Gene Therapy of Inherited Retinopathies: A Long and Successful Road from Viral Vectors to Patients

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Abstract

Inherited retinopathies (IRs) are common and untreatable blinding conditions inherited mostly as monogenic due to mutations in genes expressed in retinal photoreceptors (PRs) and in retinal pigment epithelium (RPE). Over the last two decades, the retina has emerged as one of the most favorable target tissues for gene therapy given its small size and its enclosed and immune-privileged environment. Different types of viral vectors have been developed, especially those based on the adeno-associated virus (AAV), which efficiently deliver therapeutic genes to PRs or RPE upon subretinal injections. Dozens of successful proofs of concept of the efficacy of gene therapy for recessive and dominant IRs have been generated in small and large models that have paved the way to the first clinical trials using AAV in patients with Leber congenital amaurosis, a severe form of childhood blindness. The results from these initial trials suggest that retinal gene therapy with AAV is safe in humans, that vision can be improved in patients that have suffered from severe impairment of visual function, in some cases for decades, and that readministration of AAV to the subretinal space is feasible, effective, and safe. However, none of the trials could match the levels of efficacy of gene therapy observed in a dog model of the disease, suggesting that there is room for improvement. In conclusion, these results bode well for further testing of AAV-mediated retinal gene therapy in patients with other monogenic and complex forms of blindness.

Introduction

Inherited retinopathies are untreatable blinding conditions and ideal targets for gene therapy

Inherited retinopathies (IRs) are common blinding diseases, mostly monogenic, that affect about one in 2,000 people worldwide and severely impair patients' living conditions (Berger *et al.*, 2010). Vision loss in IR patients is due to the dysfunction/degeneration of retinal photoreceptor cells (PRs; rods and cones) and/or the retinal pigment epithelium (RPE) (Fig. 1). So far, IR causative mutations have been identified in more than 150 genes expressed mostly in PRs and to a lesser extent in the RPE (Daiger *et al.*, 2011). IRs are both genetically and phenotypically heterogeneous. The different forms are commonly classified based on the genetic defect (when identified), the inheritance pattern (either autosomal dominant, recessive, or X-linked), the main cell type affected (rods, cones, or RPE), the onset and type of visual dysfunction, the disease progression rate, and the appearance of peculiar ocular fundus abnormalities (Berger *et al.*, 2010). The most frequent and severe forms of IR are retinitis pigmentosa (RP) (Hartong *et al.*, 2006), Leber congenital

amaurosis (LCA) (den Hollander *et al.*, 2008), Stargardt's disease (STGD) (Fishman, 2010), and achromatopsia (Berger *et al.*, 2010). In addition, IR can be part of a syndrome, among which the most frequent is Usher syndrome (USH) that combines RP with hearing impairment (Millan *et al.*, 2011). IRs are incurable, and no definite treatment could be envisaged before the advent of gene therapy. Indeed, IRs are ideal candidates for gene therapy because: (i) they are conditions inherited mostly as monogenic traits (Daiger *et al.*, 2011); (ii) many IR causative genes have been identified (Berger *et al.*, 2010); (iii) the affected cells (PRs and RPE) can be approached by various surgical procedures used in the clinic to treat other ocular diseases (Liang *et al.*, 2000); (iv) rodent and large animal models that mimic the human IRs are available (Chader, 2002; Lin *et al.*, 2002; Baehr and Frederick, 2009); and (v) noninvasive analyses that are currently used in the clinic to diagnose and follow up patients with IRs (Maguire *et al.*, 2008; Testa *et al.*, 2011) have been successfully adapted to animal models (Aleman *et al.*, 2004; Weymouth and Vingrys, 2008; Fischer *et al.*, 2009). This has led, in the last 20 years, to many therapeutic proofs of principle of the efficacy of gene therapy in animal models of IR, and we are currently

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FIG. 1. (A) Schematic representation of the eye structure and intraocular injection routes. (B) Cross section of the adult rodent retina stained by hematoxylin and eosin (magnification, $20\times$; scale bar = $100\ \mu\text{m}$). The retina is the light-sensitive tissue that lines the back of the eye. The retina is organized into three layers of cells: (i) the outer nuclear layer (ONL), composed of rod and cone nuclei; (ii) the inner nuclear layer (INL), composed of interneurons (bipolar, amacrine, and horizontal cells) and Müller glia cells; and (iii) the ganglion cell layer (GCL) containing ganglion and displaced amacrine cells. The retinal pigment epithelium (RPE) overlays the retinal photoreceptors (PRs), and the RPE microvilli surround the PR outer segments (OSs). The PR OS is formed by an elaborate system of stacked membranous discs that contain the photosensitive opsin proteins. The PR inner segment (IS) contains instead the cell cytoplasm and organelles. The proteins that are targeted to the discs are transported from the IS to the OS along the connecting cilium. Light reaches the retina from the vitreous side. PRs and RPE can be approached by subretinal (SR) injections, and the inner retina by intravitreal (IV) injections. ILM, inner limiting membrane.

observing the successful translation of some of them from bench to bedside. In this article, we will review the landmark developments of gene therapy for IRs, the most recent breakthroughs, as well as the current challenges that the field is facing to expand the initial clinical successes to a larger number of IRs.

Review

Efficient viral vectors are key elements for successful retinal gene therapy: advantages and limitations of adenoviral and lentiviral vectors

The success of retinal gene therapy relies on vectors that can efficiently deliver nucleic acids to target cells (mainly PRs and RPE). One of the advantages of the eye as a gene therapy target organ is that it is easily accessible from the exterior. The retina and RPE can be targeted by two routes of injection: intravitreal (IV) (Liang *et al.*, 2000) and subretinal (SR) (Liang *et al.*, 2000) (Fig. 1). Whereas the IV injection releases the therapeutic agent in the vitreous, the SR injection releases it in the subretinal space that forms between the PRs and RPE as a consequence of a reversible, injection-induced retinal detachment (Liang *et al.*, 2000). The IV injection is less invasive than the SR, but the diffusion of the therapeutic agent to the PRs and RPE is limited by several physical barriers, such as the vitreous, the inner limiting membrane (ILM), and the inner retina (Fig. 1). Thus, the SR is the preferred route used to date to target efficiently RPE and PRs. Many studies over the past years have provided evidence of the higher efficiency of viral versus non-viral vehicles (Andrieu-Soler *et al.*, 2006) for retinal gene delivery; however, recent reports on nanoparticle-mediated retinal gene therapy showed an improvement compared with previous studies with nonviral agents (Cai *et al.*, 2008). The vectors most studied and used for retinal gene transfer are those derived from adenoviruses (Kumar-Singh, 2008),

lentiviruses (Balaggan and Ali, 2012), and adeno-associated viruses (Vandenberghe and Auricchio, 2012) which are capable of infecting and transducing nondividing cells such as PRs and RPE.

Following SR delivery, adenoviral vectors (Ads) transduce the RPE efficiently, but PRs poorly (Bennett *et al.*, 1994; Li *et al.*, 1994). These vectors determine fast but short-lived transgene expression (Bennett *et al.*, 1994; Reichel *et al.*, 1998) due to the cytotoxic T lymphocyte-mediated removal of the transduced cells that express the Ad proteins encoded by the viral genome (Reichel *et al.*, 1998). Despite this limitation, several studies reported that Ad-mediated gene transfer provided short-term improvements in IR animal models (Bennett *et al.*, 1996, 1998; Kumar-Singh and Farber, 1998; Akimoto *et al.*, 1999; Vollrath *et al.*, 2001; Chen *et al.*, 2006). Notably, the helper-dependent Ad vectors (Hd-Ads) that do not encode for viral proteins (Kochanek, 1999) evade the immune response and target the RPE stably (Kreppel *et al.*, 2002). The thorough characterization of Hd-Ads in the retina of large animal models and the identification of new serotypes and/or the modification of known ones (Von Seggern *et al.*, 2003; Cashman *et al.*, 2007; Sweigard *et al.*, 2010) to improve PR transduction will hopefully lead to the re-evaluation of Ad for the gene therapy of IRs. Indeed, due to their high DNA cargo capacity (up to 36 kb), the development of Hd-Ads that efficiently and stably transduce PRs and the RPE would be particularly useful to treat those IR forms caused by mutations in genes that would not fit in adeno-associated-viral vectors (AAVs) or lentiviral vectors (LVs), as well as to test large regulatory regions (*i.e.*, endogenous enhancers and promoters) for a more physiological regulation of transgene expression.

LVs are integrating RNA retroviruses with a cargo capacity of about 8–10 kb (Balaggan and Ali, 2012). LVs are deleted of all viral genes and, thus, do not activate the immune system (Bennett, 2003; Pauwels *et al.*, 2009), but the

possibility of insertional mutagenesis, vector replication, and mobilization in human cells (Pauwels *et al.*, 2009) raises significant concerns. Although no malignant transformation has been reported in the murine eye using high-titer integrating LVs (Balaggan *et al.*, 2012), the issue of insertional mutagenesis is being addressed through the generation of nonintegrating LVs (Yanez-Munoz *et al.*, 2006), whereas vector replication and mobilization are being tackled through the use of nonprimate LVs (Pauwels *et al.*, 2009). Most studies showed that following SR delivery, LVs transduce the RPE efficiently in rodents (Auricchio *et al.*, 2001; Bainbridge *et al.*, 2001; Duisit *et al.*, 2002; Kachi *et al.*, 2009), but PRs variably and inadequately (Miyoshi *et al.*, 1997; Bemelmans *et al.*, 2005; Balaggan *et al.*, 2006). Notably, PR transduction by LVs is inversely correlated to PR differentiation (Miyoshi *et al.*, 1997; Bainbridge *et al.*, 2001; Pang *et al.*, 2006; Nicoud *et al.*, 2007). Consistent with these observations, the SR delivery of LVs to the adult retina was effective in rodent models of IR caused by mutations in genes expressed in the RPE (Vollrath *et al.*, 2001; Bemelmans *et al.*, 2006), whereas perinatal (Takahashi *et al.*, 1999; Hashimoto *et al.*, 2007; Kong *et al.*, 2008) or *in utero* (Williams *et al.*, 2006) deliveries were required to treat rodent models that bear mutations in genes expressed in PRs. These preclinical studies served as the basis to test the nonprimate equine infectious anemia virus in ongoing clinical trials to treat exudative age-related macular degeneration (NCT01301443), STGD (NCT01367444), and USH type IB (USH1B) (NCT01505062). The results from these trials are not available yet. However, the transduction of mature PRs remains the fundamental limitation of LVs and Ads as vectors for the therapy of IRs. It is possible that the large size of Ad and LV particles (60–120 nm) prevents their efficient diffusion through the dense adult interphotoreceptor matrix (IPM) (Miezewska, 1996). It is interesting that PR transduction by LVs has been reported to either positively (Balaggan *et al.*, 2006; Pang *et al.*, 2006) or negatively (Calame *et al.*, 2011) correlate with disease-associated or induced PR damage and to be improved by loosening the IPM (Gruter *et al.*, 2005). Therefore, it is possible that the degree of retinal degeneration impacts on PR transduction mediated by LVs or Ads in IR patients.

AAV as safe and most efficient vector for therapy of IRs

AAVs are currently the most favored vectors for gene therapy of IRs given their: (i) small (25 nm) size and ability to efficiently target various retinal layers; (ii) low immunogenicity, allowing the possibility to readminister the vectors to the subretinal space; (iii) excellent safety profile; and (iv) availability of over 100 different forms, the AAV serotypes. This last feature is one of the major strengths of the AAV vector platform; the versatility of the AAV production system allows the easy exchange of capsids among various AAV variants, thus creating hybrid vectors that contain a genome with the same AAV inverted terminal repeats (ITRs) (*i.e.*, those from AAV2) and the capsid from a different variant (Auricchio, 2003). AAVs obtained through this transcapsidation system are named AAV2/n, where the first number refers to the ITRs and the second to the capsid (Surace and Auricchio, 2008). Over the past years, AAV

vectors derived from naturally-occurring serotypes (Auricchio *et al.*, 2001; Gao *et al.*, 2002, 2005) or with rationally-modified (Zhong *et al.*, 2008b) or *in vitro* evolved capsids (Perabo *et al.*, 2008) have been generated, significantly expanding the potential of AAVs for the treatment of IRs. The most relevant findings have been: (i) the identification of AAV2/8 as the most efficient naturally-occurring serotype for PR transduction in mice (Allocca *et al.*, 2007; Auricchio, 2011), pigs (Mussolino *et al.*, 2011a), and nonhuman primates (NHPs) (Vandenberghe *et al.*, 2011); (ii) the availability of AAV2/7, 2/9, and 2/5 as alternatives to AAV2/8 for PR transduction (Auricchio *et al.*, 2001; Lotery *et al.*, 2003; Allocca *et al.*, 2007; Mussolino *et al.*, 2011a); and (iii) the generation of tyrosine-mutated AAV capsids (Zhong *et al.*, 2008b) that avoid targeting of AAVs to the proteasome, thus increasing the vector load that reaches the nucleus, resulting in increased transgene expression (Zhong *et al.*, 2008a). In particular: (i) the single and triple mutants from AAV2/2 (Y444F and Y444,500,730F) and 2/8-Y733F determine stronger and more widespread transduction than their wild type (wt) counterparts when delivered SR in mice (Peters-Silva *et al.*, 2009); and (ii) quadruple and pentuple tyrosine-mutant AAV2/2 vectors are capable of transducing murine PRs and RPE when injected IV (Peters-Silva *et al.*, 2011). PR and RPE transduction from the vitreous is particularly attractive for IR treatment, because it would allow a more widespread and homogeneous transgene expression and reduces the risk of surgical morbidity. However, the presence of a thick (especially in large animals) ILM limits the diffusion of vectors to the outer retina after IV administration. Indeed, the IV delivery of wt AAV2/2 in mice and NHPs allows transduction of the inner retina (Auricchio *et al.*, 2001; Yin *et al.*, 2011) and only a few scattered foveal cones (Wadsworth, 2012). However, if the retinal architecture is altered by a degenerative process, the diffusion of AAV viral particles to the outer retina from the vitreous side is enhanced (Park *et al.*, 2009; Kolstad *et al.*, 2010).

Two major limitations of AAVs in the retina are the slow onset of transgene expression due to the time-consuming conversion of the single-stranded AAV genome into a double-strand, and the vector limited cargo capacity. The first limitation can be circumvented by using self-complementary (sc) AAV vectors that package double-stranded AAV genomes (McCarty, 2008). scAAV2/2, 2/8, and 2/5 drive faster onset and stronger levels of transgene expression than their single-stranded counterparts in the retina of mice (Yokoi *et al.*, 2007; Natkunarajah *et al.*, 2008; Kong *et al.*, 2010) and dogs (Petersen-Jones *et al.*, 2009), theoretically allowing the delivery of lower vector doses to obtain similar levels of transgene expression. However, in some cases, it is not clear whether this is due to the inherent properties of scAAVs or to a systematic erroneous titration (Fagone *et al.*, 2012) that results in the administration of higher doses of scAAVs than expected. The major limitation of AAVs remains their packaging capacity, which is considered to be restricted to the size of the parental genome (4.7 kb) and thus hampers the treatment of certain forms of IR caused by mutations in genes whose cDNA exceeds 5 kb (*i.e.*, *Abca4*, *Myo7a*, or *CEP290*, mutated in STGD, USH1B, and LCA10, to name a few). Strategies to overcome AAV size restriction are based either on the generation of dual AAV vectors that reconstitute the full-length genome in transduced cells (referred to as

trans-splicing, overlapping, or hybrid dual-vector strategies) (Duan *et al.*, 2001; Ghosh *et al.*, 2008) or on packaging of oversized genomes (Grieger and Samulski, 2005; Allocca *et al.*, 2008; Hirsch *et al.*, 2010). Notably, oversized AAVs allow the expression of full-length proteins *in vitro* and in the murine retina (Allocca *et al.*, 2008), but contain highly heterogeneous genomes (Dong *et al.*, 2010; Hirsch *et al.*, 2010; Lai *et al.*, 2010; Wu *et al.*, 2010), which limit their use in clinical settings. On the other hand, the efficiency of the dual AAV systems for gene delivery to the retina still remains to be thoroughly exploited, because few studies have been performed to date (Reich *et al.*, 2003).

To silence, to replace, or to supply?

Around 40% of IR patients that present a recognizable pattern of inheritance are affected by dominant forms of the disease (Berger *et al.*, 2010). In several cases, the mutation underlying the disease exerts a toxic, gain-of-function effect. More than 150 different mutations associated with dominant RP have been described in the *rhodopsin* (*RHO*) gene alone. Dominant forms of the disease pose two main challenges: (i) gain-of-function mutations require silencing rather than replacement; and (ii) mutation-independent silencing that may be desirable in the presence of high allelic heterogeneity requires efficient replacement, as both the mutant and wt alleles will be silenced. Allele-dependent approaches have used AAV-mediated delivery of either mutation-specific ribozymes (Lewin *et al.*, 1998; LaVail *et al.*, 2000) or short-hairpin (sh) RNAs (Tessitore *et al.*, 2006) directed to the common P23H-*RHO* mutation. This provided partial (Lewin *et al.*, 1998; LaVail *et al.*, 2000) to no (Tessitore *et al.*, 2006) amelioration of PR degeneration, presumably due to the high levels of *RHO* expression in rods. Notably, mutation-independent approaches whose design is not restricted by the position of the mutation provided more potent silencing whether based on ribozymes (Gorbatyuk *et al.*, 2005, 2007b) or on shRNAs (Gorbatyuk *et al.*, 2007a; Chadderton *et al.*, 2009). Recently, Mussolino *et al.* (2011b) showed that it is possible to effectively repress the expression of the *RHO* gene in a mutation-independent way by targeting engineered zinc-finger-based DNA binding proteins to the *RHO* promoter. This is an interesting approach, as promoter repression targets the locus of a gene that is present in two copies per diploid cell, as opposed to the transcript, which can be very abundant and challenging to silence, as in the case of *RHO*. To date, the most advanced proof of concept of the "suppression-replacement" strategy for dominant *RHO* mutations has been provided by O'Reilly *et al.* (2008), Mao *et al.* (2012), and Millington-Ward *et al.* (2011), who used a single AAV2/8 vector that codelivers an shRNA directed to *RHO* and a silencing-resistant *RHO* transgene. This strategy resulted in significant delay of PR cell loss in treated *RHO*-P23H mice (O'Reilly *et al.*, 2007; Mao *et al.*, 2012) and in increased rod function (Mao *et al.*, 2012). The use of two separate AAVs, one that delivers the shRNA and the other the *RHO* transgene, allows *RHO* suppression and replacement to be modulated and optimized by varying the two vector doses (Millington-Ward *et al.*, 2011). The results achieved so far using the suppression-replacement strategy in animal models with *RHO* dominant mutations are encouraging. However, as both high and low levels of *RHO*

have been shown to be deleterious for rods (Gorbatyuk *et al.*, 2005; Mao *et al.*, 2011), further studies are required to optimize this complex approach in order to consistently achieve therapeutic efficacy and avoid toxicity. For those dominant IR forms due to mutations that exert a dominant-negative effect, this can be reduced by gene replacement, *i.e.*, by overexpressing the wt protein that competes with the mutant. Indeed, AAV-mediated delivery of wt *RHO* has been recently reported to ameliorate the phenotype of heterozygous *RHO*-P23H mice in a *Rho*^{+/+} background (Mao *et al.*, 2011), raising the possibility that P23H exerts a dominant-negative rather than gain-of-function effect.

Most forms of IR are determined by loss-of-function mutations (Daiger *et al.*, 2011), which can be treated by gene replacement, that is achieved by the delivery of the correct cDNA copy of the defective gene in the affected cells (mainly PRs and RPE) (Colella *et al.*, 2009). Historically, AAV-mediated gene replacement has been reported to be more effective in forms of IR due to mutations in genes expressed in the RPE (Smith *et al.*, 2003; Dejneka *et al.*, 2004; Surace *et al.*, 2005; Gargiulo *et al.*, 2009) than in PRs (Bennett *et al.*, 1996; Schlichtenbrede *et al.*, 2003a; Allocca *et al.*, 2011). This can be attributed to the features of the RPE (*i.e.*, transducibility), the need for fine-regulated transgene expression in PRs, or the different rates of retinal degeneration, which is usually faster in PR- than RPE-specific diseases (Smith *et al.*, 2009). To date, the most successful preclinical gene therapy study for a recessive RPE defect is represented by the AAV-mediated delivery of the 65-kDa RPE-specific isomerase (RPE65) in a dog model of LCA2 (Acland *et al.*, 2001; Narfstrom *et al.*, 2003; Le Meur *et al.*, 2007; Annear *et al.*, 2011), which has led to three independent human clinical trials in LCA2 patients, as discussed later. Gene replacement has been successfully evaluated in additional rodent models of IR caused by mutations in genes expressed in the RPE, such as *MER tyrosine kinase* (*Mertk*) (Vollrath *et al.*, 2001; Smith *et al.*, 2003; Tschernutter *et al.*, 2005) and *lecithin-retinol acyltransferase* (*LRAT*) (Batten *et al.*, 2005). Gene transfer to PRs has been enormously advanced in recent years by the development of more efficient AAV vectors; before then, attempts had been made using LV, Ad, or AAV vectors with poor PR transduction ability. Indeed, the first evidence of the benefits as well as the limitations of gene replacement was provided for two forms of RP caused by mutations in genes expressed in PRs: the *beta cGMP phosphodiesterase 6* (*PDE6-β*), which is required for rod phototransduction (Bennett *et al.*, 1996), and the *peripherin 2* (*Prph2*), which is essential for proper formation and activity of cones and rods (Ali *et al.*, 2000). Very limited improvement of rod function was achieved in the murine models of *PDE6-β* deficiency following the delivery of the *PDE6-β* by Ad (Bennett *et al.*, 1996), AAV2/2 (Jomary *et al.*, 1997), and LV (Takahashi *et al.*, 1999) vectors that inefficiently transduce PRs, whereas transient improvements (due to onward rod degeneration) were observed by using either AAV2/5 (Pang *et al.*, 2006) or AAV2/8 (Allocca *et al.*, 2011). Recently, the delivery of *PDE6-β* by AAV2/8-Y733F in *rd10* mice (which carry homozygous *PDE6-β* missense mutations) restored retinal function and preserved rods up to 6 months (Pang *et al.*, 2011), indicating that the faster onset and more widespread transgene expression provided by the tyrosine-mutant serotype 8 can significantly slow PR loss in this severe model of PR degeneration. The recent report of

successful AAV-mediated gene transfer in *rcd1* dogs (Monnier *et al.*, 2012) with *PDE6-β* deficiency suggests that the time is ripe for the clinical translation of gene therapy for this common form of recessive RP (Hartong *et al.*, 2006). Other successful proofs of concept of AAV-mediated gene replacement in models of inherited PR disease include the rescue of the *aryl hydrocarbon receptor interacting protein like-1* (*Aipl1*)-deficient mouse (Tan *et al.*, 2009; Sun *et al.*, 2010; Ku *et al.*, 2011) and the *retinitis pigmentosa GTPase regulator interacting protein* (*RPGRIP*) $-/-$ mouse (Pawlyk *et al.*, 2005). These experiments show that AAV2/8 and its tyrosine-mutant version are the most effective gene transfer vectors for rod-cone dystrophies. Both AAV2/5 and 2/8 have been successfully applied to the retina of animal models of cone-specific or cone-rod dystrophies (Michaelides *et al.*, 2004; Thiadens *et al.*, 2010; Thomas *et al.*, 2011). Mice (Alexander *et al.*, 2007; Michalakakis *et al.*, 2010; Carvalho *et al.*, 2011; Pang *et al.*, 2012), dogs (Komaromy *et al.*, 2010), and squirrel monkeys (Mancuso *et al.*, 2009) with achromatopsia or color blindness have been successfully treated long-term after single SR administrations of AAVs. Similarly, in a mouse model of the common LCA1 due to mutations in the *guanylate cyclase-1* gene (the *Gucy2e* $^{-/-}$ mouse), a single SR administration of AAV2/8 resulted in long-term cone functional rescue that was more pronounced than that observed with AAV2/5 (Boye *et al.*, 2010, 2011; Mihelec *et al.*, 2011). Overall, these data suggest that gene therapy of IRs due to mutations in genes that are expressed in rods and cones is feasible with AAV2/5 and AAV2/8, and soon we should see the clinical translation of these proofs of concept.

As PR apoptosis is the final outcome and hallmark of IRs, independent of the specific causative gene (Wright *et al.*, 2010), therapies based on “supply” of growth, neurotrophic, anti-apoptotic, anti-oxidative, and anti-inflammatory molecules have been widely exploited to sustain PR survival (Colella and Auricchio, 2010). The list of such molecules delivered by intraocular gene transfer with AAV vectors, which allow safe and sustained expression, include the fibroblast and lens-epithelium-derived growth factors (FGF and LEDGF, respectively), the ciliary and brain-derived neurotrophic factors (CNTF and BDNF, respectively), the glial-cell-derived neurotrophic factor (GDNF), the pigment epithelium-derived factor (PEDF), erythropoietin (EPO), the X-linked inhibitor of apoptosis (XIAP), and the heme oxygenase 1 (HO-1) (Colella and Auricchio, 2010). The data collected so far by using protective factors showed that it is possible to delay PR loss in a mutation-independent way; however, it is still difficult to predict which IR form would be responsive to a specific factor and to evaluate the risks and benefits related to the nonphysiological expression of factors with growth and anti-apoptotic properties (Colella and Auricchio, 2010). The safety concerns are even more critical in view of the fact that gene supply does not directly target the causative gene and does not represent a definitive cure for IRs. A paradigmatic example of gene supply is CNTF, which has been tested in a phase I safety trial (NCT00063765) by intraocular release in IR patients using encapsulated-cell implants (Sieving *et al.*, 2006). Despite minor improvements in visual acuity in the absence of severe adverse effects, significant reductions in electroretinogram (ERG) were reported (Sieving *et al.*, 2006), confirming what was previously observed in preclinical studies (Bok *et al.*, 2002;

Schlichtenbrede *et al.*, 2003b; Wen *et al.*, 2006). The detrimental effects observed following the intraocular delivery of high CNTF doses highlight the importance of regulating the expression of molecules with growth/neurotrophic/anti-apoptotic activities, which may have tight therapeutic ranges. Intraocular delivery of EPO offers an additional example of the risks and benefits associated with the use of neurotrophic molecules: EPO is a potent neuroprotective molecule (Brines and Cerami, 2005; Grimm *et al.*, 2005), but it is endowed with marked erythropoietic activity, an undesirable feature when treating IRs. The recent discovery of EPO mutants that lack erythropoietic activity while retaining neuronal protective effects (Leist *et al.*, 2004) has allowed the successful testing of AAV-mediated intraocular delivery of these derivatives in IR animal models (Colella *et al.*, 2011; Sullivan *et al.*, 2011). Another strategy that is independent of the mutation underlying the disease relies on gene delivery of light-activated ion channels or pumps to retinal neurons (either PRs, bipolar, or ganglion cells) to stimulate retinal activity (Busskamp *et al.*, 2012). As this optogenetic strategy has the potential to render light-sensitive cells that are normally not, it can be applied to end-stage retinal diseases in which PRs are lost at the time of intervention. So far, AAV-mediated delivery of microbial opsins (channelrhodopsin-2 and halorhodopsin) or of the light-gated ionotropic glutamate receptor (LiGluR) restored light sensitivity in various murine models of IRs and human postmortem retinas (Busskamp *et al.*, 2012).

Gene therapy clinical trials for LCA2

The first IR for which retinal gene therapy has been developed up to a phase I/II clinical trial is LCA2 due to mutations in *RPE65*. This is because of the excellent and consistent preclinical results obtained in small and large animal models, as well as of the LCA2 phenotype, which is ideal to test the efficacy of gene therapy: LCA2 patients have severe visual dysfunction (negligible ERGs, reduced light sensitivity, and progressive visual acuity decline), slow PR degeneration, and an adequate RPE preservation (Cideciyan, 2010). Therefore, restored expression of *RPE65* in RPE cells is predicted to result in production of 11-*cis*-retinal and in reactivation of PR function. To date, 30 LCA2 patients have been treated in three independent phase I/II clinical trials (NCT00643747, NCT00516477, NCT00481546) by SR delivery of AAV2/2 encoding for the human *RPE65* gene (Bainbridge *et al.*, 2008; Cideciyan *et al.*, 2008, 2009a,b; Hauswirth *et al.*, 2008; Maguire *et al.*, 2008, 2009; Simonelli *et al.*, 2010; Ashtari *et al.*, 2011; Bennett *et al.*, 2012; Jacobson *et al.*, 2012) (Table 1). The preliminary results from a fourth trial (NCT004815469) that is being conducted using the same AAV2-RPE65 vector as NCT00481546 have been recently published; however, we will focus on the trials with a longer follow-up. Notably, all trials showed significant improvements of both retinal and visual function (Table 1), despite the fact that: (i) the vectors used contained slightly different regulatory sequences and were independently produced, purified, and characterized; (ii) the LCA2 patients were independently selected, characterized, and followed-up at each site; and (iii) the surgical delivery was performed independently at each site. The data collected so far showed: (i) improvement of retinal light sensitivity in most treated eyes; (ii) increase of visual acuity

TABLE 1. CLINICAL TRIALS OF GENE THERAPY FOR LCA

<i>Clinical Trials.gov identifier</i>	<i>Reference</i>	<i>No. of patients</i>	<i>Promoter</i>	<i>Follow-up</i>	<i>Major results reported</i>
NCT006543747	Bainbridge <i>et al.</i> , 2008.	3 (aged 17–23 years)	Human RPE65	4 months to 1 year	1. No serious adverse events; 2. no surgery-related retinal damages; 3. no significant changes in visual acuity, peripheral visual fields, and electroretinograms; 4. significant improvement (1 patient out of 3, 1/3) of retinal sensitivity by microperimetry and dark-adapted perimetry which are accompanied by improved mobility at low luminance (4 lux).
NCT00516477	Maguire <i>et al.</i> , 2008, 2009; Simonelli <i>et al.</i> , 2010; Ashtari <i>et al.</i> , 2011.	12 (aged 8–44 years)	CBA	4 months to 2 years	1. No serious adverse events; 2. surgery-related macular hole (1/12); 3. significant improvements in the treated eyes compared with baseline measurements including: (i) nystagmus (9/9); (ii) visual fields by Goldmann perimetry (12/12); (iii) retinal sensitivity to white light by FST (5/7) and global retinal sensitivity by PLR (11/11); (iv) VA (7/12); (v) mobility (3/12) at dim-low luminances (10–4 lux) and dim luminances (50–60 lux) (1/12); 4. no retinal responses by full-field scotopic or photopic electroretinograms (12/12); 5. development of electrical responses from the injected area of the retina (macula) by mfERG (2/2); 6. significant visual cortex activation by fMRI following stimulation of the treated eye; 7. age-dependent therapeutic effects.
NCT00481546	Hauswirth <i>et al.</i> , 2008; Cideciyan <i>et al.</i> , 2008, 2009a,b; Jacobson <i>et al.</i> , 2012.	15 (aged 11–30 years)	CBA	1 month to 3 years	1. No serious adverse events; 2. surgery-related foveal damages (3/15); 3. significant improvement of retinal function in treated eyes with respect to baseline including: (i) rod (14/15) and extrafoveal cone (15/15) sensitivity by FST; (ii) global retinal sensitivity by PLR (13/15) and dark-adapted perimetry (12/12); (iii) VA in extrafoveally fixating eyes (2/3); (iv) mobility (3/6) at low luminances (0.2–4 lux); 4. no age-dependent therapeutic effects.
NCT01208389 (Follow-on study to NCT00516477)	Bennett <i>et al.</i> , 2012.	3 (aged 27–46 years)	CBA	6 months (2 nd eye); 3–4 years (1 st eye)	1. No serious adverse events; 2. no significant humoral or cell-mediated immune responses; 3. improvement of retinal function in the second treated eye with respect to the baseline including: (i) rod (2/3) and cone sensitivity (1/3) by FST; (ii) global retina sensitivity by PLR (3/3); (iii) VA (1/3); (iv) mobility (2/3) at dim-low luminances (10–5 lux); 4. significant visual cortex activation assessed by fMRI following stimulation of either the second- or the first-treated eye (3/3).

CBA, CMV enhancer/chicken beta actin promoter; fMRI, functional magnetic resonance imaging; FST, full-field stimulus testing; mfERG, multifocal electroretinogram; PLR, pupillary light reflex; RPE65, 65-kDa RPE-specific isomerase; VA, visual acuity.

in 30% of treated patients; (iii) reduction of nystagmus in 30% of patients; (iv) increase of visual fields; (v) reactivation of the visual cortex; and (vi) significant mobility improvement in navigation tasks (Table 1). A longer follow-up will be required to assess whether PR cell loss is halted and/or slowed in the AAV2/2-RPE65-treated eyes. Importantly,

Bennett *et al.* (2012) have successfully readministered AAV2/2-RPE65 to the contralateral eye of three LCA2 patients previously treated with the same vector and have observed that this is safe and effective (Table 1), confirming the immune-privileged status of the subretinal space (Anand *et al.*, 2002).

However, unlike what was observed in the preclinical studies (Acland *et al.*, 2001; Narfstrom *et al.*, 2003; Le Meur *et al.*, 2007; Annear *et al.*, 2011), full-field Ganzfeld ERGs remained negligible in all treated LCA2 eyes. In two cases, the reduced nystagmus allowed the measurement of electrical responses from the treated area by the sensitive multifocal ERG technique (Maguire *et al.*, 2009) (Table 1). The lack of full-field ERG responses in the trials suggests that either the levels of RPE65 transduction achieved are insufficient or that most of the surviving LCA2 PRs are irreversibly compromised due to the 11-*cis*-retinal deficiency. To better understand the effect of 11-*cis*-retinal deprivation on PR physiology, it would be worth testing the effect of substitute chromophores in the LCA2 retina. In fact, a clinical trial (NCT01014052) that evaluates the efficacy of systemic administration of substitute chromophores to LCA2 patients has been recently initiated, and the preliminary efficacy results are encouraging (Cideciyan *et al.*, 2012). The substitute chromophores could thus be exploited to counterbalance PR demise before RPE65 gene delivery (Batten *et al.*, 2005). On the other hand, AAV serotypes, other than AAV2, which efficiently transduce the RPE [*i.e.*, AAV2/4 (Le Meur *et al.*, 2007) and AAV2/1 (Auricchio *et al.*, 2001; Dejneka *et al.*, 2004; Jacobson *et al.*, 2005)], used in combination with improved RPE65 expression cassettes could provide higher levels of RPE65 transgene expression, thus resulting in ERG improvement.

The LCA2 trials also raised some concerns on the invasiveness of the SR vector delivery to a diseased retina, in particular regarding the foveal region (Jacobson *et al.*, 2005; Maguire *et al.*, 2008). Delivery of the vector to extrafoveal PR-rich regions (Jacobson *et al.*, 2005, 2008) should still achieve significant visual improvement, reducing the risks of damaging the remaining foveal cones in the LCA2 retina.

Conclusions and Future Prospects

The excellent safety and efficacy results from the LCA2 trials strongly argue for testing AAV-mediated gene therapy in other rare and common blinding conditions. The best candidates are retinal diseases with severe functional defects but slow PR degeneration, such as achromatopsia or LCA1. However, several IRs have a mild phenotype with a slowly-progressing retinal degeneration. The design of clinical trials for these conditions is much more challenging, as long-term observations and a detailed knowledge of the natural history of each specific form will be required to establish efficacy.

Another challenge to be overcome in the next years will be gene therapy of those IRs caused by mutation in large genes expressed in PRs, such as *Abca4* mutated in STGD. As these genes are not efficiently accommodated in single AAVs, to date the most efficient viral vector for PR transduction, the identification of Ads or LVs able to efficiently target PRs upon SR administration or improvements in dual or oversized AAV technology may satisfy this yet unmet need.

An additional major hurdle in the development of gene therapy of IRs is the extremely high genetic heterogeneity of this group of diseases, which renders particularly challenging the identification of the mutations underlying the disease in each given patient. Considering that more than 50 genes have been associated with RP (Daiger *et al.*, 2011) and more than 15 are involved in LCA (Daiger *et al.*, 2011), a classic Sanger sequencing-based method would be unfeasible to

screen the DNA sample of an RP or LCA patient for mutations in each of these genes. The recent development of high-throughput sequence technologies referred to as next-generation sequencing (NGS) (Metzker, 2010) will most likely allow the sequencing of the whole exome or genome of a patient with IR in a very limited time and at very low costs.

In vivo, noninvasive retinal imaging is also dramatically improving thanks to the development of optical coherence tomography (van Velthoven *et al.*, 2007), which allows the precise evaluation of the structure and thickness of the various human retinal layers over time. The combination of information coming from NGS and optical coherence tomography will allow to: (i) overcome the poor genotype-phenotype correlation of IR patients; (ii) select the most appropriate window of time for therapeutic intervention; and (iii) deliver the therapeutic vector to the most preserved and responsive retinal areas.

In conclusion, the time is ripe for the clinical development of gene therapy for IRs. From now on, the pace will be dictated mainly by the availability of sufficient funding supporting the translation of many proofs of concept from bench to bedside. U.S. and E.U. funding agencies are increasing the budget allocated to support the development of new therapies for rare diseases, such as IRs. Biotech and pharmaceutical companies are viewing rare diseases as important targets to test the safety and efficacy of novel therapeutic platforms, like gene therapy, before moving to more common conditions. The future looks bright for gene therapy of blindness.

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The authors have nothing to disclose.

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