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REVIEW

Gene editing for corneal disease management

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Abstract

Gene editing has recently emerged as a promising technology to engineer genetic modifications precisely in the genome to achieve long-term relief from corneal disorders. Recent advances in the molecular biology leading to the development of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and CRISPR-associated systems, zinc finger nucleases and transcription activator like effector nucleases have ushered in a new era for high throughput *in vitro* and *in vivo* genome engineering. Genome editing can be successfully used to decipher complex molecular mechanisms underlying disease pathophysiology, develop innovative next generation gene therapy, stem cell-based regenerative therapy, and personalized medicine for corneal and other ocular diseases. In this review we describe latest developments in the field of genome editing, current challenges, and future prospects for the development of personalized gene-based medicine for corneal diseases. The gene editing approach is expected to revolutionize current diagnostic and treatment practices for curing blindness.

Key words: Adeno-associated virus; Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9; Cornea; Clustered Regularly Interspaced Short Palindromic Repeat; Double strand breaks; Gene editing; sgRNA; Gene targeting; Homology directed repair; Homologous recombination; Indels; Lentiviral vector;

Protospacer-adjacent motif; Transcription activator like effector nucleases; Zinc finger nucleases

Core tip: Gene editing technology including Clustered Regularly Interspaced Short Palindromic Repeats/Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9, zinc finger nucleases, or transcription activator like effector nucleases has great potential for generating *in vitro* and *in vivo* models of corneal diseases including keratoconus and Fuchs' endothelial corneal dystrophy. Furthermore, gene editing is a powerful tool for studying molecular mechanisms mediating corneal development, pathogenesis and developing next generation innovative gene therapies including the patient-specific personalized medicine for curing corneal diseases. This review discusses current status and latest developments in the field of gene editing. Gene editing based molecular therapy has the potential to revolutionize current practices in ophthalmology clinic for curing corneal blindness.

Raikwar SP, Raikwar AS, Chaurasia SS, Mohan RR. Gene editing for corneal disease management. *World J Transl Med* 2016; 5(1): 1-13 Available from: URL: http://www.wjgnet.com/2220-6132/full/v5/i1/1.htm DOI: http://dx.doi.org/10.5528/wjtm.v5.i1.1

INTRODUCTION

According to World Health Organization ocular diseases affect about 285 million people worldwide. It is estimated that over 39 million people suffer from blindness and 246 million people have low or impaired vision worldwide. In the United States, vision impairment is among the top ten disabilities according to the Centers for Disease Control and Prevention. According to the National Eye Institute, approximately 38 million people have vision impairment in the United States with an annual cost of over $68.8 billion for medical care. If the present increasing trend in eye disease continues, it is estimated that by 2050 the patient volume with blindness will increase by 150% with a corresponding increase of 250% in direct medical costs leading to an economic burden of $717 billion. To break this increasing trend and fulfill unmet clinical needs, it is imperative to develop novel next generation gene-based molecular therapies for ocular disease.

Cornea is the transparent tissue in front of the eye. It provides two thirds of refractive power and protection to the eye[1]. Trauma, injury and/or infection to the eye are known to compromise corneal transparency and cause corneal fibrosis and/or neovascularization. Corneal diseases are the second leading cause of blindness globally with an estimated 23 million patients and nearly 80% of all corneal blindness is preventable. Corneal defects are one of the most prevalent reasons for vision impairment worldwide. About 4% of the United States population has corneal disorders and approximately 1.5 million additional people experience corneal blindness each year. It is more pronounced in developing countries especially among children due to trachoma which alone causes blindness in 4.9 million people worldwide[2,3]. The current treatments for corneal blindness offer only short-term relief, require repeated drug application, meticulous patient compliance, cause side effects, and often fail. The surgical corneal transplantation is typically used to restore vision, requiring donor corneas which are not available in many countries, and their availability in America is sharply declining due to laser surgeries, hepatitis, human immunodeficiency virus (HIV), *etc*. Therefore there is an urgent need to develop novel corneal disease models and therapeutic strategies to treat corneal diseases. Over the past several years, the major focus of our research has been on the development of novel strategies for gene therapy to treat corneal diseases using adeno-associated virus (AAV) and nanoparticles[1,4-11]. Our lab has demonstrated that various AAV serotypes could be successfully used to deliver therapeutic genes to treat corneal diseases with varying transduction efficiency without major side effects. Our ongoing research suggests that AAV and nanoparticle vectors are essential for achieving intended gene editing in the cornea.

Gene targeting by homologous recombination has been the gold standard for generating germ-line targeted gene knockout and knock-in mice[12,13]. Ocular cells represent a unique platform to investigate emerging technologies to gain an insight in to the precise molecular mechanisms underlying the disease as well as to develop novel personalized therapeutic strategies. According to clinicaltrials.gov there are currently multiple clinical studies on gene therapy and stem cell based regenerative medicine for ocular diseases. However, gene-targeting strategies in human embryonic stem (hES) and human induced pluripotent stem (hiPS) cells are relatively more cumbersome, inefficient, time consuming, expensive and challenging[14]. As a result, several studies have utilized small interfering RNA and short hairpin RNA to knockdown multiple genes. There are several major caveats of this approach including non-specificity, off target effects, altered cellular physiology, toxicity and only a transient reduction in gene expression leading to an incomplete or partial knockdown effect[15-19]. To overcome these limitations, it is imperative to modify the host genome precisely. The recent advances in gene editing have led to a widespread enthusiasm and significant improvements in this direction. In this review, we describe the current and emerging tools for gene editing, and their potential applications in the treatment of ocular diseases.

ZINC FINGER NUCLEASES

Zinc finger nucleases (ZFNs)'s belong to the first generation of gene editing tools based on the pioneering work of Kim *et al*[20-23]. ZFNs are designer nucleases that

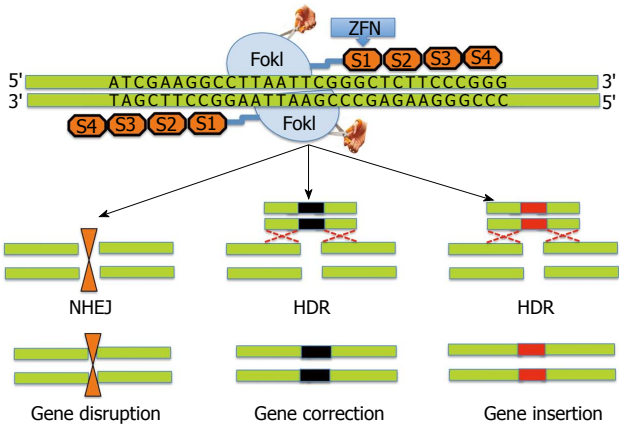

Figure 1 Schematic diagram showing structure and design of a typical zinc finger nuclease. Zinc finger nucleases (ZFNs) use a modular array of 3-6 ZFNs (4 shown) specifically designed to bind to the target DNA together with the FokI cleavage domain. The FokI cleavage domains can be engineered to function as heterodimers or homodimers to achieve desired cleavage specificity. ZFNs typically recognize 24-36 bp unique sequence within the genome to achieve target specificity. ZFN mediated cleavage of the target leads to double strand breaks, which in turn induces either non-homologous end joining pathway (NHEJ) or homology directed repair (HDR) processes. NHEJ leads to gene disruption due to small insertions or deletions (indels) while HDR leads to gene correction.

combine the DNA binding domain of eukaryotic transcription factors-zinc finger proteins with the nuclease domain of the *FokI* restriction enzyme[24,25]. In ZFNs, tandem arrays of Cys_2His_2 zinc fingers provide DNA binding specificity through recognition of approximately 3 base pairs of the target DNA. The catalytic domain of *FokI* requires dimerization to cleave the DNA at the targeted site and two adjacent ZFNs to independently bind to a specific codon with correct orientation and spacing. ZFNs work by introducing site-specific DNA double strand breaks (DSB) at a predetermined genomic locus. The DSB introduced by ZFNs undergo repair in the eukaryotic cells by either homology directed repair (HDR) process or non-homologous end joining pathway (NHEJ)[26-28]. DNA repair by homologous recombination leads to preservation of the original DNA sequence in the targeted cells rendering them vulnerable to re-cutting by ZFNs. In contrast, NHEJ can potentially lead to random insertion or deletion of nucleotides at the target break site thereby causing permanent disruption of the original DNA sequence. Figure 1 shows schematic representation of ZFN technology.

A previous study by Urnov *et al*[29] has demonstrated that ZFNs designed against X-linked severe combined immune deficiency (SCID) mutation in the *IL2R* gamma gene yielded > 18% gene-modified human cells with about 7% cells exhibiting desired genetic mutation on both X chromosomes. It has been previously demonstrated that HIV-1 uses the co-receptor CCR5, a validated target for HIV therapy[30,31]. Surprisingly, allogeneic stem cell transplant of a naturally occurring homozygous CCR5 deletion mutant (CCR5Δ32/Δ32) led to the elimination of HIV-1 in a patient[32]. Despite the low frequency of naturally occurring CCR5Δ32/Δ32 mutation, researchers have successfully harnessed the potential of ZFNs to disrupt *CCR5* gene expression in hematopoietic stem and progenitor cells using a recombinant adenoviral vector encoding CCR5-specific ZFNs[33]. Recently, ZFNs have shown potential therapeutic benefits in clinical trials[34-36]. In a recent open-label phase I clinical study, HIV patient-derived autologous CD4 T cells were subjected to ZFN-mediated gene editing to render them resistant to HIV by knocking out *CCR5* gene[36].

While the promise and feasibility of ZFN technology for gene editing has been demonstrated, multiple challenges remain. For example, ZFNs are relatively difficult to generate and are very expensive. Additionally, ZFNs can be non-specific and may result in off-target cleavage leading to multiple DSBs, which in turn can cause chromosomal rearrangements. These issues were addressed by developing ZFN variants that have ability to reduce off-target non-specific mutagenesis. The ZFN variants include a mix of two distinct ZFNs with different *FokI* domains that are obligate heterodimers, which introduce DSBs only when two distinct ZFNs are able to bind adjacent DNA regions[37-39].

TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASES

Another approach to administer gene editing has subsequently emerged through the recognition of a new class of designer nucleases termed transcription activator-like effector nucleases (TALENs). The gene editing steps associated with TALEN technology are presented in Figure 2. Transcription activator-like effectors (TALEs) are proteins secreted by *Xanthomonas* bacteria to subvert the host genome regulatory networks and can be engineered to bind any desired target sequence[40-43]. TALEs have a DNA binding module termed TAL repeat, which is used by each protein in a tandem array with 10-30 repeats to recognize extended DNA sequences with a ratio of 1 TAL repeat to 1 base pair of DNA sequence[43]. Each repeat in turn has about 33-35 amino acids with 2 adjacent amino acids [Repeat Variable Di-residue (RVD)], which confer their specificity for the DNA bases[40,44]. Decoding of the RVD has led to the development of a new class of designer nucleases called TALENs that contain an array of TAL repeats fused to *FokI* nuclease domain[45-47].

As compared to ZFNs, TALENs are relatively easier to design and generate due to their modular nature[48]. The promise of TALEN approach has been successfully demonstrated through the generation of gene-knockout animal models of *C. elegans*, rats, mice and zebra fish[49-53]. Deml *et al*[53] have developed zebrafish mutants carrying *MAB21L2* gene to model human ocular coloboma. Homozygous *mab21l2*^{Q48Sfs*5} zebrafish mutant embryos exhibit severe lens and retinal defects with complete lethality while *mab21l2*$^{R51\_F52del}$ mutants display a milder lens phenotype and severe coloboma. This study demonstrates the power of genome editing

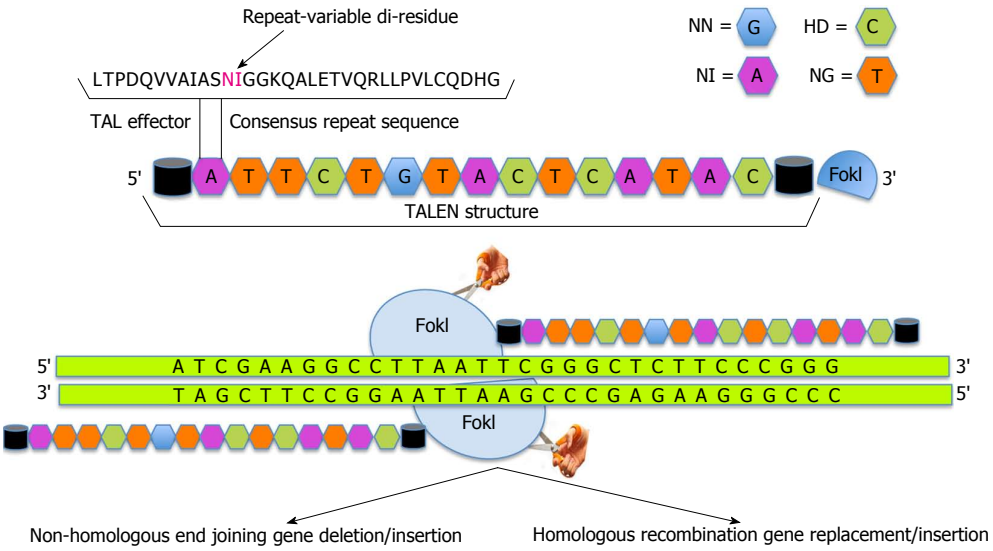

Figure 2 Transcription activator-like effector nucleases. In transcription activator-like effector nucleases (TALENs) the nuclease effector domains of FokI are fused to TALE DNA binding domains. Since FokI is active only as a dimer, pair of TALENs are constructed to position FokI nuclease domains adjacent to genomic target sites. Like zinc finger nucleases, dimerization of TALENs leads to double strand breaks that is repaired by either error prone non-homologous end joining pathway thereby leading to frameshift mutations (deletions, insertions or frameshift) if exons are targeted or homology directed repair which can be utilized to introduce non-random mutations, targeted deletion or addition of large fragments.

in model organisms for studying molecular mechanisms underlying human ocular diseases. TALENs have recently been exploited to develop genetically engineered hES cell lines, hiPS cells and mouse disease models[45,54-57]. Experimental correction of genetic defects *in vitro* has been successfully achieved by TALENs in hemophilia[54], mitochondrial diseases[58,59], and Duchenne muscular dystrophy[60]. To demonstrate the potential utility and efficiency of TALENs, Ding *et al*[61] have successfully generated mutant alleles of 15 genes in cultured somatic cells or human pluripotent stem cells. In an interesting study, Kim and colleagues have generated a library of 18740 TALEN pairs (http://www.talenlibrary.net/) to disrupt or modify every protein-coding gene for the entire human genome using a high throughput Golden-Gate cloning system[62]. In another study, Menon *et al*[63] utilized iPS cell technology and TALENs to generate a subject-specific mutant gene-corrected iPS cell lines for the treatment of X-linked SCID. It is interesting to note that while the subject derived mutant iPS cells could generate hematopoietic precursors and myeloid cells, only wild-type and gene corrected iPS cells could additionally generate mature cells and T cell precursors expressing the correctly spliced IL2R gamma. The work also suggests that TALEN technology can be employed for the manipulation of immune processes and chronic inflammatory diseases in the eye including corneal inflammatory disorders and diabetic retinopathy. Indeed, scores of further studies are needed to harness the bench-to-bedside potential of this approach and move forward towards the development of an autologous patient-based cell therapy.

The reversal of malignant phenotype *via* TALEN technology has been recently reported. Hu *et al*[64] have demonstrated that genome editing of human papilloma virus (HPV) oncogenes E6/E7 by TALENs efficiently reduced viral DNA load, restored the function of tumor suppressor p53/RB1, and reversed the malignant phenotype of host cells both *in vitro* as well as *in vivo*. In this study, HPV E6/E7 specific TALENs were effective in inducing apoptosis, inhibiting growth and reducing tumorigenicity in HPV positive cell lines. Further, direct cervical application of HPV E7 targeted TALENs efficiently mutated the E7 oncogene and reversed the malignant phenotype in K14-HPV16 transgenic mice. The study suggested two possible mechanisms for the reversal of the malignant phenotype. Firstly, TALENs specifically recognized and cleaved HPV DNA sequence in host cells leading to DSBs that directly induced apoptosis and suppressed their proliferation. Secondly, the cells that survived genotoxic stress, activated DSB repair *via* NHEJ pathway causing E6/E7 mutation. This led to the activation of E6/E7-inhibited tumor suppressor p53/RB1 and downregulation of CDK2 and E2F1. The ongoing experiments in our laboratory are attempting to generate *in vitro* and *in vivo* models and newer therapeutic approaches for corneal disorders and dystrophies using TALEN technology. This powerful gene editing approach has been particularly useful in studying keratoconus and Fuchs' endothelial corneal dystrophy.

CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS AND CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEAT ASSOCIATED SYSTEMS

Clustered Regularly Interspaced Short Palindromic

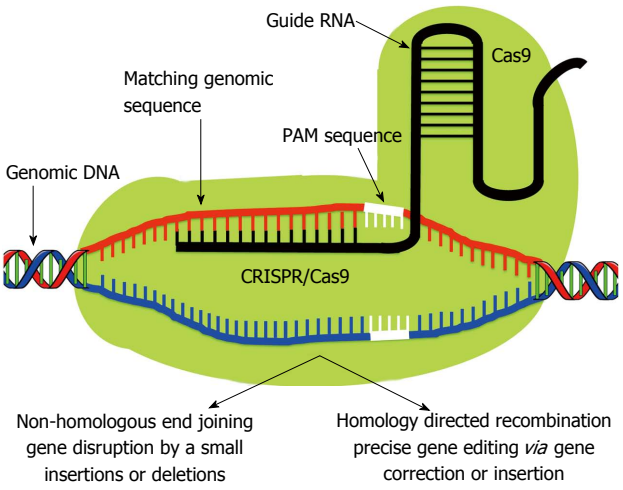

Figure 3 Clustered Regularly Interspaced Short Palindromic Repeat/Clustered Regularly Interspaced Short Palindromic Repeat Associated Systems. In contrast to Like zinc finger nucleases and transcription activator-like effector nucleases, Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated protein (Cas9) monomer possess innate nuclease activity which catalyzes double strand breaks leading to random knockout phenotypes *via* non-homologous end joining pathway. Therefore Cas9 requires a single guide RNA (sgRNA) to recognize its target site. The sgRNA is composed of two separately expressed RNAs including a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA), which are processed by endogenous bacterial machinery to yield the mature gRNA. The current CRISPR/Cas9 system employs a single chimeric sgRNA, which is a fusion of crRNA and tracrRNA. Currently used sgRNA typically contains a 17-20 nucleotide long variable region, which is complementary to the genomic target sequence. A short region immediately 3' to the target sequence known as protospacer adjacent motif has NGG sequence which is a major specificity determinant of Cas9. PAM: Protospacer-adjacent motif.

Repeat (CRISPR)/Clustered Regularly Interspaced Short Palindromic Repeat Associated Systems (Cas9), derived from the bacterial adaptive immune system, has tremendous potential for achieving precise *in vitro* and *in vivo* gene editing[65-69]. Figure 3 depicts the core principle of this approach for obtaining intended gene editing in the genome. For the sake of convenience, Figure 4 provides a side-by-side comparison between TALENs and CRISPR/Cas9 systems. CRISPR/Cas9 based gene editing relies on co-expression of the bacterial Cas9 endonuclease and a short guide RNA (sgRNA) sequence to generate DNA DSBs in eukaryotic cells. The excision occurs at genomic sites that have a short homologous sequence to the 5' end of the sgRNA followed by an NGG sequence called protospacer-adjacent motif (PAM)[66,70]. Since DNA DSB are primarily repaired through the error-prone NHEJ pathway in eukaryotes *via* small indels generated at the target sites. Therefore, CRISPR/Cas9 system provides a simple and cost-effective approach to simultaneously disrupt the open reading frames of multiple coding genes to produce loss/gain of function alleles at a high versatility[71-78]. CRISPR/Cas9 system has been successfully used for genome editing in *C. elegans*, Drosophila, mosquito, zebrafish, mouse, rat and human[79-90]. Cas9 nucleases cleave the double stranded DNA through the activity of their RuvC and HNH nuclease domains to generate DSBs. Cas9 can be engineered to cut only one strand of the DNA by catalytically inactivating either the RuvC or HNH nuclease domains[66,91,92]. These newly designed Cas9 nickases offer a unique approach to gene editing with high fidelity and specificity.

Recently, Chen *et al*[93] have successfully combined tamoxifen-inducible CRISPR/Cas-mediated genome editing with Flp/FRT and Cre/LoxP system to generate inducible gene knockout hPSC lines. They found that targeting dual sgRNA was essential for biallelic knockin of FRT sequences to flank the exon. They further developed a strategy to simultaneously insert an activity controlled recombinase-expressing cassette and removed the drug-resistance gene thereby enhancing the generation of *SOX2, PAX6, OTX2* and *AGO2* inducible knockout human ES and iPS cell lines. The target genes in these cell lines can be uniformly deleted at any given time by simple application of 4-OHT.

Wu *et al*[94] have recently reported successful correction of *Crygc* gene mutation that causes cataracts in mice. In this study, a dominant mutation in *Crygc* gene could be rescued in mouse zygotes by co-injection of Cas9 mRNA and a sgRNA targeting the mutant allele. Correction in the *Crygc* gene occurred by HDR based on an exogenously supplied oligonucleotide or the endogenous wild type allele, with only rare evidence of off-target modifications. The resulting mice were fertile and were able to transmit the corrected allele to their progeny. Similarly, Courtney *et al*[95] have examined the potential of an allele-specific CRISPR/Cas9 system for hereditary corneal dystrophies by specifically focusing on a dominant-negative mutation in KRT12, Leu132Pro which results in Meesmann's epithelial corneal dystrophy. Further, Zhong *et al*[96] have utilized the CRISPR/Cas9 system to generate *Kcnj*13 mutant mice, which mimic human *KCNJ*13-related Leber congenital amaurosis, an early form of blindness.

The studies discussed above provide proof of principle for the application of CRISPR/Cas9 system in developing models of corneal dystrophies and personalized therapeutics for treating ocular diseases.

GENE EDITING FOR CORNEAL DISEASE MANAGEMENT

Cornea is an ideal target tissue for the development of personalized therapy. Gene editing approaches can successfully be used to develop novel corneal disease models. For example, it is possible to develop disease in a dish model for corneal dystrophies using patient derived corneal tissues. However, there are multiple challenges that need to be overcome before gene editing for corneal disease management becomes a reality. One of the major challenges is the lack of an authentic *in vitro* corneal endothelial cell culture model. This is because feline and human corneal endothelial cells are extremely difficult to culture. To overcome this major limitation, we have recently established reversibly immortalized

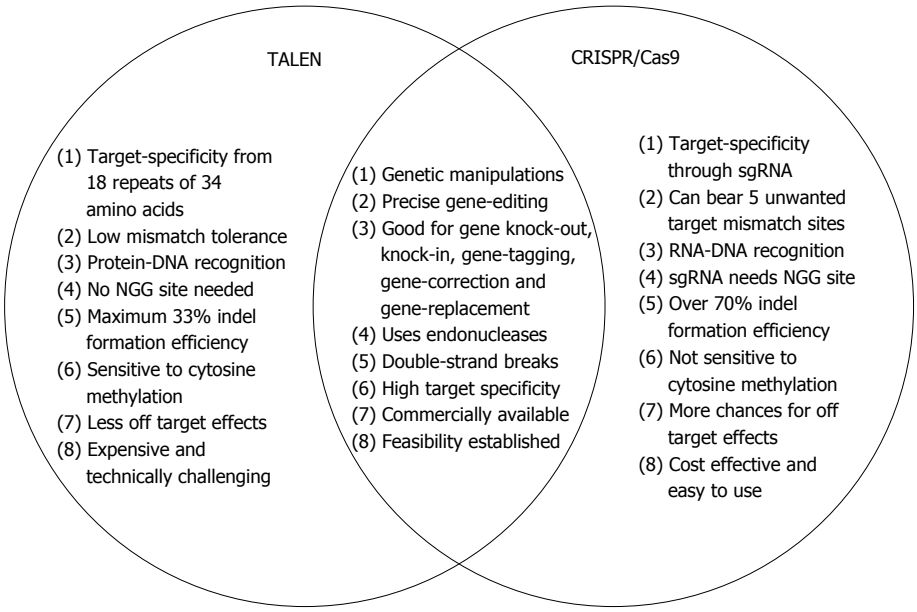

Figure 4 Venn diagram of transcription activator-like effector nucleases and Clustered Regularly Interspaced Short Palindromic Repeat. The schematic Venn diagram shows potential differences and similarities between transcription activator-like effector nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) systems. The gold standard to decipher the gene function is to selectively knockout or disrupt the gene expression and analyze the resulting phenotypes. Both TALENs and CRISPR are promising and powerful gene editing tools that allow complete loss-of-function reverse genetics approaches to study gene function. sgRNA: Single guide RNA; Cas9: Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9.

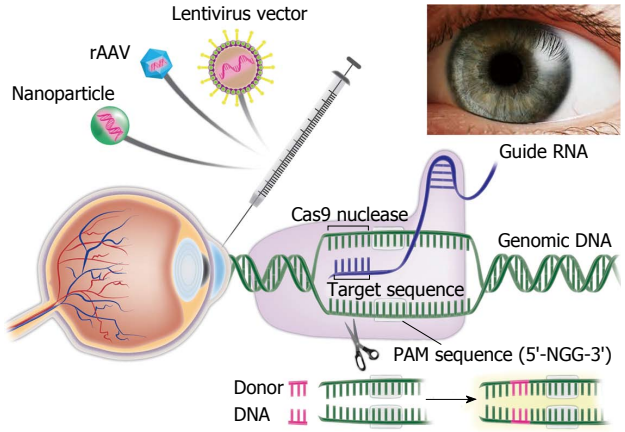

Figure 5 Application of Clustered Regularly Interspaced Short Palindromic Repeat/Clustered Regularly Interspaced Short Palindromic Repeat Associated Systems to develop novel therapies for corneal diseases. Corneal Delivery of Clustered Regularly Interspaced Short Palindromic Repeat/Clustered Regularly Interspaced Short Palindromic Repeat Associated System using recombinant adeno-associated virus, integrase deficient lentiviral vectors and nanovectors can be used to potentially target multiple corneal diseases especially Fuchs' endothelial corneal dystrophy to develop novel disease models as well as innovative personalized gene and stem cell therapies. PAM: Protospacer adjacent motif. Cas9: Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9.

feline and human corneal endothelial cell lines using Doxycycline inducible lentiviral vector system expressing human papillomavirus E6/E7 chimeric gene product. These immortalized feline and human corneal endothelial cell lines are valuable to study pathophysiology as well as molecular mechanisms regulating dystrophies and wound healing in the cornea. Currently there is no *in vivo* model for Fuchs' endothelial corneal dystrophy. We are attempting to develop novel Fuchs' endothelial corneal dystrophy models employing CRISPR/Cas9 gene editing technology and conditionally immortalized corneal endothelial cells (Figure 5). Further, gene editing can be used on patient derived iPS cells to develop novel corneal disease models. Gene editing can be used to treat corneal fibrosis and neovascularization by targeting pathologic genes, microRNAs, long noncoding RNAs, and/or signaling pathways driving corneal wound repair. Combat related traumatic corneal injuries present an ideal target where gene editing can be applied to maximize wound healing and tissue regeneration in corneal tissue without major adverse effects. Viral vectors and nanoparticles offer a novel platform to accomplish gene editing in corneal tissue. Real-time noninvasive intravital imaging will allow precise monitoring of gene editing success in an *in vivo* experimental animal model. Overall, there is tremendous potential of gene editing technology for corneal disease management as depicted in Table 1.

CURRENT CHALLENGES AND FUTURE DIRECTIONS

The current major limitations in the field of gene editing include concerns regarding specificity, efficiency, and delivery of designer nucleases (ZFNs, TALENs and CRISPR/Cas9). The non-viral delivery systems including electroporation and protein transfection of designer nucleases have shown promising results with limited applications. The cell-specific delivery of designer nucleases such as CRISPR/Cas9 could be achieved through the recombinant viral vectors including adeno-associated

Table 1 Application of gene editing for corneal disease management

Disease	Target genes for gene editing
Corneal fibrosis	BMP7, CTGF, Decorin, Hevin, Moesin, Smad2, Smad3, Smad4, Smad7, TGFβ1, TGFβR2, TRPA-1, Twist2, Vimentin
Corneal wound healing	CTGF, CNTF, EGF, EGFR1, EGFR2, Fibronectin, IGF, KGF, Laminin, Lumican, MIF, MMP-1, MMP-2, MMP-3, MMP-9, NGF, OGF, PAI-1, PAF, PDGF, rho-associated protein kinase (ROCK), TGFβ1, TGFβ2, TGFβ3, TLR4, TIMP-2, Vasohibin
Corneal neovascularization	Angiopoietin 1, Angiopoietin 2, Angiostatin, βFGF, Endostatin, FGFR-1, FGFR-2, FGFR-3, FGFR-4, FOXC1, HGF, IGF, IL-8, IL-1, Leptin, MMP-2, MMP-9, MMP-14, Netrin-1, Netrin-4, Neuropilin-2, NF-κB, PAI-1, PDGF, PEDF, PGF, Prox-1, ROCK, TNFα, TGFβ, TSP-1, Tie2, VCAM-1, VE-Cadherin, VEGF, VEGFR-1, VEGFR2, VEGFR-3
Keratoconus	BANP-ZNF469, LOX, BNIP3, CAST, CLF1, COL4A4, COL5A1, CPT1B, CPTB1B, DOCK9, IL-1A, IL-1B, IPO5, KRT72, MPDZ-NFIB, NEFL, Noxa, PMAIP1, RAB3GAP1, SLC25A2, SLC25A4, SLC25A31, SOD2, STK24, TGFB1, TIMP1, TIMP3, UCP1, UCP3, VSX1, ZEB1
Congenital hereditary endothelial dystrophy	SLC4A11
Epithelial basement membrane dystrophy	TGFBI
Francois-neetens mouchetee fleck corneal dystrophy	PIKFYVE (PIP5K3)
Fuchs' endothelial corneal dystrophy	APEX1, AGBL1, COL8A2, LOXHD1, NOX4, SLC4A11, SnaI1, TCF4, TCF8, ZEB1
Granular corneal dystrophy type 2	TGFBI, TGFBIp
Gelatinous drop-like corneal dystrophy	TACSTD2
Macular corneal dystrophy	CHST6
Meesmann epithelial corneal dystrophy	KRT3, KRT12
Posterior polymorphous corneal dystrophy	COL8A2, VSX1, ZEB1
Reis-Bücklers' and Thiel-Behnke Corneal dystrophies	TGFBI
Schnyder corneal dystrophy	UBIAD1

TGFβ1: Transforming growth factor beta 1; TGFβR1: Transforming growth factor btea receptor 2; EGF: Epifdermal growth factor; MMP-1: Matrix metalloproteinase-1; TIMP-2: Tissue inhibitor of metalloproteinases metallopeptidase inhibitor 2; TLR4: Toll-like receptor 4; IL: Interleukin; NF-κB: Nuclear factor kappa B; TNFα: Tumor necrosis factor alpha.

virus (rAAV), integrase deficient lentivirus, baculovirus, adenovirus or nanoparticle vectors. Our laboratory has successfully identified rAAV, disabled lentivirus and nanoparticle vectors for delivering therapeutic genes into keratocytes of the mouse and rabbit corneas *in vivo* and human and canine corneas using *ex vivo* organ culture models[4,97]. The restricted cloning capacity and challenges associated with packaging of the expression cassettes limit the use of current hybrid rAAV vectors. However, recently two different promising strategies have been successfully employed to overcome the packaging limitations of rAAV. A strategy developed by a commercial vendor, proposed that *Cas9* gene could be split between pAAV-Guide-it-Up and pAAV-Guide-it-Down plasmids with 1.6 kb region of homology. In this system, sgRNA sequence against the genomic sequence of interest could be cloned into pAAV-Guide-it-Down plasmid and two separate recombinant AAVs (AAV-Up and AAV-Down) could be generated and co-transduced into target cells. Due to precise homologous recombination at the site of homology, full-length Cas9 gene driven by an upstream promoter is generated in the targeted cells leading to successful genome editing. Employing a different strategy, Ran *et al*[98] have recently identified six smaller Cas9 orthologs. These authors showed that Cas9 from *Staphylococcus aureus* (SaCas9) could edit the genome with efficiencies similar to those of *Staphylococcus pyogenes* (SpCas9) despite being more than 1 kilobase shorter[98]. In these studies SaCas9 and its sgRNA expression cassette were packaged into hepatocyte tropic rAAV8 to target the cholesterol regulatory gene pro-protein convertase subtilisin/kexin type 9 (*Pcsk9*) in the mouse liver. Following systemic delivery with rAAV, > 40% genome modification accompanied by significant reduction in serum *Pcsk9* and total cholesterol levels was observed. Further, the specificity of SaCas9 was confirmed using an unbiased DSB detection method, BLESS to identify a list of candidate off-target cleavage sites. These studies highlight the potential of newer SaCas9 for AAV-mediated *in vivo* genome editing applications.

The possibility of undesired genetic modification is a major concern associated with current gene editing technologies. To minimize off-target activity of Cas9, Ran *et al*[99] have recently developed an approach that simultaneously combines a Cas9 nickase mutant with paired guide RNAs to introduce targeted DSB. Since individual nicks in the genome are repaired with high fidelity, simultaneous nicking *via* appropriately offset guide RNAs is required for DSB and extends the number of specifically recognized bases for target cleavage. This versatile strategy can reduce off target effects by 50- to 1500-fold in cell lines and therefore has a great potential for genome editing applications that require high fidelity as well as high specificity.

In yet another interesting study, Suzuki *et al*[100] have performed whole genome sequencing to evaluate the mutational load at single base resolution in individual gene-corrected hiPS cells derived from Hutchinson-Gilford progeria syndrome, sickle disease and Parkinson's disease patients. They have reported that in single cell clones, gene correction by helper-dependent adenoviral vector (HDAdV) or TALEN exhibited few off-target effects and a low level of sequence variation. Furthermore, they

Table 2 Potential applications of zinc finger nucleases, transcription activator-like effector nucleases and Clustered Regularly Interspaced Short Palindromic Repeat/Clustered Regularly Interspaced Short Palindromic Repeat Associated Systems to develop novel disease models and innovative therapeutic strategies

Target gene	Target cell	ZFN/TALEN/CRISPR	Disease	Ref.
α-Globin	Human iPS	ZFN	α-thalassemia	[104]
Tnfrsf9	NOD mouse embryo	ZFN	Diabetes	[105]
HBV	Huh7 cells	ZFN	Hepatitis B	[106]
CCR5, CXCR4	$CD4^+$ T cells	ZFN	HIV	[107]
CCR5, IL2RG	Multiple	ZFN	HIV, X-SCID	[108]
TCRα, β	T cells	ZFN	Leukemia	[109]
HBB	Human iPS cells	ZFN	Sickle cell anemia	[110]
PIG-A	Human ES, iPS cells	ZFN	PNH	[111]
gp91(phox)	Human iPS cells	ZFN	X-CGD	[112]
Albumin	Mouse hepatocytes	ZFN	Hemophilia A and B	[113]
SCN1A	Human iPS	TALEN	Epilepsy	[114]
PSIP1	HT1080, 293T, Jurkat	TALEN	HIV	[115]
HBB	Human iPS cells	ZFN/TALEN/CRISPR	Sickle cell anemia	[116]
gp91(phox)	Human iPS cells	TALEN	X-CGD	[117]
Ctnnb1, Apc	H2.35	TALEN	Hepatocellular carcinoma	[118]
hFVIII	Human iPS cells	TALEN	Hemophilia A	[119]
PLN R14del	Human iPS cells	TALEN	Cardiomyopathy	[120]
BUB1B	HCT116	TALEN	PCS (MVA)	[121]
MECP2	Monkey zygotes	TALEN	Rett syndrome	[122]
Sry, Uty	Mouse blastocysts	TALEN	NA	[123]
Dystrophin	Myoblasts	CRISPR/Cas9	DMD	[124]
FANCC	Patient fibroblasts	CRISPR/Cas9	Fanconi anemia	[125]
APC, SMAD4, TP53, KRAS, PIK3CA	Human intestinal epithelial organoids	CRISPR/Cas9	Colorectal cancer	[126]
FAH	Mouse liver	CRISPR/Cas9	Tyrosinemia	[127]
PTEN, TP53	Mouse liver	CRISPR/Cas9	Liver cancer	[128]
DMD	Mdx mouse zygotes	CRISPR/Cas9	DMD	[129]
B2M, CCR5	$CD4^+$ T and $CD34^+$ HSC	CRISPR/Cas9	NA	[130]
CFTR	CF intestinal organoids	CRISPR/Cas9	Cystic Fibrosis	[131]
C. parvum	HCT8	CRISPR/Cas9	Cryptosporidiosis	[132]
HCV	Huh7.5	FnCas9	Hepatitis C	[133]

ZFN: Zinc finger nuclease; TALEN: Transcription activator-like effector nucleases; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; Cas9: CRISPR associated protein 9; HIV: Human immunodeficiency virus; NOD: Non-obese diabetic.

have developed a TALEN-HDAdV hybrid vector, which significantly increased gene-correction efficiency in hiPS cells. Interestingly, a comparative analysis of TALENs, CRISPR/Cas9 and HDAdV revealed that HDAdVs have a clear superiority over both CRISPR/Cas9 and TALENs in gene targeting and gene correction of the *HBB* locus.

Utilizing a novel approach, Nihongaki *et al*[101] have recently developed an engineered photoactivatable Cas9 (paCas9) that enables optogenetic control of CRISPR-Cas9 genome editing by NHEJ and HDR pathways in human cells. Optogenetic paCas9 was developed by fusing the two split Cas9 fragments with photoinducible dimerization domains termed magnets. The system gets activated in response to blue light and expresses paCas9 in target cells and induces targeted genome editing which can be switched off by extinguishing the light. Development of optogenetic paCas9 will enable conditional genome editing with ultra high precision and lead to potentially innovative gene and cellular therapies for currently incurable genetic disorders.

Most recently, Zetsche *et al*[102] have now characterized Cpf1, a new single crRNA-guided endonuclease which lacks tracrRNA and utilizes a T rich PAM. In contrast to the well-established Cas9, which requires tracrRNA to process crRNA arrays as well as crRNA and tracrRNA to mediate interference, Cpf1 doesn't require tracrRNA to process crRNA arrays. Furthermore, Cpf1-crRNA complexes are capable of independently cleaving target DNA molecules without any additional RNA species to generate staggered cut with a 5' overhang unlike the blunt ends generated by Cas9. Additionally, Cpf1 has multiple advantages over Cas9 including smaller size and therefore it has a great potential to maximize high fidelity gene editing in corneal diseases.

Human germ line editing approach is currently in its infancy as its application has recently been demonstrated in China[103] and is gaining momentum in the United Kingdom. Further, CRISPR/Cas9 could be effectively used to eradicate selective group of harmful plants, animals or insects that interfere with the natural ecological balance. For example, taking a note of the fact that only female mosquitos (*Aedes aegypti*) which feed on blood are responsible for pathogenic transmission of dengue, yellow fever and chikungunya viruses, Hall *et al*[87] were able to harness the power of CRISPR/Cas9 system to knockout *Nix* gene leading to a population of largely feminized genetic males while induced ectopic expression of *Nix* resulted in genetic females with nearly complete male genitalia. This study represents a promising new approach for implementing vector-controlled strategies

wherein the disease carrier female mosquitoes can be converted into harmless male mosquitoes.

Another, pressing challenge with viral vectors especially AAV and lentiviral vectors is that they have a broad tissue tropism and efficiently transduce vast majority of cell types both *in vitro* as well as *in vivo*[4]. As a result, targeted *in vivo* genome editing of a very specific cell type in a highly complex organ like eye is extremely challenging but not impossible. Several different approaches can be used either independently or in combination to circumnavigate and bypass this critical issue. First, a highly tissue specific promoter-enhancer combination can be used to specifically limit the expression of CRISPR-Cas9 to the desired cell type. However, tissue-specific promoters often times lack fidelity and exhibit promiscuous expression in non-targeted cells. Furthermore, transgene expression driven by tissue-specific promoters may either be inadequate for therapeutic effect or supra-physiological thereby leading to toxicity. Second approach involves either AAV capsid engineering or using a specific AAV serotype to target specific cell types. In this regard, doxycycline, rapamycin, mifepristone and tamoxifen inducible expression vectors offer an excellent choice. However, caution needs to be exercised since certain drugs like rapamycin can perturb endogenous mammalian target of rapamycin pathway. Alternatively, delivery of Cas9 vectors into the target cells using episomal expression vectors, integration deficient lentiviral vectors, adenoviral vectors and nanoparticles has a tremendous potential that needs to be explored. We believe that the development of novel hybrid genome editing vectors will lead to robust high fidelity targeted genome editing and will potentially enable futuristic gene and cellular therapies for currently incurable genetic disorders an ultimate reality.

The tremendous potential to achieve intended gene editing using ZFNs, TALENs and CRISPR/Cas9 system for the development of novel disease models and innovative therapies has been well demonstrated (Table 2). However, a theoretical risk remains that this technology can be misused and exploited for bioterrorism and may have unimaginable negative consequences. Thus, it is extremely important to develop stringent guidelines to prevent the potential misuse of CRISPR/Cas9 based innovative gene editing technology. Like any other genetic engineering technology ZFNs, TALENs, and CRISPR/Cas9 technologies can be a double-edged sword. Indeed, gene editing approach is going to play a crucial role in improving human and animal health, increasing food and biopharmaceutical production, maintaining clean environment and revolutionizing medicine.

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REVIEW

Potential therapeutic targets from genetic and epigenetic approaches for asthma

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Abstract

Asthma is a complex disorder characterised by inflammation of airway and symptoms of wheeze and shortness of breath. Allergic asthma, atopic dermatitis and allergic rhinitis are immunoglobulin E (IgE) related diseases. Current therapies targeting asthma rely on non-specific medication to control airway inflammation and prevent symptoms. Severe asthma remains difficult to treat. Genetic and genomic approaches of asthma and IgE identified many novel loci underling the disease pathophysiology. Recent epigenetic approaches also revealed the insights of DNA methylation and chromatin modification on histones in asthma and IgE. More than 30 microRNAs have been identified to have regulating roles in asthma. Understanding the pathways of the novel genetic loci and epigenetic elements in asthma and IgE will provide new therapeutic means for clinical management of the disease in future.

Key words: Asthma; Immunoglobulin E; Genome-wide association studies; Epigenetics; MicroRNA

Core tip: Asthma is a complex disorder characterised by inflammation of airway. Allergic asthma is an immunoglobulin E (IgE) related disease. Severe asthma remains difficult to treat. Genetic and genomic approaches of asthma and IgE identified many novel loci underling the disease pathophysiology. Recent epigenetic approaches also revealed the insights of DNA methylation and chromatin modification on histones in asthma and IgE. More than 30 microRNAs have been identified to have regulation roles in asthma. Understanding the pathways of the novel genetic loci and epigenetic elements in asthma and IgE will provide new therapeutic means for clinical management of the disease in future.

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INTRODUCTION

Asthma runs strongly in families and has a heritability of up to 60%[1]. Allergic asthma, atopic dermatitis and

allergic rhinitis are immunoglobulin E (IgE) related diseases. The T_H2 inflammation in airway is a predominate feature of asthma. A sharp increase in the prevalence of asthma was observed in many countries in recent years and a report from the International Study of Asthma and Allergies in Childhood found that the prevalence of symptoms of asthma in children differed more than 20-fold between study centres around the world[2]. Genetic and environmental factors contribute to the prevalence of the disease. The current management of asthma relies on non-specific medication to control airway inflammation and prevent symptoms. Severe asthma remains difficult to treat.

The genetic approaches to asthma include candidate gene studies, positional cloning studies and genome-wide association studies (GWASs)[3]. The gene *FCERB* on chromosome 11 encoding high-affinity IgE receptor (FcεRI) β unit identified almost three decades ago was one of the early mile stones for genetic approaches of asthma[4]. It then turned out the genetic approaches to identify genes underlie complicated diseases were confined by many factors. Genetic associations to asthma for certain locus may be found in one population but may not always be replicated in the other populations. GWAS is powerful approach to overcome the limitations of candidate gene and positional cloning studies. In a GWAS approach the relationship between disease and allele frequencies is examined across a large number of markers spaced in the genome in a big case and control population, robust genetic effects that have substantial population risk can be identified.

Genetic approaches of asthma and IgE have brought remarkable results, but only a small component of the overall genetic contribution to asthma so far has been identified. The missing heritability may be due to rare highly penetrant mutations, multiple small effects, or epigenetic modifications of gene function and other regulating elements for the genome. Epigenetic regulation modifies gene expression that is not caused by changes in the DNA sequence but by DNA methylation, histone modification and other mechanisms. DNA methylation involves the addition of a methyl group to the DNA nucleotide cytosine and adenine which lead to gene silencing. Histones are highly alkaline proteins in eukaryotic cell nuclei that package and order the DNA into nucleosome. The major histone modifications are methylation, acetylation, phosphorylation, ubiquitination and sumoylation. Such modifications affect range from gene activation to gene silencing.

This review discusses the recent discoveries from genetic and epigenetic approaches to asthma and also summarizes the implications of specific loci or regulating elements for therapeutic intervention for asthma.

Genetic approaches

More than one hundred genes have been found to have associations with asthma by candidate gene approaches. The candidate gene approach cannot identify novel pathways[5]. Positional cloning is another genetic approach that identifies disease genes by progressive dissection of linkage regions that are consistently co-inherited with the disease. *ADAM33*[6], *PHF11*[7], *DPP10*[8], *GPRA*[9], *HLA-G*[10], *CYFIP2*[11], *IRK3*[12], *OPN3/CHML*[13] were discovered as asthma genes by positional cloning. Most associations identified by candidate gene studies and positional cloning studies were moderate. GWAS is more efficient and can be performed to investigate the entire genome simultaneously. It provides the opportunity to identify novel mechanisms of disease pathogenesis. The first GWAS study for asthma was carried out in the GABRIEL Consortium. The consortium consisted of collaborations among 35 partners across the European Community. In 2007, the consortium reported SNPs in the chromosome 17q12-q21 region to be significantly ($P < 10^{-12}$) associated with childhood asthma and asthma associated SNPs were associated with the expression levels of the ORM1-like 3 *Saccharomyces cerevisiae* (*ORMDL3*) gene[14]. Then a large consortium GWAS study also confirmed *ORMDL3* as an important asthma suspected gene. The consortium also identified *IL-18R1*, *HLA-DRBI*, *HLA-DQ*, *IL-33*, *SMAD3*, *IL-2RB*, *SLC22A5*, *IL-13* and *RORA* as asthma or IgE suspected genes[15]. To date, more than ten GWASs on asthma or asthma-relevant traits have been published. Serum YKL-40 levels were shown to elevate in patients with asthma and were correlated with asthma severity, thickening of the subepithelial basement membrane in airway, and pulmonary function[16]. Polymorphisms of *Ch13LI* were associated YKL-40 level in 753 Hutterites in a GWAS study for asthma[17]. Polymorphisms of *PDE4D*, *TLE4*, *ADRA1B*, *PRNP*, *DPP10* and *GNAI3* were found to associate with asthma in GWASs studies of different populations[18-20]. Polymorphisms of *DENND1B* and *ORMDL3* were also found to associate with asthma in a European American population GWAS study[21]. In another European GWAS study, *RAD50*, *IL-13*, *HLA-DR-DQ*, *LRP1B*, *SNX10*, *CA10*, *KCNJ2* were shown associations with asthma[22]. In the EVE Consortium, *ORMDL3*, *IL-1RL1*, *TSLP*, *RTP2*, *IL-33, PYHIN1* were found to associate with asthma[23]. Genome-wide association study identified *IL-12A*, *IL-12RB1*, *STAT4*, and *IRF2* genes associated with lung function in asthmatic patients[24]. *ORMDL3/GSDMB*, *IL-1RL1/IL-18R1* loci were also found to associate with severe asthma[25]. In a Danish GWAS study for asthma exacerbations in childhood, *GSDMB*, *IL-33*, *RAD50* and *IL-1RL1* and *CDHR3* showed association with asthma[26]. *CTNNA3* and *SEMA3D* also were associated asthma exacerbation in GWASs studies in two paediatric clinical trials in the United States[27]. IL-4R was found increased in genome-wide expression profiling in allergic asthma[28]. Genome-wide differential gene expression in response to dust mite allergen also identified *IL-5*, *IL-9* and *PRG2* to interact with environmental dust mite to increase severe asthma exacerbations in children[29]. In a Japanese GWAS study, *TSLP-WDR36* and *USP38-GAB1* loci were found to associate with asthma[30]. Lung function, particularly for forced expiratory volume in the first second [FEV(1)] and its ratio to forced vital capacity

[FEV(1)/FVC], was studied in meta-analyses of GWAS studies. It identified *HHIP, GPR126, ADAM19, AGER-PPT2, FAM13A, PTCH1, PID1, HTR4, INTS12-GSTCD-NPNT*, *THSD4* as suspected genes for lung function change[31,32].

Epigenetic approaches

Epigenetic effects are other possible causes of asthma. The patterns of gene expression become stably restricted during development, majorly through methylation of CpG sequences and gene silencing. Sex, age, environmental factors and genetic polymorphisms have all been strongly associated with altered methylation at selected loci. To asthma, allergens, microbes, tobacco smoke, diet and metabolism, fish oil, obesity and stress are important environmental factors that influence epigenetic effects in human cells[33]. CD19 (+) B lymphocytes methylation patterns and expression levels showed difference in the locus *CYP26A1* in house dust mite allergic patients[34]. Children growing up in a traditional farming environment had lower risk of allergic respiratory diseases. Demethylation of the *FOXP3* promoter was association with higher number of FOXP3 cells in cord blood mononuclear cells in an extensive farming exposure environment[35]. Hypomethylation of *ORMDL1* and *STAT6* and hypermethylation of *RAD* and *IL-13* were also found from farm children[36]. DNA methylation in the *CD14* promoter was also significantly less in farm mothers[37]. PBMC s from obese asthmatic children had lower levels of promoter methylation of the *CCL5*, *IL-2RA* and *TBX21* and higher level promoter methylation of *TGFB1* and *FCER2*[38]. Recent epigenome-wide approach identified 36 loci that had association of serum IgE level[39]. Among them, DNA methylation events have been found in cytokine signalling genes *IL-4*, *IL-5R*, transcription factor genes *ZNF22*, *RB1*, *GATA1*, *KLF1*, transmembrane or transporter genes *SLC25A33*, *SLC17A4*, *SLC43A3*, *TMEM52B*, *TMEM41A*, eosinophil associated genes *PRG2* and *PRG3*, phospholipid metabolism genes *LPCAT2*, *CLC* and *MEM86B*, and metabolic enzyme genes *L2HGDH*, *CEL*, *KEL*, *PDE6H*, *EFNA3*, *ALDH3B2*.

Noncoding RNAs emerged as novel molecules that are important in lung diseases in recent years[40]. Noncoding RNAs include housekeeping RNAs, long noncoding RNAs and small noncoding RNAs. Micro RNAs (miRNAs) are the most studied small noncoding RNAs. miRNAs are about 18-25 nucleotide long noncoding RNAs that silence target mRNA. More than 3000 human miRNA genes have been identified so far. There is a significant number miRNAs that are still uncharacterized[39]. miRNAs induce messenger RNA (mRNA) degradation and then inhibit the translation. miRNAs can target 60% of mRNAs and control the signally pathways in most cell types[41]. More than 30 miRNAs have been found to associate with asthma[42]. These miRNAs regulate epithelium cells, airway smooth muscle cells and T_H2 response.

To date, it is not reality to assume that genetic targets and regulating elements for asthma identified by genetic and epigenetic approaches can be accessed either by biologics (antibodies and proteins) or small molecules (drugs), but several genes regulate in pathways from epithelial damage to the adaptive immune system in asthma, providing a new means for effective therapies. This review focuses on the novel genes expressing on human airway epithelium cells and cytokine networks that play important roles in asthma pathophysiology. It also summarizes the miRNAs that were found to regulating asthma pathogenesis.

THE POTENTIAL THERAPEUTIC TARGETS FOR ASTHMA IN EPITHELIAL CELLS

Human airway epithelium is now believed to be central to the pathogenesis of asthma[43,44]. Several asthma candidate genes identified by genetic and epigenetic approaches may modify the inflammatory response to epithelial damage or regulate homeostatic and healing pathways. The following novel genes identified by GWASs express in the airway epithelium and understanding their pathways in inflammation response will provide unique opportunities to develop new therapeutic means for asthma (Table 1).

ORMDL3

The association signals on human chromosome 17 with asthma are maximal within an island of linkage disequilibrium that contains *ORMDL3*, *GSDMA* and *GSDMB.* Now the associations have been found in many GWAS studies. The loci were not only associated childhood asthma, but also associated with severe asthma or asthma exacerbations. ORMDL3 protein is found in the membranes of the endoplasmic reticulum (ER). ER stress is one of important stage linked to cellular responses to inflammation[45]. ORMDL3 has been found to be up-regulated in transcriptional activator XBP-1(S)[46]. *ORM* gene expression regulates sphingolipid metabolism[47]. Ceramide and sphingosine-1-phosphate (S1P) are two important bioactive signalling sphingolipids. They mediate cell survival, proliferation, apoptosis, differentiation and cell-cycle arrest[48]. Clinical observation showed that they were increased in asthmatic airways[49]. Recent study showed Ormdl3 may regulate ceramide level in epithelial cells and then regulate the inflammation response[50]. Transfection of ORMDL3 in human bronchial epithelial cells *in vitro* induced expression of many chemokines and selectively activated activating transcription factor 6, suggest an ER UPR pathway through which ORMDL3 may be linked to asthma[51]. ORMDL3 also regulates eosinophil trafficking, recruitment and degranulation[52], ORMDL3 was shown to modify SERCA in the ER and induce inflammation[53]. A recent study showed in 17q21 risk allele carrier children their mononuclear cells significantly increased IL-17 secretion[54]. ORMDL3 may influence multiple pathways in the ER that mediate inflammation during asthma and regulating ORMDL3 may have the potential therapeutic effects on inflammation disease such as asthma.

Table 1 The potential genetic therapeutic targets in airway epithelium for asthma

Genes	Chromosome location	Phenotypes	Identifying methods	Possible pathways related to asthma	Ref.
DPP10	2	Asthma	GWAS/PC	Unknown; Kv4 ion channel complex	[8,20]
TSLP	5	Asthma	GWAS	Airway remodelling; promoting TH2 inflammation	[23,30]
CDHR3	7	Asthma	GWAS	Epithelial polarity; cells interaction and differentiation	[26]
SEMA3D	7	Asthma	GWAS	Airway remodelling; angiogenesis	[27]
SMAD3	15	Asthma	GWAS	Transcriptional modulator; TGFβ pathway	[15]
ORMDL3	17	Asthma	GWAS	Sphingolipid metabolism, ER stress response	[14,15,21,23,25,26]
GSDMB	17	Asthma	GWAS	Epithelium cell growth	[14,15,21,23,25,26]
GSDMA	17	Asthma	GWAS	Cell proliferation	[14,15,21,23,25,26]

PC: Positional cloning; GWAS: Genome-wide association study; TGFβ: Transforming growth factor-beta; ER: Endoplasmic reticulum.

GSDMB and GSDMA

The human chromosome 17 locus of asthma covers a genomic area of approximately 200Kb. *ORMDL3* and *GSDMB* reside in one island of linkage disequilibrium that contains all the maximally associated SNPs. Independent associations are also detectable telomerically near the *GSDMA* which may make contributions to asthma susceptibility as well[14]. The *GSDM* family genes were first identified in mouse. They are expressed majorly in the gastrointestinal tract and expressed a lower level in the skin. The mouse syntenic homology areas including mouse *Gsdm1*, *Gsdm2* and *Gsdm3* are on mouse chromosome 11. Mouse Gsdm proteins contain DFNA5 domain of Pfam domains. They are expressed predominantly in the gastrointestinal tract and in the skin[55] in a highly tissue-specific manner[56]. In humans *GSDMA* and *GSDMB* are expressed in the gastrointestinal and bronchial epithelium. Members of the gene family may have a role in regulation of apoptosis[57]. GSDMA was shown to mediate cell-growth inhibition. GSDMB is expressed in stem cell-resided region and has a potential role in stem cell proliferation. The GSDMB-driven HSVtk expression vector had a therapeutic effect on the occult peritoneal dissemination (PD) model mice. This strategy can potentially be used to treat GC patients with PD in clinical[58]. The specific expression of *GSDMB* and *GSDMA* in epithelium may also service to therapeutic means to asthma in future.

Thymic stromal lymphopoietin

Thymic stromal lymphopoietin (*TSLP* gene) was found to associate with asthma by GWAS and SNPs in *TSLP* may have asthma risk through up-regulating its mRNA expression or the protein secretion[59]. It expresses mainly by epithelial cells at barrier surfaces (skin, gut and lung)[60,61]. TSLP plays a critical role in orchestrating the inflammatory response and a critical factor in airway remodelling in asthma. Airway remodelling is a repair process that happens after injury resulting in airway hyper-responsiveness in asthma. TSLP induces cellular senescence during airway remodelling in asthma[62,63]. Myeloid dendritic cells (DCs) are the cell populations with the highest known co-expression of the TSLP receptor and its associated subunit IL-7R. Treatment of human DCs with TSLP induces improved survival, up-regulation of major histocompatibility complex class Ⅱ and the production of a variety of chemokines[60]. It promotes TH2 cytokine-associated inflammation by directly promoting the effector functions of $CD4^+$ TH2 cells[61].

SMAD3

SMAD3 encodes SMAD (mothers against decapentaplegic homolog) family member 3 and has a role in modifying tumour growth[64,65] through the transforming growth factor-beta (TGFβ) pathway[66]. SMAD3 is concentrated in the nuclei of bronchial epithelial cells and macrophages and functions as a transcriptional modulator activated by TGFβ. The family members of TGFβ maintain of immune function in lung[67] and the TGFβ signalling pathways can be activated after allergen challenge in mild asthma[68]. A mouse knockout of *Smad3* showed accelerated wound healing and an impaired local inflammatory response[69], even though mice lacking *Smad3* may exhibit increased baseline levels of pro-inflammatory cytokines in their lungs[70]. Smad3 signalling is required for myogenic differentiation of myoblasts[71], this may be linked a role in airway smooth muscle hypertrophy.

DPP10

DPP10 was the only gene that was identified both by positional cloning and GWAS studies. *DPP10* genetic variants could affect lung function decline in aging and also associate aspirin-exacerbated respiratory disease. The DPP proteins have a β-propeller that regulates substrate access to an α/β hydrolase catalytic domain. Unlike other DPP family members, DPP10 lack of enzymatic activity is unable to cleave terminal dipeptides from asthma-related cytokines and chemokines[8]. In neurones, DPP10 forms part of the A-type K^+ (Kv4) ion channel complex and DPP10 variants accelerate channel gating kinetics. It is not clear what exact roles of DPP10 in the airway epithelial cells, the future research will focus on how DPP10 regulate inflammation response in epithelial cells in asthma by applying animal models and cellular models.

Cadherin-related family member 3

Cadherin-related family member 3 (CDHR3) is a transmembrane protein with six extracellular cadherin

Table 2 The genetic and epigenetic loci modify cytokines and receptors of asthma

Genes	Chromosome location	Phenotypes methods	Identifying and functions in asthma	Possible pathways	Ref.
IL-18R1	2	Asthma	GWAS	Activation of NF-κB, inducing TH-associated cytokines	[15,25]
IL-1RL1	2	Asthma, Eos	GWAS	Receptor for IL-33	[15,23,94]
IL-5RA	3	IgE	Epigenetics	TH2 inflammation, regulating eosinophils	[39]
IL-12A	3	Lung function	GWAS	TH1 regulation, activating IFN-γ	[24]
IL-4	5	IgE	Epigenetics	TH2 inflammation, promoting IgE class switching	[39]
IL-13	5	Asthma, IgE	GWAS/epigenetics	TH2 inflammation, promoting IgE class switching	[15,22]
IL-5	5	Asthma	GWAS/epigenetics	TH2 inflammation, regulating eosinophils	[29,36,94]
IL-9	5	Asthma	Expression profiling	Stimulates cell proliferation and prevents apoptosis	[29]
IL-33	9	Asthma	GWAS	Inducing TH-associated cytokines	[15,23,26,94]
IL-2RA	10	Asthma	Epigenetics	PI3K-Akt signalling pathway and Akt signalling	[38]
IL-4R	16	Asthma	Expression profiling	TH2 inflammation	[28]
IL-12RB1	19	Lung function	GWAS	TH1 regulation, activating IFN-γ	[24]
IL-2RB	22	Asthma	GWAS	Endocytosis and transducer mitogenic signals	[15]

GWAS: Genome-wide association study; IL: Interleukin; IgE: Immunoglobulin E; IFN-γ: Interferon-γ; NF-κB: Nuclear factor kappa-B.

domains. The biological function of CDHR3 remains. It belongs to the cadherin family of transmembrane proteins that have function roles in homologous cell adhesion. It is important for epithelial polarity, cell-cell interaction and differentiation[72]. Other members including E-cadherin of the family have been associated with asthma[73]. CDHR3 Protein structure modelling showed that the Cys529Tyr risk-associated alteration was located at the interface between two D5 and D6 membrane-proximal cadherin domains. The variant residue may interfere with interdomain stabilization, folding or conformation[26].

Semaphorin-3D

Semaphorin-3D (SEMA3D) is a member of the semaphorin class 3 signalling molecules. SEMA3A and SEMA3E are secreted transmembrane proteins involved in immune response and the recruitment of $CD4^+$ and $CD8^+$ T cells[74]. SEMA3D is responsible for endothelial cell migration[75] and has been shown to be essential for healthy angiogenesis during development[76]. Angiogenesis is also a feature of airway remodelling. It is possible that SEMA3D plays a role in airway remodelling from plausible mechanisms. It directs angiogenesis and airway epithelium migration, resulting in a reduction of epithelial cells. Like other semaphorins, it has effects on immune cell recruitment during the inflammatory response, which leads to remodelling[27].

THE POTENTIAL THERAPEUTIC TARGETS IN CYTOKINE NETWORKS FOR ASTHMA

Genetic and epigenetic approaches of asthma and IgE have revealed many cytokines and cytokine receptors that regulate the inflammation in the airways. These cytokines and cytokine networks play critical roles for inflammation response in epithelium cells and immune cells. Specific targeting the cytokines and the networks may provide new therapeutic means to asthma. The cytokines identified by GWAS and epigenetic approaches are discussed here (Table 2).

IL-33, IL-18R1 and IL-1RL1

IL-33, IL-18 and IL-1 belong to the IL-1 family of cytokines that alter host responses to inflammatory and infectious challenges. They employ their functions through a toll-like receptor-IL-1 receptor (TLR-IL-1R) superfamily. IL-1 receptor signalling activates transcription factor nuclear factor kappa-B (NF-κB), mitogen-activated protein (MAP) kinases p38, JNK, and ERK1/2[77].

IL-33 was originally identified as a nuclear factor in vascular endothelial cells[78], and was subsequently detected in airway epithelial cells[79,80]. The activities of IL-33 as a nuclear factor remain unclear[81]. IL-33 is constitutively expressed and has function as an endogenous danger signal to alert the immune system after endothelial or epithelial cell damage during trauma or infection stresses[82]. A mouse *IL-33* gene knockout has shown IL-33 works as a crucial amplifier of innate immunity[83]. IL-33 expression is induced by a range of environmental and endogenous triggers, suggesting an essential role during infection, inflammation and tissue damage[84]. IL-33 activates a herterodimeric receptor complex containing IL-1RL1 (ST2) and IL-1 receptor accessory protein (IL-1RAP), leading to activation of NF-κB and MAP kinases and then drives production of TH2 cytokines IL-4, IL-5, and IL-13[79].

The *IL-18R1* gene is located on chromosome 2q. It form a gene cluster along with four other members of the interleukin 1 receptor family [*IL-1R2*, *IL-1R1*, *IL-RL2* (*IL-1Rrp2*), and *IL-1RL1* (*T1/ST2*)] on the loci. *IL-18R1* and *IL-1RL1* flank each other with the same

orientation of translation. They are within the same island of linkage disequilibrium and it has not yet been possible to assign the genetic effects at this locus to one gene or the other. It is possible that both genes may be co-regulated. *IL-1RLI* encodes the receptor of IL-33. IL-18 is closely related to IL-33[79] and synergizes with IL-12 to induce interferon gamma and to promote T_H1 responses[85]. These loci therefore identify a pathway for the communication of epithelial damage to the adaptive immune system and a potential switch point for choosing between T_H1 or T_H2 responses.

IL-2RB

IL-2RB encodes the beta receptor of IL-2. IL-2 is secreted by antigen-activated T cells. It controls the survival and proliferation of regulatory T cells[86] and plays a prominent role in the maintenance of natural immunologic self-tolerance[87]. The IL-2 receptor has α (CD25), β (CD122) and γ chains[86]. The β chain (IL-2RB) is a signal transduction element that is also present in the IL-15 receptor. It belongs to the type Ⅰ cytokine receptor family and has no intrinsic kinase activity[88]. The receptor regulates T cell-mediated immune responses through endocytosis, whereby ectodomain shedding of IL-2Rβ generates an intracellular fragment[89]. In a mouse model of asthma, local inhibition of Il2rb restored an immunosuppressive cytokine milieu that ameliorated lung inflammation[90].

IL-4 and IL-4R

IL-4 is adjacent to *RAD50* on chromosome 5. The locus is exceptional in showing strong association to IgE in addition to doctor-diagnosed asthma[15]. The 3′ end of *RAD50* has several enhancer elements and conserved non-coding sequences that act as a locus control region for *IL-4* and *IL-13*[91]. IL-4 is one of the key T_H2 cytokines and immunoglobulin class switching in B cells. IL-4 methylation was associated with IgE production[39]. IL-4R is the best candidate allergic biomarker and shows to have association with allergic asthma in a genome-wide expression profiling study[28]. A soluble form of the IL-4 receptor can block B cell-binding of IL-4 or other IL-4R antagonists[92].

IL-5 and *IL-5RA*

IL-5 encodes a growth and differentiation factor for B cells. IL-5 also controls the activation and localization of eosinophils[93]. A SNP (rs4143832) located near *IL-5* on 5q31 showed to have association with blood eosinophil counts[94]. Eosinophils are an important source of cytokines and chemokines at the allergic inflammation sites[95] *IL-5RA* was methylation different with asthma[39]. *IL-5RA* encodes a receptor that selectively stimulates eosinophil production and activation[96]. In clinic, therapies directed at eosinophil may be effect in a subgroup of refractory asthma individuals[97].

IL-13

IL-13 encodes an immunoregulatory cytokine primarily by activated T_H2 cells. IL-13 is involved in several stages of B-cell maturation and differentiation. It up-regulates CD23 and MHC class Ⅱ expression. It also promotes IgE isotype switching of B cells. IL-13 down-regulates macrophage activity and inhibits the production of pro-inflammatory cytokines and chemokines. This cytokine is critical to the pathogenesis of allergen-induced asthma but works through mechanisms independent of IgE and eosinophils. rs20541 (Arg130Gln or IL13 + 4257GA) in the coding region of *IL-13* has been shown to be associated with asthma[98] and total serum IgE levels[99]. One GWAS study confirmed the important role of T_H2 cytokine and antigen presentation genes in asthma[22].

IL-12A and *IL-12RB1*

IL-12 is a key cytokine that regulates innate and adaptive immune responses. IL-12 is composed of the p35 subunit and the p40 subunit (encoded by *IL-12A* and by *IL-12B* respectively). The formation of the high-affinity IL-12 is led by the co-expression and dimerization of the IL-12RB1 and IL-12RB2 proteins. IL-12 activates interferon-γ (IFN-γ) production. STAT4 regulates the response of lymphocytes to IL-12; it induces the expression of IL-12RB2 and transcription factor IRF1. IRF1 is induced by IFN-α, IFN-β, and IFN-γ. IRF2 can competitively inhibit the expression of genes induced by IRF1. The IL-12-STAT4-IFN-γ signalling pathway is essential for the differentiation of naive T_H cells into T_H1 cells[24].

IL-9

IL-9 was found to interact with environmental dust mite to increase severe asthma exacerbations in children[29]. IL-9 induces cell proliferation and prevents apoptosis through the IL-9R. IL-9R activates different STAT proteins. IL-9 has been shown to promote mast cell recruitment to the lung, increase mast cell activity, and enhance airway remodelling in a murine model of asthma and also mast cells act as the main expressers of IL-9 receptor in human asthmatic lung tissue[100]. IL-9 production from bronchoalveolar lavage lymphocytes increases after an inhaled allergen challenge in atopic asthmatic patients[101] and IL-9 has been shown to up-regulate expression of eotaxin in cultured human airway smooth muscle cells[102].

miRNAs AND THEIR REGULATIONS IN ASTHMA

miRNA can act as a regulator between genetic and environmental factors in the pathogenesis of asthma. Epigenetic changes are potentially revisable and therapeutic modulation of miRNAs may provide opportunities to regulate or suppress allergic inflammation[103]. There are more than 11 miRNAs differentially expressed in human exhaled breath condensate from asthma patients compared with health subjects[104]. miRNA

Table 3 The microRNAs and their potential roles in asthma

miRNA	Possible function roles in asthma	Ref.
miR-1	Targeting Mpl to regulate TH2 inflammation and P-selectin in lung endothelium	[109]
miR-126a	Regulating TH2 inflammation, airway hyper-responsiveness, eosinophil recruitment	[110]
miR-221	Mediator IL-6 proliferation in airway smooth muscle	[42]
miR-146a	NF-κB dependent gene, control toll-like receptors and cytokine signalling	[111]
miR-146b	NF-κB dependent gene, control toll-like receptors and cytokine signalling	[111]
miR-150	Down-regulated transcription factor c-Myb to control lymphocyte development	[112]
miR-155	Targeting c-Maf to promote TH2 cells to generate IL-4, IL-5 and IL-10	[115,116]

IL: Interleukin; NF-κB: Nuclear factor kappa-B.

570-3p was found to have lower level in serum and exhaled breath condensate from asthma patient[105]. miR-221, miR-146a and miRNA146b has been found to have altered expressions in asthmatic patients airway smooth muscle[42,106]. There are number of miRNAs down-regulated or up-regulated in nasal biopsies of asthma patients[107]. Here the most potential miRNAs that could be used as therapeutic targets for asthma are discussed (Table 3).

miR-1

Vascular endothelial growth factor (VEGF) is an important regulator of pulmonary TH2 inflammation. Lung-specific overexpression of VEGF can decrease miR-1 expression in the endothelium of lung. Intranasal delivery of miR-1 inhibited inflammatory responses to allergen ovalbumin, house dust mite, and IL-13 overexpression. Myeloproliferative leukaemia (Mpl protein) is the receptor for thrombopoietin and has roles in megakaryopoiesis and hematopoietic stem cell differentiation[108]. VEGF controlled the expression of endothelial Mpl during TH2 inflammation *via* the regulation of miR-1. *In vivo* silence of Mpl inhibited TH2 inflammation. It indirectly inhibited the expression of P-selectin in lung endothelium. These experiments defined a novel VEGF-miR-1-Mpl-P-selectin effector pathway in lung TH2 inflammation. The utility of miR-1 and Mpl may be potential therapeutic targets for asthma management[109].

miR-126a

In a mouse model, blockage of miR-126 suppressed the asthma phenotype, resulting in diminished TH2 response, inflammation, airway hyper-responsiveness, eosinophil recruitment and mucus over secretion. *In vivo* activation of TLR4 by house dust mite antigens led to the induction of allergic disease, a process that is associated with expression of many small, noncoding miRNAs. miR-126 inhibition resulted in augmented expression of POU domain class 2 associating factor 1 that regulated GATA3 expression. Targeting miRNA-126a in the airways may lead to anti-inflammatory treatments for allergic asthma[110].

miR-221

The mass of airway smooth muscle (ASM) is increased as a feature of asthmatic airways. Increased miR-221 expression was found in ASM cells from individuals with severe asthma. miR-221 increased ASM proliferation and IL-6 release. In severe asthma patients the inhibition of miR-221 reduced proliferation and IL-6 release. miR-221 regulated p21(WAF1) and p27(kip1) expression levels and regulated the hyper-proliferation and IL-6 release of ASM cells from severe asthma patients[42].

miR-146a and miR-146b

miR-146a and *miR-146b* gene expressions were a pattern of induction in response to a variety of microbial components and pro-inflammatory cytokines. *miR-146a* is a NF-κB dependent gene. miR-146a/b were predicted to base-pair with sequences in the 3'UTRs of the tumor necrosis factor (TNF) receptor-associated factor 6 gene and IL-1 receptor-associated kinase 1 gene. These genes encode two key adapter molecules of Toll-like and cytokine receptors. miR-146 controls toll-like receptor and cytokine signalling. It works through a negative feedback regulation loop involving down-regulation of IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6 protein levels[111].

miR-150

miR-150 down-regulated transcription factor c-Myb that regulates lymphocyte development. MiR-150 is specifically expressed in mature lymphocytes. c-Myb is a transcription factor controlling lymphocyte development. *In vivo* miR-150 controls c-Myb expression in a dose-dependent manner over a narrow range of miRNA and c-Myb concentrations. MiR-150 and other miRNAs have evolved to control the expression of a few critical target proteins in particular cellular contexts[112]. c-Myb is an important regulator of Gata3[113]. c-Myb and GATA-3 cooperatively regulate IL-13 expression as regulate IL-13 expression[114].

miR-155

Like miR-146a, miR-155 is one of the most frequently studied miRNAs in both innate and adaptive immune response. Mice without miR-155 displayed increased airway remodelling and were unable to produce the cytokines for immune system homeostasis and function[115,116]. miR-155 targets transcription factor c-Maf, which promotes TH2 cells to generate IL-4, IL-5 and

IL-10 cytokines.

FUTURE RESEARCH DIRECTIONS

The genetic and epigenetic approaches identified many novel loci and regulating elements in human genome. The airway epithelial expressions of some loci and inflammatory cytokines in asthma provide unique therapeutic targets. Regulating elements such as miRNAs also can be served as potential therapeutic targets for the disease. RNA sequencing, deep DNA sequencing, ChiP-sequencing, exome sequencing, transcript profiling and miRNA profiling are becoming more and more powerful platforms to discover more genetic variants, regulators of transcriptions that are in the pathogenesis of asthma. Research on cellular models, animal models and pharmacological models for these novel loci and regulation elements will eventually decipher the precise functions of these targets and it will provide new therapeutic means for asthma in future.

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REVIEW

New insights in sperm biology: How benchside results in the search for molecular markers may help understand male infertility

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Abstract

The male factor is responsible for about 40% of couple infertility cases and such percentage is expected to increase in the future because of several likely factors including the presence of endocrine disruptors in the environment, changes in lifestyle habits and advanced couple aging. How such factors affect male fertility status, however, should be clarified. Most studies on male fertility status have focused on parameters analyzed using a spermiogram test, the primary diagnostic tool in the routine assessment of male infertility, which is, however, poorly predictive of both natural and medically assisted conception. For these reasons it is mandatory for the scientific community to identify new molecular markers to incorporate into the existing diagnostic tests of male fertility. Ideally, such markers would be detected in mature spermatozoa to avoid invasive procedures for the patient. This review summarizes the recent advancements in benchside approaches that appear most promising for the development of new diagnostic sperm fertility tests, or identification of therapeutic targets, and, illustrates their advantages and limits.

Key words: Sperm markers; Male infertility; Genetic and epigenetic approaches; Proteomic approach; Ion channels

Core tip: This review focuses on genetic, epigenetic, proteomic, and post-translational protein modification and ion channel studies present thus far in the literature to identify possible sperm markers that could be helpful for new diagnostic tests or represent possible therapeutic targets for male infertility.

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INTRODUCTION

Infertility is a worldwide health problem affecting about 15% of couples[1]. Although the World Health Organization (WHO, 5th edition[2]) defines it as a disease of the reproductive system, infertility also influences emotional, social and psychological spheres. The male factor is involved in about 40% of couple infertility cases, with the highest incidence rates in Eastern Europe and Africa[1]. Male infertility, affecting presently 7% of the worldwide population, is expected to double over the coming years. Some possible explanations reside in the rise in hectic lifestyles, in the increase of pollution and in socio-economic changes that delay couples in starting a new family. Currently, how all these factors affect male fertility status is not clear.

The increase in reproductive age is becoming an important social problem, which can be particularly noted in industrialized countries. The role of advanced maternal age in the lower success of natural and medically assisted reproduction has been well established[3]. A recent trend among young women is to freeze their oocytes for social reasons, such as desire to have a career, delaying the age of the first conception. Not surprisingly, some multinational American corporations offer to pay for an oocyte preservation procedure for their female employees to allow for career advancement. In contrast with the maternal age, whether paternal age affects fertility is still highly debated. Despite some authors not finding correlations between paternal age and infertility[4,5], others have shown that a forward shift in male age represents a further risk factor for the failure to conceive[6,7], for the success of assisted reproductive techniques (ART) and for the health of offspring[8]. Advanced age may lead to changes in hormonal profile[9] and germinal epithelium disorders with the consequent alterations in seminal parameters[10,11]. Decreased sperm quality may be due to alterations in the expression of some proteins[11,12], as well as an increase in sperm DNA fragmentation (sDF)[13,14] or of other types of DNA damage[11]. In addition, it has been demonstrated that the higher number of *de novo* mutations found in offspring of increasingly older fathers can mostly be attributed to paternal transmission[15-17].

As mentioned above, besides male aging, there are several other factors contributing to the decrease in male fertility potential with similar pathogenic mechanisms, such as the ever increasing presence of endocrine disrupting chemicals in the environment[18] and the changes in lifestyle with an increased prevalence of obesity and metabolic syndrome[19].

Pharmacological treatment of the male partner can only be successfully applied to non-idiopathic causes (such as hypogonadropic hypogonadism), whereas for idiopathic infertility, despite many attempts, virtually no effective treatment is currently available[1]. A recent meta-analysis has concluded that gonadotropin therapy is a possible choice to improve fertility, especially in case of post-pubertal onset hypogonadotropic hypogonadism[20]. Efforts to treat idiopathic male infertility, for instance using gonadotropins, or anti-aromatase, anti-estrogen and anti-oxidant drugs, have not demonstrated a conclusive, beneficial effect of said therapies[21]. Until robust results are obtained, ARTs remain, for idiopathic male infertility, the option with the highest chance of achieving pregnancy.

Although ARTs have expanded globally over the last few decades, these procedures remain inaccessible in many parts of the world and are quite expensive. Moreover, despite ARTs' success rate having approved greatly over the past few years, the current live birth outcome remains low, averaging just 34%[22], with important economic and psychological consequences for couples. For these reasons it is mandatory, for the scientific community, to identify the causes of infertility in order to find effective treatments and new sperm markers to improve the accuracy of diagnosis.

The primary diagnostic tool in the routine assessment of male infertility is semen analysis (spermiogram), which consists in the evaluation of the macroscopic (volume, pH, liquefaction) and microscopic (number, motility and morphology) characteristics of seminal fluid. Despite the fact that WHO issued detailed laboratory guidelines to standardize the methods and has established normal reference values[2], spermiogram has a high operator variability, high intra-individual variation[23] and is not highly predictive of the fertility status[24,25]. The diagnosis of infertility results as being accurate only in the case of azoospermia and severe oligozoospermia. Semen analysis does not provide information about the molecular status of spermatozoon and the functions necessary for oocyte fertilization. For this reason, identification of new semen or sperm molecular markers able to discriminate between fertile and infertile men is one of the main goals of current research. Markers that single out spermatozoa with a higher fertilizing ability could lead also, in the future, to a better sperm selection for ARTs. Indeed, although new advanced tools for sperm selection have been developed based on sperm surface charge, apoptotic or maturity sperm markers and sperm ultramorphology, more studies are needed before introducing advanced sperm selection methods in ART[26]. Based on current published data, sperm selection using real-time motile sperm organelle morphology examination at high magnification coupled with intracytoplasmic morphologically selected sperm injection seems to be a promising method with benefits for late ART outcomes (pregnancy, live birth and abortion rates)[27].

This review will focus on the recent advancements of benchside approaches that appear most promising for the identification of new sperm/germ cells as molecular markers of infertility.

GENETIC AND EPIGENETIC STUDIES ON TESTICULAR GERM CELLS AND MATURE SPERMATOZOA

At least 15% of male infertility cases are due to genetic alterations[28], including Y chromosome microdeletions, present in about 20% of cases of azoospermia or severe oligozoospermia[29]. Innovative approaches implying whole-genome analysis, such as the evaluation of single nucleotide polymorphisms and copy number variations, could be helpful in the search for new gene candidates having a role in male infertility[30-32]. For instance, a recent study by Yatsenko *et al*[33] identified hemizygous mutations in the *TEX11* gene as one of the causes of meiotic arrest and azoospermia in infertile men. A microarray study found a different expression of genes linked to spermatogenesis in testis RNA from non-obstructive azoospermic (NOA) men when compared to commercial RNA from normal testicular tissue[34,35]. We expect that other genes responsible for azoospermic/severe oligozoospermic phenotypes will be discovered in the future.

Whereas genetic studies are of great help in identifying the genes involved in testicular disorders that lead to severe alterations in sperm number, the search for genetic modifications leading to sperm dysfunctions in idiopathic infertility appears to be a sort of "fishing expedition". Conversely, the use of genetic, epigenetic and proteomic approaches on ejaculated spermatozoa could allow researchers to characterize the complete spectrum of sperm phenotypes present in infertile subjects better and, accordingly, to understand the leading causes of infertility in depth.

Epigenetic alterations derived from environmental pollution, toxicants and nutritional habits could impair both sperm quality and embryo development[36,37], increasing the risk in offspring of developing chronic diseases, such as type 2 diabetes, obesity, cardiovascular disease and cancer[38,39]. Evidence in animal models suggests that some epigenetic markers can be inherited by the offspring through parents' gametes[39]. Rodent studies have demonstrated that paternal diet affects pregnancy achievement and offspring metabolism[40,41]. In two recent studies evaluating genome wide sperm DNA methylation, such an epigenetic pattern was found to differ significantly between *in-vitro* fertilization (IVF) patients and normozoospermic fertile men[42] and between men achieving pregnancy within two months and men who did not obtain pregnancy within twelve months, despite similar semen quality[43]. These studies identified candidate methylation loci to be explored in future studies in order to consolidate the results. Epigenetic inheritance related to spermatozoa includes not only DNA methylation but also other epigenetic factors such as histone retention or non-coding RNA (ncRNA). In view of the recent observation that histone retention in specific loci is important for subsequent embryo development[44,45], new sperm diagnostic tests based on histone enrichment in specific genes could be developed in the future. Alterations in ncRNAs may also impair embryo development and transgenerational inheritance. Among ncRNA, the occurrence of miRNA in sperm, seminal fluid and testicular tissue has been reported recently[46]. The fundamental role of miRNA during spermatogenesis is demonstrated by the fact that the knockout of the Dicer enzyme, which is responsible for the cleavage from immature to mature forms of miRNA, leads to infertility[47]. What remains to be determined is whether miRNAs are required also for human spermatogenesis. Recently, an alteration of five miRNAs in subfertile and NOA subjects has been shown[48]. Similarly, employing next generation sequencing, Jodar *et al*[49] found a set of sperm RNA elements required to achieve live births in couples with idiopathic infertility undergoing non-invasive fertility treatments, such as timed intercourse or intrauterine insemination (IUI). However, the absence of such RNA elements does not appear to be critical when ARTs are employed.

Whereas the above described potentially new tools for male infertility diagnosis are still a long way off from use in clinical practice, sDF tests are utilized at present in many ART laboratories in support of traditional semen analysis. Many studies, summarized in the meta-analysis by Zini[13], have evaluated the effect of high sDF levels on the outcomes of both natural conception and ART. The meta-analysis concluded that pregnancy rate is negatively associated with sDF in natural insemination, IUI and IVF but not in intra-cytoplasmatic sperm injection (ICSI). These results were confirmed in a later meta-analysis[50]. Even more disturbing, the risk of miscarriage resulted as being strongly related to sDF levels in couples undergoing both IVF and ICSI[13]. Also these results were confirmed in recent meta-analyses[51,52]. Interestingly, the review by Robinson *et al*[51], pointed out the importance of the methodology used to evaluate sDF, as a subgroup analysis demonstrated that the association with miscarriage is strongest for studies employing the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

The methodology used in sDF studies represents an important issue. Among the various techniques employed to detect sDF[53], Sperm Chromatin Dispersion assay is the only standardized one and the only one for which there is enough agreement on the reference values across studies. Conversely, for the other methods, such as the widely employed TUNEL or COMET assays, standardization is lacking and established cut-off levels for fertility differ in the various studies. Recently, our group has set up a new refined flow cytometric method, TUNEL/propidium iodide, which allows a more accurate measure of sDF[54,55], eliminating all semen confounders[56]. Employing such a method, we have established a cut-off level for fertile subjects and demonstrated that sDF is able to discriminate between fertile men and patients regardless of age and semen quality[57]. sDF analysis in live sperm[58,59]

is an advancement of the TUNEL technique allowing clinicians to detect the damage in the sperm population which participates in the fertilization process. Another advancement is the possibility of assessing, in the same COMET slides, sDF and the presence of oxidative damage[60].

Despite the presence, in the literature, of many studies evaluating the impact of sDF on reproduction, a position report from the European Society of Human Reproduction and Embryology[61] and the guidelines for male infertility drafted by the American Society for Reproductive Medicine Practice Committee[62] claim that evaluation of sDF cannot be considered as a diagnostic test until "randomized, well-designed, adequately powered studies comparing infertile couples to a population of men with demonstrated recent fertility, and excluding cases with female infertility" are conducted in great number. However, as has recently been, introducing sDF among the diagnostic tests of male infertility could improve IVF success rate[63].

Finding the causes responsible for the generation of sperm DNA breaks could be the basis for the development of new therapeutic strategies to prevent the onset of sDF in infertile men. As oxidative stress is considered the main insult generating DNA damage in spermatozoa[64] and infertile men have lower levels of antioxidants and higher reacting oxygen species (ROS) amount in their semen compared to fertile men[65-67], many studies have investigated the effect of antioxidant administration on sDF. A recent Cochrane review[68] concluded that the current body of evidence does not allow for the deducing of clear conclusions regarding the role of antioxidants in the treatment of idiopathic infertility. Further well-designed randomized controlled trials are necessary in order, on one hand, to demonstrate the real efficacy of antioxidants and, on the other hand, to evaluate any eventual adverse events and their side effects[69]. Interestingly, we have recently demonstrated that sDF is mostly established in the testis as a result of an apoptotic process, whereas oxidative DNA damage occurs mostly during transit in the male genital tracts[70]. Accordingly, testis apoptosis should be the primarily target of therapies aimed to reduce sDF. Among these, treatment with follicle-stimulating hormone appears promising[71-73]. However, the complex role of apoptosis in human health makes it difficult to develop anti-apoptotic treatments for male infertility, whereas antioxidants remain an interesting object of study.

PROTEOMIC STUDIES ON MATURE SPERMATOZOA

In recent years, proteomic studies have been conducted in order to define sperm protein profiles and to characterize the role of different proteins in sperm functions. Over the years multiple strategies have been set up to study sperm proteome. In general, the first step is the isolation of spermatozoa from the complex semen matrix, then proteins are separated by various methods (Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis, two dimensional-gel electrophoresis, two dimensional fluorescence difference gel electrophoresis), analyzed by liquid chromatography-mass spectroscopy and identified by a database. Isolation of spermatozoa from semen matrix is a tricky step, representing a major limitation of these studies, as density gradient centrifugation or swim up (*i.e.*, the collection of a fraction of motile spermatozoa moving from semen to an upper medium) procedures, although they eliminate most immature germ cells and leukocytes, may lead to selection of a sperm population which is not representative of the entire sperm population present in the ejaculate. Another stumbling block in performing proteomic analysis is the poor amount of available sperm material in cases of oligozoospermia, thus leaving out a considerable portion of infertile subjects, as in many cases oligozoospermia is accompanied by other sperm defects, such as low motility and abnormal morphology.

In initial studies, few sperm proteins were detected, but the optimization of proteomic technologies has allowed, in recent years, to characterize more than 6000 proteins[74], even though proteins whose concentration is under the dynamic range of instruments remain undetected.

To investigate the roles of sperm proteins in male infertility, studies comparing proteomic profiles of different sperm samples have been performed. They compared infertile *vs* fertile subjects[75-78], asthenozoospermic *vs* normozoospermic men[79-83], male partners of couples undergoing successful ART *vs* those who failed[84-86], subjects with high sDF *vs* low sDF[87,88], men displaying elevated *vs* low ROS levels[89,90], and patients with metabolic disorders *vs* healthy men[91-93]. Overall, these studies led to the identification of a variable number of proteins, likely implicated in male infertility, that are down- or up-regulated in specific sperm defects. Results are, however, often inconsistent among the various studies, probably because of a high intra- and inter-variability of proteomic sperm profiles[94,95], the frequent use of pooled samples and problems related to sperm isolation (see above).

A further progression of proteomic studies is the isolation of proteins from specific sperm compartments leading to the association of the identified protein with its cellular localization and thus with its specific function. Using these approaches, several proteins have been assigned to the main compartments, including histone variants, transcription factors and zinc finger proteins in the nucleus[96,97], several receptors (progesterone receptor, metabotropic glutamate receptor, transforming beta growth factor receptor, Neurotensin receptor 3) to the sperm head[98] and proteins related to energetic metabolism, structure, and motility to the tail[82]. In the latter compartment, also proteins involved in lipid metabolism, mitochondrial oxidation and ADP/ATP carriers[99,100] have been found. Further studies are

needed to understand if these proteins are differentially expressed or mislocalized in spermatozoa from men with defects in motility or morphology.

POST-TRANSLATIONAL PROTEIN MODIFICATIONS IN MATURE SPERMATOZOA

Another point that increases the complexity of proteomic analysis is post-translational protein modifications (PTMs) that carry out an important role in the regulation of functions of mature spermatozoa which, being transcriptionally and translationally silent, mostly rely on PTMs to accomplish important and complex processes necessary for oocyte fertilization, such as capacitation, development of hyperactivated motility and acrosome reaction[101]. For this reason, expression levels *per se* could not have biological relevance for those proteins undergoing PTMs for their functionality. Phosphorylation is a well described PTM in spermatozoa and human phosphoproteomic studies found numerous differently regulated phosphoproteins involved in sperm capacitation[102] and motility[80]. Early studies by Buffone *et al*[103] demonstrated that spermatozoa from asthenozoospermic men showed a reduced protein tyrosine phosphorylation during capacitation *in vitro*, which may be related to a decrease in membrane fluidity leading to the inability to achieve a hyperactivated motility[104]. Among the proteins that are highly phosphorylated in tyrosine during the process of capacitation, A-kinase-anchoring proteins (for review see[105]), structural proteins of the sperm tail, represent an interesting target of these studies, in light of their involvement in motility.

Although ubiquitination is another important PTM, which most likely acts as a sperm quality control system during epididymal transit[106,107] and is related positively to normal sperm morphology[108], most ubiquitin-modified proteins in spermatozoa are still unknown. A similar PTM to ubiquitination is sumoylation, which is associated with poor motility, occurrence of DNA damage and recognition of morphologically defective spermatozoa[109,110]. Recently, Vigodner *et al*[109] identified by mass spectrometry several sumoylated proteins, whose role in sperm functions remains undefined.

Clearly, proteomic studies on spermatozoa are still in their infancy and need to be further validated in field trials before drafting a complete list of sperm proteins that may differentiate fertile and infertile subjects.

SPERM ION CHANNELS

In the attempt to find new male infertility markers, researchers have focused their attention on sperm ion channels having a central role in sperm physiology and in the fertilization process[111]. In particular, proton voltage-gated ion channels (Hv1) induce intracellular pH (pHi) modification involved in the capacitation process[112]. pHi regulation and the role of Hv1 channels has assumed importance with the discovery of two pHi- and voltage-sensitive ion channels, namely Slo3 and Cation channel of sperm (CatSper), that may be connected functionally to the regulation of important sperm activities. Slo3 is a sperm-specific potassium channel involved in mouse sperm capacitation[113], whose role in human sperm functions has yet to be defined. Recent studies have shown that Slo3 channel activity may be regulated also by intracellular calcium increase[114]. Calcium is a well-studied sperm second messenger, whose role in the fertilization process has been widely demonstrated over the last 15 years. Many different types of calcium channels have been described in spermatozoa. Among them, the CatSper calcium channel[115] appears to play a key role in intracellular calcium regulation. CatSper knock-out mice are unable to develop hyperactivated motility, and, for this reason, to reach and fertilize the oocyte[115-117]. Similarly, men with *CatSper* gene mutations leading to a lack of expression of the protein are infertile[118,119]. CatSper gained further importance when, in 2011, two independent groups of research[120,121] demonstrated that it is activated, in human spermatozoa, by progesterone which is considered the main candidate for stimulating the acrosome reaction process in the fertilizing spermatozoon[122,123]. We have demonstrated recently that sperm CatSper expression is lower in asthenozoospermic men and correlates positively with progressive and hyperactivated motility[124,125]. In addition, we found that CatSper (but none of the parameters evaluated by routine semen analysis) accurately predicts the ability of the sample to hyperactivate[125]. Conversely, the involvement of CatSper in the acrosome reaction process, although expected, is debated in the literature[124,126,127]. CatSper and Slo3 expression and activity may be related to the fertility status of the patient and may be involved in the pathogenesis of asthenozoospermia. However, introduction of CatSper or Slo3 evaluation in the diagnosis of male infertility is presently unlikely. Indeed, the techniques to evaluate their function or expression (patch clamping, flow cytometry and Western blot) are costly and/or need skilled personnel, becoming unsuitable for routine clinical practice. Studies on *CatSper* gene mutations or polymorphisms[118,128], if conducted in a large cohort of infertile men, could help to identify novel gene candidates for male infertility. In addition, both channels represent an attractive target for development of a male contraceptive[129,130], being expressed only in germ cells[114,115].

CONCLUSION

Follow-up studies reveal that ART children present an increased incidence of birth defects, prematurity and low birth weight[131], congenital malformations[132] and imprinting disorders[133] when compared to naturally conceived children. A large study conducted in Australian

Table 1 Promising sperm markers of male infertility based on so far published literature

Approach type	Main outcomes	Ref.	Advantages (+)/disadvantages (-)
Semen analysis			
	Macroscopic and microscopic evaluation of semen according WHO guidelines	[2]	(+) Established reference values (-) High operator variability (-) Poorly predictive of fertility
Genetic and epigenetic			
	NGS: Found a set of sperm RNA elements required to achieve live births	[47]	(+) Broad-spectrum analysis
	miRNA: Alteration of 5 miRNAs in subfertile and NOA subjects compared to controls	[46]	(-) Lack of validation (-) Not independently predictive of fertility
	DNA methylation: Different methylation pattern between fertile and infertile subjects	[42,43]	(-) Too early for diagnostic purpose
	sDF: Discrimination between fertile and infertile subjects	[55,56,58]	(+) Presently adopted in many ART laboratories (+) Prediction of fertility independent from semen quality (-) Employment of different techniques to detect sDF (-) Lack of agreement on cutoff values
Proteomic			
	> 6000 proteins (histone variants, transcription factors, zinc finger proteins, receptors, proteins related to metabolism, structure and motility, carriers)	[80,95-98]	(+) Broad-spectrum analysis (-) Isolation of spermatozoa (-) Low available sperm material in oligozoospermic subjects (-) Intra- and inter-variability of proteomic profiles
PTMs			
	Phosphorylation: Reduced tyrosine phosphorylation in asthenozoospermic subjects	[101]	(+) Higher biological relevance compared to gene or protein expression *per se*
	Ubiquitination: Sperm quality control system	[104]	(-) No target proteins identified
	Sumoylation: Marker of defective sperm	[107,108]	(-) Too early for diagnostic purpose
Ion channels			
	Slo3: Involved in hyperpolarization during sperm capacitation	[111,112]	(+) Analysis free from confounders
	CatSper: Involved in sperm progressive and hyperactivated motility	[123]	(-) Skilled personnel and advanced instruments are required (-) Too early for diagnostic purpose

PTMs: Post-translational protein modifications; WHO: World Health Organization; NGS: Next-generation sequencing; NOA: Non-obstructive azoospermia; sDF: Sperm DNA fragmentation; ART: Assisted reproduction technique.

ART couples demonstrated that, after multivariate adjustments for male and female factors of infertility, the risk for any birth defect retained statistical significance only for ICSI, hypothesizing that differences in male infertility factors, which lead to the use of ICSI, may underlie the phenomenon. Similarly, a recent large and well-designed retrospective study demonstrated that ICSI children have an increased incidence of neuro-developmental disorders[134]. Identifying the possible causes of male infertility may lead, in the future, to a decrease in ART children's anomalies, not only because of the possible development of new therapeutic strategies for male infertility but also because of the establishment of new technologies for a better sperm selection for ARTs. However, despite the urgency of establishing new diagnostic tests and defining new sperm markers of male infertility to be used in conjunction with semen analysis, new tests based on "omics" studies or in evaluating sDF (Table 1), are not routinely made a part of the diagnosis of infertile men, mainly because of a lack of standardized procedures, the need to validate the results, and the establishment of clinically accepted cut-off values.

Researchers' efforts should be devoted to gradually translating their acquired knowledge to clinical practice. In this respect, a continuous discussion between clinicians and researchers is desirable, so that basic research will be conducted on the real needs of the medical practice. This will allow for research innovations to be transformed into new diagnostic or therapeutic methods in order to achieve a more successful natural or assisted conception and delivery of healthy babies. The inclusion in clinical practice of new markers, employing advanced technologies, could be more expensive and may require skilled personnel compared to semen analysis, however, once such predictive markers are validated and, consequently, widely employed to diagnose male infertility, their costs will likely decrease, allowing a breakthrough in the management of infertile couples.

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REVIEW

Sphingolipid metabolism affects the anticancer effect of cisplatin

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Abstract

Cisplatin, a DNA crosslinking agent, is widely used for the treatment of a variety of solid tumors. Numerous studies have demonstrated that sphingolipid metabolism, which acts as a target for cisplatin treatment, is a highly complex network that consists of sphingolipid signaling molecules and related catalytic enzymes. Ceramide (Cer), which is the central molecule of this network, has been established to induce apoptosis. However, another molecule, sphingosine-1-phosphate (S1P), exerts the opposite function, *i.e.*, serves as a regulator of pro-survival. Other sphingolipid molecules, including dihydroceramide, ceramide-1-phosphate, glucosylceramide (GluCer), and sphingosine (Sph), or sphingolipid catalytic enzymes such as Sph kinase (SphK), Cer synthase (CerS), and S1P lyase, have also attracted considerable attention, particularly Cer, GluCer, SphK, CerS, and S1P lyase, which have been implicated in cisplatin resistance. This review summarizes specific molecules involved in sphingolipid metabolism and related catalytic enzymes affecting the anticancer effect of cisplatin, particularly in relation to induction of apoptosis and drug resistance.

Key words: Apoptosis; Sphingolipid metabolism; Drug resistance; Cisplatin; Anticancer

Core tip: Cisplatin classifies as a classical anticancer drug and DNA is identified as the most important target of cisplatin. However, increasing evidences have

testified that sphingolipid metabolism is associated with the anticancer effect of cisplatin. In this mini-review, we discussed sphingolipid signaling molecules and/or related enzymes affected the anticancer effect of cisplatin, particularly in cisplatin-induced cancer cell apoptosis and drug resistance. Targeting these sphingolipid molecules and enzymes might contribute to the development of novel anticancer strategies or to increase the sensitivity of currently used drugs.

Li YL, Lin ML, He SQ, Jin JF. Sphingolipid metabolism affects the anticancer effect of cisplatin. *World J Transl Med* 2016; 5(1): 37-45 Available from: URL: http://www.wjgnet.com/2220-6132/full/v5/i1/37.htm DOI: http://dx.doi.org/10.5528/wjtm.v5.i1.37

INTRODUCTION

The mechanisms underlying the anticancer effect of cisplatin (cis-diamminedichloroplatinum) have been extensively investigated by researchers since the discovery of its activity in 1969[1]. It is well known that DNA is the most important target of cisplatin in a variety of cancers, especially ovarian cancer, colorectal cancer, bladder cancer, testicular cancer, head and neck cancer, and lung cancer. DNA adducts of cisplatin with covalent coordinate bonds results in DNA damage and subsequent failure to maintain normal replication and ultimately induced apoptosis[2-5]. However, increasing evidences have testified that sphingolipid metabolism is associated with cancer therapies of cisplatin[6-8]. Treatment with cisplatin in several cancer cells often results in the generation of ceramide (Cer), which has been involved in regulating the cell death response. For example, cisplatin activates acid sphingomyelinase (aSMase) and induces the production of Cer in cancer cells, which triggers a series cellular response, including redistribution of CD95 and cell apoptosis[6]. In addition, sphingolipid molecules and relative enzymes have been implicated in regulating cisplatin sensitivity[7,8]. In this review, we mainly discuss the molecules of sphingolipid metabolism and relative enzymes affecting the anti-cancer effect of cisplatin, particularly in the induction of apoptosis and drug resistance.

SPHINGOLIPID METABOLISM AFFECTS THE FATE OF CANCER CELLS

Sphingolipids are membrane lipids that are important constituents of eukaryotic cells. Sphingolipid metabolism is a highly complex network that is composed of various sphingolipid molecules and enzymes that have been identified as pivotal regulators of various cellular processes, including cell growth, migration, adhesion, apoptosis, cell arrest, senescence, autophagy, and drug resistance[8-12]. Cer and sphingosine-1-phosphate (S1P) are the most essential sphingolipid molecules, followed by sphingosine (Sph), ceramide-1-phosphate (C1P), dihydroceramide, sphingomyelin (SM), and glycosphingolipids, of which glucosylceramide (GluCer), lactosylceramide, and galactosylceramide (GalCer) have been extensively studied in various sphingolipid metabolism pathways. Cer and S1P play opposite functions in the regulation of cell fate; the former is implicated in apoptosis[10,13-16], senescence[17,18], differentiation[19,20], and autophagy[11,21,22], whereas the latter promotes cell survival and proliferation, vasculogenesis, inflammation, and resistance to widely used drugs[23-25]. In addition, Sph inhibits cell cycle progression and induces apoptosis[26], whereas C1P and GluCer induce proliferation of cells and are associated with the development of resistance to cisplatin. As we all know, Cer is thought as the central in sphingolipid pathways (Figure 1), and is the precursor of several kinds of sphingolipid molecules, including SM, C1P, GluCer and GalCer. Cer and each of these sphingolipid molecules can be reversibly converted by the action of related enzymes. Cer is generated by multiple pathways, including the synthesis by *de novo*, the SM hydrolysis, or the Sph recycling[11,27,28]. The main generated route is the *de novo* synthesis pathway, which takes place in the endoplasmic reticulum (ER). Cer is synthesized from palmitoyl-CoA and serine to form 3-ketodihydrosphingosine through the catalyst, serine palmitoyltransferase[29,30]. Subsequently, the conversion of 3-ketodihydrosphingosine to sphinganine, which is condensed with fatty acyl-CoA by six specific dihydroceramide synthase (CerS 1-6) to form dihydroceramides of different lengths[31], which is then catalyzed by dihydroceramide desaturase to generate Cer. In the hydrolysis of SM pathway, SM is catabolized to Cer through the action of the neutral or SMase, but not alkaline SMase, which is mainly due to the specific forms of phospholipase C[32-35]. The catalysis of acidic and neutral SMases plays a pivotal role in apoptotic process and cell cycle arrest[32,36]. Cer is generated *via* dephosphorylation of C1P by Cer-1-phosphate phosphatase. In addition, C1P can be recovered from the phosphorylation of Cer by ceramide kinase. Cer can also be formed *via* the degradation of the glycosphingolipids, GluCer and GalCer, which each contains a single sugar molecule linked to Cer[37], and hydrolyzed by specific β-glucosidases and galactosidases, to yield Cer[38]. Inversely, GluCer is generated by GluCer synthase (GCS) in the Golgi apparatus[39].

Cer is degraded by ceramidases (CDase) to produce Sph, Sph is subsequently phosphorylated by two sphingosine kinase isoenzymes (SK-1 and SK-2) to produce S1P, which then is decomposed under the action of S1P lyase to produce ethanolamine phosphate and hexadecenal. This is the only exit pathway of this complex network. In addition, Sph and S1P can be recycled back to Cer by CerS or dephosphorylated back to Sph, respectively[40].

Recent studies have identified the sphingolipid molecule Cer and enzymes CerS, SK, and S1P lyases as important targets for developing anticaner drugs and

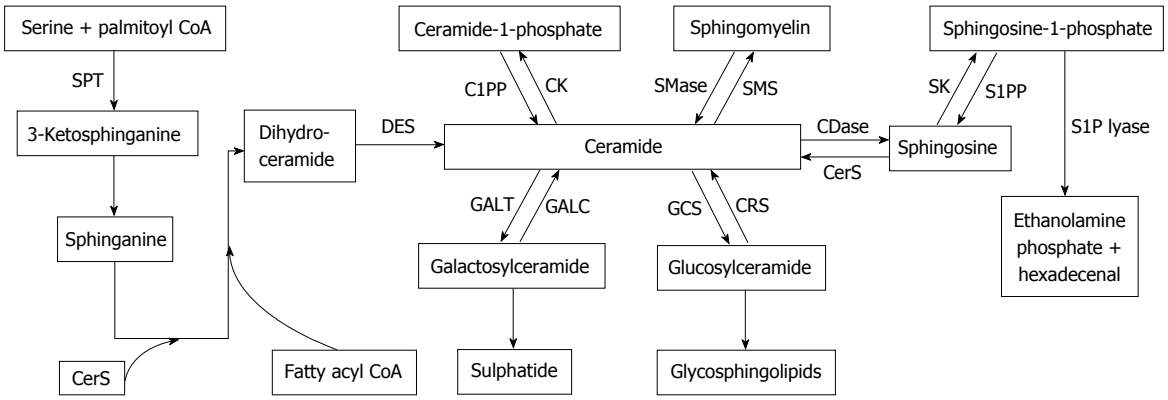

Figure 1 Sphingolipid metabolism pathways. Cer is the central in sphingolipid pathway, it is generated *via* multiple pathways, including the synthesis by *de novo*, the degradation of SM or the recycling of Sph; it is further metabolized and then produces many metabolites. Unidirectional arrows mean the generation of lipid molecules from one direction and bidirectional arrows mean mutual transformation between the two lipid molecules. SPT: Serine palmitoyl transferase; CerS: Ceramide synthase; DES: Dihydroceramide desaturase; C1PP: Ceramide-1-phosphate phosphatase; CK: Ceramide kinase; SMase: Sphingomyelinase; SMS: Sphingomyelin synthase; CDase: Ceramidase; SK: Sphingosine kinase; S1P: Sphingosine-1-phosphate; S1PP: S1P phosphatase; GALT: Galactosyltransferase; GALC: Galactosylceramidase; GCS: Glucosyl ceramide synthase; CRS: Cerebrosidase; Sph: Sphingosine.

drug resistance. Glycosphingolipids also play important role in multidrug resistance[15,41,42].

SPHINGOLIPID METABOLISM IN CISPLATIN-INDUCED CELL APOPTOSIS

Cisplatin induces apoptosis via the Cer-mediated mitochondria pathway

Although DNA is regarded as the main therapeutic target of cisplatin in various tumor cells, cisplatin induces apoptosis *via* signaling through plasma membrane lipid rafts that contain abundant sphingolipids, and these membrane lipid rafts are perhaps the targets of cisplatin-induced apoptosis[43-45]. It has been reported that sphingolipids are the major components of lipid rafts, and sphingolipids act as pivotal roles in maintaining the structural integrity of cell membranes and in modulating apoptosis *via* gene regulation and signal transduction[46]. In addition, an imbalance in sphingolipid levels results in apoptosis, which may be triggered by deviant intracellular apoptotic signaling[47]. Thus, cisplatin-induced apoptosis is closely associated with sphingolipid metabolism. However, Cer, the central molecule of sphingolipids metabolism, is involved in cisplatin-induced apoptosis. The two main apoptotic pathways include the receptor-involved extrinsic pathway and the mitochondria-associated intrinsic pathway[48]. The mechanism involved the Fas death receptor-mediated pathway that contributes to cisplatin-induced apoptosis will be discussed later. In the present section, we will talk about the Cer-played role in cisplatin-induced apoptosis in the mitochondria.

A central role in the intrinsic pathway of apoptosis is played by mitochondria. Stressors such as cisplatin, a chemotherapeutic agent, targets the mitochondria, resulting in the alteration of mitochondrial outer membrane permeabilization (MOMP) that promotes some proteins of mitochondria releasing from the intermembrane space into the cytosol. Then the caspase cascade pathway is activated and cells die within minutes. Thus, MOMP is strictly regulated and is identified as an irreversible event[49-51]. Early in 1993, Obeid *et al*[52] firstly illustrated that Cer is a potent apoptotic inducer. Subsequently, several studies have indicated that the increase in cellular Cer early in apoptosis is a common cellular response to cisplatin[8,53,54]. Research has shown that Cer, coupled with downstream Cer metabolites that participate in apoptosis, can change the function of mitochondria and give rise to increase of MOMP[49,50,55]. Accompanying the increase in cellular Cer levels, some proteins release from the mitochondrial intermembrane space to the cytoplasm, reactive oxygen species produce more in mitochondria, and the inner membrane potential of mitochondria is decreased[56-58]. Suppressing mitochondrial function can inhibit apoptosis induced by Cer[59]. In addition, the channels formation by Cer itself facilitates apoptosis in the mitochondrial membrane with elevated Cer levels[60]. Therefore, Cer is regarded as a pro-apoptotic molecule. A study in C6 rat glioma cells revealed that cisplatin-induced apoptosis links to Cer production resulting from cisplatin-mediated neutral sphingomyelinase activation. After that, cytochrome C releases from mitochondrion to the cytosol, which is dependent upon the BCL-2 family and activation of caspases-9 and caspases-3[55,61]. These post-mitochondrial events also intrinsically trigger apoptosis. Furthermore, Cers are generally synthesized from sphingoid bases, and very long (C24) or long (C16) fatty acid chains are added by specific Cer synthases. Cers containing different acyl chain lengths may affect susceptibility to cisplatin-induced apoptosis. During cisplatin-induced apoptosis, although intracellular Cer levels are not changed, C16 Cers are specifically elevated[54,62]. In addition, the function of certain proteins involved in apoptosis, including cathepsin D, PKC-ζ, PP1, PP2A, and ceramide-activated protein kinase, were modulated by Cer. These indirect mechanisms may possibly contribute to the mechanism underlying Cer-mediated apoptosis

that is involved in the mitochondria pathway[11].

Cisplatin induces apoptosis through the Cer-mediated death receptor Fas pathway

Fas (also known as CD95) use the death domain that is important for protein-protein interaction inside the cell to recruit Fas-associated death domain (FADD), subsequently to recruit the proenzyme of caspase-8[63]. It is necessary to recruit FADD (the adaptor protein) and procaspase-8 to the rafts of Fas ligation in order to initiate the signaling of Fas-mediated apoptosis, disrupting the integrity of rafts fails to initiate the Fas apoptotic signaling[64,65]. Previous reports have manifested that the death receptor Fas is localized in lipid rafts constitutively or under stimulation state, the receptor clustering in lipid rafts is necessary to the cell death mediated by Fas[66,67]. It has been reported that cisplatin causes the Fas clustering at the membrane of HT29 cancer cells derived from human colon, which in turn is inhibited by an inhibitor of aSMase, imipramine[44]. Additionally, CD95 could contribute to cisplatin-induced HT29 cell apoptosis in which redistribution of CD95 played a key role; however, a cholesterol sequestering agent, nystatin through preventing aSMase translocation and Cer production, inhibits cisplatin-induced CD95 clustering and decreases cisplatin-induced HT29 apoptosis[6]. Taking together, these results show that cisplatin triggers Fas redistribution into the plasma membrane rafts by activation of aSMase and induction of Cer production. Therefore, the contribution of Fas redistribution to cell apoptosis and cell death is clearly confirmed[6,44]. Furthermore, it has been reported that apoptosis is easy to be induced by many kinds of factors, for example cisplatin, Fas, tumor necrosis factor-1, growth factor withdrawal, or hypoxia. Several of above apoptotic stimuli can regulate Cer production, that hints us Cer plays an important role in apoptotic process[14]. In addition, the levels of Cer elevate in response to cisplatin, and the Cer increase by using inhibitors of enzymes that is responsible for metabolizing Cer or by overexpressing enzymes that account for Cer production leads to apoptosis[68]. The formation of Fas capping that involves decoupling of Fas ligand and Fas receptor at the plasma membrane enriched sphingolipids especially sphingomyelin is one mechanism in Cer-mediated apoptosis[69]. In other words, cells are resistant to mitochondria-involved apoptosis if they are not sensitive to Fas-mediated apoptotic signaling[70], suggesting that cells will lose sensitivity to death signaling if their Cer-Fas pathway is disturbed. Therefore, Cer has a tight connection with apoptosis, the Fas death receptor pathway is one of the mechanisms in which cisplatin induces apoptosis.

Cisplatin-induced apoptosis through other pathways

Several other mechanisms are responsible for the induction of cisplatin-induced apoptosis. Perrotta *et al*[71] reported that cisplatin triggers the apoptosis of dendritic cells (DCs) through increased expression and activation of aSMase, which could be inhibited by preconditioning DCs with nitric oxide donors. Further studies involving human colon cancer cells have shown that cells' acidification, which is depended on NHE1, appears early in the process of cisplatin-mediated apoptosis, subsequently leading to aSMase activation and fluidity elevation in cell membrane, which differs from cisplatin-induced DNA adduct formation[72]. Furthermore, de-N-acetyl-lysoglycosphingolipid, a hydrolyzed product of ganglioside GM1, inhibits the growth of various tumor cell lines, which occurs in synergy with cisplatin[73].

SPHINGOLIPID METABOLISM AFFECTS CISPLATIN RESISTANCE

Sphingolipid metabolism in Dictyostelium discoideum alters the sensitivity to cisplatin

Although cisplatin is an extremely effective drug that induces apoptosis in cancer cells, the efficacy of cisplatin treatment in some types of cancer is often impeded by drug resistance[74]. Therefore, intrinsic and acquired resistance to cisplatin is a vital problem when using this drug clinically. The mechanisms underlying cisplatin resistance[74-77] include some classical drug resistance mechanisms such as the decrease in the concentration of intracellular cisplatin, inactivation of the drug, increase in DNA repair, and reduction in apoptotic response. However, some resistance related genes including cyclooxygenase-2, heat shock proteins, or other cell signaling pathways and molecules also play some roles in the resistance to cisplatin. Additionally, cell membrane fluidity and lipids are also associated with cisplatin resistance[78]. To investigate the underlying molecular basis of resistance to cisplatin, Alexander *et al*[79] used *Dictyostelium discoideum* (*D. discoideum*) as an excellent eukaryotic model for studying the mechanisms underlying cisplatin drug sensitivity[79-82]. Genome sequencing of *D. discoideum* has shown that various genes and pathways are highly homologous to those in human cells[79,83]. Because the pathway of sphingolipid metabolism is highly conserved between humans and *D. discoideum*[79], mutations in sphingolipid metabolism-related genes confer cisplatin resistance in both species[78]. The role of some of the enzymes in sphingolipid metabolism (S1P lyase and SK) in the regulation of cisplatin resistance has been investigated by establishing a *D. discoideum* model[78,79,84]. S1P lyase (sglA) is highly conserved in humans and this enzyme accounts for the final metabolism in the sphingolipid pathway[85]. Although the sphingolipid metabolism pathway has been extensively investigated in mammalian cells, no previous studies have indicated the relationship between this pathway and cisplatin resistance prior to 2000. SglA was found for the first time to modulate sensitivity to anticancer drug cisplatin in *D. discoideum*[84]. Sphingolipids are involved in regulating cell fate, and the ratio of Cer and S1P levels could be used to determine whether cells enter the pathway of cell death or survival[86-89]. Various

stimuli, including γ-irradiation and anticancer drugs, have also been reported to lead Cer increase and/or to decrease S1P, which is a bioactive sphingolipid that plays a central role in apoptosis inhibition, pro-survival, or cell movement[23] in cancer cells. These effects are reversed with decreased Cer or increased S1P, which results in cell survival and proliferation. Therefore, we hypothesized that deletion of the S1P lyase increases resistance to cisplatin, whereas overexpression of this enzyme yields the opposite effect. The reports that the S1P lyase null (sglA$^{\Delta}$) and overexpressing cells (sglA^{OE}) displayed decreased or increased sensitivity to cisplatin, respectively, have thoroughly proven the above hypotheses in *D. discoideum*[7,90,91].

Two other enzymes associated with the direct regulation of the production of S1P in *D. discoideum* include the sgkA and sgkB sphingosine kinases that produce S1P from sphingosine and ATP. We thought that reducing sphingosine kinase expression leads cells are more sensitive to cisplatin, whereas over-expressing this enzyme results in resistance to this drug. *D. discoideum* sgkA and B genes mutants were generated, which harbored disrupted single or double sphingosine kinases or overexpressed the *sgkA* gene. Single or double disruption of the sphingosine kinases resulted in a reduction of growth rates, whereas overexpressing mutants presented elevated growth rates. Furthermore, these two enzymes showed a capacity to modulate sensitivity to cisplatin. The null mutants of sphingosine kinase appeared elevated sensitivity to cisplatin, whereas overexpression of SgkA in these mutants would rescue this effect. The addition of S1P or using N, N-dimethylsphingosine, a sphingosine kinase inhibitor[92], counteracts these effects[90]. The effects of sensitivity of the null or sgkA-overexpressing mutants were similar to those of another platinum-based drug, carboplatin. Taken together, these findings in *D. discoideum* allowed us to conclude that modulation of cisplatin sensitivity can be achieved through the regulation of related enzymes of sphingolipid metabolism.

Sphingolipid metabolism enhances cisplatin sensitivity in mammalian cells

Based on the above results, considerable attention has been paid to study cisplatin resistance and related mechanisms in mammalian cells. The results of studies on the mechanism underlying the resistance to cisplatin on *D. discoideum* should be confirmed in mammalian cells. Researchers have investigated the effect of overexpressing or deleting S1P lyase on cisplatin sensitivity in mammalian cells. The overexpression of S1P lyase in both human lung cancer (A549) and human embryonic kidney 293 cells resulted in an increase in cisplatin sensitivity, whereas the opposite effects were obtained with the disruption of S1P lyase[93]. The role of sphingosine kinases (SphK1 and SphK2, which are the equivalent of the SgkA and SgkB on *D. discoideum*, respectively) affecting cisplatin resistance was also examined in mammalian cells. Although these human isoenzymes generate the same product, S1P possesses different functions in cells[94-96]. Thus, SphK1 and SphK2 also had different effects on cisplatin sensitivity. Increasing the expression of SphK1 reduced cisplatin sensitivity, whereas SphK2 generated cells that with higher cisplatin sensitivity[8]. The deletion or overexpression of S1P lyase or SphKs affects the generation of S1P, indicating that the regulation of S1P is one of the mechanisms underlying cisplatin resistance.

Cer is regarded as another sphingolipid metabolism-related molecule that influences cisplatin sensitivity. Based on the bioactivity of Cer, the alteration of Cer accumulation alters a cell's sensitivity to cisplatin. Although Cer can be produced from various sphingolipids, *de novo* synthesis has proven to be the ultimate source of Cer. Each of the six key dihydroceramide synthase (CerS1 through CerS6) enzymes prefers a fatty acyl CoA with different chain length as a substrate to produce specific Cer molecules[31,97]. Three of these enzymes have yet to be tested in terms of its capacity to regulate cisplatin sensitivity. Only expression of CerS1 leads cell is more sensitive to the all tested drugs such as cisplatin, vincristine, doxorubicin, and carboplatin, accompanied by more p38 MAPK activation. Nevertheless, CerS5 expression resulted in an increased sensitivity to vincristine and doxorubicin, whereas the ovexpression of CerS4 had not similar effect on all the above mentioned reagents. The effects of CerS1 expression are implicated in its specific translocation from the ER to the Golgi apparatus, but not CerS4 or CerS5, and are reversed by the expression of SphK1, but not SphK2.

It has been previously reported that overexpression of GCS efficiently leads GluCer formation from Cer in some cancer cells, including breast cancer cells and human ovarian carcinoma cells[98-100]. Compared to sensitive cells, GluCer production is markedly higher in resistant cells[99,101,102], which is accompanied by an increase in the expression of P-glycoprotein, a membrane efflux transporter and one of the most common alterations in resistant cells[99,103,104], indicating that glucosylation of Cer is associated with drug resistance[105]. GCS is associated with multidrug resistant cancers and elevates the expression of multidrug resistance protein 1 (MDR1). Previous studies have revealed that MDR1 expression is markedly inhibited by siRNA-mediated GCS deletion, which functions as a membrane translocase and reverses drug resistance[98,99]. This finding indicates that the downregulation of GCS prevents the accumulation of glucosylceramide, which in turn increases sensitivity to anticancer drugs[15,106]. However, this phenomenon has not been observed despite the downregulation of GCS expression using specific inhibitors[107]. In addition, MDR1, as the major GluCer translocase, is required for the synthesis of neutral glycosphingolipids, but not for acid glycosphingolipids[108]. The production of glycosphingolipids with α-hydroxy fatty acids and longer carbohydrate chains is markedly higher in the human ovarian carcinoma cisplatin-resistant KF28 cells (KFr13) and taxol-resistant KF28 cells (KF28TX) compared to

that of sensitive KF28 cells, suggesting that changes in the glycosphingolipid composition of cancer cells are associated with cisplatin resistance[100]. Taken together, these results suggest that the molecules related to the sphingolipid metabolic pathway can be manipulated to a certain extent by regulating the expression of related enzymes to improve cisplatin sensitivity.

CONCLUSION

In conclusion, sphingolipid metabolism may play crucial roles in the induction of apoptosis and resistance of cisplatin. In particular, Cer is closely related to cisplatin-induced apoptosis and is considered a potential target for cancer therapeutics. To study the mechanisms underlying cisplatin resistance in sphingolipid metabolism pathways, *D. discoideum* was established as an excellent eukaryotic model. The results obtained from this model have been extensively translated to and validated in human cells. Thus far, sphingolipid molecules particularly S1P, GluCer, and related enzymes, particularly SphK, CerS, and S1P lyase have been implicated in cisplatin sensitivity. Tumor pathogenesis is considered as an intricate process; therefore, to fully understand the mechanisms underlying the use of cisplatin as an anticancer drug targeting the sphingolipid metabolism pathway, a variety of strategies should be utilized. Targeting these essential molecules of sphingolipid metabolism may contribute to the development of novel anticancer strategies or to increase the sensitivity of currently used drugs.

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MINIREVIEWS

Naked DNA in cells: An inducer of major histocompatibility complex molecules to evoke autoimmune responses?

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Abstract

The major histocompatibility complex (MHC) is the exclusive chaperone that presents intracellular antigens, either self or foreign to T cells. Interestingly, aberrant expression of MHC molecules has been reported in various autoimmune target tissues such as thyroid follicular cells in Grave's disease. Herein, we review the discovery of an unexpected effect of cytosolic double-stranded DNA (dsDNA), despite its origins, to induce antigen processing and presenting genes, including MHC molecules, in non-immune cells. Moreover, we highlight several recent studies that suggest cell injury endows thyroid epithelial cells with a phenotype of mature antigen presenting cells by inducing multiple antigen processing and presenting genes *via* releasing genomic DNA fragments into the cytosol. We discuss the possibility that such cytosolic dsDNA, in naked form without binding to histone proteins, might be involved in the development of cell damage-triggered autoimmune responses. We also discuss the possible molecular mechanism by which cytosolic dsDNA can induce MHC molecules. It is reasonable to speculate that cytosolic dsDNA-induced MHC class Ⅰ is partially due to an autocrine/paracrine effect of type Ⅰ interferon (IFN). While the mechanism of cytosolic dsDNA-induced MHC class Ⅱ expression appears, at least partially, distinct from that mediated by IFN-γ. Further in-depth are required to clarify this picture.

Key words: Cytosolic double-stranded DNA; Major histocompatibility complex molecules; Autoimmune response; Antigen presentation; Tissue injury

Core tip: We reviewed the discovery of an unexpected effect of cytosolic double-stranded DNA (dsDNA) to induce antigen processing and presenting genes including major histocompatibility complex (MHC) molecules in non-immune cells. We also focus on the

current status quo of the overall research in the field with highlight on our recent findings that suggest cell injury-induced self-cytosolic dsDNA is a potential trigger in the development of autoimmunity. Meanwhile, we provide in-depth discussion of the molecular signals responsible for the effect of dsDNA to induce MHC molecules, based on the current opinion of dsDNA-mediated signal pathways.

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INTRODUCTION

The major histocompatibility complex (MHC) is the exclusive chaperone that presents intracellular antigens (more specifically, peptides), either synthesized by the cell itself or internalized from the extracellular environment, to T cells. Upon binding to the MHC-peptide complex *via* the T cell receptor and CD4/CD8 co-receptors, T cells should in principle tolerate self antigens. In contrast, activation should occur if the T cells had not been trained to recognize the antigen during thymus positive selection. If this principle, known as immune tolerance, is violated, an autoimmune response will occur. Interestingly, aberrant expression of MHC molecules has been reported in endocrine epithelial cells in autoimmune target tissues (Table 1) such as pancreatic beta cells of insulin-dependent diabetes[1] and thyroid follicular cells of Grave's disease[2-5]. Transgenic mouse strains harboring MHC linked to insulin promoter overexpress MHC molecules in pancreatic beta cells and spontaneously develop insulin-dependent diabetes[6-8]. Many attempts have been made to artificially induce Grave's disease in mice. Although not very successful, these efforts have demonstrated that autoantibodies developed only when the animals were immunized with cells co-expressing the self antigen, thyroid stimulating hormone receptor (TSHR), and MHC class Ⅱ molecules[9]. The few successful mouse models of Grave's disease were generated by vaccination of TSHR-expressing plasmids or by infection of TSHR-expressing adenovirus[10,11]. Significantly, both DNA vaccine and adenovirus are primarily double-stranded DNAs (dsDNAs) that should be able to induce MHC molecules in the cells at the site of inoculation. Does this "adjuvant-effect" contribute to the successful generation of the autoimmune mouse model?

CYTOSOLIC DOUBLE-STRANDED DNA INDUCES MHC MOLECULES IN NON-IMMUNE CELLS

In 1999, Suzuki *et al*[12] reported that the expression of MHC molecules, including MHC class Ⅰ and MHC class Ⅱ, could be strongly induced by the transfection of dsDNA, regardless of their origins. Diverse dsDNAs were tested *in vitro,* including bacterial DNA, viral DNA, salmon sperm DNA, calf thymus DNA, host genomic DNA, plasmid DNA and artificially synthesized DNA fragments. Remarkably, all induced MHC expression, whereas single-stranded DNA (ssDNA) could not[12]. To exert this effect, dsDNA needs to be introduced into the cytosol, as free dsDNA in extracellular medium was not sufficient to induce MHC expression[13], indicating that this effect is unlikely mediated by cell surface receptors. The method of introducing dsDNA into cytosol is not critical. Different transfection procedures, including lipofection, electroporation and diethylaminoethyl-dextran similarly increased MHC levels[12]. The first study that explicitly and thoroughly described such effects specific to cytosolic dsDNA was not reported until 1999. This was surprisingly late, considering that cell culture transfection methods were developed in the 1970s and became widespread during the 1980s, although it had been previously observed that fibroblasts could somehow respond to nucleic-acids derived from pathogens or the host[14,15].

The effect of cytosolic dsDNA does not require professional antigen presenting cells (APCs). In addition to professional APCs, including primary cultures of mouse dendritic cells (DCs) and macrophages, the induction of MHC molecules by cytosolic dsDNA was reproduced in non-professional APCs such as rat thyroid follicular cells, human and mouse fibroblasts, human and mouse muscle cells and human endothelial cells[12,13,16,17]. These results imply the possibility that a potential APC pool consisting of various non-immune cells *in vivo* can be activated upon their exposure to cytosolic dsDNA (possibly derived from invasive pathogens or dying host cells). In particular, direct evidence has shown that MHC-expressing thyroid epithelial cells are potentially competent APCs to present antigens to activate T cells. MHC class Ⅱ-positive human thyroid follicular cells were able to present a influenza-specific peptide to a human T-cell clone, a reaction which was abrogated by anti-MHC class Ⅱ antibodies[3]. Lectin-induced MHC class Ⅱ-positive human thyroid cells in monolayer culture were able to induce a proliferative reaction in autologous T cells, a phenomenon not found with MHC class Ⅱ-negative cells[18]. Wistar rats are susceptible to the induction of experimental autoimmune thyroiditis. A cloned Wistar thyroid epithelial cell line was shown to be directly recognized by Wistar rat lymphoid T cells that were both MHC class Ⅰ- and class Ⅱ-restricted[19]. When CBA mouse lymphoblasts generated on co-culture with monolayer syngeneic thyroid epithelial cells were injected either intravenously or into the thyroid lobes of intact CBA recipients, thyroiditis appeared within three weeks[20]. All these evidence suggest that the potential ability of thyroid epithelial cells as APCs to directly interact with T cells in a MHC-restricted manner likely precipitates autoimmune response in the thyroid, although whether exposure to cytosolic dsDNA would

Table 1 Inappropriate expression of major histocompatibility complex molecules in autoimmune disorders

Disease	Cells with aberrant expression of MHC molecules
Insulin-dependent diabetes	Pancreatic beta cells[1]
Grave's disease	Thyroid epithelial cells[54]
Rheumatic carditis	Valvular fibroblasts[55]
Primary biliary cirrhosis	Bile duct epithelial cells[56]
Sjögren's syndrome	Salivary acinar and ductal epithelial cells[57]
Acute lymphoproliferative disorders	Bone marrow-derived mesenchymal stromal cells[58]
Asthma[1]	Bronchial epithelial cells[59]
Dilated cardiomyopathy[1]	Endothelial and endocardial cells[60]
Tubulointerstitial nephritis[1]	Renal tubular epithelial cells[61]
Biliary atresia[1]	Intrahepatic bile ducts[62]

[1]A role of autoimmunity is suggested in the pathology of the indicated diseases. MHC: Major histocompatibility complex.

substantialize such potential in non-immunes cells needs to be further tested by experiments.

Unmethylated CpG motifs, which commonly exist within bacterial DNA and viral DNA, but not in vertebrates, have been shown to activate innate immunity *via* CpG sensor toll-like receptor 9 (TLR9)[21]. However, CpG motif-containing ssDNA failed to induce MHC molecules whereas methylase-treated CpG dsDNA induced MHC expressions to a level comparable to untreated CpG dsDNA[12]. These results indicated that the induction of MHC molecules by cytosolic dsDNA is not mediated by CpG motifs. In contrast, DNase treatment, as predicted, completely abolished the induction of MHC molecules following dsDNA transfection[12,13]. Later, it was shown that the effect of cytosolic dsDNA to stimulate a host innate immune response was independent of TLRs, but required a classic double-stranded right-handed helix sense (B-DNA)[22] with a native sugar-phosphate backbone[16]. Although the effect of dsDNA on MHC appears sequence-independent, MHC expression was shown to be induced by dsDNA in a length-dependent manner[12,13]. Nevertheless, a DNA fragment as short as 25 bp was capable of exerting a reproducible concentration-dependent effect on the expression of MHC molecules[12]. It was shown in later studies that cytosolic dsDNA activated innate immune responses in a length-dependent manner. This result might indicate an increasing binding affinity by putative cytosolic dsDNA sensors for longer dsDNA[22-24].

Antigen processing and presenting is a multiple-step process involving numerous molecules with diverse functions, such as proteasome proteins responsible for antigen degradation to generate peptides, *e.g.*, LMP2 and cathepsin; molecules required for transporting and loading peptides onto MHC molecules, *e.g.,* transporter associated with antigen processing, MHC Ⅱ-associated invariant chain (Ii), and HLA-DMB; cell surface co-stimulators indispensable for fully activating T cells, *e.g.,* CD80, CD86, CD40; and cell adhesion molecules for stabilizing the binding with lymphocytes, *e.g.,* CD54 (also known as intracellular adhesion molecule 1)[25,26]. In addition to MHC molecules, many of these essential participants in antigen processing and presenting, as well as the transcription factors for MHC expression, including signal transducers and activators, interferon regulatory factor 1, nuclear factor κB (NF-κB), and class Ⅱ MHC transactivator (CⅡTA), have also been shown to be induced/activated by cytosolic dsDNA, but not ssDNA, in both professional APCs and non-professional APCs[12,13,16,17]. Overall, these results suggest that in the presence of cytosolic dsDNA, even non-immune cells can acquire full capability to present antigens (so called APC maturation). Theoretically, T cells should have a much greater chance to be activated by antigens under this condition. In agreement with such a prediction, production of interleukin-2 (IL-2) and interferon-γ (IFN-γ) in T cells was significantly increased (approximately 3-fold) by mixing with peptide-challenged DCs containing cytosolic dsDNA, compared to those without cytosolic dsDNA, or those containing cytosolic ssDNA[13].

TISSUE INJURY INDUCES APC MATURATION *VIA* CYTOSOLIC DNA

DNA, normally sequestered within the nucleus or in mitochondria, can be internalized into the cytosol of phagocytes from apoptotic bodies released from dying cells *in vivo*. Phagocytes engulf the apoptotic bodies (also the nuclei expelled from erythroid precursor cells) in extracellular medium and digest the DNA contents using DNase in the phagolysosomes[27,28]. Indeed, a large amount of cytosolic DNA has been demonstrated to accumulate in DNase-deficient murine macrophages that were presented apoptotic cells, but only a small amount in wild-type macrophages after the same treatment[29]. Defective clearance of self DNA due to mutations in DNase genes is associated with the development of human autoimmune diseases such as systemic lupus erythematosus[30,31] and Aicardi-Goutieres syndrome[32,33], thus revealing self DNA as a potential trigger for autoimmune responses.

Despite the presence of normal DNase, tissue injury, depending on the severity, can generate more dying cells than the intrinsic digestive capacity of phagocytes can process, inevitably leading to cytosolic DNA accumulation in phagocytes. It has been shown that electric pulse-mediated cell injury under sterile conditions induced cytosolic DNA in a current intensity-dependent manner in rat thyroid epithelial cells[17], supporting the notion that tissue injury is a possible cause for the presence of cytosolic DNA *in vivo*. Intriguingly, electric pulse-caused cell injury also induced the expression of MHC Ⅱ and its transactivator CⅡTA in a current intensity-dependent manner in rat thyroid epithelial cells[12]. Such cell injury endows thyroid epithelial cells with a phenotype of

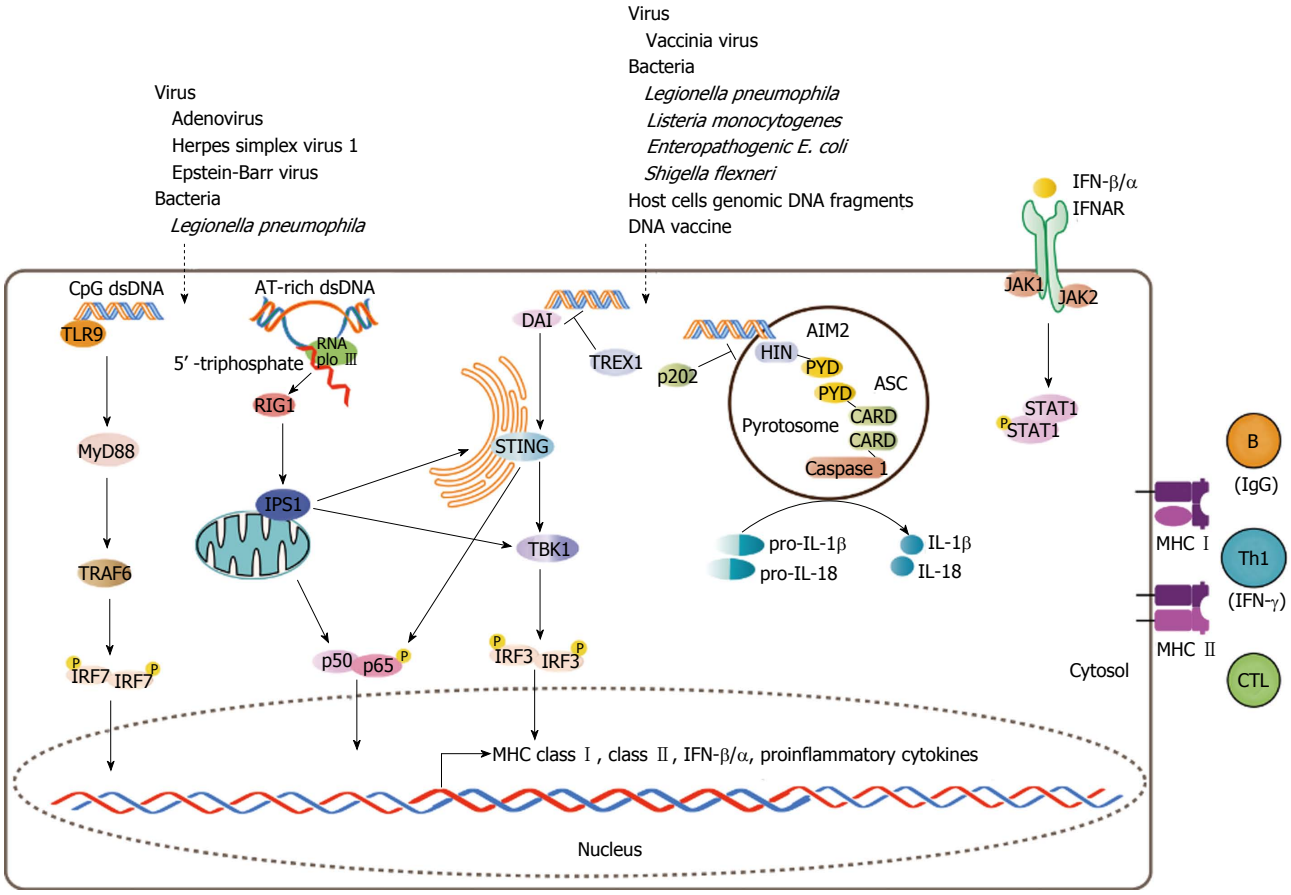

Figure 1 Cytosolic double-stranded DNA signal pathways. TLR9-dependent and TLR9-independent signal pathways have been proposed to mediate foreign- or self-derived cytosolic dsDNA signal to induce the expression of MHC molecules, type Ⅰ IFNs, and proinflammatory cytokines. At the same time, cytosolic dsDNA can trigger AIM2-mediated inflammasome formation to produce active IL-1 and IL-18. Consequently, exposure to cytosol dsDNA will increase the probability of (auto)immune response. TLR9: Toll-like receptor 9; MyD88: Myeloid differentiation primary response gene 88; TRAF6: Tumor necrosis factor receptor associated factor 6; IRF7/3: Interferon regulatory factor 7/3; RNA pol Ⅲ: RNA polymerase Ⅲ; RIG1: Retinoic acid-inducible gene 1; IPS1: Interferon-promoter stimulator 1; DAI: DNA-dependent activator of interferon - regulatory factors; TREX1: 3-5 exonuclease (also known as DNase Ⅲ); STING: Stimulator of interferon genes; TBK1: TANK-binding kinase 1; AIM2: Absent in melanoma 2; ASC: Apoptosis-associated speck-like protein complex; HIN: C-terminal HIN-200 domain; PYD: N-terminal pyrin domain; CARD: Caspase activation and recruitment domain; IFNAR: Type Ⅰ interferon receptor; STAT1: Signal transducer and activator of transcription 1; JAK1: Janus kinase 1; CTL: Cytotoxic T lymphocytes; IFN: Interferon; MHC: Major histocompatibility complex; IL: Interleukin; dsDNA: Double-stranded DNA.

mature APC by inducing multiple antigen processing and presenting genes that largely overlapped with cytosolic DNA-inducible molecules such as MHC Ⅰ, MHC Ⅱ, CD40, CD54, CD80, CD86, RFX5 and CⅡTA[12,17].

APC maturation characterized by increased expression of CD40 was also observed in primary cultures of immature murine CD11c+ bone marrow dendritic cells (BMDCs) when cultured with necrotic fibroblasts derived from the same animal[13]. Proteinase-K-treated necrotic fibroblasts reproduced the same effect, inducing APC maturation in BMDCs to a similar extent[13], indicating self protein was unlikely the essential factor. However, additional DNase treatment significantly abrogated the ability of necrotic fibroblasts to induce APC maturation[13], suggesting it is likely that the self DNA derived from the dying cells had contributed to the activation of APCs. However, the profile and kinetics of cell injury-induced genes was not completely duplicated by that induced by dsDNA transfection[12,17], indicating additional factors other than cytosolic DNA may be involved in a cell injury event.

MOLECULAR SIGNALS FOR CYTOSOLIC DNA-INDUCED MHC MOLECULES

Cytosolic DNA-mediated signal pathways have been extensively investigated (well-reviewed in[34]). Two independent responses can be simultaneously induced by cytosolic dsDNA, one characterized by the production of type Ⅰ IFNs (IFN-α and -β) as well as type Ⅰ IFN-inducible molecules, and a second pro-inflammatory response characterized by the production of IL-1β and IL-18, both of which have been implicated in the activation of the immune response[34] (Figure 1).

It is reasonable to speculate that cytosolic dsDNA-induced APC maturation is partially due to an autocrine/paracrine effect involving secreted type Ⅰ IFNs, mediated by the cell surface type Ⅰ IFN receptor (IFNAR) (Figure 1). Such a process could stimulate APC maturation in DCs and precipitate T cell activation *in vitro* and *in vivo*, concomitantly with increased expression of antigen processing and presenting genes, including MHC Ⅰ, CD40 and CD86[35-37] (Figure 1). Repeated low-

dose chemotherapy or radiation could also trigger an autoimmune response. These cellular insults can induce MHC Ⅰ expression in cancer cells *via* the IFN-β/IFNAR signal pathway[38] and enhance $CD8^+$ T cell-mediated antitumor immune responses to tumor vaccine *in vivo*[39]. As a therapeutic strategy to restore autoimmune surveillance in cancer cells, low-dose chemotherapy is given to metastatic pancreatic cancer patients before receiving a cell-based cancer vaccine[40]. Thus, cell damage-induced autoimmunity may not be entirely harmful if wisely used.

Moreover, it is possible that the induction of type Ⅰ IFNs and antigen processing and presenting genes share some upstream signals in common, such as STAT and NF-κB signal pathways that could be directly activated upon the recognition of cytosolic dsDNA as a "danger signal"[12,17,34,41]. Thus, it is likely that the signal pathways that mediate type Ⅰ IFN production and the induction of antigen processing and presenting genes cross-talk with one another (Figure 1). Stimulator of IFN genes (STING)[42,43] and TANK-binding kinase 1 (TBK1)[44,45], which have been shown to mediate cytosolic dsDNA-induced type Ⅰ IFN production (Figure 1), may also be required for the induction of antigen processing and presenting genes by cytosolic dsDNA, as DNA vaccine-mediated T cell activation was abolished in STING-knockout mice that were challenged with antigen peptides[43]. Moreover, TBK1-deficiency abrogated cytosolic dsDNA-induced APC maturation in primary mouse bone marrow macrophages[45]. Further studies are required to clarify this picture.

Cytosolic dsDNA-induced MHC Ⅱ expression should be IFNAR-independent, as type Ⅰ IFNs do not induce MHC Ⅱ, but rather suppress its expression by acting as an antagonist of IFN-γ[46], especially in non-professional APCs[47]. Indeed, cytosolic dsDNA prominently induced MHC Ⅰ rather than MHC Ⅱ in rat thyroid epithelial cells *in vitro*[12]. Knockout mice have revealed something more *in vivo*. Both MHC Ⅰ and MHC Ⅱ induction occurred in areas of tissue injury in IFN-γ-deficient mice, but with 50% less induction than that in the wild-type[48], suggesting that the IFN-γ signal contributed half of the effect to induce MHC molecules triggered by tissue injury *in vivo*, while IFN-γ-independent signals were also at play. It is possible *in vivo* that the activated T cells secrete IFN-γ (Figure 1), which in turn induces more MHC molecules on APCs to facilitate antigen presentation to further accelerate T cells activation and IFN-γ secretion, thus forming a positive-feedback loop in the area of injury.

Cytosolic dsDNA-induced production of IL-1β and IL-18 is mediated by a rather independent upstream signal pathway that involves absent in melanoma 2, apoptosis-associated speck-like protein complex and caspase 1 cleavage[49-52] (Figure 1). Nevertheless, the contribution of a pro-inflammatory extracellular milieu to the development of autoimmunity should never be underestimated.

CONCLUSION

Numerous factors must be working together to trigger an autoimmune response: Environmental stimuli (*e.g.*, those that can cause tissue injury) and genetic predisposition (*e.g.*, having a specific human leukocyte antigen haplotype increases the risk of autoimmune diseases[53]). Studies have indicated that cytosolic naked dsDNA (either foreign or self origin) could be a universal factor that activates both innate and acquired immune responses in any tissue and cell type to trigger unfavorable immune responses in autoimmune-prone individuals.

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ORIGINAL ARTICLE

Basic Study

Prednisolone inhibits SaOS2 osteosarcoma cell proliferation by activating inducible nitric oxide synthase

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Abstract

AIM: To investigate the effect of prednisolone, a synthetic glucocorticoid used in inflammatory diseases, on the growth of cultured osteosarcoma cells.

METHODS: Two osteosarcoma cell lines with different degree of differentiation were used. SaOS2 show a rather mature phenotype, while U2OS are negative for almost all osteoblastic markers. The cells were exposed to different concentrations of prednisolone (1-9 μmol/L) with or without antioxidants or the inhibitor of inducible nitric oxide synthase (iNOS) l-N^6-(iminoethyl)-lysine-HCl (L-NIL). Cell growth was assessed by counting viable cells. The production of nitric oxide (NO) was measured in the conditioned media by the Griess method. The production of reactive oxygen species was quantified using 2′-7′-dichlorofluorescein diacetate. Western blot with specific antibodies against NOSs was performed on cell extracts.

RESULTS: Prednisolone inhibited SaOS2 cell growth in a dose dependent manner. No significant effects were observed in U2OS. The inhibition of SaOS2 growth is not due to oxidative stress, because antioxidants do not rescue cell proliferation. Since high concentrations of NO inhibit bone formation, we also measured NO and found it induced in SaOS2, but not in U2OS, exposed to prednisolone, because of the upregulation of iNOS as detected by western blot. Therefore, we treated SaOS2 with prednisolone in the presence or in the absence of L-NIL. L-NIL prevented NO release induced by prednisolone at all the concentrations apart from 9 μmol/L. At the same concentrations, we found that L-NIL rescued SaOS2 growth after exposure to prednisolone. In U2OS cells, prednisolone did not induce NO production nor affected cell growth. All together, these data indicate that a link exists between increased amounts of NO and growth inhibition in response to prednisolone in SaOS2.

CONCLUSION: Prednisolone inhibited SaOS2 proliferation by increasing the release of NO through the upregulation of iNOS, while no effect was exerted on U2OS.

Key words: Osteosarcoma cells; Prednisolone; Nitric oxide; Inducible nitric oxide synthase; Endothelial nitric oxide synthase; Reactive oxygen species

Core tip: Since prednisolone, a widely used synthetic glucocorticoid, inhibits osteoblast proliferation, we evaluated its effects on osteosarcoma cells. In particular, we used two osteoblastic osteosarcoma cell lines with different degree of differentiation, *i.e.*, SaOS2, which have a rather mature phenotype, and U2OS, which are less differentiated. We found that prednisolone inhibited SaOS2 proliferation by increasing the release of nitric oxide (NO) through the upregulation of inducible NO synthase (iNOS). Indeed, pharmacological inhibition with the iNOS inhibitor l-N^6-(iminoethyl)-lysine-HCl restored the normal proliferation rate of the SaOS2. On the contrary, prednisolone did not modulate NO production nor cell growth in U2OS.

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INTRODUCTION

Osteosarcomas are aggressive primary malignant tumors of the bone characterized by the deposition of immature bone by the neoplastic cells which most likely arise from mesenchymal stem cells. Osteosarcomas mostly affect teenagers and frequently metastasize. Nowadays, systemic multidrug chemotherapy and surgery are successful in 60%-70% of patients. Therefore, novel approaches are foreseen.

Since glucocorticoids participate to the regulation of survival, differentiation, and proliferation of many cell types, including osteoblasts and bone mesenchymal stem cells[1-3], we asked whether glucocorticoids might control the growth of osteosarcoma cells. Glucocorticoids act by binding their cognate receptor which functions as a hormone-regulated transcription factor. In addition, glucocorticoids interact with transcription factors such as AP1 and nuclear factor kappa B (NF-κB) and inhibit their activity. They can also modulate some intracellular signalling pathways, one of which is the MAP kinase cascade. Because of their effects on cell cycle progression and apoptosis[4], they are also used in the treatment of lymphoid malignancy and of some solid cancers[5,6].

In this study, we evaluate the effect of prednisolone, a synthetic glucocorticoid widely used to treat inflammatory diseases, on cultured osteosarcoma cells. It is well known that cultured neoplastic cells have been the basis of cancer biology and the chase to identify drug treatments[7]. Two human osteosarcoma cell lines are particularly intriguing, *i.e.*, SaOS2 and U2OS, which are among the first generated cell lines used for anticancer research[8]. U2OS were derived from a moderately differentiated sarcoma of a 15-year-old girl, and SaOS2 from an osteogenic sarcoma of an 11-year-old girl. SaOS2 are relatively resistant to drugs because of the mutation of major oncosuppressors, *i.e.*, p53 and Rb[9], which are functional in U2OS. While SaOS2 show a mature phenotype, U2OS are negative for almost all osteoblastic markers but positive for cartilage markers like collagen Ⅱ, Ⅸ and Ⅹ and for type Ⅳ collagen, which is only expressed in very early differentiation stages but not by mature osteoblasts. These two cell lines were selected for this study because of their different degree of differentiation and gene expression.

MATERIALS AND METHODS

Cell culture

SaOS2 and U2OS (American Type Culture Collection) were cultured in DMEM containing 10% fetal bovine serum. Proliferation assays were performed on cells at low density (7000/cm^2) with different concentrations of prednisolone. After trypsinization and staining with trypan blue solution (0.4%), the viable cells were counted. In some experiments cells were exposed to apocynin (10 μg/mL), trolox (40 μmol/L), or l-N^6-(iminoethyl)-lysine-HCl (L-NIL) (100 μmol/L), a selective inhibitor of inducible nitric oxide synthase (iNOS).

Reactive oxygen species evaluation

Intracellular oxidative stress was quantified using 2′-7′-dichlorofluorescein diacetate (DCFH). Cells were seeded into black bottomed 96 plates (Greiner Bio-One) and 24 h later exposed for 30 min to different concentrations of prednisolone dissolved in a 20 μmol/L DCFH solution. The rate of intracellular oxidative stress was evaluated by monitoring the emission at 529 nm of the DCFH dye using Promega Glomax Multi Detection System[10]. Data are shown as the mean of three independent experiments in triplicate ± SD. H_2O_2 (50 μmol/L) was used as a positive control.

NOS activity

NOS activity was measured in the conditioned media by the Griess method as described[11]. Data are shown as the mean of four independent experiments in triplicate ± SD.

Western blot analysis

Western blot was performed using anti-iNOS, total endothelial nitric oxide synthase (eNOS) and p-eNOS^{Ser1177} antibodies (Cell Signalling Technology) followed by

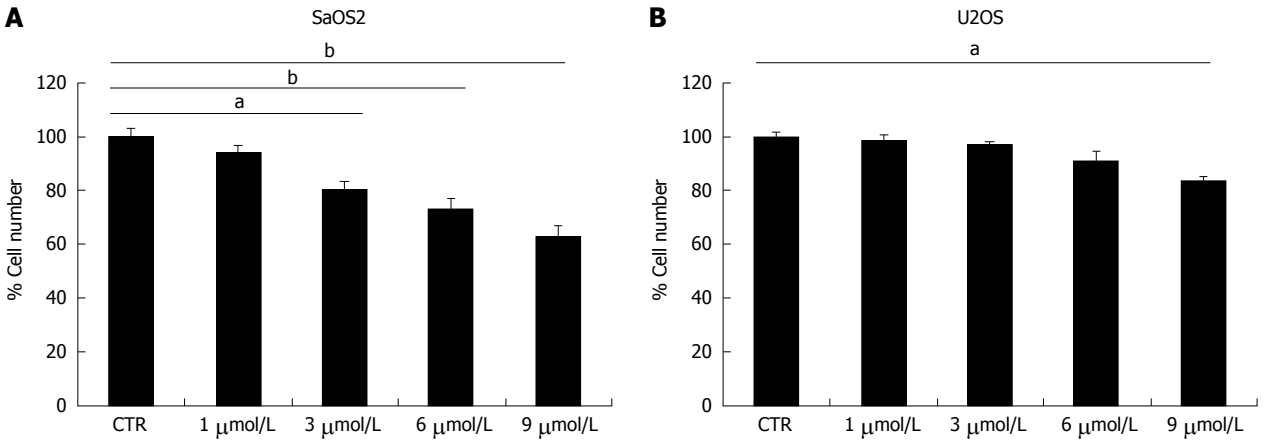

Figure 1 Prednisolone inhibits the growth of SaOS2 but not of U2OS. A: Viable SaOS2 cells were counted after 4 d in the presence of different concentrations of prednisolone ($^{a}P < 0.05$; $^{b}P < 0.01$); B: Viable U2OS cells were treated as above and counted after 96 h ($^{a}P < 0.05$). Results are shown as the mean of three separate experiments ± SD. CTR: Control.

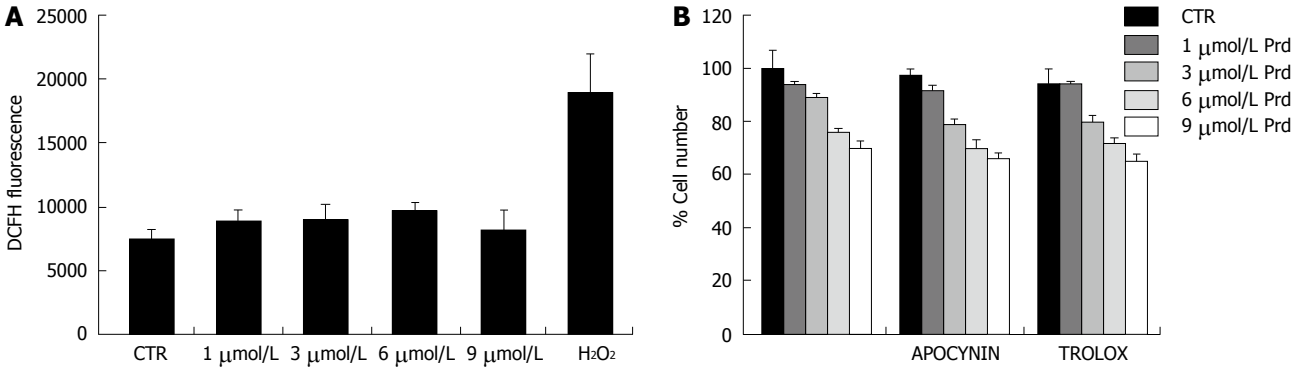

Figure 2 Prednisolone does not induce the formation of reactive oxygen species and antioxidants do not prevent growth inhibition. A: SaOS2 cells were treated with various concentrations of prednisolone. H_2O_2 was used as positive control. Reactive oxygen species generation was measured. Data are shown as the mean of three separate experiments ± SD. *P* value was calculated *vs* untreated cells and found not significant; B: SaOS2 cells were treated with apocynin (10 μg/mL) or trolox (40 μmol/L) in the presence of prednisolone (Prd). Viable cells were counted after 96 h. Results are shown as the mean of three separate experiments ± SD. CTR: Control.

incubation with secondary antibodies labelled with horseradish peroxidase (GE Healthcare). Anti-actin antibodies (Sigma-Aldrich) were used to show that equal amounts of proteins were loaded per lane. The SuperSignal chemiluminescence kit (Thermo Fisher Scientific) was utilized to detect immunoreactive proteins. Densitometry was performed using ImageJ software and results are shown as the mean ± SD of three separate experiments. A representative blot is shown.

Statistical analysis

Statistical significance was determined using the student's *t* test and set at *P* values less than 0.05. In the figures $^{a}P < 0.05$; $^{b}P < 0.01$; $^{d}P < 0.001$.

RESULTS

Prednisolone inhibits SaOS2 cell proliferation

SaOS2 were cultured in media containing different concentrations of prednisolone and counted after 4 d. Figure 1A shows that prednisolone inhibits SaOS2 cell growth in a dose dependent manner. No effect is observed in cells treated with 1 μmol/L prednisolone, while growth inhibition is significant with 3, 6, and 9 μmol/L. Similar results were obtained when the MTT assay was used (data not shown). Under the same experimental conditions U2OS were less sensitive to prednisolone than SaOS2 since a modest growth inhibition was observed only with 9 μmol/L of prednisolone (Figure 1B).

We focused on SaOS2 to understand the mechanisms underlying the inhibitory effect of prednisolone. Since the detrimental effects of glucocorticoids in osteoblasts are mediated by the induction of oxidative stress[12], we measured intracellular reactive oxygen species (ROS) by DCFH fluorescence in SaOS2. Prednisolone did not significantly affect the basal levels of DCFH-detectable ROS (Figure 2A). Accordingly, antioxidants, *i.e.*, apocynin, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, and trolox, a water soluble analog of α-tocopherol, did not prevent growth inhibition by prednisolone (Figure 2B).

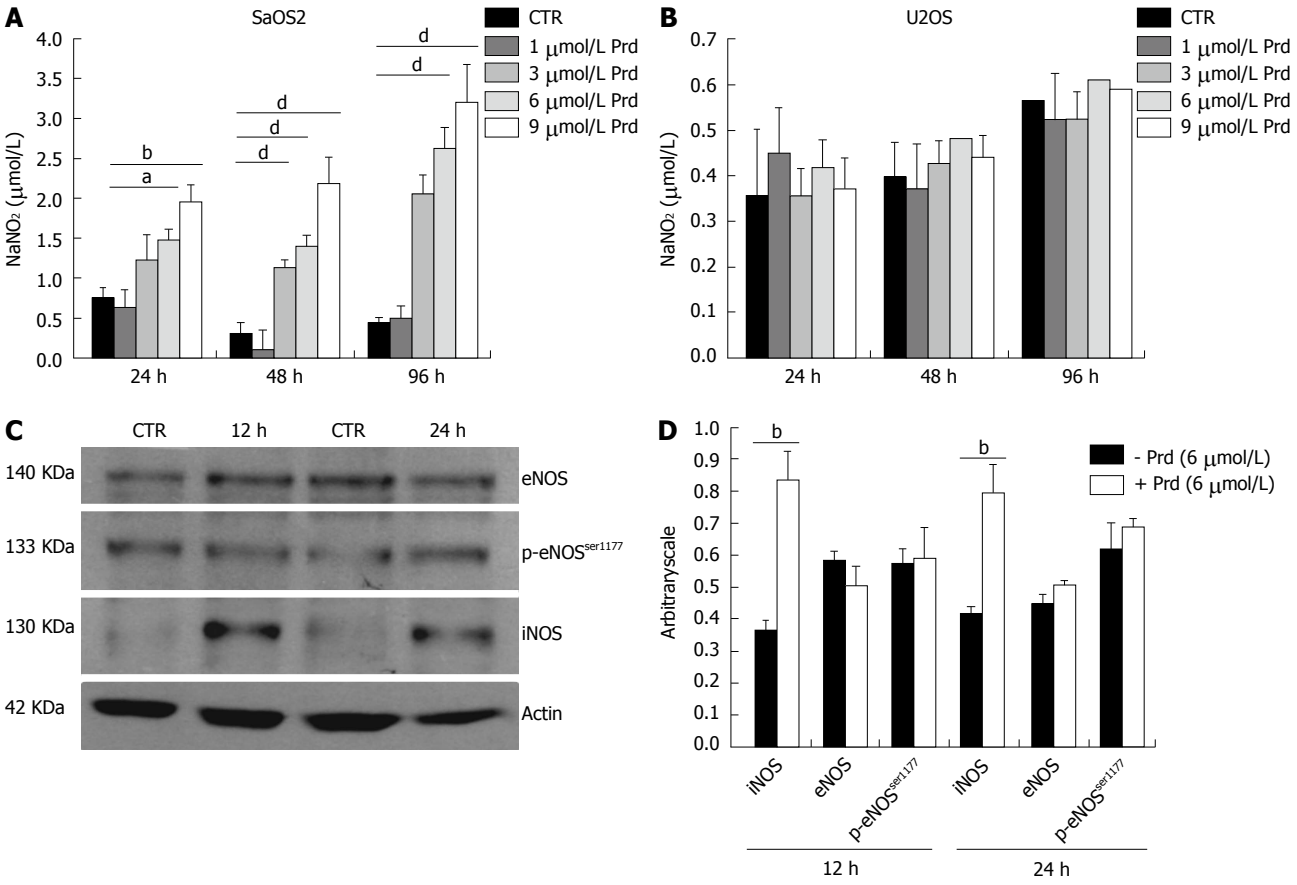

Figure 3 Prednisolone increases the release of nitric oxide in SaOS2. A: SaOS2 were cultured in the presence of different concentrations of prednisolone. Nitric oxide was measured after 24, 48 and 96 h. Results are expressed as the mean ± SD of four different experiments ($^aP < 0.05$, $^bP < 0.01$, $^dP < 0.001$); B: U2OS cells were processed as described in (A). No statistical significance was achieved; C: SaOS2 cells were exposed to prednisolone for 12 and 24 h and then lysed. 80 μg of protein extracts were loaded on SDS-PAGE. Western blots using specific antibodies against iNOS, eNOS, p-eNOS-P-Ser1177 were performed. Actin shows that equal amounts of protein were loaded per lane. The figure shows a representative blot; D: The histogram shows the quantitative evaluation of NOS/actin ratio by densitometry. Results are expressed as the mean ± SD of three separate experiments ($^bP < 0.01$). CTR: Control; iNOS: Inducible nitric oxide synthase; eNOS: Endothelial nitric oxide synthase; Prd: Prednisolone.

Prednisolone induces nitric oxide release in SaOS2

Because of the role of nitric oxide (NO) in bone homeostasis[13], we evaluated whether prednisolone affected NOS activity. After 24, 48 and 96 h of culture in various concentrations of prednisolone, we found that NOS activity was higher in SaOS2 treated with the glucocorticoid as detected by Griess assay (Figure 3A), while no increase of NO was detected in U2OS (Figure 3B). Since iNOS and eNOS were described in cultured osteoblast-like cells from various species[14], we evaluated the amounts of these enzymes by western blot in SaOS2. The phosphorylation of p-eNOS^{Ser1177} was also investigated because it enhances enzyme activity[11]. After 12 and 24 h exposure to prednisolone (6 μmol/L), iNOS was up-regulated (Figure 3C), while the amounts of total eNOS and p-eNOS^{Ser1177} remained almost unvaried.

Inhibition of iNOS activity rescues SaOS2 cell proliferation

To study whether an increased activity of NOS was responsible for SaOS2 growth retardation by prednisolone, the cells were cultured in medium containing various concentrations of prednisolone in the presence or in the absence of the iNOS inhibitor L-NIL (100 μmol/L) for 96 h. Figure 4A shows that L-NIL (100 μmol/L) prevented NO release induced by prednisolone up to 6 μmol/L, but not at 9 μmol/L. We then counted the cells and found that L-NIL prevents prednisolone-dependent growth inhibition up to 6 μmol/L (Figure 4B).

DISCUSSION

High levels of glucocorticoids impact on the generation and lifespan of osteoblasts[15]. In humans, prednisolone, even at low doses[16], causes significant bone loss and increases the risk of fractures through a direct action mainly on osteoblasts and osteocytes[17]. Because of the inhibitory effect of prednisolone on osteoblast proliferation and viability, we asked whether prednisolone might inhibit also osteosarcoma cell proliferation. Indeed, the outcome of antineoplastic therapies in osteosarcoma is not satisfactory and the quest for novel treatments continues. Here, we investigated the effects of prednisolone on two human osteoblastic osteosarcoma cell lines that reveal a different degree of differentiation, *i.e.*, SaOS2 and U2OS[8]. We found that SaOS2 are

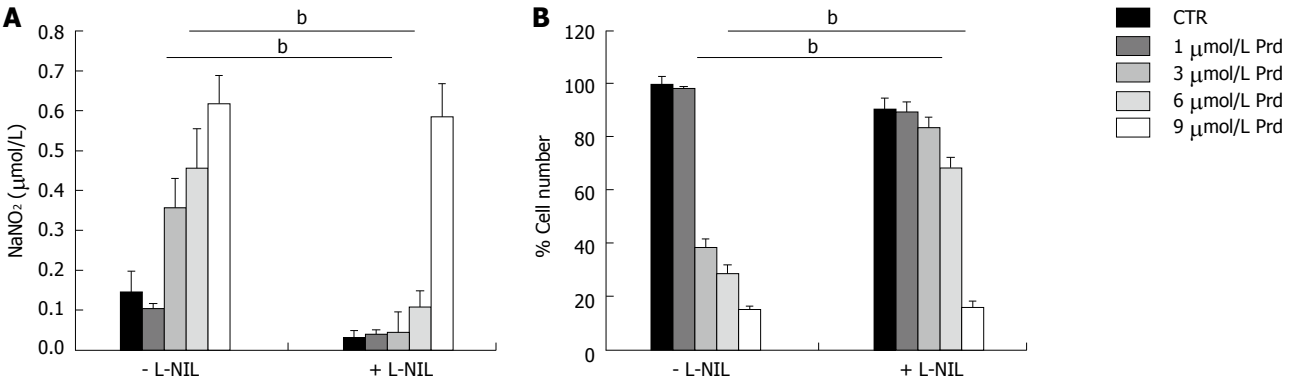

Figure 4 Pharmacological inhibition of inducible nitric oxide synthase rescues SaOS2 cell growth. A: SaOS2 were treated with prednisolone in the presence or in the absence of L-NIL (100 μmol/L). Nitric oxide was measured as described ($^bP < 0.01$); B: Viable SaOS2 cells were counted after 4 d in the presence of different concentrations of prednisolone with or without L-NIL ($^bP < 0.01$). CTR: Control; L-NIL: l-N6-(iminoethyl)-lysine-HCl.

growth inhibited by prednisolone while U2OS are not. We therefore investigated the mechanisms underlying prednisolone inhibition of SaOS2 cell growth, which also means to understand why U2OS are far less sensitive to the drug. Glucocorticoids are known to alter redox balance. Indeed, the administration of prednisolone to mice increased ROS production in the bone and dexamethasone had similar effects on osteoblastic cells *in vitro*[12]. Moreover, prednisolone enhanced the formation of superoxide by augmenting NADPH oxidase activity in pulmonary endothelial cells[18]. We found no significant induction of ROS production in prednisolone-treated SaOS2. In agreement with this result, two anti-oxidants with different mechanisms of action have no effect in preventing SaOS2 cell growth inhibition by prednisolone.

Also NO has a role in bone homeostasis. Low NO levels stimulate, while high concentrations inhibit bone formation. It is eNOS, constitutively expressed in the bone, that is implicated in maintaining the basal levels of NO[19]. Accordingly, eNOS$^{-/-}$ mice show defective bone formation and are osteopenic[11]. Also iNOS null mice show imbalances in bone osteogenesis and abnormalities in bone healing[11]. It is interesting to note that iNOS pathway is crucial in bone resorption upon inflammatory stimuli and also mediates the negative effects of estrogen depletion on bones[20]. Indeed, once activated, iNOS is capable of generating high levels of NO locally for many hours. It should be recalled that NO is also an inducer of stress signaling, owing to its ability to damage proteins and DNA. We here show that SaOS2 exposed to prednisolone upregulate iNOS and, because of this, produce higher amounts of NO than untreated cells. Indeed, pharmacological inhibition of iNOS reduced NO release to basal levels and restored the normal proliferation rate. The mechanisms implicated in iNOS induction are still a matter of investigation. It is known that iNOS is regulated through the activation of several signaling pathways among which NF-κB and MAPK. We can rule out a role of NF-κB, since glucocorticoids suppress NF-κB activity. More studies are necessary to reveal the pathways responsible for the increase of iNOS activity.

It is noteworthy that prednisolone does not induce NO in U2OS and this might account for the different behavior of the two cell lines. It is noteworthy that NO impairs also U2OS proliferation as shown in a study that links the increased activity of iNOS and the detrimental effects of benzyl isothiocyanate and phenethyl isothiocyanate on these cells[21]. It is also possible that the different response of SaOS2 and U2OS to prednisolone is due to the many differences of their proteomic profile[9,22]. Alternatively, since the glucocorticoid receptor gene generates several splice and translation protein variants that lead to different genomic and non genomic effects, the different response of U2OS and SaOS2 might result from the expression of various isoforms of glucocorticoid receptors.

We have previously shown that increased iNOS activity mediates SaOS2 growth inhibition by low magnesium[11]. Therefore NO is emerging as a relevant signaling molecule to control SaOS2 cell proliferation.

Our results indicate that prednisolone impairs SaOS2 cell proliferation through the upregulation of iNOS and consequent induction of NO release.

COMMENTS

Background

Glucocorticoids control the growth and differentiation of osteoblasts and bone mesenchymal stem cells. Little is known about the effects of glucocorticoids on osteosarcoma cells. The authors therefore evaluated the response to prednisolone of two human osteosarcoma cell lines, *i.e.*, SaOS2, which show a mature phenotype, and U2OS, which are rather undifferentiated.

Research frontiers

Prednisolone inhibited SaOS2 cell growth through the induction of inducible nitric oxide (NO) synthase with consequent increase of NO production. No effects were observed in U2OS.

Innovations and breakthroughs

NO is emerging as a relevant signaling molecule to control SaOS2 cell proliferation under different experimental conditions. This result also highlights the different sensitivity to prednisolone of osteosarcoma cells with different degree of differentiation.

Applications

More than one cell line should be used when *in vitro* experiments are performed to test the response to various compounds. The possibility of using glucocorticoids in animal models of osteosarcoma should be fostered.

Peer-review

The manuscript by Cazzaniga *et al* analyses the effects of prednisolone on two different osteosarcoma cell lines. The data are novel and the experiments have been competently performed.

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