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Gene Therapy Approaches for the Treatment of Retinal Disorders

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Abstract: There is an impelling need to develop effective therapeutic strategies for patients with retinal disorders. Gleaning from the large quantity of information gathered over the past two decades on the mechanisms governing degeneration of the retina, it is now possible to devise innovative therapies based on retinal gene transfer. Different gene-based approaches are under active investigation. They include strategies to correct the specific genetic defect in inherited retinal diseases, strategies to delay the onset of blindness independently of the disease-causing mutations, and strategies to reactivate residual cells at late stages of the diseases. In this review, we discuss the status of application of these technologies, outlining the future therapeutic potential for many forms of retinal blinding diseases.

Introduction

Retinal degenerative diseases are major causes of blindness worldwide. As a group, these diseases are characterized by the progressive loss of one or several cell types of the retina (Figure 1), resulting in irreversible vision loss. Retinal degenerations encompass multifactorial diseases such as age-related macular degeneration and glaucoma, as well as a large variety of monogenic ocular disorders, such as Leber congenital amaurosis (LCA) and retinitis pigmentosa. Currently, there is no cure for retinal degenerative diseases.

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Over the past two decades, the rapid elucidation of the molecular and genetic mechanisms involved in these diseases has led to the identification of novel targets for therapeutic development. Among the therapeutic approaches being developed to slow or stop disease progression in patients, ocular gene therapy demonstrates a lot of promise. Gene therapy is a strategy that takes advantage of the host protein synthesis machinery to locally produce therapeutic substances (Wang and Gao, 2014). It involves the transfer of nucleic acids into target cells to correct or supplant a mutated gene product or to introduce a new function that will alter the pathogenesis of the disease. Consequently, one significant advantage of gene therapy over traditional pharmacological approaches is that therapeutic benefits can be maintained over a long period of time without the need of repeated interventions. Moreover, the combination of appropriate gene delivery vectors and cell type specific promoters can limit expression of the therapeutic gene to the desired target cells, which is rarely possible with local drug delivery methods.

Due to its specific anatomical and physiological characteristics, the retina is particularly suited for gene transfer. Firstly, the retina is easily accessible for surgical injection and different injection routes can easily target either the inner retina (intravitreal injection) or the outer retina and the retinal pigment epithelium (RPE) (subretinal injection). Secondly, the retina is small and compartmentalized, which allows obtaining a therapeutic effect with a low gene/vector dose. Thirdly, the blood-retinal barrier confers immune-privilege to the tissue and also limits systemic spread of the virus. In addition, the characterization of small and large animal models recapitulating the clinical course of the corresponding human diseases has enabled rapid implementation of gene therapy strategies for dozens of ocular conditions.

Several strategies of gene therapy have been developed and tested in different animal models of retinal degenerations (Petit *et al.*, 2016). These strategies include (i) gene-specific therapy approaches that aim at targeting

the primary genetic defect in affected cells to cure the disease, and (ii) non gene-specific therapies that aim at either expanding the time of useful vision by modulating common degenerative secondary effects of the disease, or at exploiting the remaining cells to restore visual perception to the blind retina during the terminal stage of the degeneration. Here, we will review some of the studies that have developed these approaches of ocular gene therapy.

Gene-specific Therapies for Inherited Retinal Diseases

Gene replacement for recessive inherited retinal disorders

Most autosomal recessive and X-linked retinal degenerations are caused by the lack of a functional protein. Delivery of a normal copy of the defective gene to affected cells has significantly ameliorated the disease phenotype in various models of recessive retinal degenerations (Boye *et al.*, 2013; Pang *et al.*, 2012; Petit *et al.*, 2016). Of particular note are studies demonstrating long-term correction of the visual defects in animal models of LCA type 2 (LCA2) (Cideciyan, 2010), a severe form of recessive inherited retinal disease.

LCA2 is caused by mutations in a gene coding for the retinal pigment epithelium protein (RPE65), an enzyme mainly expressed in RPE cells, which is critical for retinal activity (Moiseyev *et al.*, 2005). Without a functioning RPE65 protein, RPE cells cannot recycle the visual chromophore required for light detection by photoreceptors (Redmond *et al.*, 1998). LCA2 is marked by profound vision impairment at birth, which generally progresses to total blindness by mid-adulthood as photoreceptor cells degenerate. Unlike other forms of reti-

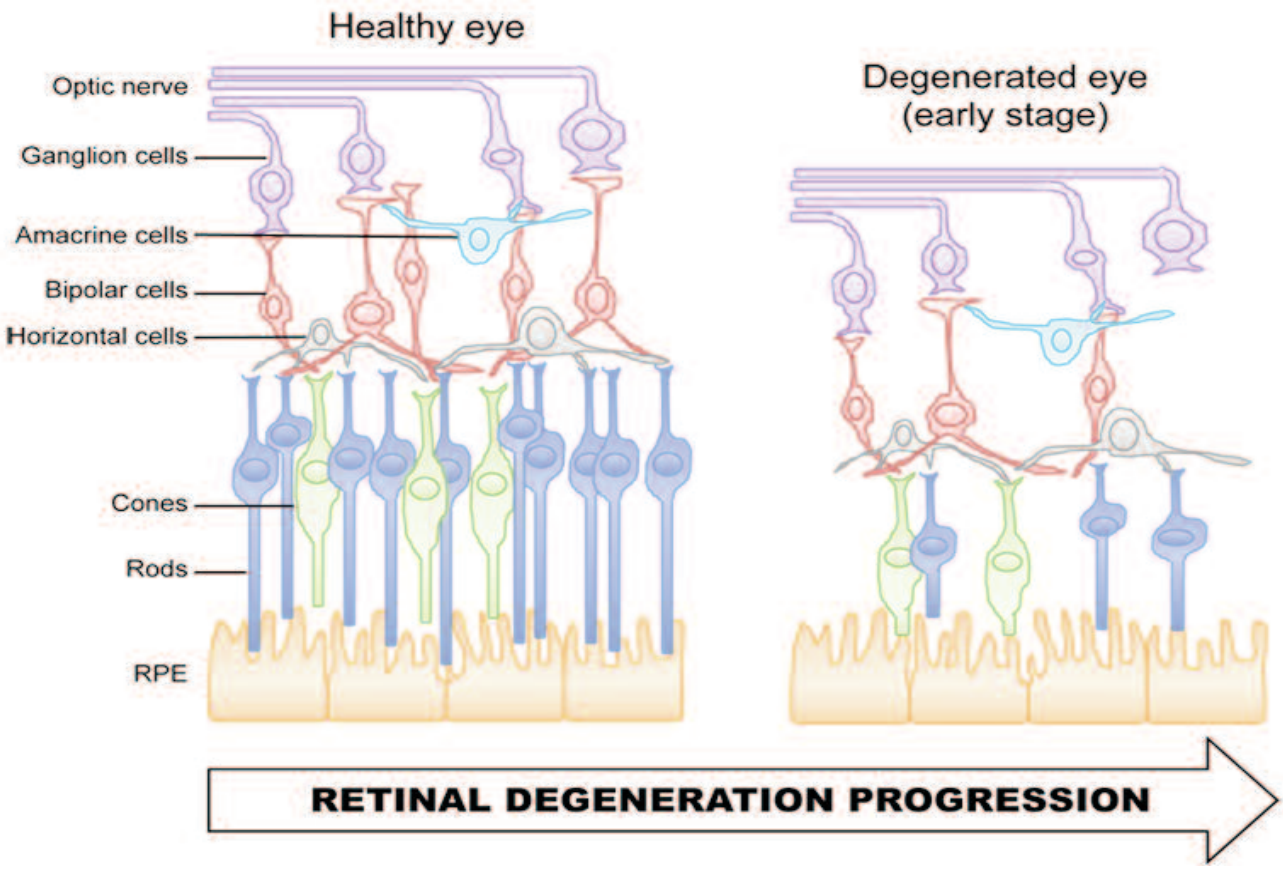

Figure 1. Schematic representation of (A) the normal organization of the mammalian retina and (B) the subsequent cell loss that occurs during retinal degeneration. (A) The retina contains five major classes of neurons. The photoreceptors, rods and cones, transduce light stimuli into electrical signals. Rods allow dim-light vision whereas cones supply central acuity and color perception in high ambient light conditions. At the synaptic terminals of photoreceptors, the electrical signals are transferred onto horizontal and bipolar cells. Bipolar cells relay signals to amacrine and ganglion cells. Ganglion cells integrate these signals and carry the information to the brain through the optic nerve. (B) The retina is the site of various diseases. Although these diseases have different causes, they are often associated with a progressive degeneration of one or several types of retinal neurons, including the photoreceptors. RPE, retinal pigment epithelium.

nal dystrophy, retinal structure in LCA2 patients is relatively well preserved for decades after diagnosis (Cideciyan, 2010), which constitutes a favorable window of opportunity for gene therapy since the most important factor for visual rescue is the presence of photoreceptors.

Initial preclinical proof-of-concepts for LCA2 gene therapy used an adeno-associated virus (AAV) as a vector to deliver the human *RPE65* cDNA (coding DNA) to the RPE cells. AAV is a particularly attractive vector for gene delivery as it is non-pathogenic; it elicits minimal immunogenicity and results in remarkably stable transduction of post-mitotic cells. Additionally, AAV vectors derived from a variety of different serotypes have been shown to mediate efficient and long-term transgene expression in different retinal cell types (Dinculescu *et al.*, 2005; Vandenberghe and Auricchio, 2012); for example, AAV2, 5, 8, and 9 efficiently transduce both RPE and photoreceptor cells after subretinal injection, whereas AAV4 specifically targets the RPE.

Subretinal injection of AAV2-*RPE65* vectors in murine and canine models of LCA2 was able to overcome the defect in retinal activity caused by RPE65 deficiency (Cideciyan, 2010). Subsequent studies with other serotypes and promoters showed stable visual benefits (Acland *et al.*, 2005; Annear *et al.*, 2013; Bainbridge *et al.*, 2015; Bennicelli *et al.*, 2008; Cideciyan *et al.*, 2013; Le Meur *et al.*, 2007; Mowat *et al.*, 2013; Narfstrom *et al.*, 2003; 2005) and demonstrated that photoreceptors can be protected from further degeneration in the vector-exposed area of the retina (Annear *et al.*, 2013; Bainbridge *et al.*, 2015; Mowat *et al.*, 2013). On the basis of these observations, six dose-escalation phase I/II clinical trials of AAV2-*RPE65* were initiated. In all of the studies reported to date, the unilateral subretinal injection of AAV2 was shown to be safe with no evidence of toxicity, serious inflammatory or immunologic responses. In addition, participants in all studies exhibited some improvements in aspects of visual function within weeks after treatment (Bainbridge *et al.*, 2015; 2008; Bennett *et al.*, 2012; 2016; Cideciyan *et al.*, 2008; 2013; Hauswirth *et al.*, 2008; Jacobson *et al.*, 2012; 2015; Maguire *et al.*, 2008; Weleber *et al.*, 2016). These remarkable improvements suggest that some subpopulations of photoreceptors remain capable of responding positively to the treatment despite advanced retinal degeneration. The findings were widely seen as a stunning success in translational gene therapy and have greatly expanded the interest for AAV-mediated applications in patients with retinal degenerations. The results opened the possibility of vector re-administration in the second untreated eye of the patients previously enrolled in the original phase I/II trial (Bennett *et al.*, 2012; 2016) and supported the development of a phase III study, which is now well underway. In addition, RPE65 trials were used as a benchmark for the clinical testing of gene therapies to combat other recessive forms of retinopathies (Carvalho and Vandenberghe, 2015; Petit *et al.*, 2016), such as retinitis pigmentosa due to mutations in *MERTK* (Ghazi et al., 2016), or choroideremia caused by mutations in *REP1* (Edwards et al., 2016; MacLaren et al., 2014).

Retinal gene replacement therapy is now progressing faster than ever with dozens of additional preclinical studies currently moving to translation (Petit *et al.*, 2016). However, the main challenge over the next decades will not just be to translate more animal findings into clinical studies, but also to maximize treatment efficacy in patients. So far, contrary to the remarkable disease rescue obtained in RPE65-deficient dogs, the level of total functional rescue obtained in LCA2 patients after gene therapy was too low to make a detectable difference by full-field electroretinography (ERG), a measure of the function of the entire retina (Cideciyan, 2010). In addition, in two of the RPE65 trials, retinal degeneration continued, alongside a decline that started between 6 months to 3 years after treatment of the initial visual improvements (Bainbridge *et al.*, 2015; Cideciyan *et al.*, 2013; Jacobson *et al.*, 2015). Several factors likely limited the quality of the treatment and thus the overall clinical benefits, including, the number of surviving RPE and photoreceptor cells at the time of treatment, the overall health of the remaining tissue and the limited levels of RPE65 expression. Of note, an AAV4-*RPE65* trial was recently completed with the goal of achieving greater RPE65 expression in RPE cells.

The progressive nature of retinal degenerations may pose challenges for delivering long-term clinical benefits because the death of mutant cells often triggers neighboring healthy cells to die (Cepko and Vandenberghe, 2013). Studies in chimeric mice in which patches of mutant rods were intermingled with wild-type rods showed that both dysfunctional and normal photoreceptors died, even when more than 40% of the retina is composed of normal photoreceptors (Huang *et al.*, 1993; Kedzierski *et al.*, 1998). Comparably in humans with retinitis pigmentosa, the death of rods, which constitute over 95% of the photoreceptors, invariably leads to the secondary degeneration of neighboring healthy cones (Punzo *et al.*, 2012). Correcting the functional defect in a few cells may thus fail to halt the continued degeneration in the long-term. In such cases, an intravitreal approach, that would the-

oretically broadly distribute the vector throughout the retina, may offer a more appropriate mode of delivery. However, it is important to note that the percentage of dying cells required to initiate a bystander effect and how this effect propagates remain to be defined. Studies in zebrafish have elegantly shown that spatial cell density may be a key factor in the ability of photoreceptors to survive in a degenerating environment (Stearns *et al.*, 2007). Interestingly, a recent study performed in a canine model of retinitis pigmentosa showed that the local correction of the genetic defect in ~50% of rods over a surface of 25-35% of the total retina completely stopped the degeneration process in the vector-exposed area for at least 30 months, despite the widespread degeneration in untreated areas (Pichard *et al.*, 2016). Another study in a dog model of X-linked retinopathy showed that degeneration could be halted with a gene replacement approach, even when treatment occurs at late stages of the disease (Beltran *et al.*, 2015). Notably, long-term follow-up of the treated dogs showed that while photoreceptors initially also degenerated in the vector-exposed area of the retina, islands of rescued cells remained as the disease progressed and retinal thickness was stabilized in the transduced area (Beltran *et al.*, 2015). The prospect of the elucidation of mechanisms of retinal degenerations in this respect is fascinating because this knowledge may permit the identification of the best 'targetable' cells and the design of the most appropriate gene therapy intervention for each patient.

Gene-specific therapy for dominant inherited retinal disorders

Compared to recessive diseases, dominant diseases pose additional challenges to their treatment by gene therapy, as in several cases the aberrant protein not only displays an impaired function but also alters the expression or the function of the wild-type protein. Treating these conditions by gene therapy ideally requires the suppression of the dominant allele to diminish the toxicity while preserving the expression of the wild-type allele to ensure proper gene function after treatment. Mutation-specific suppression of the mutant gene has been achieved at the RNA level by AAV-mediated delivery of ribozymes and RNA interference in murine models of several forms of dominantly inherited retinopathies (Farrar *et al.*, 2012). This approach, however, addresses only one pathogenic mutation at the time. Thus, it will likely not be possible to extend it to all patients because most disease genes present an extremely high degree of allelic heterogeneity and the number of patients that carry one specific mutation is very small. Specifically, rhodopsin (RHO)-linked autosomal dominant retinitis pigmentosa, the most common form of dominant retinopathy is caused by over 200 different mutations in the *RHO* gene alone. The time and cost of developing sequence-specific inhibitor for each mutation would be prohibitive, as the efficacy and safety of each new construct will need to be tested rigorously in preclinical and clinical trials. To circumvent this problem, progress has been made on ways to eliminate indiscriminately both mutated and normal RNA, while supplementing at the same time the cell with a normal RNA engineered to resist degradation. However, as a dual therapy, the gene suppression-and-replacement strategy requires optimization of each element separately. The approach must silence endogenous RNA to therapeutically relevant levels, and also, as it is the case for gene replacement therapy, drive sufficiently high levels of the resistant transgene to avoid the development of a more aggressive form of the disease. Transgenic mice expressing dominant *RHO* transgenes have been widely used for the development of mutation-independent approaches of gene therapy for autosomal dominant diseases (Farrar *et al.*, 2012). To date, significant functional and structural benefits have been obtained in these murine models, using either a single dual-component AAV vector (Mao *et al.*, 2012) or two separate AAV vectors (Millington-Ward *et al.*, 2011), strengthening the validity of this approach. However, rhodopsin constitutes over 90% of the outer segment membrane proteins and recapitulating such high level of expression after gene transfer remains a major technical challenge for the future progress of this approach toward clinical application. In this regard, it is of note that gene transfer to the retina is improving quickly with the discovery of more efficient AAV serotypes and the construction of optimized promoters (Vandenberghe and Auricchio, 2012). For example, functional rescue of a murine model of RHO knockout has been recently achieved using a novel variant of AAV and an optimized murine RHO promoter (Palfi *et al.*, 2015). Undoubtedly, new vectors will be used more extensively in the future in an attempt to optimize the treatment of dominant retinopathies and move gene suppression-and-replacement therapies to clinical reality.

Non gene-specific Therapies for Ocular Diseases

Data from the early clinical trials of gene replacement therapy and the increasing number of proof-of-concept studies illustrate the very high potential of gene-specific therapy for the treatment of retinal degenerative diseases. It needs to be noted, however, that as we write, inherited retinopathies involve mutations in over 260 different genes, some of which are too large for the currently used vectors. To broaden the applicability of retinal gene therapy, generic approaches that work

across genetic subtypes are being explored in paralle with therapies aimed at correcting the primary genetic defect.

Gene therapies modulating the secondary mechanisms of retinopathies

An alternative strategy to the correction of the primary genetic defect of the disease is to target the common causes or mechanisms concurring to photoreceptor degeneration in order to prolong the lifespan of the retinal cells and delay the onset of blindness. This approach offers the possibility to treat not only genetic diseases but also a range of more common multifactorial disorders. The idea for such an approach was based on the realization that in many forms of retinal diseases, the patterns of photoreceptor cell death are similar. For example, as mentioned above, as rods malfunction and die, there is always a secondary death of neighboring healthy cones. Interestingly, cone death always starts near the end of the rod death phase independently of the rate of rod death (Punzo *et al.*, 2009), indicating a common pathologic cause and process.

Cones represent only 5% of all photoreceptors in humans, but their role in vision is essential. It is their loss that leads to the most damaging symptoms of retinal degenerations and potentially total blindness. One strategy to delay secondary cone death involves the generic protection of rods, even without rescuing their function, as this should inhibit the onset of secondary cone death. Similarly, gene therapies directed toward prolonging cone survival would enable the treatment of a very large number of individuals. Cones persist in most patients for a prolonged period of time before they die, indicating that slowing their death down further could result in an almost permanent cure.

Several therapies have been tested to delay rod photoreceptor death before loss of cones occurs. These therapies include the delivery of genes encoding anti-apoptotic factors to inhibit the execution of a cell death program in photoreceptors (Chinskey *et al.*, 2014; Leonard *et al.*, 2007; Zadro-Lamoureux *et al.*, 2009), or the delivery of genes encoding neurotrophic factors to raise the threshold of cellular stress required for the initiation of apoptosis, such that rod death is delayed or less frequent. Overall, anti-apoptotic therapies have been shown to be less robust than neuroprotective ones, probably because they target the final step in the lifecycle of photoreceptor cells. In addition, it is now well accepted that different cell death mechanisms can be activated in photoreceptors and that such mechanisms often cooperate during cell death. Thus, photoreceptors that are physiologically ready to die may activate alternative cell death pathways to the one that is inhibited.

AAV-mediated delivery of genes coding for various neurotrophic trophic factors (e.g., erythropoietin derivatives, CNTF, or GDNF) has resulted in effective protection of photoreceptors in rodent models of induced and inherited retinal degenerations (Bok *et al.*, 2002; Colella *et al.*, 2011; Fernandez-Sanchez *et al.*, 2012; Leaver *et al.*, 2006; Liang *et al.*, 2001; Lipinski *et al.*, 2015; MacLaren *et al.*, 2014; Martin *et al.*, 2003; Rex *et al.*, 2004; Wu *et al.*, 2002). However, it is worth noting that their mode of action is not always fully understood, which makes the translation of the approach into the clinic challenging. Such therapies could have possible pleiotropic effects, depending on the dose, delivery mode, and/or the target cells, and thus be associated with undesirable side effects. Another concern is that significant rod degeneration often occurs in many patients before they first visit an ophthalmologist, resulting in missed opportunities to rescue the rods and indirectly prevent the loss of cones.

Several groups are currently trying to identify the mechanisms of cone death with the goal to directly prevent cone death. Recently, the conjunction of retinal biology, genetics, and biochemistry has led to the realization that metabolic deregulation and oxidative stress are major causes for secondary cone death (Punzo *et al.*, 2009; 2012). Rods constitute 95% of the photoreceptors and their massive death may irreversibly compromise the ability of cones to efficiently uptake the glucose that is provided by the RPE. In parallel, as rods die, cones may be subjected to increased levels of oxygen and experience oxidative damage.

Cone death was delayed after transgenic (Usui *et al.*, 2009) or viral-mediated (Xiong *et al.*, 2015) overexpression of antioxidant factors in murine models of retinal degenerations, which supported the idea that oxidative stress contributes to cone death. Moreover, gene transfer of rod-derived cone viability factor (RdCVF), a truncated thioredoxin that mediates resistance against photo-oxidative damage (Cronin *et al.*, 2010; Elachouri *et al.*, 2015; Fridlich *et al.*, 2009; Mei *et al.*, 2016) and induces glucose uptake *in vitro* (Ait-Ali *et al.*, 2015) promoted cone survival in two different models of retinopathy (Byrne *et al.*, 2015). However, oxidative stress itself is unlikely to be the sole cause governing secondary cone death in retinal dystrophy. Recent findings that activation of metabolic genes downstream of mTORC1 in cones dramatically improved cone survival in mice strongly suggest that the overall cause of cone death is nutrient shortage (Venkatesh *et al.*, 2015). This discovery directly inter-

sects with the increased oxidative stress in cones during degeneration, since cones mainly use glucose to fuel the biosynthesis of NADPH, and NADPH regulates antioxidant activity of the cells. Based on these results, boosting cone metabolism emerges as a central theme to prevent the secondary death of cones. Gene therapy methods that enhance glucose uptake or utilization in cone photoreceptors are expected have a great impact on the treatment of various forms of retinal degenerations (Venkatesh *et al.*, 2015; Zieger and Punzo, 2016).

Optogenetics

Once most photoreceptors have degenerated, other strategies need to be employed if some form of light sensitivity is to be restored. Optogenetics is a particular form of gene therapy in which photosensitivity is conferred to the remaining retinal cells by means of transgenic expression of a light-sensitive protein. A variety of different optogenetic tools have been developed (Deisseroth, 2015). In most cases these tools are light sensitive ion channels. Once these genes are expressed in retinal neurons, it becomes possible to activate (depolarization) or silence (hyperpolarization) the cells upon light stimuli. This approach has been shown to restore light perception and basic visual-guided behavior in small and large animal models of retinal degenerations, either by re-activating remaining cones compromised during disease progression (Busskamp *et al.*, 2012) or by turning inner retinal neurons into artificial photoreceptors (Bi *et al.*, 2006; Cronin *et al.*, 2014; Doroudchi *et al.*, 2011; Lagali *et al.*, 2008; Mace *et al.*, 2015; Scalabrino *et al.*, 2015; Thyagarajan *et al.*, 2010).

The big challenges of the optogenetics field are to deliver sufficient light sensitivity and to specifically target certain retinal cell types. Progress is expected thanks to the possibility to act at the molecular level to increase the sensitivity and amplitude of the light currents produced by these light-sensitive channels (Deisseroth, 2015). Additionally, novel vectors and cell type specific promoters should greatly benefit the optogenetics approach (Cronin *et al.*, 2014; Lu *et al.*, 2016; Vandenberghe and Auricchio, 2012).

Conclusion

Gene therapy for the treatment of retinal diseases is an advancing field. Gene therapy clinical trials for several forms of retinopathies have now started and demonstrated that these approaches are both safe and efficient. In parallel, progress in our understanding of disease pathogenesis continues to expand the applicability of retinal gene therapy. The continued successful implementation of ocular gene therapy into the clinic heralds a time when the treatment of many other forms of blinding disorders, which until recently have been considered to be incurable, will be available.

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Disclosure

The authors report no conflicts of interest.

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