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Authors(s)	Smith, Andrew J., Carter, Stephen P., Kennedy, Breandán
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1 **Genome editing: the breakthrough technology for inherited retinal** 2 **disease?**

8 **Abstract**

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

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

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

33 **Keywords**

34 retina, photoreceptor, retinal pigment epithelium, inherited retinal disease, gene
35 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 loss¹. 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 pigmentosa (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-linked¹ (**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

72 retina². Both recessive and dominant iRD mutations can lead to a variety of
73 biological outcomes including, but not limited to, mistrafficking and aggregation of
74 outer segment proteins activating cell stress responses, defective regeneration of
75 visual pigments for phototransduction, or loss of photoreceptor-specific gene
76 expression. Rod photoreceptors enable sensitivity to dim light and are responsible
77 for scotopic peripheral vision. In contrast human cone photoreceptors concentrated
78 in the retinal fovea are less sensitive to low light but enable detection of a broader
79 bandwidth of light intensity. Cones function in bright light, and despite lower
80 abundance than human rods, are responsible for central photopic vision^{3,4}. The RPE
81 is located adjacent to the neuroretina and is responsible for functions including:
82 phagocytosis of photoreceptor outer segment membranes, facilitation of
83 photoreceptor nutrient supply and supporting retinoid recycling⁵.

84 iRDs are typified by retinitis pigmentosa (RP). First identified in 1991⁶, 30-40% of
85 autosomal dominant RP results from dominantly inherited missense mutations in
86 the rhodopsin gene, a G-protein coupled receptor essential for phototransduction⁷.
87 Pathogenesis is associated with protein-ubiquitin aggregates in inclusion bodies.
88 Due to the impaired ability of the cell to degrade non-functioning proteins,
89 photoreceptor apoptosis is initiated. Other pathological mechanisms caused by
90 rhodopsin mutations include rod outer segment instability and defective
91 intracellular trafficking^{8,9}.

92 In 1997¹⁰, iRDs including LCA were first linked to mutations in genes expressed in
93 the RPE but not in photoreceptors. Mutations in the gene encoding retinoid
94 isomerohydrolase RPE65 result in photoreceptor degeneration due to visual cycle
95 disruption. RPE65 is an RPE-specific protein catalyzing the conversion of all-trans-
96 retinyl ester to 11-*cis* retinol. Autosomal recessive mutations disrupting the visual
97 cycle reduce the levels of 11-*cis* retinoids needed to couple with opsins to form
98 light-sensitive visual pigments^{11,12}.

99 The genetic landscape of inherited retinal disease is vast, currently amounting to
100 ~256 causative genes. Unexpectedly, identification of the causative genes has
101 generally not accelerated development of clinical interventions. Ultimately, iRD
102 mutations, whether dominant or recessive, result in retinal dysfunction and sight
103 loss. For patients, loss of vision can result in diminished independence. These
104 causative mutations are targets for corrective gene editing which has potential to
105 delete alleles carrying autosomal dominant mutations, or correct dominant,
106 recessive or X-linked mutations.

107

108 **2. Gene Editing Technologies**

109

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

shift mutations¹³. 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 template¹⁴. 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 editing¹⁵. 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 lentivirus¹⁶.

129

130 2.1 Zinc Finger Nucleases

131

Gene editing can be achieved using multiple endonuclease systems. Zinc finger proteins are a class of transcription factors that bind DNA through Cys₂-His₂ 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 $\beta\beta\alpha$ structure. Zinc finger nuclease (ZFN) technology developed from the functional independence of the DNA-binding and cleavage domains of the FokI restriction endonuclease¹⁷. 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 NHEJ¹⁸⁻²³.

144

145 2.2 TALENs

146

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 editing²⁵. Additionally, *in vivo* delivery of TALENs is hampered by their large size –approximately 3 kb for a single TALEN-

164 and the repetitive nature of TALE arrays, leading to packaging difficulties into
165 certain viral delivery systems^{26,27}.

166

167 **2.3 CRISPR/Cas**

168

169 CRISPR-Cas RNA guided nucleases function as an adaptive immune system in
170 bacteria²⁸. The three components required in bacteria for effective immune defense
171 via the type II CRISPR nuclease system include a Cas9 protein, the mature CRISPR
172 RNA (crRNA) and trans-activating crRNA (tracrRNA). Fusion of the crRNA and the
173 tracrRNA produces a guide RNA (gRNA)²⁹, and retargeting of the Cas9-gRNA
174 complex is achieved through alternating a short portion of the gRNA³⁰. The
175 formation of a DNA-RNA duplex at the matched target site in the genome allows
176 cleavage of target DNA³¹. The sequence limitation of this system is based on a
177 necessary protospacer-adjacent motif (PAM) positioned immediately 3' to the target
178 site, for example the PAM sequence 5'-NGG-3' is required for binding and cleavage
179 of DNA by the most commonly used *Streptococcus pyogenes* Cas9 enzyme²⁸. A
180 consideration in the design of sgRNA for directing Cas9 to cause DSBs is the
181 necessity to identify a PAM site in the target sequence. Engineering of modified Cas9
182 proteins, or the identification of other Cas proteins with different PAM motifs are
183 addressing this limitation. The major benefit of CRISPR/Cas9 is that unlike ZFNs and
184 TALENs, engineering of novel proteins for each DNA target site is not required.

185

186 A limitation of CRISPR is the difficulty to insert exogenous DNA in post mitotic cells.
187 Recently, a modified approach relies on homology independent targeted integration
188 (HITI), which utilises NHEJ to insert a corrective donor DNA into the host genome
189 using flanking Cas9 sites on the donor coding sequence. It is preferable for an
190 exogenous wild type copy of a disease-causing gene to be inserted into the
191 endogenous gene locus. This allows for transcriptional control under endogenous
192 promoter elements. To achieve this, the donor DNA template containing the genetic
193 element can be flanked with homology arms including sequences which have the
194 same identity as the endonuclease cut site, allowing for specific insertion^{19,32}.
195 Subsequently, NHEJ-mediated ligation of donor DNA sequence directly into a target
196 locus at the overhangs produced by the endonuclease-induced DSBs is an
197 alternative option for HDR mediated gene insertion in post mitotic
198 photoreceptors³³. NHEJ mediated HITI overcomes limitations of low frequency HDR
199 in post mitotic cells. However, there remains a lack of knowledge in photoreceptor
200 specific DNA repair mechanisms, which represents a potential barrier to the
201 development of effective therapeutics for iRDs. Ability to quickly design and
202 synthesize multiple guide RNAs targeting different genomic regions underpins why
203 this more versatile *molecular tweezers* has revolutionized genome editing.

204

205 **3. Treatment of inherited retinal disease**

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

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

213 **3.1 Overview of strategies for the treatment of retinal degenerative disease**

214

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

221 Pharmacological interventions for treatment of iRDs offers the ability to protect
222 photoreceptor and RPE dystrophy through targeting common convergent pathways
223 responsible for cell death, such as attenuating apoptotic signaling or mitochondrial
224 function or by promoting cell survival³⁴. However, drug based neuroprotection for
225 iRDs has stagnated at clinical trial phases, and to date clinical benefit to patients is
226 limited. Cell transplantation offers a regenerative treatment strategy for iRDs
227 through replacement of cells/tissues, and unlike other biological approaches may be
228 beneficial even at advanced disease stages. The technique depends on integration of
229 human retinal cells and/or tissue differentiated from induced pluripotent stem cells
230 (iPSCs) or embryonic stem cells (ESCs) following sub-retinal injection. Phase I/II
231 clinical trials evaluating human embryonic stem cell (hESC)-derived allogeneic RPE
232 grafts demonstrated the procedure to be safe³⁵. Ethical and immunogenic concerns
233 remaining for hESCs may be overcome using iPSCs. However, recently a clinical trial
234 with transplanted RPE derived from iPSC was halted due to oncogenic mutations in
235 one patient's iPSCs. Allogenic cells are now being studied as an alternative³⁶.
236 Furthermore, and of direct relevance to *ex vivo* gene editing, recent studies cast
237 doubt over the ability of transplanted photoreceptor progenitor cells to functionally
238 integrate into the retina.

239

240 Gene therapy typically relies on the identification of disease-causing genetic
241 mutations. A gene silencing strategy aims to shut down expression of a defective
242 dominant gene copy, with or without replacing a wildtype allele. Silencing can be
243 achieved through silencing RNAs (siRNA), antisense oligonucleotides binding to
244 mRNA transcripts, targeting them for degradation. A broader strategy utilizing
245 siRNAs to silence all rhodopsin gene mutations coupled with replacement of a
246 distinct functional gene transiently improve visual function in a murine iRD model
247 ³⁷. Challenges for gene silencing therapy include optimising dose to produce specific
248 gene knockdown, off-target silencing and transient effects. Kleinman *et. al.*
249 demonstrated that siRNAs induced retinal degeneration in mice by activating RPE
250 Toll-like Receptor 3 (TLR3) receptors and triggering caspases-3 dependent
251 apoptosis via nuclear translocation of interferon regulatory factor 3³⁸.

252

253 In recessive iRDs, gene replacement therapy showed promising clinical results
254 across a range of retinal indications³⁹. Recent clinical trials to treat LCA caused by
255 RPE65 mutations show significant improvement in functional vision, through
256 delivery of a replacement RPE65 gene⁴⁰. Previously, clinical trials for RPE-
257 associated LCA using alternative vectors and constructs reported in 2008 that gene
258 therapy was safe and efficacious in patients. Despite this, three-year follow-up
259 analysis found initial gains in retinal sensitivity waned over time and did not result
260 in meaningful improvements in objective measurements of visual function^{41,42}. Gene
261 therapy also has potential to deliver more generic factors, which address common
262 disease mechanisms such as modulating oxidative stress, reducing protein
263 aggregation, or delivering anti-apoptotic or neurotrophic factors. Sub-efficacious
264 dosages and irreversibility of gene therapy represent therapeutic and safety issues.
265 Gene editing technologies offers promise as an alternative approach to address
266 these treatment limitations. Iterations of gene editing technology offer the potential
267 to precisely correct mutated iRD genes, overcoming safety and efficacy barriers
268 (**Table 1**). For example, the limitation of transient silencing of dominant genes using
269 siRNAs is surmounted by persistent silencing achieved with irreversible editing of
270 the causative mutation in the genome. Editing of the endogenous gene allows its
271 expression to be controlled under “native” regulatory control mechanisms which
272 may overcome issues associated with under- and over-expression using exogenous
273 promoter fragments. Immune rejection of transplanted cells is not a concern for *in*
274 *vivo* gene editing. However, other barriers arise for gene editing including the
275 mosaicism of “corrected” and “uncorrected” cells in the retina which may mask
276 clinical benefit, the low efficiency of precise gene correction and concerns over an
277 adverse response to bacterial Cas9 expression in the eye (**Table 1**).

278 **3.2 Ex vivo gene editing for the treatment of iRDs**

279 Transplantation of gene edited ES or iPSC cells to the retina is a potential
280 therapeutic strategy for iRD (**Figure 2**). Recently Bassuk *et. al.* using CRISPR/Cas9
281 demonstrated *ex vivo* correction of an X-linked retinitis pigmentosa GTPase
282 regulator (*RPGR*) iRD mutation⁴³. Fibroblasts from a patient skin biopsy were
283 transduced to produce iPSCs harboring the c.3070G>T mutation. Introduction of
284 CRISPR gRNAs, Cas9 endonuclease and a donor homology template corrected 13%
285 of the *RPGR* gene copies, converting the premature stop codon to glutamate at
286 position 1024. This *proof-of-concept* study demonstrates capability to repair *RPGR*
287 ORF15 region using CRISPR/Cas9. A treatment milestone will be achieved if these
288 findings are safely and effectively applied to patient eyes.

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

space and exchange intracellular material with host photoreceptors⁴⁷⁻⁴⁹. 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 tissue³⁵. 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 scaffolds³⁵. 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.

333
334

335 **3.3 *In vivo* gene editing for the treatment of iRDs**

336

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

341 knockout yellow fluorescent protein (YFP) in a *Thy1-YFP* transgenic mouse retina
 342 using intravitreal delivery of an AAV2-encapsulated *Streptococcus pyogenes* Cas9
 343 (SpCas9) and a single guide RNA (sgRNA) against YFP. This work demonstrated
 344 ability to efficiently achieve targeted gene editing knockouts in mammalian retinæ.
 345 Furthermore, the mice maintained visual function 5 weeks after injection, an
 346 important *proof-of-concept* for viral-mediated retinal gene editing *in vivo*. Bakondi
 347 *et al.*⁵² demonstrated sub-retinal injection of a targeted gRNA/Cas9 plasmid in
 348 combination with electroporation generated allele-specific rhodopsin (rho)
 349 disruption in a rat retinitis pigmentosa (RP) model. In autosomal dominant disease,
 350 allele specific ablation using gene editing could restore retinal function through the
 351 activity of the remaining wildtype allele. For patients, rhodopsin hemizygosity
 352 should not manifest as haploinsufficiency as wild type rhodopsin expression from
 353 50-200% is asymptomatic⁵³. The transgenic S334ter rat displays similar phenotypes
 354 to the human class I RHO mis-trafficking mutations. Notably, CRISPR-Cas9 could
 355 selectively disrupt *Rho*^{S334} due to a PAM motif present in *Rho*^{S334} but not in *Rho*^{WT}
 356 alleles. Improved visual acuity, and retention of photoreceptor cell number or outer
 357 nuclear layer thickness compared to a control gRNA demonstrate the first effective
 358 use of retinal gene editing to target iRD mutations *in vivo*⁵². Specific ablation of a
 359 dominant allele would not restore any populations of degenerated photoreceptors,
 360 however deletion of a dominant allele in stressed cells that are functioning sub-
 361 optimally could result in gradual depletion of the dominant protein and expression
 362 of the wild type gene could enhance the functionality of these remaining cells.

363
 364 The Bakondi *et al.* study is significant for autosomal dominant retinal degeneration.
 365 However, many iRDs are characterized by loss-of-function mutations. Integration of
 366 template donor DNA for gene correction is an attractive therapeutic option for such
 367 scenarios. Recently Suzuki *et al.*⁵⁴ achieved this in post-mitotic cells using CRISPR to
 368 introduce DSBs followed by homology-independent targeted integration (HITI), an
 369 NHEJ-mediated targeted integration, allowing for robust DNA knock-in *in vivo*. The
 370 HITI method relies on NHEJ for functional integration of DNA, as opposed to
 371 previous studies, which focused largely on HDR to insert coding sequences. The
 372 technique involves donor DNA containing a gene's correct coding sequence, flanked
 373 by Cas9 cut sites, which aid in the homology independent integration of the
 374 sequence into the host genome. Post-mitotic neurons, including photoreceptors rely
 375 largely on NHEJ and not HDR as a DNA repair mechanism. For the *in vivo*
 376 applications of HITI, and to prove efficacy, AAV8 and 9 serotype vectors were used.
 377 In the RCS rat model of RP, a deletion from intron 1 to exon 2 in the *Mertk* gene
 378 disrupts RPE phagocytosis. HITI-AAV vectors, one containing a Cas9 expression
 379 system, and the second containing the coding sequence for *Mertk* exon 2, were sub-
 380 retinally injected. PCR confirmed insertion of *Mertk* exon 2 into intron 1 of *Mertk*
 381 leading to a statistically significant upregulation of *Mertk* mRNA, preservation of
 382 outer nuclear layer thickness and improved b-wave ERG amplitudes, a measure of
 383 scotopic cone vision. In comparison to a HDR-mediated gene insertion of *Mertk* exon
 384 2, HITI knock-in rodents had significantly improved visual function measured by
 385 ERG, and greater ONL thickness. This landmark study sets a precedent for the
 386 application of gene editing technologies in post-mitotic cells. Future studies using

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

389

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

415

416 With the rapid development of revolutionary gene editing technologies, and the
417 publication of *proof-of-concept* studies *in vivo*, focus is shifting to translating CRISPR
418 technologies to the clinic. The gene editing market is predicted to top US\$3.5bn by
419 2019⁵⁶. EDITAS Medicine recently announced plans to begin a clinical trial for
420 treatment of LCA using CRISPR-Cas9. Approximately 20% of LCA patients harbor
421 mutations in the ciliary protein CEP290. The most common mutation in CEP290 is
422 IVS26 c.2991+1655 A>G mutation in intron 26, introducing a novel splice donor,
423 resulting in aberrant splicing and a premature stop codon. EDITAS are employing a
424 dual cut approach by applying two gRNAs directing the *Staph. aureus* CRISPR-Cas9
425 system to excise the mutation containing region using AAV vectors^{57,58}. This
426 approach was reported to repair the mutation in LCA10 patient fibroblasts. More
427 recently, work published by Ruan *et al.*⁵⁹ similarly demonstrated the use of a dual
428 cut approach couple with either a *Staph. aureus* or a *Strep. pyogenes* Cas9 packaged
429 in an AAV5 vector. In the study using SpCas9 the group could effectively induce
430 targeted genomic deletion of wild-type mouse intron 25 of *Cep290*, which is
431 homologous to the human intron 26. Furthermore, the group used a novel approach
432 in developing a self-limiting CRISPR/Cas9 system by incorporating recognition sites

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

438

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

448

449 **4. Future Perspectives for Gene Editing as an iRD Therapy**

450

451 Despite the attention gene editing systems such as CRISPR received in recent years,
452 concerns remain as to their clinical use for iRDs. While the opportunity to correct
453 mutations responsible for RPE or photoreceptor dysfunction is attractive, fixed
454 editing of patient genomes raises safety concerns. The efficacy of targeted gene
455 editing relies on the cleavage of DNA in a site-specific manner while preventing
456 collateral damage to the genome caused by off-target effects of the gRNA.
457 Inactivation of Cas9 nuclease domains and creation of a Cas9 nickase increases
458 specificity due to the requirement of two gRNA/Cas9 complexes to cleave a single
459 strand of DNA individually, coming together at a precise distance and orientation to
460 introduce a DSB⁶¹. Additionally, novel Cas9 variants, and reducing the length of
461 complementarity between gRNA and the target site from 20 to 17 nucleotides
462 increases Cas9 DNA cleavage^{62–64}. Further optimization of gene editing techniques,
463 and the identification of novel endonucleases, such as Cpf1, or alternative RNA-
464 guided RNase functioning enzymes such as C2c2, will diversify the treatment
465 strategies and increase the targetable mutations^{65,66}. A further limitation to using
466 the well characterized SpCas9 for *in vivo* gene editing is its relatively large size for
467 AAV based gene delivery (4.9kb). It is conceivable that efficacy of *in vivo* genome
468 editing could be limited if both the Cas9 and the gRNA construct are delivered
469 separately. Alternative Cas9 orthologues with shorter coding sequences, such as
470 Cas9 from *Staphylococcus aureus*, which is approximately 1kb shorter than SpCas9,
471 could be delivered in the same vector as SaCas9⁶⁷.

472

473 Understandably, in terms of treating iRDs *ex vivo*, the major advantage is the ability
474 to validate gene correction before transplantation of differentiated cells to a patient.
475 *In vivo* gene editing is based on the principle that single editing events are sufficient
476 to treat iRDs. However, the required dosage, mechanism of delivery, and
477 pharmacokinetic profile of such an approach present significant challenges, as is the
478 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 delivery⁶⁸. 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 (RetNet⁶⁹, <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 photoreceptors⁷⁰.

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 RGCs⁷¹. 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 compounds⁷². 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 therapeutics⁷³. 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 factors⁷⁴. 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

525 forms of macular degenerative diseases⁷⁵. Due to the lack of specific disease causing
526 genetic mutations, such multifactorial conditions are not inherently treatable by
527 genome editing technologies, and other therapeutic modalities, or innovative use of
528 gene editing must be explored. While editing and correction of disease causing
529 genetic mutations does not immediately appear relevant for AMD, it would be
530 intriguing to determine if correction of mutations associated with increased risk of
531 AMD would be beneficial.

532

533 **5. Conclusion**

534

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

545

546 **6. Expert Opinion (Words: 583)**

547

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

557

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

569

570 An *in vivo* approach requires the correct genetic editing event to occur following
571 administration of a targeted nuclease, and the absence of harmful off-target
572 mutations. With progress in development of more specific nucleases with reduced
573 off-target effects, an *in vivo* approach is more likely to reach the clinic due to the
574 advances made in the safe delivery of gene therapies via AAV vectors. Factors that
575 need to be considered for iRD treatment must focus on the efficacy of treatment, and
576 its safety. For the treatment to be efficacious without off-target editing, it would be
577 advantageous to deliver Cas9 protein with the appropriate gRNA and possibly donor
578 template. With developments in manipulating Cas9 to increase its specificity, or
579 alter its function (e.g. Nickase activity), the safety profile of CRISPR is likely to
580 improve. With further changes to Cas9, the amount of potential targets is likely to
581 increase, in addition to the discovery of other Cas proteins such as Cpf1 and
582 C2c2^{65,66}, or even the discovery of novel gene editing systems such as DNA guided
583 nuclease systems, or targetable site-specific recombinases^{76,77}. Despite the
584 manipulability of these gene-editing systems, their therapeutic use is limited by the
585 identification of a causative gene. With advances in our knowledge of causative
586 genes through next generation sequencing, more mutations will become amenable
587 to gene editing. Until the time when the mutated gene has been identified, other
588 treatment options are required. Moreover, a specific genetic mutation is not present
589 in complex retinal disease such as AMD. CRISPR and other gene editing technologies
590 still have important roles to play in the elucidation of novel gene function, but also
591 in drug discovery approaches in high throughput settings, and in preclinical drug
592 testing. Genome editing may redefine gene and cell therapies for treating retinal
593 degeneration, but fundamental translational research is needed for these exciting
594 technologies to be used to their full advantage before genome editing becomes the
595 breakthrough technology to treat iRDs.
596

	General	Ex-Vivo	In-Vivo
Pros	<ul style="list-style-type: none"> • Versatility of editing approaches • Ability to correct mutations with high efficiency and specificity 	<ul style="list-style-type: none"> • Ability to identify correctly modified cells and clonally expand • Ability to identify biallelic modification • 	<ul style="list-style-type: none"> • Cell transplantation not required
Cons	<ul style="list-style-type: none"> • Potential Cas9 immunogenicity • Potential harmful off target editing effects 	<ul style="list-style-type: none"> • 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) 	<ul style="list-style-type: none"> • 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

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

599

600 **Figure Legends**

601

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

611

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

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

628

629 **Reference Annotations**

630

631 18 *Landmark paper demonstrating genome editing capabilities of Zinc Finger
632 Nucleases.

633

634 24 *Landmark paper demonstrating genome editing capabilities of TALENs
635 technology.

636

637 28,29,30 *Series of three important papers on the development of CRISPR
638 technology for gene editing.

639

640 43 **An important publication demonstrating the ability to correctly replace a
641 retinal degeneration causing mutation in patient derived stem cells.

642

643 50 *Important demonstration of the use of endonuclease in retinal pigment
644 epithelium.

645

646 47, 48, 49 **The three papers published within 4 months of each other describe the
647 modality by which stem cell derived photoreceptors do not efficiently integrate into
648 the retina, but rather fuse with host cells and exchange cytoplasmic contents.

649

650 51 *Important demonstration of the safe *in vivo* applicability of CRISPR/Cas
651 technology.

652

653 52 **Research demonstrates the correction of a retinal degenerative disease causing
654 mutation in an established autosomal dominant animal model of retinitis
655 pigmentosa *in vivo* using CRISPR/Cas9

656

657 54 **This study demonstrates the efficient replacement of a defective gene copy
658 with donor DNA, correcting retinal degeneration using Homology
659 Independent Targeted Integration

660

661 65 *Important example of identification and application of novel endonucleases for
662 genome editing.

663

664 66*Important example of identification and application of novel endonucleases for

665 genome editing.

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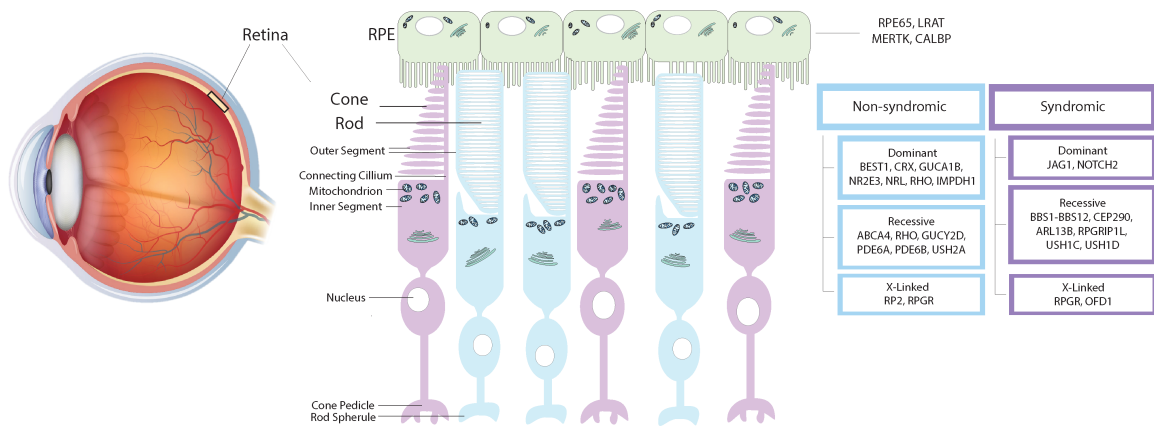
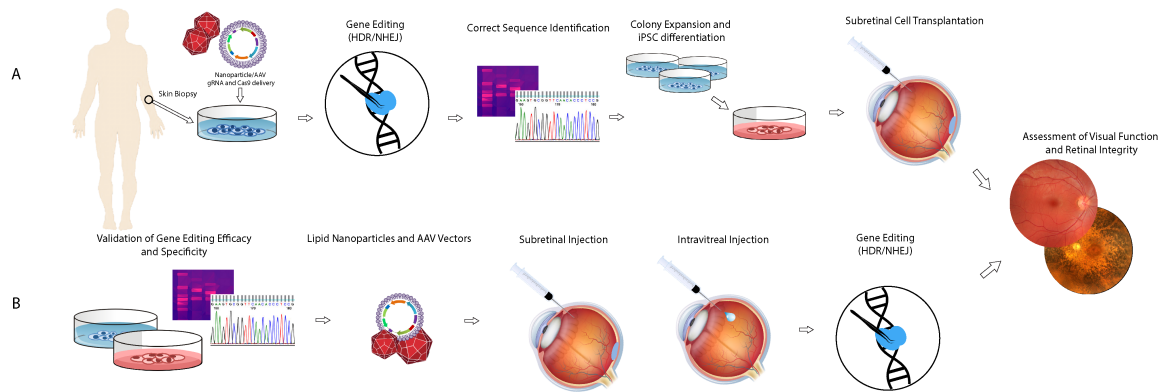


Figure. 1



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Figure. 2