

Gene and Cell-Based Therapies for Inherited Retinal Disorders: An Update

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Retinal degenerations present a unique challenge as disease progression is irreversible and the retina has little regenerative potential. No current treatments for inherited retinal disease have the ability to reverse blindness, and current dietary supplement recommendations only delay disease progression with varied results. However, the retina is anatomically accessible and capable of being monitored at high resolution in vivo. This, in addition to the immune-privileged status of the eye, has put ocular disease at the forefront of advances in gene- and cell-based therapies. This review provides an update on gene therapies and randomized control trials for inherited retinal disease, including Leber congenital amaurosis, choroideremia, retinitis pigmentosa, Usher syndrome, X-linked retinoschisis, Leber hereditary optic neuropathy, and achromatopsia. New gene-modifying and cell-based strategies are also discussed. © 2016 Wiley Periodicals, Inc.

KEY WORDS: retinal degeneration; gene therapy; stem cells; iPSCs; CRISPR; leber congenital amaurosis; choroideremia; retinitis pigmentosa; Usher syndrome; retinoschisis; leber hereditary optic neuropathy; achromatopsia

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INTRODUCTION

Inherited retinal diseases comprise a group of conditions with diverse clinical manifestations and heterogeneous genetic mutations that share a common end-result of progressive photoreceptor death and irreversible blindness. Approximately 1 in 2000 individuals worldwide are affected by inherited retinopathies [Sohocki et al., 2001]. There is currently no proven cure available for patients with inherited retinal degenerations, although management entails specialized genetic counseling, improving the use of residual vision, and treatment of complications that arise [Smith et al., 2015]. However, gene- and cell-based therapeutics hold great

promise as next-generation treatment strategies.

The eye is an ideal target for novel therapies due to its accessibility, ease of noninvasive monitoring, significant compartmentalization, immune privileged status, and optical transparency. Additionally, in translational studies and randomized control trials, the contralateral eye serves as a control, providing a more ideal outcome analysis [Lin et al., 2015]. The retina lines the posterior aspect of the inner eye and is made of multiple cellular layers involved in the signal transduction of light stimuli (Fig. 1). Inherited genetic mutations affecting protein function in cells of the retina can lead to pathologic cellular dysfunction and death, causing visual

impairment. Within the past decade, researchers have made progress in understanding the underlying pathophysiology contributing to retinal degenerations. This knowledge, along with advances in gene delivery and targeting as well as cell-based techniques, has provided hope for future treatment of inherited retinal disorders.

This review will summarize molecular and biologic strategies currently being tested in clinical trials and those demonstrating potential for clinical application in the future. We include updates regarding gene therapy and randomized control trials for inherited retinal disease, including Leber congenital amaurosis, choroideremia, retinitis pigmentosa, Usher syndrome, X-linked

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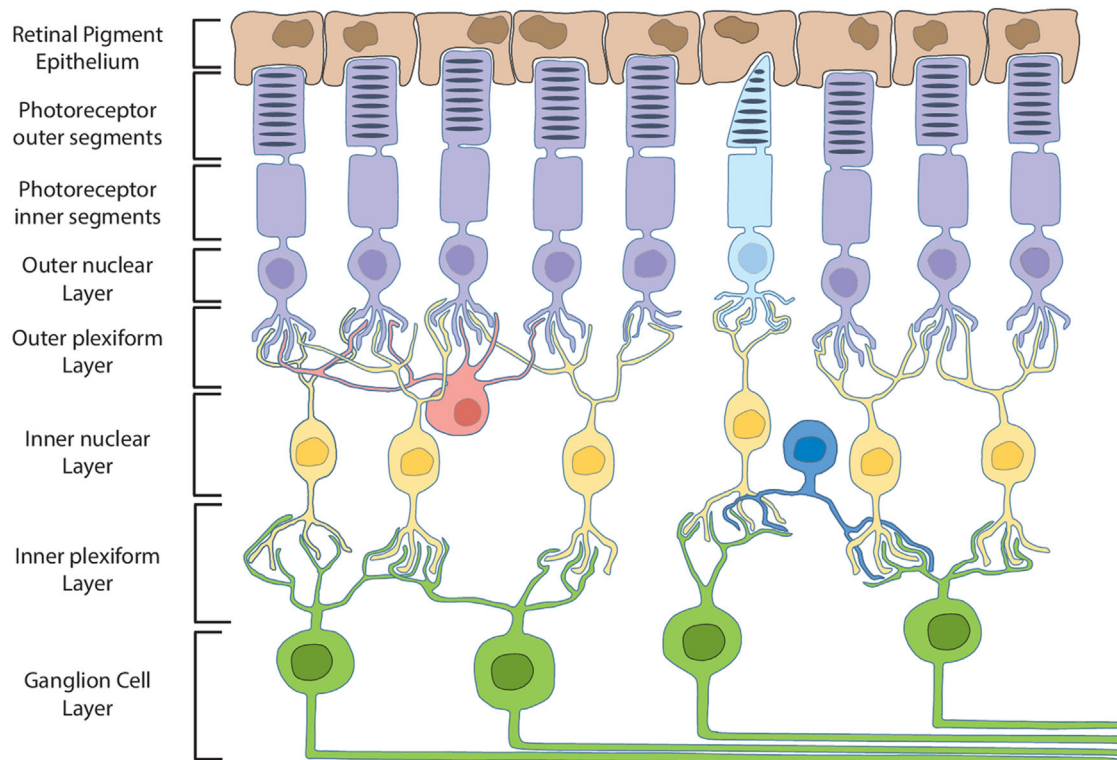


Figure 1. Schematic illustration of retinal cell layers. RPE cells (brown) maintain close contact with and phagocytose rod (purple) and cone (light blue) photoreceptor outer segments. Bipolar cells (yellow) synapse with and transfer information between photoreceptors and ganglion cells (green). Axons of the ganglion cell layer converge to form the optic nerve. Horizontal (red) and amacrine (dark blue) cells serve multiple functions by integrating and regulating signal transduction throughout the retina.

retinoschisis, Leber hereditary optic neuropathy, and achromatopsia. Additionally, the latest advances in genome-engineering techniques, such as clustered regularly interspaced short palindromic repeats (CRISPR) and induced pluripotent stem cell (iPSC) technologies, will be introduced and summarized here.

GENE THERAPY FOR INHERITED RETINAL DISORDERS

Gene therapy uses viral machinery to insert therapeutic genes into native cells. The interventional approach of the therapy is dependent on the inheritance pattern of the condition: recessive conditions, including diseases caused by haploinsufficiency, can be approached through traditional gene addition, meaning a normally functioning gene is provided therapeutically. Patients with dominant or dominant-negative

conditions, however, may require gene suppression or repair. In recessive conditions, mutations in both alleles prevent production of a functional gene product, so addition of a wild type copy of the gene can restore the normal phenotype. Adeno-associated virus (AAV) is the safest and most effective viral vector for gene delivery to the retina, and thus far, it is the only viral vector that has shown beneficial results in treating inherited retinal disease [Bainbridge et al., 2008; Cideciyan et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008; Cideciyan et al., 2009; Maguire et al., 2009; Banin et al., 2010; Simonelli et al., 2010; Jacobson et al., 2012; Testa et al., 2013; Boye and Boye, 2015]. Characteristics that make AAV a successful vector include: lack of pathogenicity, low immunogenicity and toxicity, long-term transgene expression observed in animal models, ability to match a variety of serotypes to specific tissue, and relative ease in manipulating

genetic elements [Daya and Berns, 2008; Boye and Boye, 2015].

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Vector delivery can be achieved by subretinal injection of the viral vector, which involves the formation of a transient retinal detachment that resolves spontaneously (Fig. 2) [Lin et al.,

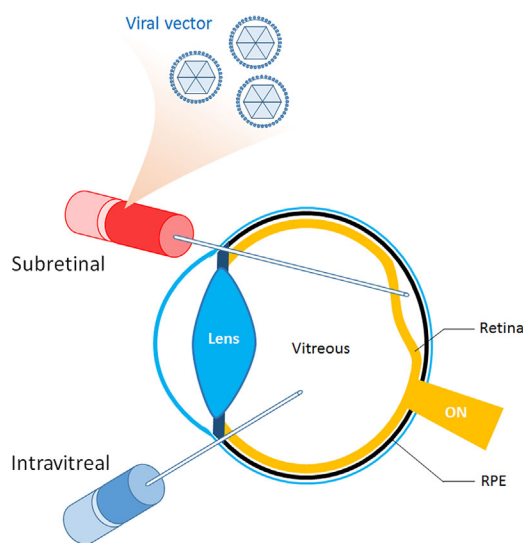


Figure 2. *Vector delivery.* Schematic illustration of subretinal and intravitreal delivery of therapeutic gene-containing vectors. Vector delivery can be achieved by subretinal injection of the viral vector, which involves the formation of a transient retinal detachment that spontaneously resolves. Intravitreal injection is less invasive and produces fewer iatrogenic complications, but may deliver therapeutic genes less efficiently. ON, optic nerve; RPE, retinal pigment epithelium.

the retinal pigment epithelium (RPE) and encodes a 65 kda protein involved in visual pigment regeneration by isomerizing all-trans- to 11-cis-retinol (Table I) [Redmond, 2009; Chacon-Camacho and Zenteno, 2015]. Patients with this mutation have marked photoreceptor dysfunction and degeneration along with severely reduced or absent electroretinogram (ERG) signal at birth or first presentation [Redmond, 2009]. Gene therapy for LCA2 is considered to be among the major milestones in precision medicine. An AAV-mediated therapeutic gene delivery was used in a naturally occurring canine model for LCA2 (*RPE65*^{-/-}) in 2001, which led to the long-term restoration of vision in treated dogs [Acland et al., 2001]. This created a platform for the development of other gene-based therapies in animal models for retinal degenerations and randomized controlled trials for patients suffering from these diseases.

Following the landmark findings of Acland et al., safety studies for *RPE65* delivery by AAV2 were performed in *RPE65*-mutant canines and normal non-human primates [Jacobson et al., 2006a,b]. Multiple phase I/II gene therapy trials assessed the effects of unilateral, subretinal administration of AAV-*RPE65*, which demonstrated short-term rescue [Bainbridge et al., 2008; Cideciyan et al., 2008; Hauswirth et al., 2008; Maguire et al., 2008; Cideciyan et al., 2009; Maguire et al., 2009; Banin et al., 2010; Simonelli et al., 2010; Jacobson et al., 2012; Testa et al., 2013]. All trials reported increased light

2015]. The vector then infects the cells of the retina, leading to expression of the therapeutic gene, as in the case of a gene addition strategy. Other methods, such as intravitreal injection, are less invasive and produce fewer iatrogenic complications, but may deliver therapeutic genes less efficiently [Yu-Wai-Man, 2016].

RPE65 Leber Congenital Amaurosis-2 (LCA2)

LCA is an inherited retinal dystrophy first described over 150 years ago by Theodor Leber. Mutations in genes

associated with multiple visual pathways have been implicated, making the molecular events that lead to LCA relatively heterogeneous. The original clinical manifestations described by Theodor Leber are still used today: significant vision loss at or soon after birth, wandering nystagmus, amaurotic pupils, and a pigmented retina [Koenekoop, 2004]. Research on a subtype of LCA, retinal pigment epithelium 65 (*RPE65*)-associated LCA (LCA2), has laid the groundwork for advances in gene therapy of ophthalmic diseases. *RPE65* is predominantly expressed in

TABLE I. Expression Patterns for Genes Discussed

Affected gene	Clinical manifestation	Location of gene expression associated with phenotype
<i>RPE65</i>	Leber congenital amaurosis	RPE
<i>GUCY2D</i>		Photoreceptor outer segments
<i>CHM</i>	Choroideremia	RPE and photoreceptors later
<i>MERTK</i>	Retinitis Pigmentosa	RPE
<i>RPGR</i>		Photoreceptor cilia
<i>MYO7a</i>	Usher syndrome	Photoreceptor connecting cilium, RPE, cochlear hair cell bundles
<i>RS1</i>	X-linked retinoschisis	Photoreceptors, bipolar cells
<i>ND4</i>	Leber Hereditary Optic Neuropathy	Retinal ganglion cells
<i>CNGB3</i>	Achromatopsia, or rod monochromatism	Cone photoreceptors

sensitivity and improved vision in some patients, without any significant adverse effects from the vector. The untreated eye of three patients in one study received therapy years later with similar results in a 6-month follow-up period [Bennett et al., 2012].

Long-term follow-up of LCA2 patients in phase I/II trials has continued. Although results initially suggested improvements in photoreceptor function among some individuals, patients continue to show retinal structural loss and degeneration [Cideciyan et al., 2013; Jacobson et al., 2015]. Identifying factors that limit the success of gene therapy is imperative to translating treatments developed in mouse models to human patients. The idea that retinal degenerations reach a “point of no return” has been entertained [Cepko and Vandenbergh, 2013]. Most pre-clinical mouse models for retinal degenerations were treated prior to disease onset, whereas patients in randomized controlled trials are treated after the onset of photoreceptor degeneration. Fortunately, studies in mouse models show that treatment is likely efficacious at both the early and late stages of disease; this maintains hope that treatment of patients after the onset of retinal degeneration may be practical and capable of preserving vision [Davis et al., 2013; Wert et al., 2014b; Hurley and Chao, 2015; Koch et al., 2015].

Spark Therapeutics (Philadelphia, PA) is sponsoring a phase III clinical trial that launched in 2013 in collaboration with the Children’s Hospital of Philadelphia and the University of Iowa (NCT00999609). Patients aged 3 years or older with retinal dystrophy caused by mutations in *RPE65* (LCA2) were administered sub-retinal AAV2-hRPE65v2 (SPK-RPE65) in both eyes. After achieving ‘breakthrough therapy designation’ in late 2014 from the U.S Food and Drug Administration (FDA), Spark announced results of their primary and secondary outcome measures in an October 2015 press release. The trial met its primary endpoint, as SPK-RPE65 gene therapy achieved statistically significant increases in functional vision compared to the control group as

measured by bilateral mobility test change score after 1 year. Two of the three secondary end-points were also met: patients receiving SPK-RPE65 had better full-field light sensitivity and favorable mobility test change scores, a measure of performance navigating in environments of various levels of lighting, compared to baseline for the first injected eye; the unmet secondary endpoint was visual acuity, which showed no statistically significant change. The company stated that they will submit a biologics license application with the FDA in 2016. In the meantime, safety of vector administration to the contralateral eye was reported in eleven patients, confirming a previous study with fewer patients [Bennett et al., 2012] and representing the first ocular gene therapy to be successfully delivered to the previously untreated eye [Bennett et al., 2016].

GUCY2D Leber Congenital Amaurosis-1 (LCA1)

LCA1 is caused by homozygous or compound heterozygous mutations in *GUCY2D*, which encodes for retinal guanylyl cyclase-1 (retGC1). RetGC1 is predominantly expressed in the outer segment of cones but is also present in rods (Table I) [Jacobson et al., 2013; Boye et al., 2015; Chacon-Camacho and Zenteno, 2015]. The normal function of retGC1 in the outer segments is to respond to low intracellular calcium levels and produce cGMP to reopen cGMP-gated channels, thus returning the photoreceptor to its pre-excitation state after a flash of light [Jacobson et al., 2013; Boye et al., 2015]. The mutation causes a severe decrease in visual acuity and produces nystagmus and marked photoreceptor dysfunction on ERG, but interestingly, patients have near-normal laminar structure of the retina, which is unique to this subtype of LCA [Walia et al., 2010; Boye et al., 2015]. *Gucy2e* is the mouse homolog of *GUCY2D*, and the *Gucy2e*^{-/-} model mimics human progression of LCA1 most closely. Multiple proof-of-principle studies have demonstrated restoration of cone function on ERG using

AAV-mediated gene delivery [Boye et al., 2010; Boye et al., 2011; Mihelec et al., 2011; Boye et al., 2013]. Because LCA1 predominantly affects foveal cones, and mice do not have a fovea, an all-cone *Nrl*^{-/-} *Gucy2e*^{-/-} mouse model was created [Boye et al., 2015]. When AAV5-GRK1-GUCY2D was delivered to this model, retinal function was restored for at least 6 months. While there are currently no clinical trials enrolling patients with LCA1, these results suggest LCA1 patients may benefit from delivery of the *GUCY2D* gene.

Choroideremia (CHM)

Truncation or deletion of the *CHM* gene causes choroideremia, an X-linked recessive retinopathy whose genetic cause was described in 1990 [Cremers et al., 1990]. Rab escort protein-1 (REP1) is encoded by *CHM* and, if deficient, can cause degeneration of the choroid, RPE and neurosensory retina (Table I) [Tolmachova et al., 2006; Zinkernagel and MacLaren, 2015]. Rab proteins are expressed in all cells and, within the eyes, are necessary for transport of proteins used for intracellular signaling in photoreceptors and for phagocytosis and breakdown of outer segment disc membranes in RPE cells [Zinkernagel and MacLaren, 2015]. REP1 facilitates lipid modification on Rab proteins by binding unprenylated Rabs and presenting them to enzymes which catalyze the addition of covalently attached hydrophobic molecules, a process termed prenylation. Prenylation is an important process that mediates the association of cytosolic proteins with biological membranes. The buildup of unprenylated Rab proteins is thought to be a major mechanism of retinal cell death [Pereira-Leal et al., 2001]. The disease shows atrophy of the choroid and thus a pale fundus due to illumination of the sclera behind the degenerating choroid (Fig. 3B). Clinically, the condition starts with night blindness and results in a progressive decrease in peripheral vision [Zinkernagel and MacLaren, 2015]. Because choroideremia progresses

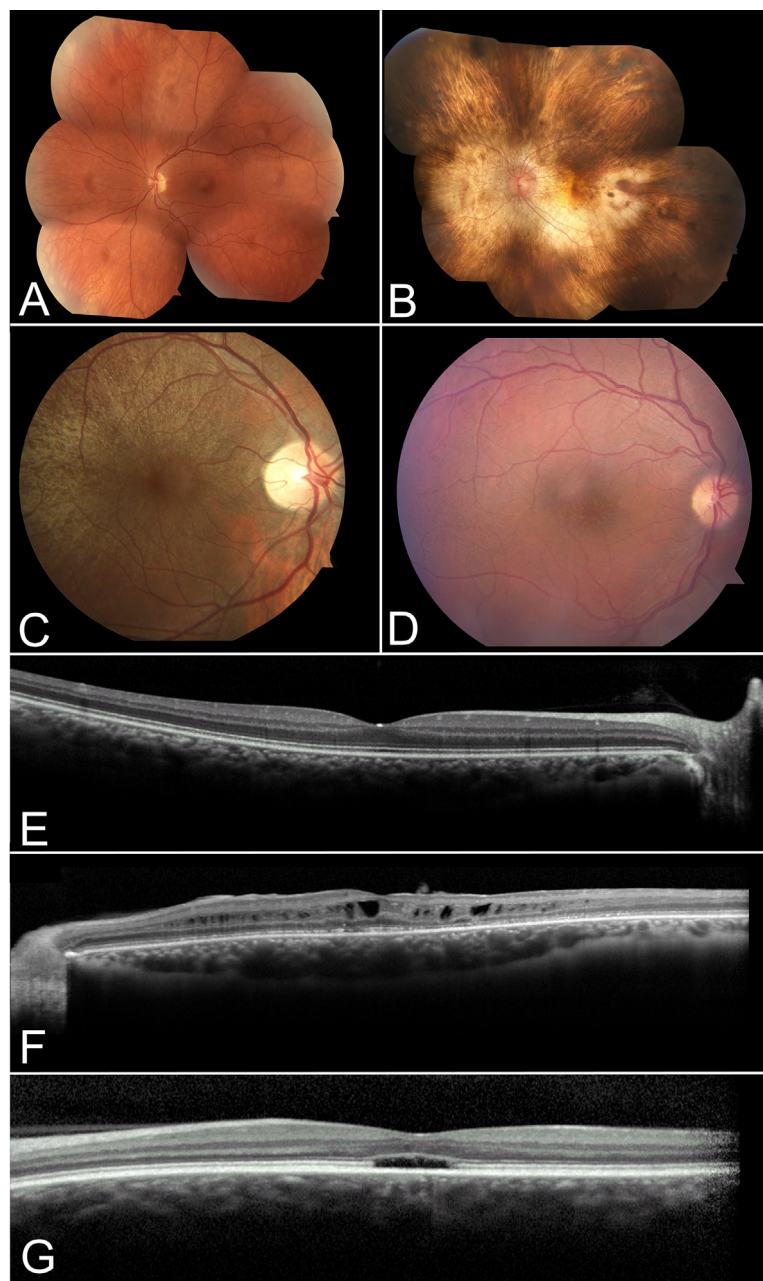


Figure 3. Retinal imaging of patients with inherited retinal disease. Color funduscopy of a normal patient (A) compared to patients with choroideremia (B), asymptomatic *RPGR*-associated RP female carrier (C) and retinoschisis (D). OCT of a normal patient depicting high resolution image of the retinal layers (E) compared to patients with retinoschisis (F) and achromatopsia (G). In B, note the characteristic pale fundus of choroideremia as a result of the sclera transilluminating through a degenerating RPE and choroid. In C, a typical tapetal-like reflex of the retina in an *RPGR*-associated RP carrier can be seen sparing the macula. In D, the subtle spoke-wheel appearance of retinoschisis is seen on color funduscopy, and characteristic foveal and perifoveal cysts are seen on OCT in F. Patients with achromatopsia can show a foveal optical gap with loss of the inner and outer segment junction on OCT as depicted in G.

replacement therapy proved achievable in *Chm*^{null/WT} mice using AAV2 in a dose-dependent fashion and in cells of patients with *CHM* mutations in vitro, providing support for clinical trials [Anand et al., 2003; Tolmachova et al., 2013]. Tolmachova et al. demonstrated that the vector produced therapeutic REP1 with prenylation in cultured fibroblasts of choroideremia patients. A subsequent phase I/II dose-escalation clinical trial which tested AAV2-REP1 in six patients was recently reported. Two patients experienced two- and four-line improvements in visual acuity, and five patients had increases in mean retinal sensitivity despite detachment of the macula (NCT01461213) [MacLaren et al., 2014]. In light of this trial, [Seitz et al., 2015] performed a retrospective cross-sectional study to define pre-treatment characteristics of choroideremia patients eligible for genetic therapy. This study was important in defining the natural progression of disease as a function of age and determined that there is a high degree of symmetry between eyes, suggesting the untreated eye is an adequate control. Additionally, it was found that the best objective measure of structural disease progression was the use of fundus autofluorescence to quantify the area of remaining RPE. Two additional trials began in 2015 to test the safety and efficacy of the same gene vector evaluated in the previous study (NCT01461213) to also treat CHM patients (NCT02077361 and NCT02341807) [Dimopoulos et al., 2015].

Autosomal Recessive Retinitis Pigmentosa (RP)

RP affects approximately 1 in 4000 individuals worldwide and has a variable inheritance pattern [Berson, 1993]. In RP, progressive atrophy of rods occurs due to an inherited gene mutation, leading to secondary death of cones [Hamel, 2006]. RP is a genetically heterogeneous disease, as over 70 mutated genes have been identified to cause this degeneration of the retina with variable phenotypes [Zhang, 2016].

slowly and has been characterized in mice [Tolmachova et al., 2006; Tolmachova et al., 2010], it is an attractive candidate for gene therapy.

Large animal models for choroideremia do not exist, eliminating the luxury that canine models for LCA2 provided [Boye and Boye, 2015]. However, *REP1*

Patients will typically present with night blindness, which progresses to tunnel vision and ultimately blindness [Fahim et al., 1993; Hartong et al., 2006; Wert et al., 2014a].

A rare form of autosomal recessive RP is caused by mutations in mer receptor tyrosine kinase (*MERTK*). Homozygous loss-of-function mutations in *MERTK* result in impaired phagocytosis of the photoreceptor outer segments by RPE cells (Table I) [Gal et al., 2000]. Debris accumulates between the outer segments and RPE over time, causing photoreceptor degeneration and vision loss. Several studies have tested the efficacy of gene therapy in the Royal College of Surgeons (RCS) rat, which was found to carry a *Mertk* mutation [Vollrath et al., 2001; Smith et al., 2003; Tschernutter et al., 2005; Conlon et al., 2013]. Conlon et al. performed pre-clinical studies with an AAV2 vector carrying *MERTK* and an RPE-specific promoter (VMD2), which was safe and effective in the RCS model [Conlon et al., 2013]. RCS rats displayed improved ERG response in the eye injected with AAV2-VMD2-h*MERTK* compared to the contralateral untreated control eye, and the subretinal injection of the vector was well-tolerated. This prompted a clinical trial by collaborators at the King Khaled Eye Specialist Hospital and King Faisal Specialist Hospital & Research Center (NCT01482195) [Conlon et al., 2013]. Safety was confirmed in preclinical studies in monkeys, and a phase I open-label, dose-escalation trial involving subretinal injection of rAAV2-VMD2-h*MERTK* to six patients with advanced *MERTK*-associated RP was initiated [Ghazi et al., 2016]. There was transient improvement of visual acuity in half of the patients, although this progress was lost in two of the three patients by the end of the 2-year follow-up.

RPGR X-Linked Retinitis Pigmentosa

X-linked retinitis pigmentosa (XLRP) accounts for approximately 10–20% of all cases of RP, with a mutation in the

retinitis pigmentosa GTPase regulator (*RPGR*) being the most common (Table I) [Hong et al., 2003; Raghu-pathy et al., 2015]. *RPGR* has been demonstrated to interact with the delta subunit of cyclic GMP phosphodiesterase, suggesting the defective protein likely causes degeneration due to detrimental effects on the processes that govern protein localization and transport [Linari et al., 1999]. Although no clinical trials for *RPGR*-XLRP exist at this point, a step toward treatment came with proof-of-concept studies in two canine models in which therapeutic AAV-*RPGR* preserved photoreceptor function and structural integrity [Beltran et al., 2012]. Other murine models also exist for *RPGR*-XLRP, but are less representative of the human condition [Hong et al., 2000; Hong et al., 2004; Brunner et al., 2010; Wright et al., 2011; Huang et al., 2012]. Applied Genetic Technologies Corporation (AGTC) (Alachua, FL) is initiating a formal preclinical testing program to provide a clinical evaluation of the gene therapy product used in the canine models.

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Although *RPGR*-XLRP almost exclusively affects males, as would be expected due to its X-linked inheritance pattern, females can also be affected [Souied et al., 1997; Churchill et al., 2013]. Heterozygous carrier females exhibit a spectrum of phenotypes, although they typically exhibit no symptoms and have a tapetal-like reflex seen on multiple imaging modalities. In

rare cases, they can develop severe phenotypes [Branham et al., 2012]. Recent data suggest that families with no male-to-male transmission who are suspected of having autosomal dominant RP may actually have XLRP instead [Churchill et al., 2013]. For females with XLRP manifesting a mild phenotype, the benefits of gene therapy likely will not outweigh the risks, and enrollment of female patients in clinical trials would require careful consideration.

Usher Syndrome (USH)

Usher syndrome is an autosomal recessive disease characterized by retinitis pigmentosa, progressive hearing loss and possible vestibular dysfunction. Previous studies estimated a prevalence of 1 in 25,000, but recent epidemiological data suggest it may be more common than originally predicted [Kimberling et al., 2010; El-Amraoui and Petit, 2014]. There are three main types that can be distinguished clinically (USH1, USH2, and USH3), but there are over fifteen known loci associated with Usher syndrome affecting both photoreceptors as well as the hair bundle and synapse of the inner ear cells. USH1 patients exhibit congenital profound hearing loss, vestibular dysfunction, and early-onset RP. USH2 patients typically have moderate hearing loss, normal vestibular function, and develop RP in early adulthood. USH3 patients have progressive hearing loss, sporadic vestibular dysfunction, and variable onset of RP [Mathur and Yang, 2015]. USH proteins are part of multiprotein complexes and are involved in actin-based intracellular trafficking, scaffolding, cell adhesion, and signaling [Williams and Lopes, 2011; Mathur and Yang, 2015]. Currently, there is no cure for Usher syndrome, and present management includes administering hearing aids or cochlear implants in those eligible [Jatana et al., 2013].

Retinal degeneration in multiple mouse models for Usher syndrome have been amenable to genetic therapies [Colella et al., 2013; Lopes et al., 2013; Zallocchi et al., 2014]. The

shaker1 mouse model of *USH1B* lacks functional myosin 7a (MYO7A) protein, which has multiple functions, including opsin delivery through the photoreceptor connecting cilium and melanosome transport in RPE (Table I) [Williams and Lopes, 2011]. Subretinal injections of EIAV-CMV-MYO7A (UshStat[®]) in the *shaker1* mouse model for *USH1B* decreased the amount of photoreceptor loss and restored the α -transducin translocation in photoreceptors. This vector was chosen in part due to its carrying capacity, as the *MYO7A* gene is large. UshStat proved to be safe and tolerable in macaques in the same study [Zallocchi et al., 2014]. Sanofi (Paris, France) is currently recruiting *USH1B* patients for a phase I/II trial assessing the safety and tolerability of UshStat in *USH1B* patients (NCT01505062).

Attempts to restore hearing and vestibular function in an *USH1* mouse model have also been undertaken. Lentz et al. restored hearing loss and balance in mice using an anti-sense oligonucleotide (ASO) identified in an in vitro

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screen. This is the first therapeutic intervention to rescue vestibular and auditory function in a mouse model of human hereditary deafness. The ASO blocks an aberrant splice site in a *USH1C* mouse model with an *Ush1c* mutation: *c.216G>A*. By blocking the aberrant splice site with an ASO to

promote correct splicing, functional harmonin protein is synthesized, which restores vestibular and hearing function. This strategy is limited to genetic defects that result in cryptic splice sites [Lentz et al., 2013; Mathur and Yang, 2015].

One challenge for Usher Syndrome is that the carrying capacity of AAV vectors makes larger therapeutic genes like *MYO7A* more difficult to deliver. To remedy this, large genes can be split in half and delivered in separate AAV vectors. Recombination then occurs in co-infected cells, leading to functional expression of the therapeutic gene [Dyka et al., 2014; Trapani et al., 2014]. The recombination of the two halves of *MYO7A* results in a sequence that is 100% identical to the predicted sequence in vitro according to one study by Dyka et al. [2014], indicating accurate homologous recombination or splicing for each dual-vector platform. Single AAV vector strategies of delivering *MYO7A* have been reported, however, which suggests that the gene may undergo spontaneous recombination when fragmentation occurs due to an unfavorable carrying capacity [Allocca et al., 2008; Colella et al., 2013; Lopes et al., 2013]. It remains to be seen which strategy will be the most efficient way to deliver large therapeutic genes in AAV vectors.

X-Linked Retinoschisis (XLRS)

XLRS is a retinal degeneration affecting between 1 in 5,000 to 25,000 individuals worldwide [Byrne et al., 2014]. The condition is characterized by splitting of the retinal layers and the presence of intraretinal fluid-filled cysts, typically in the macula [Schubert and Wissinger, 2015]. A hallmark finding in the functional assessment of XLRS is a diminished ERG b-wave with relative preservation of the a-wave, correlating to decreased synaptic transmission via photoreceptor-bipolar cell synapse [Schubert and Wissinger, 2015]. Mutations in retinoschisin (*RS1*), a gene encoding a protein thought to be important for retinal cell layer organization and synapse structure, were identified by positional cloning as the cause of

XLRS (Table I) [Sauer et al., 1997; Weber et al., 2002]. In a mouse model that lacks the homolog of retinoschisin, the human condition is mimicked, including formation of fluid-filled cavities and progressive photoreceptor death [Weber et al., 2002]. Since the identification of *RS1* as the gene associated with XLRS, progress has been made in developing gene therapies to slow degeneration in mice [Zeng et al., 2004; Min et al., 2005; Molday et al., 2006; Kjellstrom et al., 2007; Janssen et al., 2008; Takada et al., 2008; Park et al., 2009; Ou et al., 2015]. Byrne et al. developed methods to deliver *RS1* via an AAV vector to specific cell types of the retina, including photoreceptors or Müller glia. It was found that intravitreal delivery of *RS1* to photoreceptors at a minimum provided sufficient rescue when compared to gene delivery targeting Müller glia [Byrne et al., 2014]. This study demonstrated the importance of careful optimization for long-lasting gene therapy.

XLRS patients have particularly fragile retinas that may not be suitable for subretinal injections, making other routes of administration favorable [Molday et al., 2006; Park et al., 2009; Byrne et al., 2014]. Two intravitreally-injected vectors, AAV8 and recombinant AAV2 (rAAV2) carrying functional *RS1* with different promoters, proved effective in *Rs1*-deficient mouse models and are currently being studied in clinical trials. The National Eye Institute launched phase I/II clinical trials for AAV8-scRS/IRBPhRS gene transfer in participants affected by XLRS (NCT02317887). AAV8-mediated gene delivery of *RS1* induces reorganization of the photoreceptor-depolarizing bipolar cell synapse and restores function in *Rs1*-KO mice, supporting its use in a clinical trial [Ou et al., 2015]. AGTC is sponsoring a similar phase I/II study for a rAAV2tYF-CB-hRS1 therapy for XLRS patients supported by safety studies in *RS1*-deficient mice and normal cynomolgus macaques (NCT02416622) [Ye et al., 2015a,b]. Normal macaques showed a dose-dependent inflammatory response in the anterior and posterior segment

which improved over time, indicating the need for frequent observation of XLRS patients receiving the recombinant vector in clinical trials [Ye et al., 2015a].

Leber Hereditary Optic Neuropathy (LHON)

LHON is an inherited mitochondrial disorder characterized by optic atrophy, specifically of the retinal ganglion cells (RGCs) [Cwerman-Thibault et al., 2014]. The majority of LHON is caused by mutations in mitochondrial genes *ND1*, *ND4*, or *ND6* (G3460A, G11778A, and T14484C, respectively); these genes encode for complex I of the electron transport chain, a key enzyme for cellular respiration (Table I) [Cwerman-Thibault et al., 2014]. Irreversible vision loss occurs as the RGCs degenerate, leading to a central visual field defect, loss of color vision and decreased contrast sensitivity [Cwerman-Thibault et al., 2014]. There are features of LHON that make it a desirable target for gene therapy: AAV or other therapeutic agents can be administered to RGCs by injection into the vitreous cavity, and vision loss occurs bilaterally and sequentially, that is, the second eye becomes involved after the first, creating a distinctive therapeutic window [Koilkonda and Guy, 2011; Cwerman-Thibault et al., 2014].

A major limitation to studying mitochondrial diseases is the lack of animal models, partly due to the difficulty of delivering genes directly to the mitochondria [Guy et al., 2002]. Mitochondrial-encoded genes are not directly amenable to gene therapy given their intra-organellar location. Qi et al. [2007] overcame this by using “allotopic expression” to create a rodent model of LHON, which was accomplished by directing expressed mutant *ND4* to the mitochondria with a targeting sequence. Using a similar genetic strategy, therapeutic *ND4* delivery was shown to effectively rescue an LHON rat model, and intravitreal delivery of *ND4* via AAV was safe in normal mice which displayed specific expression in the mitochondria of RGCs [Ellouze et al.,

2008; Guy et al., 2009]. These results supported initiation of phase I/II clinical trials.

The University of Miami is leading a clinical trial attempting the ‘allotopic expression’ method to deliver *ND4* into patient RGCs (NCT02161380). Initial results published in November 2015 reported no serious safety issues in the first five patients enrolled and increased visual acuity in two of five patients following the delivery of the therapeutic gene [Feuer et al., 2016]. Improved visual acuity in safety studies with low sample size may be due to other factors. In this particular trial, the natural history of LHON may explain the spontaneous improvement in visual acuity, which has been reported in the m.11778G>A patient population that was studied, albeit at much lower rates [Finsterer and Zarrouk-Mahjoub, 2016]. Continued enrollment with an adequate sample size should determine if changes in visual acuity are due to therapeutic gene delivery [Lam et al., 2014]. Similar clinical trials for LHON include those led by GenSight Biologics (Paris, France) (NCT02064569) and Huazhong University of Science and Technology (Wuhan, China) (NCT01267422). Results for the latter study showed intravitreal injection of an AAV vector delivering an allotopically expressed *ND4* to be safe during the 9-month follow-up, with six of the nine patients showing increased visual acuity, enlarged visual field, and no change in the retinal nerve fiber layer cross-section on optical coherence tomography (OCT) [Wan et al., 2016].

Achromatopsia

Achromatopsia, also referred to as “rod monochromatism,” is an autosomal recessive retinal condition with a prevalence of approximately 1 in 30,000 [Zobor et al., 2015]. This condition presents early, in infancy or at birth, with pendular nystagmus and photophobia and subsequently reduced visual acuity accompanying poor color vision [Zobor et al., 2015]. There is currently no treatment, and management relies upon low vision aids such as tinted contact

lenses or glasses with cutoff filters to reduce symptoms of photophobia [Kohl and Hamel, 2013]. Approximately 50% of cases are associated with altered or truncated cyclic nucleotide-gated ion channel B3 (CNGB3) (Table I) [Kohl et al., 2005]. Other cases are associated with mutant *CNGA3*, guanine nucleotide-binding protein G subunit alpha-2 (*GNAT2*), and phosphodiesterase 6C and 6H (*PDE6C*, *PDE6H*) [Kohl and Hamel, 2013]. These genes are all crucial for proper cone phototransduction. CNG channels open in the dark and close upon light stimulation due to a decreased concentration of cGMP, which allows for further downstream photoreceptor signal transduction. Mouse models (*GNAT2*, *CNGA3*, and *CNGB3* deficiency), canine models of *CNGB3* deficiency, and a sheep model of *CNGA3* deficiency have been treated with gene replacement, yielding increased visual function [Alexander et al., 2007; Komaromy et al., 2010; Michalakakis et al., 2010; Carvalho et al., 2011; Pang et al., 2012; Banin et al., 2015]. A phase I/II clinical trial sponsored by AGTC in collaboration with the National Eye Institute (NEI) is currently recruiting patients for a safety and efficacy study for AAV-mediated delivery of *CNGB3* to patients with congenital achromatopsia caused by mutations in *CNGB3* (NCT02599922).

CHALLENGES FOR GENE THERAPY

Just 15 years after the landmark study by Acland et al. providing gene therapy for dogs with LCA2, the amount of progress in precision medicine research has continued to accelerate. However, several challenges still face researchers. Follow-up studies on the LCA2 trials showed that photoreceptor degeneration continued despite variable improvements in vision among participants [Cideciyan et al., 2013]. Treatment failure could be due to inefficient vector transduction or the timing of rescue in relation to disease onset [Cepko and Vandenbergh, 2013; Wert et al., 2014b]. Recent research approached this question using a

genome-engineered mouse model of retinal degeneration, where correction of the deficiency could be controlled pharmacologically in a temporal manner via a tamoxifen inducible *Cre-loxP* rescue allele *Pde6b^{Stop}* strategy. This study indicated that treatment is likely efficacious regardless of whether the intervention occurs pre- or post-onset, as long as there is a sufficient number of rods [Davis et al., 2013]. However, very advanced stages of retinal degeneration are not optimal for gene therapy interventions, which thus far are only able to slow disease progression. Instead, patients at advanced disease stages may benefit more from cell-based transplantation methods, which theoretically could restore visual function.

An alternative hypothesis is that rescued cells may be dying in a non-cell autonomous manner. That is, gene rescue in an individual cell can occur, but extrinsic toxic factors may still cause cell death. Studies discerning the potential factors contributing to non-cell-autonomous death are needed. If these effects prove significant, then for gene therapy to be successful, another mechanism of neuroprotective rescue must be administered in conjunction with therapeutic gene supplementation. This theory is supported by the recent use of a rod-derived cone viability factor (RdCVF). RdCVF is a truncated version of its full-length isoform, which is normally secreted by rods to promote cone survival. It was identified in 2004 via an assay assessing genes that affect cone survival. RdCVF contains a domain which prevents damage from hyperoxemia and was reported to prolong cone survival in *rd10* and *P23H* mouse models of rod-cone dystrophy [Byrne et al., 2015]. *rd10* mice model autosomal recessive RP associated with *Pde6b* dysfunction, and *P23H* mice model dominantly inherited RP associated with a mutation in rod opsin. In patients, a neuroprotective treatment, ciliary neurotrophic factor (CNTF) delivered as an intraocular encapsulated cell implant, tested alone in early- and late-stage RP patients improved cone survival but had no effect on functional vision [Birch et al., 2013].

Further studies to assess gene therapy in patients are needed, but inherited retinal degenerations are relatively rare, and achieving an adequate sample size to accurately assess the efficacy of these treatments remains difficult. A database created by the Foundation Fighting Blindness (FFB) may remedy the difficulty of low sample numbers in trials. The FFB has recently funded a registry, My Retina TrackerTM, to serve as a genetic database for patients suffering from inherited retinal degenerations and their related, unaffected family members (NCT02435940). This database is critical for successful clinical trials, as it collates longitudinal data from participants and provides researchers with institutional review board-approved projects the ability to screen or recruit registrants for clinical studies [Fisher et al., 2016].

Another problem facing clinical trials is surgical complications that hinder effective ocular gene therapy. Subretinal injection to deliver the therapeutic gene provides restricted delivery to a small area and a risk of retinal thinning and visual acuity loss [Boye and Boye, 2015]. Furthermore, diseased retinas are increasingly at risk of damage from subretinal injection. Novel ways to deliver gene therapy to the outer retina via the vitreous are in development and may prove more useful (Fig. 2) since transduction theoretically occurs uniformly across the retina [Lebherz et al., 2008; Natkunarajah et al., 2008; Dalkara et al., 2013; Kay et al., 2013].

ZFN, TALEN, AND CRISPR: THE FUTURE OF GENE THERAPY?

Multiple DNA repair and modification strategies occur naturally and have been a source of inspiration for novel gene therapy approaches. Double-strand breaks (DSBs) are considered a potentially fatal event by the cell and quickly induce two repair mechanisms [Carroll, 2014; Gilles and Averof, 2014]. Non-homologous end joining (NHEJ) results in direct reannealing of the broken strands without concern for sequence

fidelity. In this case, preserving the cell and avoiding apoptosis, which can be initiated by compromised DNA, are prioritized over accuracy of the sequence itself [Cong et al., 2013; Gilles and Averof, 2014]. While this strategy is the default method of repair, it often induces insertions or deletions (indels) that code for nonfunctional gene products. The alternative repair mechanism is homology-directed repair (HDR), wherein unbroken sister chromatids from a homologous chromosome are used as a template to ensure the correct sequence is achieved prior to ligation (Fig. 4). While scientists have been eager to implement these strategies in the laboratory, inducing DSBs at loci of interest has been challenging. Only recently has this ability been made efficient by way of protein nucleases, with zinc finger nucleases providing the first generation of gene targeting strategies.

Zinc Finger Nucleases (ZFNs)

Zinc finger binding domains were fused with the catalytic subunit of the Type IIS restriction enzyme, *FokI*, to create zinc finger nucleases [Carroll, 2014; Gilles and Averof, 2014]. Zinc fingers are composed of thirty amino acids that can be designed to target specific sequences. Each finger can bind to three nucleotides, and at least three consecutive fingers are required for sufficient binding to DNA. Because *FokI* must dimerize to cleave the DNA, two sets of three zinc fingers must bind on either side of the cleavage site. These recombinant proteins composed of zinc fingers and the *FokI* nuclease were designed to identify a DNA sequence and induce a DSB, leading to homologous recombination or re-ligation. Zinc fingers identify the target sequence and the nuclease causes a DSB. When administered with a donor DNA, this strategy theoretically has the potential to treat inherited disease by correcting the gene sequence through HDR [Greenwald et al., 2010]. ZFN technology was among the first of its kind to induce directed gene editing in eukaryotic cells, and it has since gone on to be

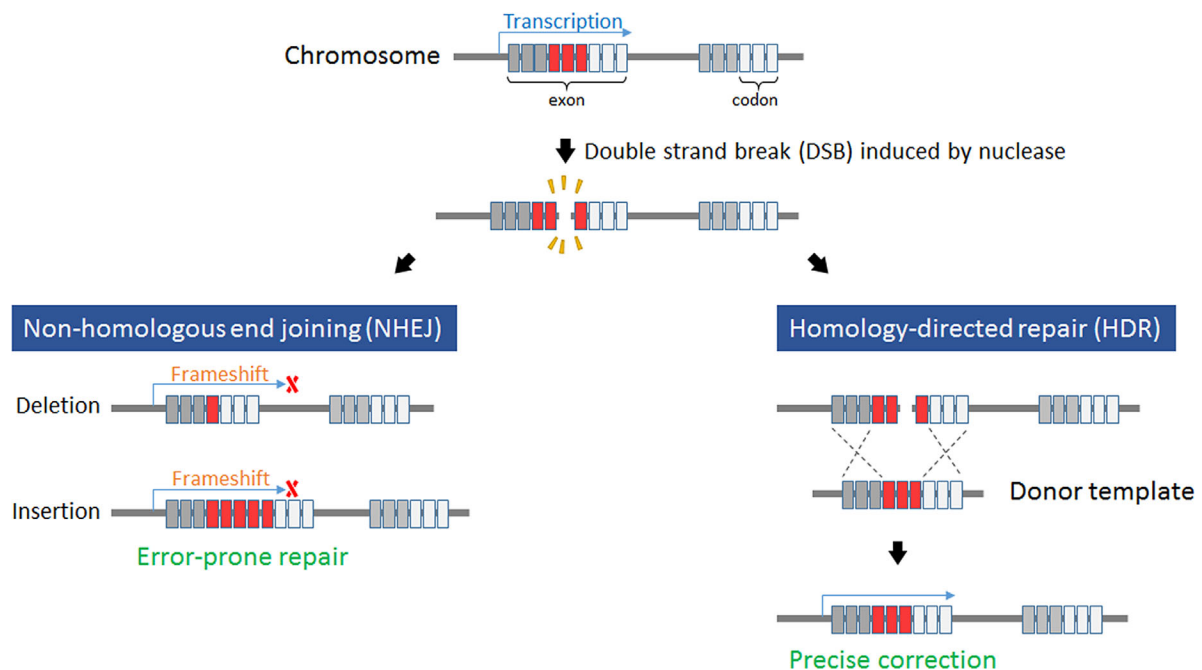


Figure 4. Non-homologous end joining (NHEJ) versus homology-directed repair (HDR). Illustration depicting differences between NHEJ (left) and HDR (right). A double strand break (DSB) may undergo subsequent error-prone NHEJ via ligation of the ends of the DNA without regard for the sequence accuracy, leading to indels. HDR occurs using a wild type donor template that is homologous to the target site and serves as a template for precise correction.

successfully applied in many tissues, including the retina. Administration of ZFNs targeted to the rhodopsin gene in embryonic retinoblast cells increased the rate of homologous recombination compared with endogenous recombination, with rates reaching as high as 17% [Greenwald et al., 2010]. In a similar study, researchers were able to achieve site-specific gene correction of the p.R31X mutation in the *Ush1c* gene following ZFN-induced DSB [Overlack et al., 2012]. These results were among the first to demonstrate the feasibility of gene targeting for retinal dystrophies with ZFN technology.

While ZFNs revolutionized the field of genetic medicine and engineering, there are several limitations that detracted from the applicability of this technology. Designing zinc fingers to bind to every combination of three base pairs has not yet been achieved, so there remain many sites that cannot be targeted. Neighboring domains within each finger may lead to interactions that affect binding specificity or affinity

[Gilles and Averof, 2014]. Thus, newer technologies were explored to build upon the foundation laid by ZFNs.

Transcription Activator-Like Effector Nucleases (TALEN)

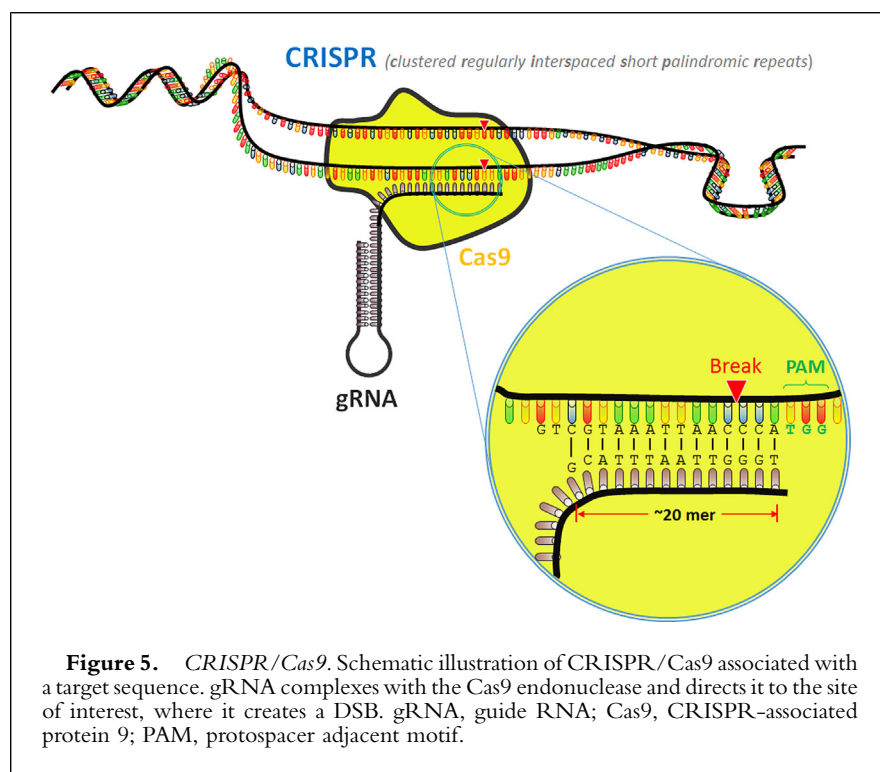
The *Xanthomonas* plant bacteria species integrates its DNA into its host genome by way of transcription activator-like endonucleases (TALENs) [Carroll, 2014; Gilles and Averof, 2014]. Researchers realized that these proteins could be exploited for gene targeting in a parallel manner as zinc finger binding domains. Fusion of TALEs with the *FokI* restriction enzyme led to the creation of TALEN (TALE effector nuclease). TALEN distinguishes itself from ZFN in that the individual TALE proteins, made of tandem repeats of thirty-four amino acids, can bind to individual nucleotides, allowing for a 1:1 ratio of TALE protein to nucleotide. The identity of the nucleotide that the TALE binds to is determined by the amino acids at sites twelve and thirteen. Simplification of the recognition code makes TALEN

much easier to design and enhances binding efficiency compared to ZFN. However, at least twelve TALEs are required for sufficient binding, and this long array is cumbersome in some assays.

TALEN has been applied to the retina. In mouse embryos expressing the *Crb1^{rd8}* mutation, TALEN was used to successfully induce HDR, leading to correction of ocular abnormalities. Single-stranded, complementary nucleotides were co-injected with TALEN to correct the mutation, and the offspring showed normal retinal phenotype [Low et al., 2014]. However, the CRISPR/Cas9 revolution has recently overtaken TALEN as the primary gene editing strategy.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

In some bacteria and archaea, immunity against viruses is achieved by breaking the viral genome into pieces that are integrated into the bacterial DNA in regions containing many clustered



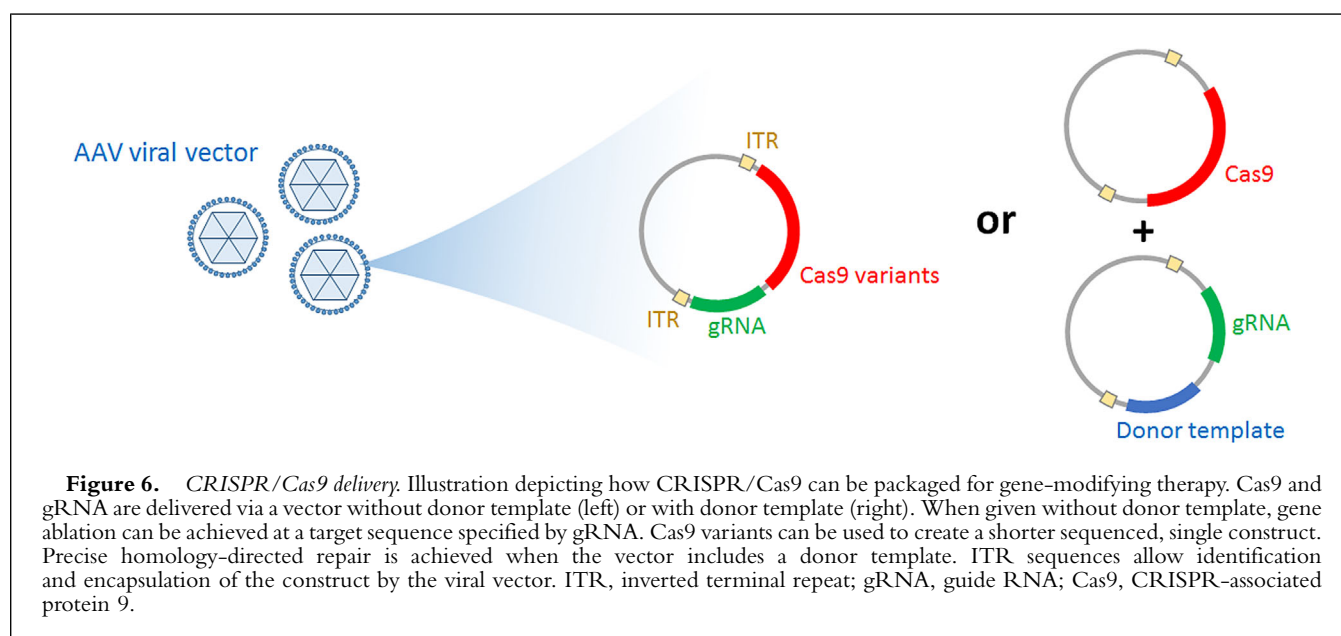
of the crRNA and tracrRNA called guide RNA (gRNA) that is equally as effective in binding to target DNA (Fig. 5) [Cong et al., 2013; Senis et al., 2014]. The gRNA has twenty amino acids at its 5' end that can be customized to target specific sequences [Jinek et al., 2012]. The 3' end contains a region that complexes with the Cas protein, and directly downstream of the cleavage site is a protospacer adjacent motif (PAM) that is required for cleavage [Doudna and Charpentier, 2014]. Thus, it is the combination of a gRNA and PAM that enables the site targeting and cleavage to occur [Ran et al., 2013a]. Previous methods for genome engineering, such as gene knockout and knock-in, have also used HDR as part of its mechanism. However, CRISPR does not rely on the low incidence of DNA damage to complete repairs; instead, it actively creates DSBs to allow for gene editing [Dow, 2015].

CRISPR/Cas-mediated NHEJ is now being applied to inherited retinal disease (Fig. 6). Recently, researchers at Cedars-Sinai Medical Center demonstrated that the mutant gene for an autosomal dominant rodent model of retinitis pigmentosa, S334ter-3 rats, could be ablated to prevent photoreceptor degeneration [Bakondi et al., 2016]. This study paves the way for

regularly interspaced short palindromic repeats (CRISPR) [Sander and Joung, 2014]. The viral genes are transcribed into RNA, also called CRISPR RNA (crRNA), which complexes with a CRISPR-associated (Cas) nuclease. When new viruses infect the bacteria, this crRNA/Cas complex can bind to

specific sequences on the invading strand, allowing the nuclease to subsequently induce DSBs and prevent infection.

The crRNA/Cas complex also requires a trans-activating crRNA (tracrRNA) to function properly, but researchers developed a chimeric fusion



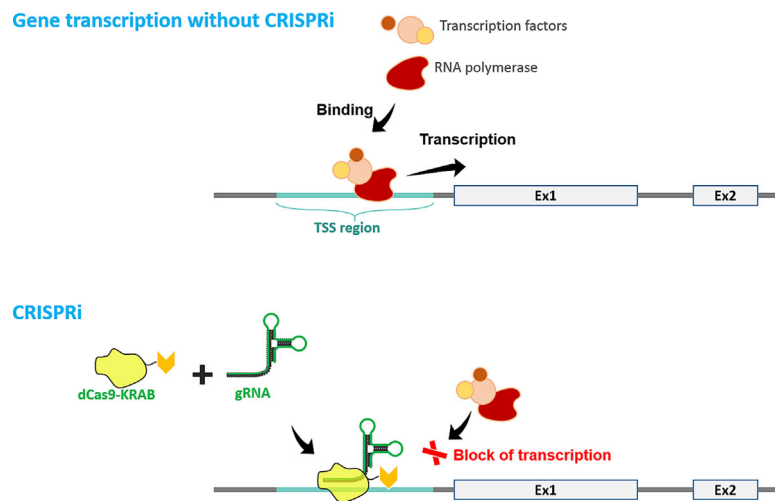
Gene transcription without CRISPRi

Figure 7. *CRISPRi strategy.* In the absence of CRISPRi, transcription factors and RNA polymerase bind to the transcription start site (TSS), which leads to gene expression. Using CRISPRi, a “dead” Cas9 (dCas9) fused to a Krüppel associated box (KRAB) domain complexes with a gRNA that is complementary to a promoter or exonic sequence. This complex does not create a DSB and instead sterically blocks gene transcription.

using gene-modifying techniques such as CRISPR for autosomal dominant diseases. Other strategies have also emerged, including a study by Bassuk et al. demonstrating that a point mutation in induced pluripotent stem cells (iPSCs) from a patient with X-linked retinitis pigmentosa could be corrected with CRISPR/Cas gene repair [Bassuk et al., 2016]. This may prove to be an efficient way of generating healthy iPSCs for future transplantation studies.

CRISPR Interference (CRISPRi): A Substitute for RNAi?

CRISPR tools are evolving quickly and many applications of this technology have been well reviewed [Dow, 2015]. Now, a nuclease-inactive “dead” Cas (dCas) developed by a team at the University of California, San Francisco is being used to decrease gene transcription through a strategy termed ‘CRISPRi’ (Fig. 7) [Qi et al., 2013]. dCas lacks endonuclease activity, so targeted blockage of transcriptional machinery occurs when dCas9 is coupled with a sequence-specific guide RNA, thus preventing RNA polymerase and transcription factors from

transcribing genes. This strategy was shown to be applicable to eukaryotes, including human cells, when fused to transcription modulators [Gilbert et al., 2013; Maeder et al., 2013; Gilbert et al., 2014]. CRISPRi is precise and affects gene expression at the DNA level with minimal off-target effects, which is an improvement upon the previous strategy involving RNAi, which decreases expression at the mRNA level [Gilbert et al., 2014]. This may provide yet another strategy for achieving gene ablation for dominantly inherited retinal degenerations.

CRISPR/Cas is becoming the preferred gene editing strategy due to its higher targeting efficiency and specificity compared with ZFN and TALEN. Unlike TALEN, CRISPR/Cas has been shown to be unaffected by DNA methylation status [Hsu et al., 2013; Ran et al., 2013b; Yaung et al., 2014]. Another recent advancement is the creation of Cas nickases, which provide single strand breaks (SSBs) instead of DSBs. This modification greatly enhances the specificity of this technique and reduces off-targeting effects, as gene correction only occurs if both strands undergo SSBs from Cas [Ran et al., 2013a; Shen et al., 2014]. Progress such

as this will continue to expand the applicability of this technology to gene editing.

CELL-BASED THERAPIES FOR INHERITED RETINAL DISEASE

The retina arises from the neuroectoderm and thus, like other central nervous system tissue, has little regenerative potential. Inherited retinal diseases that cause irreversible photoreceptor death and subsequent blindness stand to benefit from cell-based therapies that can, in theory, regenerate functional retina. In contrast to gene therapy which corrects the mutated gene in existing viable cells, cell-based therapies can replace dead cells. Research is currently focused on producing functional retinal graft tissue or generating in vitro disease models to discern disease mechanisms [Yvon et al., 2015; Wang et al., 2016].

In contrast to gene therapy which corrects the mutated gene in existing viable cells, cell-based therapies can replace dead cells. Research is currently focused on producing functional retinal graft tissue or generating in vitro disease models to discern disease mechanisms.

In the early 1990s, adult and fetal RPE tissue was first transplanted in patients with end-stage age-related macular degeneration (AMD) to replace diseased RPE [Peyman et al., 1991; Algvere et al., 1994]. More recently, two phase I/II studies sponsored by Ocata Therapeutics (Marlborough, MA) finished testing the use of embryonic stem (ES) cell-derived RPE cells to treat advanced dry-AMD and Stargardt disease (NCT01344993 and NCT01345006, respectively). A total of 18 patients were enrolled, including

nine patients with dry-AMD and nine patients with Stargardt disease. The trial reported adverse effects associated with vitreoretinal surgery and immunosuppression, including cataract progression in four patients and one case each of vitreous inflammation and culture-confirmed postoperative endophthalmitis. Importantly, no systemic or ocular effects occurred as a result of the transplanted tissue. Of the eighteen patients, best-corrected visual acuity improved in ten eyes and decreased more than ten letters in one eye compared to control eyes. Furthermore, subretinal pigmentary changes consistent with transplantation were observed in thirteen patients. OCT imaging of areas with increased pigmentation were suggestive of a layer of cells associated with parts of Bruch's membrane [Schwartz et al., 2015].

Due to the controversy and ethical considerations surrounding the use of harvested fetal tissue, emphasis was placed on use of cultured cells for transplantation. iPSCs from a patient's own fibroblasts can circumvent this issue [Lin et al., 2015]. Additionally, using autologous iPSCs decreases the risk of graft rejection. Although the eye is a relatively immune privileged organ, rejection is more likely when using cells from a non-autologous donor. iPSC-derived RPE cells were transplanted successfully in the *RPE65^{rd12/rd12}* mouse model [Li et al., 2012]. Li et al. demonstrated that the transplanted cells co-localized with native RPE. This study supports future initiatives to provide autologous iPSC transplantation for retinal degenerations with an etiology that is driven by RPE loss. The first iPSC transplantation in a human was performed in Japan under the direction of a team at the Riken Institute and the Institute of Biomedical Research and Innovation Hospital [Aoi, 2016]. The study was temporarily on hold when a mutation was believed to be found in a known oncogene during a genomic validation step. The study has since resumed, and the results will be important in determining the feasibility of iPSC transplantation in humans.

Meanwhile, the University of California, Davis is attempting other strategies for stem cell delivery. They are sponsoring a phase I trial to assess safety of autologous intravitreal bone-marrow CD34+ stem cells for various retinopathies, which is estimated to be completed in 2017 (NCT01736059). Preliminary findings showed that eyes tolerated the transplant, and cellular adaptive optics OCT imaging suggested incorporation of the transplanted cells into the macula [Park et al., 2015].

Cell-based therapies for disease characterized by an initial photoreceptor insult face larger challenges, because photoreceptors, unlike the RPE, need to correctly integrate with the neuronal circuitry [Lin et al., 2015]. Thus, the future of cell-based strategies in retinal disease with a photoreceptor-predominant pathophysiology will likely depend most on the ability of the cell-based therapy to not only become a viable photoreceptor, but correctly synapse in the complex visual processing pathway. Despite this challenge, progress has been made. Pearson et al. was able to use photoreceptor precursor cells in mice to integrate into host retina and improve vision [Pearson et al., 2012]. Another group confirmed the ability of human embryonic stem cells to form photoreceptor layers, structured graft maturation, and possible synaptic contacts between transplanted and host cells in primate models for photoreceptor degeneration [Shirai et al., 2016].

CONCLUSIONS: GENE AND CELL-BASED THERAPIES ARE PROMISING

The past decade has shown marked improvements in gene therapy and cell-based approaches as well as newer, large-impact techniques such as CRISPR. Ophthalmic conditions continue to be at the forefront of these developments, as ocular disease is more accessible to treatment strategies compared to other pathologies. Continued translational and randomized control studies are important for assessing various treatment modalities and stand to benefit

many patients suffering from irreversible blindness.

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