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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.

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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

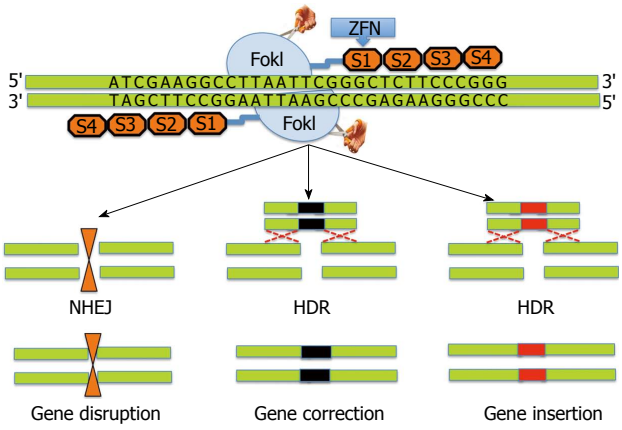

Figure 1 Schematic diagram showing structure and design of a typical zinc finger nuclease. Zinc finger nucleases (ZFNs) use a modular array of 3-6 ZFNs (4 shown) specifically designed to bind to the target DNA together with the FokI cleavage domain. The FokI cleavage domains can be engineered to function 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

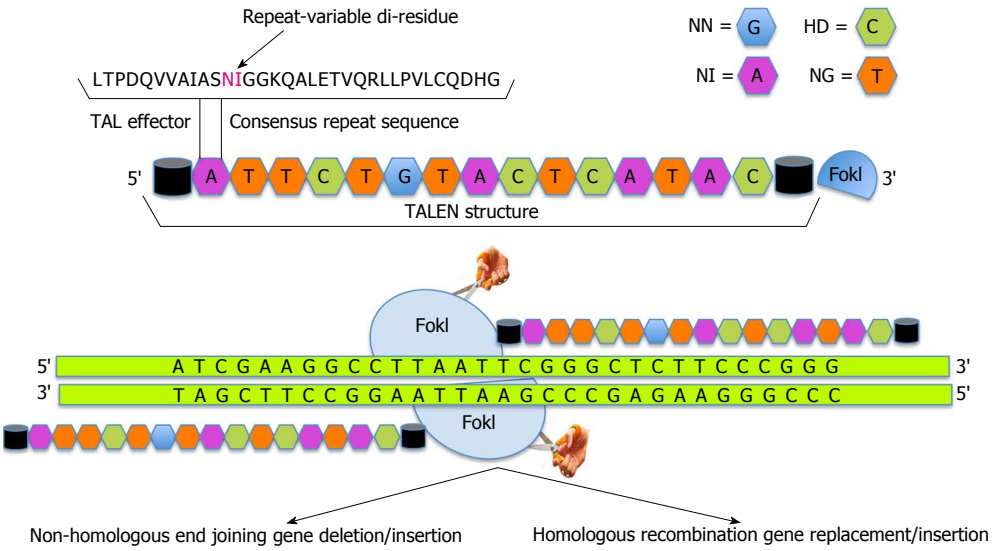

Figure 2 Transcription activator-like effector nucleases. In transcription activator-like effector nucleases (TALENs) the nuclease effector domains of FokI are fused to TALE DNA binding domains. Since FokI is active only as a dimer, pair of TALENs are constructed to position FokI nuclease domains adjacent 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

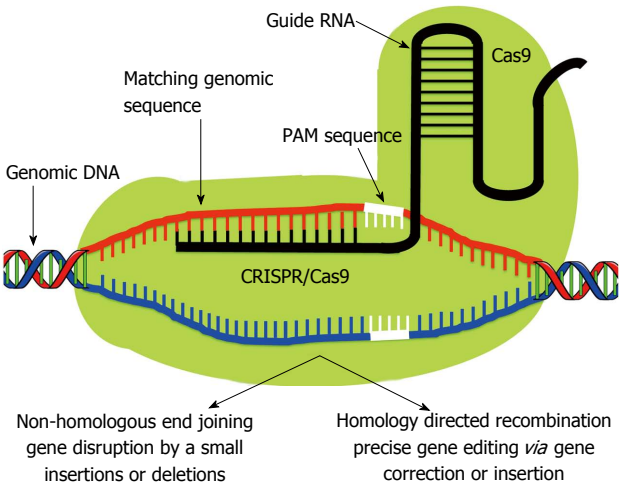

Figure 3 Clustered Regularly Interspaced Short Palindromic Repeat/Clustered Regularly Interspaced Short Palindromic Repeat Associated Systems. In contrast to Like zinc finger nucleases and transcription activator-like effector nucleases, Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)-associated 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

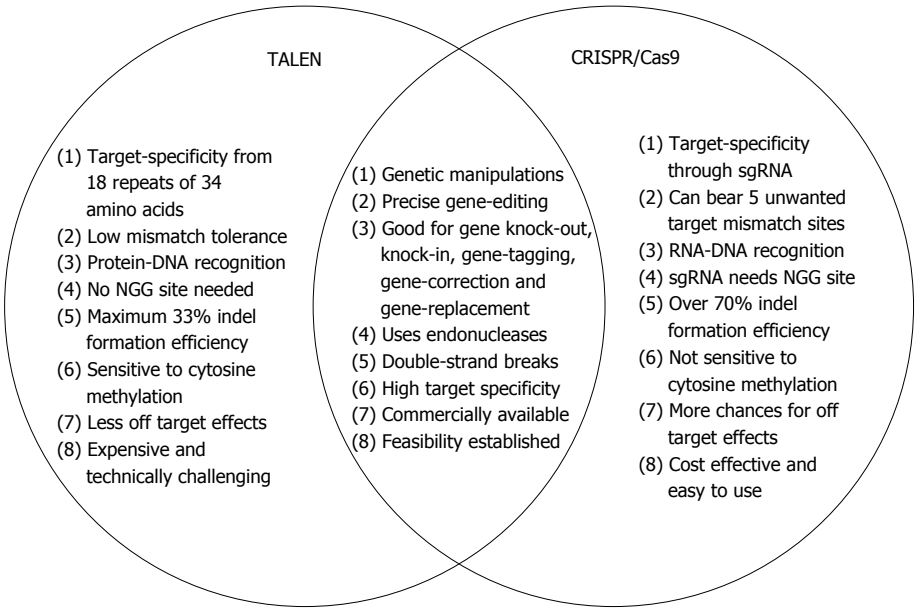

Figure 4 Venn diagram of transcription activator-like effector nucleases and Clustered Regularly Interspaced Short Palindromic Repeat. The schematic Venn diagram shows potential differences and similarities between transcription activator-like effector nucleases (TALENs) and Clustered Regularly Interspaced 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.

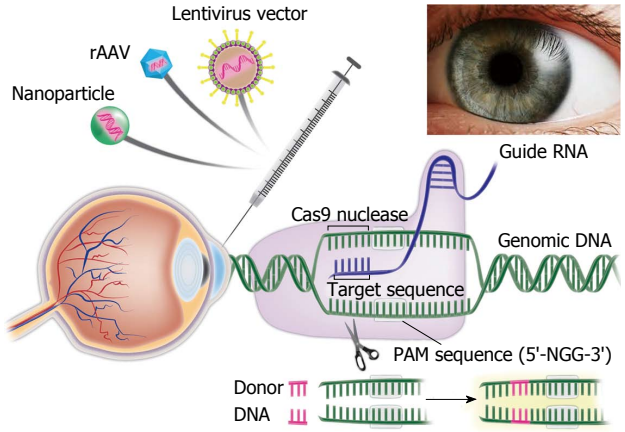

Figure 5 Application of Clustered Regularly Interspaced Short Palindromic Repeat/Clustered Regularly Interspaced Short Palindromic Repeat Associated Systems to develop novel therapies for corneal diseases. Corneal Delivery of Clustered Regularly Interspaced Short Palindromic Repeat/Clustered Regularly 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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