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Gene Therapy: A Paradigm Shift in Inherited Retinal Disease

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In this issue of *Ophthalmology Rounds*, we discuss the revolutionary impact of gene therapy for patients with inherited retinal dystrophies (IRDs). The landmark approval of voretigene neparvovec for patients with Leber congenital amaurosis (LCA) caused by mutations in *RPE65* began an era in which preventing and even reversing vision loss is the new reality. However, *RPE65* is responsible for only a fraction of IRD cases. We discuss novel therapies that could greatly expand access to treatment for IRD patients. These include therapies for 2 other severe conditions, *GUCY2D*-associated LCA and RPGR-associated X-linked retinitis pigmentosa (RP). We end by introducing a "gene agnostic" treatment, *NR2E3* helper gene therapy, which could be beneficial for RP patients with a spectrum of gene mutations.

Inherited retinal dystrophies (IRDs) are a heterogenous group of genetic diseases, many of which show progressive photoreceptor dysfunction. Most are monogenic conditions displaying classic Mendelian inheritance patterns. More than 300 causative genes have been identified, with roles in phototransduction, the retinoid cycle, photoreceptor cell structure, transcription, and ribonucleic acid (RNA) splicing.[1,2]

Before the wider availability of genetic testing, IRDs were classified by their clinical and electrophysiologic features. It is now recognized that mutations in different genes can produce almost indistinguishable clinical phenotypes. Retinitis pigmentosa (RP), for example, can be produced by mutations in 69 different genes.[3] Similarly, individual gene mutations can manifest distinct clinical features even within members of the same family.[2,4,5] While clinical characterization aids in narrowing the list of potential genetic causes, genetic testing should be offered to all patients with a suspected IRD.[6] Of note, however, even after comprehensive genetic testing no causative gene mutation will be found in 30%–40% of such patients.[7]

Traditionally, treatment of IRDs has been limited to low vision rehabilitation. However, the 2017 approval of a gene therapy, voretigene neparvovec-rzyl, for patients with retinal pigment epithelium-specific 65 kDa protein (*RPE65*)-associated retinal dystrophy has been a paradigm shift. It is now conceivable to halt and even reverse vision loss from IRDs.

Gene Therapy for Ocular Disease

Gene therapy involves delivering exogenous genetic material into tissues to replace, repair, or silence disease-causing mutations.[8] A major advantage of this treatment approach is that it may be curative. Several features of IRDs make them promising candidates for gene therapy: well-characterized molecular mechanism for many IRDs; relative ease of surgical access to the retina and RPE; the eye's relative immune privilege, which theoretically reduces the risk of systemic dissemination and significant inflammatory reaction; and the availability of noninvasive imaging modalities and functional tests for monitoring treatment effects.[9]

One form of gene therapy, termed gene replacement therapy, uses a vector to carry the corrective genetic material directly into target cells. This is particularly helpful where the disease is caused by loss-of-function mutations in disease-associated genes. In patients with a deleterious gain-of-function mutation, alternative strategies are necessary, such as gene editing or gene silencing. In this article, we restrict our focus to gene replacement therapies.

Adeno-associated virus (AAV) is the most studied gene therapy vector for ocular disease. AAVs are single-stranded deoxyribonucleic acid (DNA) parvoviruses that can infect a wide range of human tissues.[10] Wild-type serovars vary in their affinity for different human cells (termed tissue tropism). Most trials for retinal disease have used serovars 2, 4, 5, and 8, as these all efficiently transduce photoreceptors and RPE.[10,11] AVV vectors can be delivered into the eye via intravitreal, suprachoroidal, and subretinal injection, with the latter being the most effective for transfecting RPE and photoreceptors.[12] Using the same mechanism as for infection, AAV virions are internalized into host cells, the genome is transported to the nucleus, and genes in the viral genome are expressed by the host transcriptional machinery (**Figure 1**). When used for gene therapy, most of the viral genetic material is replaced with the therapeutic gene and promoter.[10]

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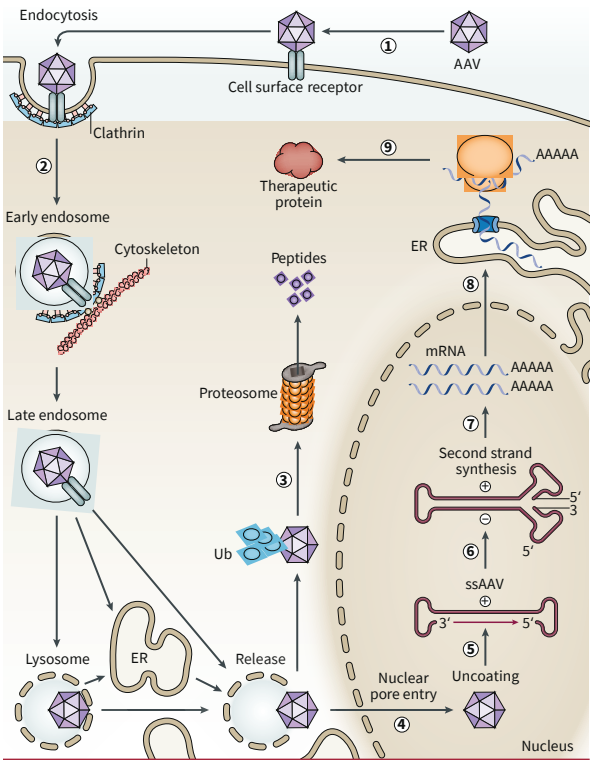
Figure 1. AAV vector transduction pathway: Adeno-associated virus (AAV) vector virions bind to receptors and co-receptors on the surface of target cells (step 1) and are taken into endosomes within these cells through endocytosis (step 2). Following their release from endosomes, AAV virions are either ubiquitylated and targeted for proteasome-mediated degradation (step 3) or intracellularly trafficked to the nucleus (step 4). Once in the nucleus, AAV virions are uncoated and the AAV genome is released (step 5). The AAV single-stranded DNA genome then is converted into double-stranded DNA (step 6), followed by transcription (step 7) and the nuclear export of mRNA (step 8) for translation and expression of the therapeutic transgene (step 9). Engineering AAV vectors to affect any step of their transduction pathway impacts their transduction efficiency.



ER, endoplasmic reticulum; ssAAV, single-stranded AAV; Ub, ubiquitin.
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Benefits of AAV as a gene therapy vector include the fact that it is not pathogenic in humans, has low immunogenicity, is capable of transfecting nondividing cells (e.g., photoreceptors), and does not integrate into the host genome. The latter reduces the risk of introducing mutations or promoting malignant transformation.[10] One limitation is their relatively small size, with a maximum packaging capacity of 4.7 kb. This is smaller than some important IRD genes including *USH2A*, responsible for Usher syndrome, and adenosine triphosphate-binding cassette subfamily A member 4 (*ABCA4*), responsible for Stargardt disease. Potential strategies to overcome this limitation include splitting large transgenes between multiple virus particles,[11] or using viruses with larger genomes such as lentivirus.[13]

Voretigene Neparvovec and the Gene Therapy Revolution

Voretigene neparvovec-rzyl is a Health Canada-approved gene replacement therapy for patients with vision loss due to inherited retinal dystrophy caused by confirmed biallelic *RPE65* mutations.[14] *RPE65* encodes for retinoid isomerohydrolase, an enzyme in RPE which replenishes the 11-cis retinal needed for phototransduction.[15] Biallelic *RPE65* mutations produce a phenotype of severe, early-onset visual impairment and nystagmus typically between birth and 5 years of age, with further visual declines occurring during adolescence.[16] Most affected individuals are legally blind by the end of their second decade of life.[16] Characteristically, retinal structure remains relatively preserved until late in the disorder, despite profoundly depressed electroretinographic (ERG) responses. This function-structure dissociation makes *RPE65* retinal dystrophy an ideal candidate for gene replacement therapy.[17]

Following successful proof-of-concept studies for AAV2-mediated *RPE65* gene replacement in a canine model,[18] several Phase I human trials were completed.[19-23] Voretigene neparvovec-rzyl was the only therapy to proceed to a Phase III trial (NCT00999609). It uses a cytomegalovirus enhancer and constitutively active promoter to drive high levels of *RPE65* expression in transfected cells. The Phase III trial enrolled 29 subjects aged ≥3 years with a molecular diagnosis of *RPE65* retinal dystrophy.[24] The study used 2 novel outcome measures: the multi-luminance mobility test (MLMT) and full-field stimulus threshold (FST). MLMT is a test of functional low vision based on the patient's ability to navigate a standardized obstacle course within 3 minutes under varying levels of ambient illumination.[25] FST measures the threshold light intensity detectable in the dark-adapted state, which has been shown to reflect the functioning of the best-preserved areas of retina.[26] FST testing does not require visual fixation, which is otherwise a challenge for patients with very low vision. Treated subjects in the Phase III trial had significantly greater improvements in MLMT and FST values than controls, as well as superior results on Goldmann visual field testing, and parent/patient reported vision-targeted quality of life (modified 25-item Visual Function Questionnaire).[24] These effects approached near maximum levels by 30 days after treatment[24] and persist for at least 4 years.[27] It is important to note that retinal degeneration appears to progress if treatment is given beyond a certain degree of photoreceptor loss.[28] For this reason, Health Canada recommends that prospective patients have evidence of sufficient treatable photoreceptors on pre-operative spectral domain optical coherence tomography (OCT).[14]

Since Health Canada's approval of voretigene neparvovec-rzyl in 2020, approximately 35 patients have received treatment at 1 of 4 Canadian centres where it is offered. **Figure 2** depicts the results of treatment for a 13-year-old patient with biallelic pathogenic missense mutations in *RPE65* (p.Leu341Ser/p.Gly187Glu) treated at our institution.

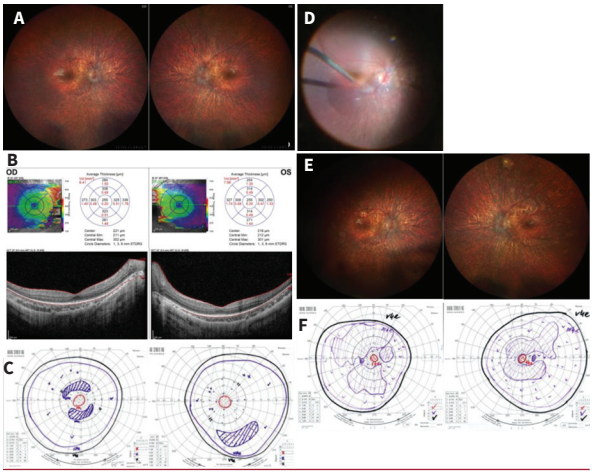
Emerging Gene Therapies for IRDs

Voretigene neparvovec-rzyl is a revolutionary treatment. However, mutations in *RPE65* cause only 1%–14% of IRD.[29] There are currently 19 gene therapy products for IRDs in clinical trials **(Table 1)**.[30] In the following sections we describe a subset of these innovative therapies that have the promise to expand treatment to significantly greater numbers of patients.

GUCY2D-associated Leber congenital amaurosis (LCA type 1)

LCA is a particularly severe form of retinal degeneration, presenting within the first year of life with severe visual impairment, nystagmus, sluggish pupils, and nonrecordable ERG, despite normal-appearing fundus.[31] LCA affects approximately 1 in 30 000–80 000 people worldwide.[32,33] More than 400 mutations in 14 genes have been implicated, most showing autosomal-recessive inheritance.[31,34] *Guanylate cyclase-1 (GUCY2D)* was the first gene to be associated with LCA,[35] hence the term LCA type 1, and accounts for 10%–20% of cases.[36] *GUCY2D* produces a photoreceptor protein called retinal guanylate cyclase-1 (RetGC-1) that replenishes cyclic guanosine monophosphate (cGMP), which is required for photoreceptor recovery to the dark-adapted state following

Figure 2. 13-year-old male patient with early childhood onset retinal dystrophy caused by biallelic RPE65 mutations. Prior to voretigene neparvovec-rzyl treatment, colour fundus photographs showing mid-peripheral retinal pigment epithelium atrophy (A), optical coherence tomograms showing extensive outer retinal loss with sparing of the subfoveal maculae (B), and Goldmann visual field testing demonstrating preservation of the III4e isopter and paracentral scotomata (C). Intra-operative photograph showing subretinal injection of voretigene neparvovec-rzyl in the right eye; left eye photo not available (D). 9 months post-treatment: colour fundus photograph showing circumscribed patches of chorioretinal atrophy (E) and Goldmann visual fields demonstrating progression of the paracentral scotomata (F) but stable macular and peripheral field sensitivity.



phototransduction.[37] Photoreceptors in patients with non-functional RetGC-1 remain hyperpolarized and unable to effectively generate light responses, akin to being in a state of constant light saturation.[35]

As with *RPE65*, LCA caused by *GUCY2D* is a model condition for gene therapy because of the remarkable degree of structure-function dissociation displayed. Despite profound ERG abnormalities from early life, normal photoreceptor anatomy persists into adulthood.[38] Guanylate cyclase-1 (*GC1*) knockout mice display a similar phenotype. Using this model, investigators tested the potential of gene therapy to restore photoreceptor function using subretinal injections of AAV5 carrying the murine wild-type *GC1* under control of the human photoreceptor-specific promoter, rhodopsin kinase (AAV5-hGRK1-mGC1).[39] They showed restored expression of *GC1* in photoreceptor outer segments and increased cone ERG amplitudes to 45% of normal by 4 weeks post-treatment. These were associated with improvements in visual behaviour to within levels demonstrated by wild-type mice.

Results of the first trial in human subjects of a similar gene therapy product, ATSN-101, were published recently.[40] ATSN-101 is a recombinant AAV5 vector carrying the human *GUCY2D* cDNA under transcriptional regulation by the human rhodopsin kinase promoter. This Phase I/II trial enrolled 15 patients with LCA and confirmed biallelic mutations in *GUCY2D* who had identifiable outer nuclear layer on OCT. Three doses from $1.0x10^{10}$ vg/eye to $1.0x10^{11}$ vg/eye were administered via single subretinal injection. No severe adverse events were related to the gene therapy product. Although most adverse events were mild, 3 severe adverse events were documented (endophthalmitis, retinal detachment and macular hole); all were related to the surgical procedure. Four patients in the high-dose cohorts developed intraocular inflammation (subretinal inflammation, vitritis, and iridocyclitis), which resolved with treatment. Eyes in the high dose cohorts (n=9) demonstrated statistically significant improvements in FST compared to untreated control eyes, starting at 28 days post-treatment and persisting to study termination at 1 year (Figure 3). Nonsignificant improvements in best-corrected visual acuity (BCVA) and MLMT times were also observed (**Figure 3**).

Table 1. Inherited retinal dystrophy gene therapy products currently in human trials[30]

Condition (Gene)	Sponsor	Stage
Achromatopsia (CNGA3)	Tubingen Hosp	Phase 1/2
Batten disease (CLN5)	Neurogene	Phase 1/2
Choroideremia (REP1)	4DMT	Phase 1/2
LCA (GUCY2D)	Atsena	Phase 1/2
LCA (LCA5, lebercillin)	Opus Genetics	Phase 1/2
RP (CNGA1)	ViGeneron	Phase 1b
RP (PDE6B)	Coave	Phase 1/2
RP (RLBP1)	Novartis	Phase 1/2
RP & LCA (NR2E3)	Ocugen	Phase 3
RP (RdCVF)	SparingVision	Phase 1/2
RP (PDE6A)	Tubingen Hosp	Phase 1/2
Retinoschisis (RS1)	Atsena	Phase 1/2
Retinoschisis (RS1)	NEI	Phase 1/2
Stargardt disease (RORA)	Ocugen	Phase 1/2
Usher syndrome (USH1B)	Aavantgarde	Phase 1/2
X-linked RP (RPGR)	Beacon	Phase 3
X-linked RP (RPGR)	Johnson & Johnson	Phase 3
X-linked RP (RPGR)	4DMT	Phase 1/2

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Retinitis pigmentosa guanosine triphosphatase regulator (RPGR) gene replacement in X-linked RP

Approximately 15% of RP is X-linked,[2,41,42] with around 70% of patients showing mutations in the RPGR gene (**Figure 4**). RPGR-associated RP is particularly severe, with patients developing visual field constriction early in childhood and profound visual acuity deficits by their third to fourth decade of life.[43] Female carriers are usually asymptomatic but may show mild RPE changes and a tapetal-like reflex on fundoscopy.

Although RPGR expression occurs throughout the body, one transcriptional variant is expressed solely in rod and cone photoreceptors. It localizes to the connecting cilium, where it may have a role in transport of materials involved in phototransduction.[44] A region of RPGR known as ORF15 is a

Figure 3. Results of Phase I/II trial of ATSN-101 for GUCY2D associated LCA: Mean change in dark adapted FST (upper panels) and BCVA (lower panel) from baseline to 1 year in treated (red) and untreated eyes (blue).

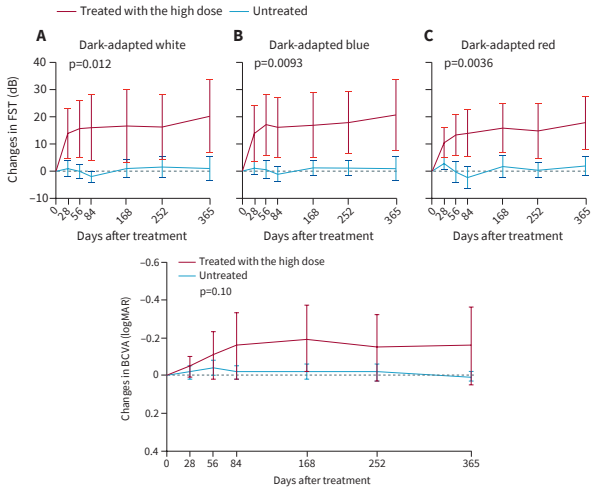

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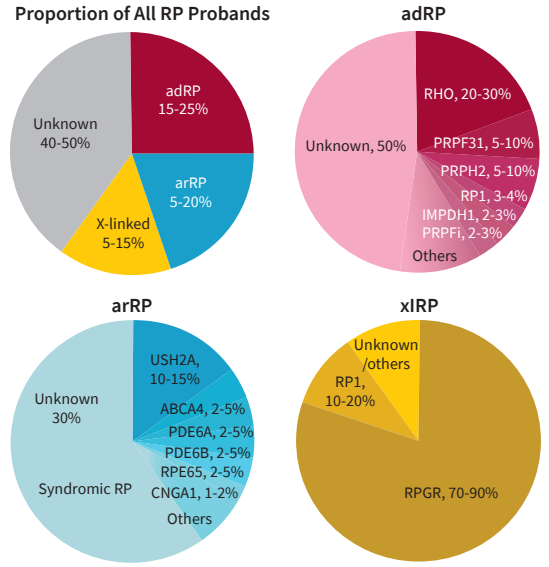
Figure 4. Causative genes and their relative proportions in retinitis pigmentosa (RP). The inheritance of RP consists of autosomal dominant (ad), autosomal recessive (ar), X-linked (xl) and unknown patterns. The causative genes for adRP are Rho, RPRF, PRPH2, RP1, IMPDH1 and PRPF8; for arRP, USH2A, ABCA4, PDE6A, and PDE6B and RPE65; for xlRP, RPGR and RP1. (Reproduced with permission from Elsevier)[2]



"mutational hotspot," where 80% of pathogenic mutations occur.[45] This region has a high concentration of purine bases and demonstrates an increased propensity for insertions/deletions, frameshift mutations, and premature stop codons.[45] Though small enough to be packaged in an AAV vector, the genetic instability of ORF15 has made RPGR a challenging target for gene therapy.[46] Researchers have found that this instability can be overcome by either randomly deleting a section of ORF15 or performing codon optimization. The latter involves modifying the nucleotide sequence to remove splice sites and reduce repeats while preserving the native protein amino acid sequence.[46] In murine and canine disease models, both strategies resulted in reduced opsin mislocalization, improved photoreceptor structure, and restored ERG function.[47-49] These promising preclinical studies have led to 3 gene therapy products entering human clinical trials.

The first RPGR molecule tested in humans, cotoretigene toliparvovec, used an AAV2/8 vector to deliver a full-length codon-optimized $RPGR^{ORF15}$ transgene. It underwent Phase I/II testing in 2017, followed by the Phase III XIRIUS study in 2018-2020 (NCT03116113). Although the Phase I/II study found no dose-limiting toxicity, there was a dose-related incidence of intraocular inflammation (7 of 9 patients in the high-dose cohorts). Two patients experienced inflammation-related visual decline, and 1 showed central photoreceptor loss.[50] The Phase I/II trial showed improvements in retinal sensitivity in 33% of eyes in the intermediate-to-high dose cohorts, which was maintained up to 12 months.[50] Unfortunately, the Phase II/III trial results were less convincing.[51] The trial included 29 male patients ≥10 years of age with RPGR-associated XLRP, who were randomized 1:1:1 to low-dose (5×10^{10} vg/eye) or high-dose (2.5×10^{11} vg/eye) cotoretigene toliparvovec, or no treatment. The primary endpoint was the percentage of participants meeting responder criteria (≥7 dB improvement in ≥5 loci) on microperimetry. At 12 months, there was no significant difference in the percentage of responders across groups (37.5% low dose, 25% high dose, 22% control).[51] However, there was a tendency toward a higher change from baseline microperimetry mean sensitivity and low light visual acuity in treated versus untreated patients, and a significantly higher proportion gaining ≥15 Early Treatment Diabetic Retinopathy Study letters in the low dose versus the control group (prespecified secondary outcomes).[51] As in the Phase I/II trial, there was a dose-related incidence of intraocular inflammation. The authors postulate that retinal inflammation from the treatment may have masked improvements in retinal sensitivity.[51]

The second RPGR therapy tested in humans was botaretigene sparoparvovec, a subretinally delivered AAV2/5 vector carrying human RPGR with a 126-codon deletion in ORF15. A Phase I/II trial in 2017 (NCT03252847) included 49 adult and paediatric patients (age ≥5 years) with XLRP-RPGR.[52] The study found acceptable safety and promising secondary efficacy outcomes. Ocular inflammation developed in 56% of treated eyes but was mostly of mild to moderate severity. Three patients developed severe ocular inflammation (uveitis or chorioretinitis), which reportedly improved or resolved by study completion. Notably, in the dose expansion stage, investigators added a perioperative injection of subtenon triamcinolone to the protocol on top of the standard oral corticosteroid prophylaxis regimen. This appeared to reduce the incidence of inflammation. Compared to a delayed treatment control group, treated subjects showed improvements at 24 weeks in low luminance vision-guided mobility, mean retinal sensitivity, scotopic microperimetry, and reading visual acuity.[52] The proportion of responders (≥7 dB improvement in ≥5 loci) on static perimetry of the central 10° was 23.8% at week 26 and 47.6% at week 52.[52] A Phase II/III trial is ongoing (NCT04671433).

A third treatment, based on a mutated AAV2 capsid carrying a different full-length codon-optimized $RPGR^{ORF15}$, underwent Phase I/II testing in 2018 (NCT03316560). Full results of this trial have not been published; interim 12-month data showed no severe medication-related adverse events, although 3/14 subjects developed vitritis.[53] At 12 months, 63% of subjects who received high-dose treatment met microperimetry responder criteria. A Phase II/III trial of this therapy is now recruiting (NCT04850118).

Nuclear Hormone Receptor 2, family E, member 3 (NR2E3) modifier gene therapy

Genetic modifiers are nonpathogenic genetic variations that can affect timing of onset, rate of progression, and severity of genetic diseases.[54] Gene therapy to enhance the expression of genetic modifiers could offer a novel broad spectrum, or gene-agnostic approach to treat IRDs.[55] One modifier gene that holds promise for patients with RP is *NR2E3*.

NR2E3 is a transcription factor involved in photoreceptor differentiation and maintenance.[54,56] The embryologic function of *NR2E3* is to promote expression

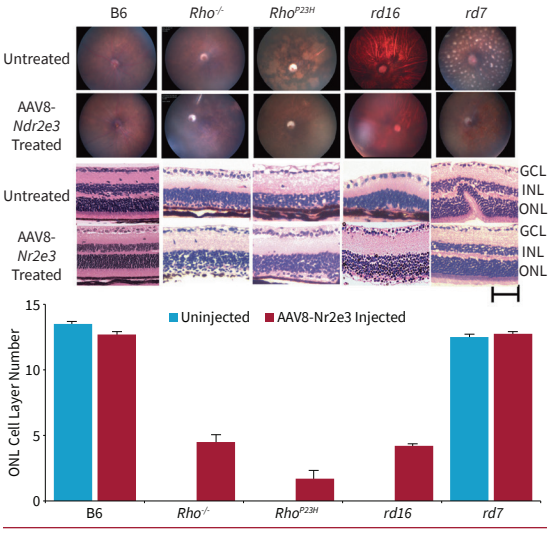
of rod photoreceptor genes and suppress those specific to cones.[54] In the mature retina, *NR2E3* appears to support photoreceptor survival.[54] Mutations in *NR2E3* are associated with a spectrum of retinal diseases, including enhanced S cone syndrome, Goldmann-Favre syndrome, autosomal-recessive and autosomal-dominant RP and clumped pigmentary retinal degeneration.[57] This phenotypic heterogeneity reflects the participation of *NR2E3* in complex interactions during photoreceptor development; however, a common feature in these patients is severely diminished rod function, sometimes associated with over-abundance of S-cones.[56]

Using 5 mouse models of retinitis pigmentosa intended to mimic the spectrum of human disease, investigators showed that *NR2E3* gene therapy increased photoreceptor numbers and improved ERG responses irrespective of the causative gene defect (**Figure 5**).[55] A Phase I/II clinical trial of this gene therapy strategy was initiated in 2022. The trial evaluated the safety of OCU400, a subretinal AAV5-based gene therapy product designed to promote expression of the wild-type human *NR2E3* gene in photoreceptors. The trial enrolled patients with autosomal-dominant or autosomal-recessive *NR2E3*-associated RP, autosomal-dominant *RHO*-associated RP, and *CEP290*-associated LCA (NCT05203939). Preliminary results from 18 adult RP patients followed for 6 months found the treatment to be generally safe, with mostly mild adverse events being attributable to the surgery.[58] One incidence of treatment-related inflammation in a patient in the medium-dose cohort recovered fully; however, a patient in the high-dose cohort developed persistent foveal detachment with permanent loss of vision. Secondary efficacy outcomes included BCVA, low-luminance visual acuity, and MLMT. Overall, 55% of treated eyes showed improvement in these endpoints compared to fellow untreated eyes, with 86% of *RHO*-associated RP patients having improvement or stabilization of their MLMT scores. Notably, patients with autosomal-dominant *NR2E3* RP appeared not to experience the same functional improvements as other patients. A Phase III trial of OCU400 is currently recruiting participants (NCT06388200). This trial has taken the unique approach of including a gene-agnostic study arm, which will enroll patients with clinically diagnosed RP of any genetic cause. Another arm will include only patients with confirmed *RHO* mutations.

Conclusion

Gene therapy offers enormous promise in halting and even reversing vision loss from IRDs. Since the landmark approval of voretigene neparvovec-rzyl, important lessons have been learned about the many challenges in this therapeutic area. As seen in studies of therapies for *RPGR*-associated RP, treatment-related ocular inflammation can be a significant concern. This highlights the need for effective prophylaxis and careful implementation of vector design techniques aimed at minimizing recognition by the host immune system. The sheer number of genes implicated in these rare diseases is another challenge for single gene replacement strategies. Therapies that augment the expression of genetic modifiers, like *NR2E3*, may circumvent this problem by being more

Figure 5. Mice injected with AAV8-*Nr2e3* at post-natal day 21 and evaluated at 2–3 months post injection. A Fundus of *Rho*$^{-/-}$, *Rho*^{P23H}, *rd16*, and *rd7* mutant animals. B Hematoxylin/eosin staining shows partial preservation of photoreceptor cells in treated mutant animals. C Cell layer numbers of outer nuclear layer were compared between AAV8-Nr2e3 treated and untreated animals in the four RP models and B6 control. Results are mean ± SEM. *N* = 7.



"broad spectrum". Other gene-agnostic treatments currently in late-stage clinical trials include optogenetics and antioxidants. Finally, gene replacement is ineffective for patients with gain of function mutations. Although not covered in this article, promising strategies like clustered regularly interspaced short palindromic repeats (CRISPR)-based gene editing and antisense oligonucleotide-based gene silencing are currently undergoing clinical trials. With such myriad advances underway, we are truly witnessing a revolution in the management of IRD.

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