Genome editing: the breakthrough technology for inherited retinal disease?

Abstract

Introduction: Genetic alterations resulting in a dysfunctional retinal pigment epithelium and/or degenerating photoreceptors cause impaired vision. These juxtaposed cells in the retina of the posterior eye are crucial for the visual cycle or phototransduction. Deficits in these biochemical processes perturb neural processing of images capturing the external environment. Notably, there is a distinct lack of clinically approved pharmacological, cell- or gene-based therapies for inherited retinal disease. Gene editing technologies are rapidly advancing as a realistic therapeutic option.

Areas Covered: Recent discovery of endonuclease-mediated gene editing technologies has culminated in a surge of investigations into their therapeutic potential. In this review, the authors discuss gene editing technologies and their applicability in treating inherited retinal diseases, the limitations of the technology and the research obstacles to overcome before editing a patient's genome becomes a viable treatment option.

Expert Opinion: The ability to strategically edit a patient's genome constitutes a treatment revolution. However, concerns remain over the safety and efficacy of either transplanting iPSC-derived retinal cells following *ex vivo* gene editing, or with direct gene editing *in vivo.* Ultimately, further refinements to improve efficacy and safety profiles are paramount for gene editing to emerge as a widely available treatment option.

Keywords

retina, photoreceptor, retinal pigment epithelium, inherited retinal disease, gene editing, CRISPR

Article Highlights

- Gene editing is emerging as an attractive novel treatment strategy for inherited retinal disease.
- Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology is currently the leading gene editing technology positioned for therapeutic intervention.
- *In vitro*, gene editing can correct mutations in patient derived induced pluripotent stem cells (iPSCs). However concerns remain over integration of transplanted retinal cells.
- *In vivo*, CRISPR-Cas9, guide RNAs and repair templates to correct specific mutations can be delivered directly into the eye. Proof of concept studies show safety and efficacy in rodent models and a clinical trial is planned for 2017.

1. Overview

Gene editing technology can be viewed as a molecular tweezers guiding targeted removal or correction of disease causing genetic mutations. Inherited retinal diseases (iRDs) comprise an extensive collection of heterogeneous mutations in over 250 genes, resulting in syndromic or non-syndromic sight loss[1]. Gene editing technology, particularly CRISPR-Cas9, offers exciting opportunities to precisely replace genetic mutations causative of disease. Emerging treatment options include *ex vivo* gene correction in patient-derived, induced pluripotent stem cells (iPSCs) followed by transplantation of retinal progenitor cells into the eye. An alternative *in vivo* approach would employ corrective gene editing directly in the patient's eye. The experimental requirements, pre-clinical progress and limitations of these approaches are reviewed below. Undoubtedly, versatile, bespoke gene correction is highly desirable to treat the heterogeneous array of disease causing gene mutations in iRD.

1.1 Introduction to Inherited Retinal Disease

In retinal disease, the inability to transmit light-triggered signals to the brain is primarily responsible for blindness. Retinal degeneration can occur as a result of risk factors including: age, diabetes, premature birth or genetics. Retinal diseases include inherited *e.g.* retinitis pigmenotsa (RP), achromatopsia and Leber's congenital amaurosis (LCA), or multifactorial forms *e.g.* age-related macular degeneration (AMD)[1]. iRDs are clinically and genetically heterogeneous in nature, where monogenic (Mendelian) disorders can present in syndromic or non-syndromic forms and disease-causing gene mutations can be dominant, recessive, or X-linked[1] (***Figure 1***).

iRDs often result from gene mutations, which perturb the development, function and/or survival of photoreceptor or retinal pigment epithelial (RPE) cells in the

retina[2]. Both recessive and dominant iRD mutations can lead to a variety of biological outcomes including, but not limited to, mistrafficking and aggregation of outer segment proteins activating cell stress responses, defective regeneration of visual pigments for phototransduction, or loss of photoreceptor-specific gene expression. Rod photoreceptors enable sensitivity to dim light and are responsible for scotopic peripheral vision. In contrast human cone photoreceptors concentrated in the retinal fovea are less sensitive to low light but enable detection of a broader bandwidth of light intensity. Cones function in bright light, and despite lower abundance than human rods, are responsible for central photopic vision[3,4]. The RPE is located adjacent to the neuroretina and is responsible for functions including: phagocytosis of photoreceptor outer segment membranes, facilitation of photoreceptor nutrient supply and supporting retinoid recycling[5].

iRDs are typified by retinitis pigmentosa (RP). First identified in 1991[6], 30-40% of autosomal dominant RP results from dominantly inherited missense mutations in the rhodopsin gene, a G-protein coupled receptor essential for phototransduction[7]. Pathogenesis is associated with protein-ubiquitin aggregates in inclusion bodies. Due to the impaired ability of the cell to degrade non-functioning proteins, photoreceptor apoptosis is initiated. Other pathological mechanisms caused by rhodopsin mutations include rod outer segment instability and defective intracellular trafficking[8,9].

In 1997[10], iRDs including LCA were first linked to mutations in genes expressed in the RPE but not in photoreceptors. Mutations in the gene encoding retinoid isomerohydrolase RPE65 result in photoreceptor degeneration due to visual cycle disruption. RPE65 is an RPE-specific protein catalyzing the conversion of all-trans-retinyl ester to 11-*cis* retinol. Autosomal recessive mutations disrupting the visual cycle reduce the levels of 11-cis retinoids needed to couple with opsins to form light-sensitive visual pigments[11,12].

The genetic landscape of inherited retinal disease is vast, currently amounting to ~256 causative genes. Unexpectedly, identification of the causative genes has generally not accelerated development of clinical interventions. Ultimately, iRD mutations, whether dominant or recessive, result in retinal dysfunction and sight loss. For patients, loss of vision can result in diminished independence. These causative mutations are targets for corrective gene editing which has potential to delete alleles carrying autosomal dominant mutations, or correct dominant, recessive or X-linked mutations.

2. Gene Editing Technologies

Gene editing techniques offer targeted modification of genome sequences with high precision. Efficient gene editing is based on endonucleases introducing DNA double strand breaks (DSBs) to stimulate one of two DNA repair pathways, namely homology-directed repair (HDR), or non-homologous end-joining (NHEJ). HDR relies on strand invasion of the broken end into a homologous sequence, and subsequent repair of the break in a template-dependent manner. NHEJ functions to repair DSBs without a template through re-ligation of cleaved ends. This is often error prone, introducing mosaic insertions and deletions (indels), producing frame

shift mutations[13]. For gene editing technology to become a mainstream therapy, a range of precise gene corrections is essential. DSBs can induce more precise editing by stimulating HDR with an exogenous DNA repair template[14]. HDR is upregulated during the G2/M cell cycle phase and in agreement enrichment of pluripotent stem cells in G2/M enhanced HDR-mediated genome editing[15]. However, as adult photoreceptor and RPE cells are post-mitotic, NHEJ-mediated editing is considered more relevant. Delivery of both a targeted nuclease and homologous DNA to the DSB site enables high efficiency HDR and NHEJ-based gene editing. Exogenous donor DNA template can be transfected into cells as a plasmid, or more recently, as single stranded oligonucleotides, and delivered through specific serotypes of adeno-associated viruses or lentivirus[16].

2.1 Zinc Finger Nucleases

Gene editing can be achieved using multiple endonuclease systems. Zinc finger proteins are a class of transcription factors that bind DNA through Cys_2-His_2 zinc finger domains. In the presence of a zinc atom, the α-helical portion of each finger makes contact with 3 or 4 base pairs in the major groove of DNA, forming a tight ββα structure. Zinc finger nuclease (ZFN) technology developed from the functional independence of the DNA-binding and cleavage domains of the FokI restriction endonuclease[17]. ZFNs function as chimeric nucleases by replacing the FokI DNA binding domain with a zinc finger domain engineered for unique binding specificity. DSBs are induced by ZFNs through the FokI nucleases acting as a dimer. Two ZFNs bind to opposite strands of DNA and are then required to induce DSBs. ZFNs can modify the genome of somatic and pluripotent stem cells through either HDR or NHEJ [18–23].

2.2 TALENs

The plant pathogen *Xanthomonas* secretes TAL (transcription activator-like) effector proteins capable of altering plant gene expression. Engineering of these DNA modifying enzymes to have endonuclease activity is achieved by fusing a TAL effector DNA binding domain to a DNA cleavage domain. This complex, termed TALENs (Transcription activator-like effector nucleases), consists of the TAL effector DNA-binding domain, composed of an almost identical 33-35 amino acid repeat units, and a cleavage domain. Similar to ZFNs, the sequence-independent FokI nuclease functions as a site-specific nuclease. TALEs have the ability to recognize specific DNA sequences through two hypervariable amino acid residues at positions 12 and 13, termed repeat-variable diresidues (RVDs)[24]. TALENs efficiently induce NHEJ and HDR in both human pluripotent stem cells and somatic cells. The only targeting restraint of TALENs is the requirement for a 5'T specified for the constant N-terminal domain, meaning TALENs can be engineered for virtually any sequence. Ultimately, the necessity to target specific sites in the genome, and the associated requirement to engineer novel proteins for each target site limits the use of TALENs, and indeed ZFNs, for gene editing[25]. Additionally, *in vivo* delivery of TALENs is hampered by their large size –approximately 3 kb for a single TALEN-

and the repetitive nature of TALE arrays, leading to packaging difficulties into certain viral delivery systems[26,27].

2.3 CRISPR/Cas

CRISPR-Cas RNA guided nucleases function as an adaptive immune system in bacteria[28]. The three components required in bacteria for effective immune defense via the type II CRISPR nuclease system include a Cas9 protein, the mature CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA). Fusion of the crRNA and the tracrRNA produces a guide RNA (gRNA)[29], and retargeting of the Cas9-gRNA complex is achieved through alternating a short portion of the gRNA[30]. The formation of a DNA-RNA duplex at the matched target site in the genome allows cleavage of target DNA[31].The sequence limitation of this system is based on a necessary protospacer-adjacent motif (PAM) positioned immediately 3' to the target site, for example the PAM sequence 5'-NGG-3' is required for binding and cleavage of DNA by the most commonly used *Streptococcus pyogenes* Cas9 enzyme[28]. A consideration in the design of sgRNA for directing Cas9 to cause DSBs is the necessity to identify a PAM site in the target sequence. Engineering of modified Cas9 proteins, or the identification of other Cas proteins with different PAM motifs are addressing this limitation. The major benefit of CRISPR/Cas9 is that unlike ZFNs and TALENs, engineering of novel proteins for each DNA target site is not required.

A limitation of CRISPR is the difficulty to insert exogenous DNA in post mitotic cells. Recently, a modified approach relies on homology independent targeted integration (HITI), which utilises NHEJ to insert a corrective donor DNA into the host genome using flanking Cas9 sites on the donor coding sequence. It is preferable for an exogenous wild type copy of a disease-causing gene to be inserted into the endogenous gene locus. This allows for transcriptional control under endogenous promoter elements. To achieve this, the donor DNA template containing the genetic element can be flanked with homology arms including sequences which have the same identity as the endonuclease cut site, allowing for specific insertion[19,32]. Subsequently, NHEJ-mediated ligation of donor DNA sequence directly into a target locus at the overhangs produced by the endonuclease-induced DSBs is an alternative option for HDR mediated gene insertion in post mitotic photoreceptors[33]. NHEJ mediated HITI overcomes limitations of low frequency HDR in post mitotic cells. However, there remains a lack of knowledge in photoreceptor specific DNA repair mechanisms, which represents a potential barrier to the development of effective therapeutics for iRDs. Ability to quickly design and synthesize multiple guide RNAs targeting different genomic regions underpins why this more versatile *molecular tweezers* has revolutionized genome editing.

3. Treatment of inherited retinal disease

While still in early-stage development, cumulative reports support applicability of gene editing to treat iRDs. Two relevant gene editing approaches include: *i)*

correcting the mutations *ex vivo* in iPSCs and subsequent transplantation of maturing photoreceptor or RPE cells into the patient retina; or *ii)* correcting the mutation *in vivo* by direct administration of the gene editing components into the eye. It is important to consider how gene editing technologies may overcome the limitations of other biological approaches to treat iRDs.

3.1 Overview of strategies for the treatment of retinal degenerative disease

There is no widely available therapeutic option for persons with iRD to access in clinic. The diverse genetic landscape, the mutation prevalence, the stage at which disease is treated, and the complexity or specificity of the therapeutic product are mitigating factors in development of iRD therapeutics. For example neuroprotection or cell replacement may offer wide applicability to patients, irrespective of the risk factor or genetic mutation.

Pharmacological interventions for treatment of iRDs offers the ability to protect photoreceptor and RPE dystrophy through targeting common convergent pathways responsible for cell death, such as attenuating apoptotic signaling or mitochondrial function or by promoting cell survival[34]. However, drug based neuroprotection for iRDs has stagnated at clinical trial phases, and to date clinical benefit to patients is limited. Cell transplantation offers a regenerative treatment strategy for iRDs through replacement of cells/tissues, and unlike other biological approaches may be beneficial even at advanced disease stages. The technique depends on integration of human retinal cells and/or tissue differentiated from induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) following sub-retinal injection. Phase I/II clinical trials evaluating human embryonic stem cell (hESC)-derived allogeneic RPE grafts demonstrated the procedure to be safe[35]. Ethical and immunogenic concerns remaining for hESCs may be overcome using iPSCs. However, recently a clinical trial with transplanted RPE derived from iPSC was halted due to oncogenic mutations in one patient's iPSCs. Allogenic cells are now being studied as an alternative[36]. Furthermore, and of direct relevance to *ex vivo* gene editing, recent studies cast doubt over the ability of transplanted photoreceptor progenitor cells to functionally integrate into the retina.

Gene therapy typically relies on the identification of disease-causing genetic mutations. A gene silencing strategy aims to shut down expression of a defective dominant gene copy, with or without replacing a wildtype allele. Silencing can be achieved through silencing RNAs (siRNA), antisense oligonucleotides binding to mRNA transcripts, targeting them for degradation. A broader strategy utilizing siRNAs to silence all rhodopsin gene mutations coupled with replacement of a distinct functional gene transiently improve visual function in a murine iRD model [37]. Challenges for gene silencing therapy include optimising dose to produce specific gene knockdown, off-target silencing and transient effects. Kleinman *et. al.* demonstrated that siRNAs induced retinal degeneration in mice by activating RPE Toll-like Receptor 3 (TLR3) receptors and triggering caspases-3 dependent apoptosis via nuclear translocation of interferon regulatory factor 3[38].

In recessive iRDs, gene replacement therapy showed promising clinical results across a range of retinal indications[39]. Recent clinical trials to treat LCA caused by RPE65 mutations show significant improvement in functional vision, through delivery of a replacement RPE65 gene[40]. Previously, clinical trials for RPE-associated LCA using alternative vectors and constructs reported in 2008 that gene therapy was safe and efficacious in patients. Despite this, three-year follow-up analysis found initial gains in retinal sensitivity waned over time and did not result in meaningful improvements in objective measurements of visual function[41,42]***.*** Gene therapy also has potential to deliver more generic factors, which address common disease mechanisms such as modulating oxidative stress, reducing protein aggregation, or delivering anti-apoptotic or neurotrophic factors. Sub-efficacious dosages and irreversibility of gene therapy represent therapeutic and safety issues.
Gene editing technologies offers promise as an alternative approach to address these treatment limitations. Iterations of gene editing technology offer the potential to precisely correct mutated iRD genes, overcoming safety and efficacy barriers (***Table 1***). For example, the limitation of transient silencing of dominant genes using siRNAs is surmounted by persistent silencing achieved with irreversible editing of the causative mutation in the genome. Editing of the endogenous gene allows its expression to be controlled under "native" regulatory control mechanisms which may overcome issues associated with under- and over-expression using exogenous promoter fragments. Immune rejection of transplanted cells is not a concern for *in vivo* gene editing. However, other barriers arise for gene editing including the mosaicism of "corrected" and "uncorrected" cells in the retina which may mask clinical benefit, the low efficiency of precise gene correction and concerns over an adverse response to bacterial Cas9 expression in the eye (***Table 1***).

3.2 E*x vivo* gene editing for the treatment of iRDs

Transplantation of gene edited ES or iPSC cells to the retina is a potential therapeutic strategy for iRD **(*Figure 2*).** Recently Bassuk *et. al.* using CRISPR/Cas9 demonstrated *ex vivo* correction of an X-linked retinitis pigmentosa GTPase regulator (*RPGR)* iRD mutation[43]. Fibroblasts from a patient skin biopsy were transduced to produce iPSCs harboring the c.3070G>T mutation. Introduction of CRISPR gRNAs, Cas9 endonuclease and a donor homology template corrected 13% of the RPGR gene copies, converting the premature stop codon to glutamate at position 1024. This *proof-of-concept* study demonstrates capability to repair RPGR ORF15 region using CRISPR/Cas9. A treatment milestone will be achieved if these findings are safely and effectively applied to patient eyes.

Despite the applicability of *ex vivo* genome editing for iRDs, fundamental safety and efficacy issues remain with downstream cell transplantation. Unfortunately, repairing severely degenerated retinas through cellular transplantation of photoreceptor progenitors suffered a setback. Previously, the migration and integration into the retina of photoreceptor progenitor cells was reported to restore visual function in preclinical models[44–46]. Recently, however, it emerged that most photoreceptors progenitors do not functionally integrate but reside in the subretinal

space and exchange intracellular material with host photoreceptors[47–49]. Tracking allogenic transplants with fluorescent reporters demonstrated cellular content transfer between graft and host photoreceptors without nuclear translocation. Material transfer may deliver functional proteins into degenerating photoreceptors by material transfer but unlikely to deliver a corrected gene to the host genome due to the lack of nuclear translocation.

Despite setbacks for photoreceptors, transplantation of autologous or allogenic RPE grafts is a feasible treatment option for specific iRDs. Indeed, clinical trials are evaluating the safety and efficacy of RPE cell transplantation. Schwartz *et. al.* conducted two prospective Phase I/II studies to assess tolerability and safety of hESC-derived RPE transplantation in Stargardt's macular dystrophy (9 patients), and atrophic-AMD (9 patients). Adverse events were associated with surgery and immunosuppression, and no adverse proliferation, rejection or serious ocular or systemic safety issues arose from the transplanted tissue[35]. Whilst evaluating visual acuity as a measure of treatment safety, promising improvements in visual function were reported in treated eyes of 8 patients. Additional trials need to validate these end-points and eliminate placebo response and the injection procedure as confounding factors. Both iPSCs and hESCs are being clinically evaluated, in some instances using cell suspensions, or scaffolds[35]. For example, future studies using gene correction in patient-derived iPSCs of RPE65, CRALBP or TIMP3 mutations could lead to personalized treatments using RPE transplants.

In complex multifactorial disorders such as advanced neovascular AMD, no single gene mutations are causative. Yet gene editing could ameliorate key pathological manifestations. The current approved treatments for neovascular AMD target-vascular endothelial growth factor (VEGF) with fusion proteins or monoclonal antibodies. Yiu *et. al.*[50] however, employed CRISPR to suppress angiogenesis by genomic disruption of VEGF-A in RPE cells. gRNAs targeting exon 1 of the VEGF-A gene were cloned into Cas9 lentiviral expression vectors. ARPE-19 cells were transfected, gene deletion confirmed and secreted VEGF was significantly decreased. This *proof-of-concept* showing VEGF reduction at a protein level could be applied for therapeutic benefit if VEGF-A deleted RPE was transplanted from patient-derived iPSCs. Hypothetically, this treatment could be delivered *in vivo* to target endogenous RPE by interrupting VEGF expression. However, invasive, expensive and complex *ex vivo* corrected cell transplants or *in-vivo* gene editing are unlikely to disrupt the relatively safe and effective anti-VEGF biologicals that currently dominate the neovascular AMD market.

3.3 *In vivo* gene editing for the treatment of iRDs

In vivo gene correction is a step-change in approach to developing treatments for iRDs. Advances in delivery methods developed for gene replacement have accelerated the pre-clinical investigations of endonucleases as an *in vivo* gene editing therapy for iRDs. Hung *et. al.*[51] demonstrated CRISPR-Cas9 to effectively

knockout yellow fluorescent protein (YFP) in a *Thy1-YFP* transgenic mouse retina using intravitreal delivery of an AAV2-encapsulated *Streptococcus pyogenes* Cas9 (SpCas9) and a single guide RNA (sgRNA) against YFP. This work demonstrated ability to efficiently achieve targeted gene editing knockouts in mammalian retinae. Furthermore, the mice maintained visual function 5 weeks after injection, an important *proof-of-concept* for viral-mediated retinal gene editing *in vivo*. Bakondi *et. al.*[52] demonstrated sub-retinal injection of a targeted gRNA/Cas9 plasmid in combination with electroporation generated allele-specific rhodopsin (rho) disruption in a rat retinitis pigmentosa (RP) model. In autosomal dominant disease, allele specific ablation using gene editing could restore retinal function through the activity of the remaining wildtype allele. For patients, rhodopsin hemizygosity should not manifest as haploinsufficiency as wild type rhodopsin expression from 50-200% is asymptomatic[53]. The transgenic S334ter rat displays similar phenotypes to the human class I RHO mis-trafficking mutations. Notably, CRISPR-Cas9 could selectively disrupt Rho^{S334} due to a PAM motif present in Rho^{S334} but not in Rho^{WT} alleles. Improved visual acuity, and retention of photoreceptor cell number or outer nuclear layer thickness compared to a control gRNA demonstrate the first effective use of retinal gene editing to target iRD mutations *in vivo*[52]. Specific ablation of a dominant allele would not restore any populations of degenerated photoreceptors, however deletion of a dominant allele in stressed cells that are functioning sub-optimally could result in gradual depletion of the dominant protein and expression of the wild type gene could enhance the functionality of these remaining cells.

The Bakondi *et al.* study is significant for autosomal dominant retinal degeneration. However, many iRDs are characterized by loss-of-function mutations. Integration of template donor DNA for gene correction is an attractive therapeutic option for such scenarios. Recently Suzuki *et. al.*[54] achieved this in post-mitotic cells using CRISPR to introduce DSBs followed by homology-independent targeted integration (HITI), an NHEJ-mediated targeted integration, allowing for robust DNA knock-in *in vivo*. The HITI method relies on NHEJ for functional integration of DNA, as opposed to previous studies, which focused largely on HDR to insert coding sequences. The technique involves donor DNA containing a gene's correct coding sequence, flanked by Cas9 cut sites, which aid in the homology independent integration of the sequence into the host genome. Post-mitotic neurons, including photoreceptors rely largely on NHEJ and not HDR as a DNA repair mechanism. For the *in vivo* applications of HITI, and to prove efficacy, AAV8 and 9 serotype vectors were used. In the RCS rat model of RP, a deletion from intron 1 to exon 2 in the *Mertk* gene disrupts RPE phagocytosis. HITI-AAV vectors, one containing a Cas9 expression system, and the second containing the coding sequence for *Mertk* exon 2, were sub-retinally injected. PCR confirmed insertion of *Mertk* exon 2 into intron 1 of *Mertk* leading to a statistically significant upregulation of *Mertk* mRNA, preservation of outer nuclear layer thickness and improved b-wave ERG amplitudes, a measure of scotopic cone vision. In comparison to a HDR-mediated gene insertion of *Mertk* exon 2, HITI knock-in rodents had significantly improved visual function measured by ERG, and greater ONL thickness. This landmark study sets a precedent for the application of gene editing technologies in post-mitotic cells. Future studies using

HITI could intervene before disease onset and assess long-term effects of gene editing on retinal degeneration and visual function.

Despite the versatility of CRISPR and the ability to produce bespoke gene editing therapies, the targeting of specific genomic mutations is an expensive, time consuming process. As cone photoreceptors are responsible for colour vision and visual acuity, their degeneration has devastating effects on a patient's life. The preservation of cone function is hugely important, and the identification of therapeutic targets common to iRDs, irrespective of the causative mutation would be desirable. Recently Yu *et. al.*[55] aimed to prevent cone degeneration by targeting *Nrl* (Neural Retina Leucine Zipper), a transcription factor responsible for determining rod cell fate during development and maintaining rod homeostasis in the adult retina. Loss of *Nrl* increases the number of photoreceptors with cone characteristics. The result of *Nrl* ablation is improved photoreceptor survival in the presence of rod-specific gene mutations. In the gene editing study, AAV8 vectors delivered expression cassettes for SpCas9 under a rhodopsin kinase promoter, and sgRNA under a U6 promoter. AAV constructs were subretinally injected at P14 into three rod photoreceptor degeneration models, *Rho*$^{-/-}$, RD10, and RHO P347S. *Nrl* knockdown resulted in the expected loss of a-wave ERG responses, a measure of rod function. However, the b-wave, a readout of cone activity was retained to much later stages indicating survival of the cone population. Furthermore, deep sequencing of the sgRNA-*Nrl* target sequence indicated 98% of total reads contained genomic changes almost exclusively at the target site. Deep sequencing of potential off-target sites even up to 9.5 months old revealed no sites with significantly higher rates of sequence alterations compared with background in control eyes. This study effectively demonstrates a common potential target for retinitis pigmentosa, the efficiency of CRISPR *in vivo* in the retina, and the safety of an active Cas9 protein late into adulthood.

With the rapid development of revolutionary gene editing technologies, and the publication of *proof-of-concept* studies *in vivo*, focus is shifting to translating CRISPR technologies to the clinic. The gene editing market is predicted to top US$3.5bn by 2019[56]. EDITAS Medicine recently announced plans to begin a clinical trial for treatment of LCA using CRISPR-Cas9. Approximately 20% of LCA patients harbor mutations in the ciliary protein CEP290. The most common mutation in CEP290 is IVS26 c.2991+1655 A>G mutation in intron 26, introducing a novel splice donor, resulting in aberrant splicing and a premature stop codon. EDITAS are employing a dual cut approach by applying two gRNAs directing the *Staph. aureus* CRISPR-Cas9 system to excise the mutation containing region using AAV vectors[57,58]. This approach was reported to repair the mutation in LCA10 patient fibroblasts. More recently, work published by Ruan *et. al.*[59] similarly demonstrated the use of a dual cut approach couple with either a *Staph. aureus* or a *Strep. pyogenes* Cas9 packaged in an AAV5 vector. In the study using SpCas9 the group could effectively induce targeted genomic deletion of wild-type mouse intron 25 of *Cep290*, which is homologous to the human intron 26. Furthermore, the group used a novel approach in developing a self-limiting CRISPR/Cas9 system by incorporating recognition sites

for the sgRNA(s) into the SpCas9 plasmid, allowing for the excision and removal of the plasmid following SpCas9 expression. This proposed "hit and go" approach is advantageous for *in vivo* gene editing as it limits the potential host immune response to the exogenous enzyme, in addition to any off target effects caused by prolonged Cas9 expression.

It remains to be seen if treatment in patients can achieve efficacy with a positive safety profile while correcting the genetic sequence. Clinical endpoints of such a therapy need to focus on reliable measures of visual function and functional vision (***Figure 2***). Long-term efficacy studies need to confirm sustained improvement, as opposed to gene replacement therapy where beneficial effects waned over time. There are distinct limitations to this method of treatment as off-target effects are difficult to identify due to the inability to obtain retinal samples for DNA sequencing approaches such as BLESS (direct in situ breaks labeling, enrichment on streptavidin, and next-generation sequencing)[60].

4. Future Perspectives for Gene Editing as an iRD Therapy

Despite the attention gene editing systems such as CRISPR received in recent years, concerns remain as to their clinical use for iRDs. While the opportunity to correct mutations responsible for RPE or photoreceptor dysfunction is attractive, fixed editing of patient genomes raises safety concerns. The efficacy of targeted gene editing relies on the cleavage of DNA in a site-specific manner while preventing collateral damage to the genome caused by off-target effects of the gRNA. Inactivation of Cas9 nuclease domains and creation of a Cas9 nickase increases specificity due to the requirement of two gRNA/Cas9 complexes to cleave a single strand of DNA individually, coming together at a precise distance and orientation to introduce a DSB[61]. Additionally, novel Cas9 variants, and reducing the length of complementarity between gRNA and the target site from 20 to 17 nucleotides increases Cas9 DNA cleavage[62–64]. Further optimization of gene editing techniques, and the identification of novel endonucleases, such as Cpf1, or alternative RNA-guided RNase functioning enzymes such as C2c2, will diversify the treatment strategies and increase the targetable mutations[65,66]. A further limitation to using the well characterized SpCas9 for *in vivo* gene editing is its relatively large size for AAV based gene delivery (4.9kb). It is conceivable that efficacy of *in vivo* genome editing could be limited if both the Cas9 and the gRNA construct are delivered separately. Alternative Cas9 orthologues with shorter coding sequences, such as Cas9 from *Staphylococcus aureus*, which is approximately 1kb shorter than SpCas9, could be delivered in the same vector as SaCas9[67].

Understandably, in terms of treating iRDs *ex vivo*, the major advantage is the ability to validate gene correction before transplantation of differentiated cells to a patient. *In vivo* gene editing is based on the principle that single editing events are sufficient to treat iRDs. However, the required dosage, mechanism of delivery, and pharmacokinetic profile of such an approach present significant challenges, as is the case with gene therapy. The duration of nuclease expression is a significant

parameter for the level of on- and off-target activity. Furthermore, the dose of donor template, in the case of loss of function mutations, is necessary to ensure efficient homologous recombination. Most *proof of concept* studies have relied on expression of plasmid DNA, which holds inherent challenges due to transfection efficiency, DNA cytotoxicity and immunogenicity. Future efforts may rely on novel nanoparticle formulations or viral mediated delivery[68]. An advantage for iRDs is the ability to locally administer the treatment either through intravitreal or sub-retinal injection, and the immune privileged nature of the eye.

While tremendous efforts are underway to apply gene editing as a direct therapeutic option for iRDs, indirect applications may also identify alternative therapies.
A requirement for corrective gene editing is prior knowledge of the disease-causing mutation. Despite intensive increases in next-generation sequencing and the identification of over 256 causative genes responsible for retinal degenerations (RetNet[69], https://sph.uth.edu/RetNet) there still remains a need to identify disease-causing mutations. Gene editing can also elucidate the function of target or disease genes in animal models. Indeed the use of a CRISPR-Cas9 vector system for tissue-specific gene disruption in zebrafish has displayed the possibility of elucidating specific gene knockout effects in photoreceptors[70].

The development of specific knockout lines has the potential to aid in the identification of novel compounds, which could be used for treating iRDs, similar to published reports for RGCs[71]. A high content screening approach has previously identified photoreceptor neuroprotective compounds. Cultured murine retinal cells are treated with a chemical insult, followed by a compound library. A fluorescent viability marker then assessed survival and identified neuroprotective compounds[72]. It is imaginable that either direct knockout of genes in cell lines, or retinal cells differentiated from stem cells of a CRISPR knockout line for a gene of interest could be generated. Indeed, the development of optic cups harboring patient mutations could be used for modeling diseases using CRISPR, and even used for identification of novel therapeutics[73]. The subsequent use of these cells for the identification of novel compounds, which promote photoreceptor cell survival is a therapeutic discovery modality with high flexibility and high throughput. Furthermore, the use of such knockout animal models will allow for *in vivo* evaluation of these compounds as potential therapeutics for the treatment of iRDs through assessment of visual behavior or function in addition to retinal morphology.

In recent decades, the elucidation of the molecular basis of retinal disease has progressed significantly, and substantial evidence for not only genetic determinants but also environmental factors grows continuously. The best example of this is age-related macular degeneration, which is not a classical monogenic disease, but involves a complex interaction of both environmental and genetic influences. In this instance, not only age is a risk factor, but smoking, hypertension, diet, obesity and chronic inflammation are possible risk factors [74]. The fact that a combination of multiple genetic loci and environmental factors are responsible for AMD development demonstrates how the etiology of AMD differs to that of monogenic

forms of macular degenerative diseases[75]. Due to the lack of specific disease causing genetic mutations, such multifactorial conditions are not inherently treatable by genome editing technologies, and other therapeutic modalities, or innovative use of gene editing must be explored. While editing and correction of disease causing genetic mutations does not immediately appear relevant for AMD, it would be intriguing to determine if correction of mutations associated with increased risk of AMD would be beneficial.

5. Conclusion

The revolutionary field of gene editing can significantly advance the elucidation of gene function, and also has potential to correct a patient's mutated gene to treat retinal disease. With advances to the technique, a diverse array of treatment options could become available. Gene editing endonucleases address concerns raised by gene therapy, primarily its' long term efficacy, and the ability to target the heterogeneity of recessive, dominant and X-linked gene mutations in iRDs. However, significant obstacles remain, particularly the low efficiency of HDR and the safety concerns related to expression of an exogenous endonuclease in the eye. Nonetheless, gene editing research is rapidly advancing towards personalized and precise treatments for iRDs.

6. Expert Opinion (Words: 583)

Gene editing holds real promise for treating a multitude of iRDs typified by specific, well-characterized genetic mutations. The application of gene editing to treating iRDs has two main treatment modalities, the first being through the *ex vivo* correction of a genetic mutation in patient derived iPSCs, and the subsequent differentiation of the cells harboring the correct gene to a retinal cell fate (*e.g.* a retinal progenitor cell) or a retinal pigment epithelial cell fate. The second approach is to deliver the targeted endonuclease such as Cas9 and the single guide RNA, and donor template if required, via lipid particles or AAV in either an intravitreal or subretinal injection.

The benefit of the *ex vivo* approach is the ability to identify and expand cell populations with the correct sequence confirmed in the genome. Thus, all transplanted cells have the gene correction and efficiency is dependent on the number of integrated cells. In contrast, for the *in vivo* approach the efficiency is dependent on the number of cells in the retina in which gene editing was successful. Following gene editing in stem cells, issues with replication and scalability may hamper its development as a therapeutic. Furthermore, much remains to be seen on the ability of donor cells to produce a therapeutic benefit through the exchange of RNA or proteins. It is likely that cell transplantation will advance as a therapeutic strategy for replacement of RPE, as these cells are amenable to *in vitro* treatments, and several clinical trials have shown them to be safe.

An *in vivo* approach requires the correct genetic editing event to occur following administration of a targeted nuclease, and the absence of harmful off-target mutations. With progress in development of more specific nucleases with reduced off-target effects, an *in vivo* approach is more likely to reach the clinic due to the advances made in the safe delivery of gene therapies via AAV vectors. Factors that need to be considered for iRD treatment must focus on the efficacy of treatment, and its safety. For the treatment to be efficacious without off-target editing, it would be advantageous to deliver Cas9 protein with the appropriate gRNA and possibly donor template. With developments in manipulating Cas9 to increase its specificity, or alter its function (e.g. Nickase activity), the safety profile of CRISPR is likely to improve. With further changes to Cas9, the amount of potential targets is likely to increase, in addition to the discovery of other Cas proteins such as Cpf1 and C2c2[65,66], or even the discovery of novel gene editing systems such as DNA guided nuclease systems, or targetable site-specific recombinases[76,77]. Despite the manipulability of these gene-editing systems, their therapeutic use is limited by the identification of a causative gene. With advances in our knowledge of causative genes through next generation sequencing, more mutations will become amenable to gene editing. Until the time when the mutated gene has been identified, other treatment options are required. Moreover, a specific genetic mutation is not present in complex retinal disease such as AMD. CRISPR and other gene editing technologies still have important roles to play in the elucidation of novel gene function, but also in drug discovery approaches in high throughput settings, and in preclinical drug testing. Genome editing may redefine gene and cell therapies for treating retinal degeneration, but fundamental translational research is needed for these exciting technologies to be used to their full advantage before genome editing becomes the breakthrough technology to treat iRDs.

	General	Ex-Vivo	In-Vivo
Pros	• Versatility of editing approaches • Ability to correct mutations with high efficiency and specificity	• Ability to identify correctly modified cells and clonally expand • Ability to identify biallelic modification •	• Cell transplantation not required
Cons	• Potential Cas9 immunogenicity • Potential harmful off target editing effects	• Doubts remain over the ability for donor cells to integrate into retina and become functional • Mosaicism of retina with endogenous (unedited) and transplanted cells (edited)	• Mosaicism of treatment effects • Irreversibility of gene editing • Inability to harvest retinal tissue for genotyping/sequencing • Difficulty in optimizing dosage for required expression level • Possible improper target site editing

Table 1. Associate pros and cons of gene editing, with specific *ex-vivo* and *in-vivo* considerations

Figure Legends

Figure 1. Inherited retinal diseases are a heterogeneous group of neurodegenerative disorders, which could be treated using CRISPR/Cas9 mediated gene editing. Genetic defects in the photoreceptor and retinal pigment epithelial cell layers of the human retina are largely responsible for both syndromic and non-syndromic retinal degenerations. Known causative genetic mutations can be identified as being dominant, recessive or x-linked. A subset of genes in which these forms of mutations have been identified to be causative in retinal degeneration is shown. RPE: retinal pigment epithelium, gRNA: guide RNA, PAM: protospacer adjacent motif.

Therapeutic modalities for the utilization of CRISPR/Cas9 for treating inherited retinal diseases. A. An *ex vivo* approach to gene editing in retinal degeneration relies on culturing patient derived induced pluripotent stem cells, and applying CRISPR/Cas9 gene editing technology using viral vectors or lipid nanoparticles. A correct and specific gene editing event must be identified before subsequent colony expansion and differentiation of iPSCs to a retinal cell fate (photoreceptor progenitor, or retinal pigment epithelial cell)[78]. Cell transplantation

occurs via subretinal injection. Visual function and retinal integrity can be assessed by OCT, ERG and Fundoscopy. **B.** An *in vivo* approach to correcting disease causing mutations in the retina begins with confirming the specificity and efficacy of the guide RNA and Cas9. Delivery via lipid nanoparticles and AAV vectors to the retina either intravitreally or subretinally occurs subsequently, allowing gene editing to occur *in vivo.* Restoration of visual function or delay of degeneration are measurable objectively. ERG: Electroretinography, AAV: adeno associated virus, HDR: homology directed repair, NHEJ: non-homologous end-joining, iPSC: induced pluripotent stem cell.

Reference Annotations

18 *Landmark paper demonstrating genome editing capabilities of Zinc Finger Nucleases.

24 *Landmark paper demonstrating genome editing capabilities of TALENs technology.

28,29,30 *Series of three important papers on the development of CRISPR technology for gene editing.

43 **An important publication demonstrating the ability to correctly replace a retinal degeneration causing mutation in patient derived stem cells.

50 *Important demonstration of the use of endonuclease in retinal pigment epithelium.

47, 48, 49 **The three papers published within 4 months of each other describe the modality by which stem cell derived photoreceptors do not efficiently integrate into the retina, but rather fuse with host cells and exchange cytoplasmic contents.

51 *Important demonstration of the safe *in vivo* applicability of CRISPR/Cas technology.

52 **Research demonstrates the correction of a retinal degenerative disease causing mutation in an established autosomal dominant animal model of retinitis pigmentosa *in vivo* using CRISPR/Cas9

54 **This study demonstrates the efficient replacement of a defective gene copy with donor DNA, correcting retinal degeneration using Homology Independent Targeted Integration

65 *Important example of identification and application of novel endonucleases for genome editing.

66*Important example of identification and application of novel endonucleases for

genome editing.

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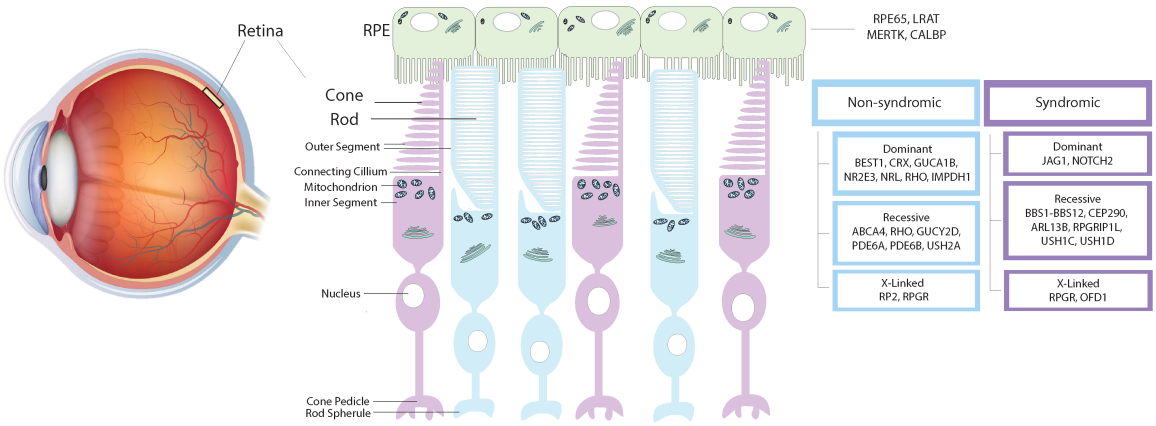
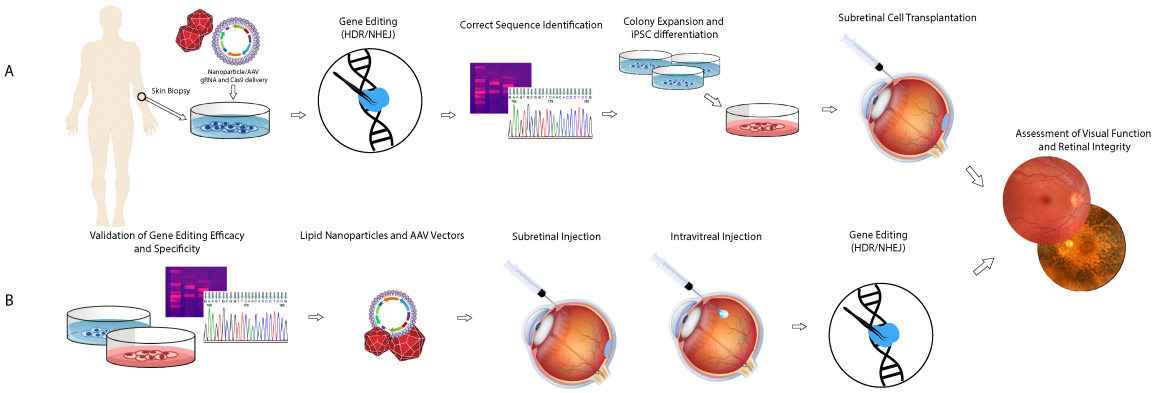

Retina
RPE
RPE65, LRAT
MERTK, CALBP
Cone
Rod
Outer Segment
Connecting Cillium
Mitochondrion
Inner Segment
Nucleus
Cone Pedicle
Rod Spherule
Non-syndromic
Syndromic
Dominant
BEST1, CRX, GUCA1B,
NR2E3, NRL, RHO, IMPDH1
Recessive
ABCA4, RHO, GUCY2D,
PDE6A, PDE6B, USH2A
X-Linked
RP2, RPGR
Dominant
JAG1, NOTCH2
Recessive
BBS1-BBS12, CEP290,
ARL13B, RPGRIP1L,
USH1C, USH1D
X-Linked
RPGR, OFD1

Figure. 1



929
930 **Figure. 2**