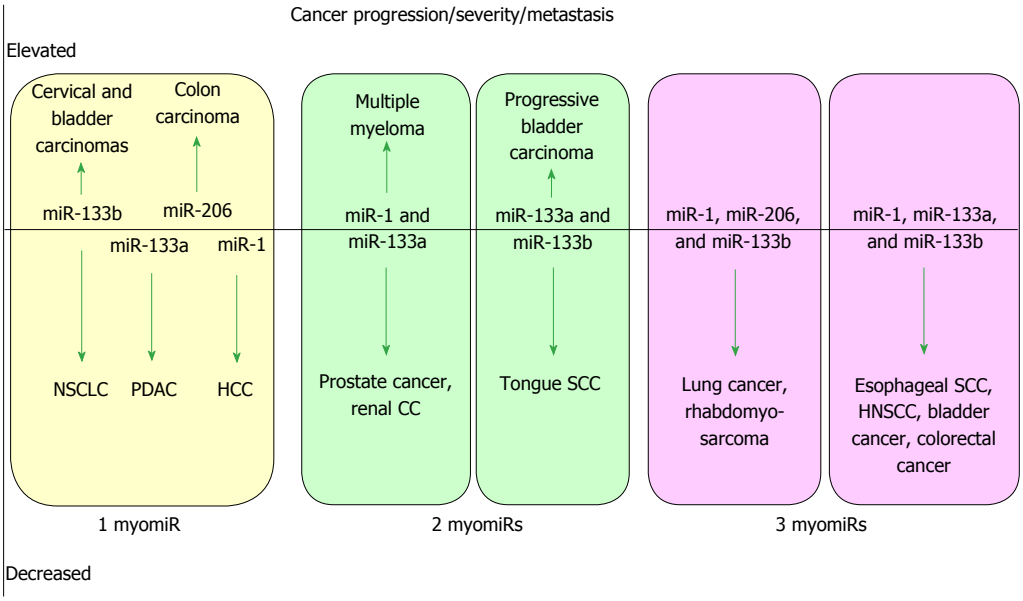


Figure 3 Roles of the myomiRs during cancer progression. Altered expression of myomiRs is associated with cancer progression and metastasis. Note that the changed level of myomiR typically contributes to cancer progression. Reduced levels of a single myomiR are associated with PDAC[103], NSCLC[105] and HCC[98], whilst an elevated single miR is associated with cervical carcinoma[121] and bladder cancer (TCC)[122]. Increased expression of miR-1/-133a is associated with t(14;16) translocation multiple myeloma[152], whilst reduced expression is associated with prostate cancer[102] and RCC[138], respectively. Reduced expression of other myomiR combinations is also associated with TSCC[111], ESCC[132], HNSCC[114,143], GIST[132], bladder cancer (TCC)[93], lung cancer[85,99], colorectal cancer[86,87] and rhabdomyosarcoma[88].

Bandyopadhyay *et al*[81] (2014) examined the effect of overexpression or suppression of miR-133a-1 in differentiated mouse THP1 cells which express the NLRP3 inflammasome, finding that miR-133a-1 overexpression suppresses expression of mitochondrial UCP2, resulting in increased caspase-1 p10 activity which subsequently causes IL-1β p17 cleavage. SiRNA silencing of UCP2 expression enhanced H_2O_2 stimulated inflammasome activity, and conversely, overexpression of UCP2 decreased the inflammasome activation, suggesting that the mechanism by which miR-133a-1 suppresses inflammasome activation involves the direct targetting of UCP2.

Further, the pulmonary cytokine GM-CSF is normally produced in lung alveolar epithelial cells (AECs) during a defense response. GM-CSF expression is suppressed by oxidative stress *via* altered mRNA turnover[82], in which miR-133a and -133b are upregulated in primary AECs. *In vitro* cell experiments confirmed that miR-133a and miR-133b bind the 3'-UTR of GM-CSF and suppress its expression, and that their inhibition can reverse oxygen-induced suppression of GM-CSF expression, suggesting that miR-133 isomers are important regulators of GM-CSF expression in AECs during oxidative stress. Studies of mouse colorectal epithelial cells involving the azoxymethane/dextran sulfate-induced mouse model of colitis-associated cancer found that the induced chronic colorectal epithelial cell inflammation downregulates miR-133a and other miRs associated with the modulation of PI3K/Akt and IGF-1 associated pathways, particularly during the earlier inflammatory stages[83].

Overall, whilst myomiRs miR-1-2, miR-133 and miR-206 have central roles muscle regeneration and (neuromuscular) repair, and in the regulation of aspects of inflammation and immunological responses during regeneration of injured and diseased muscles, miR-133a and miR-133b also appear to be involved in other regulatory processes affecting, repair, inflammation and immunological responses a number of other non-muscle cells/tissues, suggesting that these may involve common signalling pathways in a range of tissues in which myomiR play a regulatory role on the levels of signalling pathway components.

MYOMIRS AND CANCER

This review also examines the numerous cancers with prominent dysregulation of one or more of the canonical myomiRs, and the molecular events influenced by them. We focus on the deregulated myomiRs and their key target genes (up- or down-regulated) which have demonstrable effects *in vitro* on cell migration, proliferation or apoptosis, or those which are *in vivo* risk factors for cancer progression or patient survival. Although cancers characteristically have changed expression of large numbers of different protein genes and numerous different miRs which may contribute to the cancer pathology, these other deregulated genes and factors are not discussed except in relation with the myomiRs or their known targets. Table 3 lists the deregulated myomiRs reported in many different cancers. In addition, Tables 4 and 5 list targets and pathways influenced by

Table 3 The deregulated expression of the canonical myomiRs in different cancers

MiR-1	MiR-206	MiR-133a	MiR-133b	Cancer type	Ref.
		+	+	Progressive bladder cancer (TCC)	[122]
-		-		Bladder cancer (TCC)	[93,156]
-		-	-	Bladder cancer (TCC)	[92,144]
-	-	-	-	Bladder cancer	[127][1]
-	-	-	-	Bladder cancer	[128][1]
-		-	-	Muscle-invasive bladder cancer	[92]
	-			Proliferating breast cancer	[108]
	-			ERα-positive breast cancer	[109]
-			-	Breast cancer	[95]
			+	Progressive cervical carcinoma	[87,121]
-	-			Chordoma	[90]
	+			Colon cancer	[116]
-				Colon cancer	[101]
		-		CRC	[104,136]
			-	CRC	[86,87]
			+/-	Liver metastasis compared to primary CRC	[118]
-			-	Colon cancer	[95]
		-	-	Progressive GIST	[132]
		-		HNSCC	[143]
			-	HNSCC	[114]
	-			EEC	[110]
		-		ESCC	[132]
-		-	-	ESCC	[94]
	-			Laryngeal SCC cells	[113]
-		-		MSSCC	[137]
		-		TSCC	[111,112]
-				HCC	[98]
+			+	Liver cancer	[95]
-				Lung cancers: (NSCLC, adenocarcinomas, lung SCC, large cell carcinoma, and bronchoalveolar cell carcinoma)	[100]
	-			High metastasis lung tumors	[107]
		-		Lung SCC tissue; lung-SCC cell lines	[115]
			-	NSCLC	[85,105]
-	-			Lung adenocarcinomas; NSCLC cells	[99]
		-	-	Lung carcinomas	[85,115,131]
-			-	Lung cancer	[95]
-			-	Lymphoma	[95]
		+		AML	[120,151]
		+		Multiple myeloma	[152]
			+	Progressive prostate cancer	[123]
-	(-)	-		Prostate cancer	[102]
-			-	Prostate cancer	[95]
-			-	Recurrent prostate cancer compared to non-recurrent cancer	[96]
		-	-	Hormone-insensitive prostate cancer cells	[101]
-		-	-	Osteosarcoma	[91]
-			-	Ovarian cancer	[95]
		-		PDAC	[103]
		-		RCC	[138]
-	-			Rhabdomyosarcoma	[88,89]
-			-	Testicular cancer	[95]

[1]DS: Deep sequencing; TCC: Transitional cell carcinoma; CRC: Colorectal cancer; GIST: Gastrointestinal stromal tumor; HNSCC: Head and neck squamous cell carcinoma; EEC: ERα-positive endometrioid adenocarcinoma; ESCC: Esophageal squamous cell carcinoma; MSSCC: Maxillary sinus squamous cell carcinoma; TSCC: Tongue squamous cell carcinoma; HCC: Hepatocellular carcinoma; NSCLC: Non-small-cell lung cancer; AML: Acute myeloid leukaemia; PDAC: Pancreatic ductal adenocarcinoma; RCC: Renal cell carcinoma.

downregulated and upregulated myomiRs respectively, and Table 6 lists validated myomiR targets related to enhanced cancer progression.

An excellent review by Nohata *et al*[84] (2012) reported changes in the expression of the miR-1/-133a and miR-206/-133b cistron clusters in numerous cancers, typically finding reduced expression of different combinations of myomiRs, such as in lung cancer[85], colorectal cancer[86,87], rhabdomyosarcoma[88,89], chordoma[90] osteosarcoma[91], muscle-invasive bladder cancer[92], bladder cancer (transitional cell carcinoma) (TCC)[93], ESCC[94], breast cancer[95] and prostate cancer[95-97]. In different cancers (Figure 3) deregulation typically involves downregulation of one or more myomiR isomers, indicating they normally function as tumor suppressors in the tissues, limiting

Table 4 Targets and pathways influenced by the downregulated myomiRs in different cancers

Downregulated miR-1				
miR-1 downregulation influences multiple cancer-related pathway processes, and promotes cell proliferation and motility	Epigenetic promoter hypermethylation reduces miR-1/-133a expression in (a subset of) human prostate tumors	Reduced miR-1, miR-133a (and miR-206)	Human prostate tumors	[102]
Actin filament network-associated genes: FN1, LASP1, XPO6, CLCN3 and G6PD; Cell cycle and DNA damage control genes: BRCA1, CHK1, MCM7; Histone acetylation: HDAC4; Oncogenes: NOTCH3 and PTMA	miR-1 downregulation associated with upregulation of multiple cancer-related pathway processes	Reduced miR-1; Exogenous introduction of miR-1 or miR-206 caused similar inhibition of various cancer-related pathway genes	Human prostate cell lines, LNCaP, 22Rv1, PC-3 and RWPE-1	[102]
HSPB1	HSPB1 restores oncogenic pathways in prostate cancer cells	Downregulates miR-1 expression	Progressive prostate cancer PCa cells	[252]
XPO6 and TWF1 (PTK9)	Inverse expression between miR-1, XPO6 and TWF1 proteins in prostate cancer cell lines	Downregulated miR-1 expression	Prostate cancer cell cultures	[253]
CCND2, CXCR4, and SDF-1α	Inverse expression between miR-1 and CXCR4 and SDF-1α protein levels in thyroid carcinomas	Strongly downregulated miR-1 expression in thyroid adenomas and carcinomas	Thyroid adenomas and carcinomas	[254]
MET	MET upregulated	Reduced miR-1	Colon cancer	[101]
		Reduced miR-1, -133b	Colon cancer	[87]
MET, Pim-1 (Ser/Thr kinase), FoxP1 and HDAC4	miR-1 downregulated, miR-1 targets MET, Pim-1, and may regulate FoxP1 and HDAC4	MET, Pim-1, FoxP1 and HDAC4 are often upregulated in lung cancer	NSCLC tissue and A549 cell line	[100]
Fibronectin1	Fibronectin1 upregulated	miR-1 downregulated	Laryngeal SCC Hep2 cells	[255]
Met, Twf1 and Ets1 and Bag4	Met, Twf1 and Ets1 and Bag4 activities upregulated	miR-1 downregulated	Mouse cutaneous squamous cell carcinomas	[256]
Mediator complex subunit 1 (Med1) and 31 (Med31)	Med1 and Med31 activation result in increased Met activity	Reduced miR-1; miR-1 targets Med 1 and Med 31	Osteosarcoma	[257]
NOTCH3 upregulates Asef expression, activating the Asef promoter, enhancing cell migration	NOTCH3 upregulated	Reduced miR-1; miR-1 targets NOTCH3	Colorectal tumor cells	[258]
Overexpression of PIK3CA correlates with low miR-1 expression in NSCLC tissues	71% of NSCLC samples had high PIK3CA expression	69% of NSCLC samples had low miR-1 expression	Predictors of lymph node metastasis in NSCLC tissues	[259]
SLUG expression downregulated by miR-1	Transcriptional repressor of E-cadherin, or an inducer of epithelial-to-mesenchymal transition	Overexpression of miR-1 induces morphological change from a mesenchymal to an epithelial character	NSCLC A549 cell line	[260]
SLUG expression high in chordoma tissue	miR-1 inhibited cell proliferation both time- and dose-dependently in chordoma	Transfection of MiR-1 inhibited Slug expression	mR-1 transfected chordoma cells Slug overexpressed in advanced chordoma tissues and chordoma cells	[261]
MET expression high in chordoma tissue	miR-1 downregulated 97% of chordoma samples	MiR-1 directly targets MET	Decreasing miR-1 expression levels correlated with severity of clinical prognosis	[262]
SRSF9/SRp30c	Exogenous upregulation of miR-1 expression	Novel apoptosis pathway involving SRSF9/SRp30c mediates tumor suppression	Bladder cancer (TCC) cells	[263]
ANXA2 is essential for glioblastoma growth and invasion	ANXA2 is highly abundant protein in glioblastoma-derived extracellular vesicles	miR-1 directly targets ANXA2; Reduced miR-1 in glioblastoma	Human Glioblastoma cells; miR-1 orchestrates glioblastoma extracellular vesicle function	[264]
EDN1	miR-1 downregulated in gastric cancer	miR-1 causes ET-1 silencing in gastric cancer cell lines	Gastric cancer tissue compared with adjacent normal tissue	[265]
EDN1	Elevated expression of EDN1 and reduced miR-1 level	miR-1 directly targets EDN1	Human liver cancer tissues	[266, 267]
Overexpressed EDN1	Enhanced *in vitro* cell proliferation and cell migration. Upregulation of several cell cycle/proliferation- and migration-specific genes	Upregulated UPR pathway mediators, spliced XBP1, ATF6, IRE1, and PERK at both RNA and protein levels	293T cells	[267]
AKT inhibitor diminished the unfolded protein response and eliminated EDN1-induced cell migration	EDN1 effects act *via* activation of the AKT pathway	Results to enhance the UPR and subsequently activate the expression of downstream genes	293T cells	[267]

Edn1	Induced steatosis, fibrosis, glycogen accumulation, bile duct dilation, hyperplasia, and HCC	Liver-specific edn1 expression	Transgenic Zebrafish liver	[267]
API5	API5 expression upregulated thus inhibiting apoptosis	miR-1 expression downregulated	Human liver cancer tissues	[268]
	Apoptosis activated, API5 reduced	Overexpression of miR-1	HepG2 liver cancer cells	
Phosphorylation of ERK and AKT; LASP1	Overexpression of miR-1 inhibits phosphorylation of ERK and AKT and reverses EMT process *via* inhibition of MAPK and PI3K/AKT pathways	MAPK and PI3K/AKT pathways	Transgenic miR-1 expressing CRC cell lines	[269]
LASP1 expression upregulated	Upregulated LASP1 stimulates EMT resulting in cell proliferation and migration	miR-1 downregulated	Colorectal tumor tissue	[269]
PIK3CA	Increased expression of PIK3CA	Downregulated miR-1 expression in lung cancer	NSCLC tissue with poor patient prognosis	[259]
PIK3CA indirectly regulating pAKT and survivin proteins	Overexpressed miR-1 downregulated PIK3CA causing reduced pAKT and survivin proteins	Exogenously overexpressed miR-1 targets PIK3CA directly.	NSCLC A549 cell line	[270]
Signalling pathways such as TGF-β, ErbB3, WNT and VEGFA, and cell motility or adhesion	Ectopic expression of miR-1 and miR-145 downregulates VEGFA and AXL, respectively	Highly downregulated expression of miR-1, miR-133, miR-143 and miR-145 in gall bladder cancer	Gall bladder tumor samples and GBC NOZ cell line	[271]
lncRNA UCA1	Lnc RNA UCA1 upregulated in bladder cancer (TCC); Inverse relationship between miR-1 and lnc UCA1	Downregulated miR-1 expression in bladder cancer (TCC); miR-1 targets lnc RNA UCA1 for downregulation	Human bladder cancer (TCC) tissue	[156]
Downregulated miR-133a				
Moesin	Moesin upregulated	Reduced miR-133a	HNSCC	[143]
ARPC5	ARPC5 upregulated	Reduced miR-133a	HNSCC	[272]
ARPC5 and GSTP1	ARPC5 and GSTP1 upregulated	Reduced miR-133a (and miR-206)	Lung carcinoma	[115]
IGF-1R, TGFBR1, and EGFR are downregulated	Restoration of ectopic-expression of miR-133a in NSCLC suppresses metastatic capacity	miR-133a inhibits cell invasiveness and cell growth *via* suppression of IGF-1R, TGFBR1 and EGFR	NSCLCs	[131]
	Low expression of miR-133a is characteristic of pancreas tissue	Reduced miR-133a	PDAC	[103]
CDC42	CDC42 upregulated causing downstream activation of PAKs	miR-133 downregulated	Gastric cancer tissues	[273]
GSTP1	Upregulated GSTP1	Downregulation of miR-133a in cancer	Bladder cancer (TCC) cell lines	[144]
	Enforced downregulation of GSTP1 inhibits cell proliferation and growth;	Enforced upregulation of miR-133a and miR-133b induces cell apoptosis		
GSTP1 in cancer specimens	GSTP1 upregulated	Reduced miR-133a	Bladder cancer (TCC) tissue	[144]
Actin-binding protein, FSCN1	Upregulated FSCN1; Enforced downregulation of FSCN1 inhibits cell proliferation, migration and invasion	Downregulation of miR-133a; Forced UP exp of miR-133a inhibits cell proliferation, migration and invasion	Bladder cancer (TCC) tissue	[274]
EGFR/AKT signalling pathway	Upregulated EGFR; Activated pAkt-1	Downregulated miR-133a; Enforced expression of miR-133a inhibits EGRF translation; causes inhibition of Akt protein phosphorylation and its nuclear translocation	Human MCF-7 and MDA-MB-231 breast cancer cell lines	[275]
Bcl-xL and Mcl-1 expression	Upregulated Bcl-xL and Mcl-1	Downregulated miR-133a correlated with tumor progression and poor patient prognosis;	Primary human osteosarcoma tissues; Osteosarcoma cell lines	[276]
E3 ubiquitin protein ligase	Downregulation of p21 and p53 proteins	Downregulated miR-133a	Primary CRC tissues	[277]
Enhanced sensitivity to doxorubicin and oxaliplatin	Enhancing apoptosis and inhibited cell proliferation	Ectopic upregulation of miR-133a	CRC cell lines	[277]
LASP1 upregulated	miR-133a expression downregulated	miR-133a targets LASP1	CRC tissues and cell lines	[278]
FTL protein upregulated	miR-133a expression downregulated	miR-133a targets downregulation of FTL protein	Patient breast cancer tissue	[279]

Increased sensitivity to chemotherapeutic drugs doxorubicin and cisplatin	Exogenous upregulation of miR-133a expression	Downregulation of FTL protein	Human MCF-7 breast cancer cells	[279]
Poor survival during breast cancer; upregulated FSCN1	Loss of miR-133a expression	FSCN1 is a direct target gene of miR-133a	Breast cancer tissue	[280]
FSCN1 downregulated	Restoration of miR-133a expression	Inhibited breast cancer cell growth and invasion	Breast cancer cell line	[280]
lncRNA Malat1/Srf/miR-133 regulatory loop	Malat1 transcript has a functional miR-133 target site, miR-133 acts as a competing endogenous RNA, regulating Malat1 levels	*In vitro* depletion of Malat1 in C2C12 cells reduces Srf activity, Srf is an enhancer of miR-133 expression; feed-back regulation loop involving miR-133	Mouse myoblast C2C12 cells	[164]
lncRNA MALAT1	MALAT1 is overexpressed in 46% of ESCC tissues, primarily in high-stage tumors, high expression correlates with lymph node metastasis	*In vitro* depletion of MALAT1 suppresses tumor cell proliferation, cell migration and invasion; G2/M phase arrest was induced and the ratio of apoptotic cells increased	Human ESCC	[162]
WIF1/lncRNA MALAT1	WIF1 (strong tumor suppressor) is systematically downregulated in glioblastoma	WIF1 down regulation correlates with strong upregulation of MALAT1. *In vitro* depletion of MALAT1 suppresses tumor cell proliferation	Glioblastoma	[163]
Downregulated miR-133b				
Fascin-1 mRNA	FSCN1 upregulated	Reduced miR-133b	High-grade GIST tissue	[281]
BCL-2 family (MCL-1 and BCL2L2)	MCL-1 and BCL2L2 upregulated	Reduced miR-133b	Lung cancer	[85]
FAIM antiapoptotic protein and GSTP1	miR-133b directly targets FAIM and GSTP1	Downregulated miR-133b	miR-133b expression significantly downregulated in 75% of prostate cancer tumor specimens	[282]
Gli1	Gli1 upregulated	Gli1 inversely correlated with downregulated expression of miR-133b	Gastric cancer	[283]
Bcl-w and Akt1	Bcl-w and Akt1 proteins overexpressed significantly	miR-133b significantly downregulated	Bladder cancer tissues	[284]
		miR-133b downregulated in tumors compared to surrounding tissue	Gastric and esophageal adenocarcinomas	[285]
			Endometrial sarcoma, leiomyosarcoma, and mixed epithelial-mesenchymal tumors	[286]
Downregulated miR-206				
Notch3/ miR-206	Downregulated Notch3, blocking of the anti-apoptotic activity of Notch3	Forced expression of miR-206 strongly induced apoptotic cell death *via*; also inhibited cell migration and focus formation	HeLa cells	[287]
Met	Upregulated Met	miR-206 downregulated	Human rhabdomyosarcoma	[288]
HGFR	Upregulated HGFR	miR-206 downregulated	Human breast cancer cells	[289]
KLF4	Upregulated KLF4	miR-206 downregulated	RK3E breast epithelium cells	[108]
KLF4; RAS-ERK signalling	Upregulated KLF4 promotes RAS-ERK signalling Endogenous KLF4 binds the promoter regions stimulates expression of miR-206	miR-206 downregulated	TNBC cells	[290]
RASA1 and SPRED1		miR-206 inhibits translation of the RAS pathway suppressors RASA1 and SPRED1	Suppression of RASA1 or SPRED1 increased levels of GTP-bound, wild-type RAS and activated ERK 1/2	
VEGF	VEGF upregulated in Laryngeal SCC tissues	MiR-206 strongly downregulated in LSCC tissues	Laryngeal SCC cancer tissue and cells	[113]
VEGF	VEGF upregulated in ccRCC tissues	MiR-206 strongly downregulated in ccRCC tissues	ccRCC tissues assayed by Deep Sequencing	[291]
Cdc42, MMP-2 and MMP-9	Upregulated Cdc42, MMP-2 and MMP-9	miR-206 downregulated	Human breast cancer tissues	[292]
ERα	miR-206 directly targets ERα 3'-untranslated region	MiR-206 inhibited by ERα agonists, indicating a mutually (double) inhibitory feedback loop;		[293]
		miR-206 downregulated		[109]
	Upregulated ERα		MCF-7 breast cancer cells	[294]
ERα	Upregulated ERα	miR-206 downregulated	EEC tissue	[110]

K-Ras	K-Ras is direct target of miR-206; MiR-206 expression significantly downregulated and k-Ras upregulated on OSCC tissues	Low miR-206 potentiates metastases, and shorter overall survival	OSCC tissue samples and cell lines	[295]
MiR-206	Enforced upregulated of miR-206 attenuated cell proliferation, increased apoptosis and inhibited cell migration and invasion	MiR-206 strongly downregulated in lung cancer tissues	Lung cancer - tissues and cell lines	[107]
EGFR/MAPK signalling switches MCF-7 breast cancer cells from ERα-positive, Luminal-A phenotype to ERα-negative, basal-like phenotype	EGFR signalling represses estrogenic responses in MCF-7 cells by enhancing miR-206 activity Elevated miR-206 reduces cell proliferation, enhances apoptosis, and reduces numerous estrogen-responsive genes	miR-206 downregulates steroid receptor co-activators SRC-1 and SRC-3 and GATA-3 transcription factor, directly	MCF-7 breast cancer cells	[296]
Greater lymph node metastasis, venous invasion, and at a more advanced stage	miR-206 expression strongly downregulated	Correlates with tumor progression	Human gastric cancer tissue	[297]
CCND2	miR-206 expression strongly downregulated	Correlates with upregulation of CCND2 and cancer progression	Human breast cancer Human gastric cancer	[298] [299]
MET	miR-206 expression strongly downregulated	Upregulation of MET	Papillary thyroid carcinoma	[300]
Prognostic signature of metastatic colorectal cancer	miR-206 expression strongly downregulated	Prognostic signature of metastases: miRs 21, 135a, 335, 206 and let-7a	Metastatic CRC	[301]
Notch3, Hes1, Bcl-2 and MMP-9;	Exogenous upregulation of miR-206 expression;	Notch3, Hes1, Bcl-2 and MMP-9 downregulated at both mRNA and protein level;	Human HHC Hep2 cells.	[302]
p57, Bax and caspase-3	miR-206 is a potent tumor supressor	p57 and Bax upregulated, and cleaved caspase-3 protein upregulated	Reduced apoptosis, and cell migration in HepG2 cells overexpressing miR-206	
STC2, HDAC4, KLF4, IGF1R, FRS2, SFRP1, BCL2, BDNF and K-ras	Exogenous upregulation of miR-206 expression; miR-206 is a potent tumor supressor	STC2, HDAC4, KLF4, IGF1R, FRS2, SFRP1, BCL2, BDNF, and K-ras downregulated strongly in SCG-7901 cells overexpressing miR-206	Gastric carcinoma SCG-7901 cells Reduced apoptosis, and cell migration in SCG-7901 cells overexpressing miR-206	[303]
Cyclin C, CCND1 and CDK4	Cyclin C, CCND1 and CDK4 upregulated in melanoma tissue; Exogenous upregulation of miR-206 expression reduced growth and migration/invasion of several melanoma cell lines; G1 arrest in melanoma cells	hsa-miR-206 downregulated in melanoma tissue Overexpression of miR-206 in melanoma cells strongly downregulated cyclin C, CCND1 and CDK4	Human melanoma cancer tissue, and cell lines	[304]
Coronin, actin-binding protein	Silencing of coronin expression reduced tumor cell migration and altered the cellular actin skeleton and cell morphology, but did not effect cell proliferation	Downregulated miR-206 allowed upregulation of coronin, a direct target; Upregulated miR-206 reduced TNBC cell migration and cell proliferation	TNBC cell lines	[305]
RNA binding protein DEAD-END (DND1), DNA cytosine deaminase (AICDA), and APOBEC3	DND1 blocks miRNA interaction with 3'-UTR of specific mRNAs, restores protein expression; APOBEC3G binds DND1 counteracts repression and restores miRNA activity	APOBEC3G blocks DND1 to restore miR-206 inhibition of CX43 translation	Mouse cells	[306]
Advanced clinical stage, T classification, metastasis and poor histological differentiation		Significant association with decreased miR-206 expression	Paired human osteosarcoma and normal adjacent tissues	[307]
Ellagic acid inhibits E2-induced mammary tumorigenesis	Reverses the downregulation of miR-206		ACI model rat mammary tissue	[308]
Actin-like 6A (BAF53a), a subunit of the SWI/SNF chromatin remodeling complex	Elevated BAF53a	Downregulation of miR-206	Primary rhabdomyosarcoma tumors	[309]
Actin-like 6A (BAF53a)	BAF53a transcript is significantly higher in primary rhabdomyosarcomas than in normal muscle	Restoration of miR-206 expression downregulated BAF53a, which inhibits proliferation and anchorage independent growth; BAF53a and is a direct target of miR-206	Primary rhabdomyosarcoma tumors	[309]

Wnt and transcription factors Tbx3 and Lef1	Exogenous upregulation of miR-206 expression	Inhibition of Wnt, Tbx3 and Lef1 activities	Estrogen receptor alpha (ER-α)-positive human breast cancer; developing mammary buds	[310]
ANXA2 and KRAS	Stimulation of KRAS activity then induces NFKB1 expression;	Downregulated miR-206 in PDAC	PDAC tissues and cell lines	[311]
Induces NFKB1	Increased KRAS results in stimulation of cytokines CXCR2, CXCL1, CCL2, as well as CSF2 (GM-CSF) and VEGFC	Increased cell cycle progression, cell proliferation, migration and invasion		
Downregulated miR-1 and miR-133a				
PNP	PNP upregulated	Reduced miR-1, -133a	Prostate cancer	[97]
TAGLN2	TAGLN2 upregulated	Reduced miR-1, -133a	RCC	[136]
TAGLN2 and PNP	TAGLN2 and PNP upregulated	Reduced miR-1, -133a	MSSCC	[137]
PTMA and PNP	PTMA and PNP upregulated	Reduced miR-1, -133a	Bladder cancer (TCC)	[312]
LASP1	LASP1 upregulated Forced expression of each miR decreased LASP1 in cell lines	Reduced miR-1, 133a, (and miR-218)	Bladder cancer (TCC)	[313]
DNA methylation regulates miR-1-1 and miR-133a-2 cistron expression	Inverse correlation with TAGLN2 levels	CpG islands upstream of miR-1-133a hypermethylated	Colorectal carcinoma tissue and liver cancer tissue	[314]
Downregulated miR-1 and miR-133b				
	miR-1 and mir-133b have sufficient power to distinguish recurrent specimens from non-recurrent prostate cancer	miR-1 and mir-133b are significantly downregulated in recurrent prostate cancer tissue specimens	Recurrent prostate cancer tissue	[96]
Downregulated miR-1 and miR-206				
NRF2 upregulated	Downregulated miR-1 and miR-206 expression	Upregulated expression of NRF2 induces increased expression HDAC4	Primary lung adenocarcinoma; DU145 human prostate cancer cell line	[99]
Loss of NRF2	Decreased expression histone deacetylase (HDAC4)	Results in increased expression of miR-1 and miR-206; which inhibits PPP expression; Reduced PPP acts as a regulatory feedback loop stimulates HDAC4 expression	A549 human NSCLC cell line	[99]
c-Met	c-Met upregulated	miR-1 and -206 downregulated	Human rhabdomyosarcoma	[89]
ARPC5 and GSTP1	ARPC5 and GSTP1 upregulated	Reduced miR-133a (and miR-206)	Lung SCC cell lines	[115]
Downregulated miR-133a and miR-133b				
PKM2	PKM2 upregulated	Downregulated miR-133a, -133b	TSCC	[111]
FSCN1	FSCN1 upregulated	Downregulated miR-133a, -133b, (miR-145)	ESCC	[132]
		miR-133a, miR-133b downregulated	ESCC	[94]
KRT7	KRT7 upregulated	Downregulated (miR-133a and miR-133b)	Bladder cancer (TCC) and *in vitro* in BC KK47 cells	[315]
Downregulated miR-1, miR-206 and miR-133				
myomiRs	Patient to patient variation in the up or down regulation of miR expression in both tumor and matched normal tissues	In tumors strong down regulation of highly expressed miR-1/133a; (downregulation of weakly expressed miR-206/-133b)	Bladder cancer assayed by deep sequencing	[316]
Candidate tumor suppressor miRNAs in RCC	Each of miR-206, miR-1, miR-133b strongly downregulated	Restored expression strongly inhibited cancer cell proliferation,	RCC	[317]
Shorter overall survival and disease-free survival	Correlated with increased downregulated of miR-133b and/or miR-206	Both miR-133b and miR-206 significantly downregulated	Osteosarcoma tissues	[318]
Cell invasion and metastasis	miR-1, miR-133a, miR-133b downregulated	miR-133a, miR-133b involved in invasion and metastasis	ESCC	[94]

UPR: Unfolded protein response; PAKs: P21-activated kinases; EDN1: Endothelin 1; AKT1: V-akt murine thymoma viral oncogene homolog 1; HCC: Hepatocellular carcinoma; API5: Apoptosis inhibitor 5; LASP1: LIM and SH3 domain protein 1; XPO6: Exportin-6; CLCN3: Chloride channel, voltage-sensitive 3; G6PD: Glucose-6-phosphate dehydrogenase; BRCA1: Breast cancer 1, early onset; MCM7: Minichromosome maintenance complex component 7; PTMA: Prothymosin alpha; TWF1: Twinfilin actin-binding protein 1; CXCR4: Chemokine (C-X-C motif) receptor 4; HDAC4: Histone deacetylase 4; ANXA2: Annexin A2; SRSF9/SRp30c: Splicing factor serine/arginine-rich 9; ARPC5: Actin-related protein 2/3 complex subunit 5; GSTP1: Glutathione S-transferase pi 1; FSCN1: Fascin homolog 1; FTL: Ferritin light chain; BCL2L2: B-cell CLL/lymphoma 2-like 2; EGFR: Epidermal growth factor receptor; PNP: Purine nucleoside phosphorylase; PKM2: Pyruvate kinase, muscle type M2; KRT7: Keratin 7.

Table 5 Targets and pathways influenced by the upregulated myomiRs in different cancers

Factor(s)	Regulation	Regulator	Tissue/cell	Ref.
Upregulated miR-133b				
Activated p-ERK, pAKT1 cause in vitro proliferation of cervical cancer cell lines, and promote *in vivo* tumorigenesis and metastasis	Downregulation of MST2, CDC42, RHOA	Upregulated miR-133b	Human cervical carcinoma tissue compared to surrounding normal cervical tissue	[121]
Decreased patient survival		Upregulated miR-133b	Progression bladder cancer	[122]
Androgen receptor	miR-133b directly represses CDC2L5, PTPRK, RB1CC1, CPNE3	miR-133b directly upregulated by AR	Hormone-sensitive human prostate cancer (LNCaP) cells stimulated by androgen	[123]
Activatived neuroendocrine neoplasia proliferation	Mutation in von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase gene (VHL)	Upregulated miR-133b expression in VHL- deficient pheochromocytoma	Human pheochromocytoma (PCCs) and paraganglioma (PGLs) neuroendocrine neoplasias	[319]
Upregulated miR-206				
Cell reprogramming factor KLF4	KLF4 downregulated in colon cancer tissue, associated with increased miR-206	miR-206 strongly upregulated in colon cancer tissues	Human colon cancer tissue	[116]
Upregulated miR-1 and miR-133				
Decreased survival of R172 IDH2-mutated subset of CN-AML patients, increases resistance to chemotherapy	Distinctive gene and microRNA expression profiles accurately predicted R172 IDH2 mutations	Upregulated expression of miR-1 and miR-133	*De novo* CN-AML patient bone marrow and blood samples	[151]
EVI1 increases aggressive cancer growth	EVI1 expression upregulated in established patient samples ChIP assays show EVI1 binds to miR-1-2 gene promoter directly	Upregulated expression of miR-1-2 and miR-133-a-1	EVI1 expressing AML subset of patients	[120]
CCND2	miR-1 and miR-133a were specifically overexpressed in the cases with t(14;16) translocation, correlates with down-regulated CCND2 expression	Upregulated miR-1 and miR-133-a	Multiple myeloma	[152]
Secreted myomiRs				
miRs selectively released into serum (within exosome microparticles)	miR-1, miR-133a, and miR-133b selectively released		Human breast cancer	[320]
Circulating microRNA	Tumor-derived exosomes		Human non-small-cell lung cancer	[321]

AKT1: V-akt murine thymoma viral oncogene homolog 1; CN-AML: Cytogenetically normal acute myeloid leukemia.

the abundance of factors involved in aspects of cell proliferation[84].

Some cancers however show that elevation of myomiR levels promotes cancer progression (Figure 3), indicating that in such cell environments the deregulated myomiR is oncogenic and their targets here are normal tumor-suppressor cell factors. These are both regulatory roles that myomiRs normally exert, either by influencing particular cell signalling pathways that maintain a differentiated non-proliferating state in mature cell development stages, or by influencing other signalling pathways involved in cell proliferation and tissue genesis stages. Notably, the reports of deregulated myomiRs in cancers often reveal significant relation between the myomiR concentration and tumor severity, with greatest disregulation of expression statistically associated with metastasis, or with tumors of high metastatic potential, or with decreasing prospects of patient survival[88,98-102]. Yet, double knockout mice lacking both *miR-133a1/2* gene alleles[14] do not display an elevated incidence of tumors in any organs or tissues *in vivo*, similarly animals with knockout of *miR-206* expression[46], or knockdown of *miR-133b* or double knockdown of *-133b/-206*[44] are not reported to be associated with tumors, indicating that the singular lack of myomiR(s) alone is not (usually) sufficient to initiate tumorigenesis. Hence the oncogenic role of the deregulated myomiRs is a significant potentiating one, dysregulating cell signalling pathways in concert with the multiplicity of other molecular and cell environmental changes occurring in the developing tumor.

It is also notable that expression of cistronic myomiR isomers is not co-ordinated in different carcinomas, emphasizing individual deregulation of control of expression of each *myomiR* gene. For example, at least one miR of either cistronic pair is expressed in 16/20 cancers having altered miR-133 expression, showing also that each miRs has independent expression in non-muscle cells and tissues. Since the myomiRs influence numerous developmental pathways in normal muscle and other tissues, the differential expression of the myomiR isomers may potentially influence the pathology of cancers differently.

Cancer and downregulated myomiRs

Reduced expression of myomiRs was associated with numerous cancers, such as with PDAC[103] colorectal cancer[86,104], NSCLC[105], HCC[98], prostate

Table 6 Validated myomiR targets related to cancer progression

Up regulated cell factor	Down regulated cell factor	Cancer type	Ref.
Downregulated myomiRs			
Downregulated miR-1			
Mediator complex subunit 1 (Med1) and 31 (Med31) upregulated	miR-1 downregulated	Osteosarcoma	[217]
Slug expression upregulated; enhanced cell migratory and invasive activities	miR-1 downregulated	Chordoma	[128]
Slug expression upregulated; stimulation of EMT process	miR-1 reduced increasingly with cancer progression	Prostate adenocarcinoma	[260]
Downregulated miR-133a			
ARPC5 upregulated;	Downregulated miR-133a (> miR-206)	Lung SCC	[115]
		HNSCC	[272]
CAV1 upregulated;	miR-133a downregulated	HNSCC	[322]
Moesin upregulated;	miR-133a downregulated	HNSCC	[143]
FSCN1 upregulated;	miR-133a/ miR-133b downregulated	ESCC	[132]
		Bladder cancer (TCC)	[274]
GSTP1 upregulated;	miR-133a downregulated	HNSCC	[323]
		Bladder cancer (TCC)	[144]
		Lung SCC	[115]
LASP1 upregulated;	miR-133a downregulated in 83% of colorectal tumors	Colorectal cancer	[136]
CAV1 downregulated with miR-133a levels, and is lowest in metastatic cancers;	Contrastingly, higher levels of miR-133a correlate with poor prognosis and increased metastasis		
FSCN1 upregulated in non-metastatic tumors			
LASP1 upregulated;	miR-133a downregulated	Bladder cancer (TCC)	[313]
PKM2 upregulated;	miR-133a downregulated	TSCC	[111]
Moesin upregulated;	miR-133a downregulated	HNSCC	[143]
EGFR upregulated;	miR-133 downregulated	Hormone-sensitive prostate cancer cell lines	[324]
Human TERT telomerase catalytic subunit upregulated;	miR-133a downregulated		[325]
TCF7 transcription factor upregulated;	miR-133a downregulated		[325]
FSCN1 and MMP14 upregulated;	miR-133a downregulated	ESCC	[326]
Reduced miR-133a expression correlated significantly with advanced clinical stages, poor histological differentiation and lymph node metastasis	Marked downregulation of miR-133a in primary EOC tumors and OVCAR-3 cell line	Epithelial ovarian cancer (EOC), and in OVCAR-3 cell line	[327]
Downregulated miR-133b			
FSCN1 upregulated;	miR-133a/-133b downregulated	ESCC	[132]
FSCN1 mRNA upregulated;	miR-133b downregulated	Progressive GIST	[281]
BCL2L2 upregulated;	miR-133b downregulated	Lung cancer	[85]
MCL1 upregulated;	miR-133b downregulated	Lung cancer	[85]
MET upregulated;	miR-133b downregulated	Colorectal cancer	[87]
MET protein upregulated;	miR-133b downregulated	high grade osteosarcoma tumor samples and cell lines	[328]
EGFR upregulated;	miR-133b downregulated	NSCLC	[105]
Multiple cell factors elevated;	miR-133b downregulated	Prostate cancer	[282]
FGFR1 downregulated;	miR-133b downregulated	Gastric cancer	[329]
Gli1 protein downregulated by miR133b, Gli1 target genes, OPN and Zeb2, are indirectly regulated	miR-133b downregulated	Gastric cancer	[283]
TAp63 supresses metastasis; downregulation target of miR-133b	miR-133b is a transcription target of TAp63, downregulated	Colon cancer cells	[330]
Chemokine (C-X-C motif) receptor 4 protein downregulated by miR133b; upregulated in advanced cancer	miR-133b downregulated	CRC	[331]
TBP-like 1 mRNA and protein are upregulated in CRC	miR-133b downregulated in CRC	CRC	[332]
Strong additional down regulation of miR-133b aids liver metastatic niche for CRC cells	miR-133b downregulated 3 × (significant) in liver metastasis compared to primary CRC Interestingly, miR-133b is not downregulated significantly in lung metastasis compared to primary CRC	miR-133b downregulated in primary CRC compared to surrounding tissue	Metastatic cancer arising from primary hCRC	[333]
SP1 targeted directly by miR-133, causing reduced expression of MMP-9 and Cyclin D1	miR-133a and -133b downregulated	Gastric cancer	[334]
miR-133b target MMP-9 is upregulated	miR-133b downregulated	RCC	[335]

Downregulated miR-206				
	ERα	ERα downregulates miR-206	ERα-positive breast cancer;	[294]
		miR-206 downregulated	Double feedback loop	[109]
		miR-206 downregulated		[293,336]
	ERα	miR-206 downregulated	EEC tissue	[110]
	SRC-1, SRC-3 and GATA-3 proteins contribute to estrogenic signalling	miR-206 downregulated	ERα-positive breast cancer Signalling contributes to Luminal-A phenotype	[296]
	KLF4 over expressed in proliferating cells and cancers.	miR-206 levels are KLF4 dependent. KLF4 and miR-206 feedback pathway oppositely affect KLF4 protein translation	Breast cancer cells and normal cells	[108]
	FGBP1	miR-206 gene double knockdown	miR-206$^{-/-}$ mouse skeletal muscle.	[12]
	VEGF upregulated	miR-206 downregulated	Laryngeal SCC cells	[113]
	VEGF upregulated	miR-206 downregulated	CRC tumors compared to matched normal tissue; (^{1}DS assay)	[337]
	miR-206 correlates with negative ER status, negative PR status, and negative HER-2 status	Downregulated miR-206	Breast cancer tumor tissue	[338]
	miR-206 was downregulated in clinical TNBC tumor samples, one of its targets, actin-binding protein coronin was upregulated	Downregulated miR-206 associates with increased metastasis potential in breast cancers	High metastatic capacity TNBC tumors	[305]
Downregulated miR-1 and miR-133a				
	PNP upregulated	miR-1/miR-133a downregulated	MSSCC	[137]
			Prostate cancer	[97]
			Bladder cancer (TCC)	[312]
	TAGLN2 upregulated;	miR-1/miR-133a downregulated	MSSCC;	[137]
			RCC	[138]
			HNSCC	[339]
			Bladder cancer (TCC)	[93]
	PTMA upregulated	miR-1 and miR-133a downregulated	Bladder cancer (TCC)	[312]
Downregulated miR-1 and miR-206				
	MET levels correlated inversely with miR-1/206 expression	miR-1/206 downregulated	Up-regulation of MET in rhabdomyosarcoma	[89,291]
	HGFR upregulated	miR-1/206 downregulated	Breast cancer cells	[289]
	G6PD; PGD; TKT; GPD2 upregulated	miR-1/206 downregulated	Primary lung adenocarcinoma	[99]
Upregulated myomiRs				
Upregulated miR-133b				
miR-133b	miR-133b strongly upregulated	MST1, CDC42, RHOA, and DUSP1 downregulated	Cervical carcinoma	[121]
miR-133b	miR-133b is directly upregulated by AR	miR-133b represses CDC2L5, PTPRK, RB1CC1, and CPNE3	PCa prostate cancer cell line	[123]
Upregulated miR-206				
miR-206	Strongly upregulated miR-206	KLF4 downregulated	Human colon cancer tissue	[116]
Upregulated miR-1 and miR-133a				
miR-1-2 and miR-133-a-1	Upregulated miR-1-2 and miR-133-a-1	EVI1 (transcriptional activator of miR-1 and miR-133b)	AML	[120,151]
miR-1 and miR-133-a	Upregulated miR-1 and miR-133-a	Downregulated CCND2	Multiple myeloma	[152]
Up-regulation of exogenous myomiR expression in cell lines				
Reduced cell proliferation	Estrogen receptor alpha	Overexpression of miR-206 has an inhibitory effect on cell proliferation	ERα-positive breast cancer cells over expressing mir-206	[289]
miR-133b	GSTP1 downregulated	Transgenic miR-133b overexpression	HeLa cervical cancer cells	[282]
miR-133b	FAIM downregulated	Transgenic miR-133b overexpression	HeLa cervical cancer cells	[282]
Apoptosis increased	TNFα-induced cell death is activated	Transgenic miR-133b overexpression	HeLa cervical cancer cells	[282]
Increased cell proliferation and migration	Downregulation of MST2 Downregulation of CDC42 Downregulation of RHOA	Transgenic miR-133b overexpression	CaSki cervical cancer cells	[121]
Increased cell proliferation and migration	Indirect upregulation of p-AKT1 activity Indirect upregulation of p-ERK activity	Transgenic miR-133b overexpression	CaSki cervical cancer cells	[121]
RB1CC1 downregulated	Exogenous upregulation of miR-133b;	miR-133bm promotes cell apoptosis, but suppressed cell proliferation and cell-cycle progression in aggressive PC-3 cells	PC3 prostate cancer cell line	[106]
	miR-133b directly targets RB1CC1 in LNCaP cells	In contrast in low-aggression LNCaP cells, miR-133b stimulate cell proliferation and cell-cycle progression, but inhibit apoptosis	Hormone sensitive prostate cancer LNCaP cell line	

Cell proliferation decreased and apoptosis increased	Met, Twf1 and Ets1 and Bag4 activities downregulated	miR-1 expression is lower in mouse cSCCs compared to normal skin Transgenic miR-1 overexpression	Mouse cutaneous squamous cell carcinomas (cSCCs); A5 and B9 cSCCcell lines	[256]
Ets1 proto-oncogene	Repression of Ets1 expression inhibited HepG2 cell invasion and migration	Transgenic miR-1 overexpression	HCC HepG2 cells	[340]
lncRNA UCA1	Knockdown of lnc UCA1 expression phenocopied the effects of upregulation of hsa-miR-1	hsa-miR-1 decreased the expression of lnc UCA1 in bladder cancer cells in an Ago2-slicer-dependent manner	Human bladder cancer (TCC) cells	[156]
NOTCH3 signalling	miR-206 had a direct inhibition of NOTCH3 signalling and indirect interaction with other signalling pathways *via* CDH2 and MMP-9	miR-206 upregulation blocks the cell cycle, inhibits cancer cell proliferation and migration and activates cell apoptosis	SW480 (plus its metastatic strain) and SW620 colon cancer cell lines	[341]
FSCN1	miR-133b targets FSCN1 in GC cells; the direct knockdown of FSCN1 can also inhibit GC cell growth and invasion	Up regulation of miR-133b in GC cells inhibits cell proliferation, cell migration and invasion	miR-133b is significantly downregulated in GC tissues compared with adjacent normal tissues, as well as in GC cell lines	[342]
FSCN1	miR-133a targets FSCN1 in CRC cells; Overexpression of FSCN1 can reverse the inhibitory effect of miR-133a upregulation, reactivating CRC cell invasion	Up regulation of miR-133a expression and downregulation of FSCN1 protein expression both suppress colorectal cancer cell invasion	miR-133a is significantly downregulated in some colorectal cancer cell lines, as well as in colorectal cancer tissues compared with the normal adjacent tissues	[343]

[1]DS: Deep sequencing. HNSCC: Head and neck squamous cell carcinoma; ESCC: Esophageal squamous cell carcinoma; ARPC5: Actin-related protein2/3 complex, subunit 5, 16 kDa; CAV1: Caveolin-1; FSCN1: Fascin homolog 1; GSTP1: Glutathione S-transferase pi 1; TSCC: Tongue squamous cell carcinoma; EGFR: Epidermal growth factor receptor; TAp63: Tumor protein p63; CRC: Colorectal cancer; KLF4: Kruppel-like factor 4; SRC-1 and SRC-3: Steroid receptor coactivator proteins; HGFR: Hepatocyte growth factor receptor; G6PD: Glucose-6-phosphate dehydrogenase; PGD: 6-Phosphogluconate dehydrogenase; TKT: Transketolase; GPD2: Glycerol-3-phosphate dehydrogenase; RHOA: Ras homolog family member A; AML: Acute myeloid leukaemia; EVI1: Ecotropic virus integration site 1 transcription factor; MST2: Mammalian sterile 20-like kinase 2; RB1CC1: RB1 inducible coiled-coil 1; UCA1: Urothelial cancer associated 1; GC: Gastric cancer.

cancer[102,106], metastatic lung tumors[107], proliferating breast cancer[108], ERα-positive breast cancer[109], EEC[110], NSCLC[85], TSCC[111,112], laryngeal SCC[113], and HNSCC[114] (Table 3). Many of the reports identify reduction in a single myomiR, but in case studies of particular cancers often the different myomiRs have been identified as downregulated, for example in NSCLC tumor samples, miR-1[100], -133a[85,115], -133b[85,115], -206[107] or miR-1/-206[99] have been reported, by implication dysregulation of all of the myomiRs. Similarly, for colorectal cancer, downregulated miR-1[101], -133a[104], or -133b[86], or upregulated miR-206[116], while in prostate cancer downregulated miR-1[101,102] and miR-206[102], miR-133a[97,102] and miR-133b[95,96] have been variously reported (Table 3). Either these studies have examined different subclasses of the particular cancer which have different myomiR profiles, or more likely the method of miR detection or the purpose of the study (*e.g.*, relating a particular miR to a particular deregulated target gene) may have influenced the findings reported, leading to some potentially conflicting and inconsistent reports. Additionally, the mature miR isomers are difficult to distinguish by molecular assay and some reports note cross-identification of miR-133b by Taqman mature miR-133a specific probe[14], which is used in many studies. Others[114,117] also make this point, that the different miR assay platforms generate different profiles which may obscure or confuse the identity of observed molecular changes, and at minimum generate apparently different miR profiles of the same tissues or diseases[118,119].

It is essential to accurately determine the specific expression of particular myomiR isomers and their alleles to understand the control processes of particular pathological states. The expressed alleles of the myomiRs can be assessed accurately by employing pri- or pre-miR RT-PCR assays. For example, only cistron miR-1-2/-133a1 is found expressed in AML, presumably due to specific activation of that cistron[120] and amongst the myomiRs only pre-miR-133b is elevated in cervical cancer[121], in bladder cancer[122], and in progressive prostate cancer[123]. We suggest that initial microarray profiling be confirmed by pri- or pre-miRNA assay of each miR isomer independently.

Significantly, a deep sequencing profile of miR expression in mouse heart[124] found that miR-133b is expressed at about 1/6 of the level of miR-133a, reflecting that microarray-based studies may underestimate the relative levels of important miR isomers. Importantly, such deep sequencing analyses also question the canonical view that miR-133b is not expressed in cardiac tissue, reinforcing the need to employ sensitive and accurate analysis to extend our understanding of miR involvement in biological processes. Discrepancies in miR profiles detected between deep sequencing analysis of liver cancer[344] and microarray/Taqman expression profiling[95], and in comparison of level 3 expression data from the Cancer Genome Atlas (TCGA) with deep sequence data from ovarian cancer patients[119] which found only 1 out of 12 survival-associated miRs identified by sequencing correlated by the TCGA data, emphasize the need for robust analytical and computational methods for in-depth profiling of tumors. Expression profiling of

prostate tumors from individual patients by deep sequencing revealed that the expression of numerous miRs changed according to tumor stage[125]; however qRT-PCR of individual miRs at each tumor stage could not consistently confirm these alterations. A detailed survey of miRs using qRT-PCR accompanied by *in situ* hybridization to confirm the identity of the changed miR expression in matched prostate tumor tissue found less than 50% identity between major altered species when compared with deep sequencing analysis of pooled tumors by the same authors[126]. Furthermore, a deep sequencing profile of expressed miRs in bladder cancer[127] also showed that individual patient profiles varied greatly, and remarkably found also that the majority of deregulated miRs are upregulated in tumor tissue compared to matched normal tissue, with up to 3-5 × more upregulated than downregulated species in some patient tumor samples. Generally however, the myomiRs expressed in normal tissue are found downregulated in tumors when analysed by both qRT-PCR and by sequencing platforms. Significantly, another deep sequencing profile of expressed miRs in bladder cancer[128] revealed a different myomiR expression profile to the above deep sequencing study[127], yet confirmed the pattern of myomiR expression detected previously by the same group using microarray and qRT-PCR techniques[93] (Table 3). Overall, these various platform comparisons suggest the need to re-evaluate the genome-wide miR expression profiles of different cancers by use of deep sequencing and then to confirm findings using independent molecular methods.

Cell factors deregulated in cancer by myomiRs

Teicher[129] (2012) and Frith *et al*[130] (2013) noted in sarcoma tumors, which arise in diverse tissues of mesenchymal origin, that the upregulation of cMET (MET), HIF-1α, IGFR-1 or EGFR, CDK4, MCL1 or mTOR is observed with some elevated frequency in (particular) sarcomas, related to increased cancer severity and enhanced tumor progression, with the significance of each altered pathway likely due to the differentiated tissue in which the sarcoma arises. Downregulated expression of myomiRs occurs in both some sarcomas and carcinomas, linked specifically to the upregulated expression of some of the above gene targets. The different deregulated myomiRs modulate the levels of numerous mRNA/gene targets, but often particular deregulated gene targets are seen in several different cancers. For example, MET is upregulated due to reduced miR-1 levels in colon cancer[87,101], NSCLC[100] and rhabdomyosarcoma[89]. MET binds specifically with HGF, resulting in activation of pathways causing malignant progression *via* increased cell mobility, tissue invasion, and reduction of apoptosis. Reduced miR-1 targetting is only one of several cell alterations which can contribute to MET activation, yet the downregulation of miR-1 relates significantly to increased cancer severity, indicating the likely importance of miR-1 as a pleiotropic suppressor of several pathways important for tumor development.

Oncogenic membrane receptors, such as IGFR-1 and EGFR are upregulated in association with reduced miR-133 expression in NSCLC[131], and a similar EGFR upregulation occurs in hormone-sensitive prostate cancer and in breast cancer (Tables 4-6). Furthermore, a marked upregulation of cyclin C, cyclin D1 and CDK4 is seen in skin melanoma tissues associated with the reduced expression miR-206; whilst the BCL2 family of pro-survival molecules (Mcl-1 and BCL2L2) are both strongly upregulated due to a marked decrease in miR-133b levels (> 20-fold) in lung carcinoma tissue[85]. In addition, a detailed investigation of the role of myomiRs in prostate cancer[102] found that miR-1, miR-133a (and miR-206) were epigenetically supressed through promotor modification in numerous tumor samples and in prostate cancer cell lines. *In vitro* studies with prostate cancer cells showed that the downregulation of miR-1 allowed overexpression of multiple target genes that regulate key pathways affecting cell proliferation and cell migration, such as the actin filament network (FN1, LASP1, XPO6, CLCN3, G6PD), cell cycle and DNA damage control (BRCA1, CHEK1, MCM7) and histone acetylation (HDAC4); and with further downregulation of miR-1 the activation of oncogenic genes such as NOTCH3 and PTMA. Cell studies also showed that miR-206 has similar effects on prostate cancer cell biology as miR-1, suggesting that the combined downregulation of the myomiRs contributes significantly to prostate cancer progression.

In addition, FSCN1 expression is elevated in progressive metastatic ESCC, in breast cancer and in high-grade GIST, in part due to the loss of miR-133 expression[132]. FSCN1 is an actin-binding protein critical to cell adhesion, cell motility, and cell-cell interactions. In normal prenatal pig skeletal muscle, FSCN1 expression increases during major muscle developmental spurts, but postnatally it is expressed highly only in brain tissues during accelerated neural cell development[133]. The CREB pathway is often activated in different metastatic human cancers, causing the upregulation of FSCN1 expression[134]. In HNSCC patients, expression of RSK2 and FSCN1 proteins correlate closely. RSK2 protein expression potentiates filopodia formation and cell bundling, increasing cell invasiveness. Overexpression of FSCN1 can rescue the invasion phenotypes in RSK2 knockdown cells, linking RSK2-CREB signalling to the upregulation of FSCN1. In the highly metastatic PDAC[135], relative FSCN1 expression correlates with expressed HIF-1α levels, suggesting the hypoxic tumor microenvironment might induce FSCN1 expression, contributing to invasion and metastasis. Taken together, the downregulation of the myomiRs can contribute to the deregulated overexpression of oncogenic cell factors such as FSCN1, TAGLN2,

KLF4, MET (cMET), IGFR and others, each of which can potentiate dysregulation of other cell signalling pathways, enhancing oncogenesis and metastasis (Tables 4-6).

Interestingly, the oncogenic membrane receptors, IGF-1R, TGFBR1 and EGFR are upregulated in NSCLCs due to the downregulation of miR-133a, whilst NSCLC patients with highly expressed levels of miR-133a tend to survive longer[131] presumably because these target oncogenic proteins are less strongly expressed. In contrast, although downregulation of miR-133a occurs generally in colorectal tumors, tumors with higher miR-133a levels are associated with increased metastasis, adverse clinical characteristics and poor prognosis[136], suggesting that other cell factors contribute to these differences in outcome. Expression of other targets of miR-133a: LASP1, CAV1, and FSCN1 are also deregulated in a complex pattern. While LASP1 showed negative correlation with miR-133a levels, CAV1 instead had significant positive correlation, which increased in patients with distant metastases, while negative correlation of FSCN1 was only seen in non-metastatic patients. Again the relationship between the deregulated myomiR and its deregulated target(s) display a complexity which suggests the involvement of other factors in early developing and metastatic cancer stages.

The upregulation of yet other cell factors were seen with reduced myomiR expression, such as TAGLN2 with the downregulation of miR-1 and -133a in MSSCC[137], in RCC[138], and in CRC, liver cancer and in bladder cancer (TCC) (Table 6). Interestingly, TAGLN2 is a known tumor suppressor, yet its upregulated expression is associated with increased metastasis, and worse patient prognosis. This outcome may be related to findings in HCC where phosphorylation of TAGLN2 by PFTK1 (an oncogenic serine/threonine protein kinase) inactivates its actin-binding function, abrogating its suppression of cell motility[139]. Furthermore, in mouse metaphase I oocytes after IGF-1 treatment, miRNA-133b was upregulated more than 30-fold and target TAGLN2 was downregulated, stimulating growth and maturation of the oocytes[140]. These several reports indicate that both the miR-133 isomers may target TAGLN2.

OTHER PLEIOTROPIC PATHWAYS

The (semi-) homologous miRs-1 and -206 are downregulated in many cancers, acting as tumor suppressors (Table 3). The homologues share many of the same oncogenic targets, which may be released from negative regulation in tumors. Recently, Singh *et al*[99] (2013) demonstrated that downregulation of miR-1/-206 in lung adenocarcinomas (and NSCLC) have major effects on carbon flux and the activity of metabolic pathways associated with cell proliferation and growth. The inhibition of miR-1/-206 expression results (indirectly) from elevated NRF2 which in turn increases the activity of redox-sensitive HDAC4, affecting myomiR expression. Importantly, key targets of miR-1-2 and -206 include enzymes of the pentose phosphate pathway (including G6PD, PGD and TKT) and the tricarboxylic acid cycle enzyme GPD2, which increase their activities and potentiate cancer cell proliferation and compromise patient survival. Pleiotropic factors, such as KLF4, HDAC4 and IGFR-1 are also elevated in gastric carcinoma and in breast cancer cells in part due to the loss of miR-206[108], yet in contrast KLF4 is reduced in colon cancer tissue due to strong upregulation of -206[116]. KLF4 is a zinc-finger transcription factor that binds to the TGF-β1 promoter and is important during normal cellular differentiation and proliferation. KLF4 is highly expressed in normal cardiac fibroblasts where it promotes the formation of myofibroblasts[141].

Some common molecular targets of the isoforms of miR-133 are elevated in different cancers (Tables 4 and 5). Moesin, a member of the ezrin-radixin-moesin protein family which links functionally between the plasma membrane and the actin-based cytoskeleton of cells and is found in invadopodia of metastatic cells[142], is deregulated in HNSCC, breast cancer, CRC, and prostate cancer. In HNSCC cells both miR-133a/-133b target moesin specifically, and restoration of miR-133b levels in HNSCC cells, as well as in PC10 and H157 lung SCC cell lines, reduces moesin expression, inhibits cell proliferation, cell migration and invasion[143]. The phenotype is also associated with elevation of other miR-133 targets such as ARPC5 and GSTP1[115]. Both ARPC5 and GSTP1 levels are elevated in lung-SCC and elevated GSTP1 is seen in bladder cancer (TCC) associated with reduced levels of miR-133a[144].

Several large cohort studies have examined the changes in expressed miR networks in a variety of cancers. Navon *et al*[95], Hart *et al*[125], Han *et al*[127], Itesako *et al*[128] and Volinia *et al*[145] noted that the downregulated expression of the *miR-133/-1/-206* genes is associated with a variety of solid tissue cancers, with numerous other miRs also having highly significant oncogenic roles in particular cancer types. In sum, the downregulation of the various myomiRs contributes significantly to the upregulation of oncogenic proteins linked to regulation of cell cycle progression in solid tumors, sarcomas and carcinomas.

UPREGULATED MYOMIRS IN CANCER

In yet other cancers in which the upregulation of a myomiR is associated with worsening metastasis or potentiation of cancer severity, the downregulation of different tumor-suppressor factors appears to be critical. In cervical cancer the upregulation of miR-133b is associated with downregulation of Mst2 protein kinase (STE20), Cdc42 and RhoA, which in turn lead to increased p-ERK and pAKT1 signalling activity,

resulting in tumorigenesis and metastatic cancer proliferation[121]. RhoA, Cdc42 and other small Rho GTPases are components the Rho-kinase pathway which is a key controller of fundamental cellular processes such as cell motility, cell proliferation, cell division, cell differentiation, cell apoptosis, as well as morphological structure development, epithelial and skin morphogenesis, nerve system and limb development[146-148]. The downregulation of these key factors restricts cell apoptosis, favouring tumorigenesis. Interestingly, elevated levels of RhoA, Cdc42, Nelf-A/WHSC2 are also observed in hypertrophic cardiac muscle, associated with reduced levels of miR-133a[6], suggesting that both isomers of miR-133 may normally regulate these factors (in muscle). Similarly, the disruption of the Mst2 pathway is also associated with the disruption of cell apoptosis, of abrogating cell cycle regulation and with increased tumorigenesis in mouse intestinal epithelium[149], specifically by derepressing the accumulation of Yes-associated protein[150] which results in strong activation of β-catenin and Notch signalling. Elevation of Notch has also been seen in colorectal tumors associated with reduced miR-1 levels.

In AML the levels of expression of both miR-133a and miR-1 were elevated significantly[120,151]; similar to multiple myeloma where upregulation of these myomiRs was associated with the downregulation of the cyclin CCND2[152]. Wang *et al*[153] (2007) also found in patients with AML that downregulated CCND2 and CCND3 results in dephosphorylation of phospho-retinoblastoma protein and induction of G(1) cell-cycle arrest. These findings suggest that CCND2 may also be downregulated in AML in association with the upregulation of miR-133a and miR-1 levels, similar to multiple myeloma. During development or regeneration of normal muscle, the downregulation of CCND2 strongly enhances the myogenic terminal differentiation of muscle progenitor cells[154]. Taken together, the upregulation of these myomiRs in particular cancers can contribute to the deregulated repression of cell factors such MST2, RHOA, CDC42, CCND2 and others, which then also contribute to dysregulation of other cell signalling pathways, potentiating oncogenesis and metastasis. In contrast, a study of the miR network associated with altered mRNA profiles in AML[155] found that miRs other than myomiRs had highly significant roles in key deregulated pathways. Overall, the several studies suggest that myomiRs also contribute to development of AML.

LONG NON-CODING RNA

Long non-coding RNAs (lncRNAs) are emerging as important new regulators of oncogenic pathways in cancers, and miRs are emerging as important regulators of lncRNA activities. Recently Wang *et al*[156] (2014) reported that deregulated expression of lncRNA UCA1, an important new oncogene in human bladder cancer (TCC), can be downregulated by miR-1 *in vitro*. These two factors are inversely expressed in bladder cancer tissue *in vivo*. lncRNA UCA1 expression is induced by HIF-1α which stimulates bladder cancer cell proliferation, migration, and invasion under hypoxic growth conditions[157]. It is also induced by the transcription factor CCAAT/enhancer binding protein α[158], and in another route for potentiation of bladder cancer cell growth and reduction of cell apoptosis the transcription factor Ets-2 binds directly at the UCA1 promoter, stimulating UCA1 promoter activity[159]. In bladder cancer cell lines the transgenic alteration of lncRNA UCA1 levels positively influences AKT expression and activity, and cell cycle progression could be reduced by inhibition of the PI3-K pathway, indicating that lncRNA UCA1 affected cell cycle progression through CREB[160]. Taken together, lncRNA UCA1 regulates the cell cycle through CREB and *via* PI3K-AKT-dependent pathways in bladder cancer. Interestingly, several of the cellular factors which are induced by lncRNA UCA1 are also regulated at the expression level by myomiRs. miR-206 targets HIF-1α directly, hence hypoxia-induced downregulation of miR-206 promotes pulmonary hypertension *via* elevated HIF-1α in hypoxic rat model pulmonary artery smooth muscle cells (Table 6). Additionally, during regeneration of injured muscle, the AMPK-CRTC2-CREB and Raptor-mTORC-4EBP1 pathways are activated in satellite cells, which involve regulation by miR-1[58]. The involvement of myomiRs in the regulation of these cellular factors in muscle cells suggests a potential for involvement of lncRNA UCA1 in the regulation of normal cellular processes involving the above protein factors.

Recently, the lncRNA MALAT1 which is upregulated during the differentiation of myoblasts into myotubes in normal muscle biogenesis[161] was also reported to be upregulated in several non-muscle cancers associated with worsening patient outcomes[162,163]. In skeletal muscle MALAT1 expression is downregulated by myostatin[161], whilst the silencing of MALAT1 expression in the mouse myoblast C2C12 cells results in the reduction of SRF transcription factor at both RNA and protein levels as well as reduced myocyte differentiation[164]. The MALAT1 transcript has a functional miR-133 target site, thus miR-133 acts as a competing endogenous RNA, regulating MALAT1 levels, which in turn modulates SRF activity. SRF also regulates the expression of miR-133a in C2C12 cells by its binding to the miR-133a enhancer[22], indicating a complex regulatory loop involving SRF, miR-133 and MALAT1.

MYOMIR TARGETS IN MUSCLE AND TUMOURS

Considering the central role of the myomiRs in the cell biology of myogenesis and muscle cell differentiation,

in muscle metabolism, as well as in muscle remodelling and recovery from injury, it may be expected that they regulate the expression of numerous other target genes, in addition to the well documented pathway genes associated with key processes. In non-muscle-tissue cancers in which myomiRs play critical roles, the myomiRs influence expression of a variety of intermediary regulatory pathway genes, causing the potentiation of tumour development and metastasis. Literature searches (PubMed) show that essentially all myomiR targetted genes detected in different cancers have identified roles in several aspects of muscle cell biology. Thus, the myomiRs likely influence the expression of these gene targets in muscle in a normal regulatory manner, yet in non-muscle cancers the deregulated expression of myomiRs contributes to the dysregulated expression of these various gene targets, to the advancement of cancer development.

CONCLUSION

This review examines the diverse and complex regulatory functions of the cistronic myomiRs miR-133, -1 and -206 in numerous tissues. The myomiRs are intimately involved in the regulation of many processes of muscle development, muscle cell metabolism and homeostasis. Indeed some of the myomiRs are critical cell factors that commit stem cells onto the path of muscle cell differentiation and development, and their removal can elicit de-differentiation of committed muscle cells to an undifferentiated state. This centrality of cell regulatory functions suggests that, by necessity, these miRs would also be involved in redeveloping and repairing tissue after damage or injury, and that dysfunctional expression of myomiRs would play important roles during disease states. Furthermore, individual myomiRs have functional roles in the development of numerous non-muscle cells and tissues, beyond their original classification as muscle-specific factors, and hence the observation that myomiRs have roles in an increasing number of different cancer types should perhaps not be surprising. Whilst the myomiRs can display either tumor suppressor or tumor stimulator roles in different cells and tissues, independent cellular function assays confirm that the altered expression of the myomiRs typically correlates with the potentiation of cancer severity. This apparently contradictory ability of miRs to cause tumor suppression or tumor stimulation actions in different tissues relates to the multiple regulatory pathway genes targeted by each miR and the specific regulatory functions targeted in each tissue type. Interestingly, this ambiguity parallels the alternate roles of key signalling pathway regulatory genes in cancers, for example the increased expression of members of the FOX family of genes can cause either tumor suppression or tumor stimulation in different cancer types[165]. Significantly, the number of validated targets of each of the myomiRs has increased greatly in recent years, yet the extent to which each myomiR, miR-133, miR-1 or miR-206, contributes to specific tumorigenesis or tumor progression must await fuller clarification and integration with complex cellular regulatory pathway processes which are not yet fully defined.

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MINIREVIEWS

Inflammation, oxidative stress and renin angiotensin system in atherosclerosis

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Abstract

Atherosclerosis is a chronic inflammatory disease associated with cardiovascular dysfunction including myocardial infarction, unstable angina, sudden cardiac death, stroke and peripheral thromboses. It has been predicted that atherosclerosis will be the primary cause of death in the world by 2020. Atherogenesis is initiated by endothelial injury due to oxidative stress associated with cardiovascular risk factors including diabetes mellitus, hypertension, cigarette smoking, dyslipidemia, obesity, and metabolic syndrome. The impairment of the endothelium associated with cardiovascular risk factors creates an imbalance between vasodilating and vasoconstricting factors, in particular, an increase in angiotensin Ⅱ (Ang Ⅱ) and a decrease in nitric oxide. The renin-angiotensin system (RAS), and its primary mediator Ang Ⅱ, also have a direct influence on the progression of the atherosclerotic process *via* effects on endothelial function, inflammation, fibrinolytic balance, and plaque stability. Anti-inflammatory agents [statins, secretory phospholipase A2 inhibitor, lipoprotein-associated phospholipase A2 inhibitor, 5-lipoxygenase activating protein, chemokine motif ligand-2, C-C chemokine motif receptor 2 pathway inhibitors, methotrexate, IL-1 pathway inhibitor and RAS inhibitors (angiotensin-converting enzyme inhibitors)], Ang Ⅱ receptor blockers and ranin inhibitors may slow inflammatory processes and disease progression. Several studies in human using anti-inflammatory agents and RAS inhibitors revealed vascular benefits and reduced progression of coronary atherosclerosis in patients with stable angina pectoris; decreased vascular inflammatory markers, improved common carotid intima-media thickness and plaque volume in patients with diagnosed atherosclerosis. Recent preclinical studies have demonstrated therapeutic efficacy of vitamin D analogs paricalcitol in ApoE-deficient atherosclerotic mice.

Key words: Atherosclerosis; Renin-angiotensin system; Inflammation; Oxidants/antioxidants imbalance; Anti-inflammatory drugs; Renin-angiotensin system blockers

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Core tip: There are several reviews in the literature contributed to the pathophysiology, therapeutic options and clinical trials for atherosclerosis. However this is a first review to report the latest cellular and molecular mechanisms of the pathways of atherosclerosis including inflammation, renin-angiotensin system, oxidants/antioxidants imbalance and the efficacy of several therapeutic strategies in improving cardiovascular outcomes, and recent clinical trials reducing the progression of the pathogenesis of atherosclerosis.

Husain K, Hernandez W, Ansari RA, Ferder L. Inflammation, oxidative stress and renin angiotensin system in atherosclerosis. *World J Biol Chem* 2015; 6(3): 209-217 Available from: URL: http://www.wjgnet.com/1949-8454/full/v6/i3/209.htm DOI: http://dx.doi.org/10.4331/wjbc.v6.i3.209

INTRODUCTION

Atherosclerosis, a continuing chronic disease with inflammation manifesting in the vascular system of blood vessels is the primary origin of cardiovascular diseases (CVDs) in the developed countries of the globe. Its burden is higher in developing countries of Asia, Africa and South America (> 5000 per 100000) than in the developed countries of North America, Europe and Australia (< 3000 per 100000). The mortality due to this disease is anticipated to be at the first place in the globe by 2020[1]. It is represented by the development of vascular lesions or plaques in the blood vessels following inflammatory/oxidant response to endothelial damage[2,3]. The plaque mainly composed of blood cells, foam cells, lipids and proteins with calcium accumulation[3-5]. Finally it results to vascular expansion, vascular blockage, and inhibition of vascular blood flow leading to burst of the vascular wall[6,7]. In cardiovascular disease, blockage and rupture of atherosclerotic coronary arteries cause myocardial infarction, whereas blockage of carotid arteries cause stroke[2,6,8]. The impairment of the physiological functions of the endothelium is seen during initial phases of the atherosclerotic lesions due to oxidant damage. Endothelial damage is linked with heart and blood vessels risk factors such as diabetes, high blood pressure, nicotine, lipid disorders, obesity, and disorders of the metabolism[2,6,9,10]. The renin-angiotensin system (RAS) also plays an important role in the advancement of atherosclerosis by influencing on physiology of the endothelium, inflammatory reactions, thrombosis, and oxidant injury[9,10]. Ang Ⅱ causes oxidant damage in vascular system by inducing oxidant species generation *via* activation of NADPH oxidase and these oxidant species oxidize cellular biomolecules including lipids, lipoproteins and DNA leading to endothelial impairment.

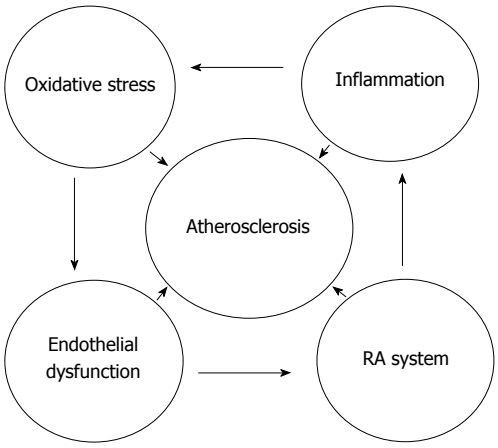

Figure 1 Relationships between inflammation, oxidative stress, renin-angiotensin system, endothelial dysfunction and atherosclerosis. RA: Renin-angiotensin.

The relationship between inflammation, oxidative stress, RAS system, endothelial dysfunction and atherosclerosis is depicted in Figure 1. This mini review presents precisely the mechanistic aspects of the events associated with atherosclerosis, implications of the inflammation, RAS and oxidative stress as well as the efficacy of several therapeutic strategies in improving cardiovascular system, physiology of the endothelium, and ameliorating the advancement of atherosclerotic events. Current clinical trials using anti-inflammatory, RAS blockers and antioxidants in attenuating the atherosclerotic lesions and preserving the pathophysiology of the endothelium is also reviewed.

INFLAMMATION AND ATHEROSCLEROSIS

Atherosclerosis is a concurrent inflammatory disease which first starts in the endothelium of the arterial wall[3,4,11]. Impairment of the endothelium is the first physiological alteration in the pathophysiology of this disorder which is manifested by enhanced vascular constriction and depressed dilatation of the vascular endothelium as well as changes in the mediators of thrombosis. Endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) plays an important role in preserving the endothelial vasodilatation and inhibiting the vasoconstriction triggered by angiotensin Ⅱ and endothelin[3]. Inflammatory processes are manifested by enhanced biosynthesis of mediators of inflammation and thrombosis. The mediators and reactions include interleukin-6, monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), endothelial-selectin, adhesion/infiltration of monocytes, oxidation of low density lipoprotein (LDL) and production of foam cells[12]. Foam cells are formed due to storage of excess cholesterol ester in the macrophages[12]. The transport of cholesterol regulated by ATP-binding cassette transporter A1 (ABCA1) and transport of

Table 1 Cytokines and factors implicated in causing inflammation in atherosclerosis

Cytokines/factors	Abbreviations
Tumor necrosis factor-alpha	TNF-α , TNF-a
Interleukine-6	IL-6
Interleukine-1beta	IL-1β, IL-1beta
Nuclear factor-kappa B	NF-κB
Monocyte chemoattractant protein-1	MCP-1
C-reactive protein	CRP
Intracellular adhesion molecule-1	ICAM-1
Vascular cell adhesion molecule-1	VCAM-1
Monocyte colony-stimulating factor	M-CSF, MCSF
Transforming growth factor beta1	TGFβ1, TGF-β1
Plasminogen activator inhibitor-1	PAI-1
Macrophage migration inhibitory factor	MIF
Cyclooxygenase-2	COX-2
Endothelin-1	ET-1
Angiotensin 2	Ang Ⅱ, ANG Ⅱ
Endothelial-selectin	E-selectin
Platelet-selectin	P-selectin
Angiotensinogen 2	ANGT 2
Leptin	LEP
Inducible nitric oxide synthase	iNOS
Matrix metalloproteinase	MMP
Low density lipoprotein	LDL
Serum amyloid A	SAA
Apolipoprotein E	ApoE
Tumor necrosis factor receptor –associated factor	TRAF
Reactive oxygen species	ROS
Angiotensin type 1 receptor	ATR1
Glutathione peroxidase 1	GPx1
Phospholipase A2	PLA2
Lipoprotein-associated phospholipase A2	Lp-PLA2
5-Lipoxygenase-activating protein (FLAP)	FLAP
5-Lipoxygenase	5-LO
Chemokine motif ligand 2	CCL2
Chemokine motif receptor 2 (CCR2)	CCR2
Toll like receptor 2	TLR2
Hear shock protein	HSP

oxidized LDL through CD36 regulate the excess of cholesterol ester in the macrophages[12]. Apart from excess foam cells, growth of smooth muscle/endothelial cells[3,13], collagens, matrix metalloproteinases (MMPs), fibronectin, and elastin are also responsible for plaque development[2,3,11]. Evidences suggest that cytokines and tissue factors also regulate pathophysiology of the endothelium due to inflammatory reactions. The influence of different cytokines and factors modulating the pathophysiology of the vessel wall is depicted in Table 1. Among the biomarkers of inflammation C-reactive protein (CRP) is important which is generated by hepatic cells and is also modulated by IL-6, IL-1 and TNF-α[14]. Evidences suggest that raised blood CRP level is one of the inflammatory biomarkers and predictors of cardiovascular diseases[15,16]. It is also implicated in the advancement of atherosclerotic lesions by regulating physiology of endothelium[3,17,18]. It enhances the production of VCAM-1, ICAM-1, selectins, and MCP-1 in the endothelium through induction of powerful constrictor of the vessels ET-1 and IL-6[3,17]. It ameliorates the synthesis of NO in the endothelium by depressing the transcription and translation of enzyme NO synthase[3,19]. It also plays a significant role in cooperating with the activities of other cytokines and factors. CRP induces the biochemical synthesis and physiological functions of PAI-1 in the endothelium[19]. PAI-1 is known to be actively involved in thrombosis during atherosclerosis process and inhibits destruction of the fibrin clot by suppressing plasminogen activation[20]. There is a good correlation between elevated blood PAI-1 concentration and death rate in patients with coronary heart diseases[20]. Apolipoprotein E as well as low-density lipoprotein (LDL)-receptor knock out animals display speedy atherosclerotic lesions[21,22]. These animals also have sizeable counts of macrophages/T cells in their plaques. Cross breading of apolipoprotein-E knock out with T-cell knock out and mice with deficient macrophages (osteoporotic op/op) revealed the influence of immune cells in the progression of atherosclerosis[23]. Inflammatory reactions are not only involved in progression of human vascular plaques generation but also have important role in the rupture of internal arterial plaques transforming chronic disorder into an acute thromboembolic disease. Several factors are implicated in the rupture of internal arterial plaques comprise of cytokines, cyclooxygenase-2, matrix metalloproteinases, and tissue factors[1,4,23]. Experimental evidences support crucial role for inflammatory reactions as a connection between risk factors for atherosclerotic disorder and pathophysiologic complexity of the disease[2]. Serum amyloid A (SAA) protein has also been implicated in the inflammatory reactions associated with atherosclerotic disorder and used as a biomarker for cardiac and vascular disorders as well as heart and vessels outcome[24]. TNF-α is one of the inflammatory cytokines involved in commencement as well as development of atherosclerosis. It induces transcription factor nuclear factor-κB (NF-κB), a key factor in the pathways of inflammation. In the process of atherosclerosis NF-κB induces the transcription of VCAM-1, ICAM-1, MCP-1, and E-selectin in smooth muscle/endothelial cells of the blood vessels[25]. TNF-α depletes NO levels in the endothelium causing decrease of endothelial dilatation leading to dysfunction of the endothelium[26,27]. TNF-α has been reported to cause apoptosis of the endothelial cells through dephosphorylation of protein kinase B (Akt) leading to endothelial damage[28,29]. Resistin exerts inflammatory reactions/vasoactive effects in cultured cells of the endothelium[8]. In atherosclerotic process resistin induces transcription of cellular factors such as VCAM-1 and MCP-1[30]. Cells from endothelium exposed to resistin deplete the levels of TNF receptor-associated factor (TRAF-3) which is a well known inhibitor of the endothelial activation[31]. It is suggested that augmented resistin concentration causes a significant dysfunction of the endothelium through activation of endothelial system. Furthermore, resistin exposure activates endothelial cells by increasing ET-1 release through induction of transcription of ET-1 indicating its role in the impairment of the endothelium[32]. Leptin

up regulates ET-1 as well as NO synthase biosynthesis in the endothelial cells and augments generation of free radicals and oxidants[33,34], causing oxidative stress[35]. Leptin also increases the cellular growth as well as migration of cells of the endothelium[36] and cells of smooth muscle[37]. It induces the synthesis of MCP-1 in the cells of the aortic endothelium[38]. It enhances the aggregation of the platelets and vascular thrombus formation through leptin receptor pathways[33,34]. It directly augments concentrations of monocyte colony-stimulating factor (MCSF)[39], increases cholesterol levels in hyperglycemia[40], and promotes new blood vessel formation[41]. We have shown in our earlier studies elevated concentrations of factors involved in inflammatory pathway namely TNF-α, MCP-1, Cox-2, TGF-β1, iNOS, and Mn-SOD in ApoE-deficient atherosclerotic mice[42,43] proving the vascular inflammation as an integral process in the atherosclerotic pathophysiology.

OXIDATIVE STRESS AND ATHEROSCLEROSIS

Oxidative stress is referred as imbalance of the cellular oxidants and antioxidants in the body. In atherosclerosis process Ang Ⅱ causes oxidants/antioxidants imbalance in the vascular system by inducing oxidant species generation *via* activation of NADPH oxidase[44,45]. Earlier studies have shown that excess superoxide generation due to NADPH oxidase activation causes inflammation and further generation of inflammatory cytokines (TNF-alpha) through NF-κB activation[46-48]. The role of NF-κB activation is also demonstrated in atherosclerosis[47,49]. These reactive oxygen species (ROS) initiate vascular membrane lipid peroxidation leading to inflammation and production of TNF-α *via* NF-κB induction[46,47] and other factors namely VCAM-1, MCP-1, TGF-β1, Matrix metalloproteinase 9 (MMP9), iNOS and Mn-SOD[50-52]. The ROS up regulate atherosclerotic events namely cell infiltration, migration, adhesion and platelet activation. These ROS oxidize cellular biomolecules including lipids, proteins and nucleic acids causing endothelial impairments[53]. However, cells are equipped with an intricate cellular defense system that includes antioxidant enzymes namely superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx), tripeptide glutathione (GSH), antioxidant vitamins A, C, and E to scavenge oxidant species thereby attenuate oxidant injury[54]. Most importantly, depletion of a major cellular antioxidant such as GSH has been reported to cause vascular dysfunction[42,55-57]. The vascular system has also been shown to be equipped with antioxidant defense to combat oxidant damage[42,55,56]. However depletion of cellular antioxidants increases oxidant species build up leading to pathological and physiological impairments[42,43,55-57]. Several studies in ApoE knock out mouse model revealed down regulation of antioxidants in atherosclerosis[42,43,58] which suggests a relationship between declined antioxidants and enhanced pathological lesions. On the contrary, over expression of catalase which hydrolyses H_2O_2, suppressed tissue injury in ApoE knock out mouse model[21] indicating the implications of H_2O_2 degrading enzyme in atherosclerosis. Furthermore, additional studies suggest defensive capacity of glutathione peroxidase (GPx1) in atherosclerosis[58]. Down regulation of erythrocyte GPx1 level was linked with enhanced risk of heart and blood vessel injury and patients with atherosclerosis of carotid artery posses declined GPx1 levels[58]. Knock down of GPx1 in animal enhanced lipoprotein oxidation and depleted NO levels causing impairment of endothelium[58]. Diabetes-related atherosclerotic events are enhanced with deficit of GPx1 through increased inflammation and thrombosis in ApoE-deficient mouse model[58]. Several studies reported a lower level of tissue antioxidants in patients with renal and cardiovascular diseases[6,59-61].

RAS AND ATHEROSCLEROSIS

The renin-angiotensin system is thought to have an important implication in the pathophysiologic injury in atherosclerosis through induction of various cellular/molecular reactions[10,61,62]. Previous studies showed that angiotensin Ⅱ (Ang Ⅱ) produced by vascular tissues increases generation of ROS and induces synthesis of several factors *via* induction of AT Ⅱ type 1 receptor (AT1R), leading to stockpile of inflammatory mediators and proliferation/migration of vascular cells[1,10,62]. These findings indicate that regional impact of AT1R activation in vascular wall plays a critical role in pathophysiology of concurrent inflammatory reactions through a direct action on local vascular cells. Several studies demonstrated an essential part of RAS in pathophysiology of heart and vascular system, including atherosclerotic disease[1,6,10,62,63]. Angiotensinogen (ANGT), a precursor of ANG Ⅱ, is synthesized in fat cells[64]. Angiotensin Ⅱ causes oxidative stress in cardiovascular systems by inducing ROS production *via* induction of NADPH oxidase[44,49]. These oxidant species begin oxidation reaction of lipids in the membranes of the blood vessels leading to inflammation and generation of inflammatory cytokines (TNF-α) through NF-κB activation[1,2,11,23,63,65]. These ROS oxidize cellular biomolecules namely lipids, proteins and nucleic acids causing oxidation of membrane phospholipids resulting in the impairments of the heart and blood vessels[1,2,11,23,57,63,65,66]. Vasoconstrictor Ang Ⅱ instantly triggers the synthesis of ICAM-1, VCAM-1, MCP-1, and macrophage colony stimulating factor (M-CSF) in the walls of the blood vessels through induction of NF-κB-regulated genetic products[67]. Moreover Ang Ⅱ stimulates production of oxidant species from NO leading to depletion of NO causing injury to blood vessels[3]. Enhanced ANGT 2 levels is associated with new blood vessels formation[68] and elevation of blood pressure[44,56,57]. These two disorders are closely linked

with impairments of the endothelium. Additionally, CRP induces ATR1-receptor transcription and translation as well as enhanced AT1-receptor levels in blood vessel wall[69]. Importantly, activation of ATR1-receptors promote Ang Ⅱ-induced ROS generation, migration/proliferation/remodeling of the cells of the blood vessels[70].

TREATMENT OF ATHEROSCLEROSIS

Use of anti-inflammatory agents

The influence of inflammatory reactions on the progression/maturation and rupture of thrombus in the vascular lumen of the blood vessels opens new therapeutic strategies for atherosclerotic disorder. One of the clinical trials (JUPITER) corroborates clinical advantage of the evaluation of the degree of inflammation in managing the therapeutic intervention to restrict the occurrence of the injury to the heart and vascular system. Academic and theoretical concepts of inflammatory reactions are being now utilized as a therapeutic tool in the clinics for the risk assessment as well as targeted therapeutics[2]. Current therapeutics effectiveness in preventing atherosclerosis such as HMG-CoA reductase inhibitors (statins), acetylsalicyclic acid (ASS), and RAS blockers deploy their influence through modulation of inflammation in the blood vessels[71]. Attentions are also focused to various therapeutic agents decreasing different modulators of inflammation process. Among these drugs are derivatives of thiazolidinedions (glitazones), HMG-CoA reductase inhibitors (statins), acetyl salicylic acid, ACE inhibitors, and ATR blockers. Glitazones are activators of nuclear peroxisome proliferator-activated receptor (PPAR)-γ. Glitazones are known to down regulate various factors involved in the process of inflammation and their activities therefore they can ameliorate the advancement of atherosclerotic events. Glitazones are also known to deplete TNF-α level in fat cells and abrogate TNF-α-induced synthesis of VCAM-1 and ICAM-1 in the endothelium of the blood vessels[8,17]. Rosiglitazone has been shown to reverse the progression of atherosclerotic activities of CRP in the endothelium of the blood vessels[8]. Glitazones also depress leptin-induced migration of the cells of the endothelium[33]. Glitazones inhibit resistin concentration in fat tissues[72,73]. Alternatively, inhibitors of matrix metalloproteinase (MMP) activity as well as vaccines are presently under developmental processes and clinical trials[23]. There are several anti-inflammatory agents have been used pre-clinically and clinically in the treatment of atherosclerotic cardiovascular diseases such as phospholipase A2 (PLA2) inhibitors[74], phospholipase A2 of lipoproteins (Lp-PLA2) inhibitors[75], 5-LO-activating protein (FLAP)[76], arachidonate 5-lipoxygenase (5-LO)[77], chemokine motif ligand 2 (CCL2), chemokine motif receptor 2 (CCR2) inhibitors[78], methotrexate[15,65], and IL-1 pathway inhibitor[79].

Use of antioxidants

Antioxidant therapy has been shown to ameliorate cardiovascular oxidative stress by scavenging excess ROS and up regulating the antioxidant defense system[54,80]. The antioxidant N-acetylcysteine is reported to abrogate accelerated atherosclerotic events in ApoE knockout mouse models[80]. Our recent study demonstrated that vitamin D analog paricalcitol ameliorated the oxidative vascular injury by suppressing ROS-generating enzyme NADPH oxidase activity and inflammatory mediators and by up regulating the antioxidant defense system in ApoE-deficient mice[42,43]. There is a novel strategy against inflammation is being used by inhibiting the oxidation of lipoproteins to stop their entry into the cells of the blood vessels. The ARISE clinical trial investigated the influence of antioxidant succinobucol (AGI-1067) in patients who had myocardial infarction/ruptured atherosclerotic plaques had significant curative effects[81]. In preclinical investigation using antioxidant enzyme GPx1 afforded significant protection in a mouse model of atherosclerosis[58].

Use of RAS inhibitors

In preclinical and clinical investigations putative drugs used for high blood pressure have been reported to adequately suppress the events of atherosclerosis[6,62,63]. The inhibitors or blockers/antagonists of RAS such as ACE inhibitors and ATR blockers act either by depleting the generation of Ang Ⅱ or by blocking the binding of Ang Ⅱ to its receptors. RAS inhibitors or blockers/antagonists have both ancillary and concurring actions that enhance the NO concentration, decrease oxidants/antioxidants imbalance, inhibit RAS-induced inflammation, as depicted by actions of these drugs on several biomarkers of inflammation[6,10,17,23,62,63,82]. The above biochemical alterations achieve amelioration in the activities of the endothelium and pathophysiology of the vascular system in atherosclerosis. Additionally above biochemical alterations are also linked with advantage over lowering the blood pressure in patients with atherosclerosis and high blood pressure. These investigations have demonstrated that olmesartan medoxomil administration suppress the advancement of atherosclerotic process and significantly ameliorate the cardiovascular dysfunctions[10,63]. ATR1 blockers have been shown to decrease the new blood vessel formation in ApoE-deficient mouse model of atherosclerosis, through toll like receptor 2 and 4 (TLR2/TLR4)-arbitrated events of inflammation and activation of MMP, consequently inhibiting the proliferation of the vascular lesions as well as rupture of the plaques[83]. Our recent studies have shown that ACEI enalapril ameliorated the oxidative vascular injury by suppressing ROS-generating enzyme NADPH oxidase activity and inflammatory mediators and by up regulating the antioxidant defense

system in ApoE-deficient mice[42,43].

Use of other agents

Preclinical as well as clinical studies have clearly demonstrated and provided the evidence that vitamin D and its analogues suppresses the various factors and biomarkers associated with disorders of vascular system and permeate cells of the immune system against the activities of the inflammation[5,9,84-87]. The knock down of vitamin D receptor (VDR) and VDR-mediated signaling pathways in animals induces hypertension by elevating renin release from the kidneys and stimulates the process of atherosclerosis conceivably through regional induction of cellular RAS[9]. The insufficiency or depletion of vitamin D causes hypertension and enhances the progression of atherosclerotic lesions in animals[5]. Our recent study demonstrated that vitamin D analog paricalcitol ameliorated the oxidative vascular injury by suppressing ROS-generating enzyme NADPH oxidase activity and inflammatory mediators and by up regulating the antioxidant defense system in ApoE-deficient mice[42,43]. In animal model when vaccines derived against oxidized LDL and heat shock protein (HSP) administered have demonstrated inhibition of inflammation and progression of atherosclerotic lesions[88]. The prospect of clinical application of vaccines for prevention or treatment of atherosclerosis is presently under exploration[23]. The antibodies raised against the oxidized lipoproteins not only reveal activity of the disorder but also confer the prevention and treatment of atherosclerosis[7,63].

CONCLUSION

The investigations delineated in the present mini review furnish to our comprehending the role of inflammatory events, oxidants/antioxidants imbalance and RAS pathways in the pathophysiology of atherosclerosis as well as targeted therapeutic intervention. Preclinical as well as clinical investigations clearly demonstrate that inflammatory reactions operates all stages of atherosclerotic events, including commencement, advancement, and the complexity of the lesions. Inflammatory process is commonly associated with several risk factors for atherosclerotic plaque formation and modified pathophysiology of the blood vessels. Alteration of implicated risk factors has a clinical advantage through abrogating the inflammatory reactions and its consequences. Convincing verifications from clinical studies authenticates the application of the drugs inhibiting the inflammation process; RAS blockers and antioxidants as a therapeutic regimen that can prevent and treat the atherosclerotic lesions.

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Techniques to elucidate the conformation of prions

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Abstract

Proteinaceous infectious particles (prions) are unique pathogens as they are devoid of any coding nucleic acid. Whilst it is assumed that prion disease is transmitted by a misfolded isoform of the cellular prion protein, the structural insight of prions is still vague and research for high resolution structural information of prions is still ongoing. In this review, techniques that may contribute to the clarification of the conformation of prions are presented and discussed.

Key words: Prion; Amyloid; Neurodegenerative disease; Protein structure; Fourier-transform infrared spectroscopy

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Core tip: Prions (proteinaceous infectious particles) are misfolded isoforms of cellular proteins that cause neurodegenerative diseases in mammals and humans. Several structural models are available for prions but a 3D-structure does still not exist. More structural information is demanded for the understanding of the conversion process and finally for the design of efficient therapeutic approaches. In this review, techniques that may contribute to the clarification of the conformation of prions are presented.

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INTRODUCTION

Prions are infectious proteins that cause fatal neurodegenerative diseases[1-5]. Rearrangements of the structure of the cellular prion protein (PrP^C) are the key of infection. PrP^C is characterized by a high α-helical content and conversion in a β-sheet rich conformer (PrP^{SC}) transforms it into an infectious protein[6,7]. According to the "prion hypothesis" the protein itself is the causative agent of disease even though several cofactors that may influence the conversion process are discussed in the literature[2,8-12]. A 3D-structure of PrP^{SC} does still not exist, albeit several structural models for

PrP^{SC} are described[13]. Definite structural information about prions is indispensable for the understanding of the conversion process and eventually for the design of new therapeutic strategies.

In the present review, several techniques to assess the conformation of prions are presented and discussed with a main focus on the application of Fourier-transform infrared spectroscopy. The intention of this mini-review article is to give an overview of current techniques used in prion research to elucidate the structure of prions. For details readers are referred to the literature cited in the text.

FOURIER-TRANSFORM INFRARED SPECTROSCOPY

Fourier-transform infrared (FTIR) spectroscopy was one of the fundamental techniques that had demonstrated the high β-sheet content of PrP^{SC} in contrast to PrP^{C}[14-16]. This technique has several advantages compared to other analytical approaches. With FTIR spectroscopy protein samples can be analyzed (1) without labeling; (2) under native conditions; (3) with only minute amounts of material (in particular in case of micro-FTIR); and (4) in a dynamic process. Moreover results can be obtained within a very short time[16,17]. FTIR spectroscopy elucidates primarily the secondary structure of proteins and originates essentially from C=O stretching vibrations of the amide groups of the protein backbone. For the structural characterization of PrP^{SC} the amide I band is the most useful infrared absorption band[18-20].

FTIR of proteins typically is performed in H_2O, D_2O or under dried conditions. For spectroscopic analyses the purity of sample material is of utmost importance[16,21]. This is particularly challenging when sample material has to be extracted from infected tissue and when the protein of interest is present in only small amounts. Improved purification protocols for the extraction of prions from tissue have been published and allowed us to analyze tiny amounts of sample material by micro-FTIR[16]. Micro-FTIR can be applied on dried protein samples using a FTIR spectrometer linked to an IR-microscope. This enables the performance of measurements on pure protein samples in the range of only a few nanograms.

Micro-FTIR has been shown to be an appropriate tool for the screening of different prion strains and *in vitro* generated prion protein (PrP)[16,17]. Particularly in case of *in vitro* generated PrP only very small amounts of prions are expected to be extractable. This could impede an analysis by other structure-sensitive techniques [*e.g.,* circular dichroism (CD) spectroscopy]. We recently recorded spectra from PrP^{SC} molecules generated by protein misfolding cyclic amplification (PMCA) using micro-FTIR[16]. PMCA mimics the conversion of PrP^{C} to PrP^{SC} *in vitro*[22,23]. Comparing spectra from native- and PMCA-derived PrP^{SC} revealed structural differences of molecules before and after PMCA. This technical progress provides the possibility to analyze the influence of cofactors on the conversion activity and putative correlating structural rearrangements in future experiments.

As mentioned above, micro-FTIR is also suitable to discriminate different prion strains[14,24,25]. In prion research, strains are defined as prion-isolates that, after inoculation into distinct hosts, cause disease with consistent characteristics, such as incubation period, distinct patterns of PrP^{Sc} distribution and spongiosis in the brain (Figure 1). A concrete example of use would be strain typing for chronic wasting disease (CWD)[17]. CWD is a prion disease occurring in cervids in North America and rapidly spreads among free ranging deer and elk[26,27]. To test whether and to which extent there are different strains for CWD in the environment or whether new strains emerge in the future, micro-FTIR (with only 100 mg of infected tissue needed to obtain a sufficient amount of protein extract for FTIR analysis) can be applied. The use of small amounts of sample material allows the preparation of multiple samples within a short time. Spectra from CWD field samples collected in the past, present or future could be compared. As different strains can correlate with different zoonotic potentials a rapid structural scan by FTIR may be suitable for a proper risk assessment.

OTHER TECHNIQUES TO DETERMINE THE STRUCTURE OF PRIONS

Electron microscopy analyses directly reveal the morphological difference between PrP^{C} and PrP^{SC} as the latter appears as amyloid fibrils[28,29]. Moreover, it is possible to distinguish between some prion strains due to their distinct morphological shape. Electron crystallography had a limited scope to predict general conformational features because prions tend to aggregate[30,31]. Atomic force microscopy (AFM) is used as an alternative technique that needs no staining of samples[16,32]. AFM can be combined with infrared- and Raman-spectroscopy suitable for site-directed sample characterization. Tip enhanced infrared- or Raman-spectroscopy principally allow measurements on defined positions within a prion fibril[33,34]. These techniques could be useful for the analyses of mixtures of prion fibrils ("quasi species") and would allow a precise biochemical characterization of prions[17,35].

The impact of specific amino acids on the prion conversion process can be determined by mutational analyses when subsequently tested in animal-, cell culture- or *in vitro* conversion assays[36-39]. By this means it could be demonstrated that specific amino acids accelerate or decelerate the conversion process and that the N-terminus is not essential for prion infectivity[40]. Limited proteolysis with Proteinase K is used to discriminate PrP^{C} from PrP^{SC} as the conversion in a β-sheet rich conformation (PrP^{SC}) prevents the

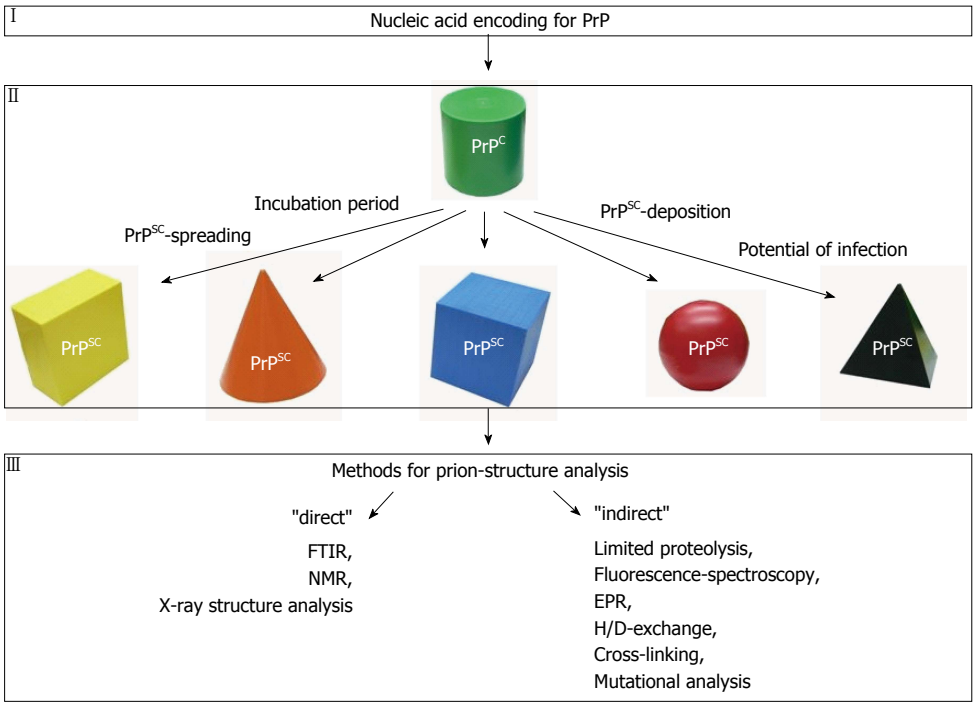

Figure 1 Different conformations of the prion protein. In mammals and humans both isoforms of prion protein (PrP) [the cellular isoform (PrPC) and the misfolded isoform (PrPSC)] are encoded by the same gene (Ⅰ). PrPC is shown in green (Ⅱ). Under specific conditions PrPC can be transferred into a misfolded and then putatively infectious conformation (PrPSC). Prion strains can adopt different conformations (indicated by different symbols and colors). When a misfolded isoform of the prion protein becomes infectious it is characterized by specific features as incubation period, PrPSC-spreading, PrPSC-deposition and a distinct potential of infection. For structural analyses of misfolded prion proteins several methods are available. While FTIR, NMR and X-ray structure analyses provide information about the secondary structure as well as 3D-structural information ("direct"), limited proteolysis, fluorescence spectroscopy, EPR, H/D-exchange, cross-linking or mutational analyses reveal more "indirect" structural information that is focused on specific residues within the protein. FTIR: Fourier-transform infrared.

C-terminus from degradation while the less structured N-terminus is cleaved off[41-46]. Limited proteolysis at different pH values, with rising amounts of protease or in the presence of chaotropic salts can give further structural information and may help to discriminate prion strains with different conformations.

Recombinant PrP can (after site-directed mutagenesis) site-specifically be labelled with fluorophores or spin labels and then analyzed by fluorescence- or EPR spectroscopy, respectively. Such studies propose a parallel in-register β-sheet structure of PrP amyloids and enable the calculation of distances between specific residues within an amyloid fibril[11,47-49]. It has to be mentioned that misfolded recombinant PrP without the addition of cofactors usually lacks infectivity and may therefore not exactly represent the *in vivo* structure of PrP^{SC}[11].

The exchange of hydrogen for deuterium ions and subsequent analysis by mass spectrometry allows the detection of unstructured and highly structured regions within PrP[50-52]. The C-terminal part of prions shows low exchange rates indicating a highly structured conformation[53].

While the above mentioned techniques reveal basic and to some extent indirect structural insights, high resolution structure information is needed to understand the conversion process of PrP. High resolution 3D-structural data from X-ray fiber diffraction and NMR are available for PrP^{C} but not for the complete PrP^{SC} molecule. Because of the insolubility and the propensity to aggregate only parts of PrP^{SC} are structurally resolved until now[54-57].

CONCLUSION

Misfolding of a cellular protein into an infectious isoform sharing the same primary structure is unique for prions. This phenomenon is currently discussed to be a general principle for other neurodegenerative diseases[58-60]. Prions lack a coding nucleic acid thus diagnosis and the detection of misfolded isoforms cannot be done by genetics but *e.g.*, by structural characterization.

A lot of structural models for PrP^{SC} exist but as discussed recently by Requena and Wille "the results and/or their interpretation remain controversial"[61]. For a better understanding of the conversion of PrP^{C} to PrP^{SC}, more structural data from biochemical and biophysical experiments are required for PrP^{SC} (Figure 1). Therapeutic strategies that aim at the prevention of this misfolding process would benefit from such a progress.

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MINIREVIEWS

Essential roles of four-carbon backbone chemicals in the control of metabolism

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Abstract

The increasing incidence of obesity worldwide and its related cardiometabolic complications is an urgent public health problem. While weight gain results from a negative balance between the energy expenditure and calorie intake, recent research has demonstrated that several small organic molecules containing a four-carbon backbone can modulate this balance by favoring energy expenditure, and alleviating endoplasmic reticulum stress and oxidative stress. Such small molecules include the bacterially produced short chain fatty acid butyric acid, its chemically produced derivative 4-phenylbutyric acid, the main ketone body D-β-hydroxybutyrate - synthesized by the liver - and the recently discovered myokine β-aminoisobutyric acid. Conversely, another butyrate-related molecule, α-hydroxybutyrate, has been found to be an early predictor of insulin resistance and glucose intolerance. In this minireview, we summarize recent advances in the understanding of the mechanism of action of these molecules, and discuss their use as therapeutics to improve metabolic homeostasis or their detection as early biomarkers of incipient insulin resistance.

Key words: Butyric acid; D-β-hydroxybutyrate; Histone deacetylases; Histone deacetylases inhibitors; Insulin resistance

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Core tip: Recent research demonstrated that the four-carbon molecule butyrate, and butyrate-related molecules (4-phenylbutyric acid, D-β-hydroxybutyrate and β-aminoisobutyric acid), act as modulators of metabolism, favoring energy expenditure. Conversely, another butyrate-related molecule, α-hydroxybutyrate, is an early predictor of insulin resistance and glucose intolerance. In this minireview, we summarize the recent progress in the understanding of the mechanism of action of these molecules and discuss their possible therapeutic use to improve metabolic homeostasis and their usefulness as early biomarkers for insulin resistance.

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INTRODUCTION

Accordingly to World Health Organisation figures, the increased incidence of overweight and obesity worldwide constitutes a major risk factor for global deaths in both developing and industrialized countries[1]. Obesity predisposes individuals to the development of several non-communicable diseases, including cardiovascular disease, dyslipidemia, insulin resistance and type 2 diabetes (T2D), and certain cancers[2]. Furthermore, obesity engenders psychological consequences in affected individuals, especially in the young[3], and recent research indicates that a predisposition to metabolic disease linked to obesity can be transmitted trans-generationally through epigenetic mechanisms[4].

Obesity is the most straightforward consequence of an energy imbalance. Indeed, body weight results from the difference in energy intake, determined by food consumption, and total energy expenditure required to sustain vital functions, including basal metabolism, thermoregulation, and exercise. If the balance is negative, stored fat is mobilized to provide calories, resulting in weight loss. Reciprocally, excessive food intake results in fat accumulation, and ultimately obesity.

Lifestyle interventions, including changes in diet and increased physical activity, are the simplest way to control body weight. Yet, these interventions are often limited by social and economic constraints, such as restricted access to healthy food and environments conducive to physical activity, especially for people already obese or overweight[5]. Consequently, extensive research has been conducted to discover therapeutic agents that might diminish or reverse the negative consequences of the western diet on body weight and associated health effects.

Interestingly, short chain fatty acids (SCFAs) and molecules related to the 4-carbon SCFA butyric acid (Figure 1) have been shown to exert beneficial effects on the control of body weight and metabolism. Here we summarize the current knowledge about these molecules, their biological source of production and physiological mode of action.

BUTYRIC ACID AND SHORT CHAIN FATTY ACIDS

SCFAs, also known as volatile fatty acids, are carboxylic acids containing up to 6 carbons in their aliphatic chain. Accordingly to the number of carbons, SCFAs include acetic (C2), propionic (C3), butyric (C4), valeric (C5) and caproic (C6) acids. SCFAs are mainly produced by the gut microbiota using dietary fibers as the major substrate. Therefore, the amount of SCFAs produced is affected by environmental, dietary and microbiological factors[6], with a plant-based diet favoring the production of acetate and butyrate[7].

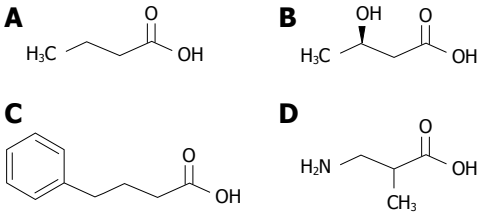

Figure 1 Molecular structures of (A) butyric acid, (B) D-β-hydroxybutyric acid, (C) 4-phenylbutyric acid and (D) β-aminoisobutyric acid.

The gut microbiota is acquired postnatally and its composition is affected by both host genetics and dietary habits. For example, in mice, a high fat and high sugar diet administered to different inbred strains induced a strain-specific propensity to obesity and strain-specific effect on the composition of their gut microbial communities[8].

The dynamic composition of the gut microbiota is a reflection of the host's physiological status[9]. A metabolic diseases like T2D, resulting from both genetic predisposition and environmental factors, is a good example of the interplay between the disease and the microbiota, as gut bacterial dysbiosis was observed in type 2 diabetic patients, with a decline in butyrate-producing bacteria[9,10].

It has been suggested that diets that favor the production of SCFAs by the microbiota may have multiple positive effects on host metabolic regulation. For example, the intake of dietary fibers, which contains indigestible and fermentable carbohydrates, exerts a positive action on the central regulation of satiety and appetite, leading to reduced weight gain and adiposity[11-13]. Some dietary fibers have been found to have a positive effect by decreasing weight gain and energy intake in mice when supplemented to a high fat diet[13], preventing insulin resistance and the development of T2D[14], and improving immune responses.

The interplay between SCFAs and the immune system

It has been shown that SCFA act on T cells to enhance the generation of Th1 and Th17 cell populations, boosting immunity to fight pathogens. At the same time SCFAs induce T cell production of IL-10 and favour the expansion of FOXP3-positive regulatory T cells, helping preventing inflammatory responses[15]. The intestinal epithelium, which separates the exterior environment from the inner organism, is constantly exposed to extrinsic pathogens: viruses, bacteria and their products, that interact with the host's immune system[16]. The gut microbiota interacts with the immune system and, depending on its composition, promotes or limits inflammatory responses in the intestine. In fact, a mixture of Clostridia strains orally administered in a mouse model of colitis, was shown to induce

T-regulatory (Treg) cells and promote the production of anti-inflammatory molecules including interleukin-10 (IL-10) and inducible T-cell co-stimulator (ICOS) in Treg cells[17]. The interplay between microbiota and immune system is mediated by bacterially produced metabolites, especially butyrate and other SCFAs. Indeed, SCFAs increase the number of Treg cells through the potentiation of their extrathymic differentiation[18]. Also, butyrate's histone deacetylase (HDAC) inhibitor properties induce histone H3 acetylation on the Foxp3 promoter in Treg cells resulting in Foxp3 transcriptional upregulation and alleviation of inflammation in the intestine[18,19]. Another SCFA, propionate, exerts the same actions of butyrate on Treg cells, also through HDAC inhibition[18].

Inflammation and the development of obesity: The existence of a correlation between obesity and a sub-clinical inflammatory state is widely accepted[20]. The chronic inflammation observed in adipose tissue in the obese is attributed to the infiltration of immune cells, mainly monocytes - which subsequently differentiate into resident macrophages - but also T cells. Such immune cell infiltration results in a sustained local production of pro-inflammatory cytokines, secreted by the infiltrating immune cells, but also by adipocytes[21]. The establishment of a pro-inflammatory environment, in turn, renders the adipocyte insulin-resistant, predisposing the individual to development of T2D in the long term.

Biological actions of butyric acid: Butyric acid is one of the most well-studied SCFAs due to its presence in dairy products such as cheese and butter[12], and its endogenous production in the human gut, mainly by butyric bacteria[22]. Indeed, plasma butyrate results from its production in the gut, which is favored by foods high in fermentable fiber[23], and impaired with high fat diets[24].

When supplemented to mice diets, particularly in the context of a high fat diet, butyrate was shown to counteract the development of obesity[25], insulin resistance and the emergence of T2D. The mode of action of butyrate was attributed to a rise in energy expenditure. In skeletal muscle, butyrate caused an increase in type Ⅰ muscle fibers along with an augmentation of ATP consumption. This effect seems to be associated with butyrate being an HDAC inhibitor and the fact that it favored type Ⅰ oxidative muscle fibers and mitochondrial biogenesis[14]. Whether the beneficial actions of butyrate are due to a direct action on tissue metabolism will require further investigation, as a clinical study investigating the effects of perfusion of short-chain fatty acids - including butyrate - on glucose metabolism in healthy men failed to show beneficial changes of glucose metabolism[26]. On the other hand, a decreased population of gut butyric bacteria has been demonstrated in type 2 diabetic patients[9,10], indicating the prominent role of butyrate in the maintenance of a healthy metabolism.

Butyrate has been shown to act as an histone deacetylase (HDAC) inhibitor[27]. In fact, its presence leads to increased histone acetylation, giving butyrate a possible action on proliferation, differentiation and regulation of metabolism[27,28].

Recent research has suggested a link between inhibition of class Ⅰ/Ⅱ HDAC by butyrate and other SCFAs[29,30], and improved metabolic responses. In liver, class Ⅱa HDACs dephosphorylate and activate FOXO, favouring the expression of gluconeogenic FOXO target genes. Thus, HDACs inhibition may provide a potential therapeutic approach to alleviate insulin resistance by decreasing gluconeogenesis[31].

Interestingly, butyric acid inhibits class Ⅰ and class Ⅱ HDACs in a competitive fashion[32], and dietary administration of sodium butyrate has been shown to improve systemic insulin sensitivity and increase energy expenditure in mice *via* up regulation of mitochondrial function in skeletal muscle and brown fat, through PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) induction and elevation of AMPK (adenosine monophosphate-activated protein kinase) activity[14]. A further mechanism accounting for the beneficial metabolic effects of butyrate is the induction of hepatocyte- and adipocyte- produced fibroblast growth factor 21 (FGF-21), a hormone promoting fatty acid oxidation in mice. Increased FGF-21 production depends on acetylated and active PPARα (peroxisome proliferator-activated receptor alpha), and inhibition of the PPARα-deacetylating enzyme HDAC3 contributes to maintaining PPARα in its active state[33].

4-PHENYLBUTYRIC ACID

4-phenylbutyric acid (4-PBA) is a chemically produced derivative of butyric acid; 4-PBA is obtained by the reaction of benzene with butyrolactone in the presence of aluminum chloride, followed by neutralization with a base (Burzynsky SR and Musial L, US patent US6372938 B1, 2002). 4-PBA was first used in therapies for urea cycle enzyme deficiencies, due to its ability to act as a chaperone for other enzymes[34,35]. 4-PBA has also been shown to have wider therapeutic indications including alleviation of endoplasmic reticulum stress and associated pathological conditions such as inflammation, hypertension and diabetes[36-40]. The chaperone activity of 4-PBA prevents protein conformational abnormalities and protein aggregation occurring in neurodegenerative diseases[41,42] and in insulin resistance/T2D - as demonstrated by the capability of 4-PBA to restore glucose homeostasis in type 2 diabetic rodent models[43]. Recent preclinical research in zebrafish and mouse models demonstrated that 4-PBA is a PDK (pyruvate dehydrogenase kinase) inhibitor[44]. PDK, inhibits the pyruvate dehydrogenase complex (PDHC) catalytic activity *via* inhibitory phosphorylation and has been used to alleviate lactic

acidosis caused by PDHC deficiency by restoring the conversion of glycolitic pyruvate into acetylCoA. Another use of 4-PBA is as an HDAC inhibitor, mostly in cancer studies[45-47]. Both 4-PBAs HDAC inhibitor activity and its chaperone-like activity might constitute upstream targets to regulate gene expression. Indeed, in metabolic diseases like insulin resistance, 4-PBA positively modulates energy expenditure by favoring the expression of key metabolic genes, including GLUT4[48]. Furthermore, in 3T3-L1 pre-adipocytes, administration of 4-PBA was shown to inhibit adipogenesis through the inhibition of the unfolded protein response, and nutritional supplementation of 4-PBA to mice lowered fat pad weight and resulted in smaller adipocytes in this tissue[49]. Considering its multiple modes of action, and in particular its activity as chemical chaperone, 4-PBA constitutes a very promising molecule to target metabolic states related to endoplasmic reticulum stress such as insulin resistance and fat over-accumulation in the adipose tissue[50]. Taken together, these studies suggest that 4-PBA favors the glycolytic utilization of glucose, while inhibiting adipogenesis and, by extension, fatty acid oxidation.

HYDROXYBUTYRATE

Ketone bodies (KB) are the organism's main source of energy in periods of fasting or prolonged physical exercise, when glucose availability is scarce[51]. KB are mainly derived from free fatty acids, although a small percentage of KB can originate from the metabolism of amino acids[52].

Essentially produced by the liver, the two main KB are acetoacetate (AcAc) and D-β-hydroxybutyrate. KB are metabolically equivalent to fat yet are water-soluble and can be transported in blood to target tissues[53]. Once in the target tissues, KB are converted back to acetyl-coA, through the sequential action of D-β-hydroxybutyrate dehydrogenase, converting D-β-hydroxybutyrate to acetoacetate; 3-ketoacyl-CoA transferase, which produces acetoacetylCoA from acetoacetate, which is finally converted to acetyl-coA by acetoacetyl-CoA thiolase. Importantly, 3-ketoacyl-CoA transferase is only present in organs using KB, and not expressed in the liver. Thus, the flow of ketone bodies is unidirectional, from the liver to peripheral organs. For a long time the liver was thought to be the only organ to produce KB, but it appears the brain can also synthetize them to a certain extent for its own energetic needs[54].

α-hydroxybutyrate (α-HB), a D-β-hydroxybutyrate isomeric but metabolically unrelated molecule, is generated in the liver (1) in the cysteine formation pathway; (2) as a byproduct during the formation of α-ketobutyrate, - the final product of methionine and threonine catabolism[55]; and also (3) under increased oxidative stress, a physiological state associated to insulin resistance. Recently, α-HB has been proposed as a marker of oxidative stress, and increased α-HB circulating levels have been shown to be early biomarkers of insulin resistance in a non-diabetic population[56]. Therefore, α-hydroxybutyrate in the blood might be a predictive sign of incipient metabolic disease[57,58], and β pancreatic cells damage[59], thus representing an ideal biomarker for early detection of predisposition to insulin resistance and T2D.

D-β-hydroxybutyrate, which is present in the serum in the micromolar concentration range under postprandial or short-term fasting conditions, can increase up to millimolar concentrations in case of fasting or intense exercise and becomes the major energy source for the brain and muscles[60,61]. D-β-hydroxybutyrate is a central metabolic intermediate that is associated to decreased free fatty acids blood concentrations and at the same time prevents from the exhaustion of fat stocks during prolonged starvation[58,61] and might also regulate the aging process, as administration of D-β-hydroxybutyrate to *Caenorhabditis elegans* has been shown to increase the worm's lifespan[62].

Recent research has demonstrated that the biological action of the ketone body D-β-hydroxybutyrate extends beyond its role as metabolite. Indeed, D-β-hydroxybutyrate has been shown to act both as a signaling molecule by activating G-protein coupled receptors (GPCRs), and as a transcriptional regulator by acting as a HDAC inhibitor.

Related to its properties as signaling intermediate, D-β-hydroxybutyrate has been shown to be an agonist for two GPCRs, PUMA-G (also named HCAR2, hydroxycarboxylic acid receptor 2, or Gpr109)[63] and the free fatty acids receptor 3 (FFA3, or Grp41)[64].

A novel and exciting research field has finally recently emerged with the discovery that D-β-hydroxybutyrate can be a key regulator of gene expression by acting as an endogenous HDACs inhibitor[60]. The HDACs inhibitory activity of D-β-hydroxybutyrate brings changes in histone acetylation and gene expression that seem to protect cells from oxidative stress[65].

β-AMINOISOBUTYRIC ACID

β-aminoisobutyric acid (BAIBA) was first identified as a catabolite derived from the breakdown of pyrimidines[66] and branched chain amino acids[67]. More recent research has demonstrated that BAIBA can act to prevent weight gain in several ways. Through an action on lipid metabolism, BAIBA reduces plasma and liver triglycerides by increasing both liver fatty acid oxidation and ketogenesis, especially by inducing β-D-hydroxybutyrate synthesis[68]. A further mode of action of BAIBA may involve the action of leptin. Leptin is an adipokine expressed by adipose tissue which contributes to the central hypothalamic regulation of food intake and energy expenditure, therefore favoring weight loss[69]. Interestingly, *ob/ob* leptin deficient mice were not responsive to BAIBA,

Table 1 Summarizing table describing the biological and metabolic effects promoted by butyric acid and its analogues molecules

Molecule	Putative molecular target or mechanism involved	Overall biological response	Ref.
Butyric acid	Increased PGC1α expression and AMPK activity	Increased type Ⅰ muscle fibers, increased insulin sensitivity, reduced adiposity	[14]
	Inhibition of HDACs	Decreased glycaemia in streptozotocin-induced diabetes. Decreased pancreatic β-cells apoptosis	[28]
	HDAC3 inhibition	Induction of FGF21, fatty acid oxidation and ketone bodies production	[33]
D-β-hydroxybutyrate	Binds and activates GPCR PUMA-G/Gpr109	Inhibition of adipocyte lipolysis	[63]
	Binds and activates GPCR FFA3/Grp41	Regulates energy consumption through FFA3/Grp41 activation in the sympathetic nervous system	[64]
	HDACs inhibition	Protection from oxidative stress	[65]
4-phenylbutyric acid	HDAC5 inhibition	Increased GLUT4 gene expression and promotion of glucose metabolism	[48]
	Inhibition of oxidative stress and endoplasmic reticulum stress	Alleviation of diabetic nephropathy in streptozotocin-induced diabetes	[40]
β-aminoisobutyric acid	Activates PPARα	Induction of hepatic β-oxidation and brown adipose tissue	[71]
	Restores normal plasma leptin levels	Increase of fatty acid oxidation and alleviation of diet-induced obesity in *ob*/+ heterozygous mice	[69]
	Induces liver ketogenesis, increasing circulating D-β-hydroxybutyrate	Decreased body fat mass	[68]

HDACs: Histone deacetylases; GPCR: G-protein coupled receptor; FGF-21: Fibroblast growth factor 21; FFA3/Grp41: Free fatty acids receptor 3; PPARα: Peroxisome proliferator-activated receptor alpha.

showing neither weight loss nor increased fatty oxidation upon BAIBA administration[68]. BAIBA, like leptin, appears to favor lipid metabolism, and at the same time, can act indirectly by promoting leptin expression in white adipose tissue[70].

Moreover, recent research has shown that BAIBA is produced in muscles and BAIBA plasmatic concentrations in rodents and humans are most likely representative of the amounts of BAIBA produced by skeletal muscle[71]. BAIBA derived from skeletal muscle can be considered as a myokine (*e.g.*, a muscle-secreted molecule with hormonal-like properties on target organs) that mediates the metabolic crosstalk between skeletal muscle and other metabolically active tissues. BAIBA production is regulated by PGC-1α, which is a general co-activator overexpressed in response to exercise[71]. As a myokine, BAIBA is capable of inducing a transition in the adipose tissue from a white adipose tissue phenotype to a tissue endowed with brown adipose tissue characteristics, thereby favoring the expression of β-oxidation and thermogenic genes, including PPARα and its target mitochondrial uncoupling protein 1 (UCP1), rather than lipogenic and lipid-storage genes[71,72]. Although the direct mechanism of action of BAIBA upstream of PPARα has not yet been elucidated, it has been proposed that BAIBA could be a possible therapeutic molecule as an anti-obesity agent, by favoring a white fat to brown fat phenotypic transition[73].

CONCLUSION

Obesity and its metabolic consequences leading to increased morbidity and mortality can be currently considered as one of the major health burdens worldwide. Extensive preclinical and clinical research is thus necessary to identify novel agents that might be therapeutically useful - along with nutritional and physical activity approaches - to counteract this pandemic.

The four-carbon backbone chemicals described in this review, through their multiple mechanisms of actions summarized in Table 1, may provide a new class of potential pharmacologically effective candidates, to be used alone or in combination to effectively reverse or alleviate obesity and the insulin resistant state.

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Current medical treatment of estrogen receptor-positive breast cancer

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Abstract

Approximately 80% of breast cancers (BC) are estrogen receptor (ER)-positive and thus endocrine therapy (ET) should be considered complementary to surgery in the majority of patients. The advantages of oophorectomy, adrenalectomy and hypophysectomy in women with advanced BC have been demonstrated many years ago, and currently ET consist of (1) ovarian function suppression (OFS), usually obtained using gonadotropin-releasing hormone agonists (GnRHa); (2) selective estrogen receptor modulators or down-regulators (SERMs or SERDs); and (3) aromatase inhibitors (AIs), or a combination of two or more drugs. For patients aged less than 50 years and ER+ BC, there is no conclusive evidence that the combination of OFS and SERMs (*i.e.*, tamoxifen) or chemotherapy is superior to OFS alone. Tamoxifen users exhibit a reduced risk of BC, both invasive and *in situ*, especially during the first 5 years of therapy, and extending the treatment to 10 years further reduced the risk of recurrences. SERDs (*i.e.*, fulvestrant) are especially useful in the neoadjuvant treatment of advanced BC, alone or in combination with either cytotoxic agents or AIs. There are two types of AIs: type Ⅰ are permanent steroidal inhibitors of aromatase, while type Ⅱ are reversible nonsteroidal inhibitors. Several studies demonstrated the superiority of the third-generation AIs (*i.e.*, anastrozole and letrozole) compared with tamoxifen, and adjuvant therapy with AIs reduces the recurrence risk especially in patients with advanced BC. Unfortunately, some cancers are or became ET-resistant, and thus other drugs have been suggested in combination with SERMs or AIs, including cyclin-dependent kinase 4/6 inhibitors (palbociclib) and mammalian target of rapamycin (mTOR) inhibitors, such as everolimus. Further studies are required to confirm their real usefulness.

Key words: Breast cancer; Endocrine therapy; GnRH-agonists; Ovarian function suppression; Tamoxifen; Selective estrogen receptor modulator; Aromatase inhibitors

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Core tip: In women with breast cancer (BC), the two major endocrine therapies (ET) available are directed at blocking estrogen receptors (ERs) on cancer cells (selective estrogen receptor modulators), or against the key enzyme in the biosynthesis of estrogens (aromatase inhibitors). The new knowledge about molecular mechanisms regulating tumor progression, suggested the existence of a strict relationship between ERs, intracellular signaling pathways, and other growth factors receptor, which justify the escape of a portion of patients from the inhibitory effects of ET. To restore endocrine sensitivity of BC cells, molecular-target therapies are giving encouraging results, but further studies are required to confirm their real usefulness.

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INTRODUCTION

Breast cancer (BC) is the most common female cancer worldwide, and the second cause of cancer death in women[1]. In the United States, the expected number of new BC cases in 2015 is approximately 231800, accounting for 29% of all cancers, with an annual change based on incidence of +0.3%[2]. In the European Union, BC seems to have a more favorable trend, with a 10.2% fall from 2009[3].

Estrogen receptors (ERs) are nuclear proteins regulating the expression of specific genes, and approximately 80% of BCs are estrogen receptor (ER)-positive, of which 65% are also progesterone receptor (PR)-positive[4]. Thus, endocrine therapy (ET) should be considered complementary to surgery in the majority of patients, inducing tumor remission and providing consistent clinical benefit. ET has represented the standard adjuvant treatment for ER+ tumors since the 1970s, although the advantages of oophorectomy, adrenalectomy, and hypophysectomy in women with advanced BC had already been demonstrated many years before[5]. ET for BC currently consist of (1) ovarian function suppression (OFS); (2) selective estrogen receptor modulators (SERMs); (3) selective estrogen receptor down-regulators (SERDs); and (4) aromatase inhibitors (AIs), or a combination of two or more drugs. The two major strategies are directed at blocking ERs of cancer cells (SERMs) or against the key enzyme in the biosynthesis of estrogens (AIs).

OVARIAN FUNCTION SUPPRESSION

For decades, in premenopausal women with operable BC in whom ovaries are the main source of estrogen and progesterone, surgical oophorectomy has represented an effective adjuvant therapy. Ovary irradiation was rarely performed for this purpose. Recently, it has been shown that the timing of oophorectomy (*i.e*., luteal phase of the menstrual cycle) does not affect disease-free survival (DFS) and overall survival (OS) of patients with BC[6]. OFS can be also obtained using gonadotropin-releasing hormone agonists (GnRHa), which are generally considered drugs with reversible actions. Although in patients with BC undergoing simultaneously GnRHa administration and chemotherapy (CT) ovarian function is rarely preserved, this treatment has largely replaced surgery[7,8].

A historic large overview of randomized trials testing the usefulness of ovarian ablation in patients with ER+ early BC, named EBCTCG (Early Breast Cancer Trialists' Collaborative Group) study, demonstrated that OFS significantly increases survival in women aged below 50 years and that these benefits are independent of axillary nodal status, compared to untreated patients[9]. The advantages of adding a GnRHa to SERMS or CT in terms of reduced recurrence and deaths rate was confirmed in other studies[10,11]. More recently, it has been shown that women who underwent adjuvant GnRHa monotherapy or standard CT regimen had similar DFS and OS, while a combination of GnRHa plus CT or SERMs significantly improved DFS and OS in comparison to CT alone[12]. Further studies suggested that, in women with ER+ early BC, adjuvant treatment with OFS plus SERMs (tamoxifen) does not lead to any advantage, while the combination of OFS and AIs (exemestane) significantly reduces recurrences compared to the other regimen[13,14].

In premenopausal women previously treated with SERMs (tamoxifen), extended therapy with GnRHa and an AI may lead to substantial adverse effects[15]. In conclusion, for patients aged less than 50 years, there is no conclusive evidence that the combination of OFS and SERMs or CT is superior to OFS alone[16].

SELECTIVE ER MODULATORS AND DOWN-REGULATORS

Tamoxifen (TAM) is the drug most extensively used when an ET is required, both in pre- and postmenopausal women, and is still considered the SERM of choice[7]. TAM (Figure 1A) was initially studied as anti-fertility drug, demonstrating soon favorable effects in patients with ER+ BC, as well as on bone tissue and cardiovascular system[17]. TAM acts as a partial nonsteroidal agonist in some tissues, such as liver, uterus and bone, but is a competitive receptor inhibitor in the breast and brain[18]. In these tissues, TAM selectively blocks signaling at the level of ERs, also inhibiting the proliferation of ductal cells[19]. TAM is hydroxylated by the cytochrome P450 enzyme system into 4-hydroxy tamoxifen (4HT), and further metabolized in the liver, thanks to a CYP2D6-mediated

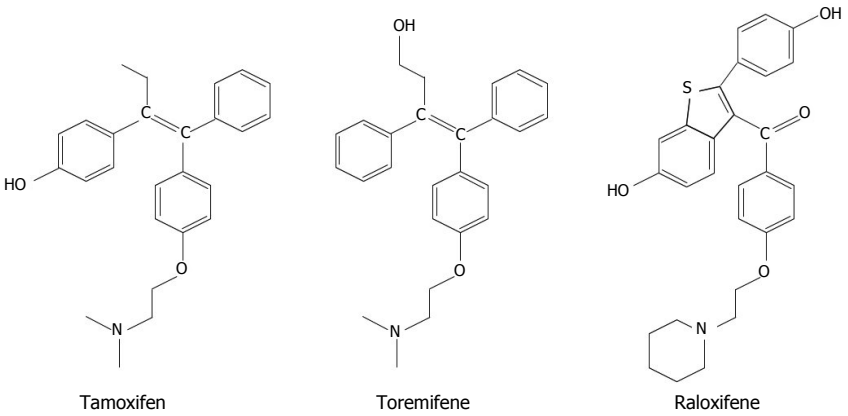

Figure 1 Chemical structures of the main selective estrogen receptor modulators.

oxidation, to endoxifen (4-hydroxy-N-desmethyl-tamoxifen)[20]. Endoxifen and 4HT are the main active metabolites of TAM, exhibit similar ability in binding to ERs, and are more potent than TAM in suppressing estrogen-dependent cancer cells[7]. The relationship between CYP2D6, endoxifen or 4HT serum levels and the response to TAM therapy has long been studied, but the hypothesized role of CYP2D6 genotype as prognostic factor affecting recurrence in patients with BC was not confirmed[21,22]. However, CYP2D6 inhibitors, such as antipsychotic drugs, selective serotonin reuptake inhibitors (SSRIs), and some cardiac medications have the potential to affect response to SERM therapy[23,24].

TAM significantly benefits only women with ER+ BC, and ASCO (American Society of Clinical Oncology) guideline recommendations consider a cut-off of 1% for immunohistochemical testing of ERs in BC cells[25]. A number of studies and meta-analyses confirm its usefulness. According to the 2011 EBCTCG study, the reduction in mortality, expressed as reduction in annual odds of death, is approximately 20% after 5-years of TAM adjuvant therapy, and the 15-year recurrence rate ranges from 4.6% to 17.4%, according to age[16,26]. The long-term mortality among TAM users was 23.9% compared with 33.1% of the control group, with limited age-related side effects, such as increased risk of thromboembolic disease (> 55 years) and endometrial cancer (> 45 years)[26,27]. Extending the treatment to 10 years, the risk of recurrence and overall mortality are further slightly reduced, and thus the 2014 ASCO guidelines recommend the use of TAM for up to 10 years in pre- or perimenopausal women[28,29].

With the aim of limiting the incidence of adverse effects of TAM, eventually improving its effectiveness, other SERMs have been proposed, such as toremifene and raloxifene, both approved by the US Food and Drugs Administration (FDA). *Toremifene* (Figure 1B) is similar to TAM in clinical activity, efficacy and tolerability, and is cross-resistant to TAM. It causes less vascular and endometrial adverse effects than TAM, but has less favorable actions on bone and lipids levels[30]. Also in patients with metastatic BC, the results of TAM and toremifene therapy did not differ significantly, and the latter represents a reliable alternative to TAM in menopausal women[31]. *Raloxifene* (Figure 1C) is another nonsteroidal SERM causing less toxicity compared to TAM, especially used for the prevention of osteoporosis in postmenopausal women[32]. However, as preventive therapy, raloxifene has been shown to be less effective than TAM, because it reduces the risk of cancer by 36% compared to 50% of the group treated with TAM[33].

In conclusion, SERMs users exhibit a reduced risk of BC, both invasive and *in situ*, especially during the first 5 years of therapy, ranging from 53% to 79% according to age and menopausal status[34].

Selective estrogen receptor down-regulators have different pharmacologic characteristics, biochemical structure, and molecular activity with respect of SERM, causing down-regulation and degradation of ERs, and inhibiting proliferation of estrogen-dependent BC cells[35]. SERDs are pure ER antagonist, blocks ERs activity and accelerate their degradation, thus exhibiting exclusively anti-estrogen effects[36]. Fulvestrant, the only SERD approved by FDA to treat patients with BC, has 100-fold the affinity of TAM for ER, in lack of adverse effect on endometrial ERs[37,38]. It is useful especially in patients with advancer BC and as second-line therapy in TAM-resistant tumors[36]. Fulvestrant sensitizes ER-negative BC to chemotherapy, showing a synergistic action with various cytotoxic agents, such as docetaxel[39,40]. In the treatment of patients with metastatic ER+ BC, the combination of fulvestrant plus an AI (anastrozole) was superior to anastrozole alone, and fulvestrant as single-drug therapy represents a reasonable alternative to AIs[41,42]. In the neoadjuvant setting, the use of fulvestrant is controversial, because of the slow response rate and possible non-response. In a phase Ⅱ randomized study, tumor response rates were 20.6% and 22.9%, according to the dosage of the drug[43].

AROMATASE INHIBITORS

The human CYP19 single-copy gene, located on chromosome 15q21.2, encodes the microsomal enzyme

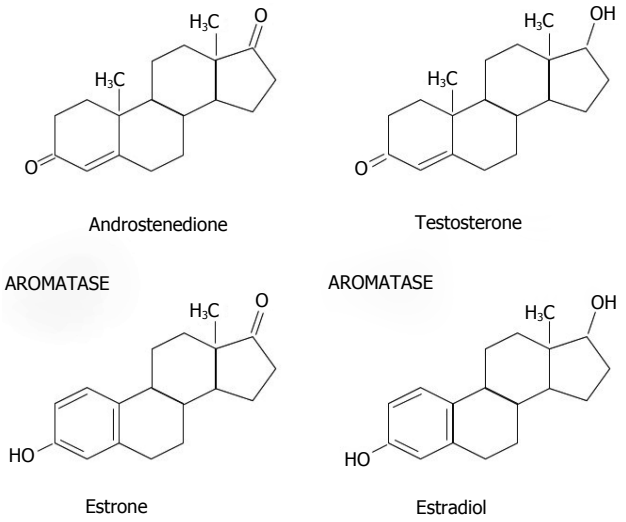

Figure 2 Actions of the aromatase on steroid hormones.

aromatase [cytochrome P450 19 (CYP19)], which catalyzes the conversion of androgens, such as testosterone and androstenedione, to estrogens, such as 17-β-estradiol and estrone[44] (Figure 2).

Aromatase activity is expressed especially in the ovary (premenopausal women), testis, placenta, brain, bone and adipose tissue (postmenopausal women)[45]. AIs block aromatase enzyme activity, safely reducing circulating estrogen levels only in postmenopausal patients. AIs are ineffective in premenopausal women with functionally active ovary in whom they increase gonadotropin secretion, and therefore estrogen production[46]. The combination of AIs and OFS, using a GnRHa to block the pituitary, is usually an effective strategy. According to the conclusions of the STAGE study, 70.4% of postmenopausal patients with early BC treated with an AI (anastrozole) plus GnRHa (goserelin) obtained complete or partial response to therapy, compared with 50.5% of the control group treated with anastrozole plus TAM[47]. Another study, did not found any difference in DFS between premenopausal women with ER+ early BC treated with TAM alone and those treated with anastrozole alone, who presented a slightly increased risk of death[48]. A correlation between elevated body mass index and reduced effect of AIs has been reported in some studies[49].

According to the chemical structure and mechanism of action, there are two types of AIs: type Ⅰ are permanent steroidal inhibitors of aromatase, while type Ⅱ are reversible nonsteroidal inhibitors (Table 1).

Aminoglutethimide, initially used as anti-epileptic drug, is the oldest nonsteroidal AI (Figure 3A). It inhibits also cytochrome P450scc, blocking the conversion of cholesterol to pregnenolone, and thus decreases the production of several steroids, including cortisol[17]. The subsequent activation of the hypothalamic-pituitary-adrenal axis, causes hypersecretion of adrenocorticotropic hormone (ACTH)

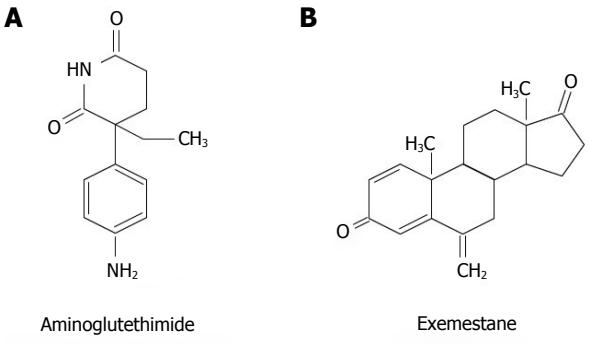

Figure 3 Chemical structures of aminoglutethimide and exemestane.

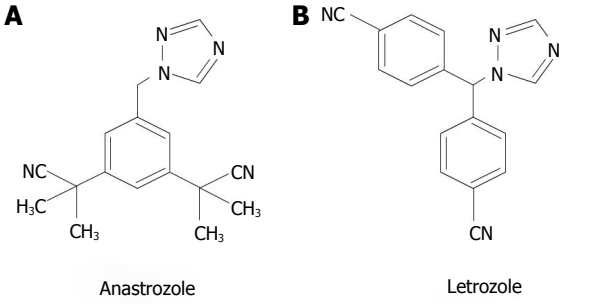

Figure 4 Chemical structures of the two main third-generation aromatase inhibitors.

leading to the need of increasing the dosage of the drug, and requires corticosteroids supplementation. Due to these side effects, aminoglutethimide has been replaced by better-tolerated and more effective AIs[7].

Exemestane (Figure 3B) is a potent irreversible steroidal AI that acts as a false substrate for aromatase and suppresses selectively estrogens at any site[50]. In some studies, exemestane plus OFS reduced the risk of recurrences in premenopausal women with ER+ early BC, while TAM plus OFS did not provided benefit[51]. In a group of postmenopausal patients at slightly increased risk of cancer according to the Gail model who underwent exemestane therapy, the annual incidence of BC was 0.33% compared to 0.77% of control group[52].

Anastrozole and letrozole (Figure 4) are two non-stedroidal third-generation AIs with similar potency and characteristics. Because their block of ERs is reversible, a continuous drug administration is required. An old comparison between anastrozole and letrozole as second-line therapy in advanced BC, reported an overall response rate of 12% *vs* 19%, respectively[53]. In postmenopausal women with advanced BC, letrozole was more effective than fadrazole, a second-generation type Ⅱ nonsteroidal AI with reversible action[54]. The more recent ACOSOG (American College of Surgeons Oncology Group) study compared exemestane, anastrozole, and letrozole as neoadjuvant therapy of women with advanced BC, and reported that the clinical response rate was 62.9%, 69.1%, and 74.8%, respectively[55]. Several meta-analysis underline the

Table 1 Main characteristics of type Ⅰ and Ⅱ aromatase inhibitors

Type	Chemical structure	Action	First generation	Second generation	Third generation
Ⅰ	Steroidal	Permanent	Testolactone	Formestane	Examestane
Ⅱ	Nonsteroidal	Reversible	Aminoglutethimide	Fadrozole	Anastrozole
					Letrozole

superiority of the third-generation AI (but not first- and second-generation AIs) compared with TAM, confirming that adjuvant therapy with AIs reduces the recurrence risk in patients with advanced BC, but does not affect mortality rate[56,57].

ENDOCRINE THERAPY AND MOLECULAR-TARGET THERAPY

The new knowledge about molecular mechanisms regulating tumor progression, suggested the existence of a strict relationship between ERs, intracellular signaling pathways, and other growth factors receptor, which justify the escape of a portion of patients from the inhibitory effects of ET[58]. To restore endocrine sensitivity of ER+ cancer cells, several other drugs have been studied, including cyclin-dependent kinase (CDK) 4/6 inhibitors (palbociclib), epigenetic modulators that inhibit histone deacetylase (HDAC), and other signal pathway inhibitors[59]. Experimentally, combined HDAC inhibition and TAM reverse ET resistance, inducing apoptosis of TAM-resistant BC cells, and in women with advanced ER+ BC and disease progression or recurrence the progression-free survival (PFS) can be reduced adding palbociclib to letrozole[60,61]. The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway and the serine/threonine kinase mammalian target of rapamycin (mTOR) are part of a signaling network regulating several cell processes, including growth, proliferation and survival of cancer cells[62]. The addition of an mTOR inhibitor (everolimus) to examestane in patients with ER+ BC and advanced disease refractory to both SERMs and AIs, can improve the progression-free survival (PFS) compared to everolimus alone[52]. A recent meta-analysis showed that exemestane and everolimus in combination was more efficacious than fulvestrant alone in patients with relapsed BC previously treated with nonsteroidal AIs[63]. Unfortunately, in women with ER+ BC, the addition of an anti-vascular endothelial growth factor (bevacizumab) to fulvestrant or letrozole did not change the resistance of the tumor to ET, and failed to improve PFS or OS[64].

METASTATIC BREAST CANCER

As in part already reported above, anti-estrogen therapy plays a major role in the prolonged remission in women with metastatic and advanced ER+ BC. Several studies showed that the inhibition of the aromatase enzyme system with third-generation AIs, such as exemestane, anastrozole and letrozole, is associated with better survival compared to SERMs[56]. In terms of both PFS and OS, there are also advantages of treating patients with AIs compared to other ET, and letrozole seems to be the drug of choice[65,66]. The adverse effects observed using letrozole or anastrozole are similar than that observed with TAM, and the time-to-progression was better[67]. The combination anastrozole and fulvestrant in metastatic ER+ BC was superior to anastrozole alone[41].

All endocrine therapies, in particular AIs, synergize their effects with several molecular target drugs, such as everolimus and palbociclib[58,60]. It has also been shown that exemestane plus everolimus were more efficacious than everolimus alone and fulvestrant alone in women with advanced BC and progressive disease[52,63]. The GINECO (Groupe d'Investigateurs Nationaux pour les Etudes des Cancers de l'Ovaire) study showed that the short-term clinical benefit rates obtained with TAM plus everolimus *vs* TAM alone were 61% and 42%, respectively, and that the time to progression increased from 4.5 to 8.6 mo[68]. However, according to the conclusion of the BOLERO-2 (Breast Cancer Trials of OraL EveROlimus-2) study, the decision of prescribing AIs and mTOR inhibitors in combination should be individualized, taking into account the characteristics of single patient and considering risks and benefits of the treatment[69]. Both CDK and HDAC inhibitors, such as palbociclib and etinostat, in combination with ET, can be useful in patients with advanced ER+ BC as third-line therapy[70].

ADVERSE EFFECTS

Unfortunately, both TAM and AIs use is associated with several adverse events (AEs) and distressing symptoms, such as hot flushes, weight gain, sexual dysfunctions, decreased bone mineral density (BMD), osteoporosis and pathologic fractures, or musculoskeletal symptoms, depending on the drug used and the duration of treatment. These effects may be severe enough to cause non-compliance due to cessation of use in a significant proportion of patients. Most of the AEs is related to the estrogen deprivation[7]. According to a systematic review, TAM was associated with more serious AEs in comparison with AIs, while exemestane caused more frequently musculoskeletal symptoms and hot flushes, but in some trials no

differences in AEs between SERMs and AIs was found[67]. Another meta-analysis showed that patients treated with AIs had increased risk of gastrointestinal symptoms, such as nausea, vomiting and diarrhea, while the incidence of thromboembolic events and vaginal bleeding were decreased compared to other ETs[66]. In several studies, AIs use was associated with a higher risk of arthralgia and decreased BMD compared to TAM, and the incidence of discontinuation ranges from approximately 12% to 14%[16]. Effects on cognitive function related to the AIs use have also been reported, similar to the so-called "chemotherapy fog" observed following prolonged cancer CT[71]. However, toxicity is related to individual treatment compliance and depends on the way in which the patient describes and reports the symptoms, and they usually resolve with discontinuation of treatment[16]. Careful patient information is recommended before starting any treatment with ETs, especially in premenopausal women.

CONCLUSION

In patients with BC and ER+ tumors, ET has several advantages, including prolonging DFS and OS, and reducing the risk of recurrence, both in patients with early BC and in those with advanced or relapsed disease. In premenopausal women, OFS and TAM has similar efficacy and there is no evidence that a regimen based on OFS, SERMs, or AIs in combination can be the strategy of choice[7,16]. In postmenopausal women, AIs are more effective than SERMs in reducing relapse of the disease, and extending the TAM administration for more than 5 year represents a useful strategy. In patients with advanced BC, molecular-target therapies are giving encouraging results, but further studies are required to confirm their real usefulness and a careful analysis of cost-benefits is also required.

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ORIGINAL ARTICLE

Basic Study

Effect of paricalcitol and enalapril on renal inflammation/oxidative stress in atherosclerosis

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Author contributions: Husain K performed the majority of the experiments, analyzed the data and wrote the paper; Suarez E performed the animal treatments, data recording and analysis; Isidro A performed physiological experiments; Hernandez W performed biochemical data and statistical analysis; Ferder L designed and coordinated the research.

Supported by A research grant from Abbott Pharmaceutical, United States.

Institutional review board statement: The animal protocol was designed to minimize pain and discomfort to the animals. The animals were acclimatized to laboratory conditions (25 ℃, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for one week.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Ponce School of Medicine (Protocol #LF-07).

Conflict-of-interest statement: There is no conflict of interest associated with any of the senior author or other coauthors contributed their efforts in this manuscript.

Data sharing statement: This is a non-clinical study hence the technical appendix, statistical code, and data sets are not available.

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Abstract

AIM: To investigate the protective effect of paricalcitol and enalapril on renal inflammation and oxidative stress in ApoE-knock out mice.

METHODS: Animals treated for 4 mo as group (1) ApoE-knock out plus vehicle, group (2) ApoE-knock out plus paricalcitol (200 ng thrice a week), (3) ApoE-knock out plus enalapril (30 mg/L), (4) ApoE-knock out plus paricalcitol plus enalapril and (5) normal. Blood pressure (BP) was recorded using tail cuff method. The kidneys were isolated for biochemical assays using spectrophotometer and Western blot analyses.

RESULTS: ApoE-deficient mice developed high BP (127 ± 3 mmHg) and it was ameliorated by enalapril and enalapril plus paricalcitol treatments but not with paricalcitol alone. Renal malondialdehyde concentrations, $p22^{phox}$, manganese-superoxide dismutase, inducible nitric oxide synthase (NOS), monocyte chemoattractant

protein-1, tumor necrosis factor-alpha and transforming growth factor-β1 levels significantly elevated but reduced glutathione, CuZn-SOD and eNOS levels significantly depleted in ApoE-knock out animals compared to normal. Administration of paricalcitol, enalapril and combined together ameliorated the renal inflammation and oxidative stress in ApoE-knock out animals.

CONCLUSION: Paricalcitol and enalapril combo treatment ameliorates renal inflammation as well as oxidative stress in atherosclerotic animals.

Key words: Atherosclerosis; Enalapril; Paricalcitol; Renal inflammation; Oxidative stress

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Core tip: Although the protective efficacy of vitamin D and angiotensin converting enzyme inhibitors (ACEIs) have been studied in the cardiovascular system of atherosclerotic mice. However this is the first report to investigate the renal protection by paricalcitol and enalapril, alone or in combination in ApoE-deficient atherosclerotic mice. This innovative study clearly shows that vitamin D, ACEI and their combo ameliorated the renal inflammation and oxidative stress in ApoE-knock out animals by depleting the inflammatory and oxidative stress markers as well as restoring the renal antioxidant defense system in atherosclerotic mice. The combination of paricalcitol and enalapril warrants the clinical usefulness in renal atherosclerotic patients.

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INTRODUCTION

Atherosclerosis is implicated of morbidity and mortality in patients with cardiorenal diseases worldwide. Epidemiological as well as clinical investigations demonstrated a strong relationship between vitamin D deficiency and cardiorenal risk factors such as hypertension, chronic kidney disease (CKD), diabetes mellitus, atherosclerosis, myocardial infarction, stroke, and congestive heart failure[1,2]. It could be suggested that vitamin D deficiency contributes to the development of renal and cardiovascular diseases *via* its strong association with risk factors such as diabetes, hyperlipidemia and hypertension[3-5]. However, direct effects of vitamin D on the renal and cardiovascular system may also be involved. Activation of vitamin D receptors not only regulates the parathyroid hormone but also alters the renin-angiotensin system (RAS), cardiac hypertrophy, inflammation, and calcium levels[6-12]. The molecular mechanism for how vitamin D may improve cardiorenal disease outcomes, specifically cardiorenal atherosclerotic lesions, is not well understood.

A number of studies documented an essential role of RAS in both cardiovascular pathophysiology, including atherosclerosis, and renal pathophysiology[13-15]. RAS inhibitors and blockers have therapeutic potentials in chronic cardiorenal dysfunctions[13,16-18]. Angiotensin II causes oxidants/antioxidants imbalance in the kidney, heart and vascular system inducing reactive oxygen species (ROS) production *via* the activation of NADPH oxidase[19,20]. These ROS initiate vascular membrane lipid peroxidation leading to inflammation and the generation of inflammatory cytokines (TNF-α) through NF-κB activation[21,22] and other mediators such as vascular cell adhesion molecule-1 (VCAM-1), MCP-1, TGF- β1, Matrix metalloproteinase 9 (MMP9), iNOS and Mn-SOD[23-25]. ROS oxidize cellular biomolecules (lipids, proteins and nucleic acids) leading to renal, heart and vascular impairments[26]. But the cellular ROS are scavenged by endogenous antioxidants including antioxidant enzymes such as SOD, catalase, and glutathione peroxidase (GSHPx), reduced glutathione (GSH) and vitamins A, C, and E[27]. Most importantly, depletion of a major cellular antioxidant such as GSH has been reported to cause renal and cardiovascular dysfunction in rats [28,29]. Antioxidant therapy has been shown to ameliorate renal and cardiovascular oxidative stress by scavenging excess ROS and upregulating the antioxidant defense system[27,30]. However, since the protective mechanisms of angiotensin converting enzyme inhibitors (ACEIs) in the atherosclerotic lesions of mouse kidney are not completely understood, we set out to investigate this aspect in the present study.

Paricalcitol, a vitamin D analog, has been associated not only with the regulation of early stages of atherogenesis but also with vascular calcification. Treatments with vitamin D analogs at therapeutic dosages ameliorate the secondary elevation of parathyroid hormones and vascular calcium levels[31-33]. In clinical studies, paricalcitol reduced proteinuria, inflammation, and the mortality rate in CKD patients[34-36]. Our recent study demonstrated that when paricalcitol combined with enalapril ameliorated the oxidative cardiovascular injury by suppressing ROS-generating enzyme NADPH oxidase activity and by upregulating the antioxidant defense system in a uremic rat model and mouse model of atherosclerosis[29,37-39]. It is not known, however, whether vitamin D analogs can protect against inflammatory and oxidative stress in the kidney of atherosclerotic mice. RAS is implicated in atherosclerosis and vitamin D suppresses it, the combination of vitamin D and RAS inhibitor most likely elevated the therapeutic potentials in the kidney of ApoE-deficient mice. Therefore, the present study aimed to investigate the protective efficacy of a vitamin D analog, *i.e.*, paricalcitol, and an ACE inhibitor, *i.e.*, enalapril on inflammation and oxidants/antioxidants

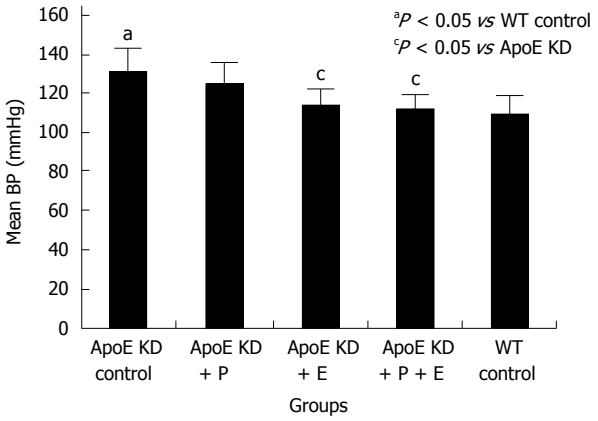

Figure 1 Effect of paricalcitol and enalapril alone and in combination for 16-wk on mean blood pressure (mmHg) in ApoE-deficient atherosclerotic mice. There was a significant ($P < 0.05$) increase in mean BP in atherosclerotic mice compared to wild type control ($n = 12$). Enalapril ($n = 12$) alone and in combination with paricalcitol ($n = 12$) significantly ($P < 0.05$) reduced mean blood pressure (BP) in atherosclerotic mice ($n = 12$). Paricalcitol ($n = 12$) slightly but not significantly decreased mean BP in atherosclerotic mice.

imbalance in the kidney of ApoE-deficient atherosclerotic mice.

MATERIALS AND METHODS

Animals

Sixty female mice (20-25 g) were procured from (Taconic Company, Hudson, NY, United States). Mice were given food and water ad libitum. They were maintained at a room temperature (25 ℃ with 50% humidity) and on 12/12-h light/dark cycle. After one week acclimatization they were separated into five groups and treated as follows:

Group 1 (ApoE deficient + vehicle): Mice were administered a vehicle (100 μL of propylene glycol, *i.p.*) 3 times a week for 16 wk ($n = 12$).

Group 2 (ApoE deficient + paricalcitol): Mice were given paricalcitol (200 ng in propylene glycol, *i.p.*) 3 times a week for 16 wk ($n = 12$).

Group 3 (ApoE deficient + enalapril): Mice were given enalapril (30 mg/kg) in their drinking water for 16 wk ($n = 12$).

Group 4 (ApoE deficient + paricalcitol + enalapril): Mice were given paricalcitol + enalapril for 16 wk ($n = 12$).

Group 5 (wild-type normal control): Wild-type control mice were given vehicle only 3 times a week for 16 wk ($n = 12$).

The use of ApoE-deficient mice as a model of atherosclerotic lesions and drug treatment were reported earlier[40,41]. BP was monitored after drug treatment using a Non-Invasive Blood Pressure System NIBP800 (Columbus Instruments, Columbus, Ohio, United States) as described earlier[37-39]. After 16 wk, mice were euthanized by decapitation and kidneys were isolated. The kidneys ($n = 6$) from each group of mice were rinsed with PBS, immediately placed in liquid nitrogen then preserved at -80 ℃. The remaining kidneys ($n = 6$) from each group of mice were fixed in buffered formalin for histological study. All Procedures involving animals were reviewed including care to minimize the pain and discomfort by the Ponce School of Medicine's Institutional Animal Care and Use Committee (IACUC). The animal protocol #LF-07 was approved by the committee. The drugs were obtained from Sigma Chemical Company (St. Louis, Mo, United States) and Abbott Pharmaceuticals (Abbott Park, IL, United States).

Determination of glutathione

Glutathione (GSH) was determined as previously described[37-39] using commercial kit (Cayman Company, Ann Arbor, MI, United States).

Lipid peroxidation assay

The end product of lipid peroxidation malondialdehyde (MDA) levels were estimated according to Ohkawa and colleague's method[42]. For the standard, 1, 1, 3, 3-tetraethoxypropane was used.

Protein assay

Total protein levels were determined using Coomassie reagent according to the method of Read and Northcole[43]. Bovine serum albumin (BSA) was used as the standard.

Protein extraction

Total protein extraction from frozen tissues was performed using M-PER buffer (Pierce, Rockford, IL, United States).

Western blot analysis

Total proteins (40 μg) was resolved by 12.5% SDS polyacrylamide gel electrophoresis (SDS PAGE) running gel and a 5% stacking gel as described previously[36-38].

Statistical analysis

The data are represented as mean ± SEM. The data were statistically analyzed by one-way analysis of variance (ANOVA) followed by a post-hoc, Schaffer test. $P < 0.05$ was considered significant. The statistical analysis of the data has been reviewed by Dr. Hernandez of Ponce School of Medicine.

RESULTS

As depicted in Figure 1, ApoE-deficient atherosclerotic mice in group 1 receiving vehicle alone developed significant hypertension (increased mean BP) compared to normal wild type controls in group 5 ($P < 0.05$) after 16 wk of treatment. Treatment with enalapril or enalapril in combination with paricalcitol for 16 wk prevented the increase in mean BP ($P < 0.05$). However, paricalcitol alone treatment for 16 wk did not

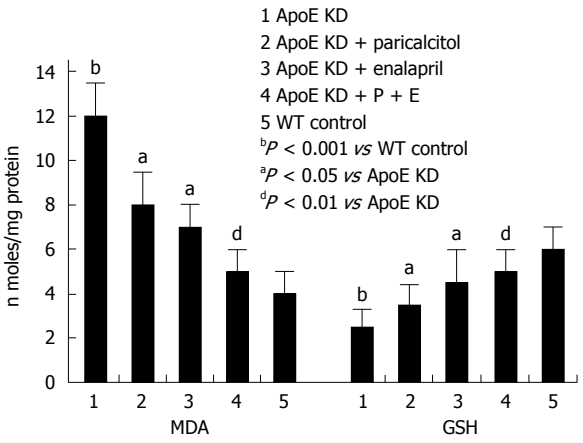

Figure 2 Effect of paricalcitol and enalapril alone and in combination for 16-wk on mono and combination for 16-wk on renal malondialdehyde and glutathione levels in ApoE-deficient atherosclerotic mice. There was a significant ($P < 0.001$) increase in the renal MDA levels in atherosclerotic mice compared to wild type control (group 5). Paricalcitol ($n = 6$) and Enalapril ($n = 6$) alone significantly ($P < 0.05$ and $P < 0.05$) reduced aortic malondialdehyde (MDA) levels. However combination of the two ($n = 6$) decreased greater and significant ($P < 0.01$) aortic MDA levels than either drug alone in atherosclerotic mice ($n = 6$). There was a significant ($P < 0.001$) decrease in aortic glutathione (GSH) levels in atherosclerotic mice compared to wild type control (group 5). Paricalcitol ($n = 6$) and enalapril ($n = 6$) alone significantly ($P < 0.05$) increased aortic GSH levels. However combination of the two ($n = 6$) increased greater and significant ($P < 0.01$) aortic GSH levels than either drug alone in atherosclerotic mice ($n = 6$).

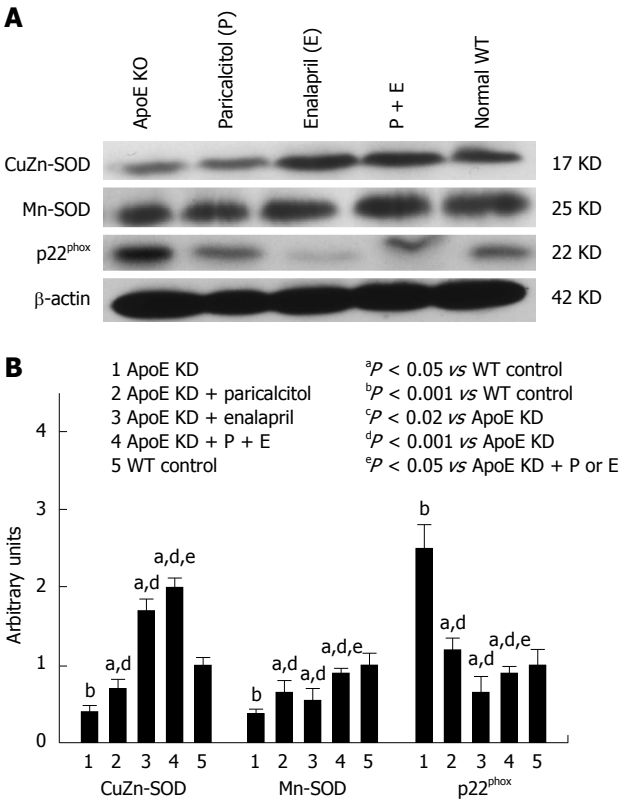

Figure 3 Western blot analysis and densitometry of protein band analysis to paricalcitol and enalapril alone and in combination for 16-wk on renal NADPH oxidase subunit p22phox, manganese-superoxide dismutase and copper/zinc-superoxide dismutase protein expression in ApoE-deficient atherosclerotic mice. A: Western blot analysis of the effect of paricalcitol and enalapril alone and in combination for 16-wk on renal NADPH oxidase subunit p22phox, manganese-superoxide dismutase (Mn-SOD) and copper/zinc-superoxide dismutase (CuZn-SOD) protein expression in ApoE-deficient atherosclerotic mice; B: Densitometry of protein band analysis show that renal NADPH oxidase subunit p22phox and Mn-SOD expression significantly increased ($P < 0.001$) whereas CuZn-SOD protein expression significantly decreased ($P < 0.001$) in atherosclerotic mice ($n = 3$) compared to controls ($n = 3$). Paricalcitol ($n = 3$), enalapril ($n = 3$) and combination of the two ($n = 3$) significantly ($P < 0.02$ and $P < 0.001$) ameliorated the oxidative stress by inhibiting the induction of NADPH oxidase and Mn-SOD expression (38%, 76% and 68%, respectively) and up-regulating the CuZn-SOD protein expression (52%, 342% and 398%, respectively) in atherosclerotic mice. The data represent mean ± SE of three independent Western blot experiments.

lower the mean BP of mice in group 2.

Figure 2 shows that the levels of the renal lipid peroxidation end product, malondialdehyde (MDA), increased by 200% ($P < 0.001$) in atherosclerotic mice in group 1 compared to wild type controls in group 5, indicating the renal oxidative injury in atherosclerosis. Paricalcitol and Enalapril alone significantly reduced renal MDA levels (33% and 58%, respectively) compared to group 1 ($P < 0.05$). Combining these two drugs, however, decreased greater MDA levels by 67% ($P < 0.01$) than either drug alone in atherosclerotic mice.

There was a marked decrease in renal reduced glutathione (GSH) (58%, $P < 001$) in vehicle-treated atherosclerotic mice in group 1 compared to wild type controls in group 5 (Figure 2) indicating an oxidative injury. Paricalcitol and Enalapril alone significantly increased renal GSH levels (40% and 80%, respectively) compared to group 1 ($P < 0.05$). Combining of the two drugs, however, increased greater GSH levels by 100% ($P < 0.01$) than either drug alone in atherosclerotic mice. All three therapies protected against the reduction in renal GSH levels, indicating an up-regulation of the renal antioxidant system.

Renal $p22^{phox}$ level was significantly elevated (150%) in ApoE-deficient mice compared to normal controls (Figure 3). Paricalcitol treatment decreased the subunit expression (38%) in the kidney compared to group 1. Enalapril and drug combo significantly decreased $p22^{phox}$ (76% and 68% of group 1) thus lowers the oxidative damage of the kidney.

As depicted in Figure 3 renal Mn-SOD level significantly elevated (98%) and CuZn-SOD level significantly depleted (55%) in atherosclerotic animals compared to group 5. Both drug separately as well as combined together depleted Mn-SOD levels (52%, 76% and 68%, respectively) and induced CuZn-SOD levels (52%, 342% and 398%, respectively) compared to group 1.

The renal inflammatory markers (TNF-α, MCP-1, TGF-β1 and iNOS) were significantly elevated (190%, 198%, 158% and 992%, respectively) and eNOS levels significantly depleted (55%) in ApoE-deficient animals compared to group 5 (Figures 4 and 5). Both drugs alone as well as combined together significantly depleted iNOS (69%, 72% and 76%, respectively),

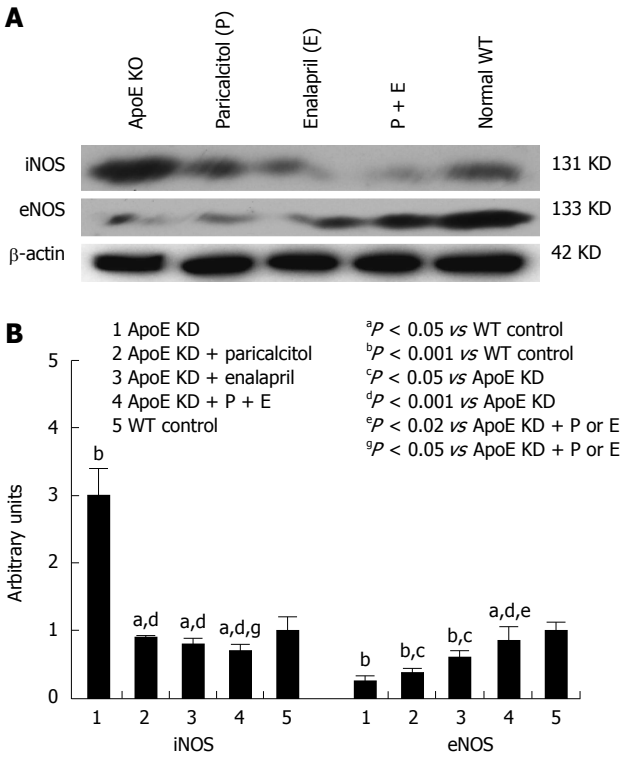

Figure 4 Western blot analysis and densitometry of protein band analysis to paricalcitol and enalapril alone and in combination for 16-wk on renal iNOS and eNOS protein expression in ApoE-deficient atherosclerotic mice. A: Western blot analysis of the effect of paricalcitol and enalapril alone and in combination for 16-wk on renal iNOS and eNOS protein expression in ApoE-deficient atherosclerotic mice; B: Densitometry of protein band analysis show that renal iNOS expression was significantly enhanced ($P < 0.001$) whereas eNOS protein expression was significantly decreased ($P < 0.001$) in ApoE-deficient atherosclerotic mice ($n = 3$) compared to controls ($n = 3$). Pricalcitol ($n = 3$), enalapril ($n = 3$) and the combination of the two ($n = 3$) significantly ($P < 0.05$ and $P < 0.001$) ameliorated the alterations in iNOS and eNOS expression in atherosclerotic mice. The data represent mean ± SE of three independent Western blot experiments.

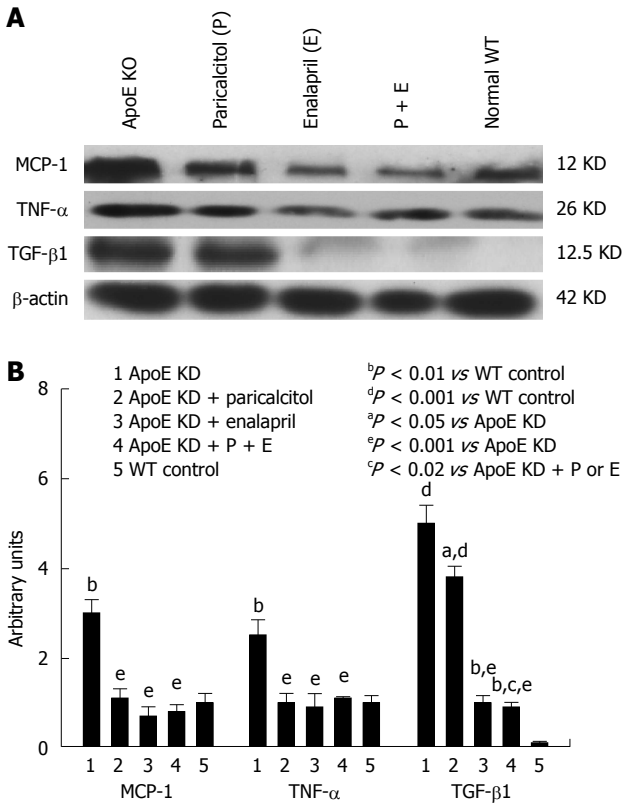

Figure 5 Western blot analysis and densitometry of protein band analysis to paricalcitol and enalapril alone and in combination for 16-wk on renal inflammatory proteins MCP-1, TNF-α and TGF-β1 expression in ApoE-deficient atherosclerotic mice. A: Western blot analysis of the effect of paricalcitol and enalapril alone and in combination for 16-wk on renal inflammatory proteins MCP-1, TNF-α and TGF-β1 expression in ApoE-deficient atherosclerotic mice; B: Densitometry of protein band analysis show that renal MCP-1, TNF-α and TGF-β1 expression was significantly enhanced ($P < 0.01$, $P < 0.01$ and $P < 0.001$) in ApoE-deficient atherosclerotic mice ($n = 3$) compared to controls ($n = 3$). Pricalcitol ($n = 3$) and enalapril ($n = 3$) alone and in combination of the two ($n = 3$) significantly ($P < 0.05$ and $P < 0.001$) exerted anti-inflammatory response by down-regulating the MCP-1, TNF-α and COX-2 expression in atherosclerotic mice. The data represent mean ± SE of three independent Western blot experiments.

MCP-1 (72%, 79%, 76%, respectively), TNF-α (79%, 88%, and 72%, respectively) and TGF- β1 (24%, 82% and 93%, respectively) compared to group 1. Both drug separately as well as combined together ameliorated the renal endothelial damage *via* induction of eNOS (98%, 147% and 342%, respectively) compared to group 1.

DISCUSSION

This study reveals the protective efficacy of the vitamin D analog, paricalcitol, and ACE inhibitor, enalapril, either alone or in combination, on renal inflammatory and oxidative stress in ApoE-deficient atherosclerotic mouse model. A number of preclinical as well as clinical studies have demonstrated the beneficial effects of RAS blockade in renal and cardiovascular diseases including atherosclerosis[13,16-18,29,37-39,44]. However, the mechanisms by which ACEIs provide protection against cardiovascular diseases are not fully understood. The role of inflammatory and oxidative injuries in the disorders of the kidney, heart and blood vessels such as CKD, renal and cardiac failure, atherosclerosis and hypertension is well known[18,34,37-39,45]. Chronically increased angiotensin II activity has been implicated in promoting oxidative stress, inflammation and atherosclerosis[46-48]. The inflammatory and oxidative stress response in atherosclerotic mice can induce the generation of oxidants and inhibit the endogenous antioxidants[27,30,37,39]. Angiotensin II has been shown to induce vascular oxidative stress through the activation of NADPH oxidase, which leads to the generation of superoxide[19,20,22,40]. The present data in an ApoE-deficient mouse model of atherosclerosis show that a profound increase in renal NADPH oxidase subunit $p22^{phox}$ protein expression corresponds to enhanced MDA levels and elevated mean BP. The down-regulation of NADPH oxidase by enalapril, paricalcitol and the combination, clearly suggests a role for RAS in the progression of renal oxidative stress. Interestingly the vitamin D analog, paricalcitol, which has been shown to be efficacious in renal and cardiovascular

diseases[12,31,34-36], has an antioxidant effect by decreasing the superoxide generating enzyme (NADPH oxidase) and MDA levels in the kidney. This action may be related to the negative regulation of RAS by vitamin D analogs[21,49]. Earlier studies have shown that excess superoxide generation due to NADPH oxidase activation causes inflammation and further generation of inflammatory cytokines (TNF-alpha) through NF-κB activation[21,22,50]. The role of NF-κB activation is also demonstrated in atherosclerosis[21,51] and in renal disease[52]. An earlier report demonstrated that 1, 25-dihydroxyvitamin D_3 blocks hyperglycemia-induced renal injury by blunting NF-κB activation[53]. Our recent studies demonstrate that renal and cardiovascular expression of pro-inflammatory substances (MCP-1, TNF-α, and COX-2) and macrophage infiltration are profoundly depressed when paricalcitol was combined with the ACE inhibitor, enalapril, in uremic rats and in atherosclerotic mice[29,38]. In preclinical studies, the treatment of animals with vitamin D ameliorated fibrosis in the kidney by inhibiting TGFβ and inducing hepatocyte growth factor[5,54]. TGFβ has been shown to inhibit interstitial fibrosis *via* down regulation of myofibroblast induction[54,55]. Preclinical report demonstrated that paricalcitol administered intravenously inhibits interstitial fibrosis in the kidneys of animals with obstructive nephropathy[35], and a clinical study has shown oral administration protected the kidney function in the CKD patients[56]. Therefore, the current study demonstrating the depletion of renal inflammatory factors by drugs treatment in atherosclerotic mice, together with previous data, suggests that the renal protection provided by paricalcitol or enalapril and combination of the two is most likely due to their anti-inflammatory actions.

Inflammation and oxidative stress in the kidney of atherosclerotic mice may also be related to an alteration in the cellular antioxidant defense system. Glutathione (GSH), a major non-protein thiol antioxidant, maintains the cellular integrity[28,57]. It directly scavenges excess ROS and also regenerates cellular antioxidant vitamins[28,48]. Our data show a significant depletion of renal GSH in atherosclerotic mice, an indicator of oxidative stress[48,57]. The depletion of GSH is known to cause failed antioxidant defense leading to lipid peroxidation[28,48,57]. The suppression of cellular glutathione by an inhibitor of glutathione synthesis causes hypertension and cardiovascular oxidative stress in rats[28]. Therefore, preservation of physiological levels of GSH is a vital cellular mechanism for the prevention of oxidative injury to the kidney. A lower level of antioxidants in the tissues of patients with renal and cardiovascular diseases has also been reported[16,18,56,58]. The data further show that both paricalcitol and enalapril alone and in combination significantly elevates renal GSH levels suggesting the antioxidant actions of the treatments. The present data show that renal mitochondrial Mn-SOD protein expression is significantly increased in ApoE-deficient atherosclerotic mice suggesting a defense mechanism for scavenge of excess superoxides. SODs are the primary scavengers of the endogenous ROS in the cells. There are three isozymes of SOD exist in mammalian cells: cytosolic CuZn-SOD, mitochondrial Mn-SOD and extracellular ecSOD and CuZn-SOD activity is inactivated by free radicals[59]. The inflammatory and oxidative stress in the renal tissues of atherosclerotic mice induced accumulation of oxidant species leading to protein oxidation and degradation of enzyme protein. Importantly, activation or inhibition of SOD activities are known to be due to cellular accumulation of oxidant species[60]. Mitochondrial Mn-SOD has been reported to be regulated by pro-inflammatory cytokine TNF-alpha[61] and both drugs paricalcitol and enalapril have anti-inflammatory actions[16,29,33,34,37-39]. Hence the combination of the two drugs would be more effective in the amelioration of renal inflammatory injury in atherosclerosis. The data further show that renal eNOS protein expression down-regulated but iNOS protein expression up-regulated in ApoE-deficient atherosclerotic mice compared to controls suggesting oxidative and inflammatory injury to the renal vascular endothelium. Cellular nitric oxide (NO) is produced by eNOS in the endothelium and by iNOS in response to inflammation[62]. Treatment with paricalcitol and enalapril attenuated the changes in eNOS and iNOS levels in atherosclerotic mice. The present data as well as our earlier studies[29,33,37-39] clearly show that vitamin D and ACE inhibitor combo ameliorated the renal inflammation and oxidants/antioxidants imbalance in animal model of atherosclerosis than either drug alone.

In summary, we demonstrated that renal inflammatory and oxidative stress in ApoE-deficient mice is due to enhanced TNF-α, TGFβ1, MCP-1, $p22^{phox}$, Mn-SOD and iNOS levels and lipid peroxidation and to a reduction in GSH levels and CuZn-SOD protein expression. Treatment with either vitamin D and ACE inhibitor alone or their combo attenuates renal inflammation and oxidative damage in ApoE-deficient animals by depleting TNF-α, TGFβ1, MCP-1, $p22^{phox}$, Mn-SOD and iNOS levels and lipid peroxidation and by preserving GSH, CuZn-SOD and eNOS levels. In conclusion, the combination of paricalcitol and enalapril is more efficacious than either drug alone in ameliorating renal inflammatory and oxidative stress in atherosclerosis.

COMMENTS

Background

Atherosclerosis is the primary cause of renal, heart and vascular diseases worldwide. There is a strong association between vitamin D deficiency and renin-angiotensin system (RAS) in atherosclerosis. Although the cardiovascular protection by vitamin D and RAS inhibitors have been reported in the atherosclerosis. However the protection against renal inflammation and oxidative stress in atherosclerosis by these agents are not reported.

Research frontiers

The study aimed to investigate the protective efficacy of a vitamin D analog, *i.e.*, paricalcitol, and an ACE inhibitor, *i.e.*, enalapril, either separately or in combo, on inflammation and oxidant damage in the kidney of ApoE-deficient atherosclerotic mice.

Innovations and breakthroughs

This study demonstrates for the first time that renal inflammation and oxidative injury is protected by the combination of paricalcitol and enalapril in ApoE-deficient atherosclerotic mice.

Applications

The study hypothesized that renal inflammation and oxidative stress (oxidants/antioxidants imbalance) in atherosclerosis is ameliorated by the combination of vitamin D and RAS inhibitor in mice. This combination is applicable to be used in the prevention and treatment of atherosclerotic renal injury in patients.

Terminology

The renal inflammatory and oxidative stress in ApoE-deficient mice is due to enhanced inflammatory markers (MCP-1, TNF-α, TGFβ1) oxidants ($p22^{phox}$, Mn-SOD and iNOS and MDA) and depletion of antioxidants (GSH, CuZn-SOD and eNOS). Administration of vitamin D and ACE inhibitor either separately or in combo attenuates renal inflammation and oxidant damage in ApoE-deficient animals by suppressing inflammatory and oxidant markers and restoring the antioxidant defense system.

Peer-review

The paper "Effect of paricalcitol and enalapril on renal inflammation and oxidative stress in mouse model of atherosclerosis" is very well written research paper. In their study they concluded that paricalcitol and enalapril combination therapy affords protection against renal inflammation and oxidative stress in atherosclerosis.

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ORIGINAL ARTICLE

Basic Study

Characterization of two alkyl hydroperoxide reductase C homologs alkyl hydroperoxide reductase C_H1 and alkyl hydroperoxide reductase C_H2 in *Bacillus subtilis*

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Abstract

AIM: To identify alkyl hydroperoxide reductase subunit C (AhpC) homologs in *Bacillus subtilis* (*B. subtilis*) and to characterize their structural and biochemical properties. AhpC is responsible for the detoxification of reactive oxygen species in bacteria.

METHODS: Two AhpC homologs (AhpC_H1 and AhpC_H2) were identified by searching the *B. subtilis* database; these were then cloned and expressed in *Escherichia coli*. AhpC mutants carrying substitutions of catalytically important Cys residues (C37S, C47S, C166S, C37/47S, C37/166S, C47/166S, and C37/47/166S for AhpC_H1; C52S, C169S, and C52/169S for AhpC_H2) were obtained by site-directed mutagenesis and purified, and their structure-function relationship was analyzed. The *B. subtilis ahpC* genes were disrupted by the short flanking homology method, and the phenotypes of the resulting AhpC-deficient bacteria were examined.

RESULTS: Comparative characterization of AhpC homologs indicates that AhpC_H1 contains an extra C37, which forms a disulfide bond with the peroxidatic C47, and behaves like an atypical 2-Cys AhpC, while AhpC_H2 functions like a typical 2-Cys AhpC. Tryptic digestion analysis demonstrated the presence of intramolecular

Cys37-Cys47 linkage, which could be reduced by thioredoxin, resulting in the association of the dimer into higher-molecular-mass complexes. Peroxidase activity analysis of Cys→Ser mutants indicated that three Cys residues were involved in the catalysis. AhpC_H1 was resistant to inactivation by peroxide substrates, but had lower activity at physiological H_2O_2 concentrations compared to AhpC_H2, suggesting that in *B. subtilis*, the enzymes may be physiologically functional at different substrate concentrations. The exposure to organic peroxides induced AhpC_H1 expression, while AhpC_H1-deficient mutants exhibited growth retardation in the stationary phase, suggesting the role of AhpC_H1 as an antioxidant scavenger of lipid hydroperoxides and a stress-response factor in *B. subtilis*.

CONCLUSION: AhpC_H1, a novel atypical 2-Cys AhpC, is functionally distinct from AhpC_H2, a typical 2-Cys AhpC.

Key words: Cysteine-dependent peroxidase; Thioredoxin; Thiol peroxidase; Peroxiredoxin; Alkyl hydroperoxide; Ortholog; *Bacillus subtilis*; Oxidative stress

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Core tip: Two alkyl hydroperoxide reductase subunit C (AhpC) homologs (AhpC_H1 and AhpC_H2) were identified by searching the *Bacillus subtilis* database. Sequence homology and phylogenetic analyses revealed that AhpC_H1 is an ortholog of *Escherichia coli* (*E. coli*) AhpC, a representative of bacterial AhpC. AhpC_H1 forms dimers consisting of atypical 2-Cys subunits, while AhpC_H2 behaves like a typical 2-Cys AhpC. These AhpC homologs may perform their respective physiological functions at different peroxide levels. Structural and catalytic differences between the enzymes indicate that AhpC_H1 is not an ortholog of *E. coli* AhpC, but a novel type of atypical 2-Cys AhpC.

Cha MK, Bae YJ, Kim KJ, Park BJ, Kim IH. Characterization of two alkyl hydroperoxide reductase C homologs alkyl hydroperoxide reductase C_H1 and alkyl hydroperoxide reductase C_H2 in *Bacillus subtilis*. *World J Biol Chem* 2015; 6(3): 249-264 Available from: URL: http://www.wjgnet.com/1949-8454/full/v6/i3/249.htm DOI: http://dx.doi.org/10.4331/wjbc.v6.i3.249

INTRODUCTION

Peroxiredoxins (Prxs) are versatile and predominantly thiol-dependent peroxidases (EC 1.11.1.15) that detoxify hydrogen peroxide and various hydroperoxides[1,2]. They are ubiquitously expressed, with multiple isoforms present in most organisms (*e.g.*, three isoforms in *Escherichia coli*, five in *Saccharomyces cerevisiae*, six in *Homo sapiens,* and nine in *Arabidopsis thaliana*)[3-13]. Prxs exhibit high reactivity with hydrogen peroxide and organic hydroperoxides and are involved in peroxide detoxification and signal transduction[14-20].

All Prxs share a common catalytic mechanism, in which an active-site cysteine (the peroxidatic cysteine) is oxidized to a sulfenic acid by a peroxide substrate, forming a disulfide linkage with a second resolving Cys, which is reduced back by the thioredoxin system consisting of thioredoxin 1 (Trx1), Trx reductase, and NADPH[1,2]. According to the number of functional Cys residues, Prxs are divided into two families: 1-Cys Prxs and 2-Cys Prxs[11,15].

The 2-Cys family contains typical and atypical groups of enzymes[21]. The disulfide bridge formed in typical 2-Cys Prxs after hydroperoxide reduction is intermolecular, whereas in atypical Prxs, it is intramolecular[22]. The founding members of the 2-Cys Prx family, the Prx1/alkyl hydroperoxide reductase C (AhpC) subfamily, including human Prxs Ⅰ-Ⅳ and bacterial AhpC proteins are well-studied and broadly distributed enzymes that form inter-subunit disulfide bonds during catalysis. The amino acid sequence and physiological function of this type of thioredoxin-dependent peroxidases (thiol-specific antioxidants) were first determined in *Saccharomyces cerevisiae*[23], and later in humans[24] and *E. coli*[3]. The members of these subfamilies are typically dimeric and form intra-subunit disulfide bonds. Another subfamily (the BCP/PrxQ group) is predominantly bacterial, although present in some eukaryotes and plants, but is absent in animals[6,12]. Most enzymes of the BCP/PrxQ group are reportedly monomeric[25], but there are two examples of dimeric proteins containing an A-type dimer (the TPx and Prx5 subfamilies). Current information suggests that BCP-PrxQ subfamily members can function as either atypical 2-Cys (α-group) or 1-Cys Prxs[26].

Moreover, there are also high molecular weight multimeric enzymes[27]. One atypical 2-Cys Prx exists as a hexamer in oxidized conditions, but dissociates to dimers upon reduction[28]; the presence of the peroxidatic Cys is critical for hexamer formation, whereas substitution of the resolving Cys does not affect oligomerization[29]. By analogy with the dimer-decamer transition of the typical 2-Cys Prx previously reported[27], the dimer-hexamer transition in atypical Prx displays a functional switch, which could be involved in signaling[28,30].

All aerobic bacteria have evolved a variety of antioxidant enzymes such as superoxide dismutases, catalases, and Prxs to cope with damaging endogenous reactive oxygen species (ROS) generated by the aerobic respiratory chain and metal-catalyzed oxidative reactions[31]. *E. coli* contains three Prxs: AhpC (Prx1/AhpC subfamily)[9], TPx (p20)[4,5], and BCP (BCP/PrxQ subfamily)[6,25].

Gene sequencing and phylogenetic studies have revealed that *B. subtilis* encodes four putative Prx enzymes with two conserved Cys residues, suggesting that they belong to different subgroups of the 2-Cys

Table 1 Oligonucleotides used for amplifying the genes expressing *Bacillus* peroxiredoxins proteins and its Cys residue-mutated proteins

	Sequence of primer for point mutation
AhpC_H1	
C37S F	AGCGTATTCTCTTTCTACCCA
C37S R	TGGGTAGAAAGAGAATACGCT
C47S F	TTCTCTTTCGTATCTCCAACTGAGCTT
C47S R	AAGCTCAGTTGGAGATACGAAAGAGAA
C166S F	CCAGGTGAAGTTTCTCCGGCTAAATGG
C166S R	CCATTTAGCCGGAGAAACTTCACCTGG
AhpC_H2	
C52S F	TTCACTTTTGTTTCTCCGACAGAAATT
C52S R	AATTTCTGTCGGAGAAACAAAAGTGAA
C169S F	ACTGGCGGACTCTCTCCGGCTAACTGG
C169S R	CCAGTTAGCCGGAGAGAGTCCGCCAGT
	Sequence of primer for protein expression
AhpC_H1	
Forward	CCGCATATGTCTTTAATCGGTAAAGAAGATC
Reverse	CCGGTCGACTTAGATTTTACCTACTAGATCAAGG
AhpC_H2	
Forward	CCGCATATGGCAGAACGTATGGTAGGTAA
Reverse	CCGGTCGACTTAAAGTGTTTTTTGGCCTGGTTT
B_BCP	
Forward	CCGCATATGACTATAGAAATCGGACAAAAAC
Reverse	CCGGTCGACTTACTTTTCAGACATTTTGAGG
B_TPx	
Forward	CCGCATATGGCTGAAATTACATTCAAAGGC
Reverse	CCGGTCGACTTACTTTCTAATGCAGCAGC

Prx family: there are two genes encoding putative AhpC-like proteins (AhpC_H1 and AhpC_H2), and two encoding TPx and BCP, respectively. AhpC_H1 has an additional N-terminal Cys residues at positions 37 (Cys37) besides two conserved Cys47 and Cys166, while ahpC_H2 possesses two conserved Cys residues (Cys52 and Cys169).

The present comparative study was designed to characterize two AhpC homologs and reveal the biological significance of the existence of two homologous AhpC enzymes in *B. subtilis*. This paper presents the first report of a detailed structure-functional analysis of the bacillus 2-Cys AhpC homologs AhpC_H1 and AhpC_H2.

MATERIALS AND METHODS

Materials

Bacterial culture media were purchased from Difco Laboratories (Detroit, MI, United States). Restriction enzymes and DNA ligase were obtained from Promega (Madison, WI, United States). Acrylamide-Bis (40% solution) was purchased from Bio-Rad (Hercules, CA, United States). Sodium dodecyl sulfate (SDS), ultrapure glycine, EDTA, dithiothreitol (DTT), Tris base, NADPH, ampicillin powder, isopropyl-b-D-thiogalactopyranoside (IPTG), H_2O_2, *tert*-Butyl hydroperoxide (*t*-BOOH) and cumene hydroperoxide (CMOOH) were from Sigma Aldrich (St. Louis, MO, United States). Iodoacetamide (IAA), N-ethyl maleimide (NEM), *tris* (2-carboxyethyl) phosphine (TCEP), 4-acetamide-4′-maleimidylstilbene-2,2′-disulfonate (AMS), and sulfhydryl-reactive DyLight 405 maleimide were purchased from Thermo Scientific, Pierce (Rockford, IL, United States).

Sequence and phylogenetic analyses

The NCBI BLAST tool (http://www.ncbi.nlm.nih.gov) was used to search for Prx amino acid homologs in the updated GenBank/EMBL and Swiss-Prot databases. Multiple sequence alignments of *B. subtilis* and *E. coli* Prxs were performed using the ClustalW 2.1 program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA *(*Molecular Evolutionary Genetics Analysis) version 6. A phylogenetic tree was generated by using the Maximum Likelihood method based on the JTT matrix-based model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches).

Bacterial strains

B. subtilis Cu1065 (*subtilis* strain 168 *attSPβ trpC2*, a derivative of *B. subtilis* subsp. subtilis str. 168) and *E. coli* XL1-blue and BL21 (DE3) strains were grown in Luria Broth (LB) medium under vigorous agitation at 37 ℃. Bacterial growth was monitored by the absorbance at 600 nm (OD_{600}), and exponentially growing *B. subtilis* cultures were exposed to different stress conditions for 30 min.

Cloning, site-directed mutagenesis, expression, and purification of recombinant proteins

The entire coding regions of *B. subtilis* genes encoding AhpC_H1, AhpC_H2, B_BCP, and B_TPx were amplified by PCR using the High Expand Fidelity kit (Roche Life Science, United States) and primers listed in Table 1. The entire coding region of the *E. coli ahpC* gene was amplified by PCR using the following primers: forward, 5′-GGG ATC CCA TAT GTC CTT AAT TAA CAC-3′; reverse, 5′-CCT CGA GTT AGA TTT TAC CAA CCA GGT-3′. To obtain Cys→Ser substitutions in AhpC_H1 and AhpC_H2, point mutations in the *ahpC_H1* and *ahpC_H2* genes were generated by site-directed mutagenesis by using complementary primers (Table 1). Gel-purified *ahpC* genes were digested with *Nde*I and *Xho*I, ligated into the pET-21a(+) expression vector by using T4 DNA ligase (Promega), and used to transform competent *E. coli* XL1-blue. Bacteria were selected on LB agar containing ampicillin (100 μg/mL), and purified plasmids were used for the sequencing of the cloned coding region by automated DNA sequencing.

The plasmids containing the confirmed sequences were used to transform *E. coli* BL21 (DE3) cells, which were then cultured at 37 ℃ overnight in 100 μg/mL ampicillin-containing LB. For protein expression, bacteria were diluted 1:250 in fresh medium, grown until OD_{600} = 0.4, and induced with 0.5 mmol/L isopropyl-L-D-thiogalactose (IPTG) for 4 h. The soluble recombinant proteins were produced in *E. coli*. Cells

Table 2 Oligonucleotides used for amplifying the DNA fusion construct to knockout *Bacillus* Prx genes

Prx null mutants	Primer names	Sequences of paired degenerated primers (5′→3′)
ΔAhpC_H1	H1_Up F	CCTTATTTCACAGATAAGCTCCA
	Kan/H1 R′	CCTATCACCTCAAATGGTTCGCTG AATGTATATTCCTCCTAAAAATGTAT
	Kan/H1 F′	CGAGCGCCTACGAGGAATTTGTATGGAGTGCATTCAATTGGTACTTG
	H1_Down R	ACGTGCAGTGTAGATCGCTGC
ΔAhpC_H2	H2_Up F	AAGCTGCGGATTTGAGTTGTCC
	Kan/H2 R′	CCTATCACCTCAAATGGTTCGCTGATGTATCCCTCCAATTTATTGTTTG
	Kan/H2 F′	CGAGCGCCTACGAGGAATTTGTAT TTCTTTCCAAGAACGAAAAGCGG
	H2_Down R	ATCTAAAATTGAGGAAAAACATCAA
ΔB_BCP	BCP_Up F	AATGGTACTGTGATCACTTCGTTT
	Kan/BCP R′	CCTATCACCTCAAATGGTTCGCTG TACGTTACCTCCGGATGTTTTTTT
	Kan/BCP F′	CGAGCGCCTACGAGGAATTTGTAT ATCTCTATGAGCCTATGCTTACTT
	BCP_Down R	AGTAGTCGATATGTGCATGCATT
ΔB_TPx	TPx_Up F	TTTTTCGCTTTGGACATGCTAT
	Kan/TPx R′	CCTATCACCTCAAATGGTTCGCTGTATAATTCCTCCCTTTTGTATGTAT
	Kan/TPx F′	CGA GCGCCTACGAGGAATTTGTAT GCAGGGAAAAAAGCTCCAGGC
	TPx_Down R	CGTCAGCTTTGAGCTCAAACG

Prx: Peroxiredoxins.

were harvested by centrifugation, suspended in 50 mmol/L Tris-HCl (pH 7.4) containing 2 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 1 mmol/L EDTA, disrupted by sonication, and centrifuged at 16000 × g for 30 min. The clarified supernatant was subjected to ammonium sulfate fractionation, size exclusion chromatography, and anion exchange chromatography. Size exclusion chromatography was performed using a Pharmacia chromatography system (Pharmacia Biotech). Protein solution was loaded onto a Sephacryl S-200 column equilibrated with 200 mmol/L NaCl-containing Tris-HCl, pH 8.0, at a flow rate of 0.2 mL/min at 4 ℃, and protein elution was monitored by the absorbance at 280 nm. Anion exchange chromatography was performed using DEAE-cellulose DE52 equilibrated with Tris-HCl, pH 8.0; protein was eluted with a linear salt gradient (0-500 mmol/L NaCl) in Tris-HCl, pH 8.0. Protein concentration was determined using the Bradford protein assay kit (Bio-Rad, United States).

Gene disruption by fusion PCR

The *B. subtilis* Prx-encoding genes were disrupted by the short flanking homology method[32]. The 5′ and 3′ regions of the *prx* genes were amplified using primers designed to obtain 0.2-kb DNA fragments (Table 2). A kanamycin-resistance determinant used as a selection marker for ΔPrx strains was amplified from the pDG780 vector using specific primers (forward, 5′-CAG CGA ACC ATT TGA GGT GAT AG-3′ and reverse, 5′-CGA GCG CCT ACG AGG AAT TTG TAT-3′). The marker was flanked by the two 0.2-kb terminal regions of the Prx-encoding genes using two-step fusion PCR. The first step was performed by fusing the 5′-terminal Prx gene fragment to the kanamycin-resistance gene. The amplified product was gel-purified and used as a template for the second round of PCR to fuse the 3′ terminal Prx gene fragment. The final PCR product was gel-purified and used for the disruption of each Prx-encoding gene in *B. subtilis* Cu1065. The null mutants were selected on 20 μg/mL kanamycin-containing LB agar, and chromosomal DNA was isolated and analyzed by PCR to confirm the replacement of the target gene.

Electrophoresis and Western blot analyses

Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 14% acrylamide gels. In reducing conditions, proteins were dissolved in loading buffer containing 100 mmol/L DTT and boiled for 3 min prior to loading; in non-reducing conditions, proteins were loaded in DTT-free buffer without boiling. After electrophoresis, gels were stained with Coomassie Brilliant Blue (Sigma-Aldrich) or processed for Western blotting. For this, PAGE-separated proteins were transferred to nitrocellulose membranes (Bio-Rad), blocked with 5% (w/v) skim milk in PBS containing 0.1% Tween 20 (PBST) at room temperature for 2 h, and probed with a manufactured rabbit polyclonal anti-AhpC_H1 (1:3000) antibodies (Young In Frontier, Seoul, South Korea) for 3 h. After three washes with PBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 1 h at room temperature, washed three times with PBST, and developed using the TMB immunoblotting detection system (Kem-En-Tec).

Further protein characterization was performed by 2D electrophoresis. Briefly, the *B. subtilis* protein extract was loaded onto 7-cm immobilized pH gradient (IPG) strips (Bio-Rad, United States) with 4-7 pH gradient (left to right), and the first-dimension separation was conducted; in the second-dimension separation, 16% reducing SDS-PAGE gels were used. For protein detection, the gels were stained with Coomassie Brilliant Blue G-250 or subjected to immunoblotting.

High-performance liquid chromatography

AhpC_H1 was purified from *B. subtilis* and a 25-μg

sample in phosphate buffer, pH 7.4, containing 150 mmol/L KCl was loaded onto a ZORBAX® Pro 10/300 GF450 column equilibrated in phosphate buffer using an automatic injector (SIL-20AC, Shimadzu, Japan). Chromatography was performed on a Shimadzu high-performance liquid chromatography (HPLC) system at the flow rate of 0.5 mL/min at room temperature, and the absorbance was monitored using a diode array detector (SPD M10A, Simadzu). The column was calibrated using protein molecular mass standards under the same conditions, and a calibration curve was obtained by plotting the Ve/Vo ratio (where Ve is the elution volume and Vo is the void volume) *vs* the logarithms of the molecular masses.

Chemical modification of cysteine sulfhydryl groups

For the reduction of protein disulfide groups, protein was reacted with chemical reducing agents such as DTT or TCEP or subjected to enzymatic reduction with *E. coli* Trx1, Trx1 reductase, and NADPH. After 30-min reaction at 30 ℃, thiol-specific modification reagent 4-acetamide-4′-maleimidylstilbene-2,2′-disulfonate (AMS) was added for another 30 min at 30 ℃.

Tryptic digestion and peptide identification

For HPLC analysis of Cys-containing peptides, sulfhydryl-reactive DyLight 405 maleimide (excitation and emission wavelengths 557 and 572 nm, respectively) was used to fluorescently label Cys residues in the intact or TCEP-denatured AhpC_H1. Proteins digested with trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK, an inhibitor of contaminating chymotryptic activity) in the presence of 2 mol/L urea were analyzed by reversed-phase HPLC. Briefly, proteins were incubated in 50 mmol/L NH_4CO_3 containing 2 mol/L urea and trypsin at the ratio 1:20 (w/w, trypsin/protein) at 30 ℃ for 12 h, and then loaded into a C18 HPLC column (2.1 mm × 25 cm). Peptides were eluted with a 5%-100% gradient of acetonitrile containing 0.2% trifluoroacetic acid (TFA) at the flow rate of 1 mL/min for 120 min. The elution of fluorescent peptides was monitored at 572 nm using a fluorescence detector (RF-10AXL, Shimadzu).

Enzyme assays

Enzymatic assays with the Trx system (NADPH, *E. coli* Trx reductase, and Trx1) and peroxides as substrates were conducted to detect peroxidase activity of the purified AhpC proteins by the decrease of absorbance at 340 nm. Briefly, the enzymes were added to the reaction mixture containing 0.14 mmol/L NADPH, 100 nmol/L *E. coli* Trx reductase, 5 μmol/L *E. coli* Trx1, 50 mmol/L Tris-HCl (pH 7.4), 0.1 mmol/L EDTA, and hydroperoxide substrates (H_2O_2 and *t*-butyl hydroperoxide) at room temperature and the absorbance was continuously monitored at 340 nm using a diode array spectrophotometer (HP 7452A). Stock solutions of *t*-butyl hydroperoxide were prepared in DMSO prior to the reaction.

General methods

Plasmid and chromosomal DNA isolation, restriction enzyme digestion, *E. coli* transformation, and DNA ligation were performed according to standard protocols[32]. The transformation of naturally competent *B. subtilis* cells was carried out as previously described[33].

RESULTS

Bioinformatic analysis of B. subtilis putative Prx homologs

Four *prx*-homologous sequences in *B. subtilis* genome were identified by searching the Data Release R16.1 (the SubtiList World-Wide Web Server, http://genolist.pasteur.fr/SubtiList/). Figure 1 shows multiple sequence alignment of three *E. coli* Prxs (E_AhpC, E_BCP, and E_TPx) and four *B. subtilis* Prxs using the ClustalW 2.1 program, and a resulting phylogenetic tree. The hallmark of Prxs is a conserved peroxidatic Cys residue located near the N-terminus. The analysis of multiple protein alignment revealed that all *B. subtilis* Prxs possessed a highly conserved peroxidatic Cys, suggesting that these enzymes belong to the Prx family. The cladogram reveals that, with the exception of B_AhpC_H2, all *Bacillus* Prxs displayed sequence similarity with the corresponding *E. coli* Prxs and were therefore named after the respective *E. coli* enzymes. Thus, E_TPx and B_TPx showed 41.5% identity in 164 overlapping amino acids and E_BCP and B_BCP demonstrated 39.7% identity in 151 overlapping amino acids. E_AhpC and B_AhpC_H1 had 65.24% identity in a 187-amino acid region, while E_AhpC and B_AhpC_H2 had 38.4% identity in a 159-amino acid sequence. Between B_AhpC_H1 and B_AhpC_H2, there was 37.0% identity in a 181-amino acid sequence.

Sequence similarity analysis indicates that AhpC_H1 is a homologue of *E. coli* AhpC, a representative of the AhpC/Prx1 subfamily. Sequence alignment of AhpC homologs revealed two conserved Cys residues, catalytic and resolving, in the N-terminal and C-terminal regions, respectively, suggesting that B_AhpC_H2 also belongs to the AhpC/Prx1 family, although its similarity to E_AhpC (38.4%) is much lower than that of B_AhpC_H1 to E_AhpC (65.2%).

Taken together, these data suggest that *B. subtilis* encodes one member of the BCP/PrxQ, one member of the bacterial TPx, and two members of the AhpC/Prx1 subfamilies.

Structural analysis of B. subtilis recombinant AhpC proteins

AhpC_H2 and AhpC_H1 each contain a pair of conserved Cys residues (Cys52/Cys169 and Cys47/Cys166, respectively); in addition, AhpC_H1 possesses an extra Cys37 (Figure 1). The members of the AhpC/Prx1 subfamily (including *E. coli* AhpC) are typical 2-Cys Prxs, which exist as homodimers formed *via* an intermolecular disulfide bond between two subunits.

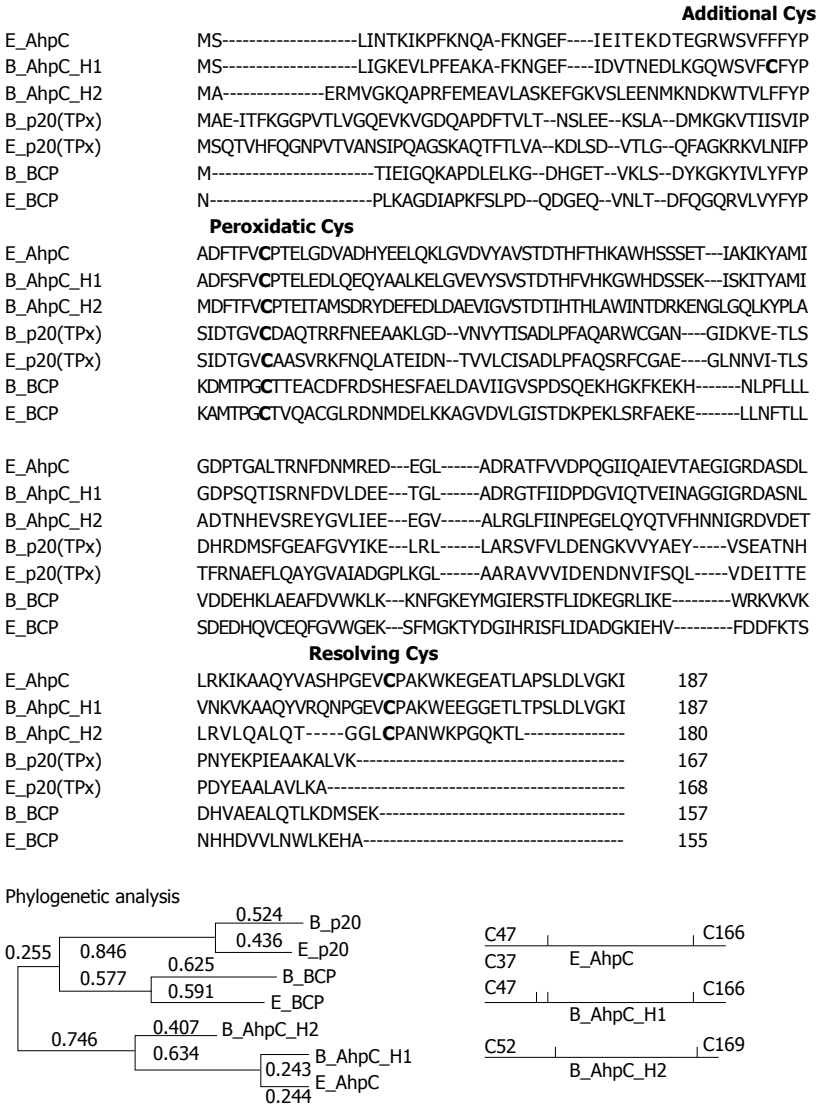

Figure 1 Amino acid sequence alignment of *Bacillus subtilis* and *Escherichia coli* peroxiredoxins. *B. subtilis* database was searched using amino acid sequences around conserved N-terminal Cys residues as a query. In addition to reported bacterial genes encoding BCP and TPx enzymes, two putative AhpC-encoding genes were identified, and four *B. subtilis* and three *E. coli* Prx sequences were aligned. The two conserved Cys residues, N-terminal peroxidatic and C-terminal resolving Cys, among the AhpC proteins are shown as shaded bold letters. Proteins homologous to *E. coli* AhpC were identified using the ClustalW 2.1 multiple sequence alignment. The phylogenetic tree was generated by using MEGA version 6 The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 7 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 143 positions in the final dataset. Position of Cys residues in AhpC proteins is shown at the bottom right panel. E_BCP, *E. coli* BCP (accession number AAC75533); E_p20 (TPx), *E. coli* TPx (accession number EDV68400); E_AhpC, *E. coli* AhpC (accession NP_415138); B_ahpC_H1, *B. subtilis* AhpC homolog 1 (accession number BAA11268); B_AhpC_H2, *B. subtilis* AhpC homolog 2 (accession number WP_019258276); B_BCP, *B. subtilis* BCP (accession number AAC75533); and B_p20 (TPx), *B. subtilis* TPx (accession NP_390827).

To examine the possible formation of a disulfide bond between the two conserved Cys residues in *B. subtilis* AhpC enzymes, Cys was replaced with Ser by site-directed mutagenesis and the purified mutant AhpC_H1 and AhpC_H2 proteins were analyzed by their migration patterns in SDS-PAGE.

In non-reducing SDS-PAGE gels, wild-type AhpC_H2 and E_AhpC appeared as single bands with the molecular masses about 48 kDa and about 40 kDa, respectively (Figure 2A), whereas single approximately 24.5-kDa and 22-kDa bands, respectively, were observed in reducing gels (Figure 2B), suggesting that in natural conditions, these enzyme exist as dimers likely held together by disulfide linkages. However, most AhpC_H1 appeared as a single band with a molecular mass of the monomer (approximately 23-kDa) under both non-reducing and reducing conditions (Figure 2A and B, respectively), suggesting the presence of monomers even in the oxidized state.

The migration pattern of AhpC_H2 mutant proteins (C52S, C169S, and C52/169S) in non-reducing SDS-PAGE gels were quite different from that of the wild-type counterpart (Figure 2A). C169S mostly existed in a monomeric form, presenting the evidence of intermolecular disulfide linkage between the two identical subunits. In non-reducing conditions, the

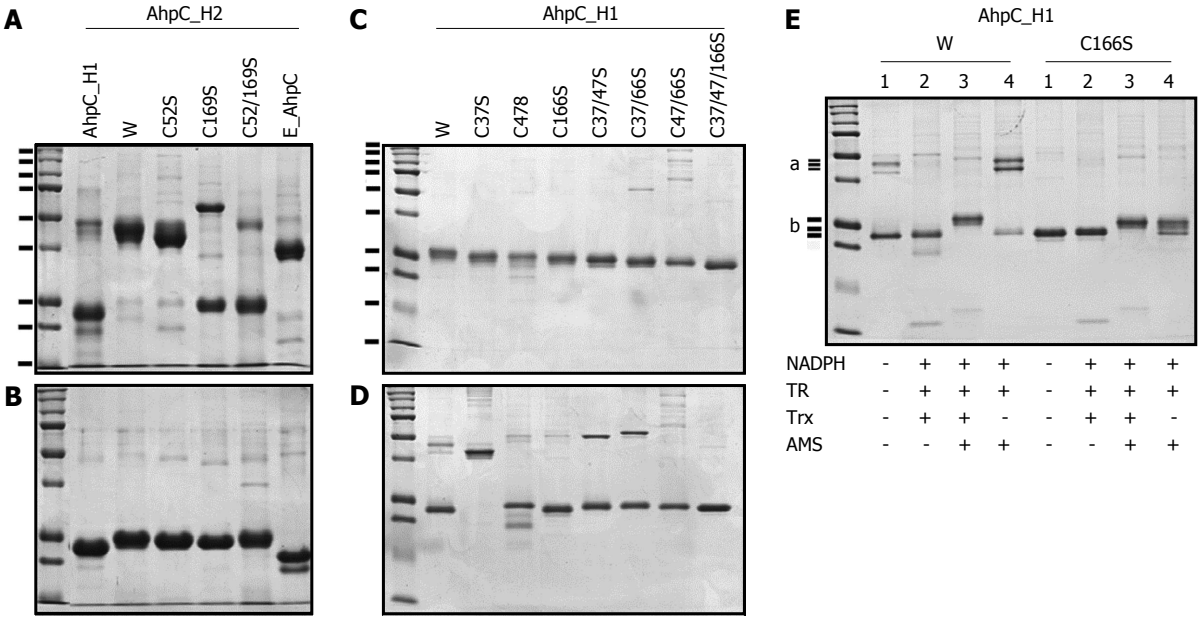

Figure 2 SDS-PAGE analyses of AhpC_H1 and AhpC_H2 proteins and their mutants carrying Cys→Ser substitutions. The wild-type (lane W) and mutated AhpC_H2 and AhpC_H1, and *E. coli* AhpC proteins were separated in non-reducing (A) and reducing (B) 12% SDS-PAGE gels; first lanes show molecular weight markers (15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa). TCEP/AMS-treated (C) and non-treated (D) wild-type (lane W) and mutated AhpC_H1 proteins were separated in non-reducing 14% SDS-PAGE gels. AhpC_H1 and its C166S mutant reduced or not with the Trx system containing Trx 1, Trx reductase (TR), and NADPH were modified with a sulfhydryl group-specific reagent AMS and separated in non-reducing 14% SDS-PAGE gels (E). a marks the position of a single AhpC_H1 band (W, lane 1) and double bands (W, lane 4). b marks three positions of the AhpC_H1 band after the treatments indicated at the bottom. First lanes in B1, B2, and C show molecular weight markers (10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa).

migration of the wild-type dimer appeared to be different from that of the mutant C52S and C169S dimers, suggesting the formation of inter-subunit disulfide bonds characteristic for typical 2-Cys Prxs such as E_AhpC.

In contrast to AhpC_H2 and E_AhpC, most of the AhpC_H1 proteins existed as monomers even in the oxidized state. The migration patterns of AhpC_H1 Cys→Ser mutants (C37S, C47S, C166S, C37/47S, C37/166S, C47/166S, and C37/47/166S) indicated that the distance of protein migration in reducing conditions depended on the number of substituted Cys residues (Figure 2C), thus confirming that all three Cys residues are susceptible to AMS modification. In non-reducing conditions, the C37S protein migrated as a dimer (Figure 2D). Considering that, in non-reducing conditions, the wild-type protein was predominantly monomeric (lane W in Figure 2D), dimerization of the C37S mutant suggests that Cys37 could participate in the formation of an intramolecular disulfide bond. In non-reducing gels, most of the proteins carrying C47S and C166S substitutions were detected at the positions slightly higher than that of the wild-type protein; however, a single C166S mutant migrated similarly to the wild-type protein, suggesting the existence of intramolecular disulfide linkage between Cys37 and Cys47 in both proteins.

Taken together, these results indicate a possibility that AhpC_H1 is an atypical 2-Cys enzyme, which forms the intramolecular disulfide linkage between Cys37 and Cys47 rather than between Cys47 and Cys166. To examine the presence of Cys37-Cys47 intramolecular disulfide bond, the wild-type AhpC_H1 and its C166S mutant were reduced using the Trx system, and analyzed by non-reducing SDS-PAGE. As shown in Figure 2E, Trx-treated C166S occupied a slightly higher position than the wild-type protein; AMS modification shifted the position of both proteins toward higher molecular weight (Figure 2E, lane 3). These results reveal that the disulfide bond can be specifically reduced by the Trx system. Furthermore, AMS modification of the wild-type AhpC_H1 in the absence of Trx1 (Figure 2E, lane 4) or TCEP (data not shown) resulted in the formation of two different dimers, but did not cause dimerization of C166S, which confirms the presence of free Cys166 in AhpC_H1. Other sulfhydryl-modifying agents such as NEM and IAA also induced AhpC_H1 dimerization (data not shown). Overall, the effect of thiol-specific modification of free Cys166 on the tertiary structure of AhpC_H1 is worth noting, although such modification may not occur *in vivo*.

Finally, we directly demonstrated the presence of free Cys166 residue in the oxidized AhpC_H1. AhpC_H1 was modified with fluorescent sulfhydryl-reactive maleimide and digested with trypsin in the presence or absence of 2 mol/L urea. The reversed-phase HPLC chromatogram of trypsin-digested native AhpC_H1 demonstrates a unique peak of a fluorescent peptide (a in Figure 3, W), which was significantly increased in the trypsin-digested denatured (2 mol/L urea-treated) AhpC_H1 (a in Figure 3, W/Urea). The

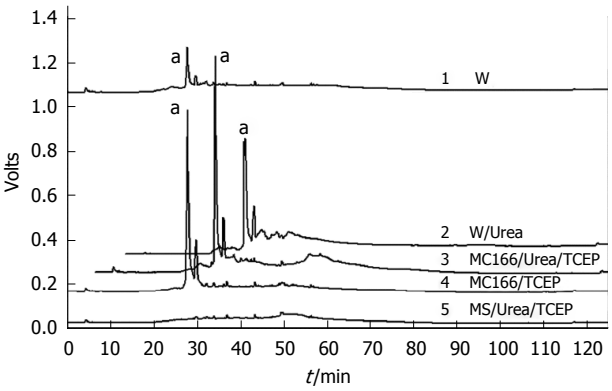

Figure 3 Reversed-phase high-performance liquid chromatography analysis of fluorescently labeled trypsin-digested AhpC_H1. AhpC_H1 was modified with sulfhydryl-reactive DyLight 405 maleimide, digested with TPCK-treated trypsin in the presence or absence of 2 M urea, and analyzed by reversed-phase HPLC; fluorescence was monitored at 527 nm. Fluorescent peak (a) corresponds to a peptide containing free Cys residue conjugated with the fluorescent dye. W: Wild-type AhpC_H1; MC166: C37/47S double mutant; MS: Cys37/47/166S triple mutant.

same peak appeared in the trypsin-digested denatured and native C37/47S double mutant (Figure 3, MC166/Urea/TCEP and M166/TCEP, respectively), which has only one free Cys166 residue. The triple mutant C37/47/166S (MS) did not produce any fluorescent peak. Taken together, these data suggest that the peak represents the peptide with free Cys166 residue, indicating the existence of free Cys166 and the intramolecular disulfide linkage between Cys37 and Cys47.

Enzymatic reduction of the intramolecular disulfide bond between Cys37 and Cys47

The modification of Trx-preincubated AhpC_H1 and AhpC_H2 with AMS, a thiol-specific reagent, resulted in the shift of the monomer to a slightly higher position (Figure 4A and B, respectively), suggesting that the Trx system (Trx1, Trx reductase, and NADPH) is able to reduce protein disulfide bonds in both AhpC_H1 and AhpC_H2.

Pre-incubation of AhpC_H1 with the Trx system containing increasing concentrations of *E. coli* Trx1 resulted in an increase of the AMS-modified AhpC_H1 in a Trx 1 concentration-dependent manner (Figure 4C), indicating that the intramolecular disulfide bond can be reduced by the Trx treatment. The dimerization of AMS-modified AhpC_H1 was also confirmed (Figure 4C). Similarly, the dimeric AhpC_H2 was gradually converted to a corresponding AMS-modified monomer in a Trx1 dose-dependent manner (Figure 4D).

To ensure functional activity of the Trx1 system to reduce disulfide bonds, we tested the Trx system in the reaction with *Bacillus* B_BCP and B_TPx as well as with Trx-supported peroxidases from other species such as *E. coli* AhpC and human Prx1 and Prx2 (Figure 4E-I, respectively). The results indicate that the reduced forms of all Prxs increased in a Trx1 concentration-dependent manner, indicating that all the tested enzymes were sensitive to the Trx system. More importantly, in non-reducing conditions, AhpC_H2, but not AhpC_H1, demonstrated a pattern very similar to that of *E. coli* AhpC (E_AhpC) (Figure 4G), suggesting that AhpC_H2 is structurally similar to E_AhpC in that they exist as intermolecular dimers linked by a disulfide bond, although amino acid sequence similarity between AhpC_H1 and E_AhpC is much higher than that between AhpC_H2 and E_AhpC (Figure 1). Taken together, these results indicate the presence of a redox-sensitive disulfide linkage between Cys37 and Cys47 of AhpC_H1, characterizing it as a member of the atypical 2-Cys subfamily.

Identification of Cys37-Cys47 intramolecular disulfide linkage in the native AhpC_H1 protein

In the previous experiments, we demonstrated that, unlike AhpC_H2, AhpC_H1 formed the intramolecular Cys37-Cys47 disulfide bond. However, it may be possible that it is characteristic only for the recombinant protein and not for the native *B. subtilis* AhpC_H1. To test this possibility, we examined the presence of the disulfide linkage in the native *B. subtilis*-purified AhpC_H1. Crude protein extract was fractionated using ammonium sulfate gradient (20% to 60%). Figure 5A shows that a major protein band of approximately 22-kDa was detected in the 40% and 45% fractions. Western blot analysis with the AhpC_H1 antibody demonstrated that the predominant band was AhpC_H1 (Figure 5B). In the 40% fraction, AhpC_H1 was reduced with TCEP, and the resulting product was specifically modified with AMS (Figure 5C). The analysis of the modified proteins by non-reducing SDS-PAGE and western blotting (Figure 5D) revealed that AMS could modify only TCEP-reduced AhpC_H1 (Figure 5D). Taken together, these results demonstrate that *B. subtilis* expresses a significant amount of oxidized AhpC_H1 with the Cys37-Cys47 intramolecular disulfide linkage.

AhpC_H1 oligomerization

Previous studies have demonstrated that the Cys37-Cys47 bond in AhpC_H1 is redox-sensitive. AhpC_H1 molecular mass was determined by HPLC. Figure 6A demonstrates that AhpC_H1 was eluted between ovalbumin (OVA, 44 kDa) and carbonic anhydrase (CA, 29 kDa); therefore, the apparent AhpC_H1 molecular mass was estimated to be approximately 38.6 kDa. Given that a theoretical AhpC_H1 molecular mass is approximately 20.5 kDa, this result indicates that AhpC_H1 exists as a homodimer. Considering that AhpC_H1 mostly appears as a 22-kDa band in non-reducing SDS-PAGE gels, this result suggests that the dimer is likely formed *via* non-covalent interactions. On the other hand, AhpC_H2 was eluted at 9.116 mL (data not shown). Given that the elution volume of apoferritin (AF, 443 kDa) was 9.514 mL, it suggests

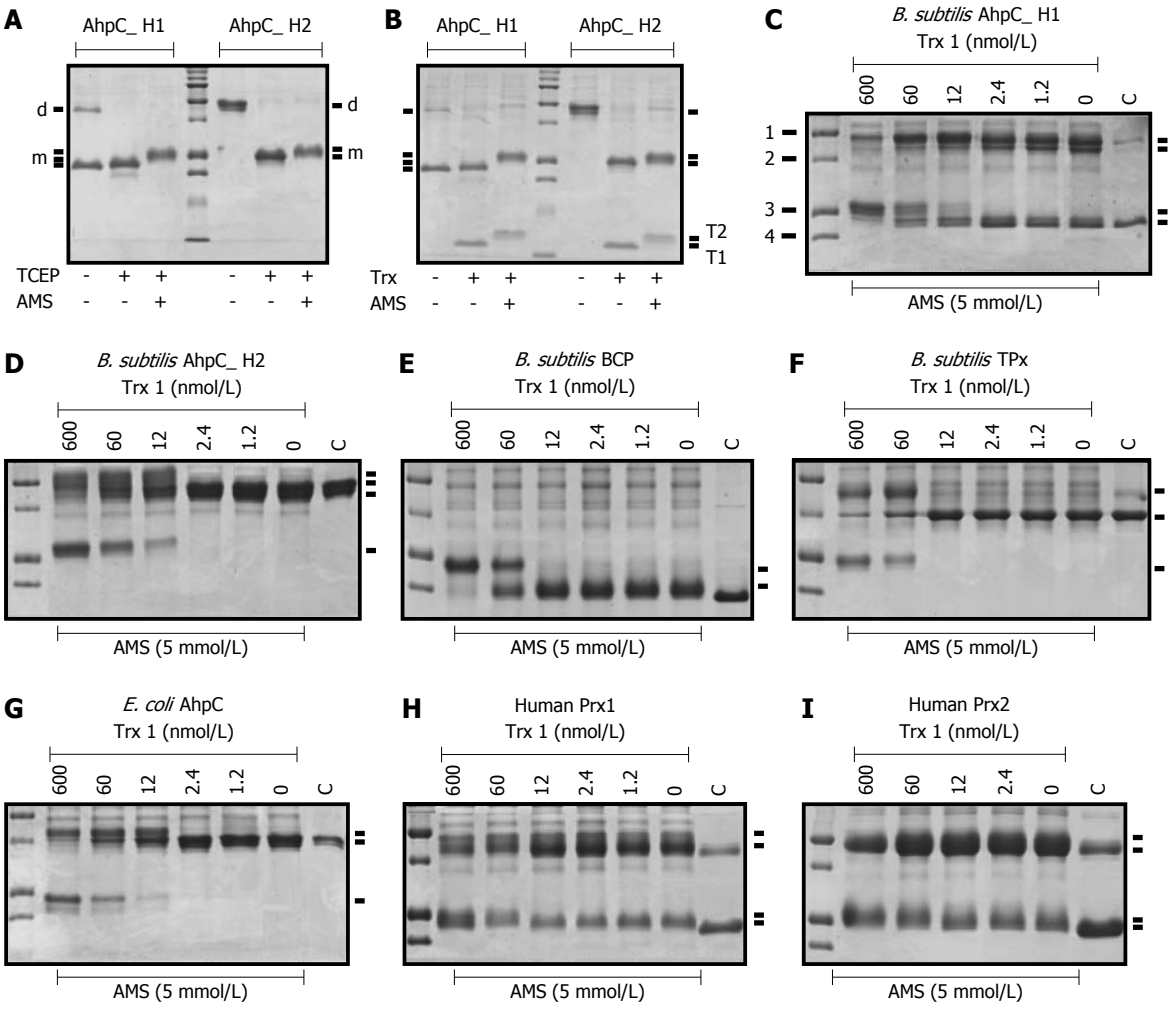

Figure 4 Non-reducing SDS-PAGE of peroxiredoxins reduced with the Trx system. (A, B) AhpC_H1 and AhpC H2 were incubated without or with 2 mnol/L TCEP (A) or Trx system (B) for 30 min at 30 ℃, followed by incubation with AMS for 30 min at 30 ℃. The reaction was terminated by adding SDS sample buffer, and proteins were separated in 16% non-reducing SDS-PAGE gels. Protein migration pattern in A is the same as in B, suggesting that the Trx system reduced disulfide bonds in both proteins; d and m mark the position of dimeric and monomeric, respectively, AhpC_H1 and AhpC_H2. T1 and T2 denote the oxidized and reduced/modified Trx 1 proteins, respectively. Middle lanes show molecular weight markers (10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa). Trx, the Trx system; (C-F) Conversion of different Prxs from the oxidized to reduced form by the Trx system. 1, 2, 3, and 4 indicate molecular weight markers of 50, 37, 25, and 20 kDa, respectively.

that AhpC_H2 oligomer has a higher molecular mass compared to AhpC_H1.

It has been reported that in some cases, Prx oligomerization is reversible and depends on the redox status of Cys residues[27-30]. To examine AhpC_H1 redox-dependent oligomerization, the molecular mass of TCEP-reduced AhpC_H1 was determined by HPLC. Figure 6B shows that the elution volume of reduced AhpC_H1 was 9.379 mL (Figure 6B, AhpC_H1/TCEP), which, considering the elution volume of 443-kDA apoferritin (9.514 mL) indicates that the apparent molecular mass of reduced AhpC_H1 was above 443 kDa. AhpC_H1 incubation with H_2O_2 did not significantly affect the molecular mass (Figure 6B, AhpC_H1/H2O2).

Taken together, these results suggest that AhpC_H2 exists as a high molecular mass oligomer, while AhpC_H1 is a homodimer. Oxidized AhpC_H1 monomers are dimerized *via* noncovalent interactions; however, after reduction, the dimers tend to assemble into high molecular mass oligomers.

Substrate-mediated inactivation of AhpC_H1 and AhpC_H2

Because some Prxs, especially of eukaryotic origin, are known to be inactivated by high substrate concentrations[1,2], we tested the response of AhpC_H1 and AhpC_H2 to elevated concentrations of their preferred substrate H_2O_2 by using the Trx1 system. Peroxidase activity was continuously monitored by the absorbance at 340 nm indicating NADPH concentration. Figure 7A and B shows a time-dependent decrease in NADPH concentration due to the peroxidase activity of AhpC_H1 and AhpC_H2, respectively. At low H_2O_2 concentrations (< 100 μmol/L), AhpC_H2 was more active than AhpC_H1, but was significantly inhibited by higher H_2O_2 concentrations, whereas AhpC_H1 was resistant even to high H_2O_2 levels, which completely

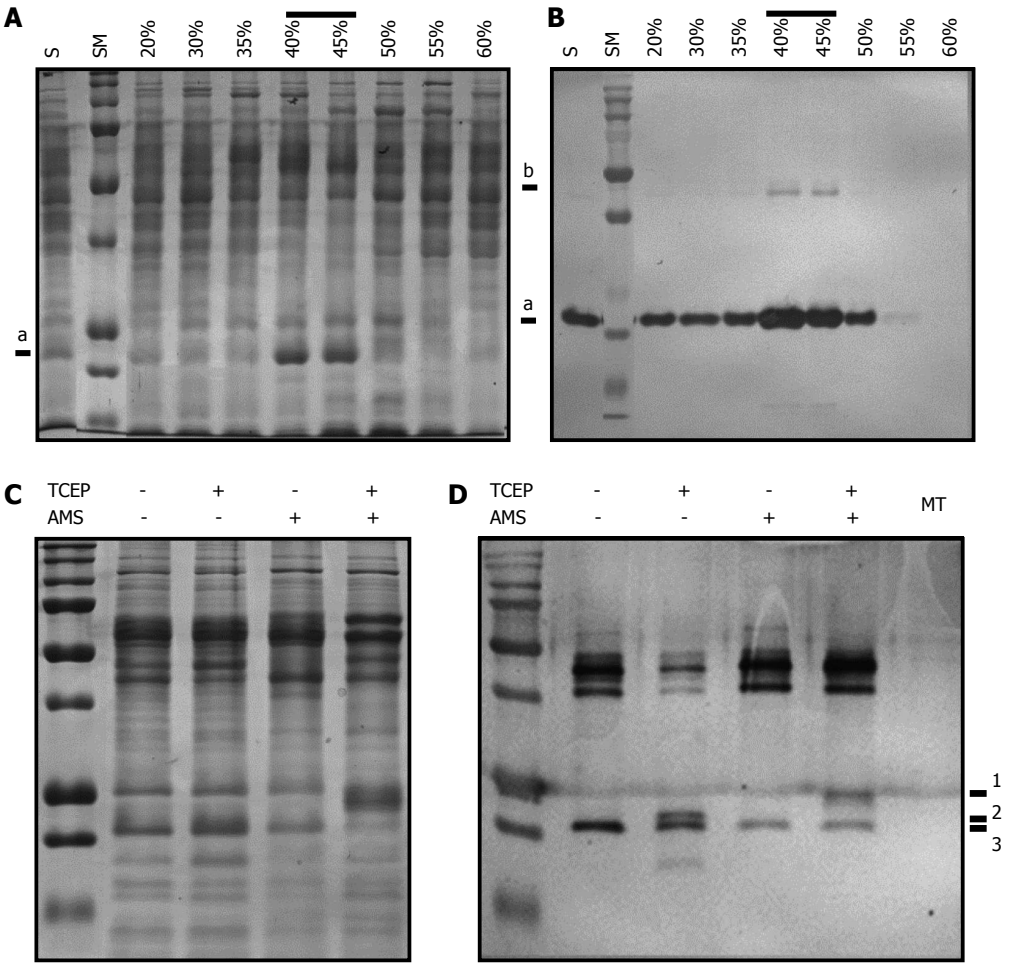

Figure 5 Identification of intramolecular disulfide linkage in AhpC_H1 from *B. subtilis*. A, B: Soluble fraction of *B. subtilis* overnight cultures was precipitated with 30%, 35%, 40%, 45%, 50%, 55%, and 60% ammonium sulfate. After dialysis of solubilized precipitates, 20 μg of each fraction was separated in 14% reducing SDS-PAGE gel, and stained with Coomassie brilliant blue (A) or processed by western blotting using anti-AhpC_H1 antibody (for western blotting, 1 μg protein was separated). Correlation of band intensities in (A) and (B) indicates that the major band marked as a is an AhpC_H1 monomer; b marks an AhpC_H1 dimer; C: Proteins (20 μg) of the 40% fraction were incubated in the presence or absence of 2 mM TCEP, reacted with 5 mmol/L of AMS for 30 min at 30 ℃, separated by non-reducing SDS-PAGE, and stained with Coomassie Brilliant Blue; D: Similarly treated proteins (100 ng) were subjected to western blotting. Triple bands (1, 2, and 3) around the position of the monomer indicate AMS modification of Cys residues after the reduction by TCEP. MT, AhpC_H1-deficient *B. subtilis*; the absence of the AhpC_H1 band indicates antibody specificity. SM, molecular weight markers (15, 20, 25, 37, 50, 75, 100, 150, 250 kDa).

inhibited AhpC_H2 activity (Figure 7C), indicating that AhpC_H1 is a much more robust enzyme compared to AhpC_H2.

Trx-dependent peroxidase activity of AhpC_H1 mutants

Figure 8 shows Trx-dependent peroxidase activity of the wild-type AhpC_H1 and its C37S, C47S, C166S mutants toward *t*-butyl hydroperoxide substrate. Compared to almost inactive C47S and C166S mutants, the C37S protein showed a substantial enzymatic activity (approximately 19.6% of that of the wild-type protein), indicating that, in addition to Cys47 and Cys166, Cys37 is also required for the peroxidation activity of AhpC_H1.

AhpC_H1 expression in B. subtilis

B. subtilis genome encodes four Prxs (B_BCP, B_TPx, B_AhpC_H1, and B_AhpC_H2) (Figure 1), and their expression as antioxidant proteins should be regulated by growth conditions in a gene-specific manner. SDS-PAGE analysis of *B. subtilis* crude extracts indicates high expression of AhpC_H1 (Figure 5). To further evaluate the regulation of Prx enzymes in *B. subtilis,* mutant strains deficient in B_BCP (ΔBCP), B_TPx (ΔTPx), B_AhpC_H1 (ΔAhpC_H1), or B_AhpC_H2 (ΔAhpC_H2) were constructed and analyzed by 2D electrophoresis. Protein patterns visualized by Coomassie Brilliant Blue staining indicate that one of the predominant spots represented the AhpC_H1 monomer (Figure 9A, a), whereas the other spot detected in the cumene hydroperoxide (CMOOH)-stressed wild-type strain (Figure 9A, W + CMOOH, b) is suggested to be the AhpC_H1 dimer.

Except for AhpC_H1, other Prxs were not detected by 2D electrophoresis (Figure 9A). As expected, AhpC_H1 (Figure 9A, a) was seen in all mutants but ΔAhpC_H1. It is worth noting that another spot corresponding to the AhpC_H1 dimer was detected

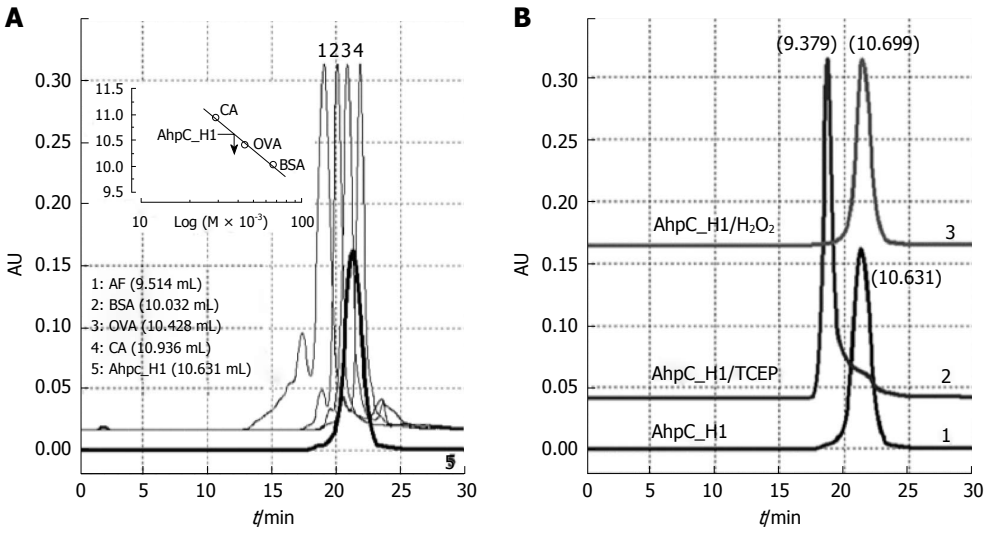

Figure 6 High-performance liquid chromatography gel-filtration chromatograms of AhpC_H1 purified from *B. subtilis*. AhpC_H1 was loaded onto a ZORBAX® Pro 10/300 GF450 column and eluted with 50 mmol/L phosphate buffer, pH 7.4, containing 150 mmol/L KCl. A: AhpC_H1 elution profile (bold line, 5); protein molecular mass standards: 1, apoferritin (AF, 443 kDa); 2, bovine serum albumin (BSA, 66 kDa); 3, ovalbumin (OVA, 44 kDa); 4, carbonic anhydrase (CA, 29 kDa). Inset shows a calibration curve used to determine AhpC_H1 molecular mass; B: The profiles of TCEP-reduced AhpC_H1 (AhpC_H1/TCEP) and AhpC_H1 treated with 2 mmol/L H2O2 for 30 min at 30 ℃ (AhpC_H1/H2O2) were compared with that of untreated AhpC_H1. Elution volume (mL) is in parentheses. Oligomeric AhpC_H1 was eluted at 9.379 mL.

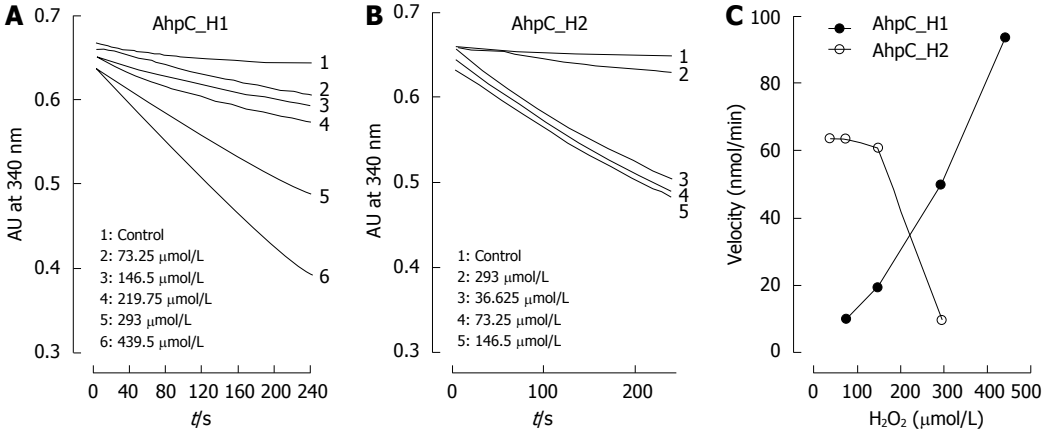

Figure 7 Susceptibility of AhpC_H1 with AhpC_H2 to inactivation by hydrogen peroxide. Peroxidase activity of AhpC_H1 (A) and AhpC_H2 (B) was continuously monitored by the decrease in 340-nm absorbance due to NADPH oxidation at 25 ℃ in the presence of 50 mmol/L of Hepes-NaOH buffer, pH 7.4, containing 1 mmol/L EDTA, 100 nmol/L of *E. coli* Trx reductase, 0.14 mmol/L of NADPH, 5 μmol/L of *E. coli* Trx, 2 μmol/L of AhpC_H1 and AhpC_H2 and varying concentrations of H2O2. Control, absorbance without H2O2; C: Changes in initial peroxidase activity rate depending on H2O2 concentration. AhpC_H1 was resistant, while AhpC_H2 was sensitive to inactivation by H_2O_2.

by western blotting analysis using anti-AhpC_H1 antibodies (Figure 9B, W/2D Western, b). Taken together, these results suggest that the expression of AhpC_H1 in *B. subtilis* is much higher than that of B_BCP, B_TPx, and AhpC_H2.

Hyperoxidation of AhpC_H1

In oxidative stress-exposed *B. subtilis*, the position of the AhpC_H1-corresponding spot at a more acidic area (Figure 9A, W+CMOOH, b) suggests that Cys residue(s) could be hyperoxidized to Cys-SO_2H or Cys-SO_3H[34]. To further investigate AhpC_H1 response to hyperoxidation, exponentially growing *B. subtilis* was treated with increasing concentrations of CMOOH, and protein migration was analyzed by 2D electrophoresis. Figure 9C shows that CMOOH treatment stimulated, at a concentration-dependent manner, the appearance of two different protein spots (b and c) at more acidic positions; at the same time, the original protein spot (a) gradually disappeared, while spot c increased. Considering that sulfinic acid (Cys-SO_3H) is more negatively charged than sulfenic acid (Cys-SO_2H), spots b and c seem to correspond to the sulfenic and sulfinic forms of AhpC_H1. The predominant appearance of the more acidic AhpC_H1 form in the oxidative stress-exposed *B. subtilis* might be considered as evidence that Cys166 residue is the primary site for hyperoxidation, provided that only

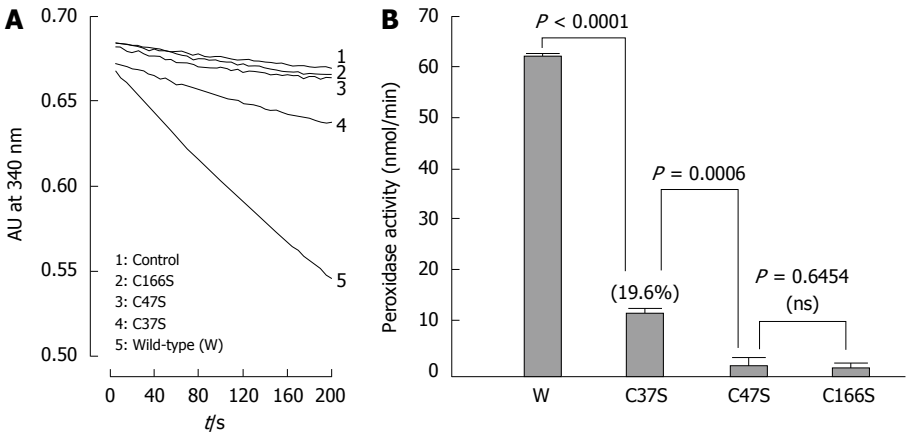

Figure 8 Trx system-supported alkyl hydroperoxide peroxidase activity of the wild-type and mutant AhpC_H1 proteins. A: Peroxidase reaction was initiated by adding 2 μmol/L of the wild-type (W) or C37S, C47S, and C166S mutated AhpC_H1 enzymes to the reaction mixture containing 50 mmol/L Hepes-NaOH, pH 7.4, 1 mmol/L EDTA, 100 nmol/L of *E. coli* Trx reductase, 5 μmol/L of *E. coli* Trx, and 0.14 mM NADPH; the activity was continuously monitored by the decrease in 340-nm absorbance at 25 ℃; B: Initial peroxidase activity rate of the wild-type and mutant AhpC_H1. The data are expressed as the mean ± standard error of three independent measurements.

Cys166 is reduced among the three Cys residues of AhpC_H1.

AhpC_H1 induction in B. subtilis exposed to alkyl hydroperoxides

AhpC_H1 expression in *B. subtilis* in response to oxidative stress was analyzed in exponentially growing cultures treated with different concentrations of H_2O_2, *t*-butyl hydroperoxide (tBOOH), CMOOH, paraquat (PQ), and diamide for 45 min. Western blotting analysis (Figure 10) indicates that AhpC_H1 levels were not significantly affected by H_2O_2, paraquat, and diamide; however, organic peroxides, especially CMOOH, markedly increased AhpC_H1 expression in *B. subtilis*, suggesting the function of AhpC_H1 as an antioxidant scavenger of lipid hydroperoxides.

Phenotype of the AhpC_H1-deficient mutant strain

To determine functional significance of four *Bacillus* Prxs, we compared the growth of the ΔAhpC_H1, ΔAhpC_H2, ΔB_BCP, and ΔB_TPx deletion mutants with that of the parental wild-type strain. All of the mutants exhibited gradual reduction of cell density in the stationary phase (Figure 11A), which could be caused by a decrease of cell viability. Figure 11B shows that among the four null mutants, ΔAhpC_H1 exhibited better survival in the stationary phase, despite much higher levels of AhpC_H1 expression in the wild-type *B. subtilis* compared to other Prxs.

DISCUSSION

Sequence homology and phylogenetic analysis performed in this study reveal that AhpC_H1 is closely related to *E. coli* AhpC (E_AhpC), a typical representative of bacterial AhpC, suggesting that AhpC_H1 may be an E_AhpC ortholog. AhpC_H1 has the highest sequence similarity to E_AhpC among the four analyzed *B. subtilis* Prxs (65.24% identity in a 187-amino acid region), although it possesses an extra Cys37 in addition to two conserved residues, Cys47 and Cys166. AhpC_H2, another AhpC enzyme in *B. subtilis*, showed 38.4% identity with E_AhpC in a 159-amino acid sequence. Our results suggest that unlike AhpC_H1, AhpC_H2 is a member of the typical 2-Cys AhpC/Prx1 subfamily, which includes *E. coli* alkyl hydroperoxide reductase. Comparative structural analysis and evaluation of peroxidase activity, resistance to inactivation by peroxide substrates, and phenotypes of null mutants have demonstrated that AhpC_H1 is a new type of bacterial AhpC, with the structure and functional activity distinct from those of AhpC_H2. Thus, our data indicate that AhpC_H2, not AhpC_H1, is an ortholog of *E. coli* AhpC. AhpC_H1 (GenBank accession number BAA11268) was first suggested to be an AhpC subunit of the *B. subtilis* alkyl hydroperoxide reductase induced in response to stressful conditions such as heat and high salinity or growth inhibition at the stationary phase[35-37]. We found a gradual decrease in the viability of Prx-deficient mutant strains in the stationary phase, which is consistent with previous reports, suggesting a functional role of these enzymes in *B. subtilis* stress response during the stationary growth phase. Together with AhpC_H1, other three *B. subtilis* Prxs are likely to be involved in bacterial defense system against toxic conditions created by increased oxidative stress[38].

Bacterial AhpC enzymes are known as electron acceptors from the Trx system, although they show much higher reactivity with their specialized flavoprotein reductase AhpF[9]. AhpC proteins from *Mycobacterium tuberculosis*[39] and *Helicobacter pylori*[40] have been identified as Trx-dependent alkyl hydroperoxide reductases. In this study, we observed that, regardless of the nature of disulfide linkages (intra- or intermolecular), all four *B. subtilis* Prxs could be easily reduced by the Trx system consisting of Trx1, Trx reductase, and NADPH,

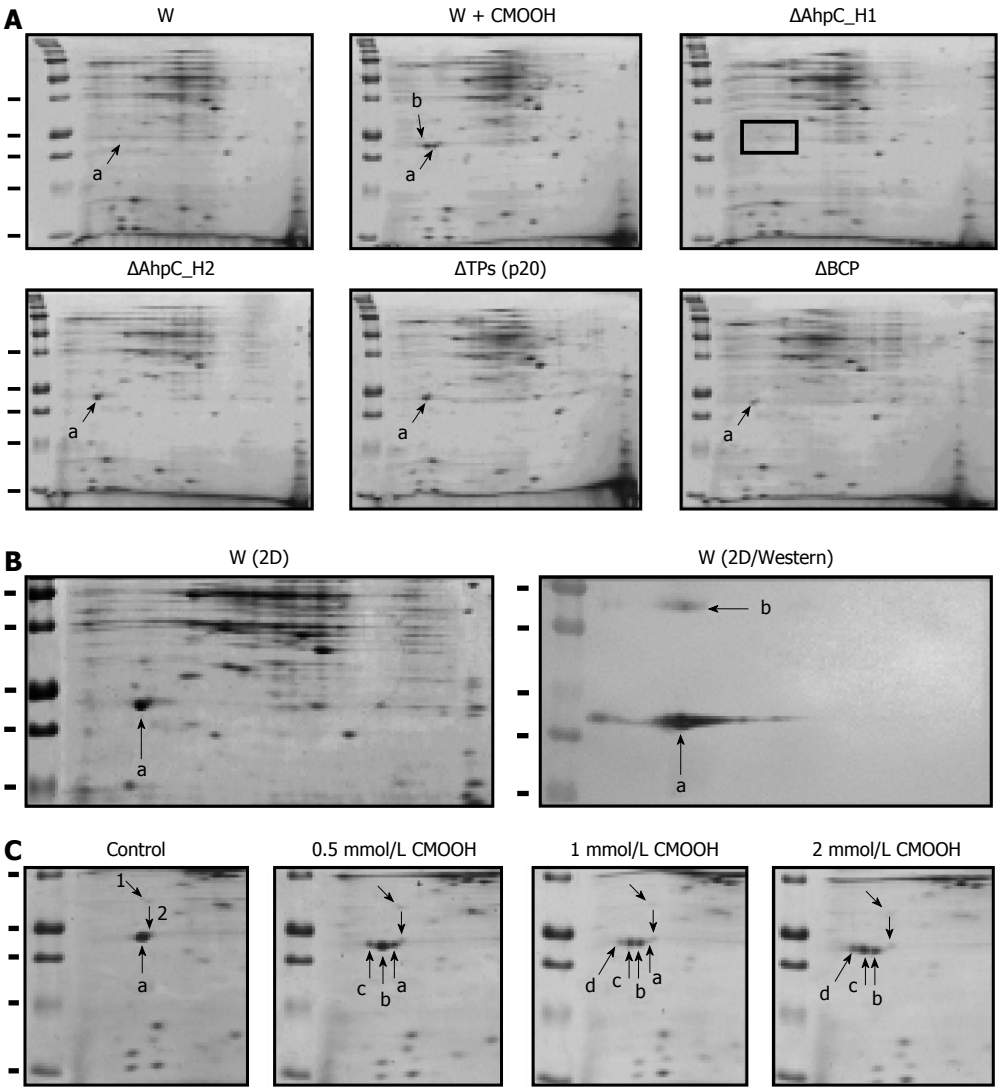

Figure 9 Expression of peroxiredoxins in the wild-type and Prx-deficient *B. subtilis* strains. A: Two-dimensional (2D) electrophoresis of soluble proteins from untreated (W) and cumene hydroperoxide (CMOOH)-treated (W + CMOOH) wild-type *B. subtilis*, and Prx-null mutant strains. Soluble protein fraction (20 μg) obtained from exponential *B. subtilis* cultures treated with or without 1 mM CMOOH for 30 min was separated in 2D gels and analyzed for AhpC_H1 expression after staining with Coomassie Brilliant Blue. a indicates AhpC_H1; b indicates AhpC_H1 shift to acidic area; in AhpC_H1 deletion mutant (ΔAhpC_H1), AhpC_H1-specific signal is absent; B: *B. subtilis* was grown and treated as in (A), and soluble proteins (1 μg) were separated by 2D electrophoresis and stained with Coomassie Brilliant Blue (W/2D) or analyzed by western blotting using polyclonal antibodies against AhpC_H1 (2D/Western); a and b indicate the position of AhpC_H1 monomeric and dimeric forms, respectively; C: *B. subtilis* cultures were grown as in (A), treated with the indicated concentrations of CMOOH for 30 min, and soluble proteins were separated by 2D electrophoresis followed by Coomassie Brilliant Blue staining. a, indicates original and b, c, and d indicate oxidized AhpC_H1 proteins; 1 and 2 denote internal standards. 2D electrophoresis was performed using 4-7 pH gradients (left to right); molecular weight markers (50, 37, 25, 20, and 15 kDa) are shown in the left lines.

although in the oxidized AhpC_H1, the intramolecular disulfide bond was formed between Cys37 and Cys47 and not between the conserved Cys47 and Cys166 residues. Conversion of the reduced AhpC_H1 from a dimeric form to a high-molecular mass oligomer raises a possibility that the disulfide bond in AhpC_H1 has a more complex role as a functional motif, which promotes conformational transitions unrelated to disulfide linkage. In the case of typical 2-Cys Prx members belonging to the AhpC/Prx1 subfamily, hyperoxidation of the conserved Cys residue(s) has been known as a covalent modification, which stabilizes higher order structures[33,41]. We have also demonstrated the existence of the hyperoxidized AhpC_H1 as a predominant form in oxidative-stress-exposed *B. subtilis*. Based on the observation of oxidation-induced dimerization (Figure 6B), we could speculate that the reduction of the disulfide bond between Cys37 and Cys47 may be the first step in the oligomerization process. Covalent modification of Cys residues could stabilize higher order structures as is the case with typical 2-Cys Prx members that lack the intramolecular disulfide bond.

Similar to AhpC_H1, *M. tuberculosis* AhpC has been reported to possess three Cys residues, two at positions 174 and 176 in the C-terminal region, and the conserved Cys61 at the N-terminus. Both Cys174 and Cys176 can form an intermolecular disulfide bond with the conserved N-terminal Cys61 during cyclic catalysis, and all three Cys residues are shown to be important for enzymatic activity[42]. In AhpC_H1, the

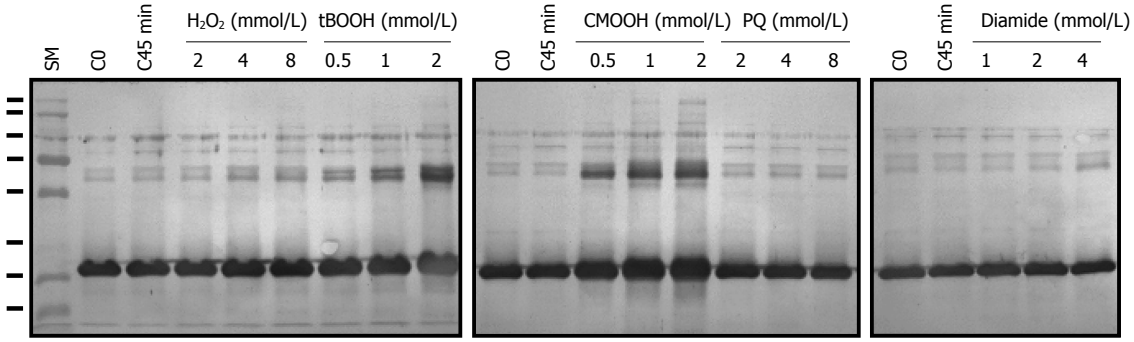

Figure 10 Effects of various oxidants on AhpC_H1 expression in *B. subtilis*. Exponentially grown bacterial cultures were treated with indicated concentrations of H_2O_2, t-butyl hydroperoxide (tBOOH), cumene hydroperoxide (CMOOH), paraquat (PQ), and diamide for 45 min at 30 ℃. Soluble proteins (5 μγ) were separated in 14% reducing SDS-PAGE gels, transferred to nitrocellulose membrane, and reacted with the polyclonal AhpC_H1 antibody. C0 and C45min indicate untreated *B. subtilis* at time 0 and after 45-min growth, respectively. SM, molecular weight markers (15, 20, 25, 37, 50, 75, 100, and 150 kDa).

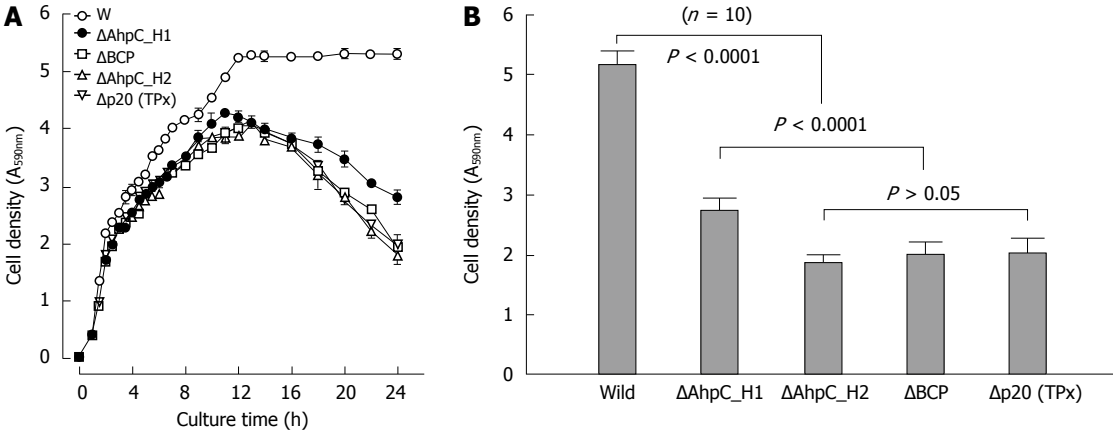

Figure 11 Growth of the wild-type and Prx-deficient ΔAhpC_H1, ΔAhpC_H2, ΔB_BCP, and ΔB_TPx *B. subtilis* strains. A: Bacterial growth was monitored by the optical density at 600 nm (OD600) for up to 25 h after addition of the same cell density of exponentially grown culture in aerobic condition; the data are expressed as the mean ± standard error of five independent measurements; B: The growth the wild-type and Prx-deficient *B. subtilis* strains after 1 d of culture. The data are expressed as the mean ± standard deviation of 10 independent measurements.

additional Cys37 was also observed to be involved in peroxidation catalysis. Distinct catalytic activities of AhpC_H1 and AhpC_H2 toward H_2O_2 (Figure 7C) may provide a rationale for the existence of two different AhpC enzymes in *B. subtilis*, which may function at different concentrations of H_2O_2.

Peroxiredoxins, a class of ubiquitously expressed cysteine-dependent peroxidases, serve not only as ROS detoxifiers but also as regulators of signal transduction pathways and peroxide responses. Thus, an intriguing feature of AhpC_H1 is redox-dependent oligomerization, which regulates enzyme activity. Other functional consequences of this dimer-oligomer conversion remain to be solved, but we hypothesize that the reduction of the intra-subunit disulfide linkage between Cys37 and Cys47 by the Trx system or other electron donors serves to stabilize the oligomeric structure, and further regulates some physiological processes. Given that AhpC_H1 is the most abundant protein among the four types of *B. subtilis* Prxs, the detailed *in vivo* function of AhpC_H1 is worth further investigation.

In summary, structural and functional differences between AhpC_H1 and AhpC_H2 revealed in this study clearly demonstrate that AhpC_H1 is not an ortholog of *E. coli* AhpC, but a novel type of atypical 2-Cys AhpC. The present data also suggest that AhpC_H2, as a typical 2-Cys enzyme, is a true member of the AhpC/Prx1 subfamily, although AhpC_H1 has the highest sequence similarity to *E. coli* AhpC among the four *B. subtilis* Prxs.

COMMENTS

Background

Peroxiredoxins (Prxs) are versatile and predominantly thiol-dependent peroxidases that detoxify hydrogen peroxide and various hydroperoxides. They are ubiquitously expressed, with multiple isoforms present in most organisms. Prxs exhibit high reactivity with hydrogen peroxide and organic hydroperoxides and are involved in peroxide detoxification and signal transduction. The present comparative study was designed to characterize two alkyl hydroperoxide reductase (AhpC) homologs and reveal the biological significance of the existence of two homologous AhpC enzymes in *B. subtilis*.

Research frontiers

This paper is the first report of a detailed structure-functional analysis of the bacillus 2-Cys AhpC homologs AhpC_H1 and AhpC_H2.

Innovations and breakthroughs

AhpC_H1 is not an ortholog of *E. coli* AhpC, but a novel type of atypical 2-Cys AhpC, and AhpC_H2, as a typical 2-Cys enzyme, is a true member of the AhpC/Prx1 subfamily, although AhpC_H1 has the highest sequence similarity to *E. coli* AhpC among the four *B. subtilis* Prxs.

Applications

Peroxiredoxins, a class of ubiquitously expressed cysteine-dependent peroxidases, serve not only as ROS detoxifiers but also as regulators of signal transduction pathways and peroxide responses. Thus, an intriguing feature of AhpC_H1 is redox-dependent oligomerization, which regulates enzyme activity. The reduction of the intra-subunit disulfide linkage between Cys37 and Cys47 by the Trx system or other electron donors serves to stabilize the oligomeric structure, and further regulates some physiological processes. Given that AhpC_H1 is the most abundant protein among the four types of *B. subtilis* Prxs, the detailed *in vivo* function of AhpC_H1 is worth further investigation.

Terminology

Peroxiredoxins (Prxs): a ubiquitous family of antioxidant enzymes that convert a harmful peroxide to water and oxygen. Peroxiredoxins are frequently referred to as alkyl hydroperoxide reductase (AhpC) in bacteria. Other names include thiol specific antioxidant (TSA). This family contains AhpC and TSA, as well as related proteins; Phylogenetic analysis: An analysis of relationships between collection of genes and proteins that are derived from a common ancestor; Orthologous proteins: any proteins found in two or more species that can be traced to a common ancestor; specif. one of two homologous genes that is descended from a common ancestor, but which has evolved in a different way; Homologous proteins: proteins having a very similar primary, secondary, and tertiary structure.

Peer-review

The paper examines the two proteins in an attempt to discover the function of these two homologous enzymes in the same organism, both of which are postulated to protect against oxidative damage in cells. These two proteins were initially identified by sequence homologies, cloned, expressed, and purified. The authors have also generated a number of site-directed mutants of the proteins and examined the role of Cys residues in the proteins. Overall, the work in the manuscript carefully examines both the biochemistry and genetics of the AhpC H1 and H2 proteins. AhpC H1levels increase in response to alky peroxides. The authors have indicated that at least AhpC H1 protects against oxidative stress. It appears that there is a significant drop in cell density. Using the mutant proteins and complementation with the AhpC H1 or H2 would have provided further proof of the role of these proteins. Also, showing actual survival of the cells in response to alkyl peroxides or hydrogen peroxide would have been useful.

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ORIGINAL ARTICLE

Basic Study

Effect of agitation speed on the morphology of *Aspergillus niger* HFD5A-1 hyphae and its pectinase production in submerged fermentation

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Author contributions: Ibrahim D designed and coordinated the research; Weloosamy H performed the majority of experiments; Lim SH analyzed the data; Ibrahim D, Weloosamy H and Lim SH wrote the paper.

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Abstract

AIM: To investigate the impact of agitation speed on pectinase production and morphological changing of *Aspergillus niger* (*A. niger*) HFD5A-1 in submerged fermentation.

METHODS: *A. niger* HFM5A-1 was isolated from a rotted pomelo. The inoculum preparation was performed by adding 5.0 mL of sterile distilled water containing 0.1% Tween 80 to a sporulated culture. Cultivation was carried out with inoculated 1×10^7 spores/mL suspension and incubated at 30 ℃ with different agitation speed for 6 d. The samples were withdrawn after 6 d cultivation time and were assayed for pectinase activity and fungal growth determination. The culture broth was filtered through filter paper (Whatman No. 1, London) to separate the fungal mycelium. The cell-free culture filtrate containing the crude enzyme was then assayed for pectinase activity. The biomass was dried at 80 ℃ until constant weight. The fungal cell dry weight was then expressed as g/L. The 6 d old fungal mycelia were harvested from various agitation speed, 0, 50, 100, 150, 200 and 250 rpm. The morphological changing of samples was then viewed under the light microscope and scanning electron microscope.

RESULTS: In the present study, agitation speed was found to influence pectinase production in a batch cultivation system. However, higher agitation speeds than the optimal speed (150 rpm) reduced pectinase

production which due to shear forces and also collision among the suspended fungal cells in the cultivation medium. Enzyme activity increased with the increasing of agitation speed up to 150 rpm, where it achieved its maximal pectinase activity of 1.559 U/mL. There were significant different (Duncan, $P < 0.05$) of the pectinase production with the agitation speed at static, 50, 100, 200 and 250 rpm. At the static condition, a well growth mycelial mat was observed on the surface of the cultivation medium and sporulation occurred all over the fungal mycelial mat. However with the increased in agitation speed, the mycelial mat turned slowly to become a single circular pellet. Thus, it was found that agitation speed affected the morphological characteristics of the fungal hyphae/mycelia of *A. niger* HFD5A-1 by altering their external as well as internal cell structures.

CONCLUSION: Exposure to higher shear stress with an increasing agitation speed could result in lower biomass yields as well as pectinase production by *A. niger* HFD5A-1.

Key words: *Aspergillus niger*; Agitation; Submerged culture; Pectinase; Microbial growth; Dissolved oxygen

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Core tip: We report the influence of agitation speed on pectinase production on our newly isolate *Aspergillus niger* (*A. niger*) HFM5A-1 in submerged fermentation. The agitation speed was found to influence pectinase production in a batch cultivation system. However, higher agitation speeds than the optimal speed (150 rpm) reduced pectinase production which due to shear forces and also collision among the suspended fungal cells in the cultivation medium. It was found that agitation speed affected the morphological characteristics of the fungal hyphae/mycelia of *A. niger* HFD5A-1 by altering their external as well as internal cell structures.

Ibrahim D, Weloosamy H, Lim SH. Effect of agitation speed on the morphology of *Aspergillus niger* HFD5A-1 hyphae and its pectinase production in submerged fermentation. *World J Biol Chem* 2015; 6(3): 265-271 Available from: URL: http://www.wjgnet.com/1949-8454/full/v6/i3/265.htm DOI: http://dx.doi.org/10.4331/wjbc.v6.i3.265

INTRODUCTION

Pectinases constitute is a unique group of enzymes that cleave the glycosidic bonds of galacturonic acid residues of pectic substances, which are the complex structural polysaccharides of plant cells. Major pectinases are refer to polygalacturonase, pectin lyase, pectate lyase and pectin esterase[1]. Pectinases are useful industrial enzyme and are largely used in various industrial processes such as for solubilization of the cell wall of plants, wood, fruit or paper[2,3].

There are several parameters influencing the maximal enzyme production in submerged fermentation, usually it consist of physical and chemical parameters. Physical parameters involving the optimization of initial medium pH, cultivation temperature, inoculum sizes and also agitation speed. Microorganisms differ in their oxygen requirement. Oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The rate of the agitation speed could influenced the extent of mixing in the shake flasks system and affected the nutrient availability as well[4]. Agitation speed has been affected many enzymes activities in different strains of bacteria and fungi[5,6] as well as microalgae[7].

Agitation provides adequate mixing, mass, heat transfer and also improving dissolved oxygen in the culture medium. At lower agitation speed insufficient oxygen in the culture medium usually affects the microbial growth, whereas higher agitation speeds sometimes also lowering the enzymes production[8]. Higher agitation speed develop shear forces among the suspended microbial cells in the culture medium and the production drops due to cell damages which results from cell collision. Shear forces also can give several effects on the fungal cell. It can cause the morphological changes to the fungal by damaging the external and internal cell structures, variation in fungal growth and yield formation[9]. Therefore, the optimal agitation speed is necessary to be determined in order to obtain maximal enzyme production. The aim of this study was to determine the influence of agitation speed on pectinase production and morphology of *Aspergillus niger* (*A. niger*) HFD5A-1 in submerged fermentation using a flask system.

MATERIALS AND METHODS

Microorganisms, culture maintenance and inoculum preparation

A. niger HFM5A-1 which was isolated from a rotted pomelo was supplied by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The fungal culture was maintained on potato dextrose agar (Merck, Germany) slant supplemented with 1.0% citrus pectin (w/v) (Sigma, Denmark) at 30 ℃ for five days aerobically (until sporulation) before storing them at 4 ℃ until further use. The cultures were sub-cultured regularly at every month to maintain viability.

The inoculum preparation was performed by adding 5.0 mL of sterile distilled water containing 0.1% Tween 80 to a sporulated culture. The spores were dislodged by using a sterile inoculation loop and shaking vigorously. The spore suspension was adjusted to 1 × 10^7 spores/mL using haemocytometer chamber

(Neubauer Germany) and 1.0 mL was taken as the inoculum.

Cultivation medium and fermentation

The cultivation medium used for fermentation was a modified medium of Maldonado and Strasser de Saad[10]. The medium consisted of (w/v): KH_2PO_4, 0.4%; Na_2HPO_4, 0.2%; $FeSO_4.7H_2O$, 0.02%; $CaCl_2$, 0.001%; peptone, 0.4%; $MnSO_4.7H_2O$, 0.007%; H_3BO_3, 0.001% and citrus pectin, 1.8%. The initial pH of the medium was adjusted to 4.5. Cultivation was carried out after inoculated with 1 × 10^7 spores/mL suspension and incubated at 30 ℃ with different agitation speed for 6 d. The samples were withdrawn after 6 d cultivation time and were assayed for pectinase activity and fungal growth determination.

Enzyme recovery and fungal biomass determination

The culture broth was filtered through filter paper (Whatman No. 1, London) to separate the fungal mycelium. The cell-free culture filtrate containing the crude enzyme was then assayed for pectinase activity.

The filter paper containing biomass was dried at 80 ℃ until constant weight and the fungal cell dry weight was obtained by deducting the weight of filter paper and the fungal biomass from the weight of filter paper alone. The fungal cell dry weight was then expressed as g/L. All the experiments were performed in triplicate and the values were reported as standard deviations.

Assay for pectinase activity

Pectinase assay was carried out by measuring reducing sugars release from pectin hydrolyzation. One aliquot of sample was added to a solution containing one aliquot of 1% pectin in 0.1 mol/L citrate buffer with pH 4.5. After incubation at 45 ℃ for 30 min, reducing sugars were determined by the dinitrosalicylic acid (DNS) method[11] using galacturonic acid as a reference. The enzymatic activity was expressed in terms of Unit (U). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of galacturonic acid per mL of culture filtrate per minute under the assay conditions.

Scanning electron microscopy studies

The 6 d old fungal mycelia were harvested from various agitation speed, 0, 50, 100, 150, 200 and 250 rpm, washed three times with sterile distilled water and then blotted dry in Whatman filter papers. The samples for scanning electron microscopy (SEM) were prepared as described previously by Darah *et al*[5] and Darah and Ibrahim[12] and observed under FESEM LEO Supra 50VP, Carl Zeiss, Germany.

Statistical analysis

In order to evaluate and determine the significant of the findings and also to compare the differences among the findings, the statistical analysis was used. One way Analysis of Variance (ANOVA) and Duncan Multiple Range Test (DMRT) with PASW Statistics 18 version were used to analyze the significant different of the mean of experimental data. A 5% confidence level or α = 0.05 were used to test all experimental data. All enzyme activities and fungal growth were made in triplicates. The error bars showed the standard deviation of triplicates.

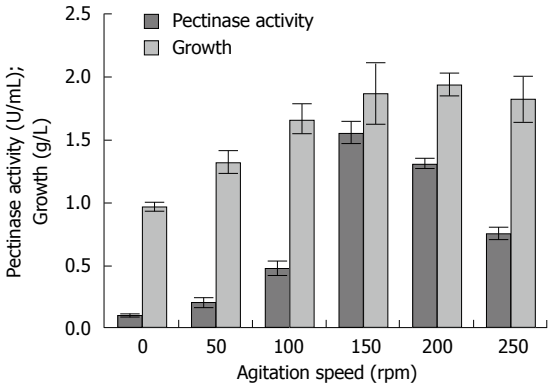

Figure 1 Effect of agitation speeds on pectinase, fungal growth and protein content by *Aspergillus niger* HFD5A-1 at 6 d cultivation time.

RESULTS

Enzyme activity rises up with the increasing of agitation speed up to 150 rpm (Figure 1), where it achieves its maximum pectinase activity of 1.559 U/mL. There were significant different (Duncan, $P < 0.05$) of the pectinase production with the agitation speed at static, 50, 100, 200 and 250 rpm. However, its highest fungal growth was showed at 200 rpm with 1.938 g/L. The agitation speed which not at 150 rpm resulted in low pectinase production. The lowest pectinase production was from the non-agitated culture (static condition) with about 0.111 U/mL and of 0.972 g/L of fungal growth.

The influence of agitation speed was found had a great impact on the morphology of *A. niger* HFD5A-1 (Figure 2). At static condition, a mycelial mat was discovered on the top of the cultivation broth (Figure 2A) and sporulation occurred all over the fungal mycelial mat. Nevertheless increased in agitation speed, the mycelial mat turned to form a single spherical pellet. It was noted that at 50, 100 and 150 rpm of agitation produced slimy mycelial clumps that were small (2.8 cm; Figure 2B), medium (3.6 cm; Figure 2C) and big (4.7 cm; Figure 2D) sizes, respectively. There were no sporulation observed at 50, 100 and 150 rpm. However, mycelial clumps formed at 150 rpm were in intense yellow in coloration. At 200 rpm, a few spherical and elongated pellet of fungal growth with the sizes between 0.6-1.0 mm were formed (Figure 2E). Besides, at 250 rpm a mixture of small and bigger sizes of fungal pellets were formed, and the small fungal pellet were those

Figure 2 Mycelial morphology of *Aspergillus niger* HFD5A-1 observed with naked eyes at different agitation speeds at 6 d cultivation time. A: Static condition; B: 50 rpm; C: 100 rpm; D: 150 rpm; E: 200 rpm; F: 250 rpm.

that detached from big pellets (Figure 2F).

In order to have a closer view of the fungal pellets, they were further analyzed under stereo light microscope at different agitation speeds (Figure 3). Figure 3A (static condition) shows loosely packed mycelium with sporulation, but the density of the mycelium were observed to increase with increasing agitation speeds from 50 rpm to 150 rpm (Figure 3B-D). Conversely, at 200 and 250 rpm (Figure 3E and F) there were decreasing in density of mycelium.

Observations under scanning electron microscope were also carried out to have a clearer view of Figure 3. SEM micrographs (Figure 4) show the mycelia of fungal are cylindrical in shape and branched with fruiting bodies. This condition occurred to the fungal culture agitated at low agitation speeds, *i.e.*, at static (Figure 4A), 100 rpm (Figure 4B) and 150 rpm (Figure 4C), whereas at higher agitation speeds of 200 rpm (Figure 4D) the fungal culture exhibited lesser branching mycelia. There was no formation of fruiting bodies at 200 rpm. In addition, fungus grown in static condition showed branched mycelia and numerous formations of fruiting bodies (Figure 4A). At 100 rpm (Figure 4B) fungal growth more on the formation of hyphae with less fruiting body. This condition can be seen in the formation of tight packing structures. However, agitation speed of 150 rpm (Figure 4C) exhibited dense and branched mycelia with increasing formation of fruiting bodies. The fungal growth was best at the agitation of 200 rpm (Figure 4D) which exhibited smoother, not well rounded of hyphae and dense with high branched mycelia. The formations of chlamydospores also observed at 200 rpm.

DISCUSSION

Generally, filamentous fungi have a typical polarized growth pattern in the form of cannular filaments name

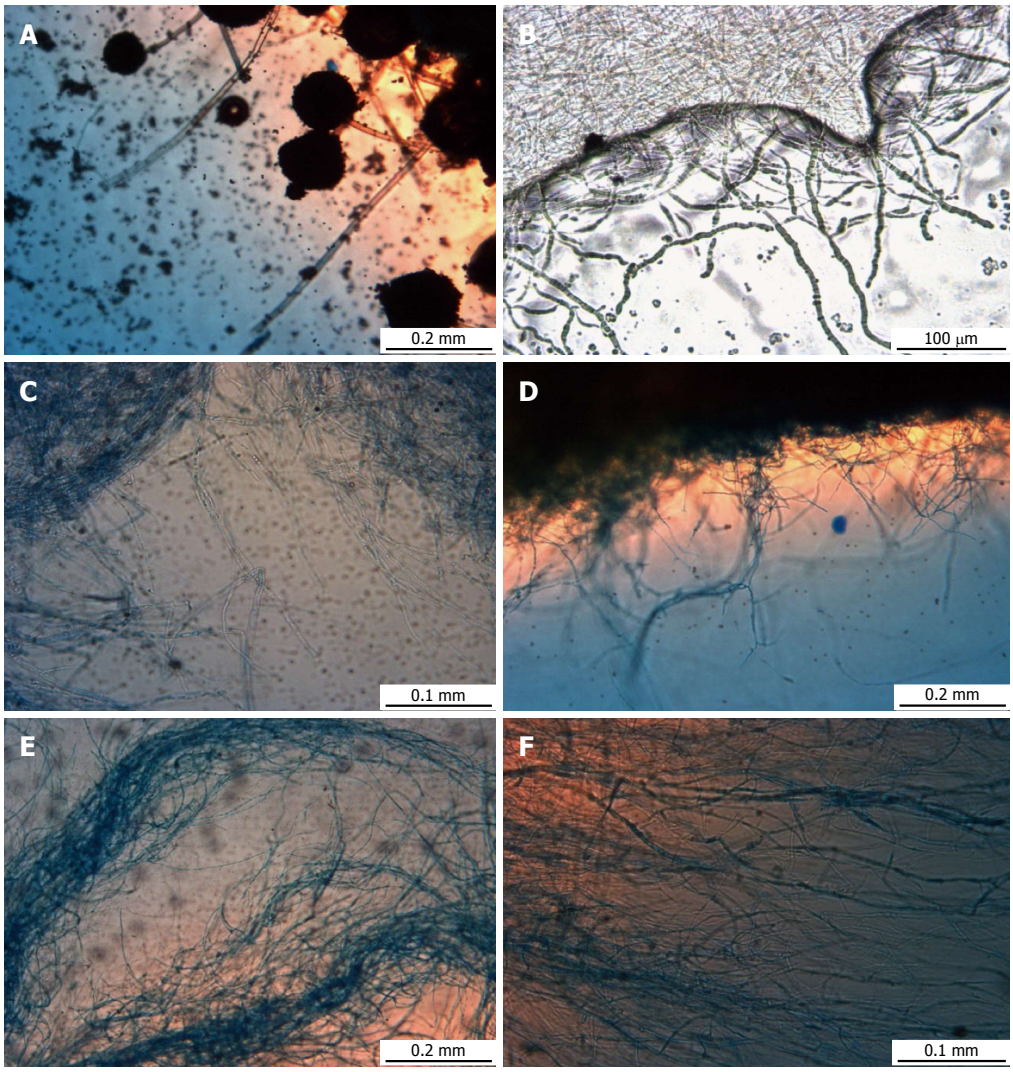

Figure 3 Mycelial morphology of *Aspergillus niger* HFD5A-1 observed under light microscope at different agitation speeds at 6 d cultivation time. A: Static condition; B: 50 rpm; C: 100 rpm; D: 150 rpm; E: 200 rpm; F: 250 rpm.

as hyphae[9]. The hyphae then grow in branches to form mycelium. In submerged cultures, the fungal culture exhibits two major morphologies observed as pellets and free mycelium. The pellets are clumps and dense agglomerates of hyphae which may be spherical or ellipsoidal agglomerates[13,14], whereas the free mycelia, are freely spread throughout the culture medium[9]. These morphologies are very much influenced by some environmental and genetic factors such as type of organism, pH and composition of the media, inoculation ratio, agitation speed and aeration rate, feeding rate and genetic factors of the culture[15,16]. The morphology of filamentous fungi in submerged fermentation has great effect on metabolite production as well as productivity. These two morphologies exhibit significant differences during the cultivation period[17].

Agitation speed has been shown to affect many enzyme productions by microorganisms. In the present investigation, *A. niger* HFD5A-1 showed highest pectinase activity at 150 rpm after 6 d of cultivation. This is probably due to aeration of the culture medium was increased and dissolved oxygen in the media was sufficient. Nutrient uptake by fungus increased resulting in increased pectinase production. At 200 rpm, the enzyme production was dropped even though the fungal growth increased considerably. The adequate aeration and nutrient might caused the fungus to grow well but shear forces and cell damage finally had negative effect on enzyme production[18]. However, the different strains of fungus demonstrated different optimal agitation speeds for maximize the enzyme production yield in submerged fermentation. Darah *et al*[19] reported that *A. niger* FETL FT3 has shown the maximum production of tannase activity at 200 rpm agitation speed instead of 150 rpm agitation speed.

Agitation speed and dissolved oxygen concentration on pectinase production are important factors leading to successful progress of fermentation. Sufficient dissolved oxygen in medium is an important for microbial cells in mass transfer characteristics example substrate and product[5]. Agitation provides adequate

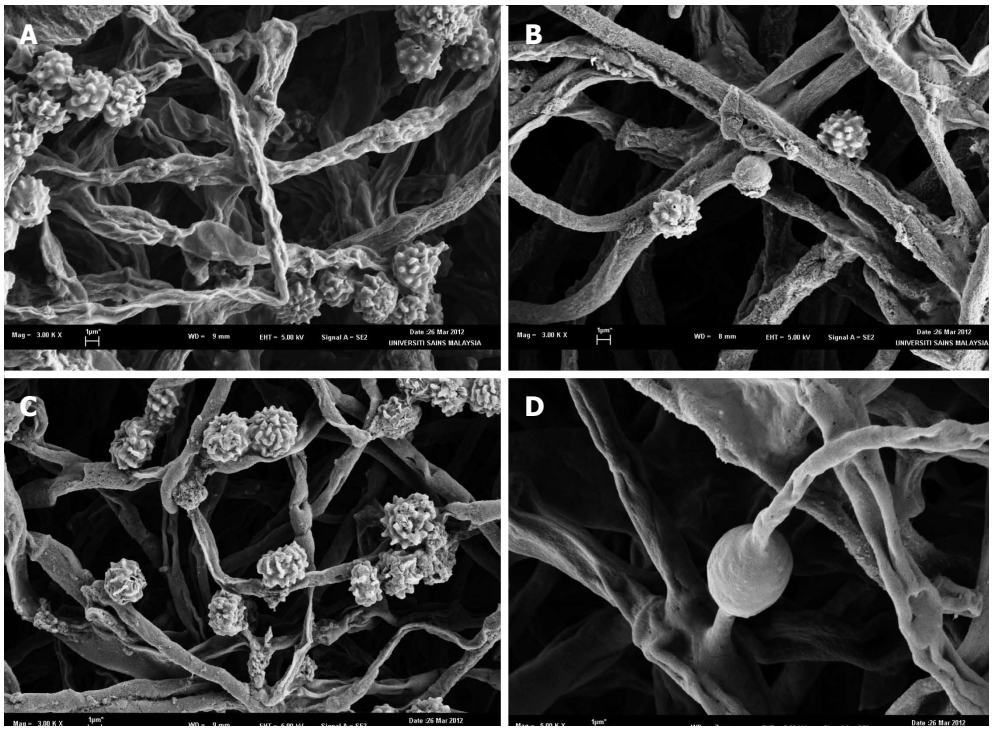

Figure 4 Scanning electron microscopy micrographs of the mycelial morphology *Aspergillus niger* HFD5A-1 at different agitation speed at 6 d cultivation time. A: 0 rpm; B: 50 rpm; C: 150 rpm; D: 200 rpm.

mixing, mass and heat transfer. However, agitation also creates shear forces, causing morphological changes, and eventually damage to the cell structure[5,20].

In industrial applications submerged cultures of filamentous fungi are widely used to produce commercially products and pellet form is usually preferred in downstream fermentation process due to the non viscosity of fermentation medium[14]. Hence, the mass transfer of oxygen and nutrients is much easier and also the separation of the pellets from the medium is noncomplex[21]. Furthermore agitation and aeration are simple technique in fermentation system, therefore the operating cost is not costly. Thus, agitation rate need to be controlled in order to obtain maximum production.

Agitation speed is an important factor in enhancing pectinase yield produce by *A. niger* HFD5A-1 in submerged fermentation. However, agitation speeds beyond than the optimum speed (150 rpm) resulted in low pectinase production.

COMMENTS

Background

Agitation speed is a crucial factor affected the enzyme production in submerged fermentation system. Therefore, the optimal agitation speed is necessary to be determined in order to obtain maximal enzyme production. The purpose of this study was therefore to evaluate the influence of agitation speed on pectinase production as well as morphology of *Aspergillus niger* (*A. niger*) HFD5A-1 in submerged fermentation using a flask system which has not been studied yet.

Research frontiers

The author aimed to to evaluate the influence of agitation speed on pectinase production as well as morphology of *A. niger* HFD5A-1 in submerged fermentation using a flask system.

Innovations and breakthroughs

This study demonstrates agitation speed plays an important role in enhancing pectinase production by *A. niger* HFD5A-1 in submerged fermentation. However, higher or lower agitation speeds than the optimum speed (150 rpm) resulted in low pectinase production. Even though agitation increases the dissolved oxygen level in the cultivation medium, higher agitation speed can cause damage to the fungal cells that was due to shear forces and collision among the fungal pellets. Therefore, the relationship among morphology and rheology as well as the factors influencing them has to be fully investigated.

Applications

The authors hypothesize that agitation speed plays an important role in enhancing pectinase production by *A. niger* HFD5A-1 in submerged fermentation. The information obtained should assist progress towards the large scale production of pectinase by submerged cultivation of *A. niger* HFD5A-1.

Peer-review

In this paper, Darah *et al* report the effect of agitation speed on the morphology of *A. nige* HFD5A-1 hyphae and its pectinase production in submerged fermentation. The findings are interesting.

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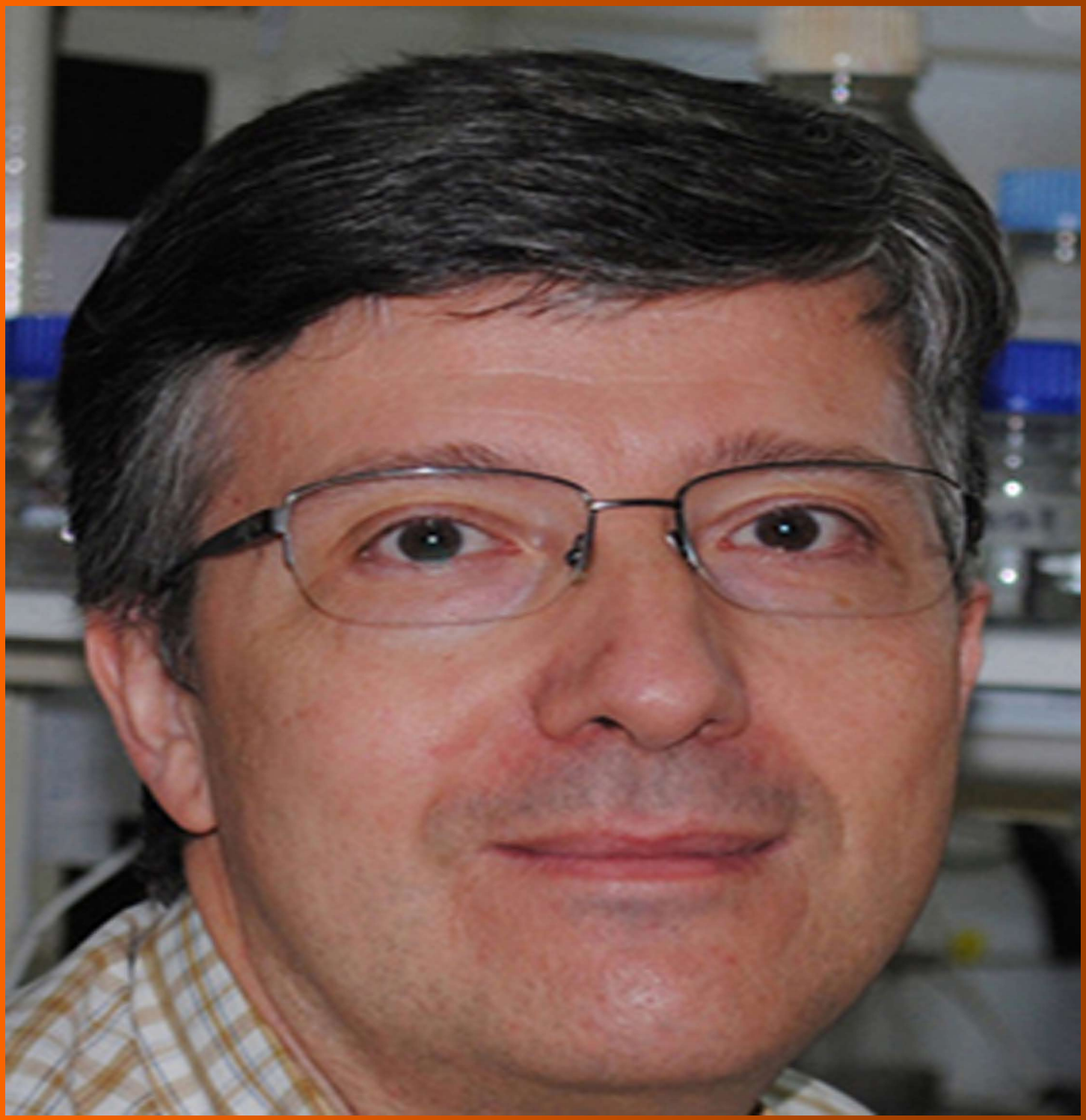

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FRONTIER

Discovery and characterization of the first non-coding RNA that regulates gene expression, *micF* RNA: A historical perspective

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Abstract

The first evidence that RNA can function as a regulator of gene expression came from experiments with prokaryotes in the 1980s. It was shown that *Escherichia coli micF* is an independent gene, has its own promoter, and encodes a small non-coding RNA that base pairs with and inhibits translation of a target messenger RNA in response to environmental stress conditions. The *micF* RNA was isolated, sequenced and shown to be a primary transcript. *In vitro* experiments showed binding to the target *ompF* mRNA. Secondary structure probing revealed an imperfect *micF* RNA/*ompF* RNA duplex interaction and the presence of a non-canonical base pair. Several transcription factors, including OmpR, regulate *micF* transcription in response to environmental factors. *micF* has also been found in other bacterial species, however, recently Gerhart Wagner and Jörg Vogel showed pleiotropic effects and found *micF* inhibits expression of multiple target mRNAs; importantly, one is the global regulatory gene *lrp*. In addition, *micF* RNA was found to interact with its targets in different ways; it either inhibits ribosome binding or induces degradation of the message. Thus the concept and initial experimental evidence that RNA can regulate gene expression was born with prokaryotes.

Key words: Non-coding RNAs; RNA/RNA interaction; Regulation of gene expression; *micF* RNA; Trans-acting RNA gene

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Core tip: The original discovery and characterization of the first non-coding RNA gene and its transcript was with prokaryotes in the 1980s. At that time the *Escherichia coli micF* RNA gene was characterized in terms of properties, its promoter region, and activation by environmental stress conditions; and the *micF* RNA transcript structure as well as the *micF* RNA/target messenger RNA duplex interaction were elucidated. This occurred over 5 years before the discovery of the first eukaryotic regulatory miRNA, which is not generally recognized. Prokaryotic and eukaryotic non-coding RNAs greatly differ in terms of RNA

processing, but the basic principle of an RNA gene locus encoding a regulatory RNA that targets gene expression *in trans via* RNA/target RNA duplex formation is similar. Thus the concept and discovery of regulatory non-coding RNAs and their functions in messenger RNA inhibition originated with prokaryotes.

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INTRODUCTION

It is now recognized that regulation of gene expression by non-coding RNAs *via* RNA/mRNA interactions is a vastly widespread phenomena that occurs in all biological domains, including viruses, and it has become a basic principle in biology. Current research on non-coding RNAs has essentially "exploded"[1-7]. Yet before the 1980s, RNAs were thought of as macromolecules that primarily supported the protein synthesis machinery, were considered inert, and the concept of RNA as a regulator was unheard of.

In this review we focus on early history of the experimental work that showed that RNA is a regulator of gene expression, and this occurred approximately 30 years ago in the 1980s. *micF* RNA was the first regulatory RNA discovered[8-11]. This preceded the discovery of regulation of gene expression by RNAs in eukaryotes, which was in the 1990s[12]. Prokaryotic non-coding RNAs that regulate gene expression are termed sRNAs; the eukaryotic RNAs are termed microRNAs (miRNAs).

The second bacterial regulatory RNA discovered was DsrA. This RNA transcript was found to mediate *rpoS* expression in 1995[13] but its mechanism of action *via* RNA/RNA binding was not determined until 1998[14,15].

ESCHERICHIA COLI 6S RNA, SPOT 42, EUKARYOTIC 7SK RNA AND CRISPRS

The discovery that RNA can serve a regulatory role was in the 1980s, however, three regulatory RNAs were found before that but were uncharacterized: *Escherichia coli (E. coli)* 6S RNA, Spot 42 and the eukaryotic 7SK RNA. These RNAs were discovered and sequenced about 40 years ago but their functions were not elucidated until decades later. The 6S RNA was first detected in 1967[16] and was sequenced by George Brownlee in 1971[17]. Its function was determined approximately 30 years later in 2000 by Wassarman *et al*[18] who showed that 6S RNA binds the RNA polymerase-sigma70 complex and inhibits sigma factor-dependent gene transcription.

The second non-coding RNA found and also uncharacterized until decades later was the transcript termed Spot 42 encoded by the *E.coli spf* gene. It was first found in 1973 as a transcript on two-dimensional gel electrophoresis by Ikemura *et al*[19], but its function as a regulatory non-coding RNA was not determined until 2002 when Valentine-Hansen's lab showed that Spot 42 binds the *galK* mRNA and inhibits the galactose operon[20]; however, more recently it has been shown to also target fourteen other operons[21].

As to eukaryotic RNAs, in 1976 Gary Zieve and Sheldon Penman found several small RNAs in HeLa cells, one was the 7SK RNA, but its function and the function of the other RNAs were unknown at the time[22]. Almost 30 years later it was shown that 7SK RNA binds to a HEXIM1 protein complex, which then binds to the transcription elongation factor P-TEFb and inhibits transcription[23,24].

There are parallels between the prokaryotic 6S RNA and the eukaryotic 7SK RNA - both were found decades before any functional roles were determined and both bind proteins resulting in inhibition of transcription. The majority of small non-coding RNAs regulate post-transcriptionally by binding target mRNAs.

Clustered regularly interspaced short palindromic repeats (CRISPRs) were discovered in *E.coli* in 1987 by Ishino *et al*[25]. CRISPRs are an array of genomic repeat sequences, which are separated by spacer sequences that originate from viral or plasmid DNA. They represent an acquired bacterial immune system. Similar to the RNAs discussed above, functions and mechanism of regulation of the CRISPR system as small RNA/target DNA inhibitors were not elucidated until about two to three decades later[26-29] (references shown represent only a partial list of contributors).

The bacterial CRISPR-specific RNA processing and targeting mechanism has been compared to the eukaryotic RNAi mechanism and they display striking similarities, although they are not homologous[30]. The CRISPR system currently represents one of the fastest moving fields in molecular biology, primarily because of its potential to alter gene structure and induce chromosomal rearrangements. On the other hand, Morange points out that some earlier pioneer work with the CRISPR system went under-appreciated[31].

COLE1 RNAI

In 1981 two labs showed that antisense RNAs can inhibit plasmid replication in *E. coli* [32,33]. These are milestone experiments as they were the first to demonstrate a regulatory function for RNA. In addition, Tomizawa[34], working with ColE1 plasmid replication, demonstrated that antisense RNA I binds to and inhibits the primer RNA II that is involved in initiating DNA replication.

Tomizawa[34] presented a model of antisense RNA/primer RNA recognition by the so-called "kissing interaction". This involves a two-step process, whereby initially there is a loop-loop interaction *via* Watson-Crick base-pairing between the short stem loops of

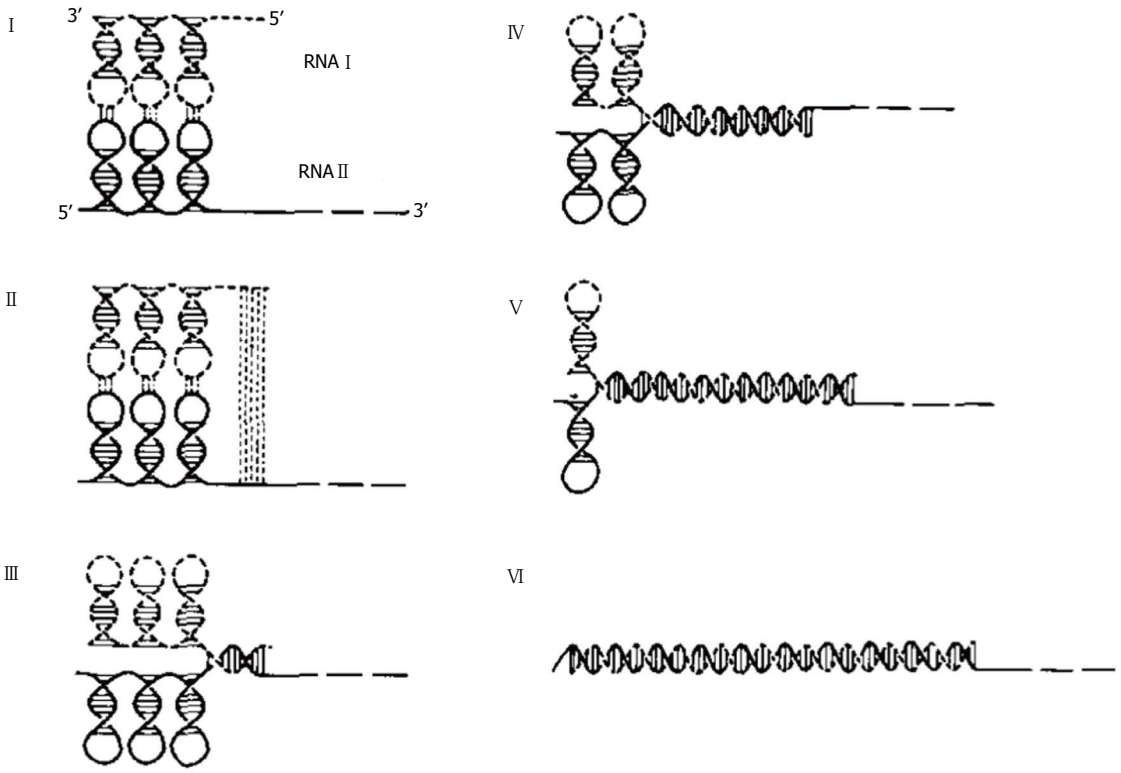

Figure 1 Model of two step interaction between antisense RNA Ⅰ and sense RNA Ⅱ-initial stem loop recognition followed by antisense/sense RNA/RNA duplex formation[34].

RNA Ⅰ and RNA Ⅱ, followed by pairing of unstructured single-stranded segments of the two RNAs and eventual melting of the stem loops and formation of an antisense/sense RNA/RNA duplex (Figure 1).

The kissing interaction was another pioneer proposal by Tomizawa[34] whereby we now know there is board significance to the initial recognition of two RNAs *via* stem loop interactions[35-39]. The loop-loop interaction has also been predicted to occur in both the prokaryotic and eukaryotic non-coding RNA/target RNA recognition process[40-42].

There were also other significant plasmid-related experiments at that time. In 1981 Rosen *et al*[43] found three RNA transcripts encoded by plasmids R100 and R1, one a counter transcript, but RNA functions were not fully elucidated. Additionally, Simons *et al*[44] and Kittle *et al*[45] showed that an antisense counter transcript from the insertion sequence of transposon IS10 inhibits transposase expression *via* RNA/RNA interactions and predicting an initial loop interaction.

MICF RNA

In 1984, Mizuno *et al*[8] were the first to present the concept of a regulatory RNA gene that controls expression of another gene *via* RNA/RNA base-pairing. This is the *E. coli micF* gene whose transcript inhibits the expression of the target gene *ompF*. This was based on multicopy plasmid repression of OmpF protein from a sequence upstream of the *ompC* gene. This sequence has complementarity to *ompF* mRNA[8]. OmpF is an outer membrane protein, a porin protein that allows small molecules to diffuse passively through the cell. Thus it is an important protein to regulate in terms of protecting the cell from deleterious molecules.

At that time it was a rather bold and innovative proposal that an RNA can regulate gene expression, There were no role models for such a function, and RNA was for the most part still considered a passive macromolecule that participates in the protein synthesis process and formed the scaffolding for ribosomal proteins, notwithstanding the previous work on antisense RNA repression of plasmid replication[32,33].

Dr. Inouye should be credited for his creativity in first proposing the existence of a regulatory RNA gene. But his lab had only multicopy plasmid effects and had not isolated the RNA transcript. An RNA molecular biologist was needed and I was asked to contribute to the work. Subsequently my lab isolated and sequenced the chromosomal-encoded *micF* RNA, found that *micF* is an independent gene with its own promoter, determined the regulatory RNA/target RNA base-pairing by structure probing, and determined its function in terms of regulation of target gene in response to environmental stress conditions[9,10].

By using *in vivo* P^{32} labeling, isolating low molecular weight RNAs and separation on gels, we found a 4.5S RNA transcript. Its sequence showed that it was *micF* RNA and that it was 93 nt long with a rho independent termination site[9]. To characterize the micF promoter

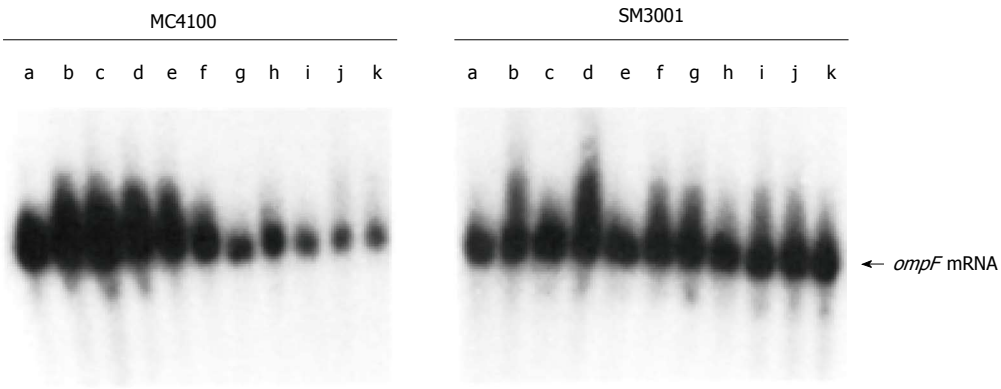

Figure 2 *ompF* mRNA levels after temperature shift from 24 ℃ to 42 ℃. Lanes marked a-k correspond to time 0-120 min. Left, RNA from strain MC4100 that has the *micF* gene; right, strain SM3001 that is a micF deletion strain. Data from Andersen *et al*[10]. Reproduced with permission.

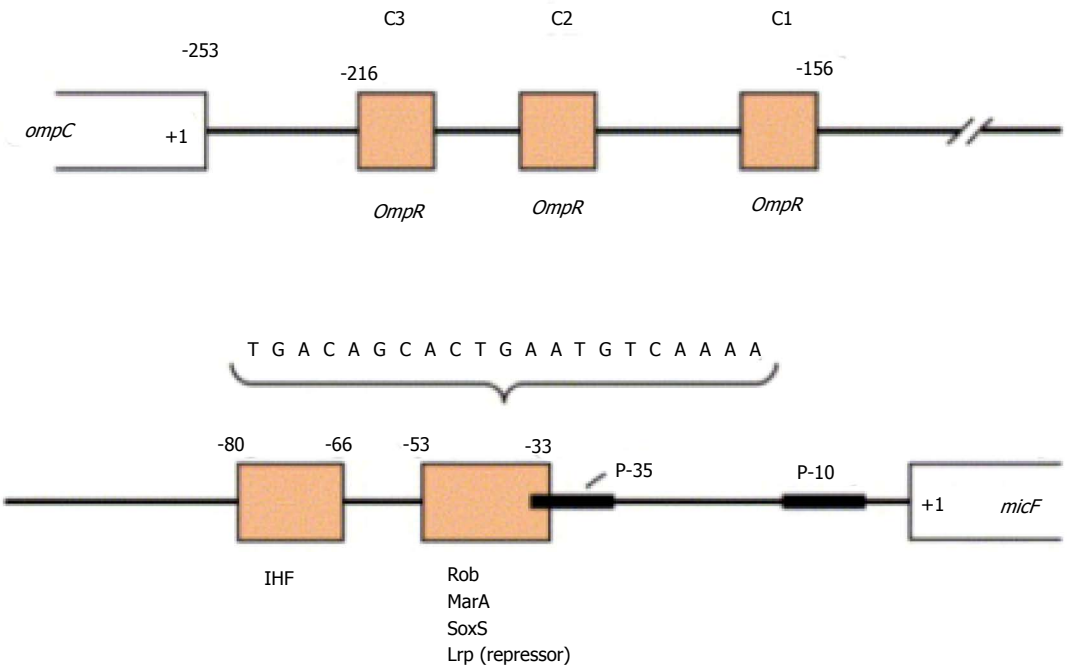

Figure 3 Schematic of *micF* gene, upstream P-10, P-35 promoter region and transcription factor binding sites. Diagram modified from Delihas *et al*[51].

regions, lacZ was fused at different sites upstream of the *micF* gene and *micF* was found to have a strong promoter based on beta galactosidase activity.

MICF RESPONSE TO STRESS CONDITIONS

A search for function was initiated by looking at the response of *micF* expression to various environmental factors. A major effect was found with temperature change and other factors. Levels of *micF* RNA increased dramatically in response to cell growth at high temperature[10]. The levels of *ompF* mRNA and the OmpF protein were also measured and found to decrease under these conditions. Figure 2 shows a marked decrease in *ompF* mRNA levels with temperature increase in the wild type strain, but no significant change in the *micF* deletion strain (SM3001). The findings revealed that *micF* RNA plays a major role in the thermal regulation of *ompF* mRNA and OmpF protein. However, the mechanism of thermal regulation of *micF* expression is still an open question, but temperature has been shown to regulate DNA supercoiling that is associated with the induction of stress related genes[46-49]. *micF* expression was also found to respond to other environmental stress conditions such as osmolarity change and exposure to ethanol[10,50].

The upstream *micF* regulatory region is complex in that it contains binding sites for a number of transcription factors; these regulate *micF* expression in response to different environmental stress factors[51]. Five transcription factors are known to bind in the upstream regulatory region (Figure 3). With respect to osmolarity change, there are three binding sites (C1-C3) for transcription factor OmpR that activates expression of *micF* in response

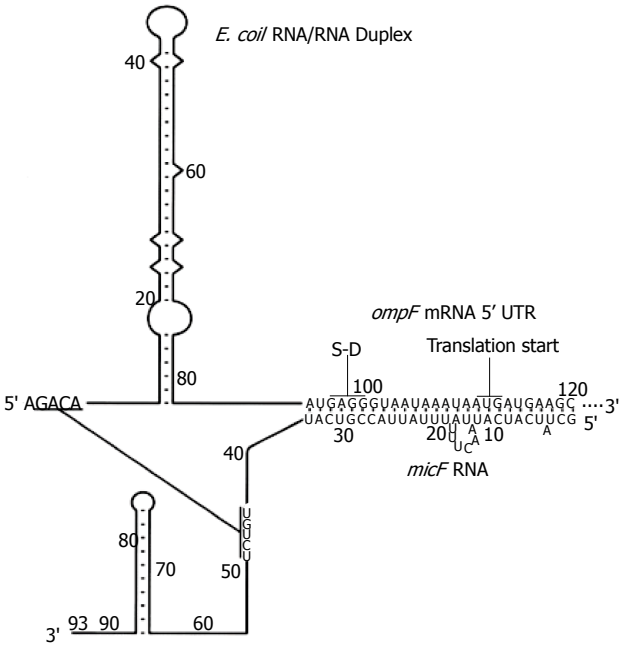

Figure 4 Model of *micF* RNA/*ompF* mRNA interaction. Duplex based on secondary structure probing; the long range pairing of five bases at the 5' end of ompF mRNA with positions 45-49 of micF RNA was based on computational analysis[66]. Reproduced from Delihas *et al*[51].

to osmolarity increase[50]. MarA responds to weak acids[52], SoxS to oxidative stress[53], Rob to peptide antibiotics[54,55] and Lrp to nutritionally poor media[56]. These four transcription factors share the same binding site on the *micF* promoter region (Figure 3). MarA, SoxS and Rob are related proteins that activate *micF*. Rowena Matthews' lab showed that Lrp represses *micF* transcription[56]; Lrp is part of the nucleoid-associated protein goup[57]. The crystal structure of transcription factor Rob bound to the *micF* promoter region has been elucidated (by Kwon *et al*[58]). The model shows one protein helix-turn-helix motif bound to the major groove of *micF* DNA. Additional biochemical studies showed a high affinity of binding[58].

micF is also regulated at the post-translational level when cells are grown in a nutrient poor medium[59]. Dorman and co-workers hypothesized that Stp induces a conformational change in *micF* RNA that makes it susceptible to degradation by nucleases[59]. Stp is also a nucleoid-associated protein and a paralogue of H-NS[60,61]. Thus *micF* gene expression is regulated at both the transcriptional and post-transcriptional levels in response to different environmental conditions.

MICF RNA/TARGET *OMPF* MRNA DUPLEX

It was demonstrated that a RNA/RNA duplex could be formed *in vitro* between *micF* RNA and the target *ompF* mRNA[62]. Full length *micF* RNA and a 150 nt 5' end fragment of *ompF* mRNA were synthesized *in vitro* and the RNA/RNA duplex was formed by annealing at 55 ℃ and 37 ℃. Subsequently, *in vitro* formed duplexes were used for secondary structure probing[63]. Single and double stranded specific RNases and chemical modification with a NiCR complex that is single strand G-specific[64] were used for probing. As nuclease probes can be sensitive to steric hindrance, chemical modification was performed using the NiCR reagent to try to gain additional information[63-65]. A schematic of the deduced *micF* RNA/*ompF* mRNA duplex base-pairing is in Figure 4.

The RNA/RNA interaction shows an imperfect duplex, and a non-canonical G_{99}-G_{29} base-pair that was determined from the absence of RNase T1 cleavage and NiCR reaction, but also the presence of double stranded specific RNase V1 cleavages[63,65]. The Shine-Dalgarno (S-D) ribosome binding site is blocked, which implies that *micF* RNA inhibits ribosome binding. *In vivo* experiments show that *ompF* mRNA levels decrease with temperature increase in an *E. coli* strain carrying *micF* (Figure 2); however, *micF* RNA has not been shown to directly participate in degradation of the message, but to block ribosome binding.

Long-range pairing based on the Maximum Weighted Matching computational program of Tabaska *et al*[66] was predicted to also contribute to the RNA/RNA interaction (Figure 4). The pairing is supported by phylogenetic comparisons and for the most part, by structure probing. Long-range pairings should contribute to the three-dimensional structure of the interacting RNAs.

MICF RNA HAS MULTIPLE TARGETS AND PARTICIPATES IN GLOBAL GENE REGULATION

Recently it has been shown that the functions of *micF* RNA are multifaceted and extend far beyond regulation of *ompF* expression. Gerhart Wagner's lab[67] showed that *micF* is part of a global regulatory network involving the leucine responsive protein (Lrp), and Jörg Vogel's lab[68] at the University of Würzburg in Germany showed that *micF* targets different mRNAs and base-pairs with these RNAs in different ways.

Approximately 10% of all genes in *E. coli* are controlled by Lrp, a transcription factor which responds to nutrient availability. Wagner's group[67] showed participation of *micF* RNA in a circular regulatory pathway, *i.e.*, Lrp regulates transcription of *micF* RNA and *micF* RNA regulate *lrp* expression post-transcriptionally, depending on the nutritional content of the cell growth media (Figure 5). Both Lrp and *micF* RNA function as repressors. *micF* RNA participates in this global regulatory network by regulating *lrp* mRNA; thus this greatly expands its role in regulation of gene expression and cell metabolism.

On the other hand, using *Salmonella* as a model, Vogel *et al*[68] demonstrated that *micF* RNA targets multiple mRNAs in addition to *ompF* mRNA and *lrp* mRNA (Figure 5). Interestingly, its mode of binding to different mRNAs is multifaceted. On the one hand, *micF* RNA can induce

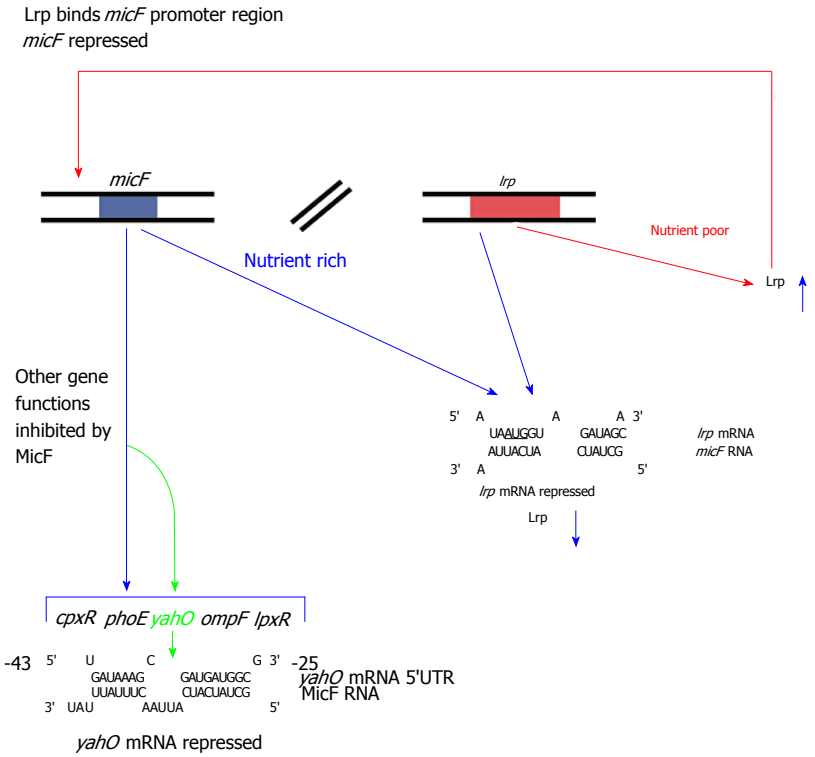

Figure 5 Multifaceted functions of micF RNA showing a "circular" regulatory process involving Lrp and *micF* RNA. Also shown are multiple mRNAs targeted by *micF* RNA. Based on work of Holmqvist *et al*[64] and Corcoran *et al*[65]. Drawing reproduced from Delihas[69].

destabilization of the *yahO* mRNA by unfolding a stem-loop structure located at the 5′-terminus, and thus make the mRNA accessible to exonuclease action. With *lpxR* mRNA, *micF* RNA binds at two sites. One site is within the coding region of the message, and when *micF* RNA binds at this site, it induces a conformational change exposing an AU region that can be targeted by the endonuclease RNase E, which then degrades the message[68]. *yahO* encodes a periplasmic protein and *lpxR* encodes an outer membrane lipid A-modifying enzyme.

HFQ PROTEIN

In vitro studies showed that *micF* RNA binds to a protein based on mobility shifts of protein-RNA complexes on non-denaturing gels[62]. UV-cross-crosslinking experiments suggested that the protein was 80 kDa, however the protein was not further characterized. It is well known that many sRNAs bind the Hfq protein and that Hfq facilitates the binding of sRNAs to their target mRNAs, including *micF* RNA[70]. The molecular weight of Hfq is 11.2 KDa[71], but the 80 kDa protein found to bind *micF* RNA[62] is inconsistent with this and there is a need for further investigation.

Hfq is a central component of the pairing of bacterial non-coding RNAs with their target messenger RNAs. The multifaceted ways that Hfq, together with sRNAs can participate in the inhibition of translation and/or mRNA degradation has been outlined[72]. Hfq also interacts with another protein, catalase HPII. The crystal structure of Hfq bound to catalase HPII has been solved[71], but the crystal structure of an Hfq-sRNA complex has not been determined.

OTHER NON-CODING RNA FUNCTIONS

In this paper we focused on initial findings involving the regulation of gene expression by RNA, however, there is a wealth of discoveries with other functions of non-coding RNAs that also occurred during the 1980s, and it is important to mention this. Table 1 lists several initial findings that include enzymatic RNA, catalytic RNA and RNA functions in protein translocation. The work on regulation of gene expression by RNA and the studies outlined in Table 1 provide a breakthrough on understanding functions of RNAs whereby before the 1980s, RNAs were largely considered inert with no dynamic functions.

CONCLUSION

The initial discovery that RNA can function as a regulatory molecule occurred in the 1980s. Table 2 summarizes the chronology. Bacterial plasmid RNAs were first shown by two labs to regulate DNA replication *via* sense/antisense RNA/RNA base-pairing[32,33] and the *E. coli micF* gene was the first found to regulate expression of another gene *in trans via* imperfect RNA/RNA base-pairing, also by two labs[8-10]. These finding were major breakthroughs that came approximately 5-10 years before the discovery of eukaryotic microRNAs and their functions. Subsequently, numerous labs added to the molecular genetics of *micF* and to functional properties of the transcript that greatly advanced our knowledge of the role of this gene in cell survival. So it was essentially work with prokaryotes that first opened the door to a new form of regulation of gene expression by RNA

Table 1 Discovery of other non-coding RNA functions

Non-coding RNA	Date discovered	Function	Ref.
RNase P RNA	1978, 1983	Catalytic RNA	[73,74]
Tetrahymena intron	1982	RNA self-splicing	[75]
snRNA	1982	Involved in RNA splicing	[76]
7SL RNA	1982	Participates in protein translocation	[77]

Table 2 Discovery of regulatory non-coding RNA functions

Non-coding RNA (prokaryotic)	Date determined to be a regulator	Function	Ref.
ColE I RNA I	1981	Inhibits DNA replication	[32,33]
micF RNA	1984/1987	Inhibits *ompF* mRNA + other mRNAs	[8-10]
6S RNA	2000	Binds RNA polymerase (transcription inhibitor)	[18]
Spot 42	2002	Inhibits galK mRNA + other mRNAs	[20]
Non-coding RNA (eukaryotic)			
lin 4	1993	Inhibits lin14	[12]
7SK RNA	2004	Binds pTEFb (transcription inhibitor)	[23]

that is now found in all biological kingdoms, including viruses. However, there are many different types of regulatory RNAs that are found only in eukaryotes (*e.g.*, piRNA, siRNA, lncRNA), and the prospects of finding new and unique functions of RNAs may lie primarily with eukaryotic RNAs. Looking towards the future, there are tens of thousands of lncRNAs that have been detected, and for the most part are uncharacterized. But the few that have been characterized display very diverse and intricate functions[78-82].

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REVIEW

Targeting amino acid metabolism in cancer growth and anti-tumor immune response

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Abstract

Recent advances in amino acid metabolism have revealed that targeting amino acid metabolic enzymes in cancer therapy is a promising strategy for the development of novel therapeutic agents. There are currently several drugs in clinical trials that specifically target amino acid metabolic pathways in tumor cells. In the context of the tumor microenvironment, however, tumor cells form metabolic relationships with immune cells, and they often compete for common nutrients. Many tumors evolved to escape immune surveillance by taking advantage of their metabolic flexibility and redirecting nutrients for their own advantage. This review outlines the most recent advances in targeting amino acid metabolic pathways in cancer therapy while giving consideration to the impact these pathways may have on the anti-tumor immune response.

Key words: Amino acid metabolism; T cells; Anti-tumor immune response; Tumor microenvironment; Cancer therapy

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Core tip: Amino acid metabolism has been a focus of increased attention by cancer researchers and immunologists due to its importance for the metabolic reprogramming of proliferating cells. Many amino acid enzymes are described as immunosuppressive in the tumor microenvironment and targeted for cancer therapy. This review addresses the metabolic control of tumor progression in the context of anti-tumor immunity and discusses current and future therapeutic approaches. Special emphasis is given to the emerging role of branched chain amino acid metabolism in cancer and immunity highlighting some recent work by our research group.

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INTRODUCTION

Two functionally distinct types of cells, cancer and T cells, undergo similar metabolic reprogramming during

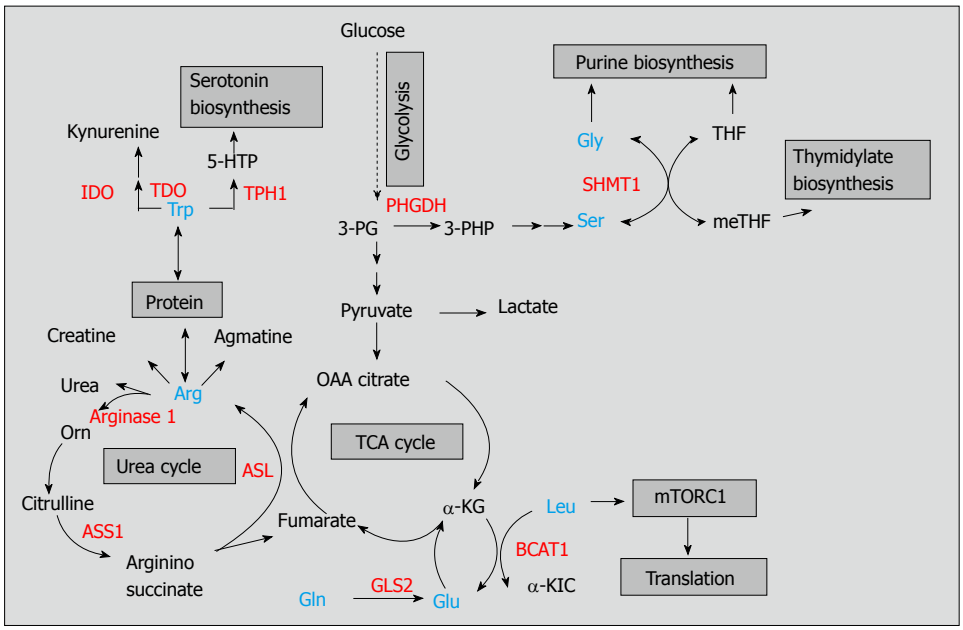

Figure 1 Schematic representation of amino acid metabolic pathways targeted in cancer therapy. The amino acid metabolism of Arg, Trp, Ser, Gly, Gln, Glu, and BCAAs interconnects with major catabolic and biosynthetic pathways shown in gray. Arg is an important precursor for creatine, urea, and agmatine synthesis while Trp is important for kynurenine and serotonin biosynthesis; Ser and Gly are major sources of methyl groups during purine and thymidylate biosynthesis; Leu is a known activator of complex 1 of mTOR pathway and Gln/Glu provide intermediates for the TCA cycle and restore glutathione to its reduced form. Metabolic enzymes catalyzing important steps in the metabolism of these amino acids are involved in clinical and/or preclinical cancer studies and are denoted in red. 5-HTP: 5-hydroxytrytophan; 3-PG: 3-phosphoglycerate; 3-PHP: 3-phosphohydroxy pyruvate; THF: Tetrahydrofolate; meTHF: Methyltetrahydrofolate; OAA: Oxaloacetate; α-KG: α-ketoglutarate; α-KIC: α-ketoisocaproate; Trp: Tryptophan; Arg: Arginine; Gln: Glutamine; Glu: Glutamate; BCAAs: Branched chain amino acids; Ser: Serine; Gly: Glycine; mTORC1: Complex 1 of the mammalian target of rapamycin; IDO: Indoleamine-2,3-dioxygenase; TDO: Tryptophan-2,3-dioxygenase; TPH1: Tryptophan hydroxylase-1; BCATc: Cytosolic branched chain aminotransferase; ASS1: Argininosuccinate synthetase 1; ASL: Argininosuccinate lyase; GLS2: Glutaminase 2; PHGDH: Phosphoglycerate dehydrogenase; SHMT1: Serine hydroxymethyl transferase 1.

proliferation to support their increased biosynthetic and energy demands[1,2]. To meet these demands, cancer cells need a continuous supply of nutrients that maintain abnormal growth and rapid division during cancer progression. Activated T cells also rely on a continuous nutrient supply to ensure proper differentiation and performance during cell-mediated immunity against pathogen attacks or while fighting cancer[3]. In areas with poor nutrient and oxygen access, such as the tumor microenvironment[4] and inflammatory sites[5], cellular metabolism must adapt to promote continued survival and function of the cells residing in and/or migrating to these areas. Not surprisingly, both cancer and T cells rely on high rates of glycolysis while retaining mitochondrial respiration, a phenomenon known as the Warburg effect[6,7]. Ever since Otto Warburg observed that cancer cells produced lactate from glucose even under non hypoxic conditions[7], subsequent reports demonstrated that high rates of glycolysis are beneficial for cell survival, providing energy and biosynthetic material for the synthesis of new proteins, lipids, and nucleic acids in proliferating cells[8-13]. Recent advances in cancer biology and cellular immunity reveal that not only glucose but also amino acids are essential to support the high metabolic demands of tumor and/or immune cells and different strategies for targeting amino acid metabolism and corresponding enzymes are now a focus of innovative treatment approaches[14-17]. The purpose of this review is to highlight the importance of amino acid metabolism in the tumor microenvironment and use this knowledge to generate more effective immunotherapies while keeping in mind that both cancer and immune cells have similar metabolic requirements for growth and function and therefore may compete for common nutrients.

ARGININE AND TRYPTOPHAN METABOLISM IN THE TUMOR MICROENVIRONMENT

The amino acid arginine has considerable nutritional and physiological significance as it is recognized as an important precursor for the synthesis of proteins, urea, and creatine as well as for the synthesis of signaling molecules such as glutamate, nitric oxide, and agmatine[18] (Figure 1). Although arginine is a dispensable (nonessential) amino acid for healthy humans, it is conditionally essential under certain physiological conditions or disease state[10,19,20]. For example, many tumors are dependent on exogenous arginine for growth as they lack the enzyme argininosuccinate synthetase 1 (ASS1)[21]. ASS1 catalyzes the conversion of citrulline into argininosuccinate in an ATP-dependent manner, completing one of the last steps in the arginine biosynthetic pathway[18] (Figure 1). Loss of ASS1 prevents the production or arginine and may lead

to arginine depletion. Osteosarcoma and bladder cancer cell lines expressing low levels of ASS1 failed to grow in an arginine-free medium, indicating that ASS1 behaves as a tumor suppressor[22,23]. However, arginine depletion in ASS1-negative tumor cells is usually associated with an aggressive phenotype and negative prognostic impact[22]. Kobayashi *et al*[22], showed that ASS1 deficiency in osteosarcoma patients correlated with the development of pulmonary metastasis in patients with osteosarcoma and can be used as a predictive biomarker for unfavorable prognosis. The aggressiveness of ASS1-negative tumors can be explained partially by the ability of these tumors to utilize more efficiently exogenous arginine coming from the tumor microenvironment. It is hypothesized that tumor associated myeloid cells (TAMCs), that consist of macrophages, monocytes, myeloid suppressor cells, and neutrophils[24], form metabolic relationship with the tumor cells in the tumor microenvironment. They provide arginine to help the tumor cells by-pass the effect of arginine deprivation[25]. In addition, TAMCs express high levels of another enzyme in the arginine metabolism, arginase 1, that hydrolyses arginine into urea and ornithine and sustains tumor growth by providing precursors for polyamine synthesis[26,27]. By producing high levels of arginase 1 in the tumor microenvironment, TAMCs reduce arginine availability for other immune cells such as T cells. However, TAMCs can arrest cytotoxic T cell proliferation and induce T cell dysfunction by more than one mechanism, including generation of nitric oxide from arginine by nitric oxide synthase[28]. Additionally, these cells can induce regulatory T cell differentiation[29] potentially promoting immune tolerance in the tumor microenvironment. Thus, TAMCs have the ability to suppress the protective anti-tumor immune response by targeting arginine metabolism and helping tumors escape immune destruction. While immunotherapeutic targeting of arginine metabolism in the tumor microenvironment is still in its infancy, several decades of substantial *in vitro* and *in vivo* studies on arginine metabolism led to the development of arginine deprivation therapy, which is currently the subject of ongoing clinical trials with several arginine depleters, such as pegylated arginine deiminase (ADI-PEG20, Polaris group) and bioengineered forms of human arginase[25,30,31] (Table 1). A small study group of patients with hepatocellular carcinoma revealed response rates to ADI-PEG20 between 25%-47% and this compound holds great potential in the arginine depravation therapy[30]. ADI-PEG20 is not limited to cancer therapy as there is ADI-PEG20 with anti-viral activity designed for treatment of hepatitis C by Polaris group.

Tryptophan is another amino acid linked to the regulation of immune tolerance and anti-tumor immune responses[32-35]. Tryptophan degradation occurs *via* the kynurenine pathway where two different enzymes, indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO), catalyze the conversion of tryptophan into kynurenine, while tryptophan hydroxylase-1 (TPH-1) converts tryptophan to 5-hydroxytryptophan and provides precursors for serotonin biosynthesis[32,36] (Figure 1). IDO is the most studied enzyme in tryptophan metabolism expressed by both immune cells (dendritic cells, macrophages) and tumor cells[33,37-39]. Due to the fact that tryptophan readily crosses the plasma membrane, dendritic cells expressing IDO are capable of depleting tryptophan in the extracellular space, limiting tryptophan supply to surrounding T cells. T cell activation is sensitive to local tryptophan concentrations, and lack of tryptophan blocks their proliferation[40]. By degrading tryptophan, IDO inhibits T cell proliferation and plays a role in autoimmunity and anti-inflammatory responses[41-43]. For example, *Apoe*$^{-/-}$ mice treated with the IDO inhibitor, 1-methyl-Trp, showed a significant increase in atherosclerotic lesions in the aortic arch and root of their hearts along with enhanced vascular inflammation[41]. Additionally, IDO-expressing dendritic cells suppressed the allograft rejection and increased the survival time of small bowel transplanted mice[44]. In the tumor microenvironment, however, IDO limits the T cell response to tumor growth[45-47]. By using an IDO-negative subline of the mouse tumor model P815B, Uyttenhove *et al*[45] elegantly showed that mice transfected with this subline completely rejected the tumor challenge while mice transfected with IDO-expressing cells developed progressive tumors and died. These results suggest that effector T cells that infiltrate the tumor microenvironment are more susceptible to the effects of IDO than are tumor cells. Indeed, immunohistochemical staining for IDO expression in endometrial cancer tissues revealed significant correlation between high IDO expression and low numbers of $CD3^+$, $CD8^+$, and $CD57^+$ immune cells, which possibly contributed to disease progression and impaired clinical outcome for patients with endometrial cancer[48]. In addition to IDO, tumors use other enzymes in tryptophan metabolism to resist immune destruction[36,49]. TDO, a well described liver enzyme, is another immunosuppressive enzyme found in bladder carcinomas, melanomas, and hepatocarcinomas[50]. In a preclinical model, Pilotte *et al*[50] demonstrated that systemic treatment of immunized mice with a TDO inhibitor prevented the growth of TDO-expressing tumor cells. Lastly, TPH-1 expressed by mast cells is also necessary for long-term graft tolerance and a suppressive anti-tumor microenvironment[51]. Therefore, more than one tryptophan catabolic enzyme contribute to the establishment of immune tolerance and could be suitable for combinatorial therapies. Thus far, IDO inhibitors, specifically designed for cancer immunotherapy, have been broadly used in preclinical and clinical trials alone or in combination with T cell checkpoint inhibitors[52-54], while systemic inhibition of TDO, although promising, may raise safety concerns[54] (Table 1). Another limitation of cancer trials targeting tryptophan metabolism is that they do not select cancer patients based on assessment of systemic IDO/TDO activities or by analysis of their metabolites in patients' serum[54]. Nevertheless, combinatorial approaches targeting tryptophan metabolism will continue to deliver novel therapeutic avenues in cancer therapy.

Table 1 Amino acid metabolic enzymes targeted in cancer therapy

Amino acid metabolism	Targeted enzyme	Drug design	Drug toxicity, adverse events	Cancer type	Response rate	Clinical studies	Ref.
Arginine	ASS1, Arginine deaminase .	ADI-PEG20	[1]Grade 3-4: Fatigue, hyperuricemia, anemia	HCC (nonresectable and metastatic)	31%-47%	Phase Ⅰ / Ⅱ	[30,94,95]
		ADI-PEG20	[1]Grade 3-4: Pain in extremity, arthralgia, pruritus, lymphedema, seizure	Melanoma (stage Ⅲ and Ⅳ)	25%	Phase Ⅰ / Ⅱ	[96,97]
	Arginase 1	rhArgIpeg5000	[1]Grade 3-4: Elevated ALT, AST, bilirubin and GGT	HCC (advanced)	26.70%	Phase Ⅰ	[31]
		Co-hArgI	Nude BALBc mouse model: Weight loss, hunch posture, lethargy, bone marrow injuries (> 15 mg/kg)	HCC (HepG2), pancreatic cancer (Panc-1); tumor site (right flank)	Smaller tumors	Preclinical studies	[98]
Tryptophan	IDO	Indoximod and docetaxel	Grade 1: Anemia, fatigue, hyperglycemia Grade 3-4: Headache, hypotension, infection	Various metastatic solid tumors	41% PD	Phase Ⅰ	[99]
		1-D-MT	Grade 1: Fatigue; Grade 2: Hypophysitis	Various metastatic solid tumors	4P (SD), 3P (PD)	Phase Ⅰ	[100]
		Indoximod and anti-CTLA4	C57BL/6 mouse model: No weight loss or acute/delayed toxicity observed	B16 F10 melanoma cell line, tumor site -flank	Delayed tumor growth	Preclinical studies	[101]
Tryptophan	TDO	Indole LM10	DBA/2 mouse model: No liver toxicity observed for 3 mo	P815B tumor cell line, tumor site –peritoneal cavity	Delayed tumor progression	Preclinical studies	[50]
Serine	PHGDH	NA	NA	Melanoma and breast cancer cell lines (SkBr3, MCF7)	NA	Preclinical studies	[15]
		NA	NA	Murine mammary fat pad tumors with MDA-MB-468 cells expressing PHGDH-shRNA	Reduced tumor growth	Preclinical studies	[63]
Glycine	SHMT1	NA	λ-Myc *Shmt1-/-* transgenic mice: no toxicity observed	Accelerated lymphomagenesis	NA	Preclinical studies	[102]
Glutamine	Glutamine –dependent enzymatic steps	L-DON[2], azaserine[2]	None reported	Various animal and human xenografted tumors	Tumor growth inhibition	Preclinical studies	[80]
		Acivicin[2]	Grade1-3: neurological toxicity Grade 2: vomiting, infections	High grade astrocytoma	Median 128 day survival	Phase Ⅱ	[103]
Leucine, Isoleucine, Valine	BCATc	NA	CD-1 nude mouse model bearing tumors with BCATc knockdown: lethargy and uncoordinated motor activity	U-87MG glioblastoma cells with BCATc-shRNA; tumor site-intracerebral transplantation	Smaller tumors	Preclinical studies	[16]
		NA	NA	Nasopharyngeal carcinoma (5-8F, 6-10B), colorectal cancer	Induced cell proliferation	Preclinical studies	[92,93]

[1]The highest grade is shown (Grade 3-4) and is summarized for either one or more studies; [2]Discontinued. ADI-PEG20: Pegylated arginine deiminase; rhArgIpeg5000: Pegylated recombinant human arginase I; HCC: Hepatocellular carcinoma; Co-hArgI: Co^{2+} substitution of the Mn^{2+} metal cofactor in human arginase I; Indoximod (NLG8189, 1-D-MT1): 1-methyl-D-tryptophan; Indole LM10, tetrazolyl-vinyl substituted (fluoro)indole; L-DON: 6-diazo-5-oxo-L-norleucine; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma glutamyltransferase; PD: Progressed disease; SD: Stable disease; P: Patient; NA: Not available.

SERINE AND GLYCINE METABOLISM IN TUMORIGENESIS

Serine and glycine metabolism are interconnected *via* the glycine cleavage system, a major metabolic pathway in one-carbon metabolism that provides cofactors for purine and pyrimidine nucleotide biosynthesis for proliferating lymphocytes, cancer cells, and/or fetal tissues[55-57] (Figure 1). Studies from the late 1980s to more recent years, strongly suggest that cancer cells have an increased capacity for *de novo* serine synthesis *via* the phosphoglycerate dehydrogenase (PHGDH) pathway. PHGDH oxidases around 10% of 3-phosphogycerate produced during glycolysis by converting it to 3-phosphohydroxypyruvate[58-61]. This compound is then transaminated, forming 3-phosphoserine, and dephos-

phorylated to yield serine (Figure 1). PHGDH along with other enzymes in the serine biosynthetic pathway were upregulated in highly metastatic breast cancer, a finding associated with overall poor patient survival[62]. Two independent studies published in 2011 reported that the gene encoding PHGDH was recurrently amplified in melanoma and breast cancers and this amplification was not associated with oncogene regulation[15,63]. Apart from PHGDH, serine hydroxymethyl transferase (SHMT), that converts serine to glycine, has also been implicated in tumorigenesis. Two isoforms of SHMT (cytoplasmic SHMT1 and mitochondrial SHMT2) were both described as targets of c-myc oncogene[64], a transcriptional factor abnormally expressed in many tumors that controls transcription of up to 15% of the genes in human cells[65]. Both SHMT1 and SHMT2 were described as downstream effectors of c-myc function and rescued the growth defects of c-myc-null cells[64]. At first glance, the role of serine and glycine metabolism in tumorigenesis may appear disadvantageous as PHGDH diverts metabolites from glycolysis to *de novo* synthesis of serine followed by conversion to glycine by SHMT (Figure 1). However, both serine and glycine are major sources of methyl groups for the one carbon pool required for a variety of biosynthetic pathways and/or DNA methylation that tumor cells use. Similar to cancer cells, immune cells are also shown to use glycolytic intermediates for serine/glycine biosynthesis with the ultimate goal of synthesizing building materials for cell growth and proliferation[66]. Preclinical studies are underway to test the efficacy of many one carbon metabolic enzymes, including PHGDH[67], as anti-tumor targets (Table 1). Dietary intervention is another strategy to target cancer metabolism and although preclinical studies restricting serine and glycine metabolism showed promising results[68,69], global or systematic interventions need to be carefully examined in the context of the immune system that rely on the same metabolic pathways for proper function.

GLUTAMINE METABOLISM, A HALLMARK OF PROLIFERATING CELLS

As early as 1951, Mider[70] described that tumors behaved as "nitrogen traps" where glutamine was the preferred nitrogen donor[70,71]. More reports form the 1980's, demonstrated that not only cancer cells but also rapidly dividing cells such as lymphocytes, thymocytes, and colonocytes had high glutamine consumption rates[72-74]. Despite the fact that glutamine is a nonessential amino acid, proliferating cells display addiction to glutamine implying that glutamine plays more roles in cell metabolism than simply being a nitrogen donor. As such, glutamine is a conditionally essential amino acid for the proliferating cells as well as critically ill humans[75]. Glutamine provides intermediates for the TCA cycle, restores glutathione to its reduced form suppressing oxidative stress, and maintains mitochondrial membrane integrity thus contributing to the survival of proliferating cells (reviewed by Wise and Thompson)[76]. Tumor suppressors (p53) and oncogenes (c-myc) were shown to regulate glutamine metabolism. Hu *et al*[77] demonstrated that p53 targeted the mitochondrial isoform of glutaminase [glutaminase 2 (GLS2)], that converts glutamine to glutamate (Figure 1). p53 increased GLS2 expression and led to enhanced mitochondrial respiration and generation of ATP along with reduction in reactive oxygen species due to increased glutathione levels[77]. Likewise, introduction of an inducible c-myc transgene in mouse embryonic fibroblasts led to induction of glutaminase 1 along with lactate dehydrogenase and glutamine transporters, suggesting that c-myc is required to support cellular dependence on glutamine[78]. Cancer "addiction" to glutamine had been explored in cancer therapeutics and three compounds, 6-diazo-5-oxo-L-norleucine (L-DON), azaserine, and acivicin, showed significant activity as glutamine analogs[79]. In early preclinical and clinical studies, these compounds showed promising results against different tumor types by inhibiting ribonucleotide biosynthesis, glutamine oxidation and reducing cell viability[80,81] (Table 1). However, due to the important role of glutamine metabolism for normal tissue physiology, these compounds were discontinued while better tumor-targeting options with less general toxicity are currently considered[76,82].

EMERGING ROLE OF BRANCHED CHAIN AMINO ACID METABOLISM IN CANCER THERAPY

Branched chain amino acids (BCAAs, leucine, isoleucine, and valine) constitute about 40% of the essential amino acid requirements of healthy individuals[83]. They play an important role in protein synthesis and serve as major nitrogen donors for alanine and glutamine synthesis[84]. Increasing evidence shows that BCAAs, especially leucine, are not merely building blocks necessary to support biosynthetic demands but also nutrient signals regulating the mammalian target of rapamycin (mTOR) pathway[85,86]. By controlling protein translation, cell growth, proliferation, and autophagy, the mTOR pathway is recognized as a critical regulator of cellular function[87]. Immune cells are particularly sensitive to mTOR regulation as mTOR pathway responds to environmental cues and coordinates immune cell differentiation and function, accordingly[88]. For example, inhibition of mTOR pathway in T cells promoted T cell tolerance and the mechanism responsible for the maintenance of tolerance was failure of T cells to upregulate mTOR activity in the presence of metabolic inhibitors including leucine antagonists[89]. In this regard, leucine appears to be an important nutrient signal that is sensed by the immune cells *via* mTOR pathway and is critical for their proliferation. Leucine supply to mTOR pathway is regulated through BCAA metabolism as shown recently in T cells[90]. The cytoplasmic branched chain aminotransferase (BCATc), that catalyzes leucine transamination, was induced in activated T cells, where

it regulated leucine supply to complex 1 of the mTOR pathway. Loss of BCATc expression eliminated cytosolic leucine catabolism leading to upregulation of complex 1 of the mTOR pathway and increased glycolysis[90]. While mTOR pathway is upregulated in many cancer types and mTOR-targeted cancer therapy has been a part of clinical research[91], a direct link between mTOR pathway and leucine/BCAA metabolism in the tumor microenvironment awaits to be explored. Similar to the tryptophan degrading enzymes, BCATc may play an immunosuppressive role in the tumor microenvironment possibly contributing to tumor escape mechanisms. Another mechanism of BCATc function in cancer was explored in glioblastomas[16]. Tönjes *et al*[16] demonstrated that BCATc and BCAA metabolism are attractive targets for the development of therapeutic approaches to treat glioma patients. The majority of gliomas show mutations in their isocitrate dehydrogenase enzyme 1 ($IDH1^{mut}$) and although IDH1 mutation status is a powerful prognostic factor, it was insufficient to induce tumors in mice alone. BCATc was overexpressed in normal (wild type) $IDH1^{wt}$ gliomas but not in gliomas with mutated IDH1, demonstrating a connection between IDH1 mutation and BCATc[16]. Additionally, BCATc was identified as a c-myc target in nasopharyngeal carcinoma and BCATc overexpression induced cancer cell proliferation and migration[92]. Although the majority of these studies imply that cancer cells require BCAA metabolism to sustain growth, and overexpression of BCATc results in increased cell proliferation[92,93], the mechanism through which changes in BCAA metabolism affect cancer growth is currently unknown. It is possible that T cells and cancer cells share similar requirements for BCAA catabolism, where mTOR pathway is dependent on leucine regulation. Thus, future use of leucine antagonists or specific inhibitors aimed at BCATc may be suitable for targeted cancer therapies.

CONCLUSION

Research on amino acid metabolism in cancer cells in the last decades has provided valuable insights on the potential impact of metabolic control and regulation in the tumor microenvironment. Amino acids are no longer regarded solely as building materials but also as nutrient signals that regulate important signaling pathways. A number of amino acid metabolic enzymes are regulated by oncogenes and tumor suppressors and have been explored as targets for cancer therapies. Design and use of inhibitors targeting tryptophan, arginine and/or glutamine metabolism either alone or in combination with anti-tumor drugs has been introduced in clinical trials. However, cancer and immune cells share similar requirements for amino acid metabolic enzymes and often compete for the same nutrients. Therefore, therapeutic interventions in the tumor microenvironment must be cautiously explored to eliminate potential negative impacts on the anti-tumor immunity. Understanding the underlying mechanisms of metabolic interplay between tumor and immune cells will provide new directions to manipulate the tumor microenvironment and unleash the anti-tumor immune response.

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REVIEW

Regulation of *MYC* gene expression by aberrant Wnt/β-catenin signaling in colorectal cancer

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Abstract

The Wnt/β-catenin signaling pathway controls intestinal homeostasis and mutations in components of this pathway are prevalent in human colorectal cancers (CRCs). These mutations lead to inappropriate expression of genes controlled by Wnt responsive DNA elements (WREs). T-cell factor/Lymphoid enhancer factor transcription factors bind WREs and recruit the β-catenin transcriptional co-activator to activate target gene expression. Deregulated expression of the *c-MYC* proto-oncogene (*MYC*) by aberrant Wnt/β-catenin signaling drives colorectal carcinogenesis. In this review, we discuss the current literature pertaining to the identification and characterization of WREs that control oncogenic *MYC* expression in CRCs. A common theme has emerged whereby these WREs often map distally to the *MYC* genomic locus and control *MYC* gene expression through long-range chromatin loops with the *MYC* proximal promoter. We propose that by determining which of these WREs is critical for CRC pathogenesis, novel strategies can be developed to treat individuals suffering from this disease.

Key words: Wnt; β-catenin; Chromatin looping; Wnt responsive DNA element; MYC

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Core tip: In colon cancer, mutations in components of the Wnt/β-catenin signaling pathway result in inappropriate *c-MYC* proto-oncogene (*MYC*) expression. To understand colorectal carcinogenesis requires the identification of Wnt responsive DNA elements (WREs) that control *MYC* expression in colorectal cancer (CRC). Through efforts to characterize *MYC* WREs, a model has emerged where several of these WREs appear largely dispensable for intestinal homeostasis, but are instead "hijacked" by oncogenic Wnt/β-catenin signaling to drive CRC. These findings raise the intriguing possibility that these WREs may be targeted therapeutically as an alternative approach to treat individuals afflicted by CRC. In this review, we summarize the literature describing the identification of *MYC* WREs and discuss how those involved in colorectal carcinogenesis may be targeted to limit progression of CRC.

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INTRODUCTION

The canonical Wnt/β-catenin signaling pathway regulates stem cell self-renewal, cell proliferation, and cell-fate decisions in the intestinal crypt microenvironment[1]. The β-catenin transcriptional co-activator governs the Wnt response, therefore, its distribution and levels within a cell are tightly regulated[2]. When cells, such as the differentiated cells in the apical regions of the intestinal crypts, are not exposed to Wnt, cytoplasmic β-catenin associates with a multi-protein "destruction complex"[3]. This complex contains adenomatous polyposis coli (APC), the axis inhibition proteins 1 and/or 2 (AXIN1/2), casein kinase 1 (CK1), and glycogen synthase kinase three beta (GSK3β). Here, APC and AXIN1/2 function as scaffolds to position β-catenin in proximity of CK1 and GSK3β. Phosphorylation of β-catenin by CK1 and GSK3β prime it for ubiquitination by the β-transducin-repeat-containing protein (β-TrCP) and subsequent degradation *via* the proteasome[4]. In the absence of Wnt, members of the T-cell factor/Lymphoid enhancer factor (TCF/Lef) family of sequence-specific transcription factors bound to Wnt responsive DNA elements (WREs) recruit transcriptional corepressor complexes. These complexes include transducin-like enhancer (TLE) and C-terminal binding protein, which associate with histone deacetylases to repress target gene expression (Figure 1A)[5].

The basal regions of the intestinal crypts contain stem cells and transit-amplifying progenitor cells[6]. These cells are exposed to Wnt ligand, secreted by the surrounding mesenchyme and differentiated Paneth cells, which binds frizzled (FZD)/low-density lipoprotein receptor-related protein 5 or 6 receptor complexes expressed on the cell surface[3]. This binding results in the subsequent recruitment of AXIN1/2 to the plasma membrane *via* interaction with dishevelled proteins (DVLs) and inactivation of the destruction complex. β-Catenin then escapes proteasomal degradation, accumulates in the cytoplasm, and translocates into the nucleus where it displaces TLE from TCF/Lef bound WREs[1]. β-Catenin/TCF/Lef complexes in turn recruit histone-modifying complexes, such as CBP/p300 protein acetyltransferases and MLL/Set methyltransferases, and chromatin remodeling complexes, including SWI/SNF, to induce expression of Wnt/β-catenin target genes and drive cellular proliferation (Figure 1B)[7].

Colorectal cancer (CRC) is the third leading cause of cancer related deaths in the United States. In 2015, 140000 individuals are predicted to be diagnosed with CRC and over 50000 patients are predicted to succumb to the disease[8]. Approximately 90% of sporadic CRCs contain mutations in components of the Wnt/β-catenin signaling pathway[9]. These mutations are found in the earliest neoplasms suggesting that this pathway serves as a critical gatekeeper to prevent colorectal carcinogenesis[10]. Most tumors contain a mutation in a single component of the pathway, although recent data from the cancer genome atlas consortium indicates that mutations in multiple components can co-occur[11]. The majority of mutations, some 85%, map to "hotspots" within APC and often lead to expression of a truncated APC protein from this allele[12]. This truncated protein is incapable of incorporating into a functional β-catenin destruction complex. Thus, when the wild-type APC is inactivated by mutation or lost through loss of heterozygosity, β-catenin levels inappropriately accumulate in the cell, leading to aberrant expression of Wnt/β-catenin target genes and the development of benign adenomas (Figure 1C)[1]. As these adenomas accumulate additional mutations in other signaling pathways, they transition into carcinomas[13]. Therefore, CRC is a disease of uncontrollable Wnt/β-catenin signaling where β-catenin/TCF complexes bound to WREs drive pathogenic expression of downstream target genes. To understand CRC initiation and progression requires identification of these genes and the WREs that control their expression.

The *c-MYC* proto-oncogene (*MYC*) was identified as a Wnt/β-catenin target gene using a differential RNA expression screen conducted in the human HT29 CRC cell line harboring mutant *APC* alleles[14]. MYC is a basic helix-loop-helix zipper (bHLHZ) transcription factor that heterodimerizes with bHLHZ factor MAX[15]. MYC: MAX heterodimers bind E-box sequence motifs to predominantly activate transcription of genes by recruiting histone modifying and chromatin remodeling complexes[16,17]. MYC: MAX regulates expression of thousands of target genes whose products control a wide-range of cellular processes including metabolism, ribosome biogenesis, and protein synthesis[18]. As such, MYC promotes cellular proliferation and cell growth[19].

MYC expression is deregulated in 50% of all cancers, including CRC[20]. In fact, *Myc* is required for tumorigenesis in mouse models of CRCs[21-25]. Given that deregulated *MYC* expression by oncogenic Wnt/β-catenin signaling is critical for colorectal carcinogenesis, several groups have sought to identify and characterize WREs that control its expression in human CRC cell lines over the years (Table 1). In this review, we summarize the literature pertaining to WREs that regulate *MYC* in CRC cells. We define a *MYC* WRE as a region of DNA that is: (1) bound by β-catenin/TCF complexes; (2) associated with histone modifications that demarcate enhancer elements, such as monomethylated lysine 4 on histone H3 (H3K4me1) and acetylated lysine 27 on histone H3 (H3K27Ac); (3) responsive to β-catenin/TCF complexes in luciferase reporter assays; and/or (4) juxtaposed to the *MYC* promoter region through chromatin loops, if it maps distal to the *MYC* gene. We then discuss

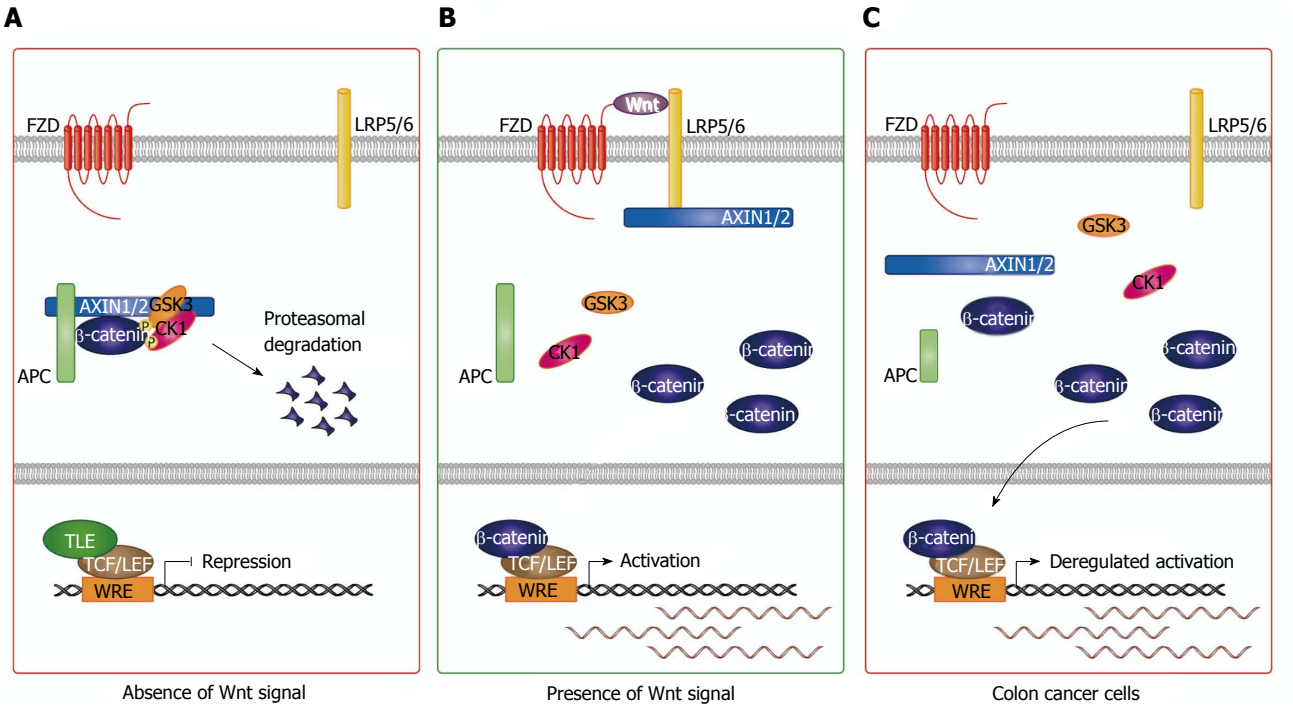

Figure 1 The Wnt/β-catenin signaling pathway. A: In the absence of a Wnt ligand, cytoplasmic β-catenin is targeted for proteasomal degradation by a multi-protein "destruction complex". Within the nucleus, TCF/Lef at WREs associates with the TLE co-repressor to repress Wnt/β-catenin target gene expression; B: Upon Wnt ligand binding to the FZD and LRP5/6 co-receptor complex, AXIN1/2 is recruited to the plasma membrane and the destruction complex is inactivated. β-Catenin subsequently translocates to the nucleus where it binds TCF/Lef assembled at WREs and recruits co-activator complexes to activate Wnt/β-catenin target gene expression; C: In CRCs, truncations of the APC protein prevent efficient targeting of β-catenin for proteasomal degradation. Therefore, nuclear β-catenin levels are elevated and aberrantly associate with TCF/Lef at WREs to drive deregulated expression of Wnt/β-catenin target genes. WRE: Wnt responsive DNA element; TCF/Lef: T-cell factor/Lymphoid enhancer factor; TLE: Transducin-like enhancer; FZD: Frizzled; LRP5/6: Lipoprotein receptor-related protein 5 or 6; AXIN1/2: Axis inhibition proteins 1 and/or 2; CRC: Colorectal cancer; APC: Adenomatous polyposis coli; GSK3: Glycogen synthase kinase three; CK1: Casein kinase 1.

Table 1 *MYC* Wnt responsive DNA element chromosomal locations

MYC WRE	Chromosomal position	Distance to *MYC* TSS	Ref.
MYC 5′ WREs	chr8: 128747425	889-bp upstream	[14,28]
MYC 3′ WRE	chr8: 128755419	7105-bp downstream	[26,27,31]
Myc 3′ WRE	chr15: 61992066	6725-bp downstream	[32,33]
MYC -335 WRE	chr8: 128413279	335036-bp upstream	[46-51,55,56]
Myc -335 WRE	chr15: 61450712	534630-bp upstream	[52]
MYC -520 WRE	chr8: 128227619	520696-bp upstream	[59,61]
MYC -517 WRE	chr8: 128231269	517046-bp upstream	[59]
MYC -513 WRE	chr8: 128235209	513106-bp upstream	[59,61]
MYC -488 WRE	chr8: 128259779	488536-bp upstream	[59,61]
MYC -470 WRE	chr8: 128278129	470186-bp upstream	[59,61]
MYC super-enhancer	chr8: 128275000	473314-bp upstream	[61,69]

TSS: Transcription start site; WRE: Wnt responsive DNA element.

future avenues of research aimed at determining which of these are the critical drivers for oncogenic *MYC* expression and how these results may be leveraged to develop new therapeutic options to manage individuals afflicted by CRC.

PROMOTER PROXIMAL *MYC* WREs

In the aforementioned study identifying *MYC* as a target of oncogenic Wnt/β-catenin signaling in human CRC cells, He *et al*[14] localized a β-catenin-responsive region within the *MYC* proximal promoter (Figure 2). This region contained two TCF binding elements, TBE1 and TBE2, which mediated β-catenin/TCF-responsiveness of this element in luciferase reporter assays. While expression of a dominant negative TCF4 protein lacking its amino-terminal β-catenin interaction domain decreased *MYC* expression in CRC cells, it was not evaluated at that time whether β-catenin/TCF complexes occupied this region at the endogenous *MYC* locus. However, this study identified the first *MYC* WRE and we have referred to it as the *MYC* 5′ WRE in our studies of WREs that control *MYC* expression in human CRC lines[26,27].

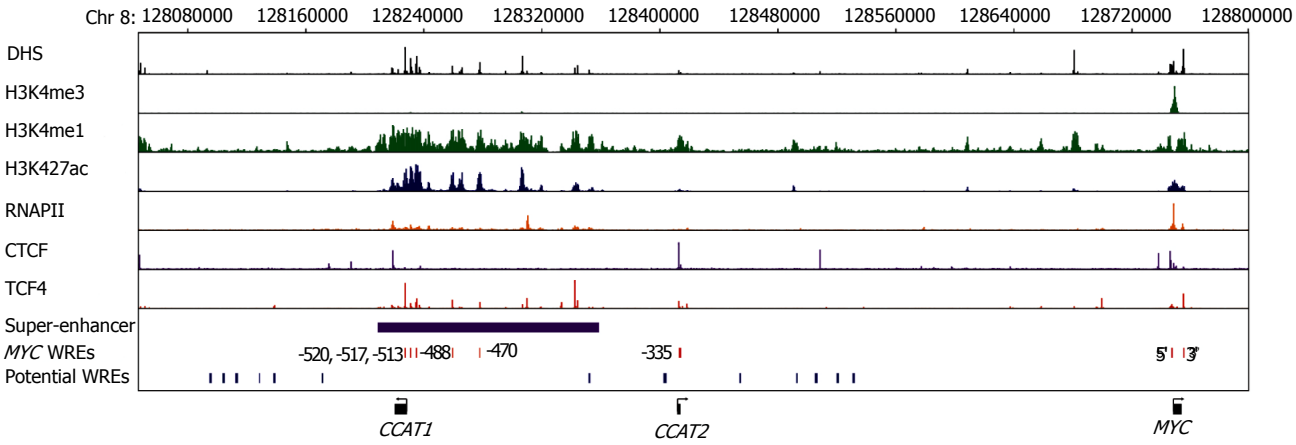

Figure 2 The *MYC* genomic locus in colorectal cancer. ChIP-Seq and DHS data in the HCT116 CRC cell line were downloaded from the WashU Epigenome Browser (http://epigenomegateway.wustl.edu/). The *MYC* distal super-enhancer is denoted as a purple rectangle, the *MYC* WREs discussed in this review are depicted as red lines, and potential WREs are denoted as blue lines[50,73]. WRE: Wnt responsive DNA element; CTCF: CCCTC-binding factor; H3K4me1: Monomethylated lysine 4 on histone H3; H3K27Ac: Acetylated lysine 27 on histone H3; RNAP: RNA Polymerase II; TCF: T-cell factor; CCAT: Colon-cancer associated transcript; DHS: DNase hypersensitivity.

Sierra *et al*[28] characterized a region mapping approximately 1200-bp upstream from the *MYC* transcriptional start site (TSS), and slightly upstream from *MYC* 5′ WRE, that harbors a third TBE, TBE3. In an extensive set of chromatin immunoprecipitation (ChIP) analyses, they found that β-catenin/TCF complex binding to this region correlated with *MYC* transcription in CRC cells. They also demonstrated that when Wnt signaling was shut-off, APC removed β-catenin and transcriptional co-activators from this site and replaced them with transcriptional corepressors to repress *MYC* expression. Interestingly, unlike wild-type APC, truncated APC found in CRC cells was unable to mediate this corepressor exchange, providing one mechanism underlying how APC mutations lead to aberrant *MYC* expression.

The identification and characterization of *MYC* promoter proximal WREs was an important step forward in our understanding of how β-catenin/TCF complexes drive *MYC* expression. However, very low levels of *MYC* transcripts are produced from a transgene in mice containing the human *MYC* genomic locus, and regions 2.3-kb upstream and 0.4-kb downstream, suggesting that additional WREs may contribute to regulating *MYC* expression[29].

THE DOWNSTREAM *MYC* PROXIMAL WRE

To identify regulatory elements that control *MYC* expression, Mautner *et al*[30] mapped DNaseI hypersensitivity sites in proximity to the *MYC* gene in the Colo320 CRC cell line. This strategy mapped a strong hypersensitivity site 1.5-kb downstream from the *MYC* transcription stop site, although it was not determined at that time whether this region demarcated a WRE. In a subsequent study, some 13 years later, a strong β-catenin binding region that overlapped this hypersensitive site was identified in the HCT116 human CRC cell line[31]. Using a combination of ChIP assays, luciferase reporter assays, and *MYC* transcript analysis, it was determined that this β-catenin/TCF binding element demarcated a WRE termed the *MYC* 3′ WRE (Figure 2)[26]. Using chromosome conformation capture (3C), it was demonstrated that β-catenin/TCF4 complexes coordinated a chromatin loop that integrated *MYC* 5′ and 3′ WREs in CRC cells[27]. This chromatin conformation was absent in quiescent CRC cells when *MYC* expression is silenced, but was induced by serum mitogens as *MYC* levels increased, indicating that loop formation accompanied *MYC* transcriptional activation. Interestingly, in HEK293 cells that contain an intact Wnt/β-catenin signaling pathway, either treatment with Wnt3A ligand or activation of the pathway with the GSK3 inhibitor lithium chloride (LiCl), failed to induce the chromatin loop even though *MYC* expression was increased. However, over-expression of oncogenic β-catenin (S45F) in these cells induced the *MYC* 5′3′ loop. Thus, the *MYC* 5′3′ chromatin conformation required elevated levels of nuclear β-catenin that typifies CRC.

In the colonic crypts of mice harboring a germ-line deletion of the *Myc* 3′ WRE, there was a 2-fold increase in *Myc* transcript levels and a 2.5-fold increase in MYC protein levels relative to levels seen in the colons of wild-type littermates[32]. This increase in MYC correlated with an increase in the number of proliferative cells and a decrease in the number of differentiated cells comprising the colonic crypt epithelium. Two approaches were taken to ascertain the role of this WRE in colorectal carcinogenesis[33]. First, *Myc* 3′ WRE$^{-/-}$ mice were bred to *Apc*$^{Min/+}$ mice that harbor a mutation in one *Apc* allele and spontaneously form intestinal tumors as the mice age and the second wild-type *Apc* allele is lost[34,35]. In comparison to *Apc*$^{Min/+}$, *Apc*$^{Min/+}$ *Myc* 3′ WRE$^{-/-}$ mice contained 4-fold more colonic tumors. Second, *Myc* 3′ WRE$^{-/-}$ mice and wild-type littermates were subjected to the azoxymethane/dextran sodium sulfate model of

colitis-associated colorectal cancer (CAC). *Myc* 3' WRE$^{-/-}$ mice subjected to this protocol contained an elevated number of tumors along the entire colonic tract, most notably in the ceca, which presented 20-fold more tumors. Thus, the primary function of this WRE is to repress *Myc* in the colonic epithelium.

DISTAL *MYC* WREs

The MYC -335 WRE

The *MYC* locus maps within a gene-poor region on chromosome 8q24[36]. Upstream of *MYC*, results from genome-wide association studies identified regions that harbor single nucleotide polymorphisms that are associated with an increased risk for the development of colon, breast, and prostate cancers[37-44]. One SNP in particular, rs6983267, has received interest in the field, with the G allele associated with increased risk of CRC over the T allele[40,42,44]. Despite this relationship, it was unknown how this SNP contributed to CRC, as *MYC*, the nearest protein-coding region, resides 335-kb downstream[45]. Using the enhancer element locator computational method, Tuupanen *et al*[46] found that a region encompassing rs6983267 was predicted to harbor a strong enhancer. Importantly, rs6983267 is adjacent to a TCF4 binding motif and was predicted to influence the binding of this factor. Indeed, the G allele conferred greater Wnt-responsiveness through this site that correlated with enhanced TCF4 binding. In an accompanying manuscript, Pomerantz *et al*[47] shed light on the mechanism for how the rs6983267 risk variant might influence *MYC* expression. They found that this *MYC* -335 WRE was physically juxtaposed to the *MYC* promoter region through a long-range chromatin loop. This result was confirmed by both Ahmadiyeh *et al*[48] and Jäger *et al*[49] in studies to identify regions of 8q24 that interact with the *MYC* -335 WRE by 3C and capture Hi-C, respectively. Furthermore, Sotelo *et al*[50] also reported evidence for this long-range interaction and found that overexpression of β-catenin and TCF4 increased the frequency of the association. However, a subsequent study found that while the SNP had no effect on the efficiency of chromatin looping, the risk-associated allele increased expression of the linked *MYC* allele[51]. Although the precise mechanistic details remain to be fully defined, these findings offer a potential explanation for how transcription factors bound to a distal regulatory element can drive oncogenic *MYC* expression in CRC.

In 2012, Sur *et al*[52] reported findings from a mouse model containing a germ-line deletion in the *Myc* -335 WRE. As was the case in *Myc* 3' WRE$^{-/-}$ intestines, deletion of the *Myc* -335 WRE did not cause gross phenotypic alterations of the intestinal architecture. Moreover, deletion of the *Myc* -335 WRE did not alter the cellular composition of the intestinal epithelium or influence expression of *Myc* in the duodenum. However, *Myc* expression in the colons of *Myc* -335 WRE$^{-/-}$ was decreased relative to levels seen in the colons of wild-type littermates. Importantly, when these mice were bred to *Apc*$^{Min/+}$ mice, deletion of the *Myc* -335 WRE led to a reduced number of tumors that formed in both the small intestines and colons. Thus, this element is a critical regulator of intestinal tumors that arise from pathogenic Wnt/β-catenin signaling.

As the *Apc*$^{Min/+}$ mouse is an important model for CRC, it is somewhat limited by the fact that tumors preferentially arise in the small intestine and not the colon[53]. A recent study suggests that this phenotype may be due to the fact that stem cells in the small intestine divide more rapidly than those in the colons and hence there is an increased chance of accumulating mutations in the small intestine[54]. In addition, tumors that arise in *Apc*$^{Min/+}$ mice are primarily adenomas, which do not progress to carcinomas because the mice become moribund[53]. Therefore, to determine whether the *MYC* -335 WRE is required in colorectal carcinomas requires deleting this regulatory element in a human CRC cell line. As part of an elegant study to functionally annotate genes whose expression is influenced by colon cancer risk SNPs, Yao *et al*[55] used clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) to delete the *MYC* -335 WRE in HCT116 human CRC cells. While *MYC* -335 WRE$^{-/-}$ cells expressed less *MYC* relative to parental cells, it was not reported whether this influenced the oncogenic properties of these cells.

In an intriguing study, Ling *et al*[56] offered an additional explanation for how the *MYC* -335 WRE influences *MYC*-driven colorectal carcinogenesis. They found that a long non-coding RNA, which they termed colon-cancer associated transcript two (*CCAT2*), is expressed from this region and contains the rs6983267 SNP. *CCAT2* expression is elevated in colon tumors relative to levels detected in adjacent and uninvolved colonic mucosa. Overexpression of *CCAT2* in HCT116 cells promoted tumorigenesis when these cells were implanted into immunocompromised mice, whereas *CCAT2* knockdown diminished the invasive capacity of the KM12SM CRC cell line. Moreover, *CCAT2* overexpression increased *MYC* expression in HCT116 cells, whereas *CCAT2* knockdown decreased *MYC* expression in these cells. *CCAT2* itself is a Wnt/β-catenin target gene, and it interacts directly with TCF4 to augment TCF4-dependent expression of Wnt/β-catenin target genes, including *MYC*. Further studies are necessary to fully understand how the rs6983267 SNP impinges upon *CCAT2* function.

In an effort to understand the function of rs6983267 in colorectal carcinogenesis, Kim *et al*[57] cloned seven lncRNAs derived from the 8q24.21 gene desert region. It was found that one of these lncRNAs, termed cancer-associated region long non-coding RNA number 5 (*CARLo5*), played an important role in driving CRC. *CARLo5* expression was elevated in CRC relative to expression in normal adjacent tissues, and knocking down *CARLo5* expression diminished CRC cell proliferation and growth of these cells as tumors in athymic nude mice. Interestingly, a region containing the

MYC-335 WRE was juxtaposed to the *CARLo5* promoter region to drive its expression in CRC cells.

The MYC distal super-enhancer

In 2010, Bottomly *et al*[58] reported results from a ChIP-Seq screen to localize β-catenin binding sites throughout the genome in the HCT116 human CRC cell line. This screen confirmed that β-catenin bound the *MYC* 5′ WRE, 3′ WRE, and -335 WRE in these cells. In addition, they noted that a cluster of β-catenin binding sites mapped to a region approximately 400-500-kb upstream from the *MYC* TSS. In a follow-up study, it was found that these regions bound TCF4, β-catenin, and RNA Polymerase Ⅱ (RNAP) and are demarcated by histones with modifications that typify enhancer regions including H3K4me1 and H3KAc[59,60]. Four of five of these distal β-catenin-bound regions formed long-range chromatin loops with the *MYC* proximal promoter region. Interestingly, these conformations were not only present in HCT116 cells, but also the non-CRC cell lines HEK293 and TIG-1, suggesting that β-catenin might use these pre-existing chromatin loops to activate *MYC* gene expression. Moreover, the interaction frequencies between two of these regions and the *MYC* proximal promoter were induced upon stimulation of the cells with serum mitogens.

Several years later, Hnisz *et al*[61] found that this distal cluster of *MYC* WREs overlapped with a super-enhancer. According to Whyte *et al*[62], super-enhancers are "…large clusters of transcriptional enhancers - formed by binding of high levels of master TFs (transcription factors) and Mediator coactivator - that drive expression of genes that define cell identity". These super-enhancers also bind high levels of the chromatin-associated protein bromodomain containing protein 4 (BRD4)[63]. Through its bromodomain, BRD4 associates with acetylated histones and recruits the positive transcription elongation factor (PTEF-b) to promote transcriptional elongation by RNAP[64,65]. BRD4 also interacts directly with the Mediator complex, and Mediator has been shown to play a role in chromatin looping[64-66]. JQ1 is a selective inhibitor of BRD4 activity that competes with BRD4 binding to acetylated substrates[67]. Interestingly, treatment of multiple myeloma cells with JQ1 led to a preferential loss of BRD4 occupancy at super-enhancers and repression of oncogene expression, including *MYC*[63,68]. A comparison of H3K27Ac patterns obtained from ChIP-Seq analysis in normal colonic mucosa and the HCT116 CRC cell line revealed that high levels of nucleosomes containing this modification map to the distal *MYC* super-enhancer in CRC cells[61]. By overlaying the ChIP-Seq profile for TCF4 binding in these cells, Hnisz *et al*[61] identified four putative WREs embedded within the distal super-enhancer that corresponded to four of the five *MYC* WREs identified previously in this region by Yochum[59]. Indeed, luciferase assays conducted with reporters containing these elements confirmed that they functioned as WREs[61].

As is the case for the *MYC* -335 WRE, a lncRNA termed colon cancer-associated transcript one (*CCAT1*), localizes within the *MYC* super-enhancer[69]. Two isoforms of *CCAT1* are expressed; a long form, *CCAT1-L*, which is 5200-bp in length, and a short form, *CCAT1-S*, which is 2600-bp in length. Both isoforms are expressed at higher levels in CRCs relative to levels in patient matched uninvolved colonic mucosa[69-71]. In contrast to *CCAT1-S*, which localizes to the cytoplasm, *CCAT1-L* localizes to the nucleus. *CCAT1-L* knockdown in CRC cells reduces *MYC* expression, while increased expression of *CCAT1-L* from its chromosomal locus enhances *MYC* expression. It was demonstrated that *CCAT1-L* is an important regulator of chromatin looping between *MYC* WREs and the *MYC* promoter. Interestingly, this lncRNA not only promoted chromatin looping between the *MYC* super-enhancer and the *MYC* promoter, but also between the *MYC* super-enhancer and the *MYC* -335 WRE. In addition, the CCCTC-binding factor (CTCF) was shown to play a role in mediating the interactions between distal *MYC* WREs and the *MYC* promoter. *CCAT1-L* interacted directly with CTCF and stabilized CTCF binding to distal WREs, providing a mechanism for *CCAT1-L*-mediated regulation of *MYC*.

ACTIVATION OF *MYC* EXPRESSION BY ONCOGENIC WNT/β-CATENIN SIGNALING

To summarize, mutations in components of the Wnt/β-catenin signaling pathway lead to aberrant *MYC* expression in CRCs through β-catenin/TCF transcription complexes bound to WREs. The *MYC* 3′ WRE, *MYC* -335 WRE, and distal super-enhancer are juxtaposed to the *MYC* 5′ WRE within the proximal promoter region through long-range chromatin loops (Figure 3)[27,47-51,59,69]. We therefore propose a model in which "hijacked" distal WREs align to the *MYC* proximal promoter and increase the local concentration of β-catenin/TCF transcription complexes to drive oncogenic *MYC* expression in CRC. Thus, the *MYC* proximal promoter serves as a "landing pad" to coordinate chromatin conformations at *MYC*. As the 3C technique represents an average interaction frequency across a population of cells, it is unknown whether these chromatin loops occur simultaneously at a single *MYC* allele[72]. It is also important to note that in several cases, these chromatin loops are not restricted to CRC cells and their formation does not depend on Wnt/β-catenin signaling[48,51,59]. Thus, the conformation itself, and not its formation, may poise the *MYC* locus to receive oncogenic signals. Finally, it is probable that additional WREs and other enhancer elements contribute to deregulated *MYC* expression in CRC cells[50,73].

CONCLUSION

Despite the identification of MYC over 30 years ago

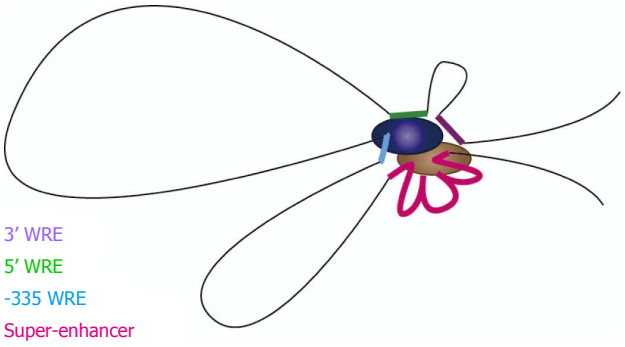

Figure 3 Model for the chromatin interaction network at the *MYC* gene locus in colorectal cancer cells with elevated nuclear β-catenin. β-Catenin "hijacks" MYC WREs in CRC cells, therefore, driving or stabilizing a distinct promoter-enhancer interaction network that is required for deregulated MYC gene expression. TCF/Lef and β-catenin are depicted as brown and purple ovals, respectively. WRE: Wnt responsive DNA element; CRC: Colorectal cancer; TCF/Lef: T-cell factor/Lymphoid enhancer factor.

and numerous reports linking deregulated *MYC* gene expression to tumorigenesis, there is still no clinically available drug that targets MYC in cancer cells[74-76]. This is in part due to the fact that, unlike other proteins, *MYC* is very rarely mutated in cancers[76]. Instead, *MYC* is overexpressed due to aberrant activation of upstream signaling pathways or due to events that trigger amplification of the *MYC* gene locus or insertions of activating sequences[77]. It is, therefore, difficult to target MYC in cancer cells *vs* normal, healthy cells where MYC is required for cellular proliferation[76]. Despite these difficulties, recent approaches to inhibit the BRD4 chromatin reader have been demonstrated to specifically and profoundly diminish *MYC* gene expression in several cancer cells[63,68,78-80]. These studies indicate that targeting *MYC* at the transcriptional level is an effective therapeutic strategy for treating cancers with deregulated *MYC* gene expression. Therefore, further characterization of the *MYC* WREs discussed in this review will likely provide new avenues for targeting *MYC* gene expression in CRC cells.

Recent reports have indicated that active DNA enhancer elements express short, bidirectional RNAs termed enhancer RNAs (eRNAs)[81-84]. Expression level changes observed for eRNAs correspond to changes in mRNA levels of the target gene, but the mechanism through which eRNAs activate target gene expression appears to vary based on cellular context. eRNAs have been found to stabilize enhancer-promoter interactions and also to relieve transcriptional pausing by inactivating the negative elongation factor complex[85,86]. It is unknown whether eRNAs are transcribed from the *MYC* WREs described in this report. It is feasible that eRNA transcripts derived from these *MYC* WREs facilitate *MYC* gene expression by stabilizing *MYC* WRE interactions with the *MYC* promoter. Additional factors could also be functioning to stabilize chromatin loops and activate *MYC* expression. β-Catenin was recently demonstrated to recruit cohesin and direct enhancer-promoter interactions in human embryonic stem cells[87]. If β-catenin also recruits cohesin at *MYC* WREs in CRC cells, it could explain how "hijacked" *MYC* WREs interact with the *MYC* promoter or suggest that β-catenin stabilizes pre-existing chromatin loops. CTCF could also be a critical factor for maintaining the genomic architecture at the *MYC* gene locus, as the interaction frequency of the *MYC* super-enhancer with the *MYC* promoter is diminished after CTCF knockdown[69]. To provide a better understanding of how β-catenin drives deregulated *MYC* gene expression in CRC cells, future work is needed to define the factors critical for *MYC* chromatin conformations and to determine the role of these conformations in activating *MYC* expression in CRCs.

Before candidate WREs can be considered as therapeutic targets, proof-of-principle experiments are required. Namely, whether these elements are dispensable in the normal colonic epithelium, but essential for colon carcinogenesis, which would suggest that a subset of WREs may be "hijacked" by oncogenic Wnt/β-catenin signaling. Indeed the mouse studies described by Sur *et al*[52] indicated that the *Myc* -335 WRE did not play a role in intestinal homeostasis, but was required for intestinal tumorigenesis caused by mutations in *Apc*. To determine whether this element was required in HCT116 human CRC cells, Yao *et al*[55] deleted it using CRISPR/Cas9 gene editing. While this deletion reduced *MYC* expression in these cells, it was not reported whether it altered the chromatin conformation at the *MYC* locus or reduced the oncogenic potential of these cells. Interestingly, the *MYC* distal super-enhancer is preferentially activated in CRC and not uninvolved colonic mucosa[61,88]. However, due to its size and complexity, it may be difficult to target using gene editing strategies. Although we reported that the *Myc* 3′ WRE suppressed colonic tumorigenesis in mice, we noted that deletion of this element decreased tumorigenesis in the small intestines of $Apc^{Min/+}$ mice[33]. Therefore, we used CRISPR/Cas9 to target the *MYC* 3′ WRE in HCT116 cells (SAR, GSY, unpublished). Using this approach, we generated clonal cell lines that harbored homozygous deletions in one of two TBEs within this element. These cells contain reduced *MYC* expression at the transcript and protein levels and also display reduced oncogenic properties.

Further investigation of the factors required for WRE-mediated transcriptional regulation of *MYC* will provide a more detailed model of how *MYC* gene expression becomes deregulated in CRCs. This model can then be applied to investigate deregulated *MYC* expression as a result of constitutive upstream signaling pathway activation in other cancers and also to identify potential therapeutic targets.

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REVIEW

Crosstalk between mitochondria and peroxisomes

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Abstract

Mitochondria and peroxisomes are small ubiquitous organelles. They both play major roles in cell metabolism, especially in terms of fatty acid metabolism, reactive oxygen species (ROS) production, and ROS scavenging, and it is now clear that they metabolically interact with each other. These two organelles share some properties, such as great plasticity and high potency to adapt their form and number according to cell requirements. Their functions are connected, and any alteration in the function of mitochondria may induce changes in peroxisomal physiology. The objective of this paper was to highlight the interconnection and the crosstalk existing between mitochondria and peroxisomes. Special emphasis was placed on the best known connections between these organelles: origin, structure, and metabolic interconnections.

Key words: Peroxisome; Mitochondrion; Beta-oxidation; Reactive oxygen species; Dynamic; Fatty acids

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Core tip: The goal of this review was to highlight the links between mitochondria and peroxisomes in terms of dynamic and metabolism. This review of the literature shows that these two organelles, even if they derive from distinct ancestors, share several common functions and coordinate their activities. The division of peroxisomes and mitochondria uses similar mechanisms, and autophagic processes are used to limit the number of both organelles. The metabolic implication of mitochondria and peroxisomes in fatty acid metabolism is remarkable, as these organelles use closely-related pathways for oxidizing fatty acid, but with different metabolic goals. All together, the available data suggest a major interconnection between these organelles.

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INTRODUCTION

Mitochondria and peroxisomes are small organelles present in almost every cell of higher organisms. They share some properties (such as size and function), but differ in terms of origin, structure, and physiological roles. With the current knowledge of the subject, it

seems clear that these two organelles communicate in the cell, and in some cases participate at the same biosynthetic pathways. They share many enzymes and mitochondrial function has been shown to regulate peroxisomal activity.

Peroxisomes and mitochondria share a common range size of 0.1 to 1 μm. However, they differ in terms of structure: Mitochondria are surrounded by a double membrane, while peroxisomes are bordered by a single membrane system. In the cell, the number of peroxisomes and mitochondria varies according to the cell type (*e.g.*, mitochondria are very abundant in brown adipose tissue, but relatively scarce in white adipocytes). Peroxisomal and mitochondrial abundance is regulated by several cellular parameters: (1) organelle formation; (2) organelle dynamic; and (3) organelle death.

ARE MITOCHONDRIA AND PEROXISOME DERIVED FROM A COMMON ANCESTOR?

Origin

Both, peroxisomes and mitochondria are very dynamic organelles. They show high plasticity and are able to adopt various shapes depending on the cell's requirements. Their number and morphology can change according to the metabolic needs of the cell and/or the physiopathological environment.

Peroxisomal origin: The origin of peroxisomes is still not fully understood. From the early days of peroxisome discovery, peroxisomes were supposedly of endosymbiotic origin[1]. However, this theory is no longer considered, as many reports have suggested that peroxisomes are instead derived from the endoplasmic reticulum[2]. This has been especially shown in experiments in which cells with no peroxisomes were able to engender new peroxisomes[3]. In fact, several studies have established that peroxisomes can be formed from pre-existing peroxisomes, as well as *de novo* from the endoplasmic reticulum (ER) under certain circumstances[4,5].

When looking at the origin of proteins present in peroxisomes, two categories of proteins can be described: those of a prokaryotic origin and those of an eukaryotic origin[6]. Eukaryotic originating proteins are essentially involved in peroxisomal biogenesis, while peroxisomal proteins originating from bacteria appear to be initially targeted to mitochondria. In accordance with the finding that peroxisomes are derived from the ER, many conserved proteins involved in peroxisome biogenesis and repairs are homologous to proteins present in the ER[7]. About 25% of proteins in peroxisomes have an origin that is difficult to precisely define. This suggested that some peroxisomal proteins have evolved from mitochondrial proteins. Many proteins are present in both organelles, which suggests that these enzymes may be retargeted from mitochondria to peroxisomes[8]. This would indicate that peroxisome formation is influenced by mitochondria.

However, if peroxisomes are derived from ER, why are mitochondrial proteins included in the organelle and why are mitochondria not assuming these functions? What are the links between ER and mitochondria/peroxisomes? These questions remain as yet unanswered.

The origin of mitochondria: The origin of mitochondria is more likely to be endosymbiotic. The endosymbiotic theory indicates that mitochondria were initially free-living prokaryotes that entered eukaryotic cells and became organelles. However, this theory has not always been accepted by the scientific community; it was first proposed for plastids at the beginning of the 20th century, but was only accepted for mitochondria for a few years before being rejected by cell biologists. It was not until the 1960's that the theory was reconsidered and more widely accepted[9].

This theory is based on several facts: (1) endosymbiotic organelles retain a small genome encoding several dozen proteins. Regardless of this genome reduction, mitochondria harbor at least 2000 proteins[10] involved in many biochemical pathways and, in particular, in energy production. The difference between the number of proteins encoded by the mitochondrial genome and the number of proteins located in this organelle is usually explained by another aspect of endosymbiotic mechanisms, *i.e.,* and (2) the endosymbiotic gene transfer. This process of gene transfer occurs during the evolution of the organism and, in time, will become a protein import mechanism, such as the mitochondrial import system for proteins. This closes the endosymbiotic process circle; the organelle carries out functions that the eukaryotic cell is unable to realize, while the eukaryotic cell provides extra proteins for the organelle. This process gives evolutionary advantages to both the eukaryotic cell and the "new" organelle[11].

Even if they share a few similarities, mitochondria and peroxisomes do not derive from the same evolutionary process, with mitochondria deriving from an endosymbiotic process and peroxisomes from an intracellular maturation process.

Protein import

Protein import in peroxisomes: Proteins present in the peroxisome are strictly dependent on nuclear genes and to an import system. Peroxisomes do not possess any DNA sequences and are not able to synthesize any proteins. They rely on cytosolic synthesis from nuclear genes and on the import of these proteins into the peroxisome. This protein import relies on the presence of a peroxisomal targeting signal in the protein sequence: either PTS1 or PTS2. The import of proteins into the peroxisomal matrix is a coordinated process involving the intervention of many proteins known as peroxins (Pex)[12]. Pex proteins control peroxisome structure, division, and inheritance. Over a dozen peroxins have

been described[13].

PTS1 is composed of the consensual sequence SKL (or their conserved variants) and is located at the C terminal domain of the protein. It is mostly used by proteins located in the peroxisomal matrix[14]. PTS1-containing proteins are recognized by Pex5 protein, whose C-terminal domain (and in rare cases its N-terminal domain) interacts with the PTS1 sequence[15,16]. This is a method of peroxisomal import unique to several species, such as the nematode *Caenorhabditis elegans*[17]. In mammals, several proteins contain another type of PTS: Type 2 PTS. This motif is recognized by the Pex7 protein, and proteins carrying this PTS2 signal are transported into the peroxisome with the same mechanisms[18].

Protein import into mitochondria: Mammalian mitochondria possess their own genome, which is a circular DNA chain of about 16000 base pairs. In animal mitochondria, the genetic code is slightly different from the "universal" code[19]. The structure of this genome is simple: there are virtually no non-coding regions and the genes are mostly adjacent to each other. The mitochondrial genome encodes for 2 rRNA, 22 tRNA, and 13 polypeptides involved in mitochondrial respiration[20].

All proteins synthesized from the mitochondrial genome participate in the respiratory chain (complex Ⅰ, Ⅲ, Ⅳ, and Ⅴ) and are located in the inner membrane of the mitochondria[21]. Among these 13 proteins, 7 are present in complex Ⅰ (NADH: Ubiquinone oxidoreductase), 1 is part of complex Ⅲ (ubiquinone: Cytochrome c oxidoreductase), 3 belong to complex Ⅳ (cytochrome c: Oxygen oxidoreductase), and 2 are part of complex Ⅴ (ATP synthase).

This means that the other proteins present in the mitochondria (*i.e.,* around 2000 proteins) are the products of nuclear genes. As for any nuclear genes, the corresponding proteins are synthesized in the cytosol, although mitochondrial proteins are subsequently imported into the mitochondria. The import of these proteins requires that they find their way to the mitochondria. The journey of these precursors throughout the cytosol is supported by mitochondrial targeting elements involved in the transport of the precursors to specific receptors on the mitochondrial surface. This mechanism also depends on cytosolic factors[22]. The most common mitochondrial targeting signal is a positively-charged sequence, known as the presequence, which is located at the N-terminus of the protein[23]. The presequence addresses proteins to the mitochondrial matrix, the inner membrane, or the mitochondrial intermembrane space. This process is universal as an important part of proteins located in the mitochondrial outer membrane, although many proteins of the inner membrane and the intermembrane space do not possess the classical presequence, and instead enclose internal cryptic targeting sequences in their amino acid sequence.

Once onto the mitochondrial outer membrane, mitochondrial protein precursors go through the lipid of this membrane with the intervention of the TOM complex (mitochondrial outer membrane preprotein translocase). The TOM complex is made of seven subunits and forms a channel that allows for the crossing of the outer membrane[24].

Dynamic

Contrarily to the nucleus that is present as a single organelle in almost all cells, numerous peroxisomes and mitochondria are present, with the actual number depending on the metabolic needs of the cells. The shape and the interconnection among and between these organelles also change depending on the metabolic environment[25].

Peroxisomal dynamic: The peroxisomes show high plasticity and a high capacity of adaptation in response to developmental, metabolic, and environmental alterations. Their number, protein content, and shape can be modulated. Peroxisome number can increase either by division of pre-existing organelles or, at least under certain circumstances, from *de novo* biosynthesis from the ER[26]. While most of the biochemical processes are involved in this dynamic process, the basic mechanisms and nature of the control of these processes are still poorly understood[27].

Mitochondrial dynamic: Mitochondria are also dynamic organelles that permanently change their morphology, size, and number. This dynamic is associated with the processes of fusion/fission that permit the fusion of two mitochondria or the division of a mitochondrion to give rise to two mitochondria, respectively[28].

In the cell, mitochondrial fusion and fission participate in maintaining an adequate mitochondrial number. The fusion process allows mitochondria to combine their whole content. This process participates in mtDNA repair, complementation of proteins, and in the balance of metabolites. Fission also participates in the dynamic of mitochondria, as this process participates in mtDNA segregation. It may also participate in the removal of altered mitochondria through the process of mitophagy. Additionally, these mechanisms of fission/fusion participate in the positive segregation of mitochondria.

The key enzyme for fission of mitochondria is Drp1. This enzyme has a GTPase activity that promotes the fission of mitochondrial lipid membrane[29]. Drp1 is the ortholog of Dnm1, a yeast enzyme. The action of Drp1 requires the translocation of this protein onto specific sites located in the outer mitochondrial membrane. Although initially only two proteins were described as docking proteins for Drp1 (Fis1 and Mff), another two (MiD49 and MiD51) were later discovered[30,31].

Drp1 is a very controlled enzyme. Its intracellular level and activity are regulated through various mechanisms, including SUMOylation and phosphorylation[32,33]. A recent review has listed and analyzed all of the factors that participate in the regulation of mitochondrial

fission[34]. The authors emphasized the regulatory role played by Bcl2 family proteins and the regulatory role of TNF-alpha and PKA.

Mitochondrial fusion involves Mfn1 and Mfn2 proteins[35]. These mitofusins are large proteins located in the mitochondrial outer membrane. They exhibit GTPase activity and allow the fusion of two outer mitochondrial membranes coming from two distinct mitochondria[35]. OPA1, on the other hand, is used in the fusion of mitochondrial inner membranes, and is located in the outer side of the inner mitochondrial membrane[36,37]. This process of fusion is highly regulated. To identify the physiological roles of these proteins, mouse mutants have been realized. Mice carrying mutations in the *OPA1* gene were created, with the resulting homozygous mice dying during gestation[38]. Mfn1- and Mfn2-deficient mice were made, which die during midgestation. Similarly, animal models carrying a deletion of the *Drp1* gene also died at the embryonic stage in mice[39].

Comparison: While it seems clear that mitochondria and peroxisomes do not derive from a common ancestor, it may be surprising that the fission machinery is in a good part conserved between these organelles. The genetic deletion of Drp1 leads to peroxisomes and mitochondria with an altered structure[39,40]. The docking proteins Mff and Fis1 are also important for both organelles[41]. This suggests that whatever the origin of these organelles, they are able to interact with each other.

Organelle degradation

Autophagy is a genetically programmed process that degrades and removes proteins present in the cell, as well as participating in the removal of damaged or excessive organelles through the initial formation of a structure known as the autophagosome; these structures will then fuse with lysosomes and have their content degraded[42].

The process of autophagy can be divided into five major steps: (1) Development of the isolation membrane; (2) Elongation of this membrane; (3) Closure of the isolation membrane with the formation of the autophagosome; (4) Fusion between the autophagosome and lysosomes; and (5) Degradation of the autophagosome content[43]. The overall mechanism is similar in yeast and mammalian cells. In the yeast *Saccharomyces cerevisiae*, nearly 30 autophagy-related proteins have been identified[44,45].

Pexophagy: An autophagic process called pexophagy is responsible for regulating the number of peroxisomes in the cell[46]. While, in mammalian cells, all aspects of this programmed peroxisome death are not yet fully characterized, some aspects of this process have been described. It has been shown that Pex11p, Pex25p, and Pex27p positively control this mechanism. It was also reported that Drp1 was directly involved in peroxisome division. The implication of Drp1a and Pex11 is not clear yet. Drp1 and Pex11-beta are overexpressed during this process, but they did not seem to act directly upon it[47].

The mechanisms involved have been extensively studied in yeast and have been recently reviewed[48].

Mitophagy: Mitophagy is a biological process that allows the elimination of mitochondria using an autophagic process[49]. During mitophagy, mitochondria are initially integrated into an autophagosome, which subsequently fuses with lysosomes and leads to the degradation of its content. This mechanism was initially described in cells undergoing starvation, but mitophagy also participates in the regulation of mitochondrion numbers[50].

METABOLIC CROSSTALK

Reactive oxygen species production and scavenging

Intense oxidation activities occur in both mitochondria and peroxisomes. These organelles are also directly implicated in the production of reactive oxygen species (ROS) through physiological and extraphysiological processes. ROS are able to activate the inflammasome system, which is a multiprotein system coupled with the caspase and interleukin activating systems. The activation of inflammasome leads to a programmed cell death process, and its dysregulation may play a significant role in various diseases[51].

On the other hand, both mitochondria and peroxisomes possess biological tools that allow for the scavenging of damaging reactive oxygen species[52]. It is important to note that ROS scavenging is crucial in limiting the cellular damage that these compounds may induce. It should also be noticed that ROS physiologically contribute to various pathways, including those involved in metabolism and signaling.

ROS production in peroxisomes: Many oxidases that produce several kinds of ROS (including nitric oxide, superoxide radicals, hydroxyl radicals, and hydrogen peroxide) are present in peroxisomes. H_2O_2 is mainly produced by oxidases that use many different types of substrates, such as lactate, urate, or oxalate[53]. Peroxisomes are also potential sources of $O_2\bullet^-$ and NO•, *via* the enzymatic activity of xanthine oxidase and nitric oxide synthase[54]. Xanthine oxidase also produces H_2O_2 and $O_2\bullet^-$ as byproducts[54]. Nitric oxide synthases (NOS) are a broad family of enzymatic proteins, with the inducible form of NOS catalyzing the oxidation of L-arginine to NO• and citrulline in response to induction by cytokines or endotoxins. This reaction requires O_2, FAD, tetrahydrobiopterin, NADPH, and FMN and, in the absence of adequate amounts of substrates, the enzyme can also produce important amounts of $O_2\bullet^-$[55]. Mammalian peroxisomes do not seem to contain enzymes that directly produce •OH or $ONOO^-$[54]. However, these ROS might be able to produce them as secondary products from H_2O_2, $O_2\bullet^-$, and NO•.

ROS production in mitochondria: The mitochondrial electron transport chain is also an important source of ROS. At the complex 1 level, superoxide ions are produced; these ions can be subsequently transformed into more potent ROS.

ROS can induce a vicious cycle that may lead to cell death. Excessive production of ROS is able to damage many macromolecules. Lipids, proteins, and DNA can become targets for ROS, and an inadequate production of ROS will damage mitochondrial enzymes and mitochondrial DNA. Subsequently, these initial damages will induce an altered functioning of the electron transport system and ultimately increase the production of ROS. In addition, overproduction of ROS in mitochondria may lead to the release of cytochrome c and the triggering of apoptosis[56].

Scavenging of ROS in peroxisomes: Several anti-oxidant systems are present in peroxisomes: catalase, Mn-SOD, Cu/Zn-SOD, peroxiredoxin Ⅰ, epoxide hydrolase, peroxisomal membrane protein 20, and glutathione peroxidase are present in the peroxisomal matrix and contribute to the defense against excessive ROS.

Catalase is the "historic" marker of peroxisomal activity and has a crucial protective function against the peroxides generated in peroxisomes and their toxicity[57]. This common marker for peroxisomes metabolizes both H_2O_2 (catalytic function) and many other substrates, such as ethanol, methanol, phenol, and nitrites, through its peroxidatic activity[58]. Catalase is targeted to peroxisomes, as it possesses modified PTS1.

The relationship between oxidative stress and the peroxisomal ROS scavenging system has been studied. It has been shown that an increase in oxygen concentration induced a moderate increase in the activity of enzymes involved in the scavenging of ROS[59]. On the other hand, low levels of enzymes involved in ROS scavenging, such as catalase, glutathione peroxidase, and Mn-SOD, are commonly observed in malignant cells. In cultured cells, it has been observed that oxidative stress (*i.e.*, UV irradiation or exposure to H_2O_2) induces pronounced elongation of peroxisomes[60], with antioxidant treatment blocking this elongation process. This elongation step seems a prerequisite for peroxisome division[61]. This suggests that peroxisomes can be activated when oxidative stress occurs, indicating that peroxisomes may actively participate in the control of ROS accumulation in the cell.

Scavenging of ROS in mitochondria: Mammalian mitochondria possess enzymes and non-enzymatic antioxidants systems for ROS scavenging[62]. Enzymes with antioxidant activities such as MnSOD, glutathione reductase, glutathione-S-transferase, and molecules with anti-oxidant properties such as thioredoxin, glutaredoxin, peroxiredoxins, cytochrome c, glutathione, and NADH are present in the mitochondria and participate in limiting oxidative damage; these aspects have been extensively reviewed by Andreyev *et al*[62]. Exposure of cells to a overproduction of ROS induces an increase in activity of the mitochondrial defense system[62].

Scavenging of ROS mobilizes both mitochondria and peroxisomes, as both organelles possess similar systems for counteracting excessive production of ROS. However, the underlying mechanisms involved in the recruitment of either system remain unknown.

Fatty acid metabolism in peroxisome and mitochondria

One of the most remarkable common features between mitochondria and peroxisome is the cooperative function for fatty acid oxidation. Fatty acids play many important role in energy production, inflammation and its resolution[63], *etc*. In mammalian cells, both peroxisomes and mitochondria contain a beta-oxidative pathway. Beta-oxidation is a key pathway for the breakthrough of fatty acids. In yeasts and plants, fatty acid oxidation occurs uniquely in peroxisomes[64], as mitochondria are not able to catabolize fatty acids. In mammalian cells, both peroxisomes and mitochondria can beta-oxidize fatty acids. These two pathways share many similarities, especially in terms of enzymatic reactions, but they differ in terms of substrates and enzymatic reactions. Furthermore, the metabolic implication and final products are not the same in mitochondria and peroxisomes[65] (Table 1).

The overall pathways are essentially the same in both mitochondria and peroxisomes: Fatty acids are first activated as acyl-CoA and then the activated fatty acid (acyl-CoA) is dehydrogenated. This represents the first step of beta-oxidation. Hydration of the double bound then occurs, and is followed by dehydrogenation and cleavage. This allows for the removal of 2 C from C:n acyl-CoA, leading to the formation of C:n-2 acyl-CoA.

Peroxisomal fatty acid oxidation: In peroxisomes, the very first step of fatty acid oxidation is the conversion reaction of fatty acid into acyl-CoA. The thus-formed acyl-CoA can be directed to the peroxisomal matrix after crossing the peroxisomal membrane, with the intervention of an ABC transporter (ABCD1) being implied[66]. Once in the peroxisomal matrix, beta-oxidation starts *via* a reaction catalyzed by acyl-CoA oxidase (ACOX), an enzyme that is often considered a key element of the process. During the reaction catalyzed by ACOX, electrons provided by FAD are transferred to oxygen, thereby leading to the formation of H_2O_2. In the mitochondrial pathway, the reaction is essentially the same, with the exception that the electrons are transferred to the respiratory chain instead. ACOX isoforms have been described in mammals and are all dimeric proteins. The next reaction is catalyzed by an enzyme known as the multifunctional enzyme (MFE), which realizes both the second and the third reactions of peroxisomal beta-oxidation. In the peroxisomal matrix, two MFE are present: MFE-1 [L-bifunctional protein (LBP)] and MFE-2 [D-bifunctional protein (DBP)]; both

Table 1 Comparison between mitochondrial and peroxisomal beta-oxidation

	Mitochondrial	Peroxisomal
Substrates	LCFA	VLCFA, branched FA, leukotrienes
Entry system	Carnitine system (including CPT1, CACT and CPT2)	ABC transporters
End products	Acetyl-CoA (and subsequently ATP)	Acetyl-CoA and MCFA (and subsequently H202)
Physiological implications	Energy production	Biosynthesis of specific fatty acids (*e.g.*, DHA)

LCFA: Long-chain fatty acids; VLCFA: Very long chain fatty acids; FA: Fatty acids; CPT: Carnitine palmitoyltransferase; CACT: Carnitine-acylcarnitine translocase; ABC: ATP-binding cassette; MCFA: Medium-chain fatty acids; DHA: Docosahexaenoic acid.

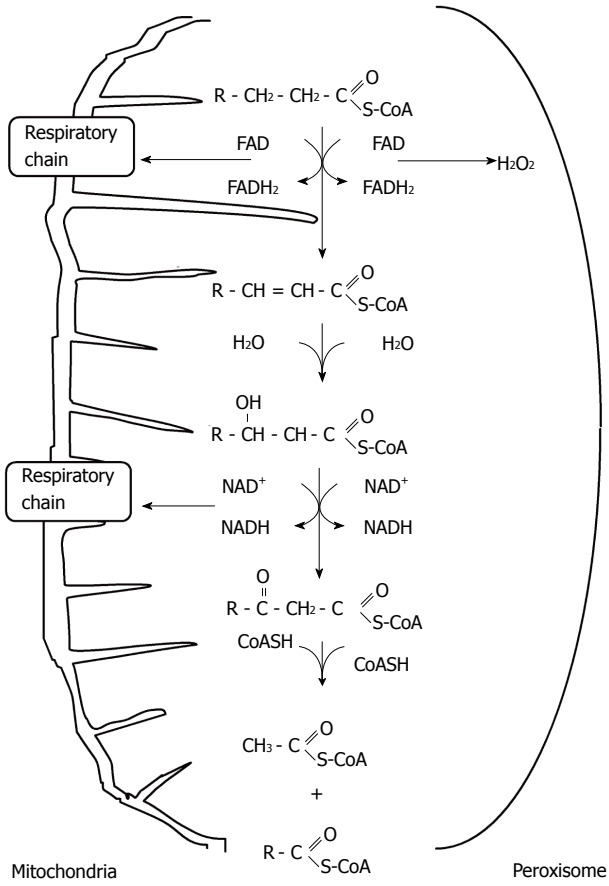

Figure 1 Mitochondrial and peroxisomal beta-oxidations. The reactions involved in mitochondria are presented on the left side of the figure, while the reactions occurring in peroxisome are on the right side of the figure.

catalyze the formation of 3-ketoacyl-CoA intermediates from substrate mirror image stereochemistry. Although the two enzymes catalyze the same reaction, they do not show any similarities in terms of structure[67]. The final reaction in this pathway is catalyzed by thiolase. This enzyme catalyzes the thiolytic cleavage of 3-ketoacyl-CoA to acetyl-CoA and Cn-2-acyl-CoA[68]. The peroxisomal beta-oxidation is incomplete, as it climaxes with shortened acyl-CoA (medium chain, with one of the common being octanoyl-CoA). These compounds are converted into acylcarnitine by carnitine octanoyl transferase, and can then leave the peroxisome[69].

Peroxisomal beta-oxidation mainly concerns very long chain fatty acids (> C22) and branched fatty acids, as well as some prostaglandins and leukotrienes. While mitochondrial beta-oxidation relies on the energy needs of the cells, peroxisomal beta-oxidation does not. Peroxisomal beta-oxidation is essentially involved in biosynthesis pathways, while the mitochondrial pathway is related to catabolism and energy production. The end product of peroxisomal oxidation of fatty acid is H_2O_2, while mitochondrial beta-oxidation is coupled with the production of ATP (Figure 1).

Mitochondrial fatty acid oxidation: Mitochondrial beta-oxidation mainly involves long chain fatty acids provided by foodstuff. This pathway supplies the acetyl-CoA used, at least in part, for ATP synthesis (Figure 1).

As for the peroxisomal pathway, mitochondrial fatty acid beta-oxidation requires the initial esterification of fatty acids into acyl-CoA and then the entry of acyl-CoA into the mitochondrial matrix. The activation of fatty acid into acyl-CoA is catalyzed by acyl-CoA synthases, which are ATP-dependent enzymes located in the cytosol. According to the size of the fatty acid, several acyl-CoA synthases with various affinities for different types of substrates carry out these reactions. The following are present in the cytosol: short-chain acyl-CoA, medium-chain acyl-CoA, and long-chain acyl-CoA. Once converted into acyl-CoA, these compounds can cross the mitochondrial membranes; short and medium-chain acyl-CoAs seem to be able to freely cross the mitochondrial membrane while, for long chain acyls, the crossing of the mitochondrial double membrane system requires the intervention of the carnitine system. This system consists of two acyl-transferases (carnitine palmitoyltransferase 1 and 2) and the transporter carnitine acylcarnitine translocase. The presence of L-carnitine is also required as an essential part of this system[70].

Four enzymatic reactions compose the mitochondrial beta-oxidation. The initial reaction is catalyzed by acyl-CoA dehydrogenase, while the subsequent steps are catalyzed by 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-oxoacyl-CoA thiolase, successively. The overall goal of mitochondrial beta-oxidation is the production of energy, mainly as ATP. The beta-oxidation itself consists of successive cycles (consisting of the four aforementioned enzymatic reactions) leading to the removal of 2C from C: n acyl-CoA, thereby generating C: n-2 acyl-CoA. The end-product of this pathway is acetyl-CoA, while mitochondrial beta-oxidation is metabolically coupled with the respiratory chain and tricarboxylic acid

cycle[71].

CONCLUSION

While peroxisomes and mitochondria do not derive from a common ancestor (the origin of mitochondria is endosymbiotic, while peroxisomes derive from the endoplasmic reticulum), several proteins are common among these organelles and they share not only a few enzymes, but also full metabolic pathways. Their divisions are closely related and use identical factors and enzymes. This suggests efficient crosstalk between peroxisomes and mitochondria.

However, the physiological function of the two organelles are different. In terms of fatty acid metabolism, mitochondria degrade the majority of long-chain fatty acids to supply acetyl-CoA for the production of ATP and for anabolic reactions, while peroxisomal beta-oxidation is more involved in anabolic processes. However, the two organelles work together for the metabolism of fatty acids. Peroxisomes and mitochondria are independent organelles but their interaction is necessary for optimal function of the cell.

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REVIEW

Modulation of the matrix redox signaling by mitochondrial Ca^{2+}

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Abstract

Mitochondria sense, shape and integrate signals, and thus function as central players in cellular signal transduction. Ca^{2+} waves and redox reactions are two such intracellular signals modulated by mitochondria. Mitochondrial Ca^{2+} transport is of utmost physio-pathological relevance with a strong impact on metabolism and cell fate. Despite its importance, the molecular nature of the proteins involved in mitochondrial Ca^{2+} transport has been revealed only recently. Mitochondrial Ca^{2+} promotes energy metabolism through the activation of matrix dehydrogenases and downstream stimulation of the respiratory chain. These changes also alter the mitochondrial NAD(P)H/$NAD(P)^+$ ratio, but at the same time will increase reactive oxygen species (ROS) production. Reducing equivalents and ROS are having opposite effects on the mitochondrial redox state, which are hard to dissect. With the recent development of genetically encoded mitochondrial-targeted redox-sensitive sensors, real-time monitoring of matrix thiol redox dynamics has become possible. The discoveries of the molecular nature of mitochondrial transporters of Ca^{2+} combined with the utilization of the novel redox sensors is shedding light on the complex relation between mitochondrial Ca^{2+} and redox signals and their impact on cell function. In this review, we describe mitochondrial Ca^{2+} handling, focusing on a number of newly identified proteins involved in mitochondrial Ca^{2+} uptake and release. We further discuss our recent findings, revealing how mitochondrial Ca^{2+} influences the matrix redox state. As a result, mitochondrial Ca^{2+} is able to modulate the many mitochondrial redox-regulated processes linked to normal physiology and disease.

Key words: Calcium transport; Signal transduction; Redox regulation; Mitochondria; Oxidation-reduction; Mitochondrial membrane transport proteins; Mitochondrial Ca^{2+} uniporter; Mitochondrial Na^+/Ca^{2+} exchanger

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Core tip: Deregulated redox signaling in mitochondria leads to mitochondrial dysfunction, associated with several disorders and disease states. Matrix Ca^{2+} rising can be linked through multiple pathways to the mitochondrial redox state. Here we describe recent progress in the field of mitochondrial Ca^{2+} handling. We further summarize how mitochondrial Ca^{2+} signals are influencing the mitochondrial redox state. This link between Ca^{2+} and redox signals is likely

of central importance in the regulation of mitochondrial function in health and disease.

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INTRODUCTION

Mitochondria are versatile multifunctional organelles best known for their contribution to cellular energy homeostasis. Mitochondria are also central regulators of cell fate. The involvement of mitochondria in a large number of biological processes is dependent on two unique characteristics. First, mitochondria are organelles able to sense and influence a number of intracellular signals (ions and small molecules, such as Ca^{2+}, ATP, pH and the redox potential). Second, these organelles are very dynamic. Mitochondria undergo morphological changes (they fragment and fuse)[1,2], and they increase or decrease in the mass due to mitochondrial biogenesis[3] and mitophagy[4]. Finally, they can move within the cell[5]. Mitochondrial dynamics combined with their ability to control the fluxes of ions and small molecules makes this organelle a central player in signal transduction. Two of the signals strongly affected by mitochondria are Ca^{2+} and redox state.

Ca^{2+} is a key intracellular messenger that coordinates a vast repertoire of cellular functions, ranging from contraction, secretion and fertilization to the control of transcription, proliferation, several aspects of development as well as learning and memory[6-8]. Cells express a large number of proteins for the precise spatial and temporal control of Ca^{2+} rising[6]. Mitochondria efficiently contribute to the shaping of Ca^{2+} signals through Ca^{2+} uptake and release[9-12]. The associated matrix Ca^{2+} rises (transient or prolonged) act as a signal *per se* that can modulate energy metabolism and cell fate[12-16]. Although the basic properties of mitochondrial Ca^{2+} handling have been established several decades ago, the molecular identities of the mitochondrial Ca^{2+} transport systems have only been revealed over the last 6 years (see section on "Molecular identification of mitochondrial Ca^{2+} transporters", below). The identification and study of these transporters has improved our understanding of the physio-pathological role of mitochondrial Ca^{2+} transport and provided researchers with new opportunities for molecular intervention.

Signals other than Ca^{2+} are generated/integrated in the mitochondrial matrix, notably redox reactions linked to the production of reactive oxygen species (ROS), and changes in the oxidation state of thiol groups in proteins (thiol switches). Redox reactions and associated changes can serve as cellular signals. Metabolites and proteins can activate specific cellular signaling pathways in a redox state-dependent manner[17,18]. Thiol switches in proteins are controlled by the balance of two opposite influences, oxidizing and reducing. ROS are able to shift the equilibrium to a more oxidized state. This is particularly relevant in mitochondria, which are a major source of ROS[19,20]. Such oxidation in mitochondria is counteracted by reducing systems that depend on the availability of NAD(P)H, which is generated by mitochondrial metabolism[18]. The oxidation/reduction of thiol groups in mitochondrial target proteins can modulate their activity, localization and stability. Such changes can regulate mitochondrial functions, including nutrient oxidation, oxidative phosphorylation, ROS production, mitochondrial permeability transition, cell death and mitochondrial morphology[21]. The study of redox switches *in vivo* has been a challenge[22]. However, the recent development of fluorescent protein redox sensors has revolutionized the study of redox processes in living cells. They allow real-time compartment-specific monitoring of thiol redox dynamics[22-25].

Excellent reviews have covered the mechanisms of ROS production[19], the importance of redox signals in the modulation of mitochondrial function, and how deregulation of these signals can lead to the development of various disease states[21,26,27]. The effect of Ca^{2+} on ROS generation and in the regulation of cellular energetics has also been reviewed recently[13,28]. Here we will review the transporters mediating and regulating the entry and extrusion of calcium across the inner membrane. We will further discuss the evidences linking mitochondrial Ca^{2+} rises to the modulation of the matrix redox signals.

MOLECULAR IDENTIFICATION OF MITOCHONDRIAL Ca^{2+} TRANSPORTERS

Mitochondria are equipped with sophisticated machinery mediating Ca^{2+} fluxes across the inner mitochondrial membrane (Figure 1). This system is composed of channels, exchangers, regulatory proteins and a poorly characterized matrix Ca^{2+} buffer system. In this section, we will focus on the recently identified Ca^{2+} channels and exchangers.

Mitochondrial Ca^{2+} uptake mechanisms

Mitochondrial Ca^{2+} uniporter: The mitochondrial Ca^{2+} uniporter (MCU) is the principal mediator of Ca^{2+} transport into the mitochondrial matrix. The MCU catalyzes the passive and unidirectional transport of Ca^{2+} across the inner mitochondrial membrane, a process that is driven by the electrochemical gradient ($\Delta\psi_{mit}$) in energized mitochondria. The inside negative potential of -180 mV is generated as electrons transferred stepwise along the respiratory chain from electron donors inside the matrix to the end-acceptor molecular oxygen. Given the large electrical gradient across the inner mitochondrial membrane, the organelle has

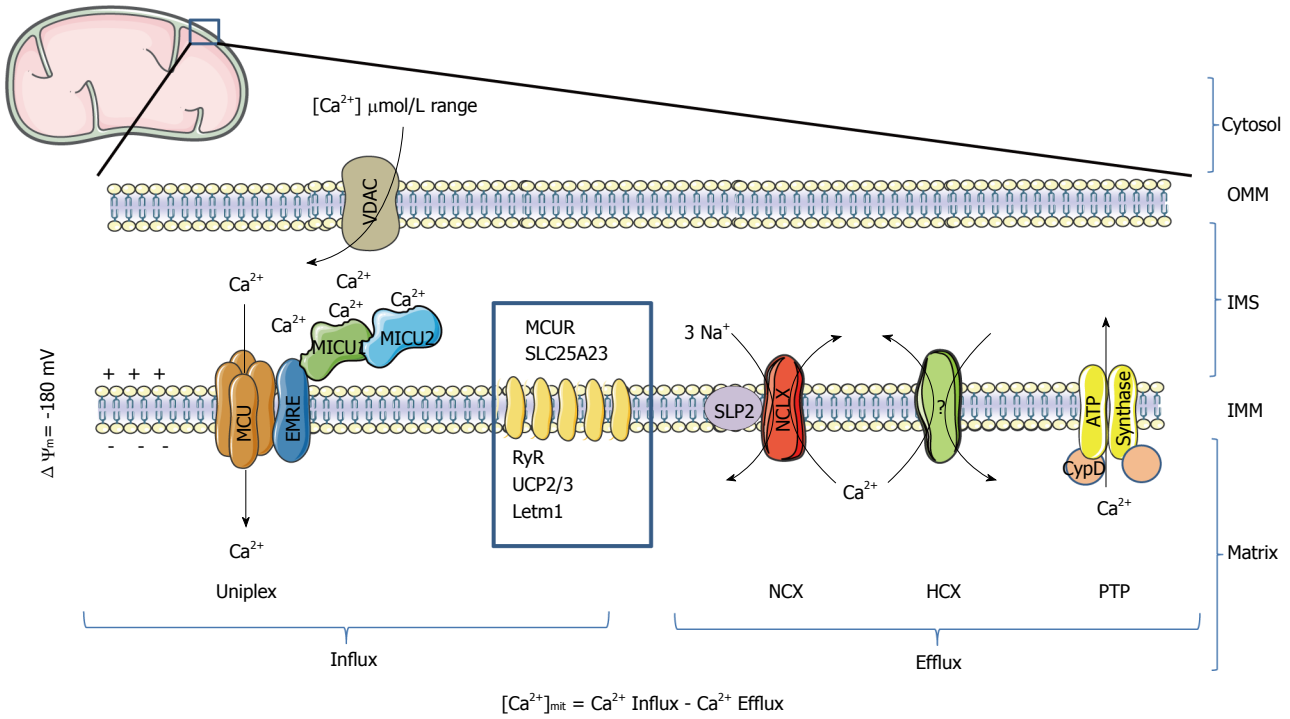

Figure 1 Ca^{2+} transport proteins of mitochondria. In mammalian mitochondria, the uptake of Ca^{2+} is mediated by the Ca^{2+}-selective channel MCU, which is part of a high molecular weight protein complex called Uniplex. At least 4 additional proteins (MCUb, MICU1, MICU2 and EMRE) regulate MCU activity. Ca^{2+} is then extruded by a sodium/calcium exchange (NCX) or proton/calcium exchange (HCX). If the protein NCLX has been confirmed to be the mitochondrial NCX, which is down-regulated by the protein SLP-2, the molecular nature of the mitochondrial HCX is still debated. Dimers of mitochondrial ATP synthase have been proposed to form the PTP, a mitochondrial channel regulated by CypD, that facilitates PTP opening by desensitizing PTP to Ca^{2+}. Besides being activated by Ca^{2+}, PTP has also been proposed to act as a reversible fast Ca^{2+} release channel. Other non-MCU mitochondrial proteins with an indirect or debated effect on Ca^{2+} transport are represented in the blue square (MCUR; SLC25A23; ryanodine receptor, RyR; UCP2; UCP3; LETM1). OMM: Outer mitochondrial membrane; IMS: Inter-membrane space; IMM: Inner mitochondrial membrane; VDAC: Mitochondrial porin; PTP: Permeability transition pore; CypD: Cyclophilin D; MCU: Mitochondrial Ca^{2+} uniporter; MCUR1: Mitochondrial calcium uniporter regulator 1; MICU1: Mitochondrial Ca^{2+} uptake protein 1; MICU2: Mitochondrial Ca^{2+} uptake protein 2; EMRE: Essential MCU regulator.

the potential to import large amounts of Ca^{2+}. At a concentration of 100 nmol/L cytosolic Ca^{2+} in a resting cells and a mitochondrial potential of -180mV, the Nernst equation would predict that the equilibrium will only be reached at 100 mmol/L mitochondrial [Ca^{2+}]. The result would be a gradient of six orders of magnitude between matrix and cytosolic calcium concentration. However, this matrix Ca^{2+} concentration is never reached under physiological conditions for three reasons: (1) the mitochondrial Ca^{2+} uptake mechanism displays low Ca^{2+} affinity, and the K_d has been estimated close to 10 μmol/L. Therefore efficient mitochondrial Ca^{2+} uptake only occurs in the μmol/L cytosolic [Ca^{2+}] range, protecting the mitochondria from Ca^{2+} overload in resting cells; (2) mitochondria activate Ca^{2+} extrusion as soon as [Ca^{2+}] rises in the mitochondrial matrix, following cell stimulation; and (3) the mitochondrial matrix contains a poorly defined high capacity Ca^{2+} buffer system, composed of PO_4^-, Ca^{2+} binding proteins and metabolites that significantly reduce free mitochondrial [Ca^{2+}].

Electrophysiological recordings in mitoplasts have revealed the existence of an inward rectifying highly Ca^{2+}-selective current across the inner mitochondrial membrane (I_{MCU})[29]. This current was shown to be reflecting mitochondrial Ca^{2+} uniport activity as it was blocked by two well-characterized pharmacological inhibitors of MCU, ruthenium red and Ru360[29]. This study was the first to define the electrophysiological properties of the MCU.

Following a long-lasting search for the proteins responsible for mitochondrial calcium uniport, two groups independently identified the essential component of the MCU in 2011[30,31]. The Mitocarta, the most complete compendium of mitochondrial proteins, was used as a starting point for the identification of MCU1 (mitochondrial Ca^{2+} uptake protein 1). Baughman *et al*[30] identified MCU1 on the basis of an integrative genomic approach combining whole-genome phylogenetic profiling, genome-wide co-expression analysis and organelle-wide protein co-expression analysis to predict proteins functionally related with MICU1. MICU1 had previously been identified as essential for mitochondrial Ca^{2+} uptake[32]. De Stefani *et al*[31] uncovered MCU1 by analyzing well-known and predicted characteristics of the mitochondrial uptake mechanism: the ubiquitous expression in mammalian cells, its absence in yeast (which lacks a ruthenium

red-sensitive Ca^{2+} uptake mechanism), its presence in kinetoplastids (which express a ruthenium red-sensitive Ca^{2+} uptake mechanism), and the presence of two or more predicted transmembrane domains.

The MCU is part of a high molecular weight protein complex called Uniplex (Uniporter Complex, Figure 1). This complex is comprised of at least 5 different proteins: MCU1[30,31], MCUb[33], MICU1[32], MICU2[34,35] (mitochondrial Ca^{2+} uptake protein 2) and essential MCU regulator (EMRE)[36]. Less well established is the interaction with and functional relevance of 2 additional mitochondrial proteins: MCUR and SLC25A23. MCU1 constitutes the pore-forming subunit. This essential component of the uniporter is sufficient for uniporter activity[37]. MCU1 contains two α-helix trans-membrane domains connected by a loop in the inter-membrane space[30]. Biochemical evidence and computational modeling predict MCU1 forming a tetramer. The trans-membrane domains build the pore of the channel. The loops, facing the inter-membrane space, constitute the mouth of the channel, which also confers Ca^{2+} selectivity. Sequence analysis of MCU led to the identification of a new MCU1 paralogue, named MCUb. This protein can replace MCU1 subunits resulting in different MCU1/MCUb ratios as observed in different tissues. MCUb can be considered a dominant negative pore-forming version of MCU1. Its presence adds a regulatory mechanism that modulates the properties of the channel[33]. MICU1 and MICU2 are Ca^{2+}-sensitive subunits of the complex[32,38]. Both carry EF-hand (helix-loop-helix) Ca^{2+} binding domains facing the inter-membrane space (Figure 1). Indirectly, these EF-hands sense cytosolic signals. MICU1 and MICU2 are likely to work as a gatekeeper defining the activation threshold of the channel, preventing the activity at resting Ca^{2+} levels (100 nmol/L) and triggering MCU activity when Ca^{2+} microdomains (several μmol/L) are generated close to the channel[39,40]. MICU1 has been shown to control cooperativity of Ca^{2+} uptake, a well-defined characteristic of the MCU. This regulatory mechanism favors the active state of the channel at high cytosolic $[Ca^{2+}]$[39]. The Ca^{2+} binding affinity of each active helix-loop-helix domain on MICU1 and MICU2 has been estimated to be in the range of 15-21 μmol/L. These values are consistent with the necessity to reach high Ca^{2+} microdomains as an essential requirement for full MCU activation[41]. The recently obtained crystal structure of MICU1 suggests that in the absence of Ca^{2+}, the protein forms hexamers that inhibit MCU1. Conversely, in the presence of Ca^{2+}, MICU1 undergoes a conformational change, forming multiple oligomers that activate MCU1[41]. Biochemical evidence suggests that MICU2 physically interacts with MICU1, which in turn interacts with MCU1. Functional interaction studies between MCU1, MICU1 and MICU2 suggest that both regulatory subunits contribute to MCU activation as a function of the amount of cytosolic $[Ca^{2+}]$. At high cytosolic $[Ca^{2+}]$, the stimulatory effect of MICU1 drives the rapid response of mitochondria to cytosolic $[Ca^{2+}]$ rises. Conversely, at low cytosolic $[Ca^{2+}]$, MICU2 is required to prevent mitochondrial Ca^{2+} uptake[34,35]. EMRE[36] was discovered as part of the mitochondrial calcium uptake machinery by quantitative mass spectrometry, and was shown to mediate MCU1-MICU1 physical and functional interaction. When EMRE was missing, the interaction between MCU1 and MICU1/2 was disrupted, despite intact MCU1 oligomers and preserved MICU1-2 interactions[36]. EMRE knock-out and knock-down models display a strong decrease in the ability to take up Ca^{2+} in permeabilized mitochondria. Furthermore, I_{MCU} Ca^{2+} current is virtually absent in mitoplasts from EMRE knocked-out cells. These results are in conflict with earlier results suggesting that MCU1 alone is sufficient for MCU activity[37].

A large variety of mitochondrial Ca^{2+} currents has been observed across different tissues and cell types[42]. Based on the current knowledge of the function of MCU components, it will be possible to study how their stoichiometry influences the variability of mitochondrial Ca^{2+} currents in different cell types.

A number of additional proteins have been shown to be important for mitochondrial Ca^{2+} uptake[43-47]. These proteins are not likely part of the Uniplex, but rather may be involved in alternative mechanisms of mitochondrial Ca^{2+} uptake. Therefore, this group of proteins is discussed separately. A genome-wide siRNA screen, designed to detect new proteins involved in mitochondrial Ca^{2+} uptake[43,44], has identified leucine zipper-EF-hand containing transmembrane protein 1 (LETM1) as a molecule able to mediate high affinity (200 nmol/L) mitochondrial Ca^{2+} uptake in exchange for H^+. Several laboratories have been able to reproduce the mitochondrial Ca^{2+} uptake defect in LETM1 knock-down cells[48,49]. Furthermore, when reconstituted in liposomes, LETM1 mainly promotes electro-neutral $Ca^{2+}/2H^+$ exchange[50]. However, LETM1 was previously proposed to exchange K^+ against H^+[51,52]. Also, thermodynamic considerations would argue against electro-neutral $Ca^{2+}/2H^+$ exchange, as this should promote Ca^{2+} extrusion rather than uptake given the proton gradient (0.8 pH units more alkaline in the matrix) across the inner mitochondrial membrane[53]. Knockout of LETM1 causes mitochondrial dysfunction, swelling and depolarization, thus reducing the driving force for mitochondrial Ca^{2+} uptake. Depolarization of the inner mitochondrial membrane as a secondary consequence of LETM1 disruption may explain impaired MCU-mediated Ca^{2+} transport[51,52,54]. The role of LETM1 in mitochondrial ion homeostasis remains controversial. MCUR1 was originally reported to be a component of the Uniplex, contributing to ruthenium red-sensitive mitochondrial Ca^{2+} uptake[45]. However, MCUR1 was not identified in mass spectrometry experiments of the purified Uniplex, arguing against a direct role in mitochondrial Ca^{2+} uptake[36]. In the absence of MCUR1 expression, oxidative phosphorylation is impaired and cellular ATP levels lower, leading to activation of AMP kinase. Recent evidence suggests that MCUR1 may instead work as a cytochrome-c oxidase assembly factor[55]. The evidence suggests that loss of MCUR1 impairs respiratory function, leading to diminished $\Delta\psi_{mit}$ and

thereby a reduction of mitochondrial Ca^{2+} uptake. Recently, the protein SLC25A23, a member of the mitochondrial Ca^{2+}-dependent solute carrier family, previously considered to be an ATP-Mg/PO_4^- carrier, has been shown to physically interact and positively modulate MCU activity. Lack of SLC25A23, as well as overexpression of the SLC25A23 EF-hand mutants, was shown to reduce MCU activity[46]. SLC25A23 may therefore be a regulatory subunit of the Uniplex. UCP2 and UCP3 have also been proposed to be essential for mitochondrial Ca^{2+} uptake[47]. This is unlikely, since mitochondria isolated from tissues of the UCP2 and UCP3 knock-out mice displayed unaltered Ca^{2+} uptake[56]. Recently, impaired mitochondrial Ca^{2+} uptake has been confirmed in UCP3 knock-out cells[57]. Even though MCU activity did not rely on the UCP3 in intact cells, the lack of UCP3 decreased cytosolic ATP available for SERCA pumps. As a result, IP_3-driven cytosolic and mitochondrial Ca^{2+} rises were reduced[57]. The contribution of UCP2 and UCP3 to mitochondrial calcium uptake is therefore *via* their impact on Ca^{2+} handling in the ER.

Studies in cardiac cells indicate that ryanodine receptors, one of the main endoplasmic reticulum Ca^{2+} release channels, contribute to an alternative mitochondrial Ca^{2+} uptake mechanism[58,59]. Consistent with this, localization of ryanodine receptors to mitochondria was demonstrated using electron microscopy and Western-blotting[58,59]. Pharmacological inhibition with ryanodine diminished inward Ca^{2+} current in mitoplasts insensitive to the MCU blocker Ru360[60]. Along the same line of evidence, single channel recordings in mitoplasts from HeLa cells after knock-down of MCU1 revealed a 2.5-fold increase in the occurrence of the extra-large conductance Ca^{2+} current[61]. These observations are consistent with an alternative and compensatory molecular mechanism for mitochondrial Ca^{2+} uptake.

Mitochondrial Ca^{2+} release mechanisms

Two main mechanisms have been proposed to account for mitochondrial Ca^{2+} release[62]: (1) Na^+-dependent, mediated by a recently identified mitochondrial Na^+/Ca^{2+} exchanger named NCLX; and (2) Na^+-independent, probably mediated by a H^+/Ca^{2+} exchanger. These two mechanisms operate to extrude Ca^{2+} during physiological mitochondrial [Ca^{2+}] transients. A third mechanism, called permeability transition pore (PTP, see below) opening gets activated under specific physiopathological conditions when mitochondria experience Ca^{2+} overload for extended periods of time[62].

The mitochondrial Na^+/Ca^{2+} exchange discovered by Carafoli *et al*[63] constitutes the main pathway for mitochondrial Ca^{2+} extrusion[64,65]. The stoichiometry of ion exchange has been estimated to be $3Na^+/Ca^{2+}$. This electrogenic export mode favors Ca^{2+} extrusion in energized mitochondria[66]. Like MCU, NCLX is highly selective for Ca^{2+} when compared with other divalent ions, but less selective for Na^+ that can be replaced by Li^+[63,67]. Although NCLX has recently been thought to localize to the plasma membrane, electron microscopy and cell fractionation experiments clearly showed that NCLX is targeted to the mitochondrial inner membrane. Na^+-dependent Ca^{2+} release was strongly reduced in NCLX knock-down cells, whereas NCLX overexpression enhanced it[68]. NCLX-driven Ca^{2+} extrusion is inhibited by 7-Chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP-37157), the most selective inhibitor of mitochondrial Na^+/Ca^{2+} exchange. Taken together, the evidence led to the conclusion that NCLX encodes the so-called mitochondrial Na^+/Ca^{2+} exchanger[68]. Several studies suggest the existence of regulatory mechanisms controlling mitochondrial Ca^{2+} export kinetics. The protein kinases PKC[69] and PINK1[70] were reported to modulate the activity of this ion exchanger. Stomatin-like protein 2 (SLP-2), which localizes to the inner mitochondrial membrane, was also shown to inhibit mitochondrial Na^+/Ca^{2+} exchange[71]. CGP-37157, an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger, was used to demonstrate that this exchange mechanism contributes to shape cytosolic [Ca^{2+}] transients[72], to mediate the Ca^{2+} transfer from the extracellular medium to the ER during IP_3-driven Ca^{2+} signaling[73], and modulates the NAD(P)H redox state[74] and ATP production[72].

Mitochondria suspended in buffers devoid of Na^+ retain their capacity to extrude Ca^{2+}, pointing to a Na^+-independent mechanism. This Ca^{2+} efflux pathway is catalyzed by a to date non-identified H^+/Ca^{2+} exchanger (reviewed in Bernardi)[62]. LETM1 has been proposed recently to exchange Ca^{2+} against H^+. LETM1 may therefore drive extrusion of Ca^{2+} from energized mitochondria[43,44]. In conflict with this interpretation, LETM1 expression in HeLa cells did not alter Ca^{2+} efflux rates, regardless of the amplitude of Ca^{2+} elevation reached during agonist stimulation[74]. These findings cast doubt on the Ca^{2+} exchanger function of LETM1.

Permeability transition pore: The PTP is a Ca^{2+} and ROS-activated, voltage-dependent and cyclosporine A-sensitive channel located in the inner mitochondrial membrane. Opening of the permeability transition pore causes a sudden increase in the mitochondrial inner membrane permeability to solutes with molecular masses up to 1500 Dalton[75-77]. Opening of PTP leads to mitochondrial permeability transition, which plays an important role in intracellular death signaling and in events ranging from tissue damage upon infarction to muscle wasting in some forms of dystrophy[75]. Given that PTP activation occurs under several pathological conditions[14,75], the channel has been extensively characterized as a pharmacological target. The proteins forming the PTP channel have been recently rediscovered. The classical model envisioned a supramolecular complex spanning the double membrane system of mitochondria including the protein voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocator in the inner membrane, cyclophilin D in the mitochondrial matrix, and also including additional

proteins such as Bax. However, genetics studies have demonstrated that permeability transition and/or even single channel activity can be observed in mitochondria devoid of the proposed components of the PTP[78-83]. Recent evidence from the Bernardi laboratory has revolutionized our view of the PTP. They propose that the pore of the channel is formed by dimers of the ATP synthase[84] (Figure 1). To be activated by Ca^{2+}, PTP has also been proposed to act as a reversible fast Ca^{2+} release channel[85]. Such transient opening of the PTP may be induced by physiological stimuli for fast mitochondrial Ca^{2+} release, preventing Ca^{2+} overload[86].

Reevaluation of the physiological role of mitochondrial Ca^{2+}

Mitochondrial Ca^{2+} uptake has been related with a plethora of cell functions, including exocytosis, gene transcription, cell cycle regulation, respiration and cell death[12,14]. Much of the evidence linked to the function of mitochondrial Ca^{2+} was obtained over the last few decades using pharmacological tools. The identification of the molecular machinery governing mitochondrial Ca^{2+} fluxes has led to a large number of genetic studies that re-evaluate the role of mitochondrial Ca^{2+} in cell physiology/pathology. Furthermore, they have allowed for the first time their role to be studied in the context of the whole organism. Recently, an MCU1 knock-out mouse has been generated. Analysis of the mouse phenotype has led to new questions regarding the importance of mitochondrial Ca^{2+} uptake. Unexpectedly, the mouse is viable. It only displays limited impairment of muscle function, even during exercise. Contrary to expectations, MCU KO hearts were also not protected from the damage induced by ischemia/reperfusion[87,88]. It is worth mentioning that the mice analyzed were obtained from a mixed genetic population (CD1) background, because disruption of the *MCU1* gene in pure C57/BL/6 inbred mice led to embryonic lethality[89]. Developmental defects were also observed in a Zebra fish knock-down model that showed defects in gastrula morphogenesis[90]. The results with MCU1 KO mice on a mixed genetic background suggest that in these animals, alternative mechanisms are able to compensate for the absence of MCU function. It has been postulated that alternative Ca^{2+} uptake mechanisms may be responsible for this compensation. This hypothesis is hard to reconcile with the absence of regulated Ca^{2+} uptake in mitochondria isolated from MCU1 KO mice[87]. Despite the negative results obtained in KO mice on a mixed genetic background, MCU activity seems to play an important role in muscle physiology. Manipulation of MCU1 expression in skeletal muscle cells *in vivo* revealed that MCU levels modulate muscle size. The phenomenon is linked to the PGC1α4 and on IGF1-AKT/PKB signaling pathways[91]. MCU-dominant negative transgenic mice showed similar heart rates compared to the wild type animals under resting conditions, but failed to increase the beating frequency upon physiological adrenergic stimulation[92]. Detailed assessment of hearts from mice lacking MCU revealed markedly impaired mitochondrial Ca^{2+} uptake. Surprisingly, the hearts of these animals appear to function relatively normally, even during stress[93]. MICU1 loss of function mutations in human fibroblasts led to a defect in mitochondrial Ca^{2+} homeostasis[94]. Patients carrying such mutations displayed neurological disorders and muscle disease.

MATRIX REDOX SIGNALING MODULATION BY MITOCHONDRIAL Ca^{2+} EXTRUSION

Energy metabolism and redox balance are regulated by mitochondrial Ca^{2+}. There is a complex relationship between Ca^{2+} and redox signaling, as Ca^{2+} promotes both oxidizing and reducing biological processes. Matrix Ca^{2+} rises stimulate respiratory chain activity[95,96], which as a side-product also produces ROS[97]. ROS production occurs at complexes Ⅰ, Ⅱ and Ⅲ of the respiratory chain and several flavoproteins in different cellular compartments[18]. In fact, it was shown that mitochondria are able to produce H_2O_2[98,99]. The peroxide is formed from dismutation of superoxide ($O_2^{\bullet -}$), which is generated within mitochondria[100,101]. By promoting ROS formation, matrix Ca^{2+} causes net oxidation of the mitochondrial redox state. On the other hand, several Ca^{2+}-activated dehydrogenases of the mitochondrial matrix form reducing equivalents, therefore favoring reduction of mitochondrial redox couples. Notably, studies in Bristol in the 1960s and 1970s led to the recognition that mitochondrial Ca^{2+} promotes the supply of reducing equivalents in the form of NADH or $FADH_2$[102-104]. Four Ca^{2+}-activated mitochondrial dehydrogenases were described: FAD-glycerol phosphate dehydrogenase (located on the outer surface of the inner mitochondrial membrane; influenced by changes in cytoplasmic Ca^{2+} concentration), pyruvate dehydrogenase, NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase (the latter three located within mitochondria and regulated by changes in mitochondrial matrix Ca^{2+} concentration). Following early studies with isolated mitochondria, the results on Ca^{2+} regulation of mitochondrial metabolism were confirmed *in situ*[13]. Recent evidence on insulin-secreting INS-1E-cells demonstrates that the interplay between mitochondrial Ca^{2+} and matrix production of reducing equivalents may be even more complex[105]. Matrix calcium signals accelerate respiration and increase cytosolic ATP levels[16]. Under the same conditions, NAD(P)H levels increased rapidly to reach a new steady-state in both INS-1E cells and human pancreatic islets[105]. Surprisingly, this substrate-dependent increase of NAD(P)H was also observed when calcium signaling was prevented. The data is consistent with Ca^{2+}-dependent control both at the level of dehydrogenases and the respiratory chain. The accelerated formation of reducing equivalents by dehydrogenases is balanced by enhanced

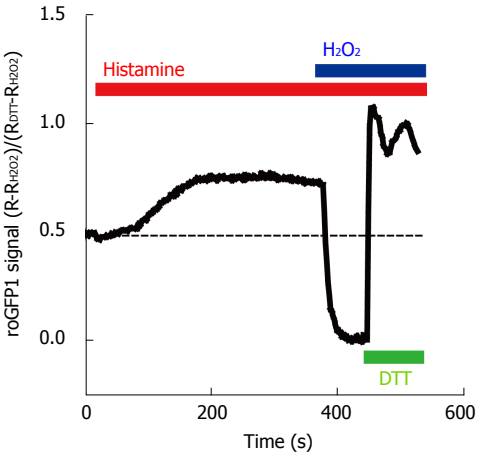

Figure 2 Ratiometric fluorescence intensity response of roGFP1 to histamine stimulation and exogenous H_2O_2 and DL-dithiothreitol in living HeLa cells. HeLa cells were transfected with the mitochondrial targeted redox sensor roGFP1. Cells were excited at 410 and 480 nm and emission was collected at 535 nm. The 410/480 fluorescence ratio (R) was normalized to the minimum of fluorescence (obtained after the addition of 1 mmol/L H_2O_2) and the maximum (after the addition of 10 mmol/L dithiothreitol, DL-dithiothreitol). The effect of intracellular Ca^{2+} was assessed by stimulating the cell with 100 μmol/L histamine. DTT: DL-Dithiothreitol.

oxidation of NADH and $FADH_2$ by the respiratory chain. Such coordinated activation of oxidative metabolism and respiratory chain activity allows the respiratory rate to change several fold with only small or no alterations of the NAD(P)H/$NAD(P)^+$ ratio[105]. These results underline the complex connection between matrix Ca^{2+} and the control of mitochondrial redox signaling.

The recently developed fluorescent green fluorescence protein (GFP)-based redox sensors can be used to further clarify the interplay between Ca^{2+} and redox regulations (see for a recent review describing in detail novel sensor variants and their utilization to understand redox biology in living cells)[22]. These roGFP sensors equilibrate predominantly with the glutathione redox couple (GSSG/2GSH)[22]. This redox balance depends on glutaredoxins, which catalyze thiol-disulfide exchange between the glutathione pool and the redox-sensitive protein[106]. By coupling human glutaredoxin to an roGFP, new redox sensors have been developed that can be used even in compartments lacking glutaredoxin activity[107]. Glutathione can be oxidized by superoxide radical, hydrogen peroxide and other oxidizing agents[108,109], therefore mitochondrial targeted roGFP1 was demonstrated to be in dynamic equilibrium with the mitochondrial redox status and to respond to membrane-permeant reductants and oxidants[24] (Figure 2).

We have recently used the mitochondrially-targeted roGFP probe to study the link between mitochondrial Ca^{2+} signals and matrix redox state. The kinetics of mitochondrial Ca^{2+} transients were analyzed by focusing on the rate of mitochondrial Ca^{2+} extrusion[74]. Following agonist-induced Ca^{2+} mobilization, maximal mitochondrial Ca^{2+} efflux rates were calculated as a function of the signal amplitude. A large heterogeneity of matrix Ca^{2+} extrusion rates was observed. Thus, only single-cell analysis is able to capture the complexity of this biological process. Manipulation of the mitochondrial Na^+/Ca^{2+} exchanger (NCLX)[68] expression has a strong impact on agonist-induced matrix Ca^{2+} transients. These experimental conditions were also used to assess the effect of mitochondrial Ca^{2+} signals on the matrix redox state (Figure 2). During HeLa cell stimulation with the agonist histamine, calcium is mobilized from the ER, leading to a mitochondrial calcium rise. Concomitant with the Ca^{2+} rise, the mitochondrial redox state was increasingly reduced as measured with a mitochondrially-targeted roGFP1. These changes likely reflect a shift of the mitochondrial glutathione pool towards the reduced form. By promoting mitochondrial Ca^{2+} extrusion in cells overexpressing NCLX, histamine-induced redox changes were completely prevented. This effect was reverted by blocking NCLX activity using CGP-37157[68,74]. Consistent with these results, NCLX expression was able to limit histamine-induced mitochondrial NAD(P)H production in HeLa cells, and this response was fully restored by CGP-37157. We conclude that the calcium rises induced by histamine stimulate matrix dehydrogenases, as reflected by the increased NAD(P)H/$NAD(P)^+$ ratio. These changes favor the formation of reduced glutathione measured by an increase of the roGFP1 signal. Mitochondrial Ca^{2+} is also a powerful activator of respiratory chain complexes. The associated acceleration of ROS production should have a net oxidizing effect on the mitochondria. Our findings demonstrate that the reducing effects are dominant during physiological calcium mobilization. Furthermore, our data establish a causal relationship between NCLX activity and matrix redox state (Figure 3).

REDOX REGULATION OF MITOCHONDRIAL FUNCTION

Redox control of mitochondrial proteins is an important topic in cell physiology and pathology because many mitochondrial functions are linked to redox reactions[21]. An increasing number of publications demonstrate a role for redox signals in the control of mitochondrial functions, including nutrient oxidation, oxidative phosphorylation, ROS production, mitochondrial permeability transition, mitochondrial morphology and cell death (reviewed in[21]). A number of reviews have covered the role of redox switches in the control of specific mitochondrial functions[21,110-113]. They highlight the fact that mitochondria harbor a unique environment that promotes thiol modifications and redox signaling. The mitochondrial proteome is very rich in protein thiols. The total concentration of such thiol groups was estimated to be in the range of 60-90 mmol/L[114]. In addition, as previously mentioned, mitochondria are a very important source for ROS (notably superoxide anion radical and hydrogen peroxide), reduced glutathione and NAD(P)H, which are required for oxidation/reduction reactions. Importantly, redox potentials are strongly

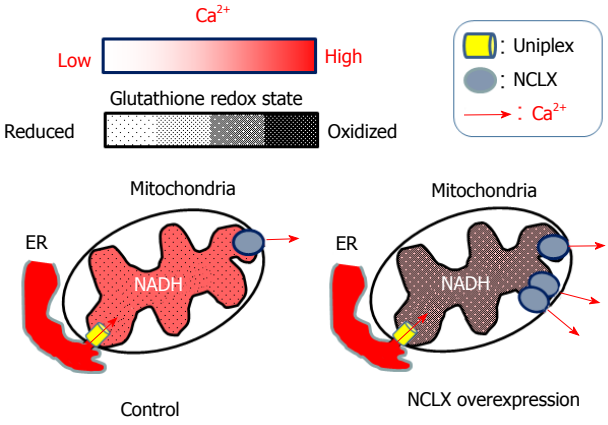

Figure 3 Proposed model for the modulation of the matrix redox signaling by mitochondrial Ca^{2+}. After cell stimulation, Ca^{2+} released from the endoplasmic reticulum (ER) enters in mitochondria by the mitochondrial uniplex (left), stimulating matrix Ca^{2+}-dependent dehydrogenases, which increase NADH levels and promoting a reduction of the mitochondrial glutathione pool. In cells over-expressing the mitochondrial Na^+/Ca^{2+} exchanger (NCLX, right) Ca^{2+} extrusion is more efficient and therefore Ca^{2+}-dependent dehydrogenases less activated. Such lowering the mitochondrial Ca^{2+} response blunts NADH formation and prevents matrix redox changes of the mitochondrial glutathione pool. NADH: Reduced form of nicotinamide-adenine dinucleotide; NCLX: Mitochondrial Na^+/Ca^{2+} exchanger.

influenced by pH, and mitochondria are able to dynamically regulate mitochondrial proton gradient during cytosolic and mitochondrial Ca^{2+} elevations[115]. Changes in mitochondrial pH seem to play an important role in physiological and pathological situations such as apoptosis, neurotransmission, pancreatic beta cells activation and insulin secretion[116,117]. Interestingly, spontaneous flashes of alkalinization have been reported in the mitochondrial matrix of living cells[118]; they can even spread between contiguous mitochondria, but their potential impact on redox potential has not yet been studied. Since the redox potential of any redox reaction involving H^+ is pH-dependent, it is likely that matrix pH influences redox reactions. By extension, pH flashes could promote localized matrix redox reactions. Given the described properties, mitochondria represent a perfect microenvironment to promote redox signaling *via* cysteine oxidation reactions.

Several types of redox modifications have been observed in mitochondria. These modifications include *S*-oxidation (sulfenylation and sulfinylation), *S*-glutathionylation and *S*-nitrosylation[21]. A proteomic method has been recently developed to profile quantitatively free cysteine thiol groups based on their intrinsic reactivity *in situ*[119]. It is noteworthy that among the 50 most reactive cellular cysteine residues listed, 19 were found in mitochondrial proteins. The most reactive mitochondrial cysteines were found in aldehyde dehydrogenases. Other examples of enzymes that undergo physiologically-relevant thiol switches have been reviewed elsewhere[18]. They include mitochondrial thiolases, creatine kinase, aconitase, homoaconitase and branched chain aminotransferase. The importance of mitochondrial thiol switches and the role of "physiological" ROS production to trigger those switches has also been highlighted by Riemer *et al*[18]. For instance, the phenotypes of knock-out mice for several mitochondrial redox-regulating enzymes (superoxide dismutases 1 and 2; glutaredoxins 1 and 2; glutathione peroxidases 1 and 4; thioredoxin 2; thioredoxin reductases 1 and 2; peroxiredoxin 3) were reviewed. The observed phenotypes in these animals range from embryonic lethality, developmental aberrations and neurodegeneration to impaired signal transduction.

Mitochondrial proteins involved in oxidative metabolism and energy production are primary targets regulated by reactive cysteines. A subunit of pyruvate dehydrogenase, which links glycolysis to the citric acid cycle, is reversibly inactivated by hydrogen peroxide[120]. The activity of several tricarboxylic acid cycle enzymes (aconitase, isocitrate dehydrogenase, ketoglutarate dehydrogenase and succinyl-CoA synthetase) is modulated by redox reactions as well[121-124]. In addition, mitochondrial respiratory chain complexes are a target of thiol-base redox regulation[125].

Glutathionylation of uncoupling protein 2 (UCP2) and UCP3, two mitochondrial protein paralogues of UCP1, has been proposed to modulate proton leak[126-128]. Interestingly, UCP2 and UCP3 modulate the activity of sarco/endoplasmic reticulum Ca^{2+} ATPases by decreasing mitochondrial ATP production[57], revealing an additional link between Ca^{2+} and redox signals.

The matrix redox state also influences mitochondrial function through sirtuins, a class of NAD^+-dependent deacetylases having beneficial health effects[129]. Among the seven members of this family, SIRT3, SIRT4 and SIRT5 are found in the mitochondria, where they govern mitochondrial processes[130]. Also, some mitochondrial transport systems have been reported to be regulated by the redox potential. For example, the carnitine/acylcarnitine carrier, required for the transport of fatty acid into mitochondria, is regulated by glutathionylation[131].

Redox regulation of mitochondrial proteins plays a crucial role also in pathology, as ROS promote the opening of the mitochondrial permeability transition pore[14,75,132]. The role of thiol oxidation during mitochondrial permeability transition has been carefully characterized[133,134]. For example, redox-active compounds belonging to the polyphenol family are able to modulate both PTP single-channel activity and PTP-dependent colloidosmotic swelling of isolated mitochondria[135]. Mitochondrial morphology has a strong impact on metabolism and cell death decisions, and this process is also modulated by redox reactions. Chronic ROS exposure promotes mitochondrial fragmentation[136]. Treatment with sublethal amounts of H_2O_2 or other acute stresses induces hyperfusion and can be prevented using antioxidants[137]. A recent genome-wide screen using RNA interference has identified ROMO1 as an essential redox-regulator, which is required for mitochondrial fusion and normal cristae morphology[138].

Deregulated mitochondrial redox signaling is associated with several diseases and condition[21]. The involvement of mitochondrial thiol oxidation has been reported in cardiovascular diseases[139]. Redox proteomics

of hearts subjected to ischemia/reperfusion indicates major changes in the redox state of thiol groups in mitochondrial proteins, including components of electron transport complexes and enzymes involved in lipid metabolism[139]. Moreover, mitochondrial PTP opening has been demonstrated to be a causative event in reperfusion damage of the heart[140]. Mitochondrial redox signals have been implicated also in neurodegenerative disorders, and deregulation of glutaredoxin-1 and thioredoxin-1 have been proposed to be important events in Alzheimer's disease pathogenesis[141]. A well-known feature of Parkinson's diseases is an imbalanced redox state[142]. Acting on mitochondrial redox signals has been suggest as an approach to attenuate oxidative stress in dopaminergic neurons of the substantia nigra in individuals with Parkinson's disease. ROS production and associated mitochondrial dysfunction may also play an important role during progression of type 2 diabetes[143]. The relevance of redox signaling in the development of type 2 diabetes has been highlighted recently[144]. In pancreatic beta cells, mitochondria are particularly important as they link nutrient metabolism to down-stream signals essential for insulin secretion. In this cell type the identification of mitochondrial proteins controlled by redox state may lead to the identification of novel signaling pathways modulating insulin secretion. Finally aging and age-related diseases in general are influenced by intracellular free radicals[145]. Disruption of mitochondrial redox signals seems to contribute to ageing[146,147] and the redox state of protein thiol group has been proposed to play key role in this process[148].

CONCLUSION

Several disorders and disease states are associated with deregulated redox signaling, including cardiovascular and neurodegenerative diseases, insulin resistance, obesity, diabetes and aging (discussed in[21]). Novel approaches are needed to rescue cellular function due to deregulated redox signaling. Polyphenols[149] are a good example as they have anti-oxidant properties and should prevent free radical damage, and thereby potentially normalize redox signaling. However, as discussed by Visioli *et al*[149], "basic and clinical science is showing that the reality is much more complex than this and that several issues, notably content in foodstuff, bioavailability, or *in vivo* antioxidant activity are yet to be resolved". Mitochondria constitute an optimal target to face those issues because they drive/modulate their functions by redox reactions. The ability of mitochondrial Ca^{2+} to modulate matrix redox state offers potential novel strategies for the manipulation of the mitochondrial redox state. Several natural compounds are known to modulate mitochondrial Ca^{2+} transport[150]. Such compounds affecting mitochondrial Ca^{2+} handling may have beneficial health effects by rescuing mitochondrial redox-related functions.

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REVIEW

Signal transducer and activator of transcription 3 regulation by novel binding partners

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Abstract

Signal transducers and activators of transcription (STATs) mediate essential signals for various biological processes, including immune responses, hematopoiesis, and neurogenesis. STAT3, for example, is involved in the pathogenesis of various human diseases, including cancers, autoimmune and inflammatory disorders. STAT3 activation is therefore tightly regulated at multiple levels to prevent these pathological conditions. A number of proteins have been reported to associate with STAT3 and regulate its activity. These STAT3-interacting proteins function to modulate STAT3-mediated signaling at various steps and mediate the crosstalk of STAT3 with other cellular signaling pathways. This article reviews the roles of novel STAT3 binding partners such as DAXX, zipper-interacting protein kinase, Krüppel-associated box-associated protein 1, Y14, PDZ and LIM domain 2 and signal transducing adaptor protein-2, in the regulation of STAT3-mediated signaling.

Key words: Janus kinase/signal transducer and activator of transcription; Signal transduction; Signal transducer and activator of transcription 3; DAXX; Zipper-interacting protein kinase; Krüppel-associated box-associated protein 1; Y14; PDZ and LIM domain 2; Signal transducing adaptor protein-2; Nuclear factor-κB

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Core tip: Signal transducer and activator of transcription 3 (STAT3) has been proposed its physiological and pathological significance in malignant and inflammatory diseases; therefore, the targeting of the STAT3 pathways is likely to be suitable for clinical application. In this review, we introduced novel regulatory molecules of STAT3 binding partners, such as DAXX, zipper-interacting protein kinase, Krüppel-associated box-associated protein 1, Y14, PDZ and LIM domain 2 and signal transducing adaptor protein-2. These proteins positively or negatively regulate critical steps of STAT3-mediated signals *via* individually unique mechanism. We hope that the information described here will help to develop a new strategy to clinically control the STAT3 activities.

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INTRODUCTION

Cytokines selectively activate Janus kinases (JAKs), which in turn activate one or more signal transducers and activators of transcription (STATs) *via* their tyrosine phosphorylation[1-3]. STATs have cytoplasmic signaling regions, such as a Src-homology 2 (SH2) domain and tyrosine phosphorylation sites. Upon cytokine stimulation, STATs are phosphorylated and dimerize *via* their SH2 domains and then move into the nucleus[4]. STAT3 is a central member of the STAT protein family, and is activated by various cytokine signals, such as interleukin 6 (IL-6)[1,5-7], which plays a role in immune regulation, hematopoiesis, inflammation and oncogenesis[6,7] (Figure 1). The majority of IL-6 functions are in turn mediated by STAT3[8,9]. Of importance, abnormal expression of STAT3 has been reported in several cancer cells as well as autoimmune diseases, suggesting the involvement of STATs in a wide range of diseases[5,10-13]. Because of important physical roles, STAT3 activity is strictly regulated by multiple molecular mechanisms. For example, the protein inhibitor of activated STAT suppresses transcriptional activities of STAT3 by interfering STAT3 from DNA binding in the nucleus[14]. Suppressor of cytokine signaling (SOCS), which is induced by STAT3, participates in the negative feedback of STAT3 activities[15,16]. Cytoplasmic tyrosine phosphatases, such as SH2-containing phosphatase 1 (SHP1), SHP2 and protein-tyrosine phosphatase 1B, function to stop STAT activities[14,15]. Nuclear tyrosine phosphatases, such as TC45, also dephosphorylate nuclear STAT3, resulting in their translocation from the nucleus to the cytoplasm[14,17]. We have identified some STAT3-interacting molecules, including DAXX[18,19], zipper-interacting protein kinase (ZIPK)[20,21], Krüppel-associated box-associated protein 1 (KAP1)[22], Y14[23,24], PDZ and LIM domain 2 (PDLIM2)[25] and signal transducing adaptor protein-2 (STAP-2)[26-28]. Here, we describe functions of each of these molecules in the STAT3-mediated signaling pathway. DAXX negatively regulates STAT3-mediated transactivation and cell proliferation through the IL-6 signal transducer gp130[18,19]. ZIPK positively regulates STAT3 transactivation through STAT3 Ser727 phosphorylation[20,21]. KAP1 negatively regulates STAT3 Ser727 phosphorylation and transactivation by interacting with HDAC3 within the nucleus[22]. Y14 is a novel type of STAT3 binding partner and influences IL-6-induced STAT3 transactivation through altering its tyrosine-phosphorylation state[23,24]. PDLIM2 acts as a nuclear E3 ligase for STAT3 and terminates STAT3-mediated signaling[25]. STAP-2 is a novel adaptor protein, composed of pleckstrin homology (PH) and SH2-like domains, and a STAT3-binding (YXXQ) motif[26-28]. Taken together, STAT3 activity is positively and negatively regulated at multiple steps.

NOVEL STAT3 BINDING PARTNERS

The STAT3 activities are strictly regulated, and recent reports have suggested several novel STAT3 regulators, whose characters are summarized in Table 1[25,26,29-33].

A nuclear STAT3 repressor, Death domain-associated protein (DAXX)

DAXX, which mainly located in the nucleus, has an ability to modulate transcription as well as cell death[34]. DAXX interacts with a number of transcription factors, including ETS1[35], PAX5[36], Glucocorticoid receptor[37], RelA[38], RelB[39], TCF4[40], SMAD4[41], C/EBP[42] and AIRE[43], and regulates their transcriptional activities. Because DAXX is also known to bind to histone deacetylases[44], DNA methyltransferases and their associated proteins[29,45,46], and the chromatin-modifying α-thalassemia syndrome protein[47,48], DAXX is likely to regulate cellular processes by regulating the transcription of specific genes *via* epigenetic modification. We found that DAXX regulates STAT3-transcriptional activity and that IFN-induced DAXX functionally links to IL-6/LIF/STAT3-mediated signaling[18]. Pretreatment of HeLa and Hep3B cells with IFN caused a decrease of IL-6-induced STAT3 transcriptional activities. Importantly, DAXX directly interacts with STAT3 in the nucleus, leading to the decreased STAT3-transcriptional activities. Indeed, knockdown of DAXX significantly enhanced STAT3 activation and gene expression after IL-6-stimulation.

The IL-6 family cytokines recognize gp130 membrane protein as a signal-transducing receptor component[8,9]. Dimerization of gp130 activates JAK family proteins (JAK1, JAK2 and TYK2), which then phosphorylate and activate STAT3. In lymphocytes, STAT3 is involved in IL-6- and/or IL-27-dependent cell growth[49,50]. In addition, STAT3 is also required for pro-B cell survival as well as efficient B lymphocyte production[51]. DAXX was reported to play a role in STAT3-mediated growth signals through gp130[19]. DAXX constitutively interacts with STAT3, leading to the impairment of STAT3 binding to the consensus DNA sequences of its target genes. In this regard, DAXX preferentially suppresses gp130-mediated Bcl-2 expression, which control cell survival. During lymphocyte apoptosis, an inverse correlation between DAXX and Bcl-2 expression levels is often observed. When progenitor B lymphocytes were treated with IFN-β, DAXX expression and nuclear localization were enhanced in parallel to Bcl-2 down-regulation[52].

Therefore, DAXX has an important function to control STAT3 activity and Bcl-2 expression during cytokine

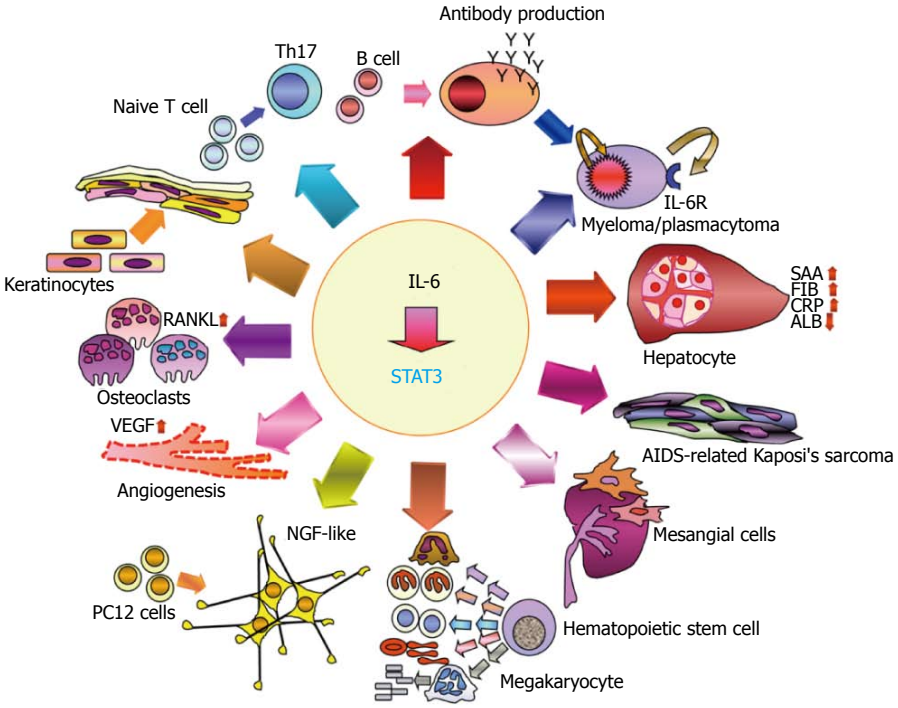

Figure 1 Interleukin 6 modulates a variety of physiological events, such as cell proliferation, differentiation, survival, and apoptosis, through signal transducer and activator of transcription 3. IL-6-STAT3 axis plays roles in the immune, the endocrine, the nervous and the hematopoietic systems, and on bone metabolism. IL-6 has been implicated in the pathology of different diseases including multiple myeloma, rheumatoid arthritis, Castleman's disease, AIDS, mesangial proliferative glomerulonephritis, psoriasis, Kaposi's sarcoma, sepsis and osteoporosis. SAA: Serum amyloid A; FIB: Fibrinogen; ALB: Albumin; CRP: C-reactive protein; NGF: Nerve growth factor; VEGF: Vascular endothelial growth factor; NF-κB: Nuclear factor-κB; RANKL: Receptor activator of NF-κB ligand; Th17: T helper type 17; IL-6: Interleukin 6; STAT3: Signal transducer and activator of transcription 3; AIDS: Acquired immunodeficiency syndrome.

stimulation.

A STAT3 Ser727 kinase, ZIPK

Tyrosine and/or serine residues of STATs are phosphorylated in response to ligand stimulation[53,54]. In the case of STAT3, a single serine phosphorylation (serine residue at the position of amino acid 727; Ser727) in the transcriptional activation domain is needed for its maximal transcriptional activity. A mutant form of S727A of STAT3, in which serine 727 was replaced by alanine, was estimated to have approximately 50% of transcriptional activity when compared with wild type[54]. To analyze the meaning of Ser727 phosphorylation *in vivo*, SA mutant mice whose STAT3 Ser727 was substituted to alanine, were produced[55]. Embryonic fibroblasts from SA/SA homozygous mice displayed approximately 50% of the transcriptional cellular responses when compared with wild-type mice; therefore, Ser727 phosphorylation is important for maximal transcriptional activities of STAT3 even *in vivo*. Serine phosphorylation increases STAT3 activity *via* the association with some cofactors, such as p300[56]. Several kinases were implicated in serine phosphorylation of STAT3, and interactions between STAT signaling and serine kinase signaling pathways have been proposed[53].

With a yeast two-hybrid screen using the C-terminal region of STAT3 as bait, we identified ZIPK as a new STAT3-binding protein[20]. ZIPK selectively bound to STAT3, but not other STAT proteins, in mammalian cells. Furthermore, the kinase domain of ZIPK interacted with the DNA binding and C-terminal domains of STAT3 although ZIPK kinase activities were not essential for their binding. Of importance, ZIPK phosphorylates STAT3 Ser727 in the nucleus, and functionally enhances STAT3-mediated transcription after IL-6- or LIF-stimulation. siRNA-mediated knock down of endogenous ZIPK expression also proposed participation of ZIPK in STAT3-mediated transcriptional activation and target gene expression after LIF-stimulation. ZIPK, a serine/threonine-specific protein kinase, binds to ATF4, which belongs to the activating transcription factor/cyclic AMP-responsive element binding protein family[57]. ZIPK aggregates *via* its leucine zipper domain to become an active enzyme form. Over-expression of wild type ZIPK, but not the kinase-inactive mutant ZIPK K42A, induces apoptosis in NIH 3T3 cells, indicating that ZIPK stimulates the apoptotic process *via* its catalytic activity[57]. The kinase domain of ZIPK shows high sequence homology to that of death-associated protein kinase (DAPK), and these proteins establish a family with DAPK2/DRP-1, DRAK1 and DRAK2, all of which are related to apoptosis[58-60]. In collaboration with DAXX and Par-4, ZIPK induces apoptosis by way of nuclear PML oncogenic domains (PODs)[61]. We previously reported that activated STAT3 enhanced ZIPK activity after IL-6- or LIF-stimulation[20,21]. In this regard, IL-6/LIF/STAT3 signaling is likely to mediate apoptotic activity *via* inducing the translocation of ZIPK into PODs, together

Table 1 Novel signal transducer and activator of transcription 3-interacting proteins and their knockout mice phenotypes

Protein	STAT3-binding site	Binding site in STAT3	Function	Reported KO mice phenotype	Ref.
DAXX	N-terminal (1-240)	DNA-BD (320-493)	STAT3 suppression	Extensive apoptosis and embryonic lethality	[29]
ZIPK	Kinase domain (1-275)	DNA-BD (320-493) and SH2-TAD (494-750)	STAT3 activation	Not yet reported	
KAP1	Not determined	Coiled-coil (138-319) and DNA-BD (320-493)	STAT3 suppression	Severe hypoproliferative anemia (hematopoietic-restricted deletion of KAP1)	[30]
				Significant expansion of immature thymocytes, imbalances in $CD4^+/CD8^+$ cell ratios, and altered responses to TCR and TGF-β stimulation (T-cell-specific Kap1-deletion)	[31]
				Male-predominant hepatosteatosis and development of liver adenoma (Liver-specific ablation of KAP1)	[32]
				Heightened levels of anxiety-like and exploratory activity and stress-induced alterations in spatial learning and memory (Conditional Deletion of KAP1 in the Forebrain)	[33]
Y14	Not determined	DNA-BD (320-493) and SH2-TAD (494-750)	STAT3 activation	Not yet reported	
PDLIM2	LIM domain	N-terminal (1-137), DNA-BD (320-493) and SH2-TAD (494-750)	STAT3 suppression	Enhanced Th17-cell dependent responses	[25]
STAP-2	SH2 domain YXXQ motif	Coiled-coil (138-319), DNA-BD (320-493) and SH2-TAD (494-750)	STAT3 activation	Reduction in LPS-induced acute phase response	[26]

STAT3: Signal transducer and activator of transcription 3; KO: Knock out; DNA-BD: DNA binding domain; SH2: Src homology 2 domain; TAD: Transactivation domain; TCR: T cell receptor; TGF: Transforming growth factor; Th17: T helper type 17; LPS: Lipopolysaccharide.

with PML and DAXX. Conversely, ZIPK induces STAT3 Ser727 phosphorylation, and enhances STAT3-mediated transcription. However, ZIPK K42A expression decreased STAT3 Ser727 phosphorylation in early but not late phase of IL-6-stimulation, suggesting that other kinases may be involved in the late phase of STAT3 Ser727 activation after IL-6-stimulation. Phosphorylation of Ser727 can increase STAT3 activity *via* associations with some co-activators, such as p300[56]. Of importance, ZIPK also interacts with p300 and forms a complex with STAT3.

Therefore, the binding of STAT3 to ZIPK in the nucleus may contribute to the stabilization of coactivator-transcription factor complexes.

A nuclear STAT3 binder, KAP1

KAP1, also known as transcriptional intermediary factor 1β and Tripartite motif-containing 28 (TRIM28), is a co-repressor of Krüppel-associated box-domain-containing zinc finger proteins[62-64]. KAP1 has an ability to coordinate various components involving in gene silencing; therefore, it can control the histone deacetylase (HDAC) complex[65-67] and a histone methyltransferase[68]. In other words, KAP1 inhibits the transcription of its target genes *via* orchestrating functions of the co-repressor complexes.

We isolated KAP1 as a STAT3-interacting protein using a yeast two-hybrid screening of a mouse embryo cDNA library[22]. Co-immunoprecipitation experiments confirmed that KAP1 binds to STAT3 in Hep3B cells. Endogenous KAP1 was present within the nucleus even in the absence of stimulation. After IL-6 stimulation, STAT3 was predominantly found in the nucleus, where it overlapped with KAP1, demonstrating that activated STAT3 translocates into the nucleus and interacts with KAP1. In Hep3B cells, KAP1 knockdown by specific siRNA significantly enhanced STAT3 activation as well as mRNA expression of SOCS3 and C/EBPδ in response to IL-6. Thus, KAP1 negatively regulates STAT3-mediated transcriptional activation and gene expression after IL-6-stimulation. Importantly, phosphorylation of STAT3 Ser727, but not STAT3 Tyr705, increased in parallel to reduction of KAP1 expression. Coincident with these data, reduction of KAP1 expression showed enhanced nuclear accumulation of STAT3 phosphorylated at Ser727. This may be in part related to the association with some cofactors, such as p300. Therefore, KAP1 is likely to recruit protein phosphatases to dephosphorylate STAT3 Ser727 in the nucleus. Alternatively, the direct interaction of KAP1 with HDACs may also be another mechanism for KAP1-mediated transcriptional repression because STAT3 has an ability to associate with HDAC3[69].

Therefore, KAP1 has a potential to suppress transcriptional activities of STAT3 in multiple ways.

A novel type of STAT3 binder, Y14

We identified Y14 as a novel associating protein with STAT3[23,24]. Y14, an RNA-binding protein, forms an exon-junction complex (EJC) with MAGOH. This complex selectively recognizes spliced forms of mRNAs immediately upstream of exon-exon junctions, and the binding is kept even after nuclear export[70,71]. In general, mRNAs produced by splicing are translated more efficiently than those from similar intronless precursors[72,73]. The EJC is in part involved in this translational enhancement because both Y14 and MAGOH recognize spliced form of mRNAs in the cytoplasm until mRNAs are translated. Human Y14 is known to shuttle mRNAs to interact with MAGOH[74]. However, only limited information is available regarding

an mRNA shuttling protein involved in the regulation of transcription factors, such as STAT3.

We found that endogenous Y14 directly binds to STAT3 in Hep3B cells and affects STAT3 transactivation activity at several steps of IL-6-mediated signaling, including the tyrosine-phosphorylation, the nuclear accumulation and the DNA-binding of STAT3[23,24]. Furthermore, MAGOH inhibits complex formation between STAT3 and Y14, and MAGOH knockdown by specific siRNA enhances IL-6-induced gene expression.

Therefore, Y14 positively regulates IL-6-induced STAT3 activation, and MAGOH interferes with this effect by displacing Y14 from STAT3.

PDLIM2, a nuclear E3 ligase for STAT3

STAT3 activation is tightly regulated at multiple levels, including the ubiquitin/proteasome-dependent degradation of STAT3[75,76]. We found that a nuclear ubiquitin E3 ligase, PDLIM2 (also known as SLIM or mystique) binds to and degrades STAT3. PDLIM2 is a nuclear protein, composed of PDZ (postsynaptic density 65-discs large-zonula occludens 1) and LIM (abnormal cell lineage 11-isket 1-mechanosensory abnormal 3) domains[77,78]. PDLIM2 promoted to polyubiquitinate and degrade STAT3 in a proteasome-dependent manner by means of its LIM domain[25]. Consistently, PDLIM2-deficiency, as well as targeted gene disruption or knockdown of PDLIM2, caused insufficient STAT3 degradation, leading to nuclear accumulation of STAT3 and enhanced STAT3-mediated gene expression.

The LIM domain of PDLIM2 is needed for the recognition of STAT3. Ubiquitination reactions require three types of enzymes: An ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2) and an ubiquitin ligase (E3). RING-type E3 ligases provide the polyubiquitin chain from E2 to their substrate by binding to E2 *via* their RING-finger domain, as well as by interacting with substrate proteins *via* the other domain[79]. The LIM domain forms a zinc finger structure and PHD domains. Proteins containing these domains generally possess ubiquitin E3 ligase activity and polyubiquitinate their target proteins[80]. Thus, the LIM domain of PDLIM2 is thought to be enough to bind to both E2 and its substrate STAT3. This possibility was consistent with the finding that the LIM domain of PDLIM2 could individually polyubiquitinate STAT3 *in vitro*. Interestingly, PDLIM2 binds to both phosphorylated and unphosphorylated STAT3, but PDLIM2 was shown to bind to phosphorylated but not unphosphorylated STAT4.

Therefore, PDLIM2 may regulate STAT3 activation *via* a different mechanism from that used on STAT4.

STAP-2 as a novel adaptor protein for STAT3

STAP-2, which we isolated as a c-*fms*-interacting protein, is composed of an N-terminal PH domain and an SH2-like domain[26]. A proline-rich region as well as a STAT3-binding YXXQ motif are also present in its C-terminal region[26]. STAP-2 is a murine homologue of an adaptor molecule BKS, which is a substrate of BRK tyrosine kinase[81]. Upon stimulation with epidermal growth factor, STAP-2 is tyrosine-phosphorylated and moves to the plasma membrane in STAP-2-overexpressing fibroblasts. IL-6 strongly induced STAP-2 mRNA in cultured hepatocytes; in addition, lipopolysaccharide-injection also induced STAP-2 mRNA in mice liver. In STAP-2-deficient hepatocytes, mRNA expression of acute-phase proteins and the tyrosine-phosphorylation of STAT3 are specifically impaired at the late phase of IL-6 stimulation. Thus, STAP-2 regulates IL-6/STAT3-mediated acute-phase protein responses during systemic inflammation. Furthermore, transient overexpression of STAP-2 mutant constructs revealed that STAP-2 enhances STAT3 activation through the YXXQ motif[26]. STAP-2 tyrosine-250 (Tyr250), a major tyrosine phosphorylation site by v-Src, JAK2 and LIF, is also required for the enhancement of STAT3 activity[27]. Indeed, the Y250F mutant, in which Tyr250 is substituted with phenylalanine, does not enhance STAT3 transcriptional activity.

As announced first, STAP-2 is a substrate of BRK[81]. BRK, also known as PTK6, is a non-receptor tyrosine kinase, composed of an SH3 domain, an SH2 domain and a tyrosine kinase catalytic domain, lacking an N-terminal myristoylation site for membrane targeting[82]. BRK is expressed by several malignant cells, such as metastatic melanomas and colon and prostate tumors as well as breast cancers[83-87]. In mammary gland, a large proportion of breast cancer cells express BRK, while normal mammary cells do not[88]. Notably, growth of breast cancer cells was impaired by siRNA-mediated down-regulation of BRK expression[89]. Our manipulation of STAP-2 expression indicates that STAP-2 plays an essential role in STAT3 activation by BRK. Indeed, STAP-2 bound to both BRK and STAT3, and STAP-2 knockdown by specific siRNA greatly decreased STAT3 activation induced by BRK in a breast cancer line T47D. Notably, an artificial STAP-2-BRK fusion protein had robust kinase activity and strongly induced activation and tyrosine phosphorylation of STAT3[28].

Therefore, STAP-2 is involved in BRK-mediated STAT3 activation and tumor cell growth.

Possible clinical utility of targeting STAT3-related molecules in future

Of note, most of these STAT3 binding proteins also directly interact with nuclear factor-κB (NF-κB) (p65/RelA) or NF-κB signaling molecules[38,78,90-92]. NF-κB, as well as STAT3, is a central signaling hub in inflammation and oncogenesis. NF-κB is also a transcription factor, which regulates gene expression of antiapoptosis as well as proinflammatory cytokines and chemokines[93,94]. Like STAT3, constitutively active NF-κB is found in many types of cancers[95]. Both STAT3 and NF-κB are also involved in the expression of target genes relating to tumor cell growth, migration and invasion[93,94,96]. Furthermore, target genes regulated by positive or negative crosstalk between STAT3 and NF-κB are gradually increasing[96,97].

In normal immune cells, activated STAT3 promotes serine-phosphorylation and subsequent proteasome-mediated degradation of IκBα, resulting in the activation of IκB kinase[98]. In cardiomyocytes as well as non-small cell lung cancer cells, the activation of NF-κB up-regulates STAT3 expression[99,100]. Importantly, STAT3 is known to directly bind to the transactivation domain of NF-κB through its DNA-binding domain[101,102]. Furthermore, it has been shown that, besides nuclear translocation after cytokine-stimulation, STAT3 continuously shuttles between the cytoplasm and the nucleus, independently of its tyrosine phosphorylation. Unphosphorylated STAT3 can interact with transcription factors, such as NF-κB, bind to DNA and drive gene expression in a distinct manner from phosphorylated STAT3[103]. Thus, direct interactions between STAT3 and NF-κB can regulate gene expression in several forms of NF-κB-dependent transcription. Therefore, STAT3-regulating molecules as well as STAT3 are likely to be key players during oncogenesis or inflammation, proposing that STAT3 could be a suitable target for malignant and/or inflammatory diseases. Although many manuscripts have showed that STAT3 has physiological and/or pathological significance, clinical meanings of the interactions with STAT3 and its binding partners should be clarified in future. STAT3 binding proteins described here are likely to have a potential to regulate STAT3 activity under some malignant or inflammatory circumstance; therefore, further experiments, including the establishment of low molecular compounds to inhibit their interaction with STAT3 could help for us to gather information about their clinical utility as well as physiological and/or pathological significance. Because STAT3 deficient mice are embryonic lethal[104], the targeting of STAT3 binding proteins may have fewer adverse effects than that of STAT 3 itself.

CONCLUSION

In this review, we summarized the functions of newly identified STAT3-interacting proteins. DAXX negatively regulates STAT3-mediated transactivation and cell proliferation through an IL-6 signal transducer, gp130[18,19]. ZIPK positively regulates STAT3 transactivation through STAT3 Ser727 phosphorylation[20,21]. KAP1 negatively regulates STAT3 Ser727 phosphorylation and transactivation by interacting with HDAC3 inside the nucleus[22]. Y14 regulates STAT3 transactivation *via* influencing tyrosine-phosphorylation after IL-6-stimulation[23,24]. PDLIM2 acts as a nuclear E3 ligase for STAT3 and terminates STAT3-signals[25]. STAP-2, a new adaptor protein, recognizes STAT3 through its YXXQ motif and stimulates STAT3 transactivation[26]. Although constitutive STAT3 activation is frequently observed in malignancies, few mutations in the *STAT3* gene have yet been described. Therefore, it is very informative to clarify the mechanism how STAT3 is activated in malignant cells. Although direct proof is lacking, STAT3-associated proteins described here may be involved in this malignant process.

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REVIEW

Global histone post-translational modifications and cancer: Biomarkers for diagnosis, prognosis and treatment?

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Abstract

Global alterations in epigenetic landscape are now recognized as a hallmark of cancer. Epigenetic mechanisms such as DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs are proven to have strong association with cancer. In particular, covalent post-translational modifications of histone proteins are known to play an important role in chromatin remodeling and thereby in regulation of gene expression. Further, histone modifications have also been associated with different aspects of carcinogenesis and have been studied for their role in the better management of cancer patients. In this review, we will explore and discuss how histone modifications are involved in cancer diagnosis, prognosis and treatment.

Key words: Epigenetics; Cancer; Diagnosis; Prognosis; Histone post-translational modifications; Treatment

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Core tip: The purpose of the review is to describe the potential of histone post-translational modifications in the field of cancer.

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INTRODUCTION

Cancer is a manifestation of both genetic and epigenetic alterations leading to the genomic instability and thus affecting several classes of genes, such as oncogenes, tumor suppressor genes, apoptotic genes and DNA repair genes. The field of cancer genetics which include the study of point mutation, deletion, insertion, gene amplification, chromosomal deletion/inversion/translocation, and allelic

loss/gain has got the attention of most cancer researchers in the last few decades. However, the appreciation of cancer epigenetics is more recent as several studies have now shown that in addition to numerous genetic alterations human cancers also harbor global epigenetic abnormalities[1,2].

Epigenetics, was initially defined by C. H. Waddington as "the causal interactions between genes and their products, which bring the phenotype into being"[3]. With time, the definition of epigenetics has evolved and is implicated in a wide variety of biological processes. The current definition is "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence". Epigenetic mechanisms include DNA methylation[4], noncoding RNA[5,6], histone variants[7] and histone post translational modifications (PTMs). These mechanisms together alter the local structural dynamics of chromatin to regulate the functioning of the genome, mostly by regulating its accessibility and compactness. All together, these mechanisms govern the chromatin architecture and gene function in various cell types, developmental and disease states[2,8-12]. Disruption in the proper maintenance of these heritable epigenetic mechanisms can result in activation or inhibition of various critical cell signaling pathways thus leading to disease states such as cancer[1,13]. Epigenetic mechanisms also cooperate with genetic alteration and work together at all stages of cancer development from initiation to progression[14]. Unlike genetic alterations, epigenetic changes are reversible in nature and can be potentially restored back to their original state by epigenetic therapy. These findings have inspired many studies aimed to understand the role of epigenetics in tumorigenesis and further explore its utility in cancer diagnosis, prognosis and therapy[15]. In recent years, research focus has been shifted to understand various post translational modifications for gaining deeper insights in to the functioning of histone/chromatin associated proteins. Information about the PTMs and the related modifying enzymes is available in the database HIstome: The Histone Infobase (http://www.actrec.gov.in/histome/)[16]. This review will discuss the role of histone post-translational modifications and its utility in cancer diagnosis, prognosis and treatment.

HISTONE PTMS: A DYNAMIC PROCESS

Histones are highly conserved and basic proteins with a globular C-terminal domain and an unstructured N-terminal tail[17]. They are also the most important proteins for converting a linear naked genome in to physiologically sensible architecture, chromatin. Nucleosomes are fundamental units of chromatin, consisting an octamer of H2A, H2B, H3 and H4 (two each) around which 146 base pairs of DNA is wrapped-. There are sequence variants of these histones which are expressed and incorporated into chromatin in a context dependent manner in normal and disease related processes. In cancer, histone H2A variants, H2A.1, H2A.Z and macroH2A have also been reported to express aberrantly[18-20]. Also, histones proteins can undergo a variety of PTMs some of which are methylation (me), acetylation (ac), ubiquitylation (ub), sumoylation (su) and phosphorylation (ph) on specific amino acid (Figure 1)[10]. Apart from these modifications, histones are also known to undergo homocysteinylation, crotonylation and glucosylation amongst others[21]. These histone modifications occur at several degrees, for example, methylation can be of monomethyl (me), dimethyl (me2) and trimethyl (me3).

Histone PTMs are added and removed from histones by enzymes called "writers" and "erasers" respectively. Histone acetyltransferases (HATs), histone methyltransferases (HMTs) and histone kinases are the examples of "writers" which add acetyl, methyl and phosphoryl groups, whereas histone deacetylases (HDACs), histone demethylases (HDMs) and histone phosphatases are examples of "erasers" which remove acetyl, methyl and phosphoryl groups, respectively (Figure 2)[22-24]. Histone-modifying enzymes are also known to interact with each other as well as other chromatin related proteins thus influencing key cellular processes such as transcription, replication and repair[10].

The mechanism behind the regulation of key cellular processes by histone post-translational modifications is not fully understood; however, it can be generalized into two categories. First, the addition of any PTM on histone protein affects inter/intra-nucleosomal interactions and their binding to DNA by steric hindrance or charge interactions. Second, addition of these PTMs to histone proteins inhibits or facilitates the binding of various proteins to chromatin[10]. These mechanisms allow a vast range of flexibility in regulating chromatin dynamics and signaling transmission and thereby regulating the gene expression. As an example of first mechanism, histone acetylation is proposed to be associated with chromatin relaxation and transcription activation, H4K16ac inhibits the formation of compact 30 nm fibers and higher order chromatin structures[25,26]. As an example of second mechanism, evolutionarily conserved specialized proteins, termed "histone readers," possess the ability to specifically bind certain histone modifications and affects a defined nuclear process such as transcription, DNA repair and replication, *etc.* (Figure 2). For example, through its evolutionary conserved chromodomain heterochromatin protein 1 recognize and gets recruited to H3K9me3 and leads to the formation of compact chromatin which in turn inhibits the access of the transcriptional machinery[27,28]. Moreover, the fact that there are different variants of each histone protein differing from few to many amino acids adds another level of complexity in functional aspects of histone PTMs. Such complicated and multilayered regulatory mechanisms of cellular processes through histone modifications have led to the hypothesis of "histone code" where a set of histone variants and modifications together perform a specific function[29]. However, due to its complexity histone code is still not fully understood[30]. Further, the status of one histone modification also regulates that of another by

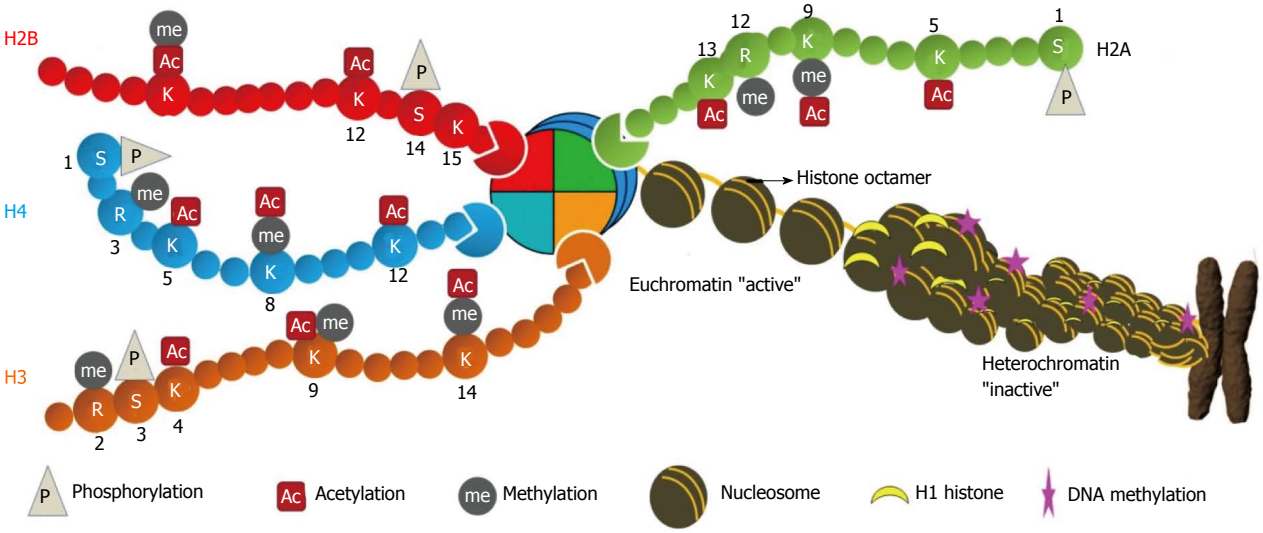

Figure 1 Chromatin architecture. The DNA is wrapped in two turns around histone octamers (nucleosomes) at intervals of about 200 bp along the DNA. Histones within the nucleosome (two each of H2A, H2B, H3 and H4) undergo numerous post-translational modifications at their N-terminal tail which protrudes from the nucleosome. Further folding of nucleosome with linker histone H1 creates a spiral structure, the heterochromatin leading to metaphase chromosome. These modifications directly regulate the chromatin structure and thus DNA-mediated cellular processes. The diagram indicates some modifications at specific residues: M: Methylation; A: Acetylation; P: Phosphorylation.

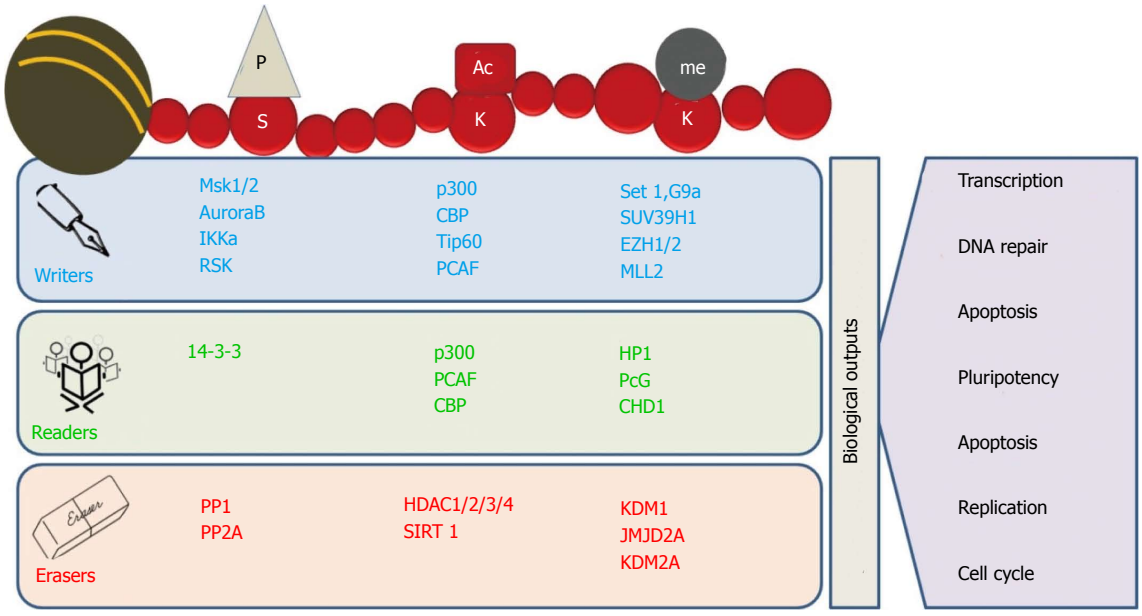

Figure 2 Readers, writers and erasers of chromatin marks. Histone modifications are highly dynamic in nature. The "writers" like histone acetyltransferases (HATs), histone methyltransferases (HMTs) and kinases add specific marks on specific amino acid residues on histone tails. These marks are identified by various proteins containing specific domains such as bromodomains, chromodomains and Tudor domain containing proteins called "readers". The written marks are removed by "erasers" like histone deacetylases (HDACs), lysine demethylases (KDMs) and phosphatases. In addition, removal and identification of these post-translational modifications on histone tails regulate various biological processes, including transcription, DNA replication and DNA repair.

cross-talk and affects chromatin remodeling and gene expression. Cross-talk between H3S10ph and H3K14ac, H2Bub and H3K4me and H3K4ac and H3K4me3 and H3K14ac are few prominent examples regulating gene expression[31]. For example, acetylation of H3K18 and H3K23 by CBP (CREB binding protein) can promote the methylation of H3R17 by Coactivator-Associated Arginine Methyltransferase 1 (CARM1), resulting in activation of estrogene-responsive genes[32].

HISTONE PTMS IN CANCER

In cancer, several histone PTMs have been reported to be misregulated; however, their involvement in cancer pathophysiological characteristics like cellular transformation, angiogenesis and metastasis *etc.*, is not well understood. Moreover, there are very few studies commenting on the cancer specific regulatory mechanism behind the alteration of histone PTMs. It has been a

decade when global loss of H4K16ac and H4K20me3 was reported for their association with cancer and considered as a common hallmark of tumor cells[33]. However, still there are no reports of their direct involvement in cellular transformation or any other cancer characteristics. Despite of the awareness of hMOF (human Male absent Of First) and HDAC4 as writer and eraser of H4K16ac, it is a recent development that low expression of hMOF has been implicated for its loss in gastric cancer[34]. Moving on to histone methylation, Lin *et al*[35] showed histone lysine demethylase KDM1A mediated loss of H3K4me2 is associated with epithelial to mesenchymal transition (EMT) in human breast cancer cells. Loss of H3ac, H3K9me3 and H3S10ph is observed at the promoters of Sfrp2, Sfrp5 and Wnt5a during genistein induced development of colon cancer in the rat model system[36]. Alterations in methylation patterns of H3K9 and H3K27 are related to aberrant gene silencing in many cancers[37,38]. Tissue microarrays done to compare the levels of H2B ub1 levels in normal mammary epithelial tissue as well as benign, malignant, and metastatic breast cancer samples have clearly shown a sequential decrease in H2B monoubiquitination with breast cancer progression and metastasis in comparision with normal epithelia[39]. A very important discovery has been made in term of phosphorylation of H3S10 as the only histone marks directly associated with cellular transformation. The knockdown and mutant (S10A) of histone H3 suppressed LMP1-induced proliferation of nasopharyngeal carcinoma cell line CNE1[40]. H3S10P has been reported to increase and has been established as indispensable for cellular transformation[41,42]. Cellular transformation by v-src constitutively activated phosphorylation of histone H3 at Ser10 in a transformation-specific manner; while, non-transforming mutant of v-src did not activate H3 phosphorylation[43]. Further, Mitogen- and stress-activated kinase 1 (MSK1) has been shown to phosphorylate H3S10 in TPA and EGF mediated cellular transformation[44]. Unpublished data from our lab has also shown increase in H3S10ph in gastric cancer, which is regulated by p38-MAPK/MSK1 pathway.

It has now been clear that acetylation, methylation and phosphorylation of histones are the most studied histone marks. In cancer, most of the studies have been done for these modifications with respect to the identification of their enzymes, regulation, effect on cellular physiology and as well as molecular biological markers for the disease management. The National Institute of Health defines a biological marker (biomarker) as a biological molecule found in blood, other body fluids, or tissues that are an objective indicator of normal or abnormal process, or of a condition or disease[45]. From the next part of the review we will see how histone acetylation, methylation and phosphorylation can be exploited as biomarkers for cancer diagnosis, prognosis and treatment.

HISTONE PTMS IN CANCER DIAGNOSIS

Diagnosis of a disease majorly depends on the analysis of physical symptoms, body fluids and fecal samples. A sensitive and specific diagnostic marker is not only useful in early diagnosis, but also helps in assessing the risk of developing the disease. Advances in the technology have enabled investigators to isolate metabolites, proteins and DNA from body fluids and fecal material and correlate them with pathophysiological symptoms of diseases including cancer.

Decades of research have discovered a battery of markers for cancer diagnosis; however, only few could reach to clinics because of issues of sensitivity and specificity. Therefore, at one side there is a need to improve techniques and on the other hand discovery of new markers is of immense importance. The discovery of the presence of DNA in fecal and urine samples[46] and circulating nucleosomes in serum[47,48] has led to the foundation of identifying epigenetic markers such as DNA methylation and histone posttranslational modification for cancer diagnosis. Ahlquist *et al*[49] demonstrated the recovery of DNA from frozen fecal samples of colorectal cancer patients which was followed by other investigators showing matching DNA methylation patterns between DNA from tissue and fecal samples of gastric and colorectal cancer patients[50-52]. Methylation pattern of DNA isolated from urine samples was also used to diagnose bladder and prostate cancer[53-57]. All these methylation studies have successfully detected global hypomethylation and gene specific hypermethylation of DNA, as established from tissue based studies.

Presence of histone proteins is not known in fecal and urine samples; therefore, histone posttranslational modifications have been utilized as cancer diagnostic markers using circulating nucleosomes (cNUCs) in serum samples. Two histone methylation marks, H3K9me3 and H4K20me3, the hallmarks of pericentric heterochromatin[58], were investigated in circulating nucleosomes by subsequent studies. Gezer *et al*[59] investigated the correlation between the H3K9me3 and H4K20me3 of cNUCs in healthy subjects and patients with colorectal cancer (CRC) and multiple myeloma and found low levels of these PTMs in cancer. Sera of patients with malignant tumors including colorectal, lung, breast, ovarian, renal, prostate cancer, and lymphoma showed high level of nucleosome concentration compared with those of healthy persons and patients with benign diseases[60]. Further, the same group showed high level ALU115 DNA sequence associated H3K9Me in multiple myeloma patients compared to healthy individuals[61]. ChIP based analysis of circulating nucleosomes in serum samples by Gloria *et al* reported a low level of H3K9me3 and H4K20me3 in patients with colorectal, pancreatic, breast and lung cancer compared to healthy control[62,63]. Moreover, H3K9me3 and H4K20me3 have been found to be lower at the pericentromeric satellite Ⅱ repeat in patients with CRC when compared with healthy controls or patients with multiple myeloma. In summary, identification of histone PTMs from serum isolated circulating nucleosomes have open the doors of immense possibility that blood samples collected by

Table 1 Global post-translational modifications of histones in cancer

Histone PTM	Writer	Eraser	Function	Cancer Diagnosis/ Prognosis/ Treatment
H3K9ac	GCN-5	SIRT-1; SIRT-6	Transcription initiation	Diagnosis: ? Prognosis: Lung, breast, ovarian Treatment: ?
H3K18ac	CBP/p300	?	Transcription initiation and repression	Diagnosis: ? Prognosis: Lung, prostate, breast, esophagus Treatment: ?
H4K5ac	CBP/P300; HAT1; TIP60; HB01	?	Transcription activation	Diagnosis: ? Prognosis: Lung Treatment: ?
H4K8ac	TIP60; HB01	?	Transcription activation	Diagnosis: ? Prognosis: Lung, Treatment: ?
H4K16ac	TIP60; hMOF	SIRT-1; SIRT-2	Transcription activation	Diagnosis: Colorectal Prognosis: Lung, breast Treatment: ?
H3K4me	SETD1A; SETD1B; ASH1L; MLL; MLL2; MLL3: MLL4; SETD7	KDM1A; KDM1B; KDM5B; NO66	Transcription activation	Diagnosis: ? Prognosis: Prostate, kidney Treatment: ?
H3K4me2	SETD1A; SETD1B; MLL; MLL2; MLL3; MLL4; SMYD3	KDM1A; KDM1B; KDM5A; KDM5B; KDM5C; KDM5D; NO66	Transcription activation	Diagnosis: ? Prognosis: Prostate, lung, kidney, breast, pancreatic, liver, Treatment: ?
H3K4me3	SETD1A; SETD1B; ASH1L; MLL; MLL2; MLL3; MLL4; SMYD3; PRMD9	KDM2B; KDM5A; KDM5B; KDM5C; KDM5D; NO66	Transcription elongation	Diagnosis: ? Prognosis: Kidney, liver, prostate Treatment: ?
H3K9me	SETDB1; G9a; EHMT1; PRDM2	KDM3A; KDM3B§; PHF8; JHDM1D	Transcription initiation	Diagnosis: Myeloma Prognosis: Kidney, pancreas, prostate Treatment: ?
H3K9me2	SUV39H1; SUV39H2; SETDB1; G9a; EHMT1; PRDM2	KDM3A; KDM3B§; KDM4A; KDM4B; KDM4C; KDM4D; PHF8; KDM1A; JHDM1D	Transcription initiation and repression	Diagnosis: ? Prognosis: Prostate, pancreas Treatment: ?
H3K9me3	SUV39H1; SUV39H2; SETDB1; PRDM2	KDM3B§; KDM4A; KDM4B; KDM4C; KDM4D	Transcription initiation and repression	Diagnosis: Colorectal, myeloma, prostate, breast and lung Prognosis: Lung, prostate, breast, leukemia, stomach Treatment: ?
H3K27me	EZH2; EZH1	JHDM1D	Transcription activation	Diagnosis: ? Prognosis: Kidney Treatment: ?
H3K27me3	EZH2; EZH1	KDM6A; KDM6B;	Transcription repression	Diagnosis: ? Prognosis: Breast, pancreatic, ovarian, prostate, stomach, Esophagus, Liver Treatment: ?
H4K20me3	SUV420H1; SUV420H2	?	Transcription repression	Diagnosis: Colorectal, myeloma, prostate, breast and lung Prognosis: Breast, lymphoma, colon, ovarian Treatment: ?

PTM: Post translational modification.

cancer patients can also be used for histone PTM based cancer diagnosis.

HISTONE PTMS IN CANCER PROGNOSIS

In cancer, to date, histones PTMs have been mostly studied for their potential as prognostic marker (Table 1). The first report in this area strongly suggested the utility of histone PTMs in cancer diagnosis and showed loss of H4K16ac and H4K20me3 in several cancers and establish these two marks as a hallmark of tumor and establishes the correlation of H4K16ac with tumor progression[33]. Further, loss of H4K20me3 is as also detected in various cancer animal models[64,65]. A study on prostate cancer showed a positive correlation of H3K18ac, H4K12ac and H4R3me2 with increasing tumor grade[66]. Another study on prostate cancer showed independently of other clinical and pathologic parameters, high rate of tumor recurrence in low-grade prostate carcinoma patients with low level of H3K4me2[66]. Loss of H3K4me2/me3 is reported in various neoplastic tissues such as non-small cell lung cancer, breast cancer, renal cell carcinoma and pancreatic adenocarcinoma serving as a predictor of clinical outcomes[67-72].

Acetylation of histone H3K9 has shown ambiguous results with the increase in some and decrease in other cancers. Decrease of H3K9ac has been linked with tumor progression, histological grading and clinical stage in prostate and ovarian tumors, hence is coupled with a poor prognosis for these patients[66,73-75]. Patients with non-small cell lung adenocarcinoma exhibited better prognosis on the reduction of the H3K9ac expression

level[68,76]. In contrast, increase in H3K9ac levels was reported in liver cancer[73]. Methylation of the same residue K9 of histone H3 requires loss of H3K9ac and is also linked to number of cancers. An association with the increase in methylation of H3K9 and aberrant gene silencing, has been found in many cancers[37,77] and its high level is associated with poor prognosis in gastric adenocarcinoma patients[77]. However, in patients with acute myeloid leukemia decrease in H3K9me3 has been found to be associated with better prognosis[78]. Decrease in H3K18ac is correlated with poor prognosis in prostate, pancreatic, lung, breast and kidney cancers[66,69,71]. It has also shown a strong correlation with tumor grade, signifying its importance in tumor progression[69]. In this regard, Kurdistani laboratory has confirmed that oncogenic transformation by the adenovirus protein E1a is associated with drastic changes in the global H3K18 acetylation pattern[79,80]. In addition, H3K18 hypoacetylation has been associated with an high risk of tumor recurrence in low-grade prostate cancer patients[66]. However, in contrast to this, low expression of H3K18ac has been correlated with a better prognosis for esophageal squamous cell carcinoma and glioblastoma patients[76,81]. This suggests that a single histone modification could predict differential prognosis in different cancers depending on it tissue specificity.

Another histone mark, H3K27me3 has been evaluated as a prognostic factor in patients with prostate, breast, ovarian, pancreatic and esophageal cancer[81-84], however, some of the results are perplexing and need further investigation. High level of H3K27me3 correlates with poor prognosis in esophageal cancers[81,84]. On the other hand H3K27me3 showed a negative correlation with overall survival time in breast, prostate, ovarian and pancreatic cancer patients[83]. Zhang *et al*[85] have identified many genes like oncogenes, tumor suppressor genes, cell cycle regulators, and genes involved in cell adhesion with significant differences in H3K27me3 pattern in gastric cancer samples in comparison to adjacent non-neoplastic gastric tissues. Further they were able to correlate changes in H3K27me3 to gene expression pattern of MMP15, UNC5B, and SHH. In non-small cell lung cancer enhanced H3K27me3 was correlated with longer overall survival (OS) and better prognosis. Moreover, both univariate and multivariate analyses indicated that H3K27me3 level was a significant and independent predictor of better survival[86]. Recently, a study showed K27M mutations of histone H3.3 variants in 31% pediatric glioblastoma tumors suggesting another level of complexity in alteration of histone PTMs in cancer which is independent of histone modifying enzymes[87]. Mass spectrometry based analysis showed high level of H3K27ac in colorectal cancer than the corresponding normal mucosa[88]. Immunohistochemical analysis on metachronous liver metastasis of colorectal carcinomas by Tamagawa *et al*[89] has correlated H3K4me2 and H3K9ac with the tumor histological type. In addition, lower levels of H3K4me2 correlated with a poor survival rate and also found to be an independent prognostic factor.

Recently DNA damage mark γH2AX also have shown its prognostic value. In triple negative breast tumors, high level of γH2AX was associated poor overall survival[90] and which was further found to be associated with shorter telomere length[91]. In colorectal cancer a high γH2AX expression in CRC tissues was associated with tumor stage and perineurial invasion. Furthermore, a high γH2AX expression was associated with poor distant metastasis-free survival (DMFS) and OS. Cox regression analysis also revealed that γH2AX was an independent predictor of DMFS and OS. A high γH2AX expression in CRC tissues is associated with a more malignant cancer behavior, as well as poor patient survival[92]. ELISA based analysis in glioblastoma multiformes tumors showed the high level of H3T6ph,H3S10p and H3Y41ph as signatures associated with a poor overall survival[93]. Increase in H3S10ph has been associated with poor prognosis in several cancers including glioblastoma multiformes[93], cutaneous nodular melanoma[94], cutaneous melanoma[95], breast cancer[96,97], esophageal squamous cell carcinoma[98], gastric cancer[99,100], melanoma[101] and nasopharyngeal carcinoma[40].

HISTONE PTM'S IN CANCER TREATMENT

Reversible nature of epigenetic changes or mechanisms has drawn major attention of scientific community to study the molecular mechanism regulating the alteration in epigenetic marks, specifically the histone post-translational modifications. Such efforts have led to the discovery of several histone modifying enzymes[102] and their chemical inhibitors[103] which has emerged as an attractive strategy in cancer treatment. Targeting these enzymes can reactivate epigenetically silenced tumor-suppressor genes by modulating the levels of histone posttranslational modifications[104]. Further, these drugs have also given additional advantage in the area of combinatorial chemotherapy[105,106].

Histone acetyl-transferases and histone deacetylases as the targets

Loss of histone acetylation has a strong correlation with aberrant gene silencing in cancer. Treatment with HDAC inhibitors reactivate silenced tumor suppressor genes by increasing histone acetylation levels and act as anti-tumorigenic agent by promoting growth arrest, apoptosis and cell differentiation[107]. Additionally, HDACi have shown their potential in reversing chemoresistance and induce antiproliferative effects on a number of cancer cell lines[108-113]. However, the question still remains whether the promise shown in the above studies by HDAC inhibitors are mainly due to their potency to alter epigenetic mechanisms or mere its effect on key cellular growth regulatory pathways.

Initial results upon treatment with HDACi like valproic acid and phenylbutyrate, as a single agent against hematologic malignancies were not encouraging[81]. However, the field showed much promise with the development of more

potent HDACi such as the class-specific inhibitors (entinostat and romidepsin) and the pan HDAC inhibitors (vorinostat, belinostat and panobinostat). The field however gained boost when in a landmark Phase IIb multicenter trial, Yu *et al*[82] have shown vorinostat as effective treatment modality for refractory cutaneous T-cell lymphoma. Further, in Phase Ⅱ multi-institutional trial, romidepsin has also been shown to have significant and durable efficacy against cutaneous T-cell lymphoma[83]. Due to their great successes in many studies, HDACi romidepsin and vorinostat have been approved by FDA as the treatment regime of cutaneous T-cell lymphoma, and romidepsin also for the treatment of relapsed peripheral T-cell lymphoma[84]. Since then many other HDACi have been under study of phase Ⅰ and/or Ⅱ trials as monotherapy, including belinostat, panobinostat, entinostat, chidamide, SB939 and LAQ824 in various cancers like ovarian, lung, soft tissue carcinoma, non-small-cell lung and breast[114-121]. However, unlike that of earlier success in treatment of lymphomas the majority of the results among solid tumor patients have been disappointing. In spite of achieving only intermittent anecdotal clinical responses, HDACi been related with severe toxicities.

Interactions between different epigenetic mechanisms have led to the foundation of research on combinatorial approach of cancer treatment using epigenetic drugs. Indeed, combinations of DNA methyltransferase and histone deacetylase inhibitors appear to synergize effectively in the reactivation of epigenetically silenced genes[107,122-124]. Such combinatorial approaches of cancer treatment have been found to be more effective than treatment with a single therapeutic agent. For example, treatment with 5-Aza-CdR and trichostatin-A in combination led to the derepression of certain putative tumor suppressor genes unlike individual treatments[107]. Pre-treatment of HDAC inhibitor SAHA relaxes the chromatin sensitizes cells to DNA damage induced by Topoisomerase Ⅱ inhibitor[125]. Similarly pretreatment of valproic acid act in synergy with epirubicine and reduces the tumor volume in breast cancer mouse model[126].

Furthermore, synergistic activity of decitabine and HDACi sodium phenylbutyrate was shown to decrease the lung cancer formation by more than 50% in comparison with decitabine alone in a murine model based study by Belinsky *et al*[124]. The same group also reported that the combination of HDACi entinostat with the DNMTi azacitidine was able to decrease tumor size and reduce the growth of K-ras/p53 mutant lung adenocarcinomas orthotopic engrafted in immunocompromised nude rats[127]. In another case HDACi sodium butyrate reduces the cell proliferation of MCF-7 cell when combine with vitamin-A[128].

Histone methyl-transferases and histone demethylases as the targets

Studies on histone methylation and their modifiers have been slow. Only few histone methylases (HMT) and demethylases (HDM) and their inhibitors have been discovered. However, studies on histone methylation could be more fruitful for their therapeutic potential because the less redundancy in HMTs and HDM compared to HATs and HDACs in targeting specific amino acid residue of histone[129]. This property of HMTs and HDMs provides exciting opportunities with more tailored treatment, while potentially minimizing side effects.

LSD1/KDM1 was among the first identified histone demethylases selectively targeting H3K4me1 and H3K4me2[130] and mediate gene repression. LSD1 has been reported to be overexpressed in many cancers like brain, breast, and prostate, thus thought to be a promising target for drug therapy[130-132]. Small molecules such as SL11144 and tranylcypromine have been developed to inhibit LSD1[133,134], Since then have shown to restore expression many silenced tumor suppressors like secreted frizzled-related protein and GATA transcription factors in many cancer cell lines. They have also been shown to possess antitumor activity in a study involving neuroblastoma xenografts model[132]. However, similar to HDACi, HDM and HMT inhibitors also have off-target effects on H3K9me2 and DNMT1 thus limiting their use[135] and further in-depth studies are required. EZH2 is another methyltransferase responsible for H3K27me3 leads to gene silencing by promoting DNA methylation[136]. EZH2 is overexpressed in head and neck, breast, and prostate cancers[137] and can be targeted by a hydrolase inhibitor called 3-deazaneplanocin A (DZNep). It induces differentiation as well as apoptosis in cancer cell lines and xenografts by countering EZH2 and inhibiting H3K27 trimethylation[138,139], while sparing normal cells.

Histone kinases and phosphatases as the targets

Compared to histone acetylation and methylation, the effort of regulating histone phosphorylation by targeting kinases and phosphatases for therapeutic uses is new. High level of several histone H3 phosphorylations such as H3S10ph, H3T6ph has been reported in a number of cancers. Unpublished data from our lab shows increase of H3S10ph in cisplatin resistance gastric cancer cell lines AGS and KATOIII. Our observation further supported the finding that p38 MAPK pathway mediated increase in H3S10ph in response to cisplatin treatment[140] in HeLa and MCF7 cells. Pacaud *et al*[93] recently reported that the kinase inhibitors like Enzastaurin (PKC-beta inhibitor), AZD1152 (Aurora-B inhibitor) and AZD1480 (Jak2 inhibitor) increases the cell death of TMZ-Irrad resistant GBM and decreases H3T3ph, H3S10ph and H3Y41ph respectively. Further, H89 (MSK1 inhibitor) treatment reduces the TPA and EGF mediated cellular transformation and by decreasing H3S10ph[44]. All these studies represent the potential of regulating histone phosphorylation for therapeutic use in cancer; however, these observations need to be further explored.

Despite of all this progress in the utilization of histone PTMs in chemotherapeutic interventions, a very little is known about their utility in monitoring the response to chemotherapy. For this purpose, levels of cNUCs and their modifications can be utilized. Because, circulating nucleosomes in serum are a result of apoptosis of

actively dividing cells; therefore, after chemotherapy/radiotherapy increase in the circulating nucleosomes correlates with progressive disease and decrease was associated with disease regression. Increase in the concentration of serum nucleosomes has been shown at 24-72 h after the first application of chemotherapy and 6-24 h after the start of radiotherapy[60]. Thus, the concentration of nucleosomes in serum might be a useful tool for monitoring the biochemical responses during antitumor therapy, particularly for the early estimation of therapeutic efficacy. Histone modifications such as H4K16ac for example, can be utilized in this regard as its loss has been reported in several cancers and also chemosensitize cancer cells[33,69,141]. Histone modifications like H3K27me3 have indeed showed perplexing results when analyzed with respect to various cancers. This can be attributed to tissue type, and indeed histone PTMs are known to be showing their abundance in a tissue specific manner[142]. This might be as because many writers and erasers utilize co-factors or substrates like acetyl CoA, SAM, NAD^+, FAD^+ or ATP which are crucial metabolites in core pathways of intermediary metabolism[143]. The cellular concentrations of these metabolites fluctuate with the metabolic status of the cells and thus, the activity of these enzymes gets affected thus the histone PTMs.

CONCLUSION AND FUTURE DIRECTIONS

The role of histone modifications in governing cellular functions has been not yet fully understood. However, with increased research over the past decade, all the organisms studied so far (from yeast to man) have bought to light the importance of chromatin environment especially histone PTMs in development and disease. These observations have revolutionized the field of epigenetics and have challenged the old hypothesis of the genetic code being the sole determinant of the pathophysiology of any disease. In cancer, especially this is further established with the discovery of small molecule inhibitors targeting histone modifying enzymes, which can restore the expression of various genes to normal and can induce apoptosis of transformed cells. The best studied examples of these drugs are HDACi, which have proven to be highly effective anticancer drugs, thus are in clinics. Although the exact nature of the mechanism by which these drugs act is not understood yet, still these drugs are faring better against cancer. Future studies need to be directed more towards understanding these mechanisms and increasing the potency of these drugs. Though many histone PTMs are known to change during cancer, less is understood regarding the significance and mechanistic details of the change observed. Much of the work done in this direction has been hindered due to technical limitations. However with the advent of new technologies, and also decrease in the cost of high throughput technologies like ChIP-seq and TMA amongst other global approaches, it is a matter of time we have more knowledge of these mechanisms. Also, new targets for development of more potent drugs need to be explored by careful understanding of an already existing chromatin atlas of various cancer cell lines and tissues. Further work in the next decade may gain deeper understanding of the global patterns of histone posttranslational modifications and their corresponding changes which will hopefully reveal many molecular targets that can be employed as new weapons in long fought battle against cancer.

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Cyclin A2: At the crossroads of cell cycle and cell invasion

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Abstract

Cyclin A2 is an essential regulator of the cell division cycle through the activation of kinases that participate to the regulation of S phase as well as the mitotic entry. However, whereas its degradation by the proteasome in mid mitosis was thought to be essential for mitosis to proceed, recent observations show that a small fraction of cyclin A2 persists beyond metaphase and is degraded by autophagy. Its implication in the control of cytoskeletal dynamics and cell movement has unveiled its role in the modulation of RhoA activity. Since this GTPase is involved in both cell rounding early in mitosis and later, in the formation of the cleavage furrow, this suggests that cyclin A2 is a novel actor in cytokinesis. Taken together, these data point to this cyclin as a potential mediator of cell-niche interactions whose dysregulation could be taken as a hallmark of metastasis.

Key words: Cyclin; Mitosis; Mesenchymal transition; Metastasis; Autophagy; RhoA; Phospholipase C

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Core tip: Cyclin A2, as an essential regulator of the cell division cycle, is commonly associated to dividing cells and, like Ki67, is usually taken as a marker of cell proliferation. However, the level of this cyclin does not always correlate with the aggressiveness of the tumor, more particularly with respect to its invasiveness. Surprisingly, recent data suggest that it plays with RhoA

also a role in the late phase of mitosis during which it is degraded by autophagy. Moreover, its dysregulation appears to be associated with the epithelial to mesenchymal transition.

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INTRODUCTION

The cell division cycle is controlled by the coordinated expression of regulatory proteins whose degradation is orchestrated by specific and timely phosphorylation/dephosphorylation events. Cyclins, as binding and activating partners of cyclin-dependent kinases (CDK) constitute a key subset of the regulatory components of the cell cycle engine. In animal cells, cyclins A and B play a central role in the control of mitosis, with cyclin A being degraded before cyclin B by the proteasome, just after the nuclear envelope breakdown. In mammalian cells there are two A-type cyclins: Cyclin A1 that is specifically expressed in the testis, and cyclin A2, that is ubiquitously expressed. Accordingly, cyclin A2 is usually linked to cell proliferation and as such is often found expressed at a high level in human cancers[1]. However, the level of this cyclin does not always correlate with the aggressiveness of the tumor, more particularly with respect to its invasiveness[2].

A wealth of information has accumulated suggesting that some cell cycle regulators play a more general role in cellular transactions[3,4], and along these lines, cyclin A2 has been shown to participate in the control of cytoskeleton dynamics and cell motility[5].

CYCLIN A2: A LATE ACTOR OF CYTOKINESIS?

Using cyclin A2-EGFP to study its ubiquitylation by FRET, we recently demonstrated that it starts in foci in prometaphase and then spreads throughout the cell in metaphase[6]. Indeed, endogenous cyclin A2 colocalized within these structures with Cdc20, a key regulator of its ubiquitylation, as well as with active proteasome, as detected with DQ-ovalbumin[6]. This unprecedented observation revealed two unexpected aspects of cyclin A2 expression: (1) not all foci colocalized with Cdc20 or DQ-ovalbumin; and (2) cyclin A2 foci persisted until late mitosis, *i.e.,* when its proteasomal degradation is thought to be complete. Since this cyclin is absent at the beginning of the G1 phase of the next round of cell division, this led us to see whether autophagy, another major intracellular proteolysis pathway, could also be involved in its degradation.

Autophagy is a degradation pathway that eliminates misfolded, damaged, or superfluous cell components, whether individual molecules or organelles, into recycled pools of biomolecules. The operating structures of autophagic degradation are the lysosomes that contain many hydrolases that break down proteins, lipids as well as nucleic acids[7]. Interestingly, some endogenous cyclin A2 foci colocalized with light chain 3-B protein (LC3-B), a marker of autophagosomes, in metaphase as well as with p62[6], a receptor for ubiquitylated proteins necessary for their degradation by selective autophagy[8]. Furthermore, our data suggest that, in prometaphase, cyclin A2 foci colocalized mainly with Cdc20 or activated proteasome, while in metaphase, they colocalized mainly with LC3-B, p62, or lysosomes[6].

Characterized first as a general recycling process for defective structures, autophagy is now proposed to participate in regular cellular regulatory pathways. Accordingly, our observation showing that it plays a complementary role in cyclin A2 degradation to prevent its accumulation at the end of mitosis, points to a potential novel and unexpected function for this cyclin late in cytokinesis. Indeed, formation of the mitotic cleavage furrow depends upon the activity of RhoA *via* its exchange factors such as Ect2[9] and GEF-H1 that localizes to the mitotic apparatus[10,11]. Our previous observation unveiled the potentiating effect of cyclin A2 on RhoA GTP loading by its exchange factors[5]. Thus, formation of the contractile ring, which is dependent upon local activation of the GTPase, would appear to result from its concomitant interaction with Ect2 and/or microtubule-activated GEF-H1 and cortical cyclin A2.

Finally, autophagy has already been shown to participate in abscission of the cleavage furrow after telophase and to promote the degradation of active RhoA[12]. This suggests that cyclin A2 may be degraded by autophagy with the GTPase (in the same complex?) when localized to cortical membranes, whereas degradation of its soluble fraction would occur mainly through proteasomal activity (Figure 1).

EPITHELIAL TO MESENCHYMAL TRANSITION AND CYCLIN A2 DYSREGULATION

The epithelial to mesenchymal transition (EMT) is a normal biological process that is important in organogenesis during early development and in wound healing. It has recently become a leading concept to explain metastasis[13]. This process entails the trans differentiation of epithelial cells to mesenchymal cells, with an associated increase in invasive properties. Cyclin A2 depletion in fibroblasts leads to an increase in cell motility and cooperates with oncogenic transformation to increase invasiveness in collagen matrices[5,14], a phenomenon that is associated with its cytoplasmic localization and independent of its association with the CDKs. When performed in epithelial cells, cyclin

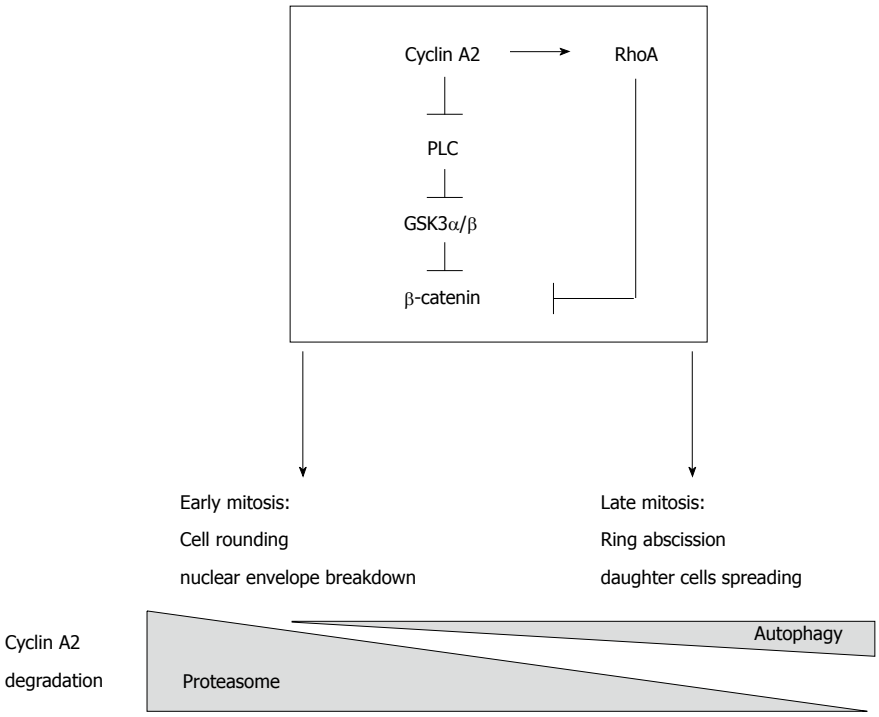

Figure 1 Presumptive regulatory network involving cyclin A2 and RhoA in the control of mitosis and epithelial to mesenchymal transition. Cyclin A2 degradation by the proteasome in mid mitosis is essential for mitosis to proceed. However, recent observations show that a small fraction of cyclin A2 persists beyond metaphase and is degraded by autophagy. Cyclin A2 participates in the modulation of RhoA activity; since this GTPase is involved in both cell rounding early in mitosis and later, in the formation of the cleavage furrow, this suggests that cyclin A2 plays also a role in cytokinesis. Taken together, these data point to this cyclin as a potential mediator of cell-niche interactions, its down regulation leading to EMT *via* PLC and RhoA converging pathways. EMT: Epithelial to mesenchymal transition; PLC: Phospholipase C.

A2 knockdown induces an EMT in RasV12-transformed mammary epithelial cells, and increases their invasiveness *in vitro* as well as in chicken embryos[14] (discussed in 15). Interestingly, these cells form tumorospheres and are more resistant to anoikis under non-adherent conditions. Whereas the Rho GTPases, RhoA and RhoC, were shown to be responsible for the invasion phenotype, with RhoA involved in the maintenance of cell-cell junctions, they do not fully account for the observed EMT phenotype[15].

Oncogenic EMT, which is thought to arise during tumorigenesis, is generally characterized by a decrease and/or a delocalization of epithelial markers (E-cadherin, occludin), upregulation of mesenchymal markers (N-cadherin, vimentin) and transcriptional factors (Zeb1/2, Slug, Snail, Twist1/2, *etc.*), as well as functional attributes such as increase in migration, invasion, cell scattering and resistance to anoikis[16].

Multiple signalling pathways are known to be dys-regulated during tumorigenesis and EMT. Among them, the WNT pathway[17] has been shown to play a major role in metastasis and promotion of EMT in breast cancer, in which expression of WNT ligands or suppression of their inhibitors induces EMT and metastasis[18]. The canonical WNT signalling pathway is initiated by binding of a WNT ligand to a frizzled (FZD) receptor and its associated co-receptors, low-density lipoprotein receptor–related proteins 5/6. This inactivates glycogen synthase kinase-3β (GSK-3β) *via* phosphorylation, followed by the release, dephosphorylation, and nuclear translocation of β-catenin, where it binds to T-cell factor/lymphocyte enhancer factor components and activate transcription of target genes, such as the mesenchymal marker, fibronectin[19].

β-catenin is also known to be regulated independently of the WNT pathway, through inactivation of GSK-3β and/or delocalization of E-cadherin, which ultimately liberates β-catenin to undergo dephosphorylation and nuclear translocation. Some reports have shown that WNT-independent β-catenin regulation can occur through transduction components such as extracellular signal-regulated kinases 1 and 2, protein kinase A, protein kinase B (AKT1), as well as phospholipases A and C isoforms. Recently, phospholipase C (PLC) isoforms have been shown to be major players in EMT, mainly in metastatic breast cancer cells, due to their roles in regulating cytoskeletal organization, differentiation and other signal transduction pathways[20]. Interestingly, isoforms of PLC are known to be activated by Rho GTPases[21,22], and PLC-γ is also regulated by the E-cadherin/β-catenin complex at the plasma membrane[23].

Our recent observations suggest that inhibition of the β-catenin pathway and/or PLC might lead to a reversion of EMT induced by cyclin A2 depletion in Ras-transformed cells, with a concomitant increase in E-cadherin expression and localization to cell membrane, as well as decrease in mesenchymal traits in the same cellular context[24]. Thus, EMT induced by cyclin A2 depletion could well be dependent on PLC and β-catenin activation.

Interestingly, a previous report showed that the

integrity of adherens junctions results from the balance of the antagonistic activities of RhoA and RhoC[25], a low level of cyclin A2 displacing the equilibrium toward the escape of β-catenin from the junction. That the two processes are linked or belong to synergistic pathways remains to be established. In any case, a down regulation of cyclin A2 expression appears to promote EMT and invasive properties. Accordingly, cyclin A2 protein levels have been shown to be much lower in human samples obtained from metastatic sites in comparison to matched primary colon adenocarcinomas, which suggest that a decreased expression level of cyclin A2 could be linked to cancer metastasis[25].

CONCLUSION

Metastasis relies on the acquisition by the candidate cell of invasive properties based on morphological changes that allow it to modify its interactions with its neighbours and, more generally, to reinterpret cues emanating from its surrounding niche. Mitosis offers such an opportunity to a cell embedded within an epithelial structure to change its fate. Indeed, while it is associated to a drastic change in cell shape, it also entails dramatic alterations of intracellular structures such as chromosome condensation and nuclear envelope breakdown. It is thus not surprising to find that dysregulation of cyclin A2 and RhoA, two major actors of its control, involved in both its early step, cell rounding, and then later, the spreading of the two daughter cells, be instrumental in giving rise to cells that escape niche controls. Interestingly, recent studies on circulating tumor cells have revealed that a large number of them harboured a phenotype that supports the idea that they were generated through an EMT-like mechanism[26]. Thus, promoting a mechanism such as EMT is nothing else but another example of how a cancer cell co-opts fundamental cellular mechanisms that are important in the early development or in wound healing.

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MINIREVIEWS

Klotho in cardiovascular disease: Current and future perspectives

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Abstract

Protein Klotho, beyond its role as a regulator of the phosphatemia, is also involved in the maintaining of the cardiovascular health, being associated its alterations with the development of cardiovascular damage and increased morbi-mortality. For all this, nowadays Klotho is the subject of a thorough research which is focused on uncover its intimate mechanisms of action, and in analyzing the utility of its modulation as a potential strategy with clinical applicability. Molecular mechanisms of Klotho are not well understood but an emerging research area links Klotho deficiency with vascular pathology. Changes in this protein have been associated with cardiovascular-related complications like inflammation, vascular calcification, and endothelial dysfunction. All this is particularly relevant if considering the recent discovery of Klotho expression in vascular tissue.

Key words: Klotho; Cardiovascular disease; Chronic kidney disease; Mechanism of action; Therapeutics

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Core tip: Protein Klotho, beyond its role as a regulator of the phosphatemia, has also been involved in the maintaining of the cardiovascular health. For all this, is the subject of a thorough research which focused on uncover its intimate mechanisms of action, and in analyzing the utility of its modulation as a potential strategy for clinical applicability. Emerging research links Klotho deficiency with vascular pathology. Changes in this protein have been associated, among others cardiovascular-related

complications, with inflammation, vascular calcification, and endothelial dysfunction.

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INTRODUCTION

The protein Klotho (formerly called α-Klotho to distinguish it of a homologue subsequently discovered and called β-Klotho) has opened an extraordinarily wide research area because of its implication in diverse biological processes, many of them related to human longevity. Although the molecular mechanisms of action of Klotho are not well understood, but current knowledge points to the high pleiotropy of this protein with diverse biological functions and downstream targets. Functions of Klotho include regulation of energy metabolism, anti-inflammatory and anti-oxidative effects, modulation of ion transport, and regulation of mineral metabolism[1]. Many of these effects have been related to the maintaining of the vascular health and the mismatch of Klotho levels has been related with the onset of cardiovascular disease (CVD).

In the organism, protein Klotho is present as a transmembrane form, which participates in the signal transduction of the phosphatonin fibroblast growth factor (FGF) 23, and as an endocrine soluble factor, detectable in blood, urine, and cerebrospinal fluid[2]. Soluble form predominates in humans[2], declines with age[3], and it can be generated through different ways including an alternative RNA splicing[2,4], which generates a short soluble form of 65 kDa, and a proteolytic cleavage by membrane-anchored A Disintegrin and Metalloproteinases (ADAM)-17 and ADAM-10 and by the beta-site amyloid precursor protein-cleaving enzyme 1[5,6] which generates a longer soluble form of 130 kDa. This longer form predominates in humans.

Klotho protein deficiency was first related to CVD in the Klotho-deficient mice aging-model, which manifests many human progeroid symptoms including accelerated arteriosclerosis, associated with extensive medial calcification of the aorta, and both medial calcification and intimal thickening of medium-sized muscular arteries[7]. In addition, they exhibit impaired angiogenesis[8] and endothelial dysfunction[9] which can be ameliorated by *in vivo* gene delivery of the *Klotho* gene or by parabiosis with the Klotho wild-type specimen[10,11].

Most of the research in Klotho has been focused in its role as renal cofactor for the binding of FGF23. The presence of Klotho in the kidneys elicits the phosphaturic effect of FGF23 and the inhibition of the synthesis of the active form of the vitamin D[12,13]. However, the existence of a soluble form of Klotho[4,5] and, importantly, its recently discovered expression in vascular tissue[14,15] and in blood[16], has allowed to consider this molecule as a novel factor able to exert important effects in multiple organs including the cardiovascular (CV) system.

KLOTHO AND CVD

The kidneys are probably the main source of soluble Klotho and are also the target of multiple mineral metabolism-related effects of Klotho. Interestingly, both genetic and chronic kidney disease (CKD) Klotho deficiencies generate very similar systemic manifestations including CV lesions. This has prompted the recent appearance of numerous investigations focused on the association of Klotho deficiency with the extremely high CV morbidity and mortality in the renal patient[17,18]. This is a particularly relevant research area if we consider that mortality from CVD disease in a 20-year-old dialysis patient is the same as in a 80-year-old person not on dialysis[19], and that the traditional risk factors, highly prevalent in CKD, are not enough to account for this incidence. In any case, studies focused on studying the relationship of Klotho with CVD should consider the presence of many confusing factors including age, kidney function, active vitamin D, FGF23, parathyroid hormone (PTH), medications, and Ca^{2+} and Pi levels which affect blood soluble Klotho levels in these patients.

CVD is the leading cause of mortality in CKD patients which universally suffer from vascular calcification, inflammation, endothelial dysfunction and oxidative stress[20,21]. Reduced Klotho, in addition to traditional and CKD-related risk factors, has been proposed as a novel contributor to the appearing of these complications[22-24].

The absence of Klotho in murine models causes accelerated aging syndrome and atherosclerosis, vascular calcifications[15], and defects in angiogenesis[8], and endothelial dysfunction[23], which is reversed by administering the *Klotho* gene or by parabiosis with the wild-type. More recent studies have confirmed the protective effects of Klotho on the vascular system, including its participation in the maintenance of endothelial homeostasis and vascular functionality[9,23], correlating their absence with the appearance of endothelial dysfunction and vascular calcifications[23,24]. Clinical studies have shown that low serum Klotho is associated with arterial stiffness in CKD patients[25] and independently associated with severity of coronary artery disease (CAD) in patients with normal kidney function[26]. In addition, genetic variation studies have demonstrated that Klotho gene polymorphisms might be also associated with longevity and CAD[27-29]. In particular, the KL-VS allele, characterized by six SNPs in a region of 800 bp in exon 2 and flanking sequence, is prevalent in the population and is associated with a reduced longevity[27]. In a study where two different groups of healthy siblings were tested, Arking *et al*[28] found that this functional variant of *Klotho* gene is an independent risk factor for CAD. This risk is modulated by

modifiable risk factors, such as hypertension, increased high-density lipoprotein cholesterol levels or smoking[28]. Likewise, in an Ashkenazi Jew group it was found that homozygous KL-VS individuals were at higher risk of stroke than wild-type subjects[30]. In the case of G-395A polymorphism, the A allele has been found to be an independent predictor of atherosclerotic CAD but not of vasospastic angina in Japanese population[29]. This polymorphism affects the promoter of the *Klotho* gene, so that the G→A substitution impairs protein binding to the region and consequently affects gene expression[31] and soluble Klotho levels. Similarly, Jo *et al*[32] observed an association of the G-395A allele with CAD but not with coronary artery calcification in Korean patients. Besides, subjects with the T allele for the C1818T polymorphism (located in exon 4) have lower prevalence of CAD than those with CC genotype[33].

CAD is mainly caused by established coronary arteriosclerosis derived from endothelial dysfunction which could be developed by low Klotho levels. Stimulation of nitric oxide (NO) synthesis by Klotho, verified in experimental models[34], could be essential for this endothelial protective effect. Consistent with this, the deficiency in Klotho expression in rats reduces the ability of arterial vasodilatation, with a decrease in the excretion of NO urinary metabolites[11]. Furthermore, this situation can be reversed by parabiosis with wild-type specimens, and also through administration of the *Klotho* gene[9,11]. Moreover, *Klotho*-deficient mice exhibit impaired angiogenesis which depends on endothelium-derived NO[35].The mechanism by which Klotho regulates NO synthesis remains to be determined.

Klotho protein also appears to be associated with the inflammatory process. Thus, the proinflammatory cytokines tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and TNF-α cause the NFκB-dependent decrease in the expression of Klotho, both *in vitro* and *in vivo*[36], while in human umbilical endothelial cells, the addition of Klotho is able to suppress the expression of adhesion molecules in the endothelium induced by TNF-α[37]. Moreover, the soluble form of Klotho is capable of inhibiting the Wnt signaling[38]. Although this signal is essential to ensure the proliferation and survival of stem cells, an excess exposure to it can contribute to depletion and accelerated senescence[39]. Therefore, the attenuation of the Wnt signal by Klotho contributes to the anti-aging properties of this protein.

Ectopic calcifications in soft tissues, including cardiac valves and aorta, have been also related to low Klotho soluble levels[7,24,40]. Vascular calcification is prominent in the Klotho mutant mice, similarly to CKD subjects, and can be reversed by Klotho overexpression through adenoviral delivery of *Klotho* gene[10]. Albeit in CKD the release of Ca^{2+} and Pi, by disturbed PTH, low calcitriol and soluble Klotho and high FGF23, may trigger or accelerate vascular calcification[41], the existence of direct effects of Klotho and also of FGF23 is the subject of extensive research at present.

PLAUSIBLE MECHANISMS OF ACTION OF KLOTHO IN THE VASCULATURE

Many vascular molecular actions are proposed to explain Klotho vascular-protective effects. Klotho is expressed in the vasculature[14,15] and is probably that the formation of both, the membrane and the soluble form, coexists in the vascular beds. Since membrane Klotho protein has an extremely short intracellular domain, it is unlikely that displays a signal transduction by itself. More probably, membrane Klotho solely serves as a correceptor for the binding to FGF receptors (FGFRs) and as a source of a soluble form[42]. However, it is still unknown what is the contribution of the vascular bed to the blood levels, and which are the regulatory mechanisms of Klotho shedding. Serum Klotho levels determined by ELISA varies between 200 and 740 pg/mL[43].

Similarly, the mechanisms of action of soluble Klotho remain unknown and the existence of a receptor for this protein is a matter of debate. Because FGFRs are quite ubiquitous, the binding of soluble Klotho to these receptors has been proposed[44] as an explanation to the pleiotropic effects of Klotho. Theoretically, soluble Klotho can bind to this FGFRs but there is no evidence that this activates signalling events[44]. Another possibility is that soluble Klotho does not act as an hormone sensu stricto, since it lacks of a specific receptor. In this scenario, the hypothetical functions of soluble Klotho would be based on its putative enzymatic abilities. Different works point to the glycan-modifying activity of circulating Klotho. Thus, a putative activity as sialidase which removes terminal sialic acids from N-linked glycans of glycoproteins in the cell surface has been proposed to explain the effect of circulating Klotho in preventing the endocytosis of the transient receptor potential cation channel, subfamily V, member 5 (TRPV5) and the renal outer medullary potassium channel 1 in the kidneys[45,46], thereby increasing renal reabsorption of Ca^{2+} and excretion of K^{+}, respectively. This enzymatic activity is also responsible of promoting the endocytosis of the NaPi-2a cotransporter in the renal proximal tubule[47]. But circulating Klotho acting as a glycan-modifying enzyme could also exert a broad range of effects beyond the kidneys; the addition of recombinant soluble Klotho protein to rat vascular smooth muscle cells (VSMCs) cultures is able to decrease high Pi-induced calcification by diminishing expression and activity of Na/Pi cotransporters type 3 (Na/Pi-3, also known as Pit 1 and Pit 2)[24,47]. Na/Pi-3 cotransporters are widely expressed in tissues such as intestinal epithelium, liver, lung, heart and VSMCs[48,49], but to date there is no evidence for an effect of Klotho on Pit-1 and 2 in the vascular beds. Finally, the aminoacidic sequence of Kotho shares homology with a β-glucosidase. However, this glucosidase activity has not been confirmed[7,50].

Another possible explanation for some of the CV disorders observed in situations of low levels of Klotho comes from FGF23. In this sense, vascular calcification might be an off-target effect of FGF23 levels. Lim *et*

al[15] proposed that vascular Klotho acts not only as an endogenous inhibitor of vascular calcification in VSMCs but also, as a cofactor required for vascular FGF23 signaling. In this study, restoration of Klotho expression in knock-down cells unmasked FGF23 anticalcific effects[15]. Finally, a second effect of FGF23 on CVD derives from the extremely high levels of this hormone observed in primary (mainly genetic) and secondary (commonly in CKD) Klotho deficiency states[7,24,51-55]. In CKD, uremic cardiomyopathy, characterized by cardiac hypertrophy and fibrosis, is a principal cardiovascular pathological feature and includes left ventricular hypertrophy (LVH). Novel risk factors for LVH in uremic cardiomyopathy are low levels of soluble Klotho and high levels of FGF23[55,56]. In fact, FGF23 causes LVH after intramyocardial and intravenous administration in wild-type mice[55]. Since Klotho is not normally expressed in the ventricles, the effects caused by Klotho deficiency might be due to other parameters. One of the parameters considered is FGF23. Some *in vivo* experiments show that Klotho influences FGF23 production[57], however, there are no *in vitro* data supporting this effect.

Klotho is also involved in the modulation of inflammation. The mechanism of regulation of inflammation by Klotho may be based in the inhibition of NF-κB activity in the endothelium[37] by unknown mechanisms. Similarly, secreted Klotho protein is also able to suppress the Wnt biological activity[38,58]. In this case the binding of Klotho to various Wnt family members results in the signal inhibition.

Finally, Klotho has been related to the regulation of the intrinsic generation of reactive oxygen species (ROS) which play a role in aging and longevity determination. Yamamoto *et al*[59] determined that cell surface-bound Klotho inhibited FOXO3a phosphorylation and promoted its nuclear translocation. FOXO3a is a member of the O subclass of the forkhead family of transcription factors which are characterized by a fork head DNA binding domain. Nuclear translocated FOXO3a then became bound to the MnSOD promoter and upregulating its expression which results in suppressing ROS formation. Moreover, Ohta *et al*[60] investigated the effect of Klotho gene delivery on blood pressure and oxidative stress *in vivo* finding an upregulation of MnSOD expression and total SOD activity in the aorta of mice, enhanced NO production, and downregulated lipid peroxide concentration in serum of mice. It was concluded that Klotho gene infusion into the tail vein of mice and rats suppressed ROS formation in animals.

ABOUT THE THERAPEUTIC UTILITY OF KLOTHO

Injection of soluble Klotho produces biological effects in experimental animal models with kidney injury[24,61,62], which is a state of Klotho deficiency, and in intact animals[44,47]. This supports the idea of a therapeutic approach based on the administration of soluble Klotho. In CKD, one of the earliest events is Klotho deficiency, and establishing whether Klotho has therapeutic potential treating this disease and its complications is a critical step. However, no study has documented the therapeutic effect of Klotho in humans with renal disease.

Another approximation is based on the stimulation of Klotho expression. Experimental studies show that calcitriol administration promotes expression of Klotho *via* the activation of vitamin D receptor[63,64]. A recent work has demonstrated that administration of alfacalcidol, a vitamin D receptor activator, promoted an up regulation of *Klotho* gene expression in the kidney of nephrectomized spontaneously hypertensive rats[65]. According this, Klotho variants associated with lower *Klotho* gene expression have been associated with a decrease in survival of dialysis patients, more pronounced among patients not treated with active forms of vitamin D[66].

Strategies aimed to increase extrarenal Klotho production might be of particular importance in end-stage renal disease patients with a significant loss of functional renal tissue. In these patients, administration or stimulation of synthesis of Klotho may potentially reverse or retard the disease progression. Therapeutic effects of Klotho may result from the modulation of circulating hormones, regulation of mineral parameters or from direct effects on target organs, leading to induction of phosphaturia, anticalcifying, antifibrotic, and antioxidative effects, and antagonism of angiotensin Ⅱ effects.

Another therapeutic approach to increase Klotho levels could be based on the recently examined relationship between inflammation and Klotho expression, showing that the inflammatory cytokines TWEAK and TNFα promote the NFκB dependent downregulation of Klotho expression both *in vitro* and *in vivo*[36]. Activation of these inflammatory cytokines and the subsequent reduction of Klotho expression could contribute to organ damage. In this sense, regulation of Klotho expression by anti-inflammatory therapeutic treatments, including statins, could suppose a new approach to delay the progression of renal damage.

More specifically, Klotho expression in the vasculature may also become an important option in therapeutics. If resident vascular Klotho protects the vasculature against vascular disorders, either as a transmembrane or as a soluble form in paracrine and/or autocrine manner, upregulation of vascular synthesis could be reflected in the prevention of many vascular disorders such as endothelial dysfunction, arterial stiffness and vascular calcification.

CONCLUSION

Nowadays, the major interest to the clinical application of Klotho research is related to CKD, where FGF23 and Klotho have been linked with CV morbidity and mortality independently of phosphatemia[67]. Importantly, these associations remain in population without impaired kidney function. Thus, Klotho has been proposed as a

potential therapeutic option. Another open question is the elucidation of the contribution of soluble vascular Klotho in advanced stages of CKD, where low serum Klotho levels are frequent. Thus far, measurements of vascular Klotho mRNA and protein in humans have been limited. It is unknown if vascular expression is also lowered in advanced CKD stages or remains at physiologic levels. Anyway, it is probably that vascular derived Klotho plays a more important role in these patients since renal contribution is dramatically diminished. What we know is that vascular expression of Klotho, along with blood soluble levels, are decreased in CAD patients without renal disease[26].

Although depicted results clearly point to Klotho as a potential therapeutic agent in mineral-cardiovascular disorders, further studies are needed to evaluate the reliability and practical utility of this protein. Despite the controversies regarding Klotho expression and its effects, current data point to a pathogenic role of Klotho deficiency the CVD. Overall, the recent discovery of FGF23 and Klotho, and the elucidation of their regulatory effects on the phosphate homeostasis, has been accompanied by significant contributions to the CVD researching field. These discoveries will revert in the development of new targets and therapies with potential clinical applications.

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MINIREVIEWS

Caldecrin: A pancreas-derived hypocalcemic factor, regulates osteoclast formation and function

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Abstract

Caldecrin was originally isolated from the pancreas as a factor that reduced serum calcium levels. This secreted serine protease has chymotrypsin-like activity and is also known as chymotrypsin C; it belongs to the elastase family. Although intravenous administration of caldecrin decreases the serum calcium concentration even when its protease activity is blocked, this effect does require cleavage of caldecrin's pro-peptide by trypsin, converting it to the mature enzyme. Ectopic intramuscular expression of caldecrin prevented bone resorption in ovariectomized mice. Caldecrin inhibited parathyroid hormone-stimulated calcium release from fetal mouse long bone organ cultures. Furthermore, caldecrin suppressed the formation of osteoclasts from bone marrow cells by inhibiting the receptor activator of nuclear factor-κ B ligand (RANKL)-stimulated phospholipase Cγ-calcium oscillation-calcineurin-nuclear factor of activated T-cells, cytoplasmic 1 pathway. Caldecrin also suppressed the bone resorption activity of mature osteoclasts by preventing RANKL-stimulated Src activation, calcium entry, and actin ring formation. *In vivo* and *in vitro* studies have indicated that caldecrin is a unique multifunctional protease with anti-osteoclastogenic activities that are distinct from its protease activity. Caldecrin might be a potential therapeutic target for the treatment of osteolytic diseases such as osteoporosis and osteoarthritis. This mini-review describes caldecrin's historical background and its mechanisms of action.

Key words: Serine protease; Osteoclasts; Hypocalcemia; Chymotrypsin; Bone resorption; Calcium signaling

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Core tip: Caldecrin (also known as chymotrypsin C) reduces serum calcium levels. This activity is distinct from its protease activity but also requires trypsin-mediated cleavage of the pro-peptide, converting caldecrin to its active form. Ectopic intramuscular expression of caldecrin prevented bone resorption in ovariectomized mice. Caldecrin inhibited parathyroid hormone-stimulated calcium release from fetal mouse long bones. Furthermore, caldecrin suppressed receptor activator of nuclear factor-

kappa B ligand-induced activation of intracellular calcium signaling, thereby reducing osteoclast formation and bone resorption. Caldecrin is a unique multifunctional protease that possesses anti-osteoclastogenic activity, resulting in reduced serum calcium levels.

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INTRODUCTION

Calcium homeostasis is controlled by intestinal calcium absorption and calcium resorption in the kidney, as well as by bone formation and resorption. Clinical and experimental observations have also linked the pancreas to calcium homeostasis. Pancreas-derived glucagon[1,2], amylin[3,4], and calcitonin gene-related peptide[5,6] have been shown to regulate calcium homeostasis, while acute and chronic pancreatitis have been shown to associate with hypocalcemia[7].

In the 1960's, the pioneering work of Takaoka *et al*[8,9] demonstrated that a porcine pancreatic extract had hypocalcemic activity. In 1992, we first successfully purified a hypocalcemic factor named caldecrin from a pancreatic extract using chromatographic separation techniques including ion exchange, gel filtration chromatography, and high-performance liquid chromatography[10]. To identify caldecrin, each fraction was intravenously administered to overnight-fasted mice and serum calcium concentrations were measured 4 h post-injection. In addition, the samples were assayed for their inhibition of parathyroid hormone-stimulated calcium release from fetal mouse long bone organ cultures. Caldecrin is an anionic protein (pI: 4.5) with a molecular weight of about 28 kDa; it was found to be a serine protease with chymotryptic activity.

In 1995, we isolated rat caldecrin cDNA from pancreatic cDNA expression library by immunoscreening with an anti-caldecrin antibody[11]. A partial amino acid sequence of caldecrin purified from rat pancreas was completely matched with that encoded by the cDNA. The nucleotide sequence was almost identical (except for three nucleotides) to that of a PCR clone referred as elastase IV (ELA4)[12]. Comparison of the amino acid sequences encoded by these two cDNAs indicated that the central region of caldecrin differed from that of ELA4 due to a frame shift caused by this minor nucleotide change (Figure 1). The amino acid sequences of the purified caldecrin fragments, including the central region, were consistent with the deduced amino acid sequence of caldecrin but not with that of ELA4. Over-expression of the ELA4 PCR clone in Sf9 cells caused a complete loss of secretion, low expression levels, and much lower protease activity[13]. Furthermore, the rat genomic DNA sequence matched that of the caldecrin cDNA, but not that of the ELA4 clone[13]. Therefore, the ELA4 PCR clone may be a cloning artifact or represent a mutant caldecrin gene. In 1995, the crystalline structure of bovine chymotrypsinogen C was reported[14-16] and its amino acid sequence was very close to that of rat caldecrin, thereby suggesting a similarity between caldecrin and chymotrypsin C (CTRC). It is now known that CTRC, caldecrin, and ELA4 are the same protein, which is encoded by the *CTRC* gene and known officially as CTRC (caldecrin), according to the *HUGO* Gene Nomenclature Committee. Table 1 compares the amino acid sequence of rat caldecrin with that of other members of the rat and human pancreatic chymotrypsin and elastase families. Caldecrin shows a greater similarity with elastase than with chymotrypsin. In addition, expressed recombinant human caldecrin also showed serum calcium-decreasing activity, even following phenylmethylsulfonyl fluoride treatment to abolish its protease activity[17].

Table 1 Amino acid sequence similarity with rat caldecrin

Species	Pancreatic protease	Identity (%)	Similarity (%)
Rat	Caldecrin	100	100
	Chymotrypsin B	41	55
	Elastase I	51	67
	Elastase II A	59	72
	Elastase III B	57	71
Human	Caldecrin	78	88
	Chymotrypsin B	41	56
	Elastase II A	61	74
	Elastase II B	56	70
	Elastase III A	57	70
	Elastase III B	55	69
Cow	Chymotrypsin A	39	57

Sequence identity: Percent of same amino residues in a sequence alignment between 2 sequences; Sequence similarity: Percent amino acid sequence identity and percent positive substitutions between 2 sequences.

In 1996, another research group purified a calcium metabolism-regulating factor from the porcine pancreas by determining its stimulatory effects on proliferation of the osteosarcoma MG-63 cell line and its inhibition of 1, 25 vitamin D_3-stimulated calcium release in organ cultures[18]. The terminal sequence of the 28-kDa protein that was isolated corresponded to that of human elastase III B. Recombinant elastase III B decreased interleukin-1-induced hypercalcemia and this effect was dependent on its protease activity. Although both have been isolated from the pancreas, caldecrin and elastase III B were found to be different molecules that exerted their hypocalcemic effects *via* different mechanisms of action.

PROTEIN STRUCTURE AND PROTEASE ACTIVITY OF CALDECRIN

The human *CTRC* gene maps to chromosome 1p36.21. The homologous mouse and rat genes are located on chromosomes 4E1 and 5q36, respectively. The *CTRC* genes consist of 8 exons in these species. Northern blot

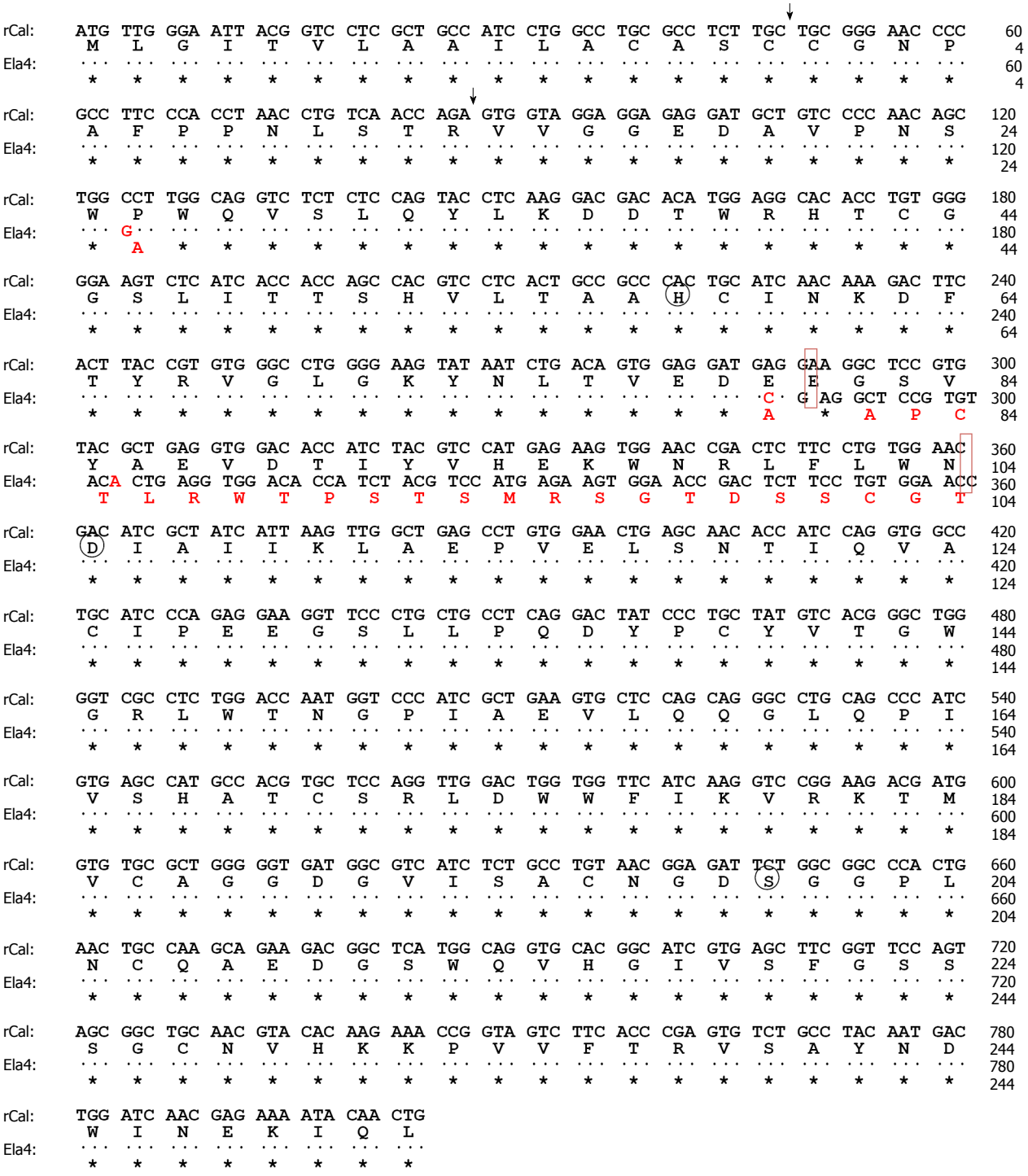

Figure 1 Nucleotide and deduced amino acid sequences of rat caldecrin (rCal) and elastase IV (Ela4). The nucleotide (upper row) and amino acid (lower row) sequences of the indicated molecules are shown. The dots and asterisks indicate nucleotides and amino acid residues, respectively, that are conserved between rCal and Ela4. Circle: Charge-relay system; Vertical arrowhead: Proteolytic cleavage site.

analysis has indicated that caldecrin is mainly expressed in the pancreas (Figure 2A).

CTRC (caldecrin) is a single protein consisting of 268 amino acids, with a signal peptide (16 amino acids), pro-peptide (13 amino acids), and the mature protein (239 amino acids; Figure 2B). The three-dimensional structure demonstrated that five disulfide bridges were formed at Cys1-Cys125 (according to the chymotrypsin numbering), Cys43-Cys59, Cys139-Cys206, Cys170-Cys186, and Cys196-Cys227 (Figure 2B). CTRC (caldecrin) was shown to have a two-barrel structure, each composed of 6-7 β-sheets and a C-terminal α-helix long tail[14-16] (Figure 2C). Following tryptic cleavage at Arg13-Val14, the caldecrin pro-peptide remains associated with the mature enzyme *via* the Cys1-Cys125 disulfide bridge; this generates a structure resembling those of chymotrypsin A and B, as

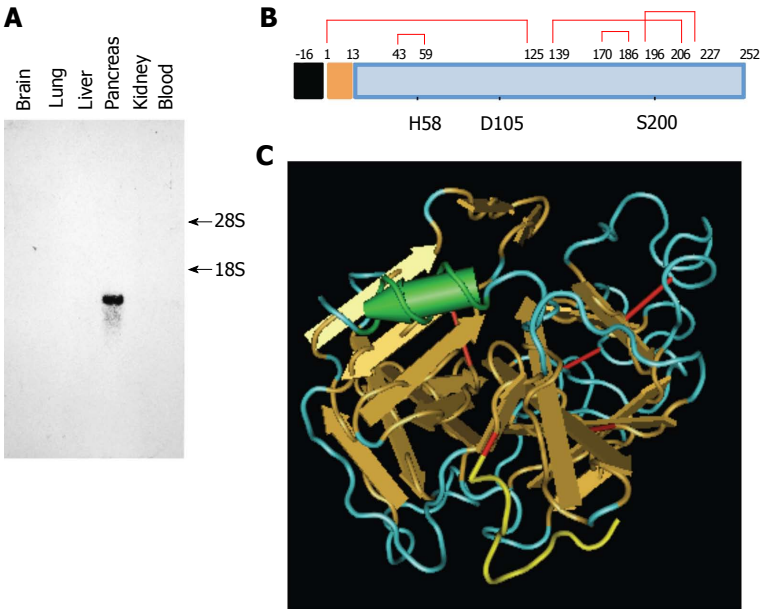

Figure 2 Caldecrin expression and protein structure. A: Caldecrin expression was analyzed by Northern blot. 18S, 28S: 18S, 28S ribosomal RNA; B: Domain structures of caldecrin. Black box: signal peptide; orange box: pro-peptide; blue box: mature protein; red line: disulfide bridges with cysteine number; the H (histidine), D (aspartic acid), S (serine) catalytic triad; C: Ribbon diagram of the crystal structure of human caldecrin (adapted from PDB ID: 4H4F, prepared from [16]). Red line: Disulfide bridge; Yellow line: Pro-peptide; Arrow: β-sheet structure; Cylinder: α-helix structure.

well as elastase ⅡA, but not those of elastase Ⅰ, ⅢA, and ⅢB, where the pro-peptide is removed from the mature enzyme after tryptic activation[11,14-16].

CTRC (caldecrin) is a serine protease with the characteristic charge-relayed catalytic triad (His58, Asp105, and Ser200), located in the active site cleft between the barrel structures[14-16]. After tryptic activation, caldecrin changes its structure to a substrate-accessible catalytic cleft form. Active caldecrin hydrolyzes the leucyl bond (*e.g.*, in the N-Succinyl-Ala-Ala-Pro-Leu-p-nitroanilide substrate) more efficiently than chymotrypsin A and B; Caldecrin also cleaves the phenylalanyl bond (*e.g.*, in the N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide substrate) and the tyrosyl bond (*e.g.*, in the N-Succinyl-Leu-Leu-Val-Tyr-p-nitroanilide substrate)[10,19-21]. The protease activity of caldecrin is inhibited by serine protease inhibitors (phenylmethylsulfonyl fluoride or diisopropyl fluorophosphate), chymotrypsin inhibitor (chymostatin), and the Bowman-Birk trypsin and chymotrypsin inhibitor. The amino acid sequence and protease activity of caldecrin indicate that it is a hybrid of chymotrypsin and elastase.

CALDECRIN AND BONE METABOLISM

Caldecrin produces dose-dependent decreases in serum calcium concentrations[10]. The administration of purified porcine and rat caldecrin *via* the tail vein of mice decreased their serum calcium concentration dose-dependently and the maximum effect was attained 2-4 h post-injection with 20-100 μg (about 0.7-3.6 nmol)/kg body weight. The hypocalcemic potency of caldecrin was almost equivalent to that of porcine calcitonin (1 nmol/kg body weight, Tomomura *et al*[10] data not shown). The caldecrin proform (pro calderon), purified from the porcine pancreas in the presence of diisopropyl fluorophosphate, appeared to show time- and concentration-dependent chymotryptic activity following cleavage by trypsin. Administration of activated caldecrin reduced the serum calcium level in mice, even after treatment with the serine protease inhibitor, phenylmethylsulfonyl fluoride, which abolished the chymotryptic activity. However, administration of procaldecrin did not decrease serum calcium levels[22]. Recombinant rat[11] and human[17] caldecrin also decreased serum calcium levels. In addition, rat protease activity-deficient caldecrin mutants (with His58Ala or Ser200Ala substitutions) decreased the levels of serum calcium. Therefore, the effect of caldecrin on serum calcium levels *in vivo* requires its activation by trypsin cleavage. An intramolecular responsive region required for this calcium decreasing activity may therefore be exposed by trypsin activation.

The caldecrin-induced serum calcium decrease occurred concomitantly with a decrease in the serum concentration of hydroxyproline, which is a marker of bone resorption. This observation suggested that this serum calcium decrease may be due to the suppression of bone resorption[10]. The effects of caldecrin on osteoclast function have also been investigated; recombinant wild-type and protease activity-deficient mutant caldecrin produced concentration-dependent suppression of bone resorption in isolated rabbit mature osteoclasts[23].

Osteoclasts execute bone resorption, which is modulated by macrophage colony-stimulating factor and receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL), produced by osteoblasts and osteocytes. An imbalance between bone formation and resorption leads to bone diseases, including osteoporosis. Osteoclast differentiation and maturation involves the following three steps: (1) Osteoclast precursor cells are generated from bone marrow cells in response to macrophage colony-stimulating factor; (2) osteoclasts begin to differentiate from the precursor cells following stimulation by RANKL; and (3) at the later stage of differentiation, osteoclasts fuse to become multinucleated giant cells, leading to the cytoskeletal actin ring formation required for bone resorption. These processes are tightly regulated to

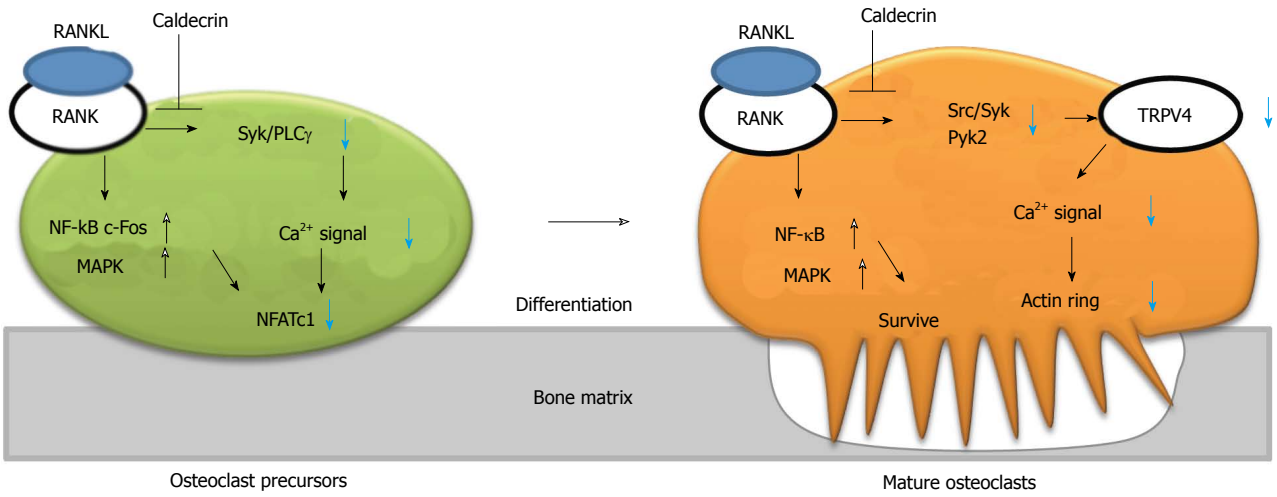

Figure 3 Caldecrin suppresses RANKL-induced osteoclast differentiation and bone resorption. RANKL binds to its receptor (RANK) on the osteoclast precursor, leading to simultaneous activation of two pathways: the NF-κB/ MAPK/c-Fos axis and the Syk/PLCγ-calcium oscillation- NFATc1 axis. In mature osteoclasts, RANKL also activates the NF-κB/MAPK and Src/Syk/Pyk2-TRPV4 channel–calcium entry–actin ring formation axes. Caldecrin inhibits the latter pathways (but not the NF-κB/MAPK pathway) in the precursor and mature osteoclasts. TRPV4: Transient receptor potential vanilloid channel 4; MAPK: Mitogen-activated protein kinase; RANKL: RANK ligand; PLCγ: Phospholipase Cγ; Pyk2: Proline-rich tyrosine kinase 2; NFATc1: Nuclear factor of activated T-cells cytoplasmic 1; NF-κB: Nuclear factor-kappa B; Syk: Spleen tyrosine kinase.

maintain bone homeostasis, and many molecules are involved in osteoclast differentiation[24-26]. The key molecule involved in osteoclastogenesis is RANKL, which is a member of the tumor necrosis factor superfamily that is expressed by osteoblasts and osteocytes in membrane-bound and secreted forms[27-33]. RANKL induces osteoclast differentiation by activating two signaling pathways: the mitogen-activated protein kinase (MAPK), NF-κB, and c-Fos activation axis and the phospholipase C γ (PLCγ)-mediated calcium oscillation-calcineurin-nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) axis. Caldecrin did not inhibit macrophage colony-stimulating factor-induced osteoclast progenitor formation from bone marrow cells but did inhibit RANKL-induced osteoclast differentiation, even in the absence of protease activity[34]. Caldecrin inhibited the RANKL-stimulated spleen tyrosine kinase- and PLCγ-induced calcium oscillation, leading to an inhibition of calcineurin and NFATc1 activity (Figure 3). Caldecrin also inhibited the RANKL-mediated actin ring formation in mature osteoclasts, which is associated with RANKL-evoked calcium entry *via* the transient receptor potential vanilloid channel 4[35]. Caldecrin significantly inhibited RANKL-stimulated phosphorylation of c-Src in association with spleen tyrosine kinase, which is upstream of transient receptor potential vanilloid channel 4 and actin ring formation. On the other hand, caldecrin did not inhibit RANKL-mediated stimulation of MAPK, NF-κB, and c-Fos activation in osteoclast precursors or mature osteoclasts[34,35]. Therefore, caldecrin antagonized the RANKL-stimulated calcium signaling pathway involved in both osteoclast differentiation and activation.

Caldecrin is a therapeutic target in osteoporosis. The ovariectomizedmouse provides a model of postmenopausal osteoporosis and exhibits an increased serum calcium level due to elevated bone resorption. This is evidenced by an increase in the bone surface to bone volume ratio, increased trabecular separation, decreased bone volume density, and decreased trabecular thickness and number. Expression of the caldecrin plasmid vector, which harbors the wild-type rat caldecrin cDNA, in the femoral muscle of this mouse model reversed this increase in serum calcium levels and restored bone resorption parameters to normal levels[36].

An important, but unaddressed, question relates to how the caldecrin released from the pancreas targets the bone. Recently, osteocalcin, which is osteoblast-derived, stored in the bone matrix, and then released by osteoclastic bone resorption, was shown to increase insulin secretion from pancreatic islets[37,38]. This activity appears to provide a physiological link between bone and pancreas, in relation to the regulation of energy metabolism. It is possible that some of the caldecrin derived from the pancreas enters the circulation and then inhibits osteoclasts, in order to regulate calcium homeostasis. The physiological activation and functions of caldecrin are not defined; however, considering its obvious effects on serum calcium levels and osteogenesis, caldecrin might be an intrinsic calcium regulating factor. The expression and distribution of caldecrin, peptide fragments of caldecrin, and its binding proteins should be explored in order to determine their physiological roles in bone metabolism.

OTHER BIOLOGICAL ASPECTS

The *CTRC* gene modulates risk for pancreatitis. Rosendahl *et al*[39] reported that *CTRC* gene mutations were significantly associated with hereditary chronic pancreatitis. Masson *et al*[40] also identified a *CTRC* mutation in patients with idiopathic chronic pancreatitis. CTRC hydrolyzes the pro-peptide and calcium-binding

loop of the trypsinogens, enhancing their activation and degradation, respectively[41-43]. Loss-of-function *CTRC* variants increase the risk for chronic pancreatitis. *CTRC* is also a susceptibility gene for tropical calcific pancreatitis, which is a juvenile form of chronic nonalcoholic pancreatitis that occurs in Asians and Africans and is associated with nearly 90% pancreatic calcium deposition[44].

It is of clinical interest that five decades ago, Takaoka *et al*[8,9,45] administered pancreatic extract to patients diagnosed with myasthenia gravis and muscular dystrophy. The symptoms of the patients treated with the extract improved progressively, suggesting that the hypocalcemic effect of the extract could have contributed to protecting them against the development of muscular dystrophy. The effect of caldecrin was also investigated in the dy/dy muscular dystrophic mouse model[46]. These mice genetically lack M-laminin and exhibit defective muscle basement membranes. Peritoneal administration of caldecrin protein or intramuscular expression of a caldecrin vector inhibited muscular destruction in the dy/dy mice. This indicated that caldecrin was responsible for the effects of the pancreatic extract on muscular dystrophy.

In 2011, Lacruz *et al*[47] found that CTRC (caldecrin) was expressed by ameloblasts and was up-regulated during enamel maturation, suggesting that caldecrin might be involved in tooth development.

CTRC (caldecrin) has been reported to be associated with pancreatic cancer, where its expression is drastically reduced. Individuals with chronic pancreatitis who show low or no activity of caldecrin show an increased risk for pancreatic cancer[48]. Furthermore, Wang *et al*[49] demonstrated that overexpression of CTRC (caldecrin) downregulated the migration of human pancreatic adenocarcinoma Aspc-1 cells, whereas the knockdown of CTRC (caldecrin) increased cell migration. It would be interesting to explore the potential use of caldecrin in pancreatic cancer diagnosis and treatment. In addition, breast cancer is highly associated with osteolytic metastatic disease. RANKL is important in mammary gland development and also in the progression of metastatic breast cancer cells[50,51]. RANKL may partly contribute to the activation of metastatic breast cancer *via* the calcineurin/NFAT pathway[52], which is modulated by caldecrin. It would therefore be interesting to investigate whether caldecrin suppresses RANKL-dependent tumor metastases.

CONCLUSION

The serum calcium-decreasing factor, caldecrin, was discovered in the pancreas. Caldecrin inhibits osteoclast differentiation and bone resorption in mature osteoclasts *via* inhibition of RANKL-induced intracellular calcium signaling. This effect occurs independently of its inherent protease activity. Therefore, caldecrin might be a potential therapeutic target for the treatment of osteolytic diseases such as osteoporosis and osteoarthritis.

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ORIGINAL ARTICLE

Basic Study

Disruption of NAD$^+$ binding site in glyceraldehyde 3-phosphate dehydrogenase affects its intranuclear interactions

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Abstract

AIM: To characterize phosphorylation of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and mobility of GAPDH in cancer cells treated with chemotherapeutic agents.

METHODS: We used proteomics analysis to detect and characterize phosphorylation sites within human GAPDH. Site-specific mutagenesis and alanine scanning was then performed to evaluate functional significance of phosphorylation sites in the GAPDH polypeptide chain. Enzymatic properties of mutated GAPDH variants were assessed using kinetic studies. Intranuclear dynamics parameters (diffusion coefficient and the immobile fraction) were estimated using fluorescence recovery after photobleaching (FRAP) experiments and confocal microscopy. Molecular modeling experiments were performed to estimate the effects of mutations on NAD$^+$ cofactor binding.

RESULTS: Using MALDI-TOF analysis, we identified novel phosphorylation sites within the NAD$^+$ binding center of GAPDH at Y94, S98, and T99. Using polyclonal antibody specific to phospho-T99-containing peptide within GAPDH, we demonstrated accumulation of phospho-T99-GAPDH in

the nuclear fractions of A549, HCT116, and SW48 cancer cells after cytotoxic stress. We performed site-mutagenesis, and estimated enzymatic properties, intranuclear distribution, and intranuclear mobility of GAPDH mutated variants. Site-mutagenesis at positions S98 and T99 in the NAD^+ binding center reduced enzymatic activity of GAPDH due to decreased affinity to NAD^+ (Km = 741 ± 257 μmol/L in T99I *vs* 57 ± 11.1 μmol/L in wild type GAPDH. Molecular modeling experiments revealed the effect of mutations on NAD^+ binding with GAPDH. FRAP (fluorescence recovery after photo bleaching) analysis showed that mutations in NAD^+ binding center of GAPDH abrogated its intranuclear interactions.

CONCLUSION: Our results suggest an important functional role of phosphorylated amino acids in the NAD^+ binding center in GAPDH interactions with its intranuclear partners.

Key words: NAD^+; Binding site; Fluorescence recovery after photobleaching; Nuclear proteins; Mutation; Glyceraldehyde 3-phosphate dehydrogenase; Anticancer agents

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Core tip: We detected the phosphorylated amino acid residues Y94, S98, T99 within the NAD^+ binding center of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Substitution of these amino acids with non-phosphorylated alanine residues did not abrogate intranuclear localization of GAPDH. Instead, such mutations altered the molecular dynamics parameters of intranuclear GAPDH probably by hindering its interactions with yet to be identified nuclear biomolecules. Our molecular modeling experiments invoke an important structural feature -T99-E97 H-bond likely involved in stabilization of NAD^+ binding center.

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INTRODUCTION

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an intriguing example of moonlighting proteins, which performs multiple functions in several unrelated cellular pathways[1]. Participation of GAPDH in diverse biochemical pathways inside distinct cellular compartments poses a problem from evolutionary point of view. Because energy metabolism of cancer cells relies mainly on glucose utilization *via* the glycolytic pathway, rather than on oxidative phosphorylation (the Warburg effect), GAPDH is important for glycolysis-dependent energy supply. Depletion of GAPDH arrests cell proliferation, and induces accelerated senescence in cancer cells[2-4].

In response to stress, GAPDH acts as a signaling molecule linking stress factors and cellular apoptotic machinery[5-7]. S-nitrosylation of Cys149 in the GAPDH catalytic center induces its intranuclear translocation and activation of apoptosis-related signaling cascade[5,8].

An additional level of complexity in regulating intracellular functions of GAPDH comes from the fact that multiple GAPDH isoforms are detected in cytosolic and nuclear fractions. Seo *et al*[9] identified multiple posttranslational modifications of GAPDH (19 types of modification on 42 sites), whose physiological role still remains to be elucidated.

Details of GAPDH-mediated intranuclear functions are incompletely known. Several lines of evidence suggest involvement of GAPDH in chemotherapy-induced DNA damage response. First, GAPDH demonstrates uracil-DNA-glycosylase activity contributing to DNA repair activity[10]. Viability of human leukemia cells treated with 6-mercaptopurine correlates with intranuclear level of GAPDH; the higher level of intranuclear GAPDH was detected in cells with lower viability[11]. Next, GAPDH was demonstrated to be a component of DNA-protein complexes formed on short DNA duplexes containing inserted modified nucleosides cytarabine, fluorouridine, and thioguanosine[12]. Finally, GAPDH binds DNA covalently linked to saframycin, a natural product with potent antiproliferative effect[13]. DNA alkylation with an antitumor drug S23906-1 results in chromatin binding with GAPDH and its protein partner HMGB1[14].

In the present study, we detected post-synthetic modifications in three amino acid residues encompassing the NAD^+ binding center in GAPDH. In order to elucidate the functional significance of these modifications, we prepared the mutant forms of GAPDH, and assessed enzyme properties, intracellular localization, and intranuclear interactions of the mutated proteins. Our results indicate that NAD^+ binding center in GAPDH is an important structural element which regulates GAPDH interactions with other nuclear components.

MATERIALS AND METHODS

Cell cultures, drug treatment and plasmids

Lung carcinoma cells A549 were obtained from the ATCC collection (ATCC, Manassas, VA). HCT116-1640 ($p53^{+/+}$) and SW48-297 ($p53^{+/+}$) cells were a generous gift from Dr. Vogelstein (John Hopkins University, Baltimore, MR). Cells were maintained in RPMI1640 medium (A549 cells; ATCC, Manassas, VA) or in McCoy's A5 (HCT116 and SW48 cells; Lonza, Allendale, NJ) at 40%-80% confluence. Cells were treated with araC {cytarabine, cytosine arabinoside, 4-amino-1-[(2R,3S,4R,5R)-3,4-dihydroxy-5-(hydroxymethyl) oxolan-2-yl] pyrimidin-2-one} dissolved in water as 500-1000 × stock solutions; drug concentrations were determined spectrophotometrically (araC, ε_{272} = 9259; The Merck Index, 2001). Human GAPDH cDNA was inserted in frame with enhanced green fluorescent protein (EGFP) into pcDNA3.1 as described

Table 1 Mutations introduced to glyceraldehyde 3-phosphate dehydrogenase cDNA ORF

GAPDH variant	Mutagenic primers[1]	Nucleotide change	Codon change
Y94A	CGATGCTGGCGCTGAG**GC**CGTCGTGGAGTCCACTGG CCAGTGGACTCCACGACG**GC**CTCAGCGCCAGCATCG	280-281	UAC- > GCC
S98A	GAGTACGTCGTGGAG**G**CCACTGGCGTCTTCACC GGTGAAGACGCCAGTGG**C**CTCCACGACGTACTC	290	UCC- > GCC
T99A	AGTACGTCGTGGAGTCC**G**CTGGCGTCTTCACCACC GGTGGTGAAGACGCCAG**C**GGACTCCACGACGTACT	295	ACU- > GCU
T99I	GAGTACGTCGTGGAGTCCA**T**TGGCGTCTTCA TGAAGACGCCA**A**TGGACTCCACGACGTACTC	296	ACU- > AUU

[1]Mutated nucleotides are indicated in bold font; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

earlier[3] and verified by sequencing. Site-mutagenesis of GAPDH ORF was performed using QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene, CA), per manufacturer's instructions. Oligonucleotides used for mutagenesis are listed in Table 1. Wild type and mutated GAPDH cDNA were inserted into pET28a plasmid, expressed in BL21 (DE3) Escherichia coli (*E. coli*) expression system (Novagen, WI) and purified using Ni-NTA chromatography.

Two dimensional gel electrophoresis

Separation of cytosolic and nuclear fractions of cells was performed with NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Rockford, IL), per manufacturer's instructions as described earlier[3]. The extracts (cytoplasmic fraction and nuclear extract) were snap-frozen on dry ice and stored at -80 ℃.

Protein extracts were precipitated by acetone, and re-dissolved in 2D buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 40 mmol/L Tris, 60 mmol/L DTT, and 1 × protease inhibitor cocktail (Roche Applied Science). Further procedures for sample preparation, the first and the second dimension separations were performed according to the published protocol[15]. Briefly, samples were diluted to 120 μL with rehydration buffer, applied on Immobiline Drystrip (7 cm) with 3-11 or 6-11 pH range (Amersham Biosciences) overnight. Isoelectric focusing was performed by applying a voltage gradient as follows: 30 min to 250 V, 1 h to 1500 V, 1 h to 3000 V and then 3 h to 8000 V. After IEF, the strips were incubated for 30 min in equilibration buffer and treated with iodoacetamide. The second dimension (SDS-PAGE) was performed on 12% Mini-PROTEAN® TGX™ Precast Gels (456-1041, Biorad). Separated proteins were stained with SYPRO® Ruby protein gel stain (Life Technologies) or transferred onto the nitrocellulose membrane by electroblotting procedure[16]. Membranes were developed with mouse monoclonal anti-GAPDH antibody at 1:10000 dilution (Millipore, CA) or phospho-T99-GAPDH antibody (Rockland Immunochemicals, PA). Polyclonal rabbit anti-pT99 antibody was raised by immunization with KLH-conjugated phosphopeptide AEYVVES [pT] GVFT corresponding to the sequence 92-103 in human GAPDH. Antibody was further purified by affinity chromatography, and used at a 1:10000 dilution. Bands were visualized by treatment with secondary antibody - IRDye 800CW donkey anti-rabbit antibody or IRDye 680 goat anti-mouse antibody (Rockland, Gilbertsville, PA) at 1:10000 dilution, and quantified by Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE) using two-color fluorescence detection at 700 and 800 nm.

Proteomics protocols

Preparation of protein samples and in-gel trypsin digestion: Protein spots were processed for gel electrophoresis-liquid chromatography-mass spectroscopy (GeLC-MS/MS) proteomics analysis. After visualization with SYPRO staining, gel images were acquired in an image reader FLA-5000 (FujiFILM) and analyzed densitometrically using the multi gauge v3.0 (FujiFILM).

The GAPDH spots were extracted and diced into - 1 mm × 1 mm. After destaining with 50% v/v Acetonitrile (ACN) in 25 mmol/L ammonium bicarbonate buffer (bicarbonate buffer), proteins within gel pieces were reduced with 10 mmol/L DTT in bicarbonate buffer, and alkylated by incubation with 50 mmol/L iodoacetamide. After gel dehydration with 100% ACN, the gel pieces were covered with approximately 40 μL of 12.5 μg/mL trypsin in bicarbonate buffer. In-gel digestion was performed at 37 ℃ for 12 h, trypsin was inactivated with formic acid at 2% final volume, and peptides were extracted and cleaned-up using C18 Tip column (ZipTips®) as previously described[17].

GeLC-MS/MS proteomics analysis: Peptides were dried in a vacuum centrifuge, then resuspended in 30 μL of 0.1% v/v TFA/H_2O. Peptide samples were loaded onto 2 μg capacity peptide traps (CapTrap; Michrom Bio-resources) and separated using a C18 capillary column (15 cm 75 mm, Agilent) with an Agilent 1100 LC pump delivering mobile phase at 300 nL/min. Gradient elution using mobile phases A (1% ACN/0.1% formic acid, balance H_2O) and B (80% ACN/0.1% formic acid, balance H_2O) was as follows (percentages for B, balance A): Linear from 0 to 15% at 10 min, linear to 60% at 60 min, linear to 100% at 65 min. The nano ESI MS/MS (electrospray ionization mass spectrometry) was performed using a HCT Ultra ion trap mass spectrometer (Bruker). ESI was delivered using distal-coating spray

Silica tip (id 20 μm, tip inner id 10 μm, New Objective, Ringoes, NJ). Mass spectra were acquired in positive ion mode, capillary voltage at -1200 V and active ion charge control trap scanning from 300 to 1500 m/z. Using an automatic switching between MS and MS/MS modes, MS/MS fragmentation was performed on the two most abundant ions on each spectrum using collision-induced dissociation with active exclusion (excluded after two spectra, and released after 2 min). The complete system was fully controlled by HyStar 3.2 software.

MS data analysis and post-translational modifications: Mass spectra processing was performed using BrukerDaltonics esquire 6.1 Data-Analysis (Version 3.4). The generated de-isotoped peak list was submitted to an in-house Mascot server 2.2.07 for searching against the Swiss-Prot database (Release 2011-06) (version 56.6, 536029 sequences). Mascot search parameters were set as follows: species, Homo sapiens (20413 sequences); enzyme, trypsin with maximum 2 missed cleavage sites. Post-translational modifications were analyzed specifically for GAPDH: Fixed modification, cysteine carboxymethylation; variable modifications: Methionine oxidation, phosphorylation of serine, threonine and tyrosine. All peptides matches were filtered using an ion score cutoff of 30.

Transfection with pEGFP-GAPDH and fluorescence recovery after photobleaching experiments

About 25000 cells/dish were seeded in 35 mm glass bottom Petri dishes (MatTek, MA) and transfected with pEGFP (Clontech, Palo Alto, CA) or pEGFP-GAPDH plasmid using FuGene6 transfection reagent (Roche, NJ). Alternatively, HCT116-4016 cells were transiently transfected by electroporation (Neon Transfection System, Invitrogen, CA). The next day, cells were treated with 1-10 μmol/L araC and incubated for 24 h.

After incubation, fluorescence recovery after photobleaching (FRAP) experiments were performed on a Leica TCS SP2 AOBS confocal microscope equipped with a 63 × 1.4 N.A. oil immersion objective at 37 ℃, as described earlier[3]. Briefly, pre-bleaching plateau was defined by acquiring 20 single section images with 6 × zoom on an area 7 × 7 μm, with acquisition speed 287 msec/frame. Bleaching was performed with 3 pulses using the 458, 476, and 488 nm lines of Ar laser. Fluorescence recovery was monitored collecting 40 single-section images at 287 msec intervals with low laser intensity (5% of the bleach intensity with the single 488 laser line, detection 495-600 nm). Quantitative analysis was performed after background subtraction, correction for laser fluctuations and acquisition photobleaching, and normalization as described by Rabut and Ellenberg[18]. 5-10 cells were analyzed on each dish; all experiments were repeated 4-5 times. Diffusion coefficient *D* value was calculated using the equation $D = 0.88*w^2/(4t_{1/2})$ where w is a radius of bleached area, with the assumptions that the bleached area is a disc and that diffusion occurs only laterally[19,20]. The immobile fraction was calculated after correction for loss of signal due to photobleaching[18].

Glycolytic assay

GAPDH glycolytic activity was measured by spectrophotometric assay at 340 nm as described earlier[21]. Briefly, the assay was carried out in 0.015 mol/L sodium pyrophosphate, 0.03 mol/L sodium arsenate (Sigma), pH 8.5, in the presence of 3.5 mmol/L DTT, 0.26 mmol/L NAD^+ and 0-2 mmol/L glyceraldehyde-3-phosphate (Sigma), or 0.51 mmol/L glyceraldehyde-3-phosphate, and 0-2 mmol/L NAD^+ catalyzed by wild type or mutated GAPDH expressed in *E. coli*. Human GAPDH (Sigma) was used as control.

Molecular modeling of the effects of mutation on NAD^+ cofactor binding

Molecular modeling experiments were performed utilizing the Sybyl molecular modeling environment. The X-ray crystal structure of human GAPDH at 1.75 Angstrom resolution (1U8F.pdb) was obtained from the Protein Data Bank and preprocessed for virtual mutagenesis studies by initial extraction of the crystallographic water molecules from the tetramer, selection and extraction of the P chain monomer with its corresponding NAD^+ cofactor, and final extraction of the cofactor as separate files. The resulting monomer was then subjected to a localized energy minimization (annealing) routine involving the region around the amino acid of interest (T99). The subset of residues within 8 Å of T99 were considered to be the "hot" region, while a radius of 14 Å comprised the "interesting" region for the annealing process, and the region was minimized to a gradient of 0.05 utilizing the AMBER7FF99 force field and charges. The resulting structure was saved, and T99 was mutated to Ala and to Ile, the resulting structures then undergoing the same local annealing process after substitution. The structures for the native T99, and the mutant forms T99A and T99I were prepared for submission to a canonical (NVT) molecular dynamics calculation utilizing the same force field and charges as above.

Statistical analysis

The statistical analyses were carried out using Student's *t* test with Statistica software program (StatSoft, OK), and non-linear regression analysis with GraphPad Prizm 4.0 software (GraphPad software, CA). A P value < 0.05 was considered statistically significant. Data are presented as the mean ± SE.

RESULTS

Proteomics analysis of intranuclear GAPDH after genotoxic stress

Nuclear and the cytosolic fractions were extracted from either untreated A549 cells, or cells treated 1 μmol/L araC for 24 h. Nuclear and cytosolic GAPDH isoforms were detected by one- or two-dimensional Western blot with

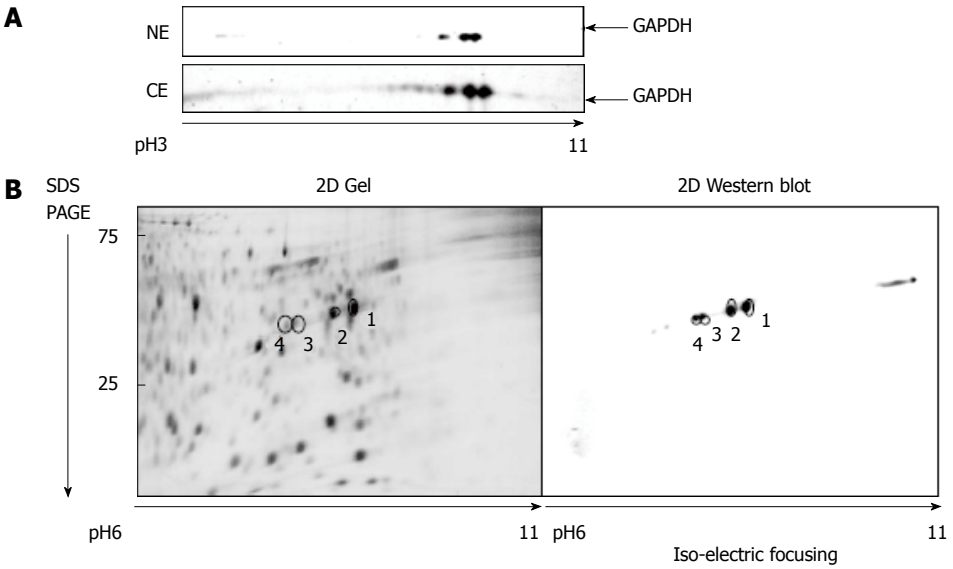

Figure 1 Glyceraldehyde 3-phosphate dehydrogenase isoforms in A549 cells. A: 2D gel separation (pH range 3-11) of proteins from the nuclear (NE) and the cytosolic (CE) fraction of A549 cells after 1 μmol/L araC treatment for 24 h. The membrane was developed with anti-GAPDH antibody as described in "Materials and Methods"; B: Proteins from the cytosolic fraction were separated by 2D gel (pH range 6-11). Separated proteins were stained with SYPRO® Ruby (left panel), or developed with anti-GAPDH antibody (right panel). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; 2D: Two dimensional.

anti-GAPDH antibodies. Because intranuclear GAPDH in untreated cells was below detection level[3], we analyzed the nuclear fraction of A549 cells after treatment with araC. Figure 1A demonstrates 2D gel separation (pH range 3-11) of the nuclear (upper part) and the cytosolic (bottom part) fractions of A549 cells treated with araC. Western blot analysis of cytosol revealed the presence of multiple GAPDH isoforms in cytosolic fraction. Only basic isoform accumulated in the nuclear fraction of treated A549 cells (Figure 1A). 2D separation (pH range 6-11) of the cytosolic fraction from untreated A549 cells is shown on (Figure 1B). The left panel shows the gel stained with SYPRO® Ruby, and the right image depicts proteins detected with anti-GAPDH antibody. Proteomics analysis of spot 1 and spot 2 on Figure 1B identified a peptide WGDAGAEYVVESTGVFTTMEK corresponding to the amino acid sequence 87-107 of human GAPDH. Further analysis of this peptide resulted in identification of phosphorylated amino acids at position 94, 98 and 99 as revealed by MS detection of the precursor ion 850.51 m/z (MS1) (Figure 2A), and the subsequent fragmentation ions (MS2) (Figure 2B).

The presence of phosphorylated T99-GAPDH in the cytosolic and nuclear fractions of treated A549 cells was confirmed with anti-phospho-T99-GAPDH antibody (Figure 3). In cytosol of araC- treated A549 cells, we detected the basic forms of GAPDH with phosphorylated T99 (Figure 3A). We also observed accumulation of basic isoforms in the nuclear fraction of treated A549 cells (Figure 3B upper image). Consecutive immuno staining of 2D gel with anti-GAPDH antibody and anti-phospho-T99-GAPDH antibody revealed phosphorylated T99 in the spots co-localized with the basic GAPDH isoforms in the nuclear fraction (Figure 3B merged image).

Site-specific mutagenesis of NAD+ binding domain in GAPDH polypeptide

Phosphorylated amino acid residues identified in our proteomics experiments are localized within the NAD+ binding center of GAPDH, as demonstrated by high-resolution structural analysis of human GAPDH[22]. To elucidate the functional role of newly found phosphorylated amino acids in NAD+ binding domain of GAPDH in chemotherapy-induced stress response, we prepared plasmids coding for wild type- and mutated forms of GAPDH polypeptide with mutations that prevented phosphorylation at position 94, 98, and 99 of GAPDH.

Plasmids coding for (His)-tagged GAPDH variants Y94A, S98A, T99A, and T99I were used for heterologous expression of mutated GAPDH polypeptides in a bacterial expression system. Enzymatic activity of purified GAPDH proteins was assayed *in vitro*. Constructs coding for GAPDH variants Y94A, S98A, T99A, and T99I in-frame with EGFP cDNA under CMV promoter were generated from pEGFP-GAPDH as described earlier[3], and these constructs were used in transfection experiments with human lung carcinoma cells A549, human colorectal carcinoma cells HCT 116-4016, and SW48-297.

Mutations in NAD+ binding domain of GAPDH inhibit glycolytic activity

Enzymatic properties of wild type and mutated forms of GAPDH were assessed spectrophotometrically using the varying concentrations of glyceraldehyde-3-phosphate or NAD+[21]. Results of these experiments are shown on Figure 4, and summarized in Table 2. All GAPDH mutant proteins had affinity for glyceraldehyde-3-phosphate close to that of wild type enzyme. In contrast, mutated polypeptides manifested decreased affinity to NAD+

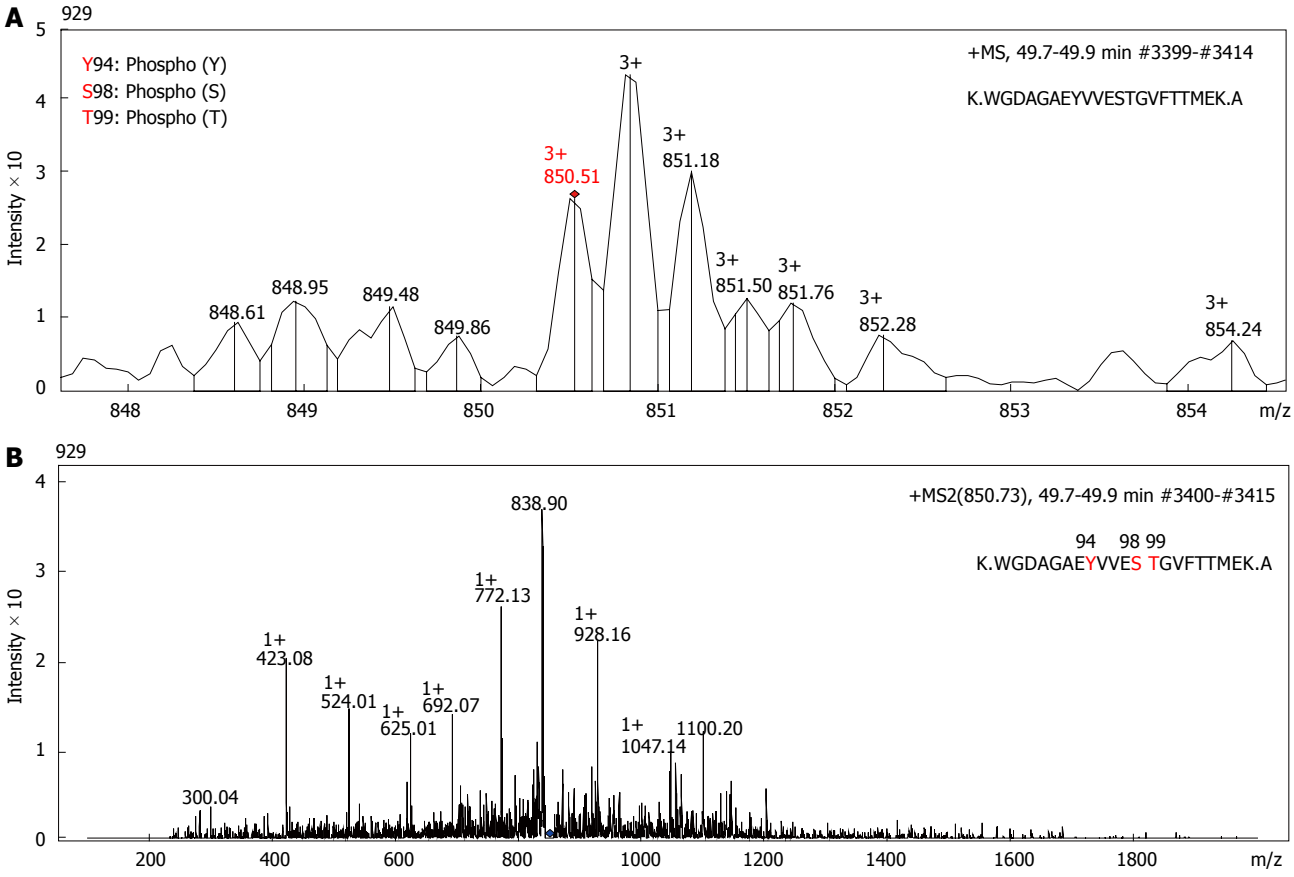

Figure 2 The tandem mass spectrometry (MS/MS) profile of glyceraldehyde 3-phosphate dehydrogenase peptides. Proteomics characterization of a basic GAPDH isoform (spot 1 from Figure 1B) from the cytosolic fraction of A549 cells by mass spectroscopy. A: Spectrum profile of the precursor ion 850.51 m/z (MS 1) selected for further fragmentation; B: Spectrum profile of the fragmentation ions (MS 2) of the peak 850.51 m/z from panel A. Retention time and number of scans where the peptide was detected are shown in the right top part of each panel. GAPDH: Glyceraldehyde 3-phosphate. dehydrogenase.

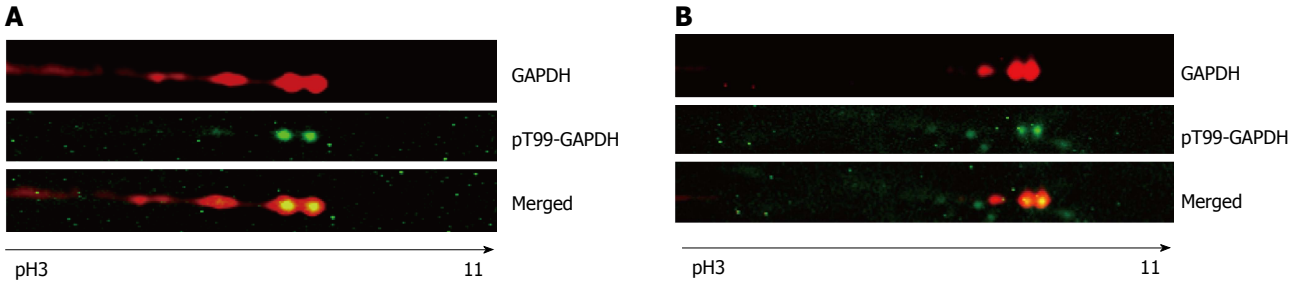

Figure 3 Phospho-T99-glyceraldehyde 3-phosphate dehydrogenase was detected in nuclei of A549 cells after genotoxic stress. A: 2D gel analysis of the cytosolic fraction extracted from A549 cells. Only a section of the membrane containing GAPDH isoforms is shown. Membranes were developed with anti-GAPDH antibody or anti-phospho-T99-GAPDH antibody in untreated and araC-treated cells. pH gradient is indicated under the bottom image; B: The nuclear fraction from A549 cells after treatment with araC. Only a section of the membrane containing GAPDH isoforms is shown. Membranes were developed with anti-GAPDH antibody (top image) or anti-phosphoT99-GAPDH antibody (bottom image). pH gradient is indicated under the bottom image. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; 2D: Two dimensional.

compared with wild type protein (Table 2). T99I variant had the lowest affinity to NAD^+, its K_m (NAD^+) was increased by more than an order of magnitude compared with wild type GAPDH (741 ± 257 *vs* 57 ± 11.1 μmol/L). This low binding of NAD^+ explains why T99I variant has low saturation under conditions of experiment (*i.e.*, at NAD^+ concentration 260 μmol/L), as depicted on Figure 4A. Extremely low binding of T99I-GAPDH to NAD^+ coenzyme accounts for decreased velocity of glycolytic reaction catalyzed by this mutated enzyme (Figure 4).

Mutations in NAD+ binding domain do not affect nuclear accumulation of EGFP-GAPDH in response to genotoxic stress

As we demonstrated earlier, endogenous GAPDH or EGFP-GAPDH fusion polypeptides localized predominantly in cytoplasm of untreated A549, SW620, and DLD1 cultured cells[3,21]. To assess the effect of mutation in NAD^+ binding

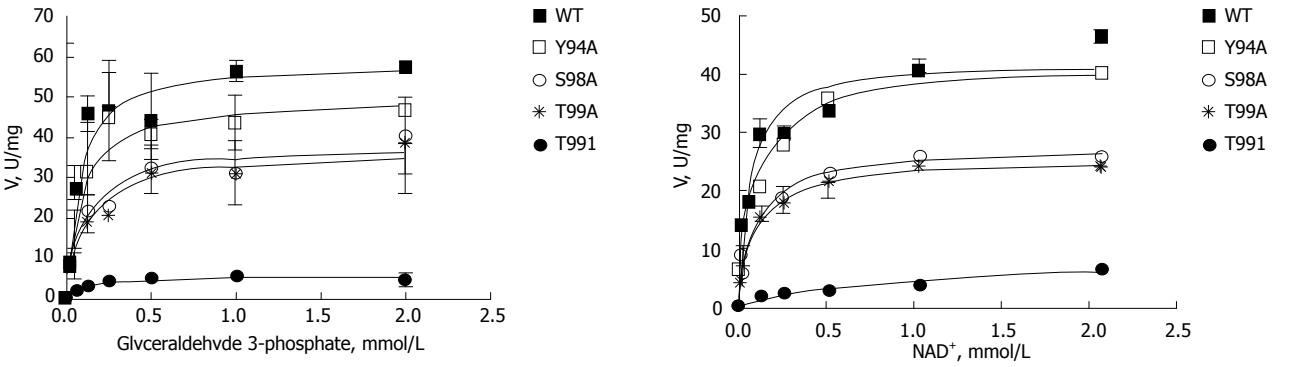

Figure 4 Kinetic analysis of wild type and mutated forms of Hisx6-tagged glyceraldehyde 3-phosphate dehydrogenase prepared in BL21 (DE3) *Escherichia coli* expression system. Conditions for the glycolytic assay are indicated in "Materials and Methods". A: Reaction was performed with 0.26 mmol/L NAD^+ and varying concentrations of D-glyceraldehyde-3-phosphate (0-2 mmol/L); B: Reaction was performed with 0.51 mmol/L D-glyceraldehyde-3-phosphate and varying concentrations of NAD^+ (0-2 mmol/L). Vmax and Km were calculated by non-linear regression analysis with GraphPad Prizm 4.0 as described in Materials and Methods. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

Table 2 Kinetic parameters of wild type and mutated glyceraldehyde 3-phosphate dehydrogenase variants

GAPDH variant	Km (G3P) (μmol/L)	Km (NAD^+) (μmol/L)
Wild type	77 ± 25.9	57 ± 11.1
Y94A	87 ± 33.1	97.4 ± 11
S98A	100 ± 36.7	101 ± 7.2
T99A	112 ± 44.7	105 ± 17.7
T99I	106 ± 38.3	741 ± 257

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

center on intracellular localization of GAPDH, we used confocal microscopy and image analysis to compare GAPDH distribution between cytoplasmic and nuclear compartments in HCT116-4016 and SW48-297 cells expressing EGFP-GAPDH (wild type), or mutated fusion proteins. After genotoxic stress caused by araC treatment, wild type EGFP-GAPDH as well as all mutated fusion proteins accumulated in the nuclei. Figure 5A shows the distribution of EGFP-GAPDH wild type, T99A and T99I mutated forms in HCT116-4016 cells before and after treatment. The basal (*i.e.*, before the genotoxic stress) intranuclear level of mutant forms was higher compared to wild type form (up to 15% *vs* 2.3%). Neither mutation at positions 94, 98, or 99 prevented GAPDH nuclear accumulation after genotoxic stress. In HCT116-4016 cells, T99I demonstrated the most prominent nuclear accumulation (45% of total EGFP-GAPDH, Figure 5B). Similar results were received in p53-proficient SW48-297 cells (not shown).

Mutations within the NAD+ binding domain alter intranuclear mobility of GAPDH

FRAP technique provides important information about dynamic properties of fluorescent proteins in the cytoplasmic and nuclear compartments of the live cells[3,23]. After short-term photo bleaching of the preselected spots inside the cytoplasmic or nuclear compartments of the cells, recovery of fluorescence intensity in the cytosolic and nuclear compartments was measured as described in "Materials and Methods". Recovery of fluorescence in the nuclear and cytosolic fractions of EGFP-GAPDH in HCT116-4016, and SW48-297 occurred differently depending on the nature of mutation in the NAD^+ binding center. As shown on Figure 6, recovery of the fluorescent signal in cytosol for all EGFP-GAPDH fusion proteins was fast and reached plateau after approximately 7.5 msec (Figure 6B, open symbols). The rate of fluorescence recovery in the nuclei of cells expressing mutant EGFP-GAPDH fusion proteins was similar to fluorescence recovery in cytosol (Figure 6B, closed symbols). In contrast, recovery of wild type EGFP-GAPDH was slow, and didn't reach plateau even after 12.5 msec (Figure 6B). Importantly, only wild type nuclear EGFP-GAPDH demonstrated about 10 times lower *D* value and about 3 time higher immobile fraction ($1-M_f$) compared with cytosolic EGFP-GAPDH (Table 3).

Molecular modeling of the effects of mutation on NAD+ cofactor binding

To probe the impact of site-directed mutagenesis on the affinity of GAPDH for NAD^+, we performed molecular modeling experiments. The X-ray crystal structure of human GAPDH at 1.75 Angstrom resolution (1U8F.pdb) was obtained from the Protein Data Bank (Figure 7A)[22]. Because of the high degree of conserved secondary structure near the region of interest, only the loop containing amino acid at position 99 was subjected to the dynamics calculation, the rest of the protein being held fixed as an aggregate. We determined the loop region to extend from E97 (end of the beta sheet) through T104 (first residue in the attached alpha helix). The in vacuo dynamics calculation was then submitted starting with a random initial velocity for a total duration of 100 ps at 300 K for the native and each mutant construct.

The results of the trajectory calculations are shown in Figure 7B, with the average structure for each simulation being shown overlaid in green (T99), yellow (T99A), and red (T99I), along with the cofactor as initially bound in the active cleft. In the native T99, an intramolecular H-bond between T99 and E97 is likely to exist, and this

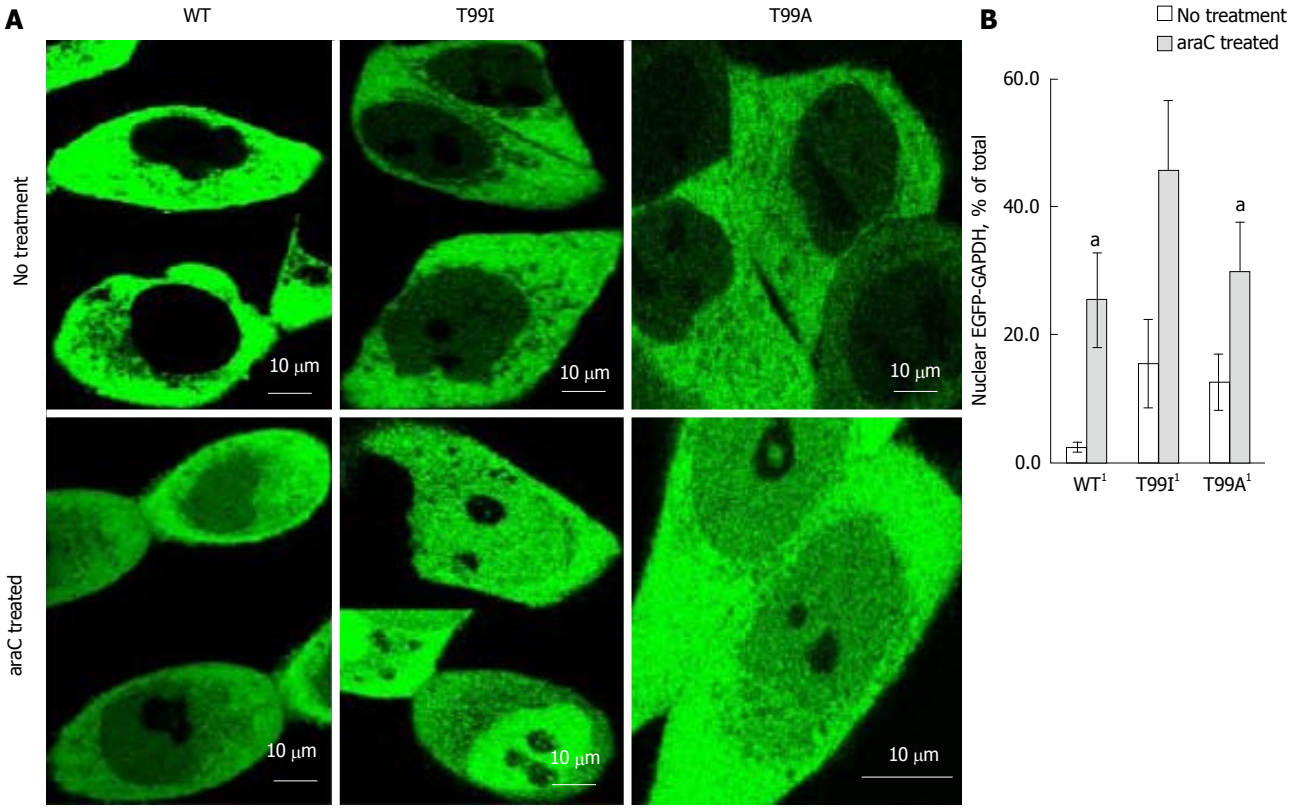

Figure 5 Nuclear accumulation of wild type and mutated enhanced green fluorescent protein-glyceraldehyde 3-phosphate dehydrogenase in live HCT116-4016 cells after transient transfections with plasmids coding for enhanced green fluorescent protein fusions with WT-glyceraldehyde 3-phosphate dehydrogenase, T99I-glyceraldehyde 3-phosphate dehydrogenase, and T99A-glyceraldehyde 3-phosphate dehydrogenase polypeptides. A: Distribution of EGFP-GAPDH variants in HCT116-4016 cells before (upper images) and after (lower images) treatment with 1 μmol/L araC for 24 h; B: Quantitative analysis of images shown in Panel A was performed using ImageJ 1.48 v software (NIH, United States). For statistical evaluation, pixel analysis of 20-25 cells was performed for each experiment (mean ± SE). [1]Significant ($P < 0.03$) alteration in nuclear GFP-GAPDH between cells treated with araC and untreated cells. EGFP: Enhanced green fluorescent protein; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GFP: Green fluorescent protein.

Table 3 Fluorescence recovery after photobleaching analysis of dynamic properties of wild type and mutated enhanced green fluorescent protein-glyceraldehyde 3-phosphate dehydrogenase

Protein	Compartment	(1-Mf)	$t_{1/2}$, s	D, μm²/s
EGFP-GAPDH-WT	cyto	0.18 ± 0.038	0.19 ± 0.055	27.17 ± 1.632
EGFP-GAPDH-Y94A	cyto	0.17 ± 0.129	0.09 ± 0.014	43.76 ± 7.158
EGFP-GAPDH-S98A	cyto	0.20 ± 0.009	0.12 ± 0.016	29.15 ± 3.885
EGFP-GAPDH-T99A	cyto	0.11 ± 0.062	0.11 ± 0.023	35.52 ± 8.133
EGFP-GAPDH-T99I	cyto	0.17 ± 0.06	0.22 ± 0.041	17.46 ± 1.733
EGFP-GAPDH-WT	nuclei	0.62 ± 0.048	1.41 ± 0.033	2.91 ± 0.331
EGFP-GAPDH-Y94A	nuclei	0.23 ± 0.127	0.09 ± 0.034	39.05 ± 6.291
EGFP-GAPDH-S98A	nuclei	0.35 ± 0.136	0.13 ± 0.018	26.57 ± 3.747
EGFP-GAPDH-T99A	nuclei	0.38 ± 0.120	0.11 ± 0.027	33.01 ± 9.209
EGFP-GAPDH-T99I	nuclei	0.29 ± 0.028	0.21 ± 0.059	14.97 ± 1.855

Cyto: Cytoplasmic compartment; nuclei: Nuclear compartment; EGFP: Enhanced green fluorescent protein; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; FRAP: Fluorescence recovery after photobleaching.

H-bond was maintained throughout the simulation. This H bond has a stabilizing effect holding the loop in a restricted conformation, thereby permitting the cofactor to fully occupy the upper region of the cleft as illustrated by Figure 7C. Mutation of T99 to A99 removes the stabilizing H-bond, and the resultant loop is able to expand somewhat into the cleft and potentially affect the binding affinity of the cofactor to GAPDH. This hypothesis is most clearly demonstrated in the mutation to I99, where the average structure places the terminus of the sidechain well within the Van der Waals radii of the adenine ring of NAD^+, thereby displacing or blocking the cofactor from its initial association with GAPDH, as illustrated by the surfaces shown in Figure 7D.

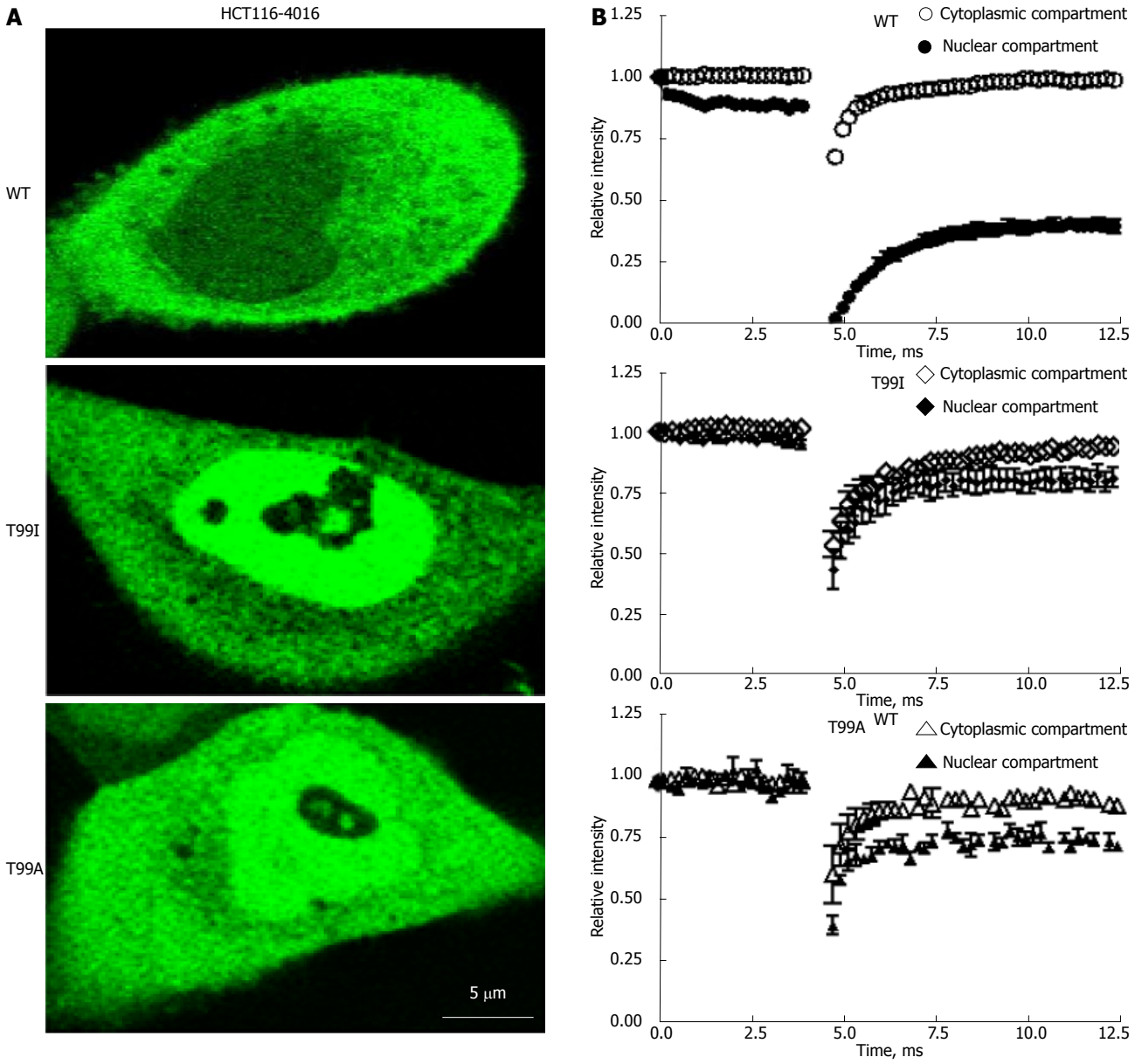

Figure 6 Intranuclear mobility of wild type and mutated variants of enhanced green fluorescent protein-glyceraldehyde 3-phosphate dehydrogenase in HCT116-4016 cells after genotoxic stress. A: FRAP analysis of fluorescent fusion protein EGFP-GAPDH in subcellular compartments of HCT116-4016 cells. Confocal images were collected from transfected cells expressing WT-EGFP-GAPDH (WT), EGFP-GAPDH-T99I (T99I), and EGFP-GAPDH-T99A (T99A) after treatment with 1 μmol/L araC for 24 h; B: Recovery rate of wild type and mutant EGFP-GAPDH fusion proteins after photo bleaching. Open symbols, cytoplasmic compartment, and closed symbols, nuclear compartment. The dynamic parameters mobile fraction (1-Mf), half-time of equilibration *t*(1/2) (s), and diffusion coefficients D were calculated from FRAP experiments, as described in "Materials and Methods" and summarized in Table 3. Where not seen, the error bars are smaller than the data point symbols. EGFP: Enhanced green fluorescent protein; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GFP: Green fluorescent protein; FRAP: Fluorescence recovery after photobleaching.

DISCUSSION

Multiple GAPDH isoforms characterized by different isoelectric points have long been known to exist in human and non-human tissues, as summarized in[24]. Because no kinetic differences were detected among the GAPDH isozymes, their role remains poorly understood. Nuclear isoforms of GAPDH are differentially regulated during apoptosis indicating important, yet to be characterized intranuclear functions[25]. In line with these data, our observations (Figure 1) also indicated the presence of post-translational modifications in GAPDH polypeptide[9]. Proteolytic digestion and subsequent MS analysis of the basic GAPDH forms with pI 8.3 revealed phospho amino acids within peptide 87-107, which were further identified as Y94, S98, and T99[26]. While multiple phosphorylation sites in GAPDH have been predicted or experimentally demonstrated, their functional role still remains obscure.

Western blot analysis with anti-GAPDH or anti-phosphoT99 antibody revealed two basic phosphorylated GAPDH isoforms in cytosol of the cells (Figure 3A). Consistently, GAPDH was excluded from the nuclei of untreated cells, but after treatment with araC, we found GAPDH phosphorylated at position T99 in the nuclei

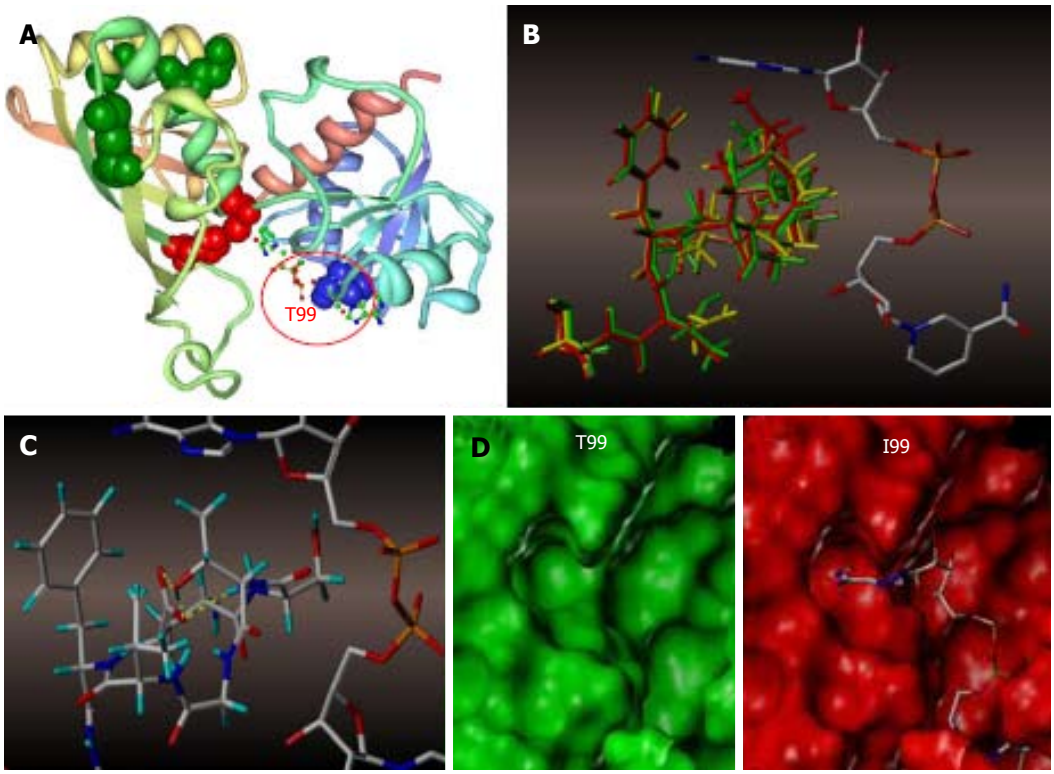

Figure 7 Molecular modeling of NAD+ binding site in human glyceraldehyde 3-phosphate dehydrogenase. A: NAD+ binding site within a GAPDH polypeptide (circled). Only one of four GAPDH subunits is shown. T99 is shown as a space-filled amino acid; B: The results of the trajectory calculations, with the average structure for each simulation shown overlaid for T99 (green), A99 (yellow), and I99 (red), along with the NAD+ molecule bound in the active cleft. Isoleucine side chain (red) of the T99I polypeptide acquires the closest position relative to the purine ring; C: In the native T99, an intramolecular H-bond between T99 and E97 (highlighted yellow) is likely to exist which holds the loop in a restricted conformation. This hydrogen bond could be a stabilizing factor permitting NAD+ to fully occupy the upper region of the cleft; D: Side-by-side comparison of T99 (green) and I99 (red) NAD+ binding site surfaces. In I99, the average structure places the terminus of the side chain well within the Van der Waals radii of the adenine ring of NAD+, thereby displacing or blocking the cofactor from its initial association with GAPDH. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

(Figure 3B).

Peptide 87-107 is a part of NAD+ binding center, as demonstrated by X-ray analysis[22]. Specifically, T99 is involved in non-polar interactions with the adenine residue of NAD+, while S98 forms electrostatic interactions with 5'-0-phosphate adjacent to nicotinamide nucleoside. The same NAD+ binding center is also involved in binding of GAPDH with RNA[27].

In order to characterize the role of phosphorylated amino acids in NAD+ binding center, we performed alanine scanning by consecutive substituting Y94, S98, and T99 with Ala. Additionally, we prepared T99I mutated GAPDH. T99I mutation results from a single nucleotide variation rs11549329 found in NCBI dbSNP database, which is likely a rare mutation.

Kinetic parameters of wild type and four mutated GAPDH variants were quite different depending on the nature of mutation. The most dramatic changes were detected in T99I variant where K_m for NAD+ was increased by more than an order of magnitude compared with wild type GAPDH. In line with these findings, the rate of the reaction catalyzed by T99I was very low (Figure 4). Mutation T99A had less effect on GAPDH parameters, with Km (NAD+) about 2 times higher, and Km (G3P) about 1.5 times higher compared with wild type GAPDH. Mutation S98A at position adjacent to T99 showed an effect similar to that of T99A. Interestingly, Y94A mutation only slightly affected kinetics of the enzyme, while its effect on molecular dynamics parameters of nuclear GAPDH was quite prominent (Tables 2 and 3). The results of the molecular modeling experiments were in good agreement with the observed changes in enzyme kinetics of the mutated polypeptides (Figure 7).

Intracellular distribution of wild type and mutant GAPDH fused to EGFP was assessed in p53-proficient HCT116-4016 and SW48-297 cells using confocal microscopy and image analysis. Unexpectedly, mutations in the NAD+ binding center changed distribution of fused EGFP-GAPDH proteins between cytoplasmic and nuclear compartments. Consistent with our previous results, wild type EGFP-GAPDH was excluded from nuclei of untreated cells, and showed intranuclear accumulation following genotoxic stress. In contrast, T99A and T99I mutant proteins were already notable in the nuclei of untreated cells. Upon incubation with araC, significantly higher levels of intranuclear T99A, and T99I GAPDH were observed far exceeding the level of wild type intranuclear GAPDH under stress conditions (Figure 5B). Both mutated proteins contained non-phosphorylated amino acids in the NAD+ binding center, and manifested decreased affinity to NAD+.

It remains to be elucidated what molecular events alter the intracellular distribution of the fusion proteins. Earlier, we demonstrated that GAPDH export from the nucleus was facilitated by CRM1[21]. We hypothesize that the redistribution of mutated GAPDH between the cytoplasmic and nuclear compartments could result from less effective interaction of mutated GAPDH polypeptides with the CRM1-mediated nuclear export system.

In our previous studies, we estimated the diffusion coefficient and the immobile fraction of GAPDH inside the living A549 cells[3]. In the present study, we performed FRAP analysis of live HCT116-4016 and SW48-297 cells treated with araC. The results of these experiments in HCT116-4016 are shown on Figure 6, and summarized in Table 3. Similarly to our experiments on intracellular distribution of wild type and mutated polypeptides, we detected profound difference in molecular dynamics characteristics of GAPDH variants. After photo bleaching, the wild type GAPDH fusion protein demonstrated ten-fold lower mobility in the nuclear compartment of HCT116-4016 cells under genotoxic stress, compared to cytoplasm. The fraction of immobile wild type EGFP-GAPDH was 0.62 ± 0.048 in the nuclei of araC-treated cells *vs* 0.18 ± 0.055 in cytoplasmic compartment. This observation parallels our findings for wild type EGFP-GAPDH in the A549 cell line[3]. Our results indicate that, inside the nucleus of the stressed cells, GAPDH loses it mobility, and significant fraction of polypeptides remains immobile presumably due to strong binding to as yet unidentified macromolecule or macrostructure. In parallel to our findings, intranuclear GAPDH in dexamethasone-treated S49 cells was found resistant to extraction with high salt or DNase treatment, consistent with our FRAP experiments[28]. David-Cordonnier's group reported binding of GAPDH with chromatin following DNA alkylation with antitumor drug S23906-1[14].

The immobile fraction was notably lower in Y94A, S98A, T99A, and T99I (Table 3). All four mutated variants had similar diffusion coefficients in cytoplasmic and nuclear compartments, in contrast to wild type nuclear GAPDH which had lower D and higher (1-Mf) values. These results indicate that mutated polypeptides are involved in less tight intranuclear interactions than their wild type counterpart, with as yet unknown nuclear components (Table 3). We conclude that the phosphorylated amino acids or functionality of NAD^+ binding center is important for GAPDH intranuclear immobilization.

Tight interaction of intranuclear GAPDH with nuclear component(s) is an intriguing observation, considering the fact that multiple stress factors promote intranuclear accumulation of this protein. At least two groups of intranuclear proteins, sirtuins and ADP-ribosyl transferases, which control stress response and DNA repair, are NAD^+-dependent. Both groups are present in the nucleus, and require NAD^+ for their activity. Importantly, GAPDH was demonstrated to directly interact with NAD^+-dependent Sir2 histone deacetylase, and poly (ADP-ribose) polymerase-1[29,30]. Therefore, we speculate that GAPDH could provide a nuclear NAD^+ reservoir, and deliver it to these proteins following the stress stimulus.

T99I variant is especially interesting as it is not an entirely artificial construct. The cDNA clone containing missense rs11549329 leading to T99I mutation was prepared from melanotic melanoma cells (GenBank: BM904848.1). This mutated variant manifests very low affinity to NAD^+ (Figure 4 and Table 2), high level of intranuclear accumulation (Figure 5), and a modest immobile fraction after genotoxic stress (Figure 6). Treatment with genotoxic drug does not alter mobility (diffusion coefficient) of this mutated form inside the nucleus or cytosol (Table 3).

Molecular modeling of NAD^+ binding site in human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed differential effects of amino acid changes at position 99 (Figure 7A). Interestingly, our molecular modeling experiment suggested the existence of a hydrogen bond between T99 and E97. Such intramolecular H-bond is likely to stabilize the loop extending from E97 (end of the beta sheet) through T104 (first residue in the attached alpha helix). This H-bond suffices to hold the loop in a restricted conformation, thereby permitting the cofactor to fully occupy the upper region of the cleft (Figure 7C).

Though T99A mutation does not prevent NAD^+ binding, decreased T99A GAPDH catalytic activity is best explained by the disruption of such T99-E97 intramolecular H-bond. Mutation of T99A removes the stabilizing H-bond, and the resultant loop is able to expand somewhat into the cleft and potentially affect the binding affinity of the cofactor to GAPDH. This hypothesis has been supported by our kinetic experiments with mutated GAPDH proteins *in vitro*, as Km of the mutant forms did decrease by 2-10 fold (Table 2). Further confirmation for T99-E97 interaction requires additional testing, for example by mutational scanning at E97. Finally, analysis of the average potential energies of the dynamics trajectories also indicates that the system exhibits decreased stabilization (higher average potential energy and total energy) upon mutation and concomitant expansion of the loop into the cleft (Table 4).

Our model predicts T99I to have the strongest effect on NAD^+ binding and catalytic activity of GAPDH. Indeed, insertion of Ile at position 99 brings its side chain in close proximity with adenine of the cofactor (Figure 7). Modeling of the van der Waals surfaces inside the NAD^+ binding center shows sterically unacceptable interaction so that such interaction either displaces, or blocks cofactor binding by T99I GAPDH.

In conclusion, we detected the phosphorylated amino acid residues Y94, S98, T99 within the NAD^+ binding center of GAPDH. Substitution of these amino acids with non-phosphorylated alanine residues did not abrogate intranuclear localization of GAPDH. Instead, such mutations altered the molecular dynamics parameters of intranuclear GAPDH probably by hindering its interactions with yet to be identified nuclear biomolecules. Our

Table 4 Average potential energy, kinetic energy, total energy and temperature calculated from dynamic simulations

Polypeptide variant	Average potential (kcal)	Average kinetic energy (kcal)	Average total energy (kcal)	Average temperature (℃)
T99	-5021.8	97.1	-4924.7	293.4
A99	-5007.3	93.7	-4913.6	293.7
I99	-4997.8	101.4	-4896.4	293.3

molecular modeling experiments invoke an important structural feature -T99-E97 H-bond likely involved in stabilization of NAD$^+$ binding center.

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COMMENTS

Background

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an important enzyme of the glycolytic pathway, and an intriguing moonlighting protein involved in multiple, seemingly unrelated biochemical processes. It is known to exist in multiple isoforms, biological role and physiological significance of which remain enigmatic. Distribution of GAPDH between cellular compartments reveals complex patterns, and is regulated by partially characterized mechanisms.

Research frontiers

Among multiple functions ascribed to GAPDH, its contribution to the nuclear processes remains obscure. The role of intranuclear GAPDH isoforms in maintaining the integrity of genome is a new direction of study.

Innovations and breakthrough

This is the first study in which the role of phosphorylation sites in a NAD$^+$ binding center of GAPDH has been explored by site-mutagenesis, and the dynamics parameters of nuclear GAPDH have been directly measured in fluorescence recovery after photobleaching experiments.

Applications

These results indicate an unexpected, important role phosphorylated amino acids within the NAD$^+$ binding center play in intranuclear functions of GAPDH, and suggest a single mechanism by which GAPDH contributes to formation and functioning of its multiple binding partners.

Terminology

Fluorescence recovery after photobleaching is an optical technique for quantifying the diffusion of the fluorescently labeled biological molecules inside the living cells.

Peer-review

In this study, Phadke *et al* investigate the phosphorylated amino acid residues Y94, S98, T99 within the NAD$^+$ binding center of glyceraldehyde 3-phosphate dehydrogenase. Substitution of these amino acids with non-phosphorylated alanine residues did not abrogate intranuclear localization of GAPDH. Instead, such mutations altered the molecular dynamics parameters of intranuclear GAPDH probably by hindering its interactions with yet to be identified nuclear biomolecules. Molecular modeling experiments suggest an important structural feature -T99-E97 H-bond likely involved in stabilization of NAD$^+$ binding center.

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ORIGINAL ARTICLE

Basic Study

Connective tissue growth factor differentially binds to members of the cystine knot superfamily and potentiates platelet-derived growth factor-B signaling in rabbit corneal fibroblast cells

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Author contributions: Pi L and Chung PY are co-first authors and share equal contribution; Pi L performed yeast two-hybrid analysis; Chung PY carried SPR experiments; Rahman MM contributed to SPR analysis; Sriram S established rabbit corneal fibroblast cell culture; Pi L, Chung PY, Scott EW, Petersen BE, and Schultz GS substantially contributed to the design of the study, acquisition, analysis and interpretation of data; Pi L and Chung PY wrote the paper; all authors made critical comments related to the intellectual content of the manuscript, and approved the final version of the article to be published.

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Abstract

AIM: To study the binding of connective tissue growth factor (CTGF) to cystine knot-containing ligands and how this impacts platelet-derived growth factor (PDGF)-B signaling.

METHODS: The binding strengths of CTGF to cystine knot-containing growth factors including vascular en-

dothelial growth factor (VEGF)-A, PDGF-B, bone morphogenetic protein (BMP)-4, and transforming growth factor (TGF)-β1 were compared using the LexA-based yeast two-hybrid system. EYG48 reporter strain that carried a wild-type *LEU2* gene under the control of LexA operators and a lacZ reporter plasmid (p80p-lacZ) containing eight high affinity LexA binding sites were used in the yeast two-hybrid analysis. Interactions between CTGF and the tested growth factors were evaluated based on growth of transformed yeast cells on selective media and colorimetric detection in a liquid β-galactosidase activity assay. Dissociation constants of CTGF to VEGF-A isoform 165 or PDGF-BB homo-dimer were measured in surface plasma resonance (SPR) analysis. CTGF regulation in PDGF-B presentation to the PDGF receptor β (PDGFRβ) was also quantitatively assessed by the SPR analysis. Combinational effects of CTGF protein and PDGF-BB on activation of PDGFRβ and downstream signaling molecules ERK1/2 and AKT were assessed in rabbit corneal fibroblast cells by Western analysis.

RESULTS: In the LexA-based yeast two-hybrid system, cystine knot motifs of tested growth factors were fused to the activation domain of the transcriptional factor GAL4 while CTGF was fused to the DNA binding domain of the bacterial repressor protein LexA. Yeast co-transformants containing corresponding fusion proteins for CTGF and all four tested cystine knot motifs survived on selective medium containing galactose and raffinose but lacking histidine, tryptophan, and uracil. In liquid β-galactosidase assays, CTGF expressing cells that were co-transformed with the cystine knot of VEGF-A had the highest activity, at 29.88 ± 0.91 fold above controls ($P < 0.01$). Cells containing the cystine knot of BMP-4 expressed the second most activity, with a 24.77 ± 0.47 fold increase ($P < 0.01$). Cells that contained the cystine knot of TGF-β1 had a 3.80 ± 0.66 fold increase ($P < 0.05$) and the ones with the cystine knot of PDGF-B had a 2.64 ± 0.33 fold increase of β-galactosidase activity ($P < 0.01$). Further SPR analysis showed that the association rate between VEGF-A 165 and CTGF was faster than PDGF-BB and CTGF. The calculated dissociation constant (KD) of CTGF to VEGF165 and PDGF-BB was 1.8 and 43 nmol/L respectively. PDGF-BB ligand and PDGFRβ receptor formed a stable complex with a low dissociation constant 1.4 nmol/L. Increasing the concentration of CTGF up to 263.2 nmol/L significantly the ligand/receptor binding. In addition, CTGF potentiated phosphorylation of PDGFRβ and AKT in rabbit corneal fibroblast cells stimulated by PDGF-BB in tissue culture condition. In contrast, CTGF did not affect PDGF-B induced phosphorylation of ERK1/2.

CONCLUSION: CTGF has a differential binding affinity to VEGF-A, PDGF-B, BMP-4, and TGF-β. Its weak association with PDGF-B may represent a novel mechanism to enhance PDGF-B signaling.

Key words: Connective tissue growth factor; Vascular endothelial growth factor A; Platelet derived growth factor B; Transforming growth factor-β; Bone morphogenetic protein-4; Yeast two-hybrid analysis; Surface plasma resonance; Rabbit corneal fibroblast cells; Platelet-derived growth factor receptor β; Platelet-derived growth factor-B signaling

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Core tip: The relative binding strength of connective tissue growth factor (CTGF) was vascular endothelial growth factor-A > bone morphogenetic protein-4 > transforming growth factor-β1 > platelet-derived growth factor (PDGF)-B. CTGF binding could potentiate PDGF-B signaling as evidenced by enhanced phosphorylation of PDGF receptor β and downstream AKT molecules in rabbit corneal fibroblast cells. Our findings provide deep insight into CTGF action in fine-tuned regulation of extracellular signaling mediated by different cysteine knot containing growth factors.

Pi L, Chung PY, Sriram S, Rahman MM, Song WY, Scott EW, Petersen BE, Schultz GS. Connective tissue growth factor differentially binds to members of the cystine knot superfamily and potentiates platelet-derived growth factor-B signaling in rabbit corneal fibroblast cells. *World J Biol Chem* 2015; 6(4): 379-388 Available from: URL: http://www.wjgnet.com/1949-8454/full/v6/i4/379.htm DOI: http://dx.doi.org/10.4331/wjbc.v6.i4.379

INTRODUCTION

Connective tissue growth factor (CTGF), also termed as CCN2, is a secreted cysteine-rich protein in the CCN (Cyr61/CTGF/Nov) family involved in a diverse variety of biological processes ranging from angiogenesis, chondrogenesis, osteogenesis, and tissue repair, to cancer development. CTGF was originally isolated from human umbilical vein endothelial cells as a 349-amino acid mitogen with biological activities similar to platelet-derived growth factor (PDGF)-B[1]. CTGF protein contains a modular architecture starting with an N-terminal signal peptide followed by an insulin-like growth factor-binding (IGFBP) domain, a von Willebrand factor type C domain, a thrombospondin type I homology domain, and a C terminal cystine knot motif[2]. These domains enable CTGF interaction with a broad repertoire of proteins including cell surface proteins, extracellular matrix proteins, and growth factors. Through these interactions, CTGF functions as an extracellular signaling modulator and regulates cell adhesion, migration, activation and differentiation[3].

The cystine-knot motif is structurally conserved in various kinds of growth factors, ligands, and mucins[4,5]. Consensus sequences of this structure can be identified by a pattern of six cysteine amino acids with a defined space consisting of three intertwined disulfide bridges. Two of the disulfide bonds and their adjacent amino acids

form a ring that is passed by the third disulfide. This structure forces exposure of many hydrophobic residues in monomers and favor the formation of homo- or hetero-dimers to decrease hydrophobicity in an aqueous environment. These dimers represent active states of the cystine knot-bearing growth factors able to elicit signal transduction *via* their cognate receptors. Interestingly, many members of this superfamily including transforming growth factor (TGF)-β, bone morphogenetic protein (BMP)-4, BMP-2, vascular endothelial growth factor (VEGF)-A, PDGF-B, von Willebrand factor (vWF), the neuronal guidance cue Slit3, the growth and differentiation factor 5 (GDF5), and GDF15 are able to interact with CTGF[6-11]. It has been shown that CTGF binding can sequester VEGF-A from VEGF receptor 1 and inhibit VEGF-A stimulated angiogenesis[6]. Moreover, the high affinity of CTGF inhibits the presentation of BMP-4 to BMP receptor type Ia and the phosphorylation of this receptor and downstream molecule Smad1, whereas its low affinity potentiates TGF-β1 binding to TβR Ⅱ receptor and enhances phosphorylation of Smad2[7]. So far, little has been determined concerning the effect of CTGF on PDGF-B activity and signaling. In this study, we compared the binding strengths of CTGF to VEGF-A, PDGF-B, TGF-β1 and BMP-4 using the LexA-based yeast two-hybrid system. The effect of CTGF on PDGF-B presentation to PDGFRβ receptor was determined in surface plasma resonance (SPR) analysis. The modulation of CTGF on PDGF-B signaling was investigated using rabbit corneal fibroblast cells.

MATERIALS AND METHODS

Yeast two-hybrid analysis

Yeast two-hybrid assays were performed according to the Clontech yeast protocol handbook. Synthetic dropout minimal medium (SD) and supplements used in this study were purchased from Clontech (Mountain View, CA). Dropout supplements containing nucleotides and amino acid residues as well as additional supplements of glucose, galactose (Gal), and raffinose (Raf) were prepared according to the manufacturer's instructions. CTGF, cystine knot motifs in VEGF-A and PDGF-B were described previously[9]. The cystine knot motif of TGF-β1 was amplified using primers 5′ AGGTCGACCTCCTGGCGATACCTCAGCAACGC 3′ (sense) and 5′ TTGCGGCCGCTGCACTTGCAGGAGCGCAC 3′ (antisense) from a human cDNA clone (Genebank Accession # NM_000660.5). The cystine knot motif of BMP-4 was amplified using primers 5′ AGGTCGACC GGACACCTCATCACACGACTA 3′ (sense) and 5′ TTGCG-GCCGCGGCATCCACACCCCTCTAC 3′ (antisense) from a mouse cDNA clone (Genebank Accession #NM_007554.2). All the constructs contained restriction enzyme sites *Sal I* and *Not I* that facilitated their cloning into the prey pB42AD vector carrying an activation domain (AD) of VP16 or the bait pLexA vector containing a LexA-DNA binding domain (BD).

In yeast two-hybrid analysis, *Saccharomyces cerevisiae* EGY48 was used as the reporter strain, which carried a wild-type *LEU2* gene under the control of LexA operators and a *lacZ* reporter plasmid p80p-*lacZ* containing eight high affinity LexA binding sites. SD medium that lacked uracil (Ura) was used for the maintenance of p8op-*lacZ* plasmids. BD or AD plasmids containing CTGF and cystine knot motifs of tested growth factors were co-transformed into EGY48(p8op-*lacZ*) and selected using histidine (His; a selection marker for pLexA plasmid) and tryptophan (Trp; for selection of pB42-AD plasmids). Co-transformants were initially selected on SD medium lacking Ura, His, and Trp but supplemented with glucose, which repressed fusion protein expression. Then these co-transformed cells were grown overnight prior to dilution into SD media that contained Gal and Raf but lacked Trip, His, and Ura. Gal and Raf supplements were added to induce fusion gene expression. The diluted cells were grown to mid-log phase with optical densities of 0.5-0.8 units. One-half of each cell culture was used to determine activation of the *Leu2* reporter gene by growth assay based on survival in SD medium contained Gal and Raf but lacked Trp, His, Ura, and leucine (Leu). The remaining mid-log-phased cells was pelleted, washed and resuspended in Z buffer (60 mmol/L Na_2HPO_4, 40 mmol/L NaH_2PO_4, 10 mmol/L KCl, 10 mmol/L $MgSO_4$, pH = 7.0). β-galactosidase activity was measured by O-nitrophenyl β-D-galactopyranoside (ONPG) cleavage in a liquid assay (Pierce Biotechnology, Rockford, IL). The results were obtained by reading absorbance at 420 nm and normalized against cell density (A_{600}) and reaction time. Values are the average folds of activation over pB42 vector alone (arbitrarily set to 1) from three independent experiments. Statistical analysis was carried out using Student *t* test in Microsoft Excel software.

SPR analysis

Biacore 2000 and CM4 dextran sensor chips (GE healthcare, Piscataway, NJ) were used to characterize protein-protein interactions based on SPR technology. Filtered and degassed HBS-EP buffer (pH = 7.4) containing 0.01 mol/L HEPES, 0.15 mol/L NaCl, 3 mmol/L EDTA, and 0.005% Tween 20 was used as running buffer. Prior to the kinetic measurements, the sample channel was immobilized with carrier free recombinant human CTGF protein (BioVendor, Asheville, NC), and the reference channel was blocked with ethanolamine. Then, serial concentrations of recombinant human homodimeric PDGF-BB protein (Shenandoah, Warwick, PA) and recombinant human VEGF-A variant 165 ($VEGF_{165}$) (Abcam, Cambridge, MA) in HBS-EP buffer were injected over the sampling and reference channels simultaneously at 30 μL/min for 3 min. The sensorgrams acquired from the reference channels were deducted from those of CTGF-immobilized channels to eliminate the responses caused by the refractive index changes. The dissociation was then followed by injecting HBS-EP buffer for 10 min. The difference in SPR response unit (RU) between the baseline and the signal after the dissociation reflects the amount of bound analytes. Before starting the next injection, the chip surface was regenerated by exposing

the chip surface with 10 mmol/L of cysteine-HCl buffer pH 2.0 at 30 μL/min for 30 s.

To determine the effect of CTGF on the binding between PDGF-BB and PDGFRβ, a mixture of PDGF-BB (2 nmol/L) and varied concentrations of CTGF (0 to 263.2 nmol/L) was injected over the PDGFRβ-immobilized channel and reference channel. Amine coupling strategy was used to immobilize the recombinant human PDGFRβ Fc chimera protein (R&D Systems, Minneapolis, MN) on the sampling channel. Briefly, a mixture of 0.4 mol/L 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 0.1 mol/L N-hydroxysuccinimide in equal proportion was used to activate the surface of a CM4 dextran sensor chip at a flow rate of 10 μL/min for 7 min. Then, PDGFRβ was injected over separate sampling channels for 7 min to covalently immobilize on the surfaces. Remaining active groups were blocked with 1 mol/L ethanolamine-HCl at pH 8.5 on both sampling and reference channels. The sensorgrams acquired from the reference channels were deducted from those of the PDGFRβ-immobilized channels to eliminate the responses caused by the refractive index changes. All data were analyzed with the BIAevaluation software for calculating dissociation constants.

Determination phosphorylation of PDGFRβ, ERK1/2 and AKT in Western analysis

Primary rabbit corneal fibroblast cells were isolated as described previously[12]. Cells were cultured to 100% confluence and serum-starved for 20 h in DMEM with 0.1% FBS. PDGF-B (1.6 nmol/L), CTGF (6.6 nmol/L), or a combination was added to the prepared cells. At 10 min and again at 1 h after stimulation, cells were collected and lysed in RIPA buffer (150 mmol/L NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mmol/L Tris, pH = 8.0) containing proteinase inhibitor (Sigma, St Louis, MI). Total protein lysates (20 μg) were separated on 10% SDS-PAGE gels followed by transferring to polyvinylidene difluoride membranes. The primary antibodies for anti-phospho-PDGFRβ, anti-PDGFRβ, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-AKT, and anti-AKT were purchased from Cell Signaling (Danvers, MA). HRP conjugated rabbit or mouse secondary antibodies were used followed by detection with the ECL kit (GE Health Biosciences).

RESULTS

Differential binding ability of CTGF to VEGF-A, PDGF-B, TGF-β, and BMP-4 in the yeast two-hybrid LexA system

To compare the relative binding activity of CTGF to VEGF-A, PDGF-B, TGF-β1, and BMP-4, we took the yeast two-hybrid approach and expressed the cystine knot motifs of these specific growth factors as AD fusion proteins under the control of the GAL1 promoter in the pB42-AD plasmids. In addition, full-length CTGF cDNA was expressed in fusion with LexA BD under the control of an alcohol dehydrogenase promoter in pLexA plasmids. These plasmids and their corresponding empty vectors were transformed into the EGY48 (p8op-*lacZ*) reporter strain (Figure 1A). CTGF itself was not able to self-activate in this LexA system since yeast cells that were co-transformed with the BD:CTGF plasmid and pB42 vector did not grow without Leucine supplementation and did not turn blue in β-galactosidase assays. Nor were cystine knot motifs of tested growth factors because they also failed to trigger *Leu2* and *LacZ* reporter genes when co-transformed with pB42-AD empty vector. In contrast, co-transformation with CTGF allowed survival of cells that expressed cystine knot motifs of all four growth factors on selective medium lacking leucine, supporting our previous observations that CTGF has broad binding capabilities to members of cystine knot superfamily[9]. In addition, we carried out an ONPG-based assay and assessed relative β-galactosidase activity in comparison to control cells that carried plasmids for CTGF and pLexA vector. As shown in Figure 1B, CTGF expressing cells that were co-transformed with the cystine knot of VEGF-A had the highest β-galactosidase activity, at 29.88 ± 0.91 fold above controls. Cells containing the cystine knot of BMP-4 expressed the second most β-galactosidase activity, with a 24.77 ± 0.47 fold increase. Cells that contained the cystine knot of TGF-β1 had a 3.80 ± 0.66 fold increase and the ones with the cystine knot of PDGF-B had a 2.64 ± 0.33 fold increase. These observations indicated that VEGF-A and BMP-4 were strong interactors whereas PDGF-B and TGF-β were weak binding proteins for CTGF.

Binding kinetics of CTGF to VEGF-A and PDGF-B in SPR analysis

SPR analysis was performed to characterize the affinity of CTGF to $VEGF_{165}$ and PDGF-BB. Initial experiments indicated that injecting homodimeric PDGF-BB over a CM5 dextran sensor chip caused a significant non-specific response on the reference channels, which may be due to the electrostatic interaction between the cationic growth factors and negatively charged carboxymethyl dextran covered on the chip. Therefore, we chose a CM4 dextran sensor chip with a lower degree of carboxymethylation to avoid the non-specific interaction. The kinetic analyses between PDGF-BB/CTGF and $VEGF_{165}$/CTGF complexes were carried out by injecting different concentrations of PDGF-BB (19.3 to 385 nmol/L) or $VEGF_{165}$ (3.879 to 52.267 nmol/L) over the CTGF-immobilized channel and reference channel simultaneously at 30 μL/min for 3 min. The dissociation process was followed by injecting HBS-EP buffer at 30 μL/min for 10 min. The higher flow rate was required to reduce the mass transfer effect and obtain kinetic parameters accurately. As shown in Figure 2A-C, the association rate between $VEGF_{165}$ and CTGF was faster than PDGF-BB and CTGF. The calculated dissociation constant (K_D) of CTGF to $VEGF_{165}$ and PDGF-BB was 1.8 and 43 nmol/L respectively. These results indicated that CTGF had higher affinity to $VEGF_{165}$ than PDGF-BB.

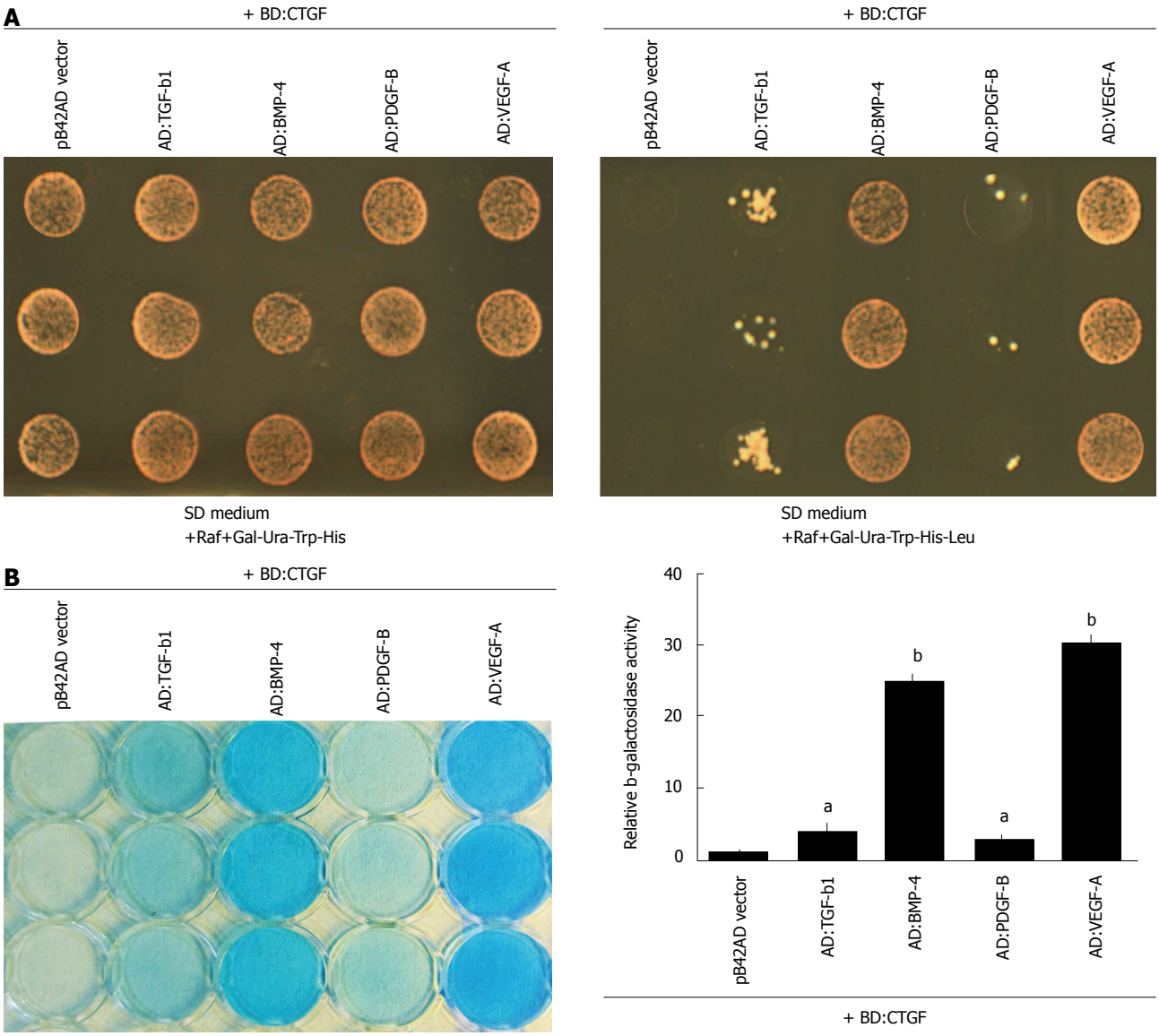

Figure 1 Determination of connective tissue growth factor binding to cystine knot motifs of vascular endothelial growth factor-A, platelet-derived growth factor-B, transforming growth factor-β1, and bone morphogenetic protein-4 in yeast two-hybrid LexA system. A: CTGF binding to cystine knot motifs of tested growth factors was assessed using growth assay on selective SD medium lacking leucine (lower panel); B: The liquid β-galactosidase assay was also carried out determining the binding strength of CTGF to cystine knot motifs of tested growth factors. The relative β-galactosidase activity was quantified in graph on the right. $^{a}P < 0.05$; $^{b}P < 0.01$. Values are the average -folds of activation over pB42-AD vector alone (arbitrarily set to 1) from three independent experiments performed in triplicate ± SD. CTGF: Connective tissue growth factor; VEGF: Vascular endothelial growth factor; BMP: Bone morphogenetic protein-4; TGF-β1: Transforming growth factor-β1; PDGF-B: Platelet-derived growth factor-B.

CTGF promotes the binding of PDGF-B to PDGFRβ determined by SPR analysis

PDGF-BB ligand and PDGFRβ receptor formed a stable complex indicated by a low dissociation constant 1.4 nmol/L in our SPR analysis (Figure 3A). To determine whether CTGF protein influenced the formation of this ligand/receptor complex, we injected a mixture of CTGF and PDGF-BB over the PDGFRβ-immobilized channel and measured the SPR response unit. As shown in Figures 3B and 4, the presence of CTGF was able to potentiate the binding of PDGF-BB to its receptor. Increasing the amount of CTGF further promoted this effect, especially when the concentration of CTGF was more than 3.3 nmol/L. Specifically, two-fold more ligand/receptor binding was observed when 26.3 nmol/L CTGF was mixed with 2 nmol/L PDGF-BB in comparison to the same amount of PDGF-BB alone. Moreover, three and a half-fold more ligand/receptor binding was seen when 263.2 nmol/L CTGF was mixed with 2 nmol/L PDGF-BB. In other words, seven-fold more ligand/receptor binding response was seen when CTGF to PDGF-BB ratio in molar concentration was 131.6 in comparison to PDGF-BB alone. To validate if the binding was indeed attributable to PDGF-BB, we also injected pure 263.2 nmol/L CTGF over PDGFRβ-immobilized channels as a control. We found no binding interaction (Figure 5).

CTGF potentiates activation of PDGFRβ and downstream signaling molecule AKT

PDGFRβ is recognized by PDGF-B ligand and deletion

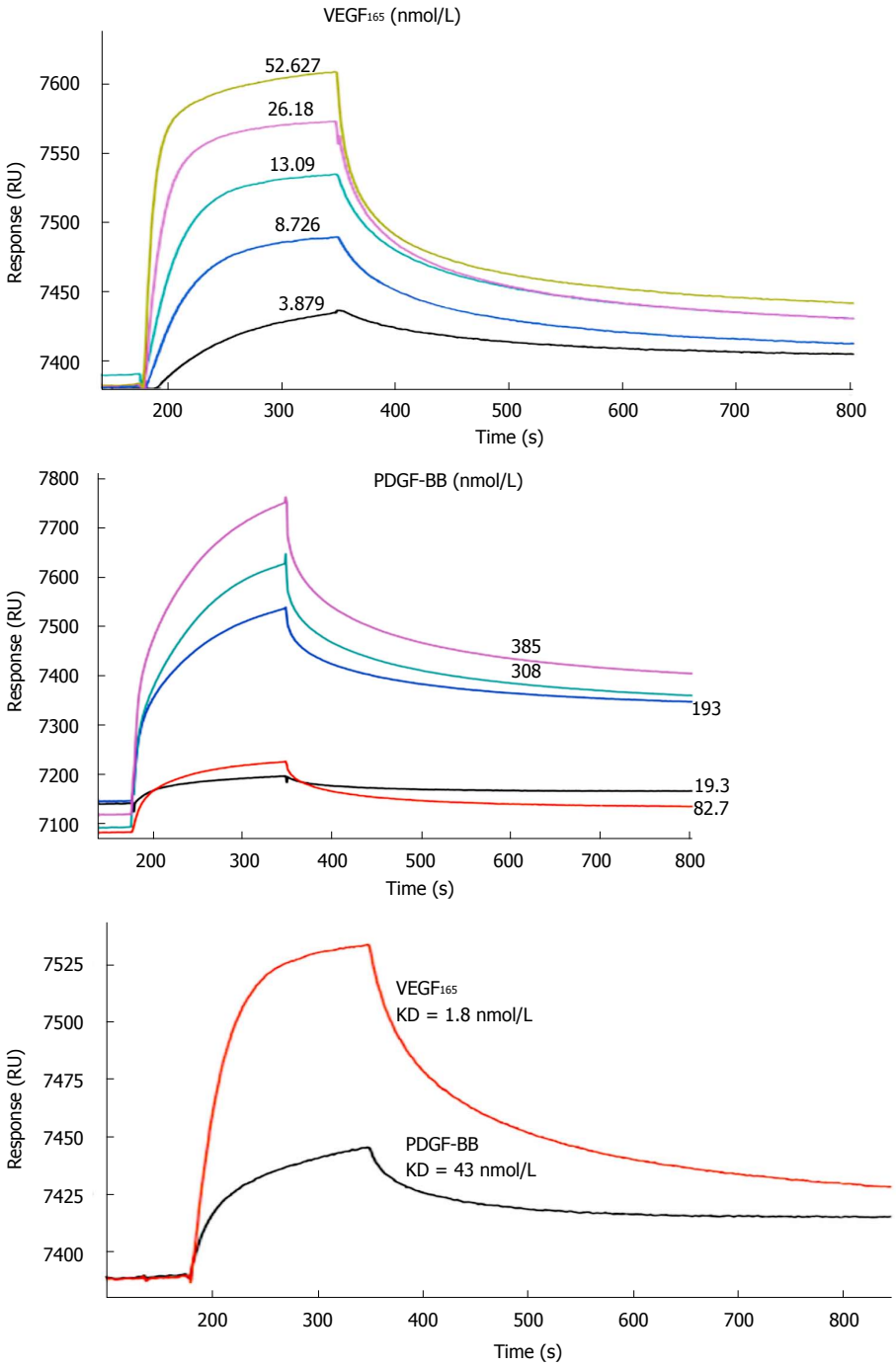

Figure 2 Quantitative measurement of connective tissue growth factor binding to vascular endothelial growth factor 165 and platelet-derived growth factor-BB in surface plasma resonance analysis. The binding kinetics of CTGF to VEGF165 (A) or to PDGF-BB (B) were determined by injecting VEGF165 ranging from 3.879 nmol/L to 52.267 nmol/L or PDGF-BB ranging 19.3 nmol/L to 385 nmol/L over CTGF immobilized channel and reference channel at 30 μL/min for 3 min simultaneously. The reference sensorgram has already deducted to eliminate the signal from a bulk refractive index shift; C: Sensorgrams of SPR analysis showed that the equilibrium dissociation constant for CTGF binding to PDGF-BB or VEGF165 was 43 nmol/L or 1.8 nmol/L respectively. CTGF: Connective tissue growth factor; VEGF: Vascular endothelial growth factor; PDGF-B: Platelet-derived growth factor-B; SPR: Surface plasma resonance.

of this receptor gene affects key fibroblast functions in wound healing[13,14]. PDGF-BB binding to the extracellular region of PDGFRβ induces dimerization and activation of intracellular kinase domain of this receptor. As a result, PDGFRβ receptors undergo auto-phosphorylation in critical tyrosine residues and gain full enzymatic activity to phosphorylate substrates. These phosphorylation regions can function as docking sites and recruit signal molecules containing SH2 and SH3 domains. For instances, Tyr751 in the kinase-insert region of PDGFRβ is the docking site for PI3 kinase, which in turn activates downstream molecules including the serine/threonine kinase AKT[15]. We used cultured rabbit corneal fibroblast cells and detected that PDGF-BB (1.6 nmol/L) quickly stimulated PDGFRβ phosphorylation at Tyr751 within minutes (Figure 6). This phosphorylated PDGFRβ gradually decreased probably due to a rapid turnover of this receptor in cultured rabbit corneal fibroblast cells. This pattern was correlated with phosphorylation of AKT and ERK1/2 in PDGF-BB stimulated cells. The phosphorylation of both PDGFRβ and AKT induced by 1.6 nmol/L PDGF-BB was enhanced by the addition of CTGF (6.6 nmol/L) to rabbit corneal fibroblast cells. In contrast, CTGF alone was unable to cause significant changes of PDGFRβ receptor and AKT levels in the cultured cells. Moreover, phosphorylation of ERK1/2 stimulated by PDGF-BB was not influenced by presence of CTGF. These results indicated that CTGF could potentiate some PDGF-

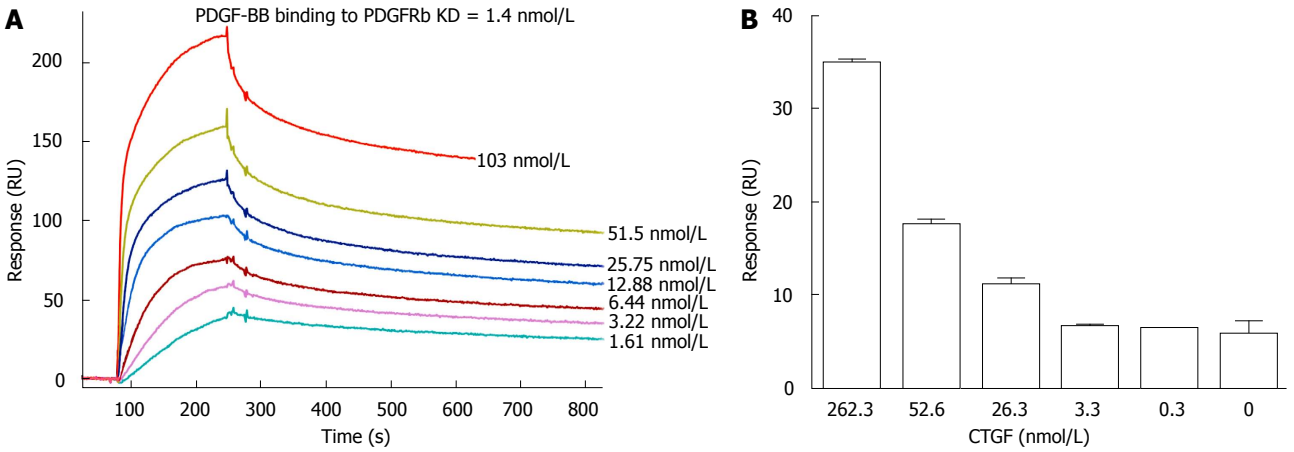

Figure 3 Connective tissue growth factor is able to enhance the binding of platelet-derived growth factor-BB to platelet-derived growth factor receptorβ. SPR assay was carried out to quantitatively measure the binding of PDGF-BB to PDGFRβ. A: Sensorgrams of SPR analyses showing the binding dissociation equilibrium constant of PDGF-BB and PDGFRβ; B: Measurement of SPR response unit showed that CTGF promoted PDGF-BB/PDGFRβ interaction. A mixture of PDGF-BB (2 nmol/L) with different concentrations of CTGF (0-263.2 nmol/L) was run over PDGFRβ sensor chips. CTGF: Connective tissue growth factor; PDGF-B: Platelet-derived growth factor-B; SPR: Surface plasma resonance; PDGFRβ: Platelet-derived growth factor receptor β.

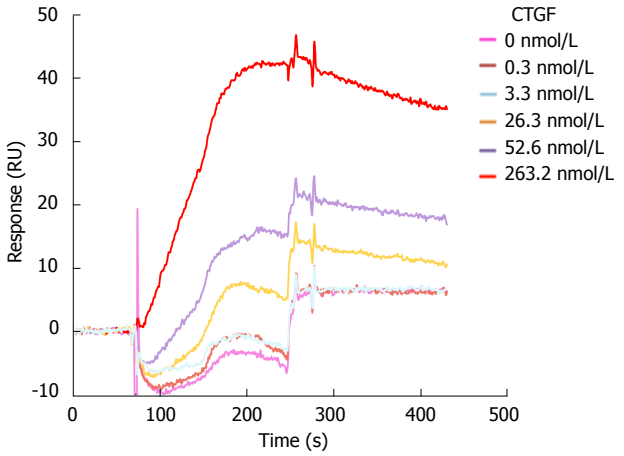

Figure 4 Connective tissue growth factor enhances platelet-derived growth factor-BB binding to platelet-derived growth factor receptor β in surface plasma resonance analysis. PDGF-BB (2 nmol/L) was mixed with different concentrations of CTGF (0-263.2 nmol/L) and run over PDGFRβ sensor chips. SPR response units were measured and used for quantitative comparisons of interaction between PDGF-BB and PDGFRβ in presence of different concentrations of CTGF proteins in Figure 3B. Representative sensorgrams from two independent trials are shown. CTGF: Connective tissue growth factor; PDGF-B: Platelet-derived growth factor-B; PDGFRβ: Platelet-derived growth factor receptor β.

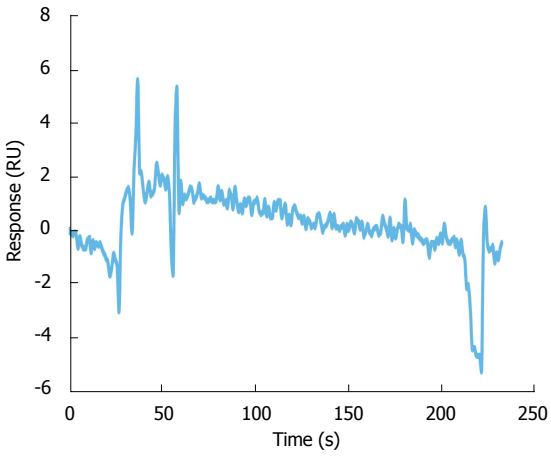

Figure 5 Connective tissue growth factor is not associated with platelet-derived growth factor receptorβ in surface plasma resonance analysis. CTGF protein (263.2 nmol/L) was run over PDGFRβ sensor chips and the SPR response unit was measured. Sensorgrams showed little binding response between CTGF and PDGFRβ proteins. CTGF: Connective tissue growth factor; PDGF-B: Platelet-derived growth factor-B; SPR: Surface plasma resonance; PDGFRβ: Platelet-derived growth factor receptor β.

BB mediated signaling including activation of PDGFRβ and AKT in rabbit corneal fibroblast cells.

DISCUSSION

The yeast two-hybrid system is designed to measure protein-protein interaction by reconstitution of a functional transcription factor that activates receptor genes. Previously, we used a GAL4 system and revealed CTGF's broad ability to bind with multiple cystine knot-bearing factors using *His3* reporter[9]. This is a qualitative assay with potential variation dependent upon the expression levels of the fusion proteins under investigation. In this study, we took advantage of LexA-responsive *LEU2* and *lacZ* reporter genes, and carried out quantitative assessments according to both colony survival and β-galactosidase activity to discriminate high- and low-affinity interactions. Our results suggested that CTGF strongly bound to VEGF-A and BMP-4 but loosely interacted with PDGF-B and TGF-β1. These observations were consistent with published results about measurement of dissociation constants for BMP-4, TGF-β1, PDGF-BB, and VEGF-A using SPR or I^{125} labeled solid phase protein binding assays[7,10]. This study further supported a previous report that the degree of protein interaction determined by yeast two-hybrid approaches correlates well with those examined in biochemical analysis[16].

The cystine-knot superfamily is composed of

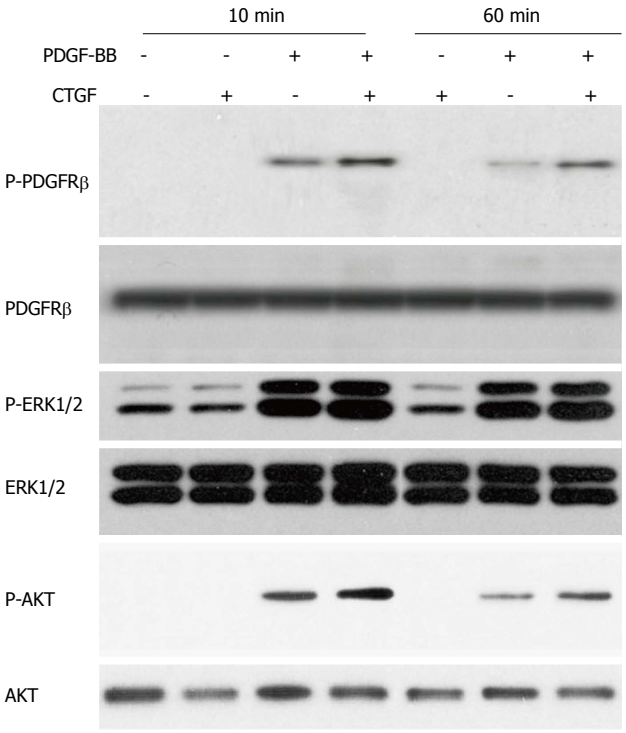

Figure 6 Connective tissue growth factor potentiates platelet-derived growth factor-BB stimulated activation of platelet-derived growth factor receptorβ and downstream signal molecule Akt. Western analysis was carried out to determine phosphorylated or total PDGFRβ, ERK1/2 and AKT in rabbit corneal fibroblast cells stimulated by CTGF (6.6 nmol/L), PDGF-B (1.6 nmol/L) or combination. CTGF: Connective tissue growth factor; PDGF-B: Platelet-derived growth factor-B; PDGFRβ: Platelet-derived growth factor receptor β.

several subgroups of proteins involved in a diverse variety of pathophysiological functions including cell growth, organogenesis, embryonic development, cell-to-cell communication, differentiation, tissue repair and cancer progression. In mammals, the PDGF subfamily has at least 10 members (*i.e.,* VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E from the VEGF family; PDGF-A, PDGF-B, PDGF-C, and PDGF-D from the PDGF family; and placenta growth factors PlGF-1 and PlGF-2)[14]. The TGF-β subfamily has about 37 members (BMP2-7, BMP8A, BMP8B, BMP10, BMP15, GDF1-3, GDF5-11, GDF15, Activin βE, Inhibin α, Inhibin βA, Inhibin βB, Inhibin βC, TGF-β1-3, Nodal, Anti-Mullerian hormone, LEFTY-A, LEFTY-B, GDNF, Neurturin, Persephin, and Artemin-2I)[17]. The Slit subfamily has three members (Slit1-3). The mucin-like subgroup include nine BMP antagonists (Follistatin, FSTL-3, Cerberus, Chordin, Gremlin, Noggin, SOST/Sclerostin, TSG, and USAG-1)[18], five mucin related protein (Norrin, mucin-2, MUC5AC protein, secretory mucin), and vWF[4]. So far, CTGF is found to interact with at least one member in each subgroup of the cystine knot superfamily. This broad binding spectrum of CTGF may be due to unique structure organization of members of the CCN family. Based on small angle X-ray scattering data in CCN3 and CCN5, CCNs seem to form long extended structures able to move and flex to allow multiple domains to bind a ligand[19]. It is conceivable that the four modular structures of CTGF protein form long extended and flexible scaffolds that "glue" the cystine knot proteins from destruction by cleavage in extracellular space.

Differential binding activity of CTGF to cystine knot-bearing ligands appears to give rise to disparate regulatory mechanisms for the ligand presentation to their receptors. One mechanism is through a high affinity/interaction with CTGF. Examples are VEGF-A, BMP-4, and BMP-2, which are strong interactors for CTGF. Our study, together with others[10] show that VEGF-A has a dissociation constant for CTGF in SPR assays around 1.8-4.8 nmol/L. BMP-4 and BMP-2 have dissociation constants of 5 and 0.77 nmol/L with CTGF respectively[7,10]. CTGF binding sequesters VEGF-A from its receptor and inhibits downstream signaling leading to anti-angiogenesis[6]. Similarly, CTGF binding inhibits BMP-4 from BMPRIa receptor and downstream signaling leading to altered development of *Xenopus* embryo[7]. BMP-2 activity on the proliferation of chondrocytes is also inhibited by CTGF[12]. Hence, we speculate that strong interaction with CTGF causes sequestration of the bound ligands from their receptors thereby preventing signal transduction.

In contrast, other cystine knot-bearing growth factors such as TGF-β1 and PDGF-B are weak interactors and their signal transduction pathways are not inhibited by CTGF[7]. The dissociation constant for CTGF binding to TGF-β1 is 30 or 64.7 nmol/L measured by two different groups[7,10]. We found that CTGF bound to PDGF-BB with a dissociation constant 43 nmol/L. Much lower binding kinetics between CTGF and PDGF-BB (K_D = 230 nmol/L) is observed by Khattab *et al*[10]. Variable factors including purity of tested recombinant proteins and variation of Biacore SPR systems may contribute to these discrepancies. Nevertheless, the measured dissociation constants for these weak interactors are often more than 10^{-10} mol/L. Despite of this low affinity, CTGF seems to increase the activity of TGF-β[7]. We found that CTGF was able to potentiate PDGF-BB binding to PDGFRβ and enhance activation of PDGFRβ and downstream AKT. Given that CTGF is a matricellular protein and can trigger adhesive signaling through association with cell surface proteins such as integrins[3], its binding to TGF-β1 or PDGF-B may be part of crosstalk for integration of these different signal pathways. Lack of overlapping in some downstream signaling between CTGF and PDGF-BB may explain little enhancement of ERK1/2 activation in cultured rabbit corneal fibroblast cells.

TGF-β1, PDGF-B and CTGF are known profibrotic partners for fibroblast activation[20]. Low affinity of CTGF may account for its synergistic effect on PDGF-BB and TGF-β in promoting chronic fibrosis. In particular, TGF-β1 can transcriptionally activate CTGF expression through SMAD responsive elements in the CTGF promoter[21]. In mice, co-injection of CTGF and TGF-β results in sustained, persistent fibrosis, whereas subcutaneous injection of just TGF-β causes a transient fibrotic response[22]. In addition, PDGF is a major regulator for vascular cells and its deficiency causes vascular abnormalities including

capillary dilation and microaneurysms during mouse embryonic development[23]. VEGF-A is critical for the proliferation and survival of vascular endothelial cells. Loss of VEGF-A expression results in severe abnormalities in the vasculature and causes embryonic death in heterozygous mice[24]. Given the importance of PDGF-B and VEGF-A in the assembly of blood vessels, the inherent stickiness of CTGF to PDGF-B and VEGF-A may represent a fine-tuned regulation of vascular smooth muscle cells, pericytes, and vascular endothelial cells during angiogenesis. Future studies about the nature of CTGF binding to VEGF-A, PDGF-B, and TGF-β may help the development of therapeutic strategies for pathological processes involving angiogenesis and fibrosis.

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COMMENTS

Background

Connective tissue growth factor (CTGF), also termed CCN2, is one prototype member in the Cyr62/CTGF/Nov family of matricellular proteins. It contains a four conserved modular structure and regulates cell proliferation, migration, differentiation, apoptosis, and survival through interaction with extracellular matrix protein, growth factors, and cell surface proteins. CTGF exhibits a broad binding ability to a structurally conserved motif characteristic of the cystine knot superfamily. However, molecular action of CTGF in modulation of the cystine knot-containing growth factors has not been fully understood.

Research frontiers

CTGF binding can sequester some of the cystine knot-containing growth factors such as vascular endothelial growth factor (VEGF)-A and bone morphogenetic protein (BMP)-4 from their cognate receptors x and inhibit their signaling. For other growth factors such as transforming growth factor (TGF)-β1, CTGF has distinct effects and potentiates TGF-β1 mediated downstream signal transduction pathway. Correlation between the binding strength of CTGF and its action on the majority of cystine knot-containing growth factors has not been investigated.

Innovations and breakthroughs

This paper combined LexA-based yeast two-hybrid system with surface plasma resonance (SPR) analysis and quantitatively assesses the binding strengths of CTGF to VEGF-A, platelet-derived growth factor (PDGF)-B, BMP-4, and TGF-β1. CTGF had high affinities with VEGF-A and BMP-4 but only weakly interacted with TGF-β1 and PDGF-B. These binding strengths were correlated with inhibition or potentiation by CTGF on signal transduction pathways mediated by these cystine knot-containing growth factors, implicating that stickiness to CTGF results in sequestration of the ligands from their cognate receptors while weak association facilitates ligand/receptor presentation. Our findings support that CTGF is a critical modulator of extracellular signaling and functions to integrate different pathways into signaling networks resulting in tight regulation and fine-tuning of cellular responses during diverse physiological and pathological processes.

Applications

Many cystine knot-containing growth factors are key regulators in angiogenesis and tissue repair. Understanding of the binding of CTGF to these growth factors may hold promise as a strategy of rational therapeutic design for the prevention or treatment of pathological processes in cancer, vascular and fibrotic disorders of many organs.

Terminology

Cystine knots refer to a common overall topology that was initially discovered in structure of nerve growth factor solved by Tome Blundell using X-ray crystallography in 1991. This tertiary structure favors dimer formation and is unique in extracellular signaling molecules of multicellular organisms since it is not found in the unicellular yeast genome. All cysteine knot growth factors are believed to be homo- or hetero- dimers and initiate signal transduction through dimerization of their cognate receptors.

Peer-review

CTGF is known as an important factor in regulating diverse biological functions, including cell adhesion, migration, tissue wound repair, fibrotic diseases, and cancers. In this paper, the author demonstrated that CTGF had different binding strengths to VEGF-A, PDGF-B, BMP-4, and TGF-β, which regulated these growth factors and triggered downstream signaling pathways. The topic of this work is of high interest. The authors approach to the study of the interactions between CTGF and a number of its interactors using the LexA-based yeast two-hybrid system and SPR analysis, and tried to explain the results using some functional aspects.

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ORIGINAL ARTICLE

Basic Study

DNA microarray unravels rapid changes in transcriptome of MK-801 treated rat brain

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Data sharing statement: The obtained gene expression data deposited at the NCBI, Gene Expression Omnibus (GEO) site (accession number GSE63639) is freely available to the scientific community (http://www.ncbi.nlm.nih.gov/geo/).

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Abstract

AIM: To investigate the impact of MK-801 on gene expression patterns genome wide in rat brain regions.

METHODS: Rats were treated with an intraperitoneal injection of MK-801 [0.08 (low-dose) and 0.16 (high-dose) mg/kg] or NaCl (vehicle control). In a first series of experiment, the frontoparietal electrocorticogram was recorded 15 min before and 60 min after injection. In a second series of experiments, the whole brain of each animal was rapidly removed at 40 min post-injection, and different regions were separated: amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain and ventral striatum on ice followed by DNA microarray (4 × 44 K whole rat genome chip) analysis.

RESULTS: Spectral analysis revealed that a single systemic injection of MK-801 significantly and selectively augmented the power of baseline gamma frequency (30-80 Hz) oscillations in the frontoparietal electroencephalogram. DNA microarray analysis showed the largest number (up- and down- regulations) of gene expressions in the cerebral cortex (378), midbrain (376), hippocampus (375), ventral striatum (353), amygdala (301), and hypothalamus (201) under low-dose (0.08 mg/kg) of MK-801. Under high-dose (0.16 mg/kg), ventral striatum (811) showed the largest number of gene expression changes. Gene expression changes were functionally categorized to reveal expression of genes and function varies with each brain region.

CONCLUSION: Acute MK-801 treatment increases synchrony of baseline gamma oscillations, and causes very early changes in gene expressions in six individual rat brain regions, a first report.

Key words: Dizocilpine; Dye-swap; Gene expression; Microarray; MK801; N-Methyl-D-aspartate receptors

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Core tip: N-Methyl-D-aspartate receptors (NMDAr) are involved in multiple physiological functions and neuropsychiatric disorders. Dizocilpine (commonly referred to as MK-801) is a well-known non-competitive NMDAr antagonist with psychotomimetic properties. A combination of electrophysiological and molecular analyses reveals not only the increased synchrony of baseline cortical gamma oscillations by MK-801, but also more importantly new insight into differential gene expressions in the cerebral cortex, midbrain, hippocampus, ventral striatum, amygdala, and hypothalamus regions after acute low-dose (0.08 mg/kg) MK-801 treatment; only the ventral striatum showed increased gene expression at a high dose (0.16 mg/kg) of MK-801. We believe that our present study will contribute in the understanding of the pathogenic mechanisms of neuropsychiatric disorders.

Kobayashi Y, Kulikova SP, Shibato J, Rakwal R, Satoh H, Pinault D, Masuo Y. DNA microarray unravels rapid changes in transcriptome of MK-801 treated rat brain. *World J Biol Chem* 2015; 6(4): 389-408 Available from: URL: http://www.wjgnet.com/1949-8454/full/v6/i4/389.htm DOI: http://dx.doi.org/10.4331/wjbc.v6.i4.389

INTRODUCTION

N-Methyl-D-aspartate receptor (NMDAr) is an ionotropic subtype of glutamate receptors, one of the excitatory amino acids, forming high calcium (Ca^{2+})-permeable cation channels that specifically accept NMDA as a selective agonist[1]. The NMDAr is a tetramer, composed of assemblies of NMDAR1 (NR1) and NMDAR2 (NR2) subunits[2]. NMDAr integrates chemical and electrical stimuli into a Ca^{2+} signal, and plays crucial roles in synaptic plasticity, which may be involved in learning, memory, and motion. Dysfunction of NMDAr has been suggested to be involved in stroke, Parkinson's disease, and schizophrenia[3-6]. Decreased Ca^{2+} influx in the response to glutamate activation leads to the impairment of NMDAr function, disrupting intra- and extracellular communication. Impaired NMDAr function in the prefrontal cortex causes injury to learning and memory formation, probably causing impulsivity, hyperactivity, and attention deficit, seen in attention-deficit hyperactivity disorder (ADHD)[7]. The state of NMDAr inhibition is also similar to the schizophrenia model[8,9].

It has been demonstrated that NMDAr play potential roles in nociceptive (the neural processes of encoding and processing noxious stimuli) transmission, particularly in the spinal cord[10]. NMDAr antagonists reduced the successive increase in response to repetitive stimuli, namely wind-up, in dorsal and ventral horn neurons[11]. Because of the psychotomimetic action of NMDAr antagonists, it was suggested that human psychosis and NMDAr blockade are correlated[12]. The drugs that non-competitively block NMDAr simulate schizophrenic psychopathology in healthy humans[13]. Of importance, using fMRI it has recently been demonstrated that acute ketamine administration in healthy subjects increases global brain functional connectivity[14], creating a state resembling that recorded in patients during the early stages of schizophrenia but not that recorded in patients with chronic (since several years) schizophrenia[15]. The acute ketamine effects are quick and reversible. These findings are consistent with preclinical studies demonstrating that in rodent's ketamine or MK-801 transiently increases the power of baseline gamma oscillations in cortical and subcortical structures[16,17]. Furthermore, there is proof that NMDAr antagonists induce a broad range of symptoms, behaviors, and cognitive deficits that resemble aspects of endogenous psychoses, particularly schizophrenia and dissociative states[9].

Phencyclidine (PCP), a well-known NMDAr antagonist, is one of the most effective psychotropic drugs. The PCP-elicited psychosis symptoms closely resemble schizophrenia such as positive/negative symptoms and cognitive deficits[8,18,19]. It should be noted that the glutamate hypothesis is one of few hypotheses of etiology and/or pathophysiology of

schizophrenia. Javitt hypothesized that hypofunction of NMDAr is involved in the pathogenic mechanism of schizophrenia[4]. As part of that evidence, it was reported that there is a (1) decrease in the phosphorylation level of NR1 subunit in post-mortem brains of schizophrenia patients[20]; and (2) occurrence of abnormal behavior similar to schizophrenia symptom in the NR2A subunit (GRIN2A) lacking mice[21,22]. Other than PCP, dizocilpine (commonly referred to as MK-801) is another well-known non-competitive NMDAr antagonist. These molecules have psychotomimetic properties. Intraperitoneal (ip) injection of MK-801 induces hyperlocomotion, ataxia, abducted hindlimbs, flat body posture, and stereotyped behavior such as head weaving in rat[23]. It should be emphasized that these conditions are also included in the ADHD symptoms. It has been proposed that ADHD-like symptoms can be produced by stopping development of the dopaminergic neuron[24]. Moreover, it has been demonstrated that NMDAr expression was enhanced in the striatum in ADHD model animals, where activities of the dopaminergic neurons were inhibited[25].

MK-801 has been used in studies of NMDAr in schizophrenia and psychosis[26-28]. Systemic administration of MK-801 causes dysfunction of learning and memory[29]. Acute ip injection of MK-801-induced transient behavior, and which correlated with schizophreniform psychosis in the rat. MK-801-treated animals progressed to an acute episode involving abnormal behavior reminiscent of symptoms of schizophrenia[16,17,30]. Furthermore, significant reproducible behavior was found, such as increased locomotion, stereotyped sniffing and ataxia[31,32]. These behavioral changes are dose-dependent, varied with age and sex, and represents a rat EAA hypofunction model of psychosis[33].

The overall goal of the present study was to identify gene expression patterns along rat chromosomes in different brain regions after a single injection of MK-801, which exerts a longer acute effect than ketamine on ongoing brain activities[16]. The brain regions (amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain and ventral striatum) of MK-801-treated rats were subjected to a genome-wide transcriptome mapping analysis (4 × 44 K). The present study should contribute in the understanding of the pathogenic mechanisms of neuropsychiatric disorders. To note, a previous study in 2004, was the first study on uncovering gene expressions using rat model to investigate the effects of both memantine and MK-801 in adult rat brain[34]. In that study, a cDNA-based microarray chip carrying 1090 well-characterized transcripts was used to profile gene expression changes in the posterior cingulate and anterior retrosplenial cortices of the rat brain following 5 to 50 mg/kg memantine and 1 mg/kg MK-801 brain[34], but which is different from our study in that six brain regions were sampled following low-dose (0.08 mg/kg) and high-dose (0.16 mg/kg) single injection of MK-801.

MATERIALS AND METHODS

Ethics

In this study, all animal care procedures were achieved in accordance with European Union Guidelines (Directive 2010/63/EU) and with CREMEAS, the National and Regional Ethics Committee. The second part of the study (for genome-wide analysis experiment) was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at the National Institute of Advanced Industrial Science and Technology (AIST), Japan.

Animals, MK-801 injection, and electrophysiology

Four adult (3-6 mo old) male Wistar rats (280-380 g body weight, BW) were used for the electrophysiology experiment performed in the French laboratory. All animal care procedures were achieved in accordance with European Union Guidelines (Directive 2010/63/EU) and with CREMEAS, the National and Regional Ethics Committee. Rats were implanted under deep anesthesia in a stereotaxic frame. For bilateral recordings two stainless steel screws were implanted extradurally over the left and right frontoparietal cortices (from bregma: 1 mm posterior and 2 mm lateral). Two other screws were fixed: in the frontal bone for ground connection and in the bone covering the cerebellum for reference. The screws were connected to a subminiature connector fixed to the skull with dental acrylic. Following the surgery, the rats were housed in separate cages with food and water *ad libitum*. Recording sessions began after 1 wk of recovery. Every rat had a 15 min habituation period before the recording session. It was gently stimulated to ensure it did not fall asleep. During this habituation period, each rat was injected with vehicle (NaCl, 0.9%, 1 mL/kg) and MK-801 (0.08 mg/kg, intraperitoneal, ip; low-dose) and the recordings were carried out about 60 min after injection[16]. The electrocorticogram (ECoG) signals were processed with a bandpass of 0.1-800 Hz and digitized at 10 kHz. Spectral analysis of ongoing spontaneously occurring activity was done with Fast Fourier Transformation (FFT). The total power was the sum of all FFT values computed between 30 and 80 Hz (resolution 2.4 Hz).

Animals, treatment with MK-801, dissection of brain, and preparation of fine brain tissue sample powders

This part of the experiment was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at the National Institute of AIST, Japan. Nine male 10-wk-old Wistar rats (300-350 g BW) were housed in acrylic cages (3 per cage) at 24 ℃ and given access to tap water and laboratory chow *ad libitum*. The rats were divided into two groups, and each group rats received ip injection of 0.08 (low-dose) and 0.16 (high-dose) mg/kg of MK-801, respectively. Three rats were treated with saline as sham (vehicle control group) using the same method (see the experimental strategy in Figure 1). After 40 min post-injection, the whole brain of each animal was rapidly removed and put on ice, and brain regions were separated according to the method of Glowinski and Iversen (1996)[35], with minor modifications[36,37]. Each brain region was placed in a sterile 2 mL Eppendorf microtube, quickly immersed in liquid nitrogen before being stored in -80 ℃ prior to further analysis. The

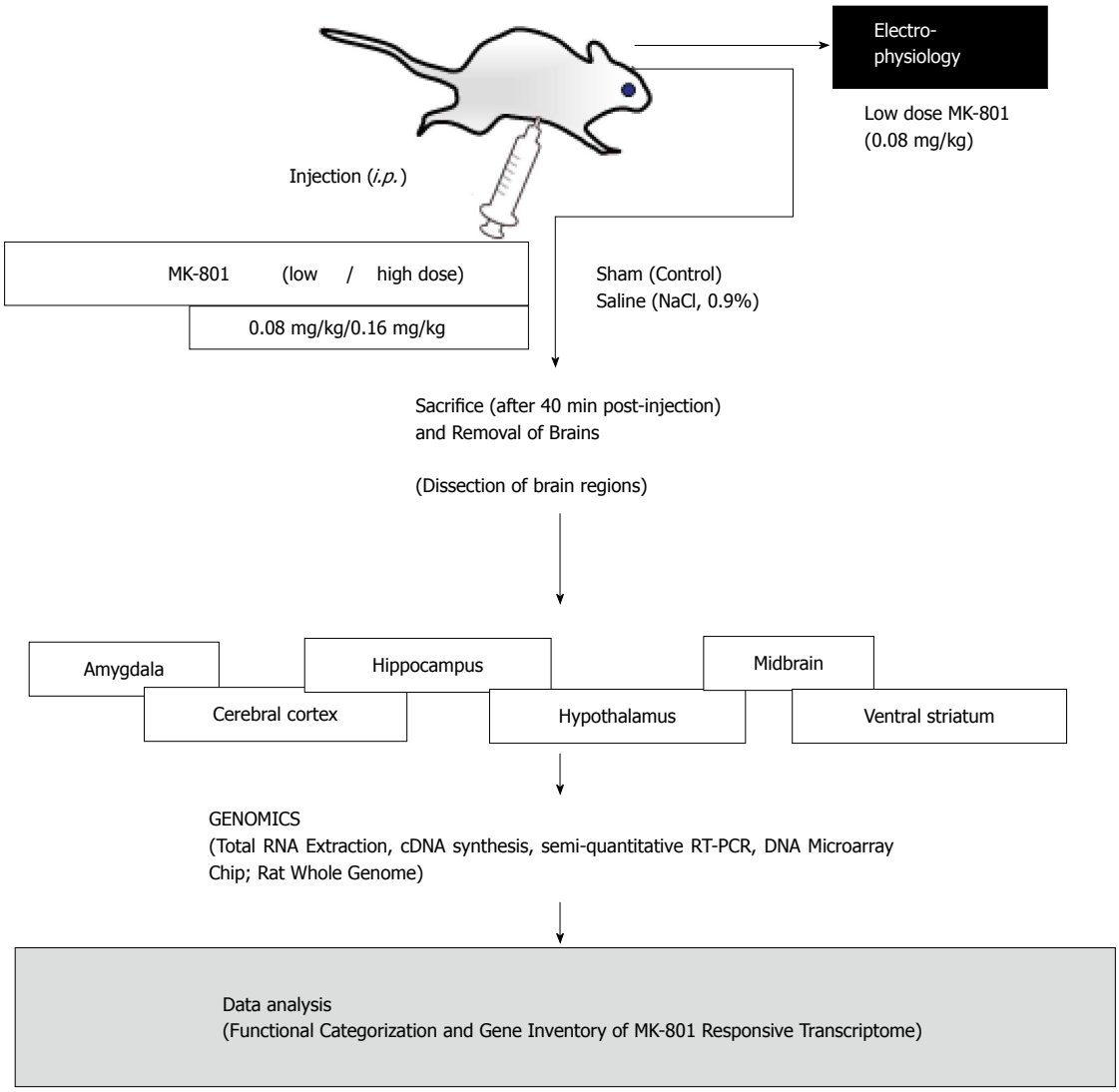

Figure 1 Experimental strategy and design to investigate the MK-801 effects on rat brain. Details are mentioned in the Materials and Methods section.

deep frozen brain regions (amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain and ventral striatum) were transferred to a pre-chilled (in liquid nitrogen) mortar and pestle and ground to a very fine powder (for details see protocol by Masuo *et al*[38,39]). The powdered samples (aliquots of 70 mg) were transferred to sterile 2 mL Eppendorf microtubes (pre-chilled in liquid nitrogen) and stored at -80 ℃ till used for extraction of total RNA.

Total RNA extraction, cDNA synthesis, and reverse transcription-polymerase chain reaction

Total RNA was extracted from about 70 mg sample powder using the QIAGEN RNeasy Mini Kit (QIAGEN, Maryland, United States). To verify the quality of this RNA, the yield and purity were determined spectrophotometrically (NanoDrop, Wilmington, DE, United States) and visually confirmed using formaldehyde-agarose gel electrophoresis. To validate the total RNA quality and subsequently synthesized cDNA, reverse transcription-polymerase chain reaction (RT-PCR) was carried out using two commonly used house-keeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin as positive controls[40,41]. The 3'-UTR gene-specific primers were designed in-house and are listed in Table 1.

Briefly, total RNA samples were first DNase-treated with RNase-free DNase (Stratagene, La Jolla, CA, United States). First-strand cDNA was then synthesized in a 20 μL reaction mixture with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, Agilent Technologies, La Jolla, United States) according to the protocol provided by the manufacturer, using 1 μg total RNA isolated from each control and treated samples. The reaction conditions were: 25 ℃ for 5 min, 42 ℃ for 5 min, 55 ℃ for 40 min and 95 ℃ for 5 min. The synthesized cDNA was mixed up to a volume of 50 μL with sterile water supplied in the kit. The reaction mixture contained 0.6 μL of the first-strand cDNA, 7 pmols of each primer set and 6.0 μL of the Emerald Amp PCR Master Mix (2 × premix) (TaKaRa Shuzo, Shiga, Japan) in a total volume of 12 μL with sterile water supplied in the kit. Thermal-cycling (S1000 Thermal Cycler, Bio-Rad, Tokyo, Japan) parameters

Table 1 Primer design for reverse transcription-polymerase chain reaction validation experiment

Accession (Gene)	Gene symbol	Description	Nucleotide sequence (5′-3′): Left	Nucleotide sequence (5′-3′): Right	Product size (bp)
X02231 X00972	*Gapdh*	Glyceraldehyde-3-phosphate dehydrogenase	TCCCTCAAGATTGTCAGCAA	AGATCCACAACGGATACATT	308
NM031144	*β-actin*	β-actin	CCTGTATGCCTCTGGTCGTA	CCATCTCTTGCTCGAAGTCT	260
NM_031327	*Cyr61*	Cysteine rich protein 61	GTCCTTGTGGACAACCAGTGTA	CCTTTAGTCCCTGAACTTGTGG	341
NM_001010970	*Amy1a*	Amylase, alpha 1A (salivary)	CTTCTGACAGAGCCCTTGTCTT	AATGGTCACTTCTTTGGTTGCT	254

Gene-specific (rat) primers are designed in-house and original to our group.

A

Sample	ng/μL	A260/280	A260/230
MK-801 1	666.1	2.15	2.25
MK-801 2	598.9	2.15	2.22
MK-801 3	688.9	2.15	2.18
MK-801 4	528.9	2.16	2.25
MK-801 5	583.7	2.15	2.23
MK-801 6	528.1	2.16	2.19

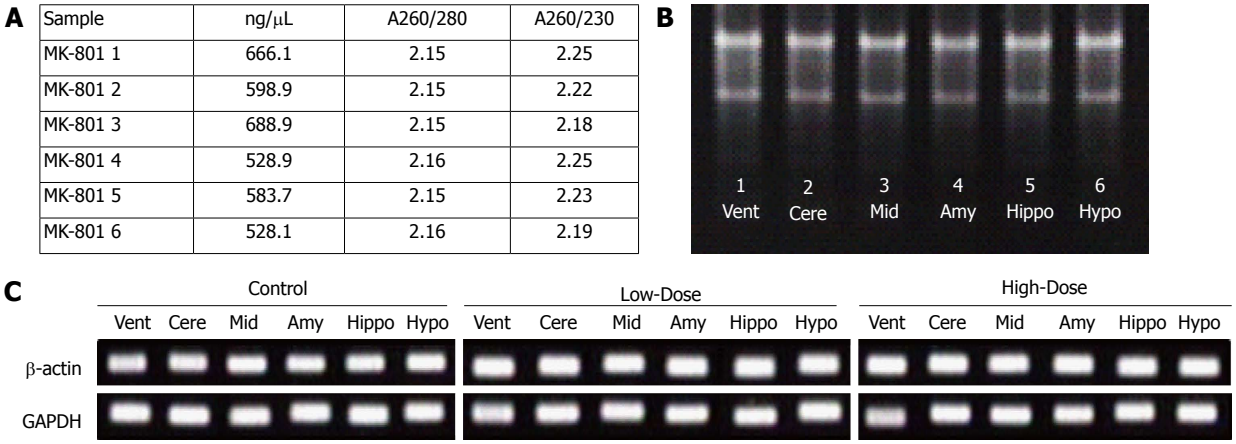

Figure 2 Total RNA quality and quantity check, and reverse transcription-polymerase chain reaction. Total RNA quality was confirmed spectrophotometrically (A) and by formaldehyde agarose-gel electrophoresis (B). Stable expression levels of GAPDH and β-actin genes are shown (C). For further details, see the Materials and Methods section. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

were as follows: after an initial denaturation at 97 ℃ for 5 m, samples were subjected to a cycling regime of 20 to 40 cycles at 95 ℃ for 45 s, 55 ℃ for 45 s, and 72 ℃ for 1 min. At the end of the final cycle, an additional extension step was carried out for 10 min at 72 ℃. After completion of the PCR the total reaction mixture was spin down and mixed (3 μL) was loaded into wells of a 1.8% agarose [Agarose (fine powder) Cat no. 02468-95, Nacalai Tesque, Kyoto, Japan] gel. Electrophoresis was then performed for about 22 min at 100 Volts in 1 × TAE buffer using a Mupid-ex electrophoresis system (ADVANCE, Tokyo, Japan). The gels were stained (8 μL of 10 mg/mL ethidium bromide (EtBr) in 200 mL 1 × TAE buffer) for about 7 min and the stained bands were visualized and quantified using an UV-transilluminator (ATTO, Tokyo, Japan). Each gene expression analysis was performed at least twice as independent PCR reactions and electrophoresis on gel, and one of the images was presented as a representative data for each gene in the respective figures (Figures 2 and 3) for no change or up- and down-regulated expressions.

Rat whole genome DNA microarray analysis

A rat 4 × 44K whole genome oligo DNA microarray chip (G4131F, Agilent Technologies, Palo Alto, CA, United States) was used for global gene expression analysis. Total RNA (800 ng) was labeled with either Cy3 or Cy5 dye using an Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent). Fluorescently labeled targets of control as well as treated samples were hybridized to the same microarray slide with 60-mer probes. A flip labeling (dye-swap or reverse labeling with Cy3 and Cy5 dyes) procedure was followed to nullify the dye bias associated with unequal incorporation of the two Cy dyes into cRNA[41-45]. Briefly, the same total RNA (800 ng) samples were labeled twice with Cy3 or Cy5: A Cy5-labeled treatment (T^{Cy5}) and a Cy3-labeled control (C^{Cy3}) were hybridized on a slide and then a Cy3-labeled treatment (T^{Cy3}) and a Cy5-labeled control (C^{Cy5}) were reversely hybridized on another slide. Hybridization and wash processes were performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray scanner G2565BA. For the detection of significantly differentially expressed genes between control and treated samples each slide image was processed by Agilent Feature Extraction software (version 9.5.3.1). This program measures Cy3 and Cy5 signal intensities of whole probes. Dye-bias tends to be signal intensity dependent therefore, the software selected probes using a set by rank consistency filter for dye-normalization. Said normalization was performed by LOWESS (locally weighted linear regression) which calculates the log ratio of dye-normalized Cy3- and Cy5-signals, as well as the final error of log ratio. The significance (*P*) value based on the propagate error and universal error models (Agilent). In this analysis, the

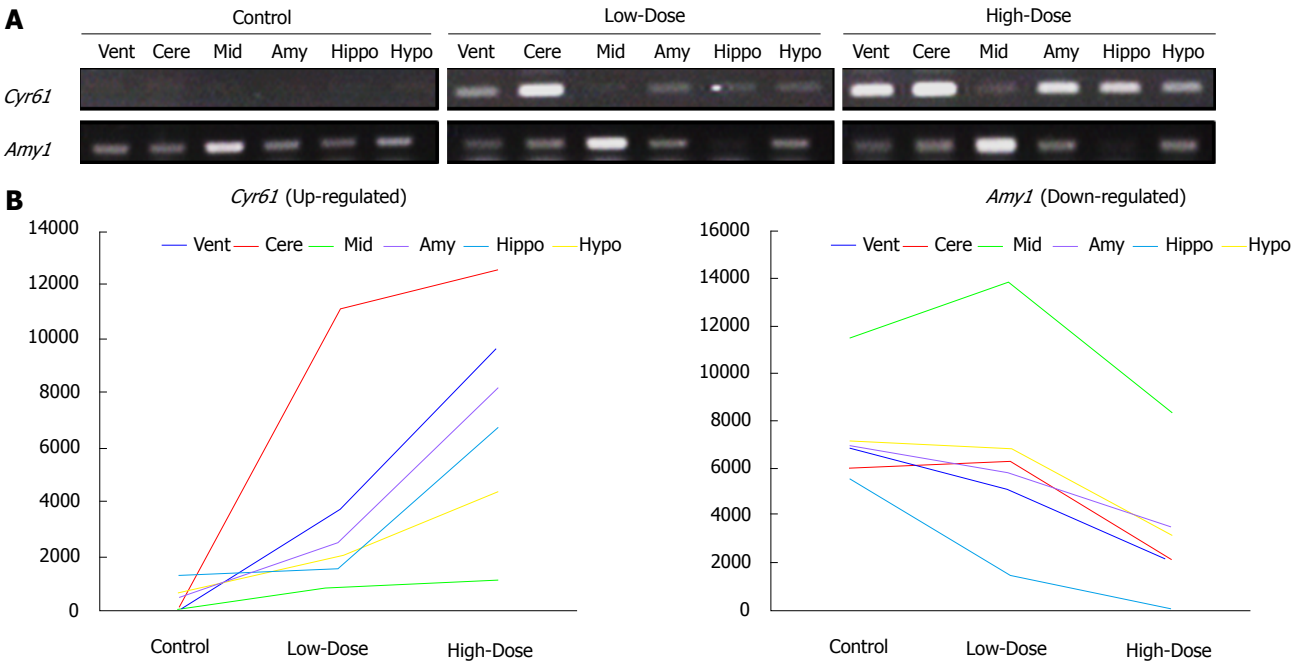

Figure 3 Reverse transcription-polymerase chain reaction analyses of Cyr61 and Amy1 genes that are differentially expressed in the six (6) brain regions by MK-801 treatment. For the stable expression levels of GAPDH and β-actin genes, please see Figure 2C. For further details, see the Methods and Materials section. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

threshold of significant differentially expressed genes was < 0.01 (for the confidence that the feature was not differentially expressed). In addition, erroneous data generated due to artifacts were eliminated before data analysis using the software. The differentially expressed gene lists (up- and down-regulated genes) were generated and annotated using the GeneSpring version GX 10 (Agilent). The outputs of microarray analysis used in this study are available under the series number GSE63639, at the NCBI Gene Expression Omnibus (GEO) public functional genomics data repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63639).

To validate the microarray data, RT-PCR analysis was performed on two selected up- and down-regulated genes using 3'-UTR specific gene primers (Table 1).

RESULTS

MK-801 increases the power of spontaneously-occurring cortical gamma oscillations

To examine the neurophysiological impact of MK-801, we conducted bilateral high-resolution ECoG recordings in free-moving awake rats during the resting state. Spectral analysis revealed that a single subcutaneous administration of MK-801 at a low (0.08 mg/kg) or higher (0.16 mg/kg) dose significantly and dose-dependently augmented the power of baseline gamma oscillations (Figure 4). The MK-801 effects lasted more than one hour and were transient[16]. It is worth mentioning that a single injection of a psychotomimetic dose of a non-competitive NMDAr antagonist (MK-801 or ketamine) transiently induces persistent aberrant gamma oscillations in multiple cortical and subcortical structures, including the prefrontal cortex, accumbens, amygdala, basalis, hippocampus, striatum and thalamus[17].

Quality of total RNA and expression level of GAPDH and β-actin genes in the brain regions

To investigate global changes in gene expression in the brain regions, the quantity and quality of the total RNA is a critical factor in further downstream analyses, and was confirmed as described in Materials and Methods section. The quantity and quality of total RNA was shown in Figure 2. This RNA was then used for synthesizing cDNA. Prior to DNA microarray analysis, we examined the expression of two commonly used house-keeping genes, namely GAPDH and β-actin in all the six brain regions. We used these two genes as positive controls rather than simply loading or using internal controls[40]. This simple test of gene expression showed that the mRNAs for GAPDH and β-actin were expressed almost uniformly across regions and conditions (Figure 2C). Following this preliminary but extremely important analysis of sample quantity and quality, we proceeded to conduct a DNA microarray analysis.

Genome-wide transcriptome analysis reveals numerous and early changes in gene expression

The differentially expressed genes in each brain region, amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain, and ventral striatum were analyzed based on their up- or down-regulations. Genes were up-regulated if they displayed a fold-change ratio greater than or equal to 1.5, whereas genes were down-regulated if they showed a fold-change ratio less than or equal to 0.75 in both the chips carrying different Cy3 and Cy5

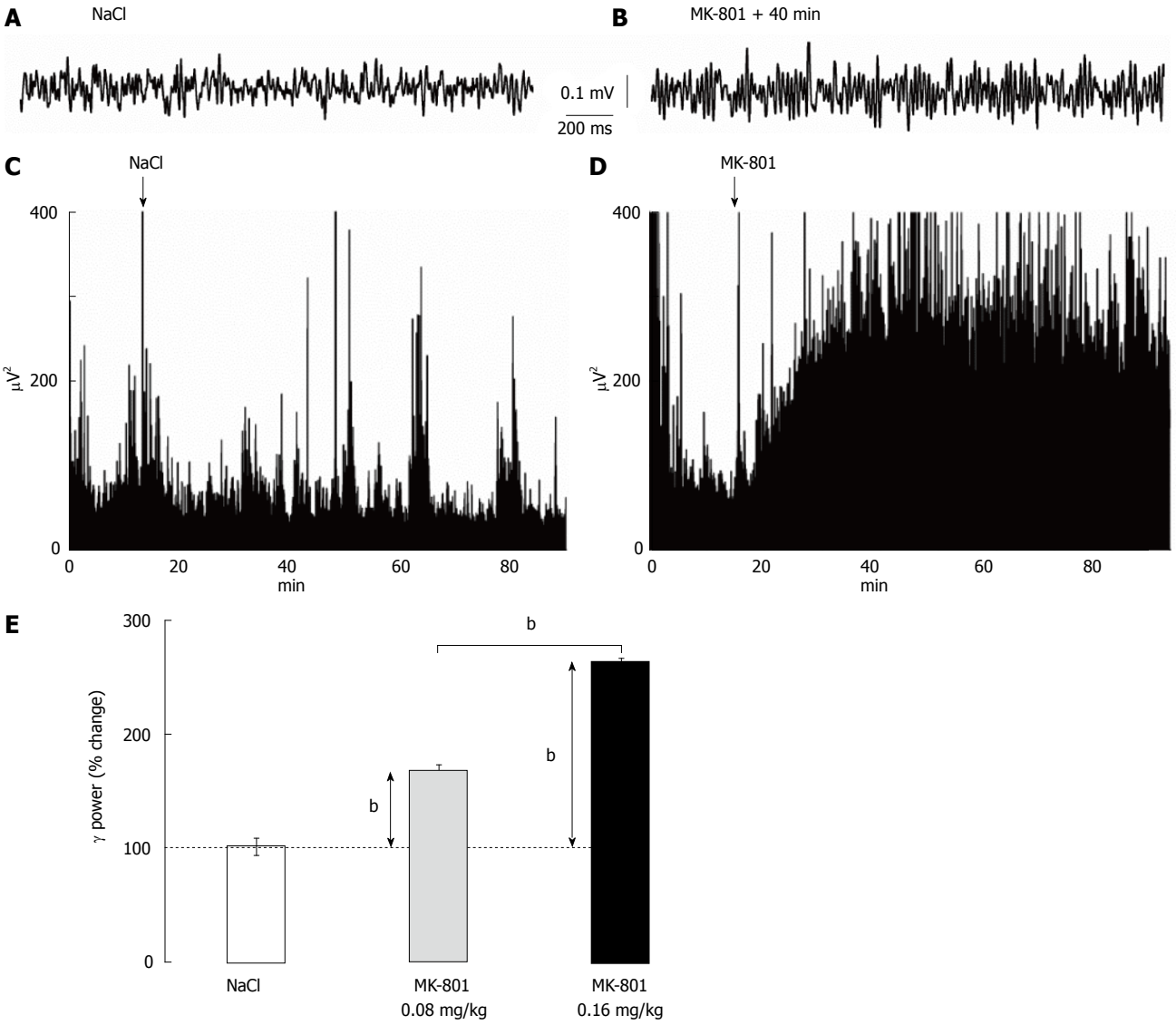

Figure 4 MK-801 increases the power of spontaneously-occurring gamma (30-80 Hz) oscillations. A-B: Typical 2-s bouts of frontoparietal electrocorticogram (bandpass: 20-80 Hz) recorded in a free-moving awake rat under control (vehicle: NaCl, 0.9%, 1 mL/kg, A) and MK-801 (about 40 min after injection, 0.08 mg/kg, B) conditions; C-D: Each chart shows the total power (resolution: 2.4 Hz; average of 8 x 2.5-s epochs) of oscillations during a 90-min recording session, during which the rat received an injection [NaCl (C) or MK-801 (D)] at 15 min (arrow). Note that MK-801 induces a dramatic increase in power, which is still present at the end of the recording session; E: The histogram shows that MK-801 significantly (*t*-test, $^{b}P < 0.001$) and in a dose-dependent manner increases the power during the 30 to 40 min post-injection period. All values are means ± SEM (5 rats > 100 values/rat, spectral analysis 2.5 s epochs).

labels; *i.e.*, dye-swap experiment[41-45]. Each brain region showed different patterns and numbers of changed gene expressions, reflecting the different responses of the brain regions to MK-801 treatment. Moreover, differential gene expression was also observed with the dose of applied MK-801, suggesting a clear dose-dependent effect of the antagonist. In Figure 5A and B, the numbers of changed genes in each region under low- and high-doses of MK-801, respectively, are shown. Results show that under low-dose of MK-801, the largest number (up- and down-regulations) of gene expressions was affected in the cerebral cortex (378) followed by midbrain (376), hippocampus (375), ventral striatum (353), amygdala (301) and hypothalamus (201) (Figure 5A). Results showed that under high-dose of MK-801, the largest number (up- and down-regulations) of gene expressions were affected in the ventral striatum (811) followed by midbrain (689), hippocampus (443), cerebral cortex (341), hypothalamus (325) and amygdala (269) (Figure 5B). The common genes between regions were numbered and indicated over the lined arrows (Figure 5A and B).

As the only other study available to date was the 2004 report by Marvanová *et al*[34] who showed gene expression changes post-MK-801 injection in adult rat brain, we compared those genes (34) with our gene inventory for all the six brain regions. Two genes were found to be in common, namely *C-fos* or *Fos* (NM_022197, FBJ osteosarcoma oncogene; an immediate early gene encoding a nuclear protein involved in signal transduction: http://www.ncbi.nlm.nih.gov/nuccore/NM_022197), and *RL/IF-1* or *Nfkbia* (NM_001105720, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor,

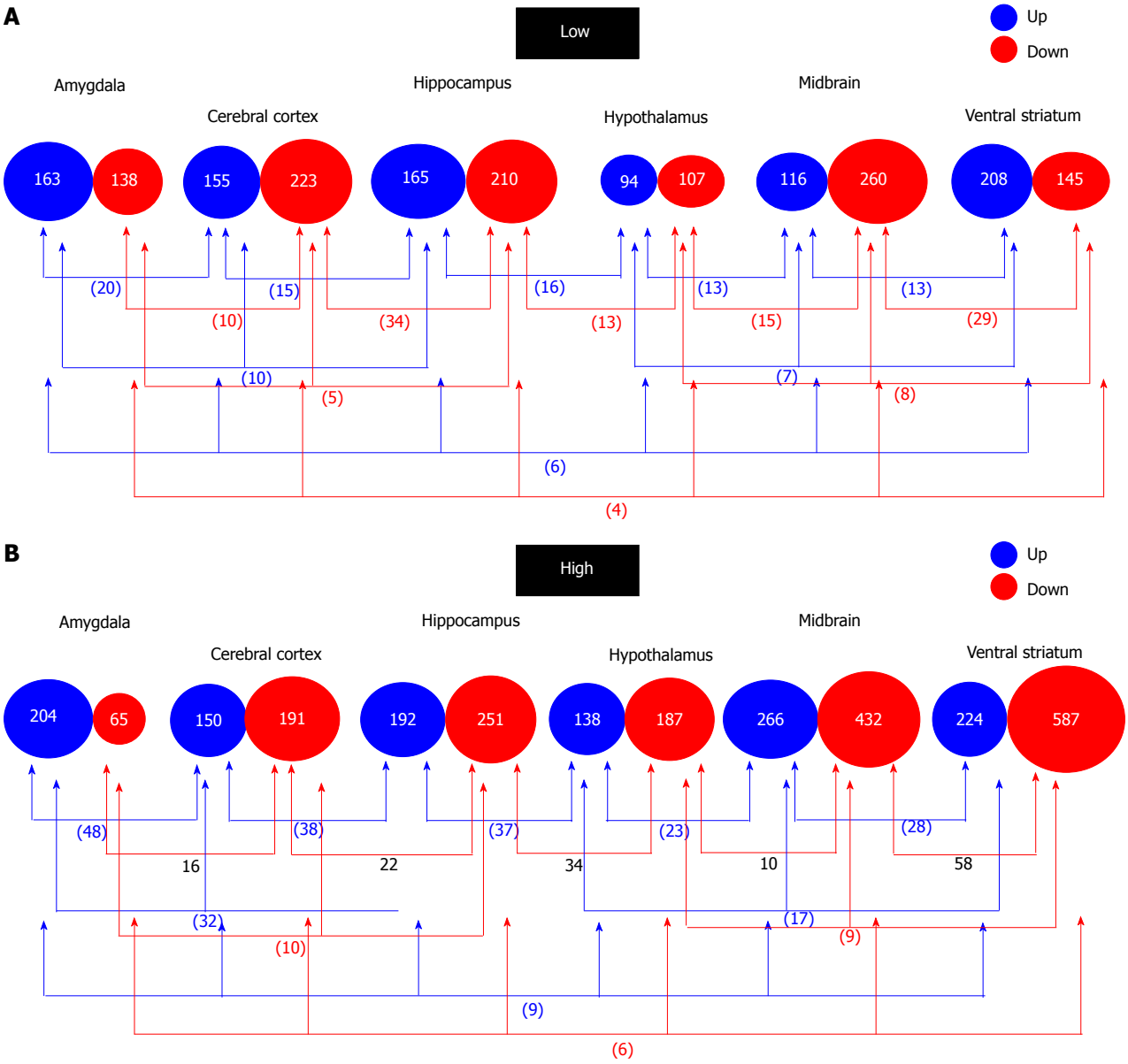

Figure 5 Number of changed genes in each brain region and the number of common genes among each region. A: Low-dose of MK-801; B: High-dose of MK-801.

alpha; inhibitor of NF-kappa-B/binds NF kappa B and retains it in the cytoplasm: http://www.ncbi.nlm.nih.gov/nuccore/NM_001105720). The *Fos* gene was found to be up-regulated in the cerebral cortex (1.7 fold), amygdala (1.87 fold), and hippocampus (1.55 fold) whereas *Nfkbia* gene was up-regulated in midbrain (1.79 fold), and only under high dose (0.16 mg/kg) MK-801. Although the *Nfkbia* gene expression may be a generalized response to the MK-801 injection as also suggested by Marvanová *et al*[34], the up-expression of the *Fos* gene may have some meaning in the brain, as it is also a marker of neuronal activation. A recently published study showed significant increase in the c-Fos protein in amygdala, hippocampus and thalamus, after local injection of MK-801 leading those authors to suggest that local blockade of NMDA receptors in the medial prefrontal cortex could lead to the activation of diverse downstream structure[46]. These low commonalities of gene expressions between the two studies are ascribed to: (1) use of posterior cingulate and anterior retrosplenial cortices of the rat brain over six distinct regions used in our study; (2) 1090 cDNA probes *vs* 41090 gene probes used here; and (3) 1 mg/kg MK-801 dose administration compared to low-dose (0.08 mg/kg) and high-dose (0.16 mg/kg) MK-801 used in our study. Nevertheless, the 2004 study contributed to the identification of 34 (and 28 following memantine injection) well characterized transcripts to delineate molecular pharmacologic effects of NMDA/glutamate receptor antagonists in the rat brain[34].

Confirmatory RT-PCR on two candidate genes

To confirm alterations in gene expression observed by DNA microarray, we selected two genes expressed differentially among brain regions and doses, namely

the up-regulated gene (*Cyr61*) and the down-regulated gene (*Amy1*) (Table 1 lists the gene-specific primers used for these two genes). The results of the RT-PCR analysis of these two genes, *Cyr61* and *Amy1* are presented in Figure 3 as both EtBr-stained PCR gene products and as a graph for clarity. Results from RT-PCR revealed that the intensity of bands were higher than control in all regions under both low- and high- dose, especially under high-dose for the *Cyr61* gene. For *Amy1*, the intensity of bands was lower than control in all regions under high-dose. These results imply that the microarray data can be re-confirmed using appropriate primer design followed by RT-PCR. The possible function of these two genes in context of MK-801 action is discussed below under up- and down-regulated genes.

Functional categorization of differential gene expressions

As a next step, all the genes in each region (low- and high- doses) were functionally characterized based on the Gene Ontology (GO), and are presented in Figures 6 to 11. These genes were divided into 38 functional categories in total. We selected some remarkably differentially expressed genes showing up-/down-regulations in all regions examined in this study, along with some genes with very high-/low-fold values and those with having characteristic functional annotations related to this study. The functional categories were annotated by NCBI database or Rat Genome Database (RGD, http://rgd.mcw.edu/). These genes are discussed below in up-regulated and down-regulated categories, respectively.

Up-regulated genes

Cyr61: Cysteine-rich, angiogenic inducer 61; an extracellular heparin binding protein involved in supporting smooth muscle cell (SMC) adhesion, promoting cell migration and enhancing growth factor-stimulated mitogenesis; NM_031327. The expression of this gene was found to be up-regulated in all regions [amygdala (30.92 fold), cerebral cortex (22.85 fold), ventral striatum (20.89 fold), hippocampus (13.41 fold), hypothalamus (4.61 fold) and midbrain (2.04 fold)] with high-dose of MK-801. Under low-dose, the expression was up-regulated in five regions [cerebral cortex (16.84 fold), ventral striatum (7.98 fold), amygdala (4.78 fold), hippocampus (2.88 fold) and hypothalamus (1.85 fold)]. *Cyr61* is prominent in extracellular matrix binding (molecular function, MF), cell adhesion (biological process, BP) and extracellular region (cellular component, CC) categories. This gene induced angiogenesis and vascular SMC chemotaxis; increased protein levels were detected after balloon angioplasty[47]. Ito *et al*[48] confirm that subcutaneously (s.c.) injection of PCP induced remarkable up-regulation of this gene expression after 60 min in the neocortex of the rats at post-natal day 56. MK-801 also caused a prominent up-regulation of neocortical expression of this gene in adult rats. It has been suggested that this gene or protein could be implicated in a molecular cascade associated with the age-dependent onset of schizophrenia. Our data indicate that up-regulation of *Cyr61* expression by the injection of NMDA receptor antagonist is induced rapidly in the adult rat brain. This gene may act as a chemotactic factor that influences neurite outgrowth in response to a stimulating acetylcholine signal[49]. Thus, the up-regulated *Cyr61* expression might be related to neurodegenerative diseases.

***Verge*:** The synonym of Apold1, apolipoprotein L domain containing 1; may regulate endothelial cell differentiation, activation and signaling; NM_001003403. The expression of this gene was found to be up-regulated in all regions [cerebral cortex (10.47 fold), hippocampus (6.63 fold), amygdala (6.31 fold), ventral striatum (5.11 fold), hypothalamus (2.90 fold) and midbrain (2.35 fold)] with high-dose of MK-801. Under low-dose, the expression was up-regulated in four regions [cerebral cortex (6.59 fold), ventral striatum (2.45 fold), amygdala (2.34 fold) and hippocampus (2.11 fold)]. This gene mainly belongs to lipid binding (MF), angiogenesis (BP) and extracellular region (CC) categories. The *Verge* mRNA and protein are induced selectively in the endothelium of adult vasculature by chemical seizures. Further, this gene may function as a dynamic regulator of endothelial cell signaling and vascular function[50]. Thus, MK-801 treatment may have an effect on the signaling related to angiogenesis.

***Ccl2*:** C-C motif (chemokine) ligand 2; a chemokine involved in leukocyte taxis and inflammation; associated with many diseases; NM_031530. The expression of this gene was found to be up-regulated in the cerebral cortex (3.80/7.06 fold), with both low- and high-dose of MK-801. Under high-dose, the expression was up-regulated in four regions [amygdala (4.28 fold), ventral striatum (4.10 fold), hippocampus (3.94 fold) and midbrain (2.02 fold)]. *Ccl2* mainly functions in CCR2 chemokine receptor binding, G-protein-coupled receptor binding, chemokine activity, immune response, inflammatory response (BP), cell soma, cytoplasm, extracellular region and extracellular space (CC) and brain injuries, brain ischemia, and bipolar disorder (pathway) categories. Drexhage *et al*[51] reported that increased serum *Ccl2* levels in the schizophrenia patients are significantly higher than in healthy adult human. Independent of the use of antipsychotic medication, both regarding the class of drug and the individual drug *Ccl2* is associated with divergent diseases, including schizophrenia. In the brain, the change of *Ccl2* expression in the cerebral cortex during early stage of schizophrenia was seen. Application of *Ccl2* on dopaminergic neurons increases their excitability, dopamine release and related locomotor activity[52]. Therefore, characteristic function of this gene may be related to increased locomotor activity which is included in the positive symptoms in schizophrenia.

***Klf4*:** Kruppel-like factor 4 (gut); a transcription factor

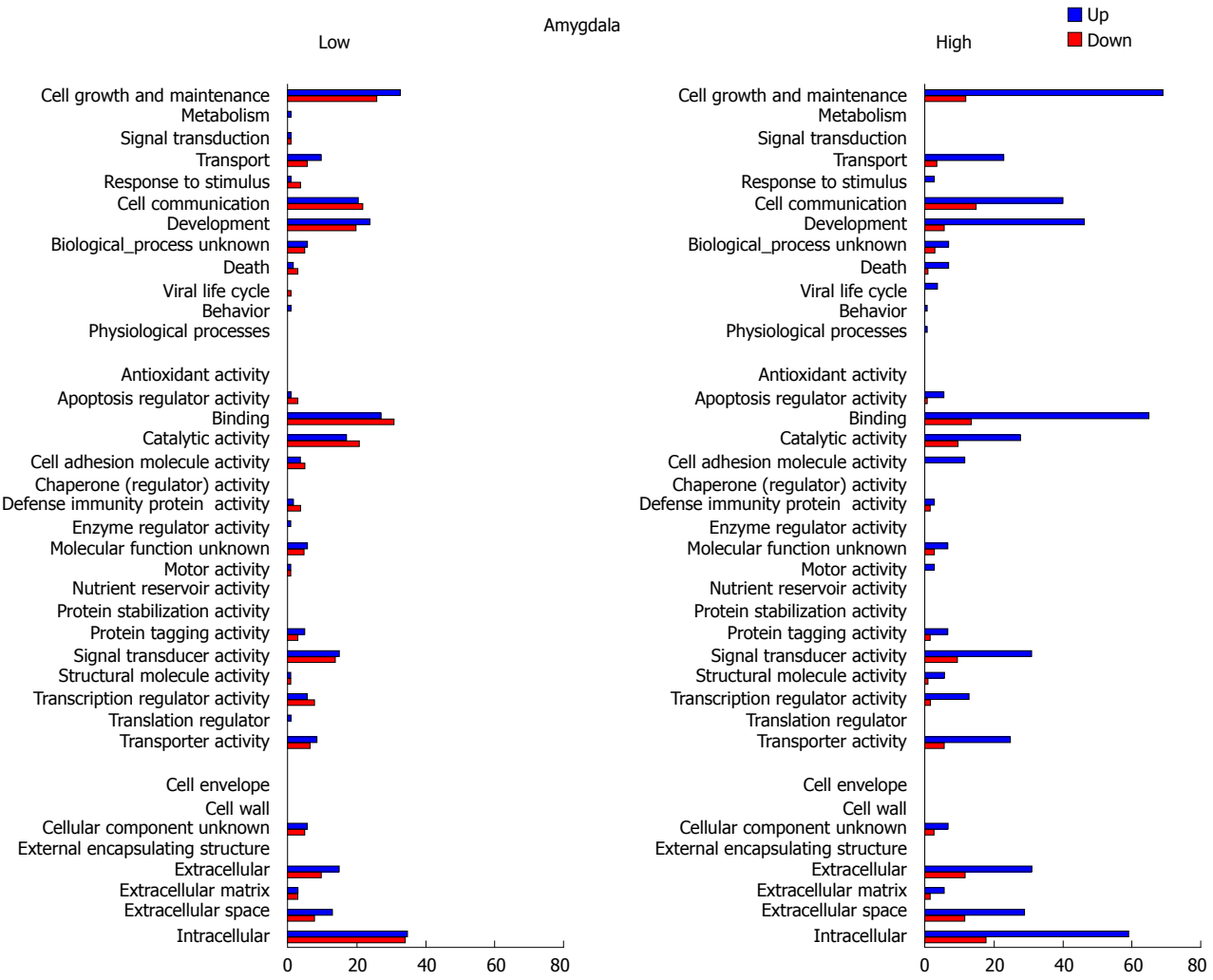

Figure 6 Functional categorization of the differentially expressed amygdala genes based on Gene Ontology. Changed genes divided into 38 function categories in the amygdala.

that works with Sp1 to activate the Laminin gamma1 chain gene; NM_053713. The expression of this gene was found to be up-regulated in the cerebral cortex (3.09/4.22 fold), with both low- and high-dose of MK-801. In other regions, the expression was up-regulated under low-dose in the ventral striatum (1.93 fold), under high-dose in four regions [amygdala (3.11 fold), ventral striatum (3.03 fold), hippocampus (2.82 fold) and hypothalamus (1.87 fold)]. This gene belongs to RNA polymerase II transcription factor activity (MF), cell differentiation (BP) and chromatin (CC) categories. Zhu *et al*[53] suggested that this gene can be up-regulated by activation of NMDA receptors and such up-regulation is dependent on calcium. MK-801 completely abolished NMDA-induced *Klf4* expression in their experiment. From these results, it is implied that NMDA-induced *Klf4* mRNA expression is dependent on Ca^{2+} influx, and is completely blocked by MK-801. Thus, this gene can be suggested to be related to NMDA receptor function in the episode of schizophrenia. Coming back to the study of Zhu *et al*[53], it is difficult to compare those data obtained from cultured neurons and brain slices, with our present study, and also whether the MK-801 concentration used (20 μmol/L) is equivalent to the amount of MK-801 available in brain tissue following a low- of high-dose of MK-801 administered ip. However, in the study of Zhu *et al*[53] it seems that the concentration of MK-801 is appropriate for measuring glutamate excitotoxicity with excessive Ca^{2+} influx. The up-regulation of the *Klf4* found in the present study might reflect a hyperglutamatergic state that would be induced following GABAergic disinhibition (at least of pyramidal neurons) subsequent to NMDAr blockade on GABAergic interneurons[54,55]. On the basis of an *in vitro* investigation and biophysical stimulation of a hippocampal disynaptic recurrent inhibitory circuit, it is generally thought that the blockade or hypofunction of NMDAr by ketamine or MK-801 or PCP would initially attenuate the excitation of PV+GABAergic interneurons, which are strongly excited by NMDAr activation[56] and which are more sensitive to NMDAr antagonists than glutamatergic neurons[57]. This difference might be due to a difference in NMDAr subunit assembly between local GABAergic interneurons and projection glutamatergic neurons.

***Adamts1*:** ADAM metallopeptidase with thrombospondin type 1 motif, 1; disintegrin and metalloprotease that

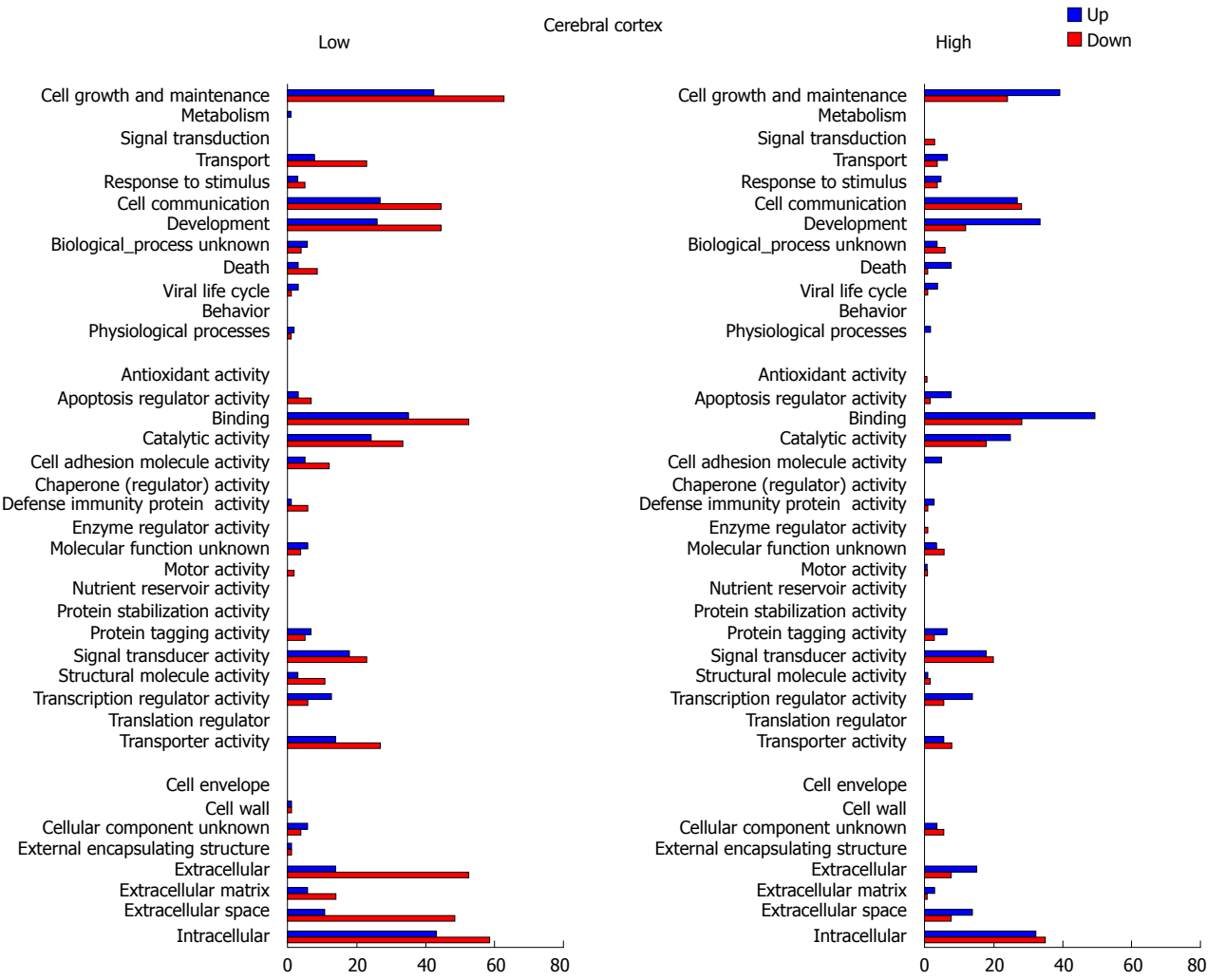

Figure 7 Functional categorization of the differentially expressed cerebral cortex genes based on Gene Ontology. Changed genes divided into 38 function categories in the cerebral cortex.

may be necessary for normal kidney morphology and function; NM_024400. The expression of this gene was found to be up-regulated in three regions [cerebral cortex (5.26/3.53 fold), amygdala (3.92/1.81 fold) and ventral striatum (3.33/1.58 fold)] with both low- and high-dose of MK-801. Under high-dose, the expression was up-regulated in two regions [hippocampus (2.21 fold) and hypothalamus (1.55 fold)]. The functional categories are heparin binding, metal ion binding, metalloendopeptidase activity and peptidase activity (MF), proteolysis (BP) and extracellular matrix and extracellular region (CC). It is reported that the expression of *Adamts1* gene increases during a physical or toxic injury[58,59] and in neurological diseases, such as Alzheimer's disease and Parkinson disease[60]. It seems that the changed gene expressions associated with models of neurodegenerative disorders may be a sensitive molecular response to the pathological circumstances of the brain areas[61]. In that study, *Adamts1* expression was up-regulated dose-dependently in three regions. These results suggest that *Adamts1* gene expression might be modulated in response to cranial nerve diseases.

***Zfp36*:** Zinc finger protein 36; acts as a transcriptional activator; NM_133290. The expression of this gene was found to be up-regulated in the cerebral cortex (2.35/3.50 fold), with both low- and high-dose of MK-801. In other regions, the expression was up-regulated under high-dose in the amygdala (1.52 fold), under low-dose in the ventral striatum (2.09 fold). The functional categories are AU-rich element binding (MF), mRNA catabolic process (BP) and cytoplasm, cytosol and nucleus (CC). Itokawa *et al*[21] determined that a microsatellite repeat in the promoter region of the GRIN2A gene suppresses transcriptional activity and correlates with the symptom severity in chronic schizophrenia patients. Furthermore, mice lacking the GRIN2A are known to show abnormal behavior similar to symptoms in schizophrenia. A 2001 study on schizophrenia drug-gene interactions revealed some bridge genes that included GRIN2A, GRIN3B, GRIN2C, and GRIN2B[62]. Therefore, *Zfp36* may act as a transcriptional activator in the schizophrenia patients. Of note, in our study, we also identified a *Grin3b* gene that was slightly down-regulated in the hippocampus and only under low-dose MK-801 treatment.

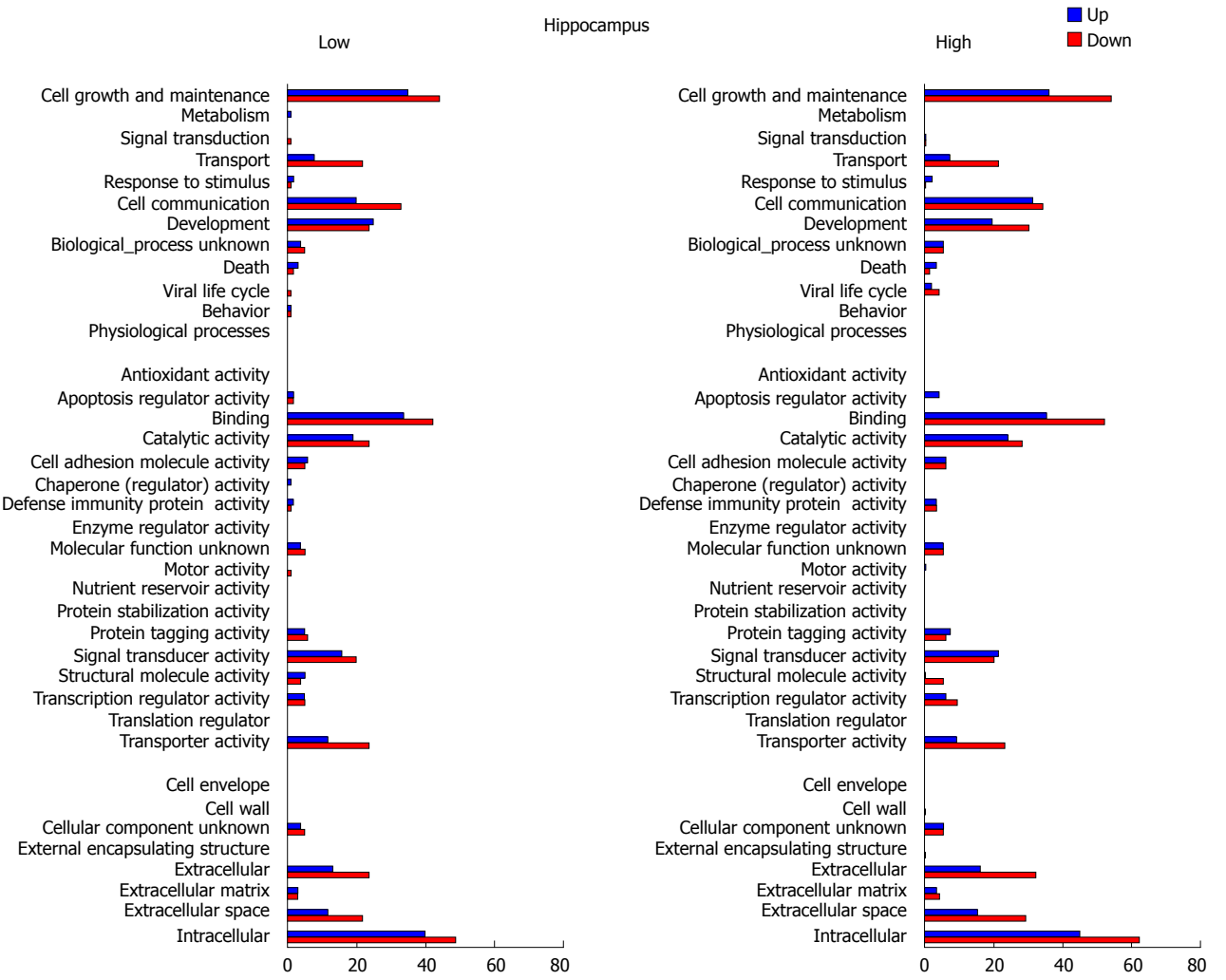

Figure 8 Functional categorization of the differentially expressed hippocampus genes based on Gene Ontology. Changed genes divided into 38 function categories in the hippocampus.

***LOC685106*:** Similar to ribosomal protein L6; XM_001062312. The expression of this gene was found to be up-regulated in all regions [hypothalamus (13.25 fold), hippocampus (12.90 fold), amygdala (10.82 fold), cerebral cortex (9.40 fold), ventral striatum (8.54 fold) and midbrain (7.67 fold)] with low-dose of MK-801. Under high-dose, the expression was not changed. It functions as a structural constituent of ribosome (FC), translation (BP) and ribosome (CC), and is therefore related to protein synthesis. Thus, MK-801 treatment might cause enhanced protein synthesis due to increased gene expression.

Down-regulated genes

Amy1: The synonym of Amy1a, amylase, alpha 1A (salivary); key enzyme in the digestion of starches and glycogen; NM_00101970. The expression of this gene was found to be down-regulated in all regions [ventral striatum (0.12 fold), cerebral cortex (0.28 fold), midbrain (0.48 fold), amygdala (0.53 fold), hippocampus (0.63 fold) and hypothalamus (0.65 fold)] with high-dose of MK-801. Under low-dose, the expression was down-regulated in the cerebral cortex (0.56 fold). This gene shows alpha-amylase activity (MF), carbohydrate metabolic process (BP), extracellular space (CC) and starch and sucrose metabolic pathway (pathway) functions. The *Amy1* gene function in the brain remains unknown, but it may be involved in glycogen degradation under normal conditions, and therefore affected by treatment with MK-801.

***Avp*:** Arginine vasopressin; a neuropeptide hormone involved in the regulation of natriuresis, vasoconstriction, cell growth and proliferation, and various behaviors; associated with hypertension, diabetes, and epilepsy; NM_016992. The expression of this gene was found to be down-regulated in the amygdala (0.13/0.19 fold) with both low- and high-dose of MK-801. In the ventral striatum, the expression was up-regulated under high-dose (1.80 fold). *Avp* shows V1A vasopressin receptor binding, V1B vasopressin receptor binding and V2 vasopressin receptor binding (MF), G-protein coupled receptor protein signaling pathway (BP), dendrite, extracellular region, extracellular space and secretory granule (CC), cerebrovascular accident, dehydration, dementia (disease) and abnormal anxiety-related response, abnormal coping response,

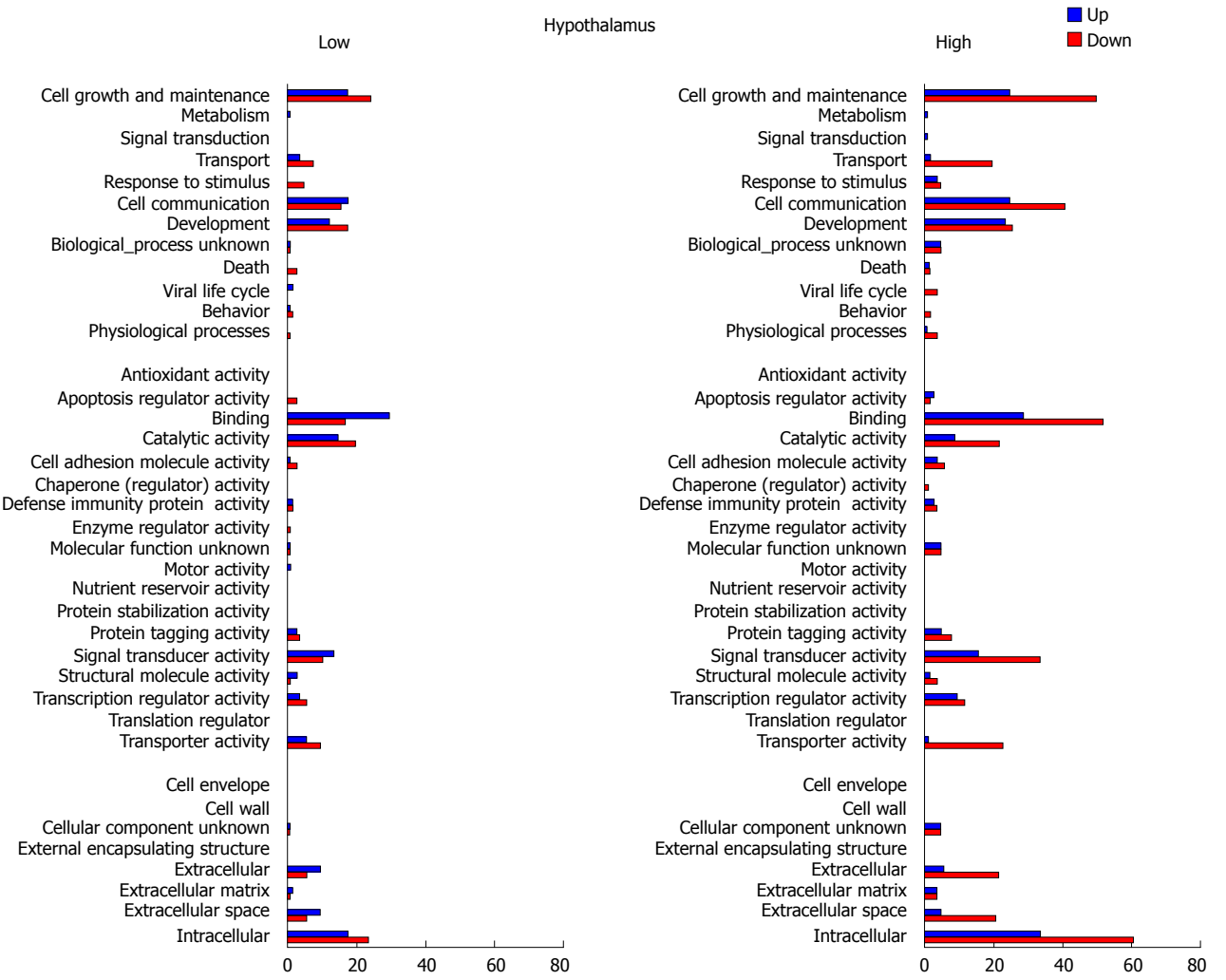

Figure 9 Functional categorization of the differentially expressed hypothalamus genes based on Gene Ontology. Changed genes divided into 38 function categories in the hypothalamus.

abnormal depression-related behavior, abnormal emotion/affect behavior, hyperactivity (phenotype) and vasopressin signaling pathway (pathway) functions. The *Avp* was shown to be involved in the regulation of brain water content and cerebral edema[63]. The *Avp* protein is synthesized in and secreted by the suprachiasmatic nucleus (SCN) in a circadian pattern and is expressed in the inner medulla[64]. The *Avp* gene expression in the SCN is mediated by MAPK signaling pathway[64]. Further, *Avp* activated by the protein kinase C activator, phorbol 12-myristate 13-acetate, and sodium channel blocker of *Avp*, tetrodotoxin (TTX), greatly decreases heteronuclear RNA levels and suppresses rhythmicity. Matsuoka *et al*[65] reported that *Avp* expression was down-regulated in the amygdala of MK-801 treated rats. Their results match our present data obtained from DNA microarray analysis. Furthermore, it was also proposed that *Avp* is related to water intoxication which is a symptom in schizophrenia patients[66]. It has been suggested that *Avp* receptors, V1a and V1b, may be implicated in the psychiatric disorders associated with dysfunction of social behavior such as schizophrenia and autism[67]. The result of this study was consistent with the previous reports. We believe that the *Avp* expression is rapidly affected in the amygdala by MK-801, and therefore might be an important factor in neurodegenerative disorders such as schizophrenia and autism.

***Sostdc1*:** Sclerostin domain containing 1; may be involved in the onset of endometrial receptivity for implantation/sensitization; NM_153737. The expression of this gene was found to be down-regulated in the hippocampus (0.20/0.19 fold) with both low- and high-dose of MK-801. In other regions, the expression was up-regulated under both low- and high-dose in the amygdala (1.50/3.18 fold), and down-regulated under high-dose in the hypothalamus (0.40 fold). The major functional categories for Sostdc1 are protein binding (MF), Wnt receptor signaling pathway and negative regulation of bone morphogenetic protein (BMP) signaling pathway (BP) and extracellular region and extracellular space (CC). *Sostdc1* belongs to a family of BMP antagonists and encodes a secretory protein called uterine sensitization-associated gene-1 or ectodin[68]. Interestingly, a recent study on altered brain gene

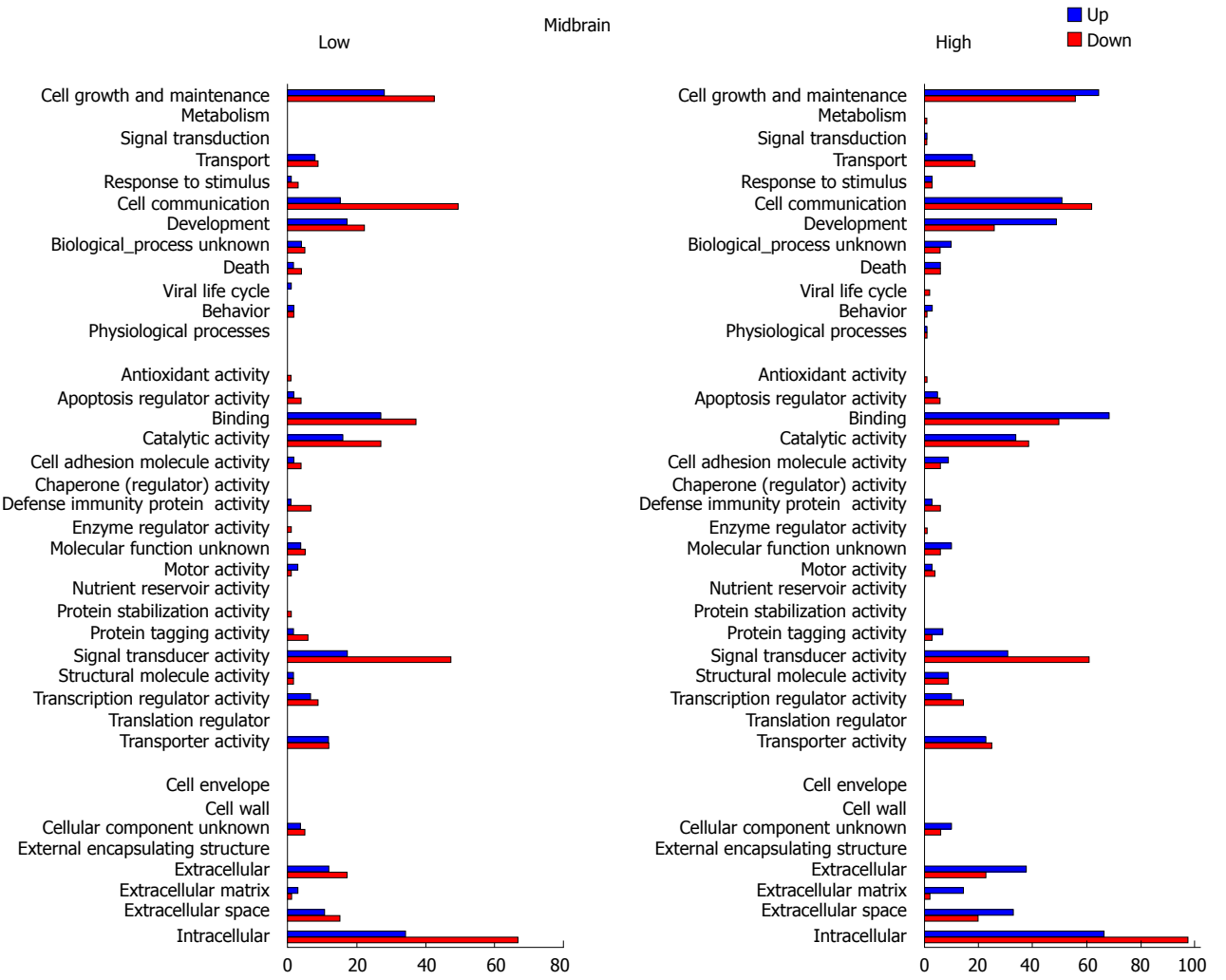

Figure 10 Functional categorization of the differentially expressed midbrain genes based on Gene Ontology. Changed genes divided into 38 function categories in the midbrain

expression profiles associated with the pathogenesis of phenylketonuria (PKU) in a mouse model revealed its up-regulation in the PKU mouse than in the wild-type[69]. It was proposed that the further study of this gene may provide new insights into the mechanisms involved in neurological damage in the PKU brain. The function of this gene in respect to MK-801 treatment remains unknown, but will also benefit from further studies.

***Kcnj13*:** Potassium inwardly-rectifying channel, subfamily J, member 13; inwardly rectifying K+ for the Na+,K(+)-ATPase; NM_053608. The expression of this gene was found to be down-regulated in the hippocampus (0.08/0.15 fold) with both low- and high-dose of MK-801. In other regions, the expression was up-regulated under high-dose in two regions [midbrain (2.21 fold) and amygdala (1.86 fold)], and down-regulated under low-dose in three regions [cerebral cortex (0.32 fold), midbrain (0.55 fold) and vent (0.74 fold)], under high-dose in the hypothalamus (0.25 fold). It mainly shows inward rectifier potassium channel activity, potassium ion binding and voltage-gated ion channel activity (MF), ion transport, potassium ion transport (BP) and integral to membrane and membrane (CC) functions. The *Kcnj13* was found to be expressed in thyroid, intestine and choroid plexus[70]. MK-801 is an open channel blocker, which inhibits only the ion channel site which is opened by ligand binding. Thus this gene with function of voltage-gated ion channel activity, also responded to MK-801 in this study.

***Ttr*:** Transthyretin; binds thyroxine (T4) and 3,5,3' tri-iodothyronine (T3); plays a role in thyroid hormone transport in serum; NM_012681. The expression of this gene was found to be down-regulated in the hippocampus (0.18/0.24 fold) with both low- and high-dose of MK-801. In other regions, the expression was up-regulated under high-dose in two regions [amygdala (2.79 fold) and ventral striatum (2.31 fold)], and down-regulated under high-dose in the hypothalamus (0.28 fold), under low-dose in the midbrain (0.46 fold). The functional categories are hormone activity, hormone binding and thyroid hormone transmembrane transporter activity (MF), thyroid hormone metabolic process and transport (BP), extracellular region and extracellular space (CC)

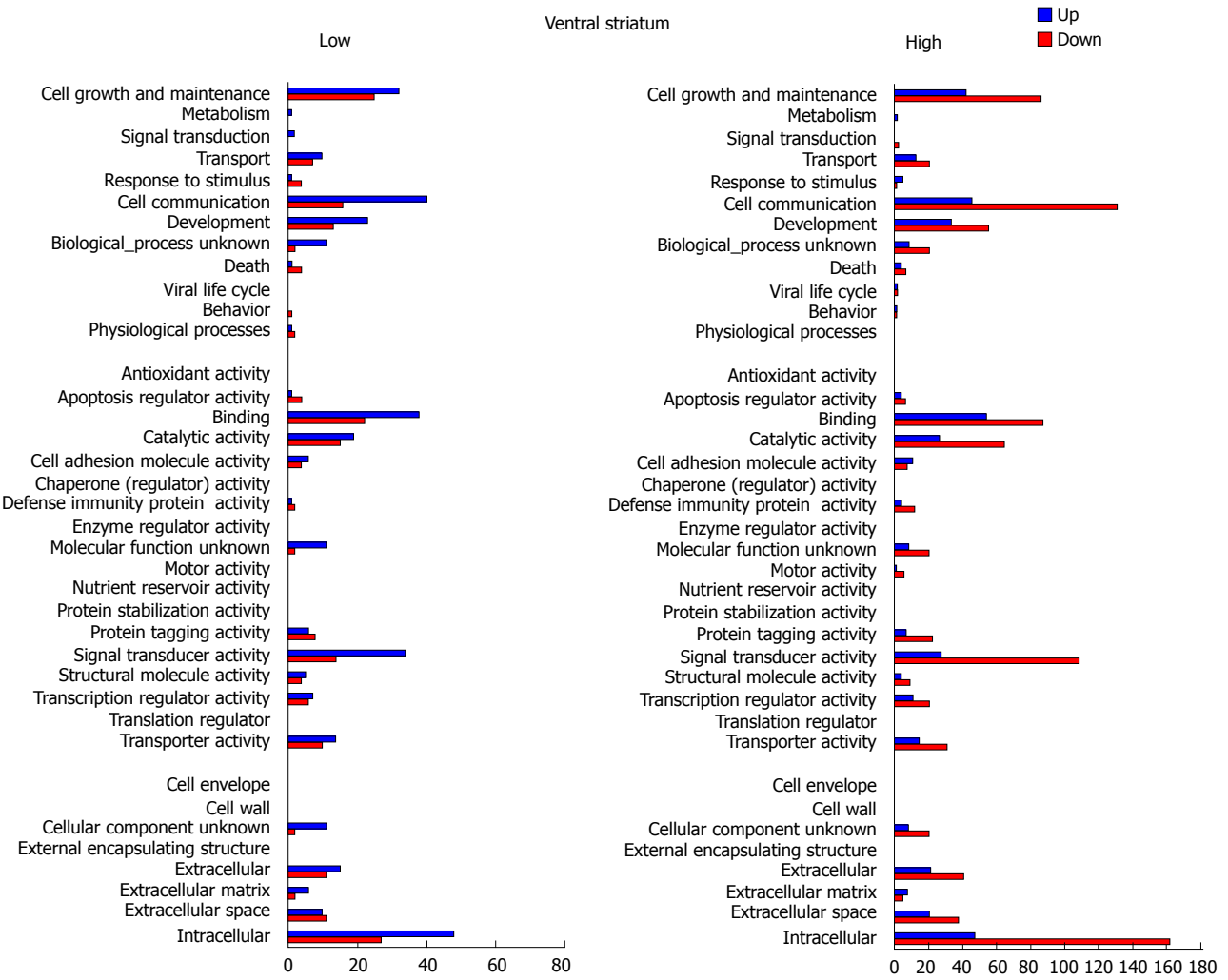

Figure 11 Functional categorization of the differentially expressed ventral striatum genes based on Gene Ontology. Changed genes divided into 38 function categories in the ventral striatum.

and Alzheimer's disease, amyloid neuropathies and brain ischemia (disease). It has been reported that *Ttr* protein expression in cerebrospinal fluid (CSF) of schizophrenia patients was down-regulated in contrast to that in plasma. *Ttr* tetramer is a retinoid transporter, and the dysfunction of retinoid may be involved in pathology of schizophrenia[71]. It was confirmed that *Ttr* expression, which is specifically expressed in the choroid plexus in that study, was completely decreased in the brain of adult rat treated with maternal separation during the neonatal period used as a model of depression[72]. Tsai *et al*[73] also suggested that the change in the expression level of this gene is correlated with the pathogenesis of the Alzheimer's disease. Thus, it is suggested that this gene may related to the psychiatric disorders. In this study, *Ttr* gene expression was also down-regulated in hippocampus, hypothalamus (high-dose) and midbrain (low-dose). Taken together with previous reports, *Ttr* may be involved in the etiology of schizophrenia in the intracerebral substances. To note, in the present study we find the *Ttr* gene down-regulation after acute MK-801 treatment, which is a model of first stages of schizophrenia, in contrast to its up-regulation after chronic MK-801 treatment, which is a model from chronic schizophrenia[65]. Interestingly, a recent study on altered brain gene expression profiles associated with the pathogenesis of PKU in a mouse model revealed up-regulation of *Ttr* in the PKU mouse than in the wild-type[69]. The authors suggested that this might be due to dopamine deficiency.

***Pmch*:** Pro-melanin-concentrating hormone; cyclic neuropeptide; induces hippocampal synaptic transmission; NM_012625. The expression of this gene was found to be down-regulated in the hypothalamus (0.12/0.28 fold) with both low- and high-dose of MK-801. Major functional categories are melanin-concentrating hormone activity and type 1 melanin-concentrating hormone receptor binding (MF), regulation of neuronal synaptic plasticity (BP) and extracellular region and extracellular space, nucleus (CC). The *Pmch* regulates appetite, and it activates Y1 receptor which in turn mediates Npy[74]. *Pmch* may play a role in retrograde facilitation on the inhibitory avoidance[75]. It plays a role in regulation of feeding behavior[76]. The *Pmch* gene is expressed in the lateral hypothalamus and

zona incerta of the central nervous system[76] and is found in the brain lateral hypothalamic area[74]. In schizophrenia patients treated with antipsychotic agents, the symptom weight gain is observed. It is suggested that increased *Pmch* is associated with weight gain[77-79]. However, in this study, *Pmch* expression was down-regulated in the hypothalamus. The *Pmch* expression may be down-regulated in schizophrenia patients without treatment, whereas it may be up-regulated by treatment with antipsychotic agents, where it is involved in weight gain.

Slc6a3: Solute carrier family 6 (neurotransmitter transporter, dopamine), member3; acts as a sodium-dependent dopamine transporter; may play a role in regulation of dopamine metabolism and signaling; NM_012694. The expression of this gene was found to be down-regulated in the hypothalamus (0.01/0.09 fold) with both low- and high-dose of MK-801. It belongs to multiple functional categories of dopamine binding, dopamine transmembrane transporter activity and dopamine: sodium symporter activity (MF), dopamine biosynthetic and catabolic processes (BP), integral to plasma membrane, plasma membrane and synaptosome (CC), Parkinsonian disorders (disease), abnormal movement/ locomotion, abnormal spatial learning, hyperactivity (phenotype), ADHD (behavior) and dopamine signaling pathway (pathway). *Slc6a3* is sodium-dependent dopamine transporter[80-82]. *Slc6a3* is expressed in substantia nigra and ventral tegmental area[80]. Sáiz *et al*[83] reported that their study provides evidence that possible interaction between dopamine-3 receptor (DRD3) and *Slc6a3* genes are associated with schizophrenia. It was also reported previously that the variations at *Slc6a3* are important determinants of schizophrenia susceptibility, with additional risk due to some variant genes. These genes encode proteins that regulate synaptic dopamine concentrations[84]. It has been proposed that the hyperenhancement of the dopamine function is involved in the positive symptom of schizophrenia. Adriani *et al*[85] found that the dopamine transporter overexpressing rats showed increased impulsivity in the absence of general locomotor effects. In this study, the expression of *Slc6a3*, one of the genes encode proteins that regulate synaptic dopamine concentrations, was down-regulated in the hypothalamus by the injection of MK-801. Therefore, the dopamine function might have been hyper-enhanced. Thereby, dopamine would modulate the excitability of neurons, for instance *via* its action on GABAergic neurons[86,87]. This dopaminergic action would interfere with MK-801 action since GABAergic neurons are thought to be the first target of NMDAr antagonists[88].

***Lect1*:** Leukocyte cell derived chemotaxin 1; cartilage-specific angiostatic factor; stimulates growth of chondrocytes and inhibits tube formation of endothelial cells; NM_030854. The expression of this gene was found to be highly down-regulated in the midbrain (0.09 fold) with low-dose of MK-801. Under high-dose, the expression was up-regulated in the hypothalamus (2.26 fold). The functional categories are cartilage development, cell differentiation, multicellular organismal development, negative regulation of angiogenesis and negative regulation of vascular endothelial growth factor receptor signaling pathway (BP) and extracellular region and integral to membrane and membrane (CC). In this study, the expression of genes having the function of positive regulation of angiogenesis, *Cyr61* and *Verge*, were up-regulated. While this gene (*Lect1*), having the function of negative regulation of angiogenesis, was found to be down-regulated. Therefore, it can be suggested that MK-801 treatment leads to a signaling related to angiogenesis (see *Verge*).

DISSCUSSION

Our study provides an exhaustive inventory of gene expressions, and shows that MK-801 is highly effective in causing large number of gene expressions as early as 40 min after treatment in rat brain. Using a high-throughput DNA microarray screening approach, in the core of this study we compare and provide, for the first time, transcriptome profiles in six brain regions, namely amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain and ventral striatum, after MK-801 treatment using rat model. It should be mentioned that one of the goals of the study was also to identify potential biomarkers closely associated with neurological damage in rat brain after MK-801 treatment. Our investigation has provided not only new insight into the differential genes expressed upon MK-801 treatment but also has identified several genes which are related to psychiatric disorders, such as schizophrenia or depression. This is interesting since the NMDAr antagonist ketamine has not only psychotomimetic but also antidepressant effects[89]. Moreover, as we pooled brain samples to decrease inter-animal variation during DNA microarray analysis, this may have decreased the specificity of the potential biomarkers. However, the approach used here has allowed us to present these candidate biomarkers to the scientific community, and undoubtedly, further functional analysis will be needed to determine their importance. Finally, detailed bioinformatics analysis, such as utilizing the Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com) tool, will be essential to reveal predominant pathways and networks of genes affected by MK-801 providing new meaning to this vast gene resource presented in this study.

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COMMENTS

Background

N-Methyl-D-aspartate receptor (NMDAr) hypofunction is thought to be involved in various neuropsychiatric disorders, including schizophrenia. Non-competitive NMDAr antagonists, like phencyclidine, ketamine and MK-801 (or dizocilpine), are suitable pharmacological tools used to understand the pathogenesis of psychosis, as they disturb - in both humans and rodents - behavior, brain connectivity and oscillations, cognition and sensory-perceptual processes which, together, resemble aspects of schizophreniform psychoses.

Research frontiers

Combining pharmacological, electrophysiological and DNA microarray approaches is a great challenge to understand the initial genetic-to-molecular mechanisms and gene-gene interactions responsible for the NMDAr hypofunction thought to underlie the pathogenesis of neurobiological disorders.

Innovations and breakthroughs

The present study used two low doses (0.08 and 0.16 mg/kg) of MK-801, which increases in a dose-dependent manner the power of spontaneously occurring gamma (30-80 Hz) and higher frequency oscillations in the adult rat cortex and subcortical structures. Using genome DNA chip (4 × 44 K), it provides, from MK-801-treated rats, a detailed gene inventory and resource from six brain regions (amygdala, cerebral cortex, hippocampus, hypothalamus, midbrain and ventral striatum). The present study reveals several genes identified as being potential biomarker candidates for schizophrenia and depression.

Applications

As this study primarily provides a gene resource, the outputs of microarray analysis are freely available to the public scientific community under the series number GSE63639, at the NCBI Gene Expression Omnibus public functional genomics data repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63639). These data can be downloaded and analysed for further bioinformatics and targeted functional analysis.

Terminology

Genome-wide transcriptome analysis is the analysis of the total set of transcripts present in a cell, tissue or organism, usually by DNA microarray technique. The mRNA abundance or expression of genes could be unraveled in the brain regions examined at the set point of 40 min post-injection MK-801.

Peer-review

This study and analysis of genes genome wide, in particular could help in the potential identification of biomarkers associated with neurological and neuropsychiatric illnesses.

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ORIGINAL ARTICLE

Basic Study

JAK3 inhibitor VI is a mutant specific inhibitor for epidermal growth factor receptor with the gatekeeper mutation T790M

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Abstract

AIM: To identify non-quinazoline kinase inhibitors effective against drug resistant mutants of epidermal growth factor receptor (EGFR).

METHODS: A kinase inhibitor library was subjected to screening for specific inhibition pertaining to the *in vitro* kinase activation of EGFR with the gatekeeper mutation T790M, which is resistant to small molecular weight tyrosine kinase inhibitors (TKIs) for EGFR in non-small cell lung cancers (NSCLCs). This inhibitory effect was confirmed by measuring autophosphorylation of EGFR T790M/L858R in NCI-H1975 cells, an NSCLC cell line harboring the gatekeeper mutation. The effects of a candidate compound, Janus kinase 3 (JAK3) inhibitor VI, on cell proliferation were evaluated using the MTT assay and were compared between T790M-positive and -negative lung cancer cell lines. JAK3 inhibitor VI was modeled into the ATP-binding pocket of EGFR T790M/L858R. Potential physical interactions between the compound and kinase domains of wild-type (WT) or mutant EGFRs or JAK3 were estimated by calculating binding energy. The gatekeeper residues of EGFRs and JAKs were aligned to discuss the similarities among EGFR T790M and JAKs.

RESULTS: We found that JAK3 inhibitor VI, a known inhibitor for JAK3 tyrosine kinase, selectively inhibits EGFR T790M/L858R, but has weaker inhibitory effects on the WT EGFR *in vitro*. JAK3 inhibitor VI also specifically reduced autophosphorylation of EGFR T790M/L858R in NCI-H1975 cells upon EGF stimulation, but did not show the inhibitory effect on WT EGFR in A431 cells. Furthermore, JAK3 inhibitor VI suppressed the proliferation

of NCI-H1975 cells, but showed limited inhibitory effects on the WT EGFR-expressing cell lines A431 and A549. A docking simulation between JAK3 inhibitor Ⅵ and the ATP-binding pocket of EGFR T790M/L858R predicted a potential binding status with hydrogen bonds. Estimated binding energy of JAK3 inhibitor Ⅵ to EGFR T790M/L858R was more stable than its binding energy to the WT EGFR. Amino acid sequence alignments revealed that the gatekeeper residues of JAK family kinases are methionine in WT, similar to EGFR T790M, suggesting that TKIs for JAKs may also be effective for EGFR T790M.

CONCLUSION: Our findings demonstrate that JAK3 inhibitor Ⅵ is a gatekeeper mutant selective TKI and offer a strategy to search for new EGFR T790M inhibitors.

Key words: Epidermal growth factor receptor; Tyrosine kinase inhibitor; Gatekeeper mutation; Non-small cell lung cancers

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Core tip: Non-small cell lung cancers caused by mutations in the epidermal growth factor receptor (EGFR) initially respond to tyrosine kinase inhibitors (TKIs). However, the therapeutic efficacy of EGFR-TKIs is limited by drug-resistant mutations such as the gatekeeper mutation T790M. Our present study rediscovered JAK3 inhibitor Ⅵ, a known TKI for Janus kinases (JAKs), as a selective EGFR T790M inhibitor. Our structural analysis revealed similarities among EGFR T790M and JAKs, offering a possible strategy to search for EGFR T790M inhibitors from known kinase inhibitors. Repositioning of the existing therapeutics may facilitate solving clinical problems such as drug resistance and toxicity.

Nishiya N, Sakamoto Y, Oku Y, Nonaka T, Uehara Y. JAK3 inhibitor Ⅵ is a mutant specific inhibitor for epidermal growth factor receptor with the gatekeeper mutation T790M. *World J Biol Chem* 2015; 6(4): 409-418 Available from: URL: http://www.wjgnet.com/1949-8454/full/v6/i4/409.htm DOI: http://dx.doi.org/10.4331/wjbc.v6.i4.409

INTRODUCTION

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib and erlotinib are clinical therapeutics effective on non-small cell lung cancers (NSCLCs) with activating mutation in EGFR[1,2]. Most frequent activating mutations in EGFR, a point mutation L858R and an exon19 deletion (delE746-A750), increase affinity for EGFR-TKIs relative to that for wild type (WT)[3,4]. However, the development of drug-resistant mutations such as the gatekeeper mutation T790M limits clinical efficacies of the EGFR-TKIs and is the major cause of acquired resistance in NSCLC patients[5,6].

An irreversible EGFR-TKI with a reactive acceptor group covalently binds to a conserved cysteine residue in the EGFR kinase domain (Cys 797) *via* a Michael addition reaction. The covalent bond formation increases stability of the complex formation between a TKI and the ATP-binding site of the kinase domain in comparison with reversible inhibitors. Therefore, irreversible EGFR-TKIs provide the ability to inhibit EGFR T790M[7-12]. However, EGFR T790M inhibition by the quinazoline-based irreversible TKIs causes simultaneous inhibition of the WT EGFR because of similar ATP affinity between WT and the T790M mutant, resulting in adverse effects such as skin rash and diarrhea. Therefore, the use of irreversible EGFR-TKIs has been limited in gefitinib- or erlotinib-resistant NSCLCs and by the dose-limiting toxicity[13,14].

Here we report that Janus kinase 3 (JAK3) inhibitor Ⅵ[15], a known TKI for a tyrosine kinase JAK3, selectively inhibits EGFR T790M/L858R, but has less inhibitory effect on the WT EGFR *in vitro*. JAK3 inhibitor Ⅵ also suppresses the proliferation of NCI-H1975 cells, an NSCLC cell line harboring EGFR T790M/L858R. This inhibitor is modeled into the ATP-binding pocket of EGFR T790M/L858R and forms potential hydrogen bonds with the kinase domain of EGFR T790M/L858R. The gatekeeper residue of JAK3 is methionine in WT, and the overall structure of the catalytic domain of JAK3 is closely related to that of EGFR. Our findings indicate that a JAK3 inhibitor is a mutant-selective reversible TKI for EGFR T790M.

MATERIALS AND METHODS

Reagents and cell lines

JAK3 inhibitor Ⅵ and JAK inhibitor I were purchased from Calbiochem (San Diego, CA). Gefitinib was purchased from JS Research Chemical Trading (Wedel, Germany). SCADS inhibitor kit 3 was provided by Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology, Japan. Antibodies against EGFR, pEGFR (Y1045), pEGFR (Y1068), and STAT3 were purchased from Cell Signaling Technology (Beverly, MA). Anti-pSTAT3 (Y705) was purchased from BD (Franklin Lakes, NJ). HRP-conjugated anti-phosphotyrosine antibodies PY20 and 4G10 were purchased from BD and Millipore (Billerica, MA). NCI-H1975 cell line was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 2 mmol/L L-glutamine and 10% Fetal Bovine Serum at 37 ℃ in a humidified atmosphere of 5% CO_2.

In vitro kinase assay and screening for kinase inhibitors

One hundred nanograms of recombinant cytoplasmic domains (amino acid residues 696 to the C-terminus) of the WT EGFR, L858R, or T790M/L858R (Cell Signaling Technology) were preincubated with kinase inhibitors in 25 μL of kinase reaction buffer (120 mmol/L HEPES, pH 7.5; 10 mmol/L $MgCl_2$; 10 mmol/L $MnCl_2$; 6 μmol/

L Na_3VO_4; and 2.5 mmol/L DTT) at 25 ℃ for 30 min. Subsequently, 25 μL of ATP/substrate solution containing 2 or 6 μmol/L ATP and 6 μmol/L poly (Glu-Tyr) biotinylated peptide (Cell Signaling Technology) was added to the preincubation mixture. The kinase reaction was performed at 25 ℃ for 30 min and terminated by adding 50 μL of stop buffer (50 mmol/L EDTA, pH 8.0). Kinase activity was estimated using ELISA with avidin-coated 96-well plates and anti-phosphotyrosine antibodies (PY20 and 4G10).

Phosphorylation analysis

NCI-H1975 or A431 cells were pretreated using JAK3 inhibitor Ⅵ for 1 h in 0.2% serum conditions and were stimulated using 200 ng/mL EGF for 15 min. Alternatively, NCI-H1975 cells were treated with kinase inhibitors at the indicated concentration for 16 h. Cells were lysed in RIPA buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 1 mmol/L NaF; 1 mmol/L Na_3VO_4; and protease inhibitors) and cleared by centrifugation. SDS sample buffer was added to the supernatants. Protein samples were separated using SDS-PAGE and analyzed using Western blotting with antibodies against phospho-specific or total proteins.

Proliferation assay

Cells were cultivated in a flat-bottomed 96-well plate at 1×10^4 cells per well in 150 μL media containing various concentrations of kinase inhibitors for 72 h. The inhibitor-treated cells were incubated in the presence of 0.5 mg/mL 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) in a CO_2 incubator for 4 h. One hundred microliters of 20% SDS was added to each well for dissolving the insoluble purple formazan product into a colored solution. The absorption at 570 nm was measured using a spectrophotometer. The means of data values from at least three independent experiments were calculated.

Docking model analysis

All starting templates, except the WT EGFR, were prepared by superimposing the crystal structure of the WT EGFR (PDB ID: 4WKQ) onto crystal structures of EGFR-LRTM (PDB ID: 4I22) and JAK3 (PDB ID: 3LXL) without ligands. Docking studies were performed to analyze interactions of some inhibitors in the binding site of the WT EGFR, EGFR-TMLR, and JAK3 through PyRx[16] using AutoDock 4.2.6[17]. The adopted standard protein preparation protocol involves the addition of missing hydrogen atoms to the starting template and the assignment of an ionizable state to each charged group. The docking grid of 11 Å × 12 Å × 14 Å was generated based on the observed binding site of the WT EGFR using gefitinib (PDB ID: 4WKQ). Schematic representations were generated using the UCSF Chimera package[18].

Statistical analysis

All data are presented as the mean ± SE. The Mann–Whitney *U*-test was used to determine statistically significant differences.

RESULTS

To profile sensitivities of EGFR mutants against different classes of kinase inhibitors, we assayed *in vitro* recombinant cytoplasmic domains of the WT EGFR, EGFR L858R, or EGFR T790M/L858R for 95 kinase inhibitors (Table 1). Several compounds such as Ellagic acid, Damnacanthal, and RAF1 kinase inhibitor I inhibited all three EGFR kinases activity, but other kinase inhibitors showed selectivity in their inhibitory activity against WT or mutant kinases. Among inhibitors with selectivity, JAK3 inhibitor Ⅵ[15] and Gö7874[19] reduced kinase activity of EGFR T790M/L858R. Gö7874 is a known PKC inhibitor and has an indolocarbazole structure that is also seen in staurosporine, a classical kinase inhibitor (Figure 1B). Because it has been reported that indolocarbazole compounds selectively inhibit EGFR T790M but show limited effects on the WT EGFR[20], JAK3 inhibitor Ⅵ was used in further studies. JAK3 inhibitor Ⅵ, a 3′-pyridyl oxindole compound (Figure 1A), inhibited EGFR T790M/L858R (IC_{50} = 1.43 μmol/L) mutant, but weaker effects were observed on the L858R mutant (IC_{50} > 10 μmol/L) and WT EGFR (IC_{50} > 10 μmol/L) (Figure 1C).

Next, we tested whether JAK3 inhibitor Ⅵ decreased EGFR signaling in the EGFR T790M/L858R-positive NCI-H1975 cells. EGF-induced EGFR autophosphorylation at Y1045 was significantly reduced in 10 or 5 μmol/L JAK3 inhibitor Ⅵ-treated NCI-H1975 cells (Figure 2A). In contrast, JAK3 inhibitor Ⅵ had no effect on the WT EGFR Y1045 phosphorylation in A431 cells (Figure 2B). These data confirm the mutant EGFR-specific blockage by JAK3 inhibitor Ⅵ in the T790M/L858R positive NCI-H1975 cells.

Although JAK 3 inhibitor Ⅵ specifically inhibited EGFR T790M/L858R in cells, it was not clear whether the T790M/L858R inhibition was a direct effect on the EGFR kinase or an indirect effect *via* JAK3 inhibition. To clarify this point, we analyzed phosphorylation levels of STAT3, a direct JAK3 substrate. The STAT3 phosphorylation level was unchanged even after stimulation using 200 ng/mL EGF, which efficiently elevated EGFR Y1045 phosphorylation (Figure 2A and C). In addition to the effects of JAK3 inhibitor Ⅵ on the EGF-stimulated phosphorylation of EGFR T790M/L858R, those on the basal phosphorylation of EGFR T790M/L858R and STAT3 were analyzed pertaining to NCI-H1975 cells in a 10% serum culture condition. In contrast to a decrease in the phosphorylation level of EGFR T790M/L858R at 5 μmol/L JAK3 inhibitor Ⅵ, STAT3 Y705 phosphorylation was apparent at 5 μmol/L and began to decrease from a higher concentration, 10 μmol/L (Figure 2D). Another JAK inhibitor, JAK inhibitor I, significantly reduced STAT3 Y705 phosphorylation at 2.5 μmol/L, but did not affect EGFR Y1068 phosphorylation at higher concentrations such as 5 or 10 μmol/L (Figure 2D). These data suggest that JAK3 inhibitor Ⅵ reduces the phosphorylation level

Table 1 List of kinase inhibitors used in a screening for compounds effective on epidermal growth factor receptor T790M/L858R

Target	Compound name
AK	ABT-702
AKT (PKB)	Akt Inhibitor Ⅳ, Akt Inhibitor Ⅷ, Akt Inhibitor XI
AMPK	compound C
ATM	ATM/ATR kinase inhibitor, ATM kinase inhibitor
Aurora kinase	Aurora kinase/CDK inhibitor, aurora kinase inhibitor Ⅱ, Aurora kinase inhibitor Ⅲ
Bcr-abl	AG957
BTK	LFM-A13, terreic acid
CAMKII	KN-93, KN-62, Lavendustin C
CDK	Kenpaullone, purvalanol A, Olomoucine, Alsterpaullone, Cdk1/2 inhibitor Ⅲ, Cdk2/9 inhibitor, NU6102, Cdk4 inhibitor, NSC625987
Chk	SB218078, Isogranulatimide, Chk2 inhibitor, Chk2 inhibitor Ⅱ
CK	Ellagic acid (dihydrate), TBB, DMAT, D4476
Clk	TG003
DGK	Diacylglycerol kinase inhibitor Ⅱ
DNA-PK	IC60211
eEF2 kinase	TX-1918
EGFR	BPIQ-Ⅱ, AG1478, AG490
FGFR	SU4984, SU5402
Flt-3	Flt-3 Inhibitor
Fms	cFMS Receptor Inhibitor
Fyn	SU6656
GSK	GSK-3 inhibitor IX, 1-Azakenpaullone, Indirubin-3′-monoxime
HER2	AG825
IGF-IR	AG1024, AGL 2263
IKK	BMS-345541, IKK-2 inhibitor Ⅵ
IRAK	IRAK-1/4 inhibitor
JAK	JAK Inhibitor I, JAK3 Inhibitor Ⅵ
JNK	SP600125, JNK inhibitor Ⅷ
Lck	Damnacanthal, PP2
MAPK	ERK inhibitor Ⅱ
MEK	PD98059, U-0126, MEK inhibitor I
Met	SU11274
MLCK	ML-7
p38 MAPK	SB202190, SB239063
PDGFR	AG1296, SU11652, PDGF receptor inhibitor V, PDGF receptor inhibitor Ⅳ
PI3K	LY-294002, Wortmannin
PKA	H-89, 4-cyano-3-methylisoquinoline
PKC	Bisindolymaleimide I, Go7874
PKG	Rp-8-CPT-cGMPS, KT5823
PKR	PKR inhibitor
Raf	RAF1 kinase inhibitor I, ZM 336372
ROCK	H-1152, Y-27632
Hsp90	radicicol
Src	PP1 analog
Syk	Syk inhibitor
TGF-bRI	SB431542, TGF-beta RI kinase inhibitor Ⅱ
Tpl2	Tpl2 kinase inhibitor
TrKA	TrkA inhibitor
VEGFR	VEGFR receptor inhibitor Ⅱ, VEGF recptor 2 inhibitor I, SU1498

AK: Adenosine kinase; AKT(PKB): Protein kinase B; AMPK: 5′-adenosine monophosphate-activated protein kinase; ATM: Ataxia telangiectasia mutated; Aurora: Aurora kinase; Bcr-abl: Breakpoint cluster region-Abelson fusion kinase; BTK: Bruton's tyrosine kinase; CAMKII: Calcium/calmodulin-dependent protein kinase II; CDK: Cyclin-dependent kinase; Chk: Checkpoint kinase; CK: Casein kinase; Clk: Cdc2-like kinase; DGK: Diacylglycerol kinase; DNA-PK: DNA-dependent protein kinase; eEF2 kinase: Eukaryotic elongation factor 2 kinase; EGFR: Epidermal growth factor receptor; FGFR: Fibroblast growth factor receptor; Flt-3: Fms-related tyrosine kinase 3; Fms: Feline McDonough sarcoma (Colony-stimulating factor-1 receptor); GSK: Glycogen synthase kinase; HER2: Human epidermal growth factor receptor 2; IGF-IR: Insulin-like growth factor I receptor; IKK: IκB kinase; IRAK: Interleukin-1 receptor-associated kinase; JAK: Janus kinase; JNK: Jun N-terminal kinase; Lck: Lymphocyte-specific protein tyrosine kinase; MAPK: Mitogen activated protein kinase; MEK: Mitogen-activated protein/extracellular signal-regulated kinase kinase; MLCK: Myosin light chain kinase; p38 MAPK: P38 mitogen-activated protein kinase; PDGFR: Platelet-derived growth factor receptor; PI3K: Phosphatidylinositol-4-Kinase; PKA: Protein kinase A; PKC: Protein kinase C; PKG: Protein kinase G; PKR: Double-stranded RNA-activated protein kinase; Raf: Rapidly accelerated fibrosarcoma; ROCK: Rho-associated protein kinase; Hsp90: Heat shock protein; Syk: Spleen tyrosine kinase; TGF-bRI: Transforming growth factor-beta type I receptor; Tpl2: Tumor progression locus 2; TrKA: Tyrosine kinase receptor A (tyrosine kinase receptor for nerve growth factor); VEGFR: Vascular endothelial growth factor receptor.

of EGFR T790M/L858R without JAK3 inhibition, and that JAKs are not major contributors to EGFR phosphorylation in NCI-H1975 cells.

Next, we performed MTT assays to test the effects

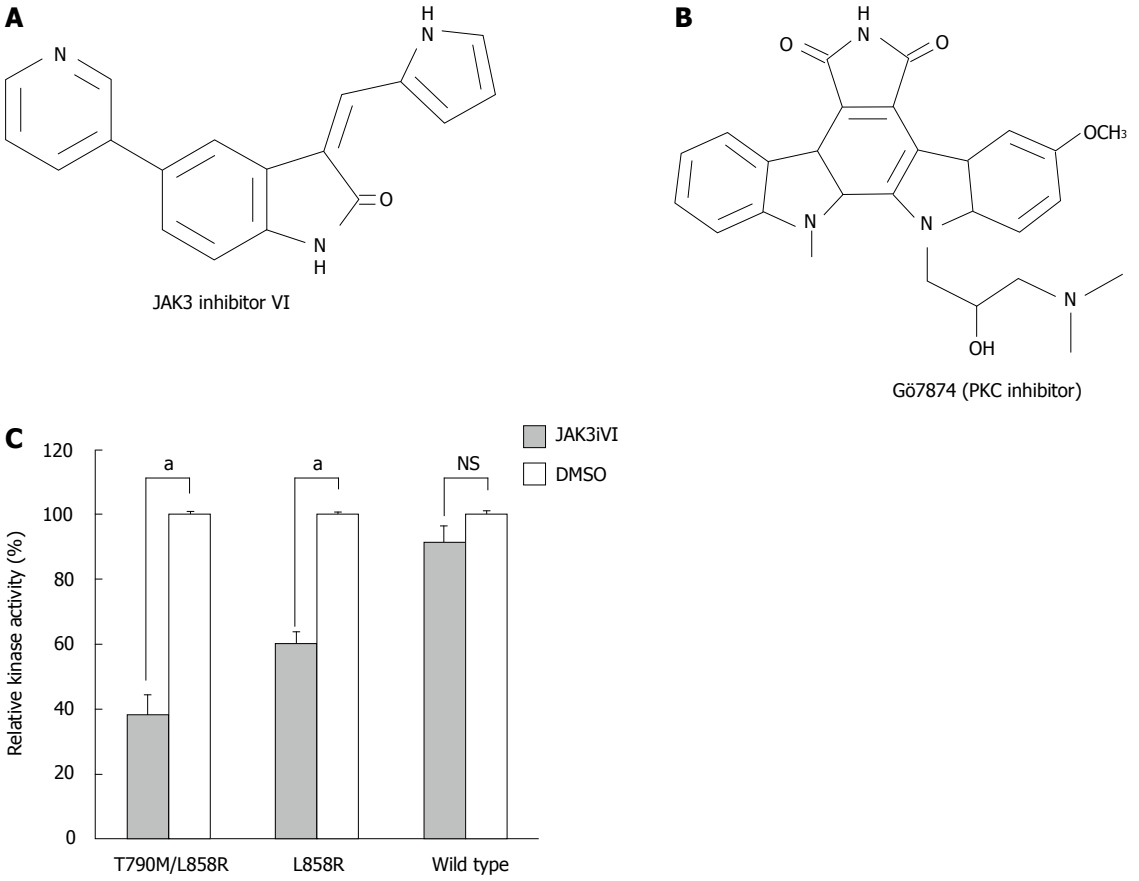

Figure 1 Janus kinase 3 inhibitor Ⅵ selectively inhibits kinase activity of the epidermal growth factor receptor gatekeeper mutant T790M/L858R *in vitro*. A, B: Chemical structures of JAK3 inhibitor VI (A) and Gö7874 (B) are shown; C: Effects of JAK3 inhibitor Ⅵ on kinase activities of WT, L858R, and T790M/L858R EGFRs were compared. Recombinant EGFR kinase domains were treated using JAK3 inhibitor Ⅵ at 10 μmol/L, and phosphorylation was initiated by adding ATP in the presence of an artificial substrate peptide. Phosphorylation levels were measured by ELISA using an anti-phosphotyrosine antibody. Error bars indicate SEM (n = 4); [a]P < 0.05. NS: Not significant; EGFR: Epidermal growth factor receptor; JAK3: Janus kinase 3.

of JAK3 inhibitor Ⅵ on proliferation of EGFR T790M/L858R positive NCI-H1975 cells. NCI-H1975 cells were cultured in 96-well plates for 3 d with or without different concentrations of compounds. JAK3 inhibitor Ⅵ showed growth suppression at 10 and 30 μmol/L, but gefitinib or JAK inhibitor I did not (Figure 3A). The effects of JAK3 inhibitor Ⅵ on proliferation of the WT EGFR expressing A431 (Figure 3B) and A549 (Figure 3C) cells were also analyzed. These cells were less sensitive to JAK3 inhibitor Ⅵ than NCI-H1975 cells.

To obtain structural insight, docking simulations between JAK3 inhibitor Ⅵ and kinases were performed. In the model structure, JAK3 inhibitor Ⅵ bound to the ATP-binding pocket of EGFR T790M/L858R and formed hydrogen bonds with M793 and T854 residues (Figure 4A). Estimated binding to EGFR T790M/L858R was more stable than that to the WT EGFR and was equivalent with that to JAK3 (EGFR T790M/L858R: -7.38 kcal/mol; JAK3: -7.59 kcal/mol; WT EGFR: -6.23 kcal/mol). Amino acid sequence alignments of the EGFR T790 flanking regions and the corresponding regions of JAKs revealed that the gatekeeper residues of WT JAKs are methionine, similar to those of EGFR T790M (Figure 4B).

DISCUSSION

EGFR T790M limits the clinical efficacy of EGFR-TKIs. Irreversible TKIs for EGFR T790M are associated with toxic effects due to simultaneous inhibition of the WT EGFR. In the present study, we found that JAK3 inhibitor Ⅵ selectively inhibits EGFR T790M/L858R and had a lesser inhibitory effect on the WT EGFR *in vitro*. JAK3 inhibitor Ⅵ also suppressed the proliferation of NCI-H1975 cells harboring EGFR T790M/L858R. Thus, our findings demonstrate that a JAK3 inhibitor is a mutant selective TKI for EGFR T790M/L858R and offer a potential strategy to develop EGFR T790M-specific inhibitors by applying known inhibitors for JAK family kinases closely related to the ErbB family.

JAK3 inhibitor Ⅵ selectively inhibits the gefitinib-resistant EGFR T790ML858R. JAK3 inhibitor Ⅵ (10 μmol/L) inhibited kinase activity of EGFR T790M/L858R; however, it was ineffective on the activity of the WT EGFR *in vitro* kinase assay (Figure 1C). Furthermore, JAK3 inhibitor Ⅵ efficiently reduced autophosphorylation of EGFR T790M/L858R in gefitinib-resistant lung cancer cells, NCI-H1975 (Figure 2A), in contrast to resistance of the WT EGFR in A431 cells (Figure 2B). Dosage restriction

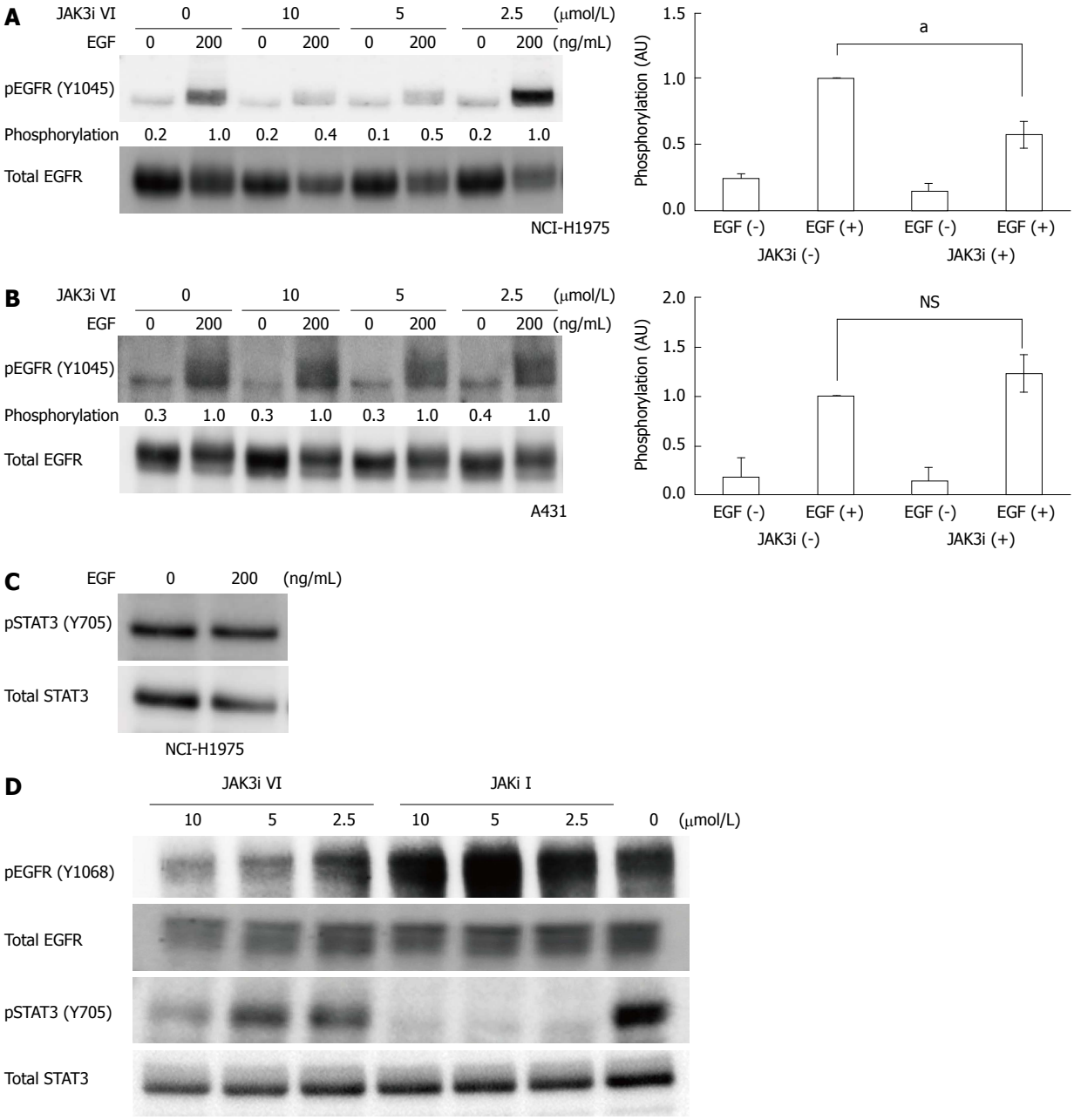

Figure 2 Janus kinase 3 inhibitor Ⅵ reduces epidermal growth factor receptor autophosphorylation in NCI-H1975 cells harboring epidermal growth factor receptor T790M/L858R. A, B: NCI-H1975 (A) or A431 (B) cells were treated with the indicated concentration of JAK3 inhibitor Ⅵ and were stimulated using 200 ng/mL EGF. Phosphorylation levels of EGFR Y1045 were analyzed using Western blotting. Phosphorylation levels of EGFR were normalized against total EGFR levels. Relative phosphorylation levels to the sample treated with DMSO and 200 ng/mL EGF are indicated. Right panels show the averages of relative phosphorylation levels from three independent experiments; C: NCI-H1975 cells were stimulated using 200 ng/mL EGF. Phosphorylation levels of STAT3 Y705 were analyzed using Western blotting; D: NCI-H1975 cells were treated using JAK3 inhibitor Ⅵ or JAK inhibitor I, as indicated. Phosphorylation levels of EGFR Y1068 and STAT3 Y705 were analyzed using Western blotting with phospho-specific antibodies. Error bars indicate SEM (n = 3); $^{a}P < 0.05$. NS: Not significant; EGF (-): 0 ng/mL EGF; EGF (+): 200 ng/mL EGF; JAK3i (-): 0 μmol/L JAK3 inhibitor VI; JAK3i (+): 10 μmol/L JAK3 inhibitor VI; EGFR: Epidermal growth factor receptor; JAK3: Janus kinase 3.

of quinazoline-based EGFR-TKI treatments is attributable to the toxicity derived from simultaneous inhibition of the WT EGFR. As a possible solution for the issue, Zhou *et al*[21] reported that compounds with a non-quinazoline structure, pyrimidine, were specifically effective on mutant EGFRs. Staurosporine-related indolocarbazole compounds such as Gö6976 and PKC412 are also more selective for EGFR T790M than quinazoline-based EGFR inhibitors[19]. Therefore, applications of non-quinazoline compounds can confer T790M mutant specific inhibition without affecting the WT EGFR. Our data indicate that JAK3 inhibitor Ⅵ, an indol-based compound, provides an additional example of EGFR T790M-specific structures.

JAK3 inhibitor Ⅵ suppresses proliferation of NSCLCs harboring the gatekeeper mutation EGFR T790M. EGFR T790M/L858R-expressing NCI-H1975 cells showed reduced proliferation in the presence of JAK3 inhibitor Ⅵ, but not in the presence of gefitinib or JAK inhibitor

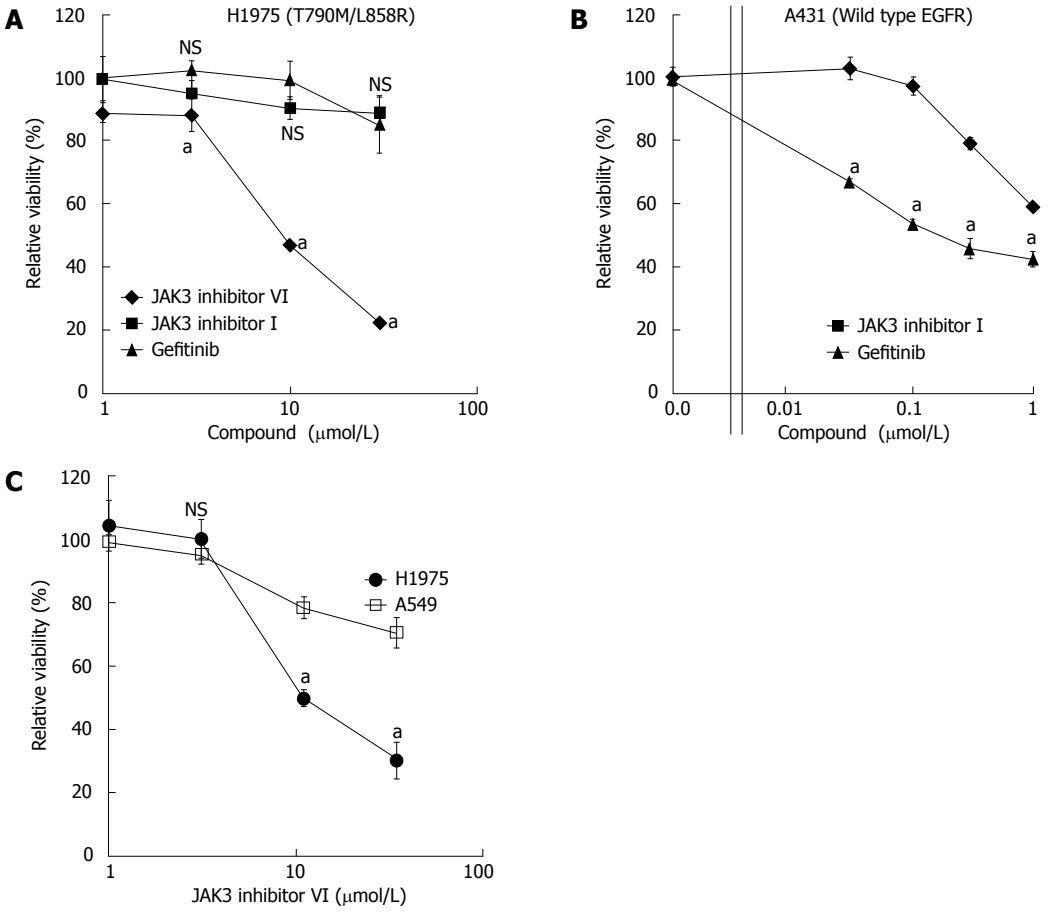

Figure 3 Janus kinase 3 inhibitor VI suppresses proliferation of NCI-H1975 cells. A-B: NCI-H1975 (A) or A431 (B) cells were treated with JAK3 inhibitor VI, JAK inhibitor I, or gefitinib. Effects of indicated concentration of kinase inhibitors on cell proliferation were analyzed using the MTT assay; C: Effects of JAK3 inhibitor VI on proliferation were compared between NCI-H1975 and A549 cells using the MTT assay. Error bars indicate S.E.M. (n = at least 3); $^{a}P < 0.05$. NS: Not significant; JAK3: Janus kinase 3; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide.

I (Figure 3A). Furthermore, WT EGFR-expressing cell lines, A431 and A549, were less sensitive to JAK3 inhibitor Ⅵ in comparison with NCI-H1975 cells (Figures 3B and C), suggesting specific inhibition of EGFR T790M by JAK3 inhibitor Ⅵ. Because JAK3 inhibitors block JAK3 that may phosphorylate EGFR, indirect effects *via* JAK3 inhibition may cause a reduction in autophosphorylation of EGFR T790M/L858R in NCI-H1975 cells. However, EGFR T790M/L858R inhibition was observed in lower concentrations of JAK3 inhibitor Ⅵ than those of JAK3 inhibition. Another JAK inhibitor, JAK inhibitor I, did not show EGFR T790M/L858R inhibition. Furthermore, T790M kinase inhibition was observed in an *in vitro* assay using recombinant kinases. Therefore, the decrease in autophosphorylation of EGFR T790M/L858R seems to be caused by direct inhibition in EGFR T790M/L858R rather than by JAK3 inhibition-mediated indirect effects.

Structural characteristics of EGFR T790M mutants may be analogous to those of JAK3. In the docking study, JAK3 inhibitor Ⅵ bound to the ATP-binding pocket of EGFR T790M/L858R with an additional hydrogen bond (Figure 4A). Binding energy calculations predicted that JAK3 inhibitor Ⅵ would more stably bind to EGFR T790M/L858R than to the WT EGFR with an equivalent stability to the simulated binding to JAK3 (Figure 4A). Furthermore, EGFR and JAK family kinases are located in neighboring clans in the human kinome[22], indicating that overall structures of the catalytic domains of JAKs are closely related to those of EGFRs. The gatekeeper residues of WT JAKs are methionines, which are identical to the gatekeeper mutant EGFR T790M (Figure 4B). Sequence alignments of the gatekeeper flanking regions between BCR-ABLs and Aurora kinases indicated that the corresponding residues in Aurora kinases are isoleucines, similar to the gatekeeper mutation T315I. These findings suggest a hypothesis in which inhibitor sensitive kinases such as EGFR L858R, exon19-deleted EGFR, and BCR-ABL obtained structural similarities to other kinases during acquisition of drug resistance. In fact, Aurora kinase inhibitors are effective on BCR-ABL T315I[23,24] in addition to our findings of JAK3 inhibitor Ⅵ as an EGFR T790M inhibitor. Therefore, it may be a rational strategy to screen compounds effective on different kinases structurally similar to gatekeeper mutants in the fight against drug resistant kinases.

ACKNOWLEDGMENTS

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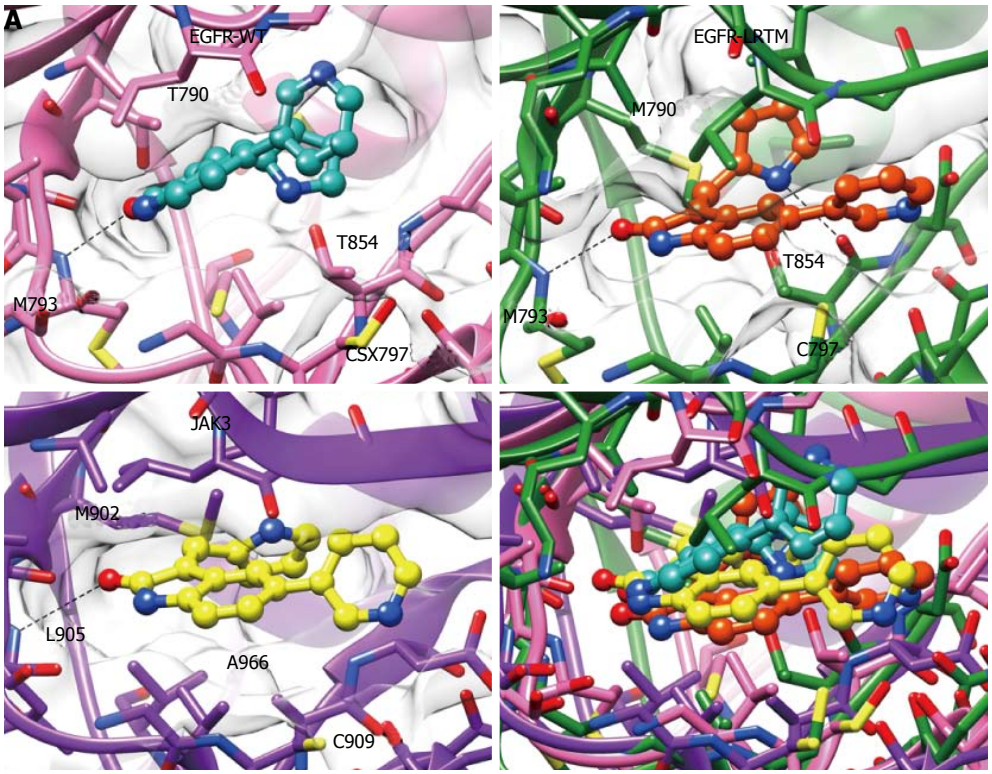

B

	Gatekeeper *	
EGFR WT	786 VQLITQLMP	794
EGFR T790M	786 VQLIMQLMP	794
JAK1	952 IKLIMEFLP	960
JAK2	925 LKLIMEYLP	933
JAK3	898 LRLVMEYLP	906
BCR-ABL WT	311 FYIITEFMT	
BCR-ABL T315I	311 FYIIIEFMT	319
Aurora kinase A	205 RVYLILEYA	213
Aurora kinase B	149 RIYLILEYA	157
Aurora kinase C	115 RVYLILEYA	123

Figure 4 Structural analysis of janus kinase 3 inhibitor VI binding to the epidermal growth factor receptor T790M. A: JAK3 inhibitor VI (cyan in EGFR WT: top left, red in EGFR T790M/L858R: top right, or yellow in JAK3: bottom left) was modeled into EGFR WT, EGFR T790M/L858R (LRTM), or JAK3. A superimposed image is also shown (bottom right). The dotted lines indicate putative hydrogen bonds; B: Amino acid sequence alignments of EGFRs and JAKs, and BCR-ABL and Aurora kinases. Asterisk indicates the gatekeeper residues. EGFR: Epidermal growth factor receptor; JAK: Janus kinase; WT: Wild type.

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COMMENTS

Background

Tyrosine kinase inhibitors against the epidermal growth factor receptor (EGFR-TKIs) are initially effective on non-small cell lung cancers (NACLCs) caused by EGFR mutations. However, drug-resistant mutations such as the gatekeeper mutation T790M limit the clinical efficacy of EGFR-TKIs.

Research frontiers

Irreversible EGFR-TKIs that covalently bind to the EGFR kinase domain have provided a way to inhibit the gatekeeper mutant EGFR T790M.

Innovations and breakthroughs

EGFR T790M inhibition by the quinazoline-based irreversible TKIs causes simultaneous inhibition of the WT EGFR, resulting in adverse effects such as skin rash and diarrhea. The present study rediscovered Janus kinases 3 (JAK3) inhibitor VI as a reversible EGFR-TKI selective for EGFR T790M.

Applications

The authors' structural analysis revealed similarities among EGFR T790M and Janus kinases (JAKs). This may offer a new strategy to screen for EGFR T790M inhibitors from known kinase inhibitors.

Terminology

JAKs are protein tyrosine kinases, whose catalytic domain structures are closely related to those of EGFRs.

Peer-review

In current manuscript entitled "JAK3 inhibitor VI is a mutant specific inhibitor for EGFR with gatekeeper mutation T790M", the authors demonstrated that JAK3

inhibitor VI could specifically inhibit EGFR gatekeeper mutation (T790M) in non-small cell lung cancers.

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