Gene Therapy for Leber's Congenital Amaurosis Due to *RPE65* Mutations

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2.1 Description of the Disease

Leber's congenital amaurosis (LCA) refers to a group of hereditary, early-onset retinal degenerative conditions characterized by severe impairment in retinal and visual function. Diagnosis is usually made during the first few months of life in infants who present with severely impaired vision, abnormal eye movements (nystagmus), and abnormal electroretinograms (ERG) reflecting decreased retinal function. The (poor) vision that is present at birth progressively deteriorates through loss of photoreceptors, typically leading to total blindness by the third or fourth decade of life (Aleman et al. 2004; Lorenz et al. 2000; Simonelli et al. 2007; Perrault et al. 1999).

LCA is usually inherited as an autosomal recessive trait, and mutations in at least 18 different genes have been reported so far (RetNet 2014). At present, there are no approved treatments available for LCA.

This development program focuses on one form of LCA, *LCA2*, caused by mutations in the gene encoding the human retinal pigment epithelium 65 kDa protein, *hRPE65* (Morimura et al. 1998; Thompson et al. 2005). The *RPE65* gene encodes an enzyme (retinal pigment epithelium 65 kDa protein (RPE65)), produced by the retinal pigment epithelium (RPE), retinal isomerohydrolase. This enzyme is necessary for production of a vitamin A derivative, 11-cis retinal, which in turn is necessary for vision (Redmond et al. 1998). Without 11-cis retinal, rhodopsin cannot be formed, and light stimuli exposing the retina cannot be transformed to electrical signals (Redmond et al. 1998; Redmond and Hamel 2000). The biochemical blockade of the visual cycle resulting from *RPE65* deficiency causes a profound impairment in visual function and visual perception. Further, there is a slow degeneration of retinal photoreceptors which may result, in part, from toxicity due to buildup of the 11-cis retinal (retinoid ester) precursors in the RPE cells.

At present, there are no approved treatments available for LCA. The avenue that is being explored is gene augmentation therapy, whereby the wild-type version of the human *RPE65* cDNA is delivered to retinal pigment epithelium (RPE) cells, allowing these cells to then produce the RPE65 protein. The *hRPE65* cDNA is delivered through a one-time exposure to recombinant adeno-associated virus (AAV). LCA2 is an excellent candidate for a gene augmentation therapy approach: (i) molecular testing is available to identify individuals with mutation(s) in the *RPE65* gene; (ii) the route of administration is based on existing standard human retinal surgery techniques; (iii) small volumes of gene transfer

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material can be delivered to the subretinal space thereby preferentially exposing the diseased cells; (iv) there is minimal systemic exposure to the gene transfer agent. This reduces the potential of systemic complications and thus of a toxic immune response (Acland et al. 2005; Bennicelli et al. 2008; Jacobson et al. 2006; Maguire et al. 2008, 2009; Hauswirth et al. 2008); (v) the relative immune-privilege enjoyed by the eye may facilitate prolonged expression; (vi) the target cells in the retina are terminally differentiated at birth, and therefore it is unlikely that the reagent would be diluted by cell division; (vii) proof of concept of gene augmentation therapy has been demonstrated in both large and small animal models using the human gene (Acland et al. 2001, 2005; Dejneka et al. 2004a; Narfstrom et al. 2001, 2003a, b, c; Bennicelli et al. 2008). Those studies documented rapid onset of improvement in retinal and visual function in a stable fashion with a high level of safety; (viii) improvement of retinal function has been reported for multiple subjects through AAV-mediated *RPE65* delivery in three separate Phase I clinical studies, and several other Phase I trials are in progress (Table 2.1) (Maguire et al. 2008, 2009; Hauswirth et al. 2008; Jacobson et al. 2012; Cideciyan et al. 2008, 2009a, b; Bainbridge et al. 2008; Banin et al. 2010). The early reports from the first three contemporaneous trials reveal a high degree of safety and demonstrate efficacy as judged by increase in light sensitivity, improved visual acuity and visual fields, improved pupillary light reflex and improved mobility (Maguire et al. 2008, 2009; Bainbridge et al. 2008; Hauswirth et al. 2008). Two of the trials have reported long-term results, and the results indicate that the initial gains in function have been maintained (Bennett et al. 2012; Simonelli et al. 2010; Jacobson et al. 2012; Cideciyan et al. 2013). The majority of the studies employed an AAV serotype 2 vector delivering the wild-type human *RPE65* cDNA subretinally to the RPE in one eye (Table 2.1), but the studies differed in terms of dose, inclusion criteria, type of promoter, location of injection, and outcome measures; (ix) there is also evidence of improvement in retinal function in a follow-on Phase I/II study, carried out at the Children's Hospital of Philadelphia (CHOP) (Bennett et al. 2012). This study involved readministration of the vector to the contralateral eye of eligible individuals involved in the initial Phase I dose-escalation study (Table 2.1).

2.2 The Road to Gene Therapy for LCA

As technology developed allowing one to clone and manipulate DNA, and demonstration was made in animals that delivery of cloned genes into the germ line could alter the phenotype of the animals, the obvious next step was to test somatic gene delivery for the amelioration or even cure of disease. It took several decades, however, for all of the necessary tools/reagents to be assembled. The retina became an interesting target once the first two blindness-associated genes were identified, choroideremia (CHM), implicated in an X-linked retinal degenerative condition, and rhodopsin (RHO), most frequent cause of retinitis pigmentosa (RP) (Cremers et al. 1990; Nathans and Hogness 1984; Dryja et al. 1990; Humphries et al. 1990).

I had been aiming in the 1980s to develop gene transfer approaches for systemic diseases, but realized once the retinal genes were identified, that monogenic diseases of the retina were excellent targets. My first experiments with the retina aimed to develop safe and stable methods of retinal gene transfer. There were two parts to this problem: one surgical and one relating to gene transfer efficiency and stability. The surgical approaches were developed through work carried out with long-term collaborator, Albert M. Maguire, MD. Dr. Maguire, while a fellow in retina surgery, received a pilot grant from Fight for Sight; simultaneously, I received a career development award from the then "Retinitis Pigmentosa Foundation," currently Foundation Fighting Blindness (FFB). With the support of these patient-oriented organizations, we developed surgical methods in large and small animal models that could be extrapolated eventually to humans. These approaches initially used physicochemical methods to transfect the *LacZ*

Table 2.1 Details of vectors and clinical trials used evaluating gene augmentation therapy for LCA2. Details were obtained from the www.clinicaltrials.gov listing, media reports, and/or published results

Surgery site	Clinical trials.gov ID	PI	Phase	Initiation date	Name of vector	Source of vector	AAV capsid	Promoter	Poly (A)	Dose/Eye (vg)	Volume (μl)	# Subjects	Lowest baseline VA (LogMAR)	Additional
Moorefields (London, UK)	NCT00643747	R.R. Ali	I/II	2008	AAVAAG76 (rAAV2/2. hRPE65p. hRPE65)	TG	2	hRPE65; 1400 bp	bGH	≤1.00E11	≤1,000	9	1.52	Small detachment generated before injecting AAV
CHOP; Philadelphia, PA, USA	NCT00516477	A.M. Maguire	I/II	2008	AAV2-hRPE65v2	CCMT/CHOP	2	CBA	bGH	1.5E10-1.5E11	150–300	12	3.5[a]–0.96	Pluronic F-68 in Excipient
UGainesville, Gainesville, FL, USA	NCT00481546	S.G. Jacobson	I/II	2008	AAV2-CBSB-hRPE65	AGTC	2	CBA (short CMV)	SV40	5.96E10-1.788E11	150–450	15	1.96 (most were ~1.1)	Some received multiple injections
Hadassah-HUMC, Jerusalem, Israel	NCT00821340	E. Banin	I	2010	AAV2-CB-hRPE65	AGTC	2	CBA (long CMV)	SV40	1.19E11	300	NA	NA (≤0.4)	
OHSU; UMass, Portland, OR; Worcester, MA, USA	NCT00749957	J.T. Stout	I	2011	rAAV2-CB-hRPE65	AGTC	2	CBA (long CMV)	SV40	1.8E11 6E11	450	12	NA (≤0.48)	
Nantes U Hospital, Nantes, France	NCT01496040	M. Weber	I	2011	rAAV2/4. hRPE65	Genethon	4	NA	NA	NA	400–800	9	NA (<0.32)	
CHOP; Philadelphia, PA; UIowa, Iowa City, IA, USA	NCT00999609	A.M. Maguire, S.R. Russell	III	2012	AAV2-hRPE65v2	CHOP	2	CBA	bGH	1.50E11	300	24	NA (<0.48)	Pluronic F-68 in Excipient
CHOP; Philadelphia, PA, USA	NCT01208389	A.M. Maguire	I/II FO	2010	AAV2-hRPE65v2	CHOP	2	CBA	bGH	1.50E11	300	12	3.5[a]–0.45	Pluronic F-68 in Excipient

CBA chicken beta actin promoter with cytomegalovirus (CMV) enhancer, *CB* CBA promoter with long CMV enhancer, *CBSB* CBA promoter with short CMV enhancer, *TG* targeted genetics, *CCMT/CHOP* Center for Cellular and Molecular Therapeutics at the Children's Hospital of Philadelphia, *Moorefields* Moorefields Eye Hospital, *UGainesville* University of Gainesville Hospital, *Hadassah-HUMC* Hadassah-Hebrew University Medical Center, *Nantes* Nantes University Hospital, *OHSU* Oregon Hospital State University (Rojas-Burke 2011), *UMass* University of Massachusetts, Worcester, *UIowa* University of Iowa Hospital, *vg* vector genomes, *bGH* bovine growth hormone, *SV40* simian virus 40, *NA* not available

[a]Visual acuity was between hand motion and light perception and so was assigned LogMAR 3.5

reporter gene, which encodes histochemically detectable beta-galactosidase (β-Gal).

Simultaneously, other investigators had been developing recombinant adenovirus vectors, which transduce respiratory cells efficiently, and thus might have been useful for gene therapy studies of cystic fibrosis (CF). We used our newly developed surgical techniques to evaluate retinal somatic gene transfer using first-generation recombinant adenoviral vectors. Because little was known about the safety of Ad vectors, our studies were carried out using biohazard level 3 facilities – i.e., facilities with air locks, full body protective apparel, and sequestration of the animals. [Animal studies using recombinant Ad vectors are usually now carried out using level 2 facilities.] Subretinal injection of Ad.CMV.LacZ led to expression of high levels and early-onset (within 48 h) expression of the β-Gal reporter gene in RPE and Muller cells of adult mice and in photoreceptor precursor cells in neonatal mice (Bennett et al. 1994; Li et al. 1994). An Ad vector, in which the βPDE cDNA was exchanged for the β-Gal cDNA, and the retinal degeneration (rd) mouse model, was then used to demonstrate the first proof of concept of in vivo retinal gene therapy (Bennett et al. 1996). Because of its immunogenic potential, its inefficient transduction of mature photoreceptors, and the lack of stability of transgene expression, we and others began to search for alternative recombinant viral vectors. The first retinal studies with adeno-associated virus (AAV) demonstrated the advantages of AAV over adenovirus: efficient and stable transduction of retinal cells with a favorable immune profile; (Fig. 2.1; Ali et al. 1996; Bennett et al. 1997; Flannery et al. 1997). AAV serotype 2 (AAV2) was the first AAV serotype identified and thus the first to be studied. AAV is a nonpathogenic, single-stranded DNA genome-containing, helper virus-dependent member of the parvovirus family. AAV particles are small (~26 nm diameter) non-enveloped, icosahedral virions (Carter 1996). Jomary was the first to use AAV to demonstrate proof of concept of gene therapy in an animal model (the rd mouse model of RP) (Jomary et al. 1997).

From 2001 to 2005, I undertook countless discussions with small and large pharma to determine whether there was corporate interest in supporting a potentially expensive Phase 1 gene therapy clinical trial for LCA2. Although there was great interest and the leaders were genuinely impressed with the proof-of-concept data, the fact that LCA2 is an ultra-orphan disease had a negative impact on decisions to support a study financially. The outlook changed, though, in July 2005, when Dr. Katherine High presented me/my team an invitation to carry out a clinical trial at CHOP. She had just established a Center for Cellular and Molecular Therapeutics (CCMT) at CHOP, complete with a GMP vector core and relevant expertise in regulatory affairs. She had recruited world-renowned experts in design of gene therapy clinical studies from a gene therapy company (Avigen) that had just folded. With the regulatory/vector expertise and financial backing secure, we were able to join forces and move forward to carry out LCA2 clinical studies without delay.

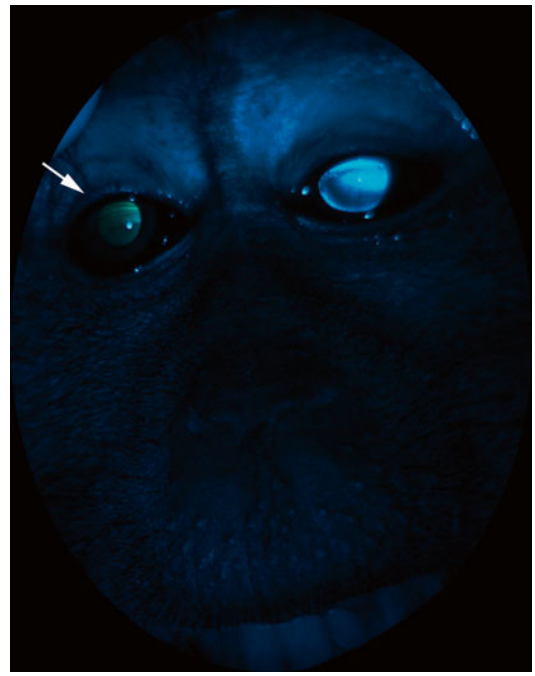

Fig. 2.1 Representative retinal transgene expression 3 months after subretinal delivery of 1E10 vector genomes (vg) AAV.CMV.GFP in a non-human primate (Vandenberghe et al. 2013). Green fluorescent protein (GFP) expressed by retinal cells make the retina glow green (*arrow*) after illumination with a blue light

2.3 Major Milestones in the Discovery

2.3.1 Identification of *RPE65*-Mediated Disease in Humans and Animal Models

Mutations in the *RPE65* gene were first identified as the cause of Leber's congenital amaurosis (LCA) in 1997. These included missense, point mutations, and rearrangements (Gu et al. 1997; Marlhens et al. 1997). This gene then became a candidate gene for the retinal disease found in Swedish Briard dogs that had previously been diagnosed with congenital stationary night blindness by Dr. K. Narfstrom et al. (1989). The Swedish Briard *RPE65* mutation, a 4 bp deletion causing a frameshift and a premature stop codon, was identified in the late 1990s (Aguirre et al. 1998; Veske et al. 1999). In the *RPE65−/−* dog, the mutation was found to cause retinal and visual dysfunction and RPE accumulation of lipid vacuoles. An appealing feature of this disease with respect to gene therapy was that the gene was expressed in RPE cells, which are very efficiently transduced by viral vectors, and that there was a slowly progressive retinal degeneration, thereby allowing a window of opportunity for gene therapy-based intervention. It thus became logical to consider testing the possibility of AAV-mediated rescue of LCA2 in the *RPE65* dog model. The dog model was available prior to the engineered mutant *Rpe65−/−* mouse (1998) (Redmond et al. 1998), and a spontaneous mutant *Rpe65−/−* ("rd12") mouse was (2005) (Pang et al. 2005).

2.3.2 Identification of the Delivery Vector

Although the first recombinant virus evaluated, adenovirus, efficiently transduces RPE and Muller cells and leads to both a rapid onset and high level of transgene expression, it quickly became apparent that this virus is highly immunogenic and that transduction results in only transient transgene expression (Maguire et al. 1995; Borras et al. 1996; Hoffman et al. 1997). The subsequent death in one human injected with Ad systemically in a study of gene therapy for ornithine transcarbamylase deficiency (Wade 1999) made this virus even less attractive. In 1996–1997, when several groups demonstrated efficient and stable transgene expression after delivery of recombinant AAV2 vectors to retina (see above), focus quickly shifted to this vector. AAV vectors do not carry any virus open reading frames and thus do not deliver any virus-specific proteins. This is an advantage (compared to adenovirus) as it limits the potential of development of a harmful immunogenic response to foreign antigens. Recombinant AAV vectors also target a more diverse set of cell types than other vectors and do not carry a high risk of insertional mutagenesis (since they rarely integrate) (Carter 1996). Once the AAV infects the cell, the DNA travels to and persists in episomal fashion in the nucleus of the target cells. Expression from the transgene cassette persists for the life of small animals (rodents) and was later shown to persist for significant periods of time (at least 11 years in dogs) after subretinal injection (Acland et al. 2005; Cideciyan et al. 2013) When using AAV to deliver the jellyfish-derived green fluorescent protein (GFP) reporter gene, one can visualize transgene expression in the retina long after injection (Fig. 2.1). A disadvantage of AAV vectors is that they have a relatively limited cargo capacity (4.8 kb); however, that is not a limitation for the *RPE65* cDNA. Thus, AAV2 rapidly became the vector of choice for retinal gene delivery for LCA2.

2.3.3 The Construct

The constructs used in the three original trials were similar in that they all used the *hRPE65* cDNA and AAV2; however, they (and the ensuing trials) differed in other variables (Table 2.1). [Only one of the more recent trials has used a different AAV capsid (AAV4).] The AAVs differed in details of the promoter/enhancer, presence of a Kozak sequence, and whether or not there was a stuffer sequence in the proviral plasmid (Fig. 2.2). The latter modification prevents reverse packaging from the

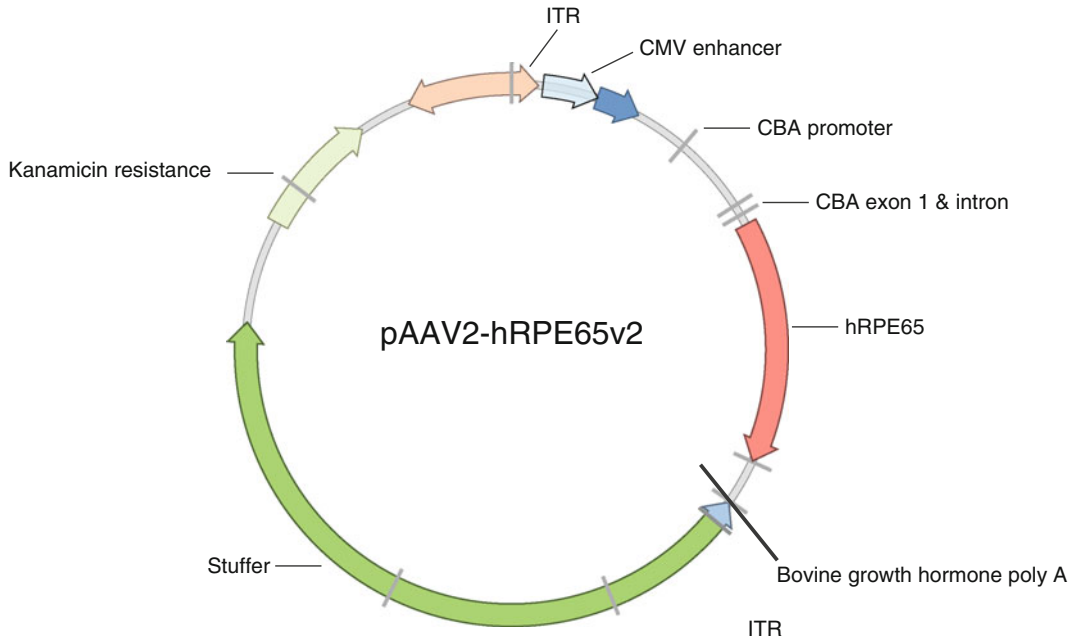

Fig. 2.2 Map of the proviral plasmid used to generate AAV2.hRPE65v2 by the team at the Children's Hospital of Philadelphia. The human RPE65 cDNA is driven by a chicken beta actin (CBA) promoter and a cytomegalovirus (CMV) enhancer, and there is a bovine growth hormone poly(A). Only the segment between the inverted terminal repeats (ITRs) is packaged in the virus. The proviral plasmid also contains a kanamycin resistance gene for selection and a noncoding stuffer sequence, used to prevent reverse packaging (Bennicelli et al. 2008). This minimizes the formation of empty capsids

AAV inverted terminal repeats (ITRs), possible when the size of the vector plasmid backbone is less than the packaging limit of AAV. Reverse packaging would result in empty vector (i.e., vector lacking the transgene cassette). This feature was thus thought to enhance safety while maximizing therapeutic effect. The AAV vector that we have used at CHOP, pAAV.CMV.CβA.hRPE65v2, contains a cytomegalovirus (CMV) immediate early enhancer, a chicken β-actin (CβA) promoter, the human *RPE65* cDNA (including intron and open reading frame), and a downstream bovine growth hormone poly A sequence (Fig. 2.2). This plasmid backbone has the following characteristics besides the stuffer sequence: (1) it contains a kanamycin resistance gene for selection and growth; (2) it contains a bacterial origin of replication; and (3) it contains inverted terminal repeats from AAV2. The excipient for the CHOP vector was phosphate-buffed saline containing 0.001 % Pluronic F68. Pluronic F68 is a surfactant that prevents absorption of vector to inert surfaces (such as the insides of storage vessels and injection devices) and thus allows accurate dosing (Bennicelli et al. 2008). None of the other studies used surfactant in the excipient.

2.3.4 In Vitro Data

We first tested AAV.RPE65 vectors in vitro in primary canine RPE cell cultures using a canine cDNA (cloned by Jharna Ray) and demonstrated that the treated *RPE65−/−* cells were able to produce the wild-type RPE65 protein after infection (Acland et al. 2001). In vitro studies revealed no evidence of toxicity to the exposed cells and no signs of abnormal cell division or increased amounts of apoptotic cell death after transduction

with AAV.RPE65. Later studies using a human RPE65 cDNA (cloned by Nadine Dejneka) and the additional modifications described in Table 2.1 showed similar results (Bennicelli, unpublished data). Importantly, the in vitro data demonstrated that transduction of RPE cells with the preclinical vector results in dose-dependent expression of the *hRPE65* cDNA without any significant cell toxicity/death (Bennicelli et al. 2008).

2.3.5 Preclinical (In Vivo) Data

Two groups simultaneously explored the possibility of gene augmentation therapy-based rescue in the *RPE65* dog model using AAV. One team was at the University of Pennsylvania with collaborators in New York and Florida (Acland et al. 2001, 2005) and the other at the University of Missouri with collaborators at the University of Western Australia (Narfstrom et al. 2003a, b, c, 2005). Similar studies were also later carried out in Europe (Rolling et al. 2006) and, later, at the Children's Hospital of Philadelphia (CHOP) in collaboration with the University of Missouri (Bennicelli et al. 2008).

The results of all groups showed dramatic restoration of vision after a single subretinal delivery of AAV.RPE65. A summary of the results follows. All institutional and national guidelines for the care and use of laboratory animals were followed. All dogs evaluated, which received a successful subretinal injection before the age of 14 months, showed treatment success for rod and cone function by ERG (Bennicelli et al. 2008; Acland et al. 2001, 2005; Narfstrom et al. 2003a, b, c, 2005). Visual behavior could be observed by 1 month after vector administration. Improvement in visual function was dramatic and persisted for the duration of the study (Bennicelli et al. 2008; Acland et al. 2001, 2005; Narfstrom et al. 2003a, b, c, 2005). Behavioral studies showed a significantly improved ability of animals that received the appropriate subretinal dose to navigate through an obstacle course. Ocular motility studies showed signs that the treatment significantly reduced nystagmus corresponding to improved fixation and visual acuity (Jacobs et al. 2006, 2009).

Dogs were euthanized and enucleated at various times after treatment, ranging from 3 months to more than 11 years (Acland et al. 2005; Bennicelli et al. 2008; Cideciyan et al. 2013; Narfstrom et al. 2003c) to demonstrate persistence of transgene expression. Both transgene expression and efficacy persisted through the longest time points. These are significant periods of time with respect to both safety and stability of expression.

In studies of Rpe65−/− mice, AAV2 vectors did not initially result in efficacy after subretinal injection of AAV2.hRPE65. Efficacy was not identified unless AAV serotype 1 vectors were used (Dejneka et al. 2004b). With AAV1 vectors, subretinal injection performed up to 4 months of age resulted in significantly enhanced restoration of function whereas subretinal injection in aged mice (>15 months old) resulted in only minimal improvement in function (Jacobson et al. 2005). Subsets of eyes were analyzed both biochemically (for 11-cis retinal) and histologically for presence of RPE65 protein and the production of rhodopsin. Rhodopsin was identified only in the subretinally injected eyes (Dejneka et al. 2004b). This finding is important as this molecule would not be formed in these animals without delivery of the RPE65 protein and its subsequent role in production of the rhodopsin moiety, 11-cis retinal (Redmond et al. 1998). In the animals in which rhodopsin was produced, the ERG of the injected eye resembled that seen in normal sighted animals. In contrast, the control-injected eyes had little or no recordable responses even to the highest stimulus intensities.

It was not until the transgene cassette was further optimized (introduction of a Kozak sequence, etc.) that AAV2-mediated rescue was observed in Rpe65−/− mice (Bennicelli et al. 2008). It was fortunate that the initial studies in LCA2 were carried out in canine models rather than murine models, as investigators might not have proceeded to work with canine models after seeing negative results in the *Rpe65*−/− mice!

2.4 Description of the Trial

2.4.1 Results of the Studies at the Children's Hospital of Philadelphia (CHOP)

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (http://www.wma.net/en/30publications/10policies/b3/). Our study was the first to enroll pediatric subjects in a gene therapy trial for a nonlethal disease (see below). The informed consent/assent process was carried out through a series of discussions and review of written and auditory materials. Informed consent or assent and parental permission was obtained from all subjects included in the study.

Participants were injected subretinally in the eye with worse vision in a dose-escalation study. Doses ranged from 1.5×10^{10} to 1.5×10^{11} vector genomes (vg) of the AAV2 vector (AAV2.hRPE65v2) (16, 18). Each one of the subjects showed improvement in multiple measures of retinal and visual function in the injected eye. Most of the subjects showed improvement in full-field light sensitivity and pupillary light reflex (PLR) (Maguire et al. 2008, 2009). About half of the subjects showed significant improvement in visual acuity, and all showed a trend toward improvement in visual fields. Five of the 12 patients (including all pediatric subjects age 8–11 years) developed the ability to navigate a standardized obstacle course (Maguire et al. 2008, 2009). The improvements were measured as early as 2 weeks after treatment and persisted through the latest time point (now >6.5 years for the initial subjects) (Maguire et al. 2008, 2009; Simonelli et al. 2010). Functional magnetic resonance imaging (fMRI) studies carried out in subjects after they had received the injection also showed that the visual cortex became responsive to retinal input after this unilateral gene therapy, even after prolonged visual deprivation (Ashtari et al. 2011). Both the retina and the visual cortex became far more sensitive to dim light and lower-contrast stimuli.

The success of the unilateral injections begged the question of whether further benefit would result from injection of the second eye. The main concern had been that the initial injection would incite a harmful immune response and that this would prevent benefit in the second injected eye and/or result in inflammation in the initially injected eye. Prior to evaluating the safety of AAV2-hRPE65v2 in humans, sequential subretinal readministration of high-dose (1.5E11 vg) AAV2-hRPE65v2 was tested in both Briard (affected) dogs as well as unaffected nonhuman primates (NHPs) that had been previously systemically exposed to AAV (Amado et al. 2010). There were no safety concerns with respect to readministration in either the initially injected eye or the second (contralateral) eye (Amado et al. 2010). An additional preclinical toxicology study, designed in consultation with FDA, examined the effects of readministration in unaffected NHPs of doses that were twofold and fivefold higher than the high dose of the Phase 1 human trial and 20-fold and 50-fold higher than the low-dose cohort. There was no indication of ocular toxicity, and there were no test article-related clinical signs of systemic toxicity (Amado et al. 2010). Thus, the results from the animal studies were reassuring with respect to the potential safety of readministration to the contralateral eye in humans.

Because results from animal studies are not always predictive of the effects in humans, the human readministration studies proceeded cautiously. This "follow-on" study entailed injection of a single dose/volume (10^13 vg in 300 μl) of AAV2.hRPE65v2 to the second (contralateral) eye. As an extra precaution and in order to optimize the risk-benefit ratio, each of the first three patients receiving readministration was an adult and was deemed least likely to benefit, based on the number of remaining retinal photoreceptors in each eye. There was a 2-month stagger between each of the 3 patients, and each patient was evaluated weekly in the clinic using a battery of ophthalmic and immunologic studies during the initial 3-month follow-up phase (Bennett et al. 2012).

Clinical examinations, immunology studies, and retinal/visual function tests following the initial contralateral eye injections demonstrated

the safety and efficacy of the bilateral approach in the first three individuals, even with a delay between vector administrations (Bennett et al. 2012), consistent with findings in nonclinical studies (Amado et al. 2010). Administration of AAV2-hRPE65v2 to the contralateral eye was well tolerated; there were no cytotoxic T-cell responses to either vector (AAV2) or transgene product (RPE65) in any of the subjects. Neutralizing antibody (NAb) responses to AAV2 and RPE65 protein remained at or close to baseline in the postoperative period (Bennett et al. 2012). Most importantly, each one of the subjects showed improvements in retinal and visual function, including the finding that two of the subjects who had previously been unable to navigate the mobility course became able to complete the course accurately in dim light (Fig. 2.5) (Bennett et al. 2012). The follow-on study has proceeded to enroll the remaining subjects eligible for participation (Bennett et al., unpublished data).

2.4.2 The Approval Process

In the USA, there are a number of regulatory hurdles that must be negotiated before embarking on a gene therapy clinical trial. The first steps included a "Pre-Investigational New Device (IND) meeting" with the US Food Drug Administration (FDA) and a review by the NIH Office of Biologic Activities (OBA) Recombinant DNA Advisory Committee (RAC). The NIH established the RAC in 1974 in response to public concerns regarding the safety of manipulating DNA. This committee is a federal advisory committee that provides recommendations related to basic and clinical research involving recombinant or synthetic nucleic acid molecules. The NIH RAC decides whether to hold a public review. Because our trial was the first to enroll children (a "vulnerable population") for a gene therapy study for a nonlethal disease, it was the subject of focus of a public meeting (see Human gene transfer protocol #0510-740, http://www.webconferences.com/nihoba/13_dec_2005.html). Reviews from the hospital Institutional Biosafety Committee (IBC) and Institutional Review Board (IRB) followed along with review and approval from a Data Safety Monitoring Board (DSMB), and finally, the Investigational New Device (IND) was submitted and reviewed by the US Food and Drug Administration (FDA). A similar process was carried out in order to undertake the follow-on study and the Phase III clinical trial that is in process.

2.4.3 The Manufacturing

AAV2-hRPE65v2 employs AAV as a delivery vehicle for normal human *RPE65*. The gene therapy material used at CHOP was manufactured under current Good Manufacturing Practices (cGMP) using a characterized HEK 293 cell line. The method that was used to generate the recombinant AAV vectors involved co-transfection of HEK 293 cells with three plasmids: the AAV vector plasmid pAAV.CMV.CβA.hRPE65v2 (containing the CBA-hRPE65 expression cassette flanked by AAV2 inverted terminal repeats (ITRs)) (Bennicelli et al. 2008), an AAV packaging plasmid providing AAV2 rep and cap sequences required for vector packaging, and an adenovirus helper plasmid providing minimal adenovirus sequences required for recombinant AAV packaging (E2A and E4 genes and RNA from serotype 2 adenovirus). The vector was purified through microfluidization, filtration, cation-exchange chromatography, density gradient ultracentrifugation and final diafiltration into phosphate-buffered saline containing 0.001 % Pluronic F68 (Bennicelli et al. 2008; Maguire et al. 2008, 2009). The Pluronic prevents subsequent losses of vector to product contact surfaces during storage and administration and thus assures accurate dosing (Bennicelli et al. 2008) (see above).

2.4.4 The First Subject

The first subject at CHOP was NP-01, a 26-year-old Caucasian mother of two children, and one of the three children in her family affected with LCA2. She and her siblings had been legally blind since birth. Subject NP-01 was referred by Dr. F. Simonelli, the Second University of Naples (SUN) in Italy. Molecular diagnosis of a

mutation in the *RPE65* gene was initially performed at the Telethon Institute of Genetics and Medicine (TIGEM) and confirmed by the CLIA-approved Carver Laboratory at the University of Iowa (Maguire et al. 2008). The informed consent process occurred at both SUN and CHOP, and unilateral subretinal administration of AAV2-hRPE65v2 occurred at CHOP on October 11, 2007. Both SUN and CHOP carried out baseline and postinjection studies and found similar results. NP01 has been followed for 6.5 years since injection of her first eye.

NP-01 was also one of the first three subjects to participate in the CHOP follow-on (second eye readministration) study approximately 3.5 years after administration to her first eye (Table 2.1). She was 29 years of age at her second surgery (Bennett et al. 2012). NP01 was truly the pioneer for this study, volunteering as one of the first individuals for both initial administration and second eye administration studies. NP01 received the lowest dose in the Phase I escalation study (1.5E10 vg) and received the highest dose (1.5E11 vg) in the readministration study.

2.4.5 The Tests

Baseline and follow-up testing include a battery of safety assessments as well as child-friendly assessments of retinal and visual function. Testing included the following:

2.4.5.1 Safety Assessments

Ophthalmic exams included vision testing, slit lamp biomicroscopy, intraocular pressure measurements, fundoscopy with indirect ophthalmoscopic exam, fundus photography, and fundus biomicroscopy (optical coherence tomography (OCT)). Kinetic visual fields were measured using Goldmann perimetry and electroretinograms (ERGs) were performed. The presence and character of any nystagmus was monitored. Systemic safety was measured using complete blood counts and serum chemistries (including liver and renal function panels). Peripheral blood and tear fluid were evaluated for evidence of vector exposure through quantitative (Q)-PCR. Immunologic studies evaluated humoral response to the transgene product (the RPE65 protein), neutralizing antibodies to V2, and T-cell responses to the V2 capsid and to the RPE65 protein (Maguire et al. 2008, 2009).

2.4.5.2 Retinal and Visual Function Assessments

Visual acuity was measured with Early Treatment Diabetic Retinopathy Study (ETDRS) testing. Goldmann perimetry was used to measure visual fields. Pupillary light reflex (PLR) responses were recorded simultaneously in both eyes with a Procyon P2000 pupillometer and PupilFit4 software (Monmouthshire, UK). Test paradigms involved both unilateral stimuli and stimuli that were presented alternatively to one eye and then the other. Light sensitivity was evaluated using full-field threshold sensitivity testing (FST) and stimuli included white, red, and blue lights. Characteristics of nystagmus were evaluated by videotaping the eye movements for qualitative clinical analysis of the subject's oscillation and strabismus. Navigational abilities were evaluated using a standardized "obstacle course." This mobility test is designed to mimic the types of obstacles that a visually impaired individual must navigate on a daily basis. The subject enters the course at a designated spot and follows arrows on the tiles such that there are a series of choices of movements to maneuver around or over the obstacles. Performance under different light levels was evaluated (Fig. 2.5). The size of the test course is within the constraints of the clinical examining room, and the course was modified from session to session so that the subject could not "learn" the course (Maguire et al. 2008, 2009).

2.4.5.3 CHOP Results: Phase I/II Trial Safety Profile

Immunologic responses were benign and no serious adverse events occurred relating to the vector. Serum antibodies to the *RPE65* transgene product were not detected after vector administration. There were mild increases in serum neutralizing antibodies to AAV2 immediately postinjection in some individuals; however, levels diminished quickly and returned to baseline

levels by day 365 after vector administration. The vector was found in samples of tears and blood only transiently after surgery.

Except for the first subject, the target area was the macula for those individuals who had sufficient retinal cells in this region. There were three individuals with substantial atrophy in the macula and in whom macular exposure was thus limited. There were some (not unexpected) surgical complications, including a macular hole in one of the subjects at d14 (despite significant improvement in retinal/visual function). A foveal dehiscence was noted at the time of injection in another individual as some of the vector escaped from the foveal defect.

All of the retinal detachments had resolved by first postoperative visit (within 14 h after surgery), and foveal abnormalities were noted in only the one patient (NP02, see above), with optical coherence tomography. The foveal dehiscence in patient CH10 had completely resolved with no evidence of a macular hole after surgery at the first assessment. All the other postoperative retinal assessments were unremarkable. In order to minimize future surgical complications (such as macular hole), the surgical procedure was modified to minimize the stress on the fovea. The PIs of the other clinical trials were contacted immediately and advised about steps to minimize this potential complication.

Efficacy

All 12 individuals in the CHOP Phase I/II study reported improved vision in dimly lit environments in the injected eyes starting by 2 weeks after surgery. Improvements in visual acuity were substantial in more than half the subjects. The improvement was not associated with age; however, the baseline visual acuity was higher in children than in adults (Maguire et al. 2008, 2009).

Although visual field tests in patients with severe impairment show substantial variability, there was a trend to improvement and the enlargements exceeded the variation in the contralateral non-injected eye. The extent of improvement in visual fields correlated roughly with the amount of salvageable retina that was targeted (Maguire et al. 2008, 2009).

All individuals had bilaterally diminished full-field sensitivity at baseline. After injection, a large interocular difference (i.e., difference in sensitivity between injected and non-injected eyes) in full-field sensitivity was noted (Fig. 2.3). Only the injected eyes showed improved sensitivity. Improvements in full-field sensitivity were

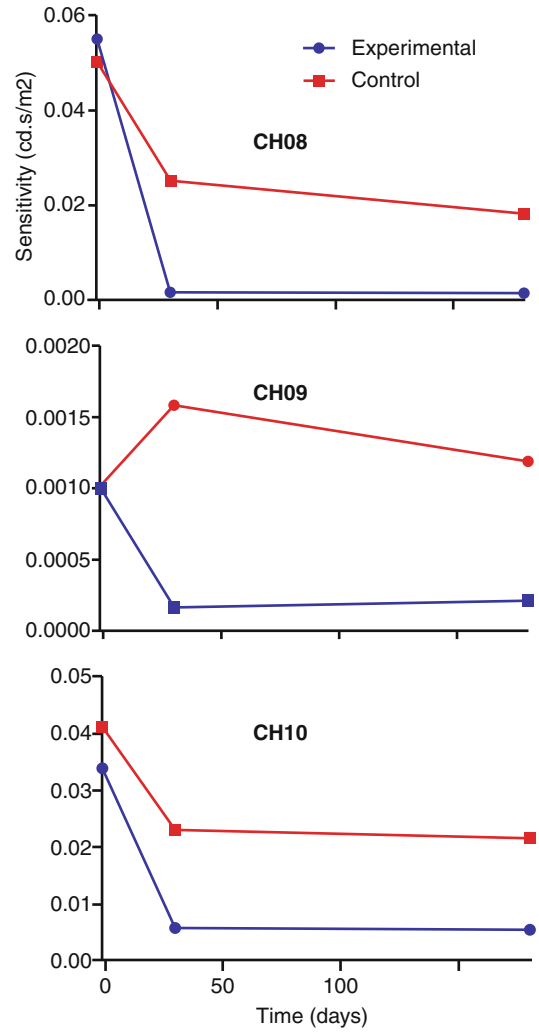

Fig. 2.3 Examples of before and after FSTs in the first three children enrolled in the Phase I study at the Children's Hospital of Philadelphia (Maguire et al. 2009). Note the increase in sensitivity of the experimental (injected) eye by day 30 after subretinal injection. Sensitivity is maintained through the latest time point shown (day 180). There can be changes in sensitivity of the uninjected (control) eye; however, these are not as large or as stable as those in the injected eye. Day 0, day of subretinal injection

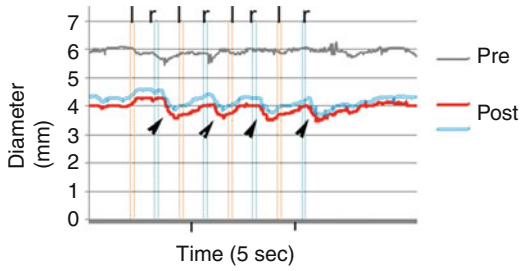

Fig. 2.4 Example of improvement in pupillary light reflex after subretinal injection of AAV2.hRPE65v2. Pupillary light reflex before (pre) intervention is shown in gray for the right pupil for one subject enrolled in the Phase 1 clinical trial at The Children's Hospital of Philadelphia (Maguire et al. 2008). Pupillary light reflex after intervention in the right eye (post) is shown in blue for the right pupil and red for the left pupil. *Arrowheads* indicate the improved responses only in the treated (right) eye. *Vertical dashed columns* indicate the stimuli presented to the left (l) vs right (r) eye

substantial in the youngest patients, who gained several log units of sensitivity (Maguire et al. 2009).

Pupillary responses improved in the injected eyes of all 11 individuals tested.

When the injected eye was illuminated with light, both pupils constricted; when the control, non-injected eye was illuminated with light, minimum constriction of the pupil was seen (Fig. 2.4). There were substantial differences between the injected and control eyes in the amplitude and velocity of constriction (Maguire et al. 2009).

The amplitude and frequency of nystagmus was reduced in several of the subjects after intervention of one eye. In some cases, this may have contributed to improved visual acuity in the non-injected eye (Maguire et al. 2008, 2009; Simonelli et al. 2010).

When patients were tested for their ability to navigate a standardized obstacle course before administration of AAV2-hRPE65v2, the majority had great difficulty, especially in dim light, as assessed by the number of errors and time taken. After injection, all of the children (CH08, CH09, CH10, and NP15) given AAV2-hRPE65v2 had substantial improvement in their ambulation when using only their injected eye. They were unable to navigate the course accurately using their non-injected eye (Maguire et al. 2009). Similarly some of the adults who were unable to complete the mobility test with their initially injected eye were able to complete the course after the second eye was treated (with higher-dose vector; Fig. 2.5).

All individuals tested with functional magnetic resonance imaging (fMRI) showed restoration of cortical responses based on the known anatomic connections between the retina and the visual cortex and the area of retinal exposure to the gene therapy reagent (Ashtari et al. 2011). fMRI testing confirmed the increased sensitivity of these individuals to light and to objects of reduced contrast (Ashtari et al. 2011).

CHOP Results: Phase I/II Follow-On Trial

Results from the first three subjects enrolled in the follow-on study have been published (Bennett et al. 2012). In those three individuals, all of whom were adults, there was no inflammation resulting from readministration of vector and immune responses were benign. The originally injected eye maintained the function it had gained after the first injection, and the second eye gained function as judged by pupillometry and full-field light sensitivity (Bennett et al. 2012). Two of the individuals had previously received a lower dose in their initially injected eyes. The data provided a suggestion of a dose effect, with the high-dose-treated eye showing even better function than the initially treated (low dose or medium dose) eye (Bennett et al. 2012). Two of the individuals who had received lower doses in their initially injected eyes became able to navigate the mobility course after their second eye was injected. fMRI testing also showed the predicted improvement in visual cortex activation (Bennett et al. 2012).

2.5 Future Plans at CHOP

At present, we are conducting a Phase III (pivotal) trial at CHOP. The goal is that AAV2-hRPE65v2 be approved as a drug for treatment of LCA2. This multicenter (CHOP and University of Iowa) Phase III study involves bilateral administration

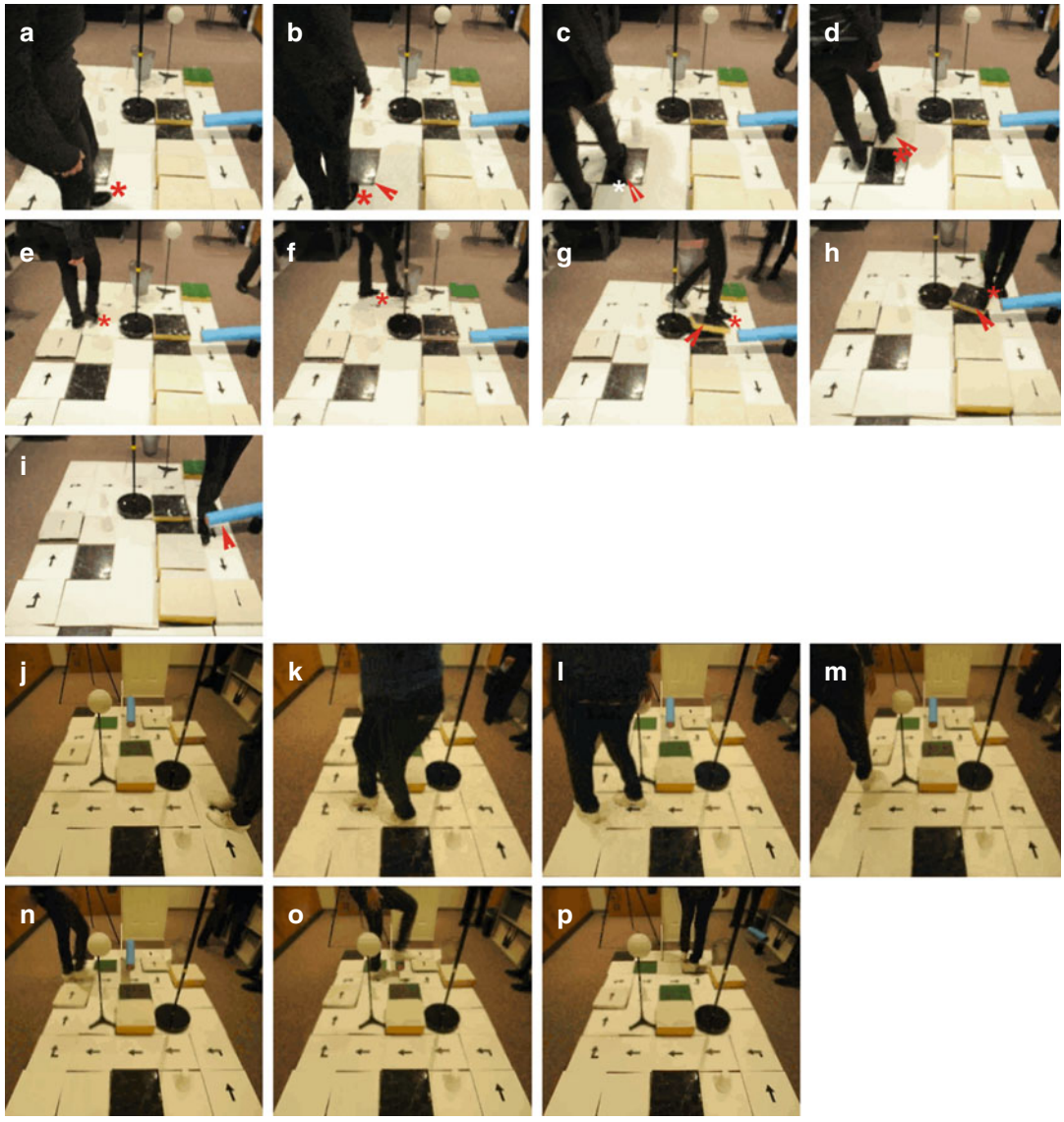

Fig. 2.5 Example of mobility test results, before and after readministration of AAV2.hRPE65v2. Frames from videos where subject NP01 undergoes obstacle course testing prior to (a–**i**) and 30 days following (**j**–**p**) intervention (Maguire et al. 2009). Light intensity was 50 lux for baseline testing and 5 lux for post-intervention testing. At baseline, NP01 goes off course repeatedly (*), collides with every obstacle (*arrowheads*), and takes 39 s to complete the course. After intervention, she completes the course in 14 s and does so without going off course or colliding with any obstacle

of AAV2.hRPE65v2 at 1.5E11 vg to the subretinal space in eligible individuals ages 3 years and higher. Subjects are randomized 2:1 to the intervention or control group, respectively. The individuals randomized to control crossover to the intervention group at year 1. Grading of primary endpoints is carried out by individuals masked as to whether the subjects have been assigned to the intervention vs the control arm of the study. Given maintenance of the current timeline, continued safety and efficacy, and no setbacks, such approval could be granted in 2016. All of the studies leading up to and including the Phase III trial stem from the efforts of a very large group

of talented scientists who designed and carried out the relevant multifaceted studies. The success of translational studies depends not on the efforts of one person but instead on the integrated efforts of molecular geneticists, experts in animal models, astute clinicians, surgeons, geneticists, virologists, clinical coordinators, regulatory experts, administrators, data analysts, and, of course, the patients themselves.

Assuming that AAV2.hRPE65v2 is ultimately approved as a treatment for LCA2, we will undertake a training program for retinal surgeons on surgical details that could optimize the outcome. We hope that the CHOP data will pave the way for safe and rapid development of other gene-based interventions for inherited and acquired retinal disease. The "de-risking" of subretinal AAV2 delivery (at least for doses up to 1.5E11 vg of purified vector) may allow more rapid development of additional retinal gene therapy strategies. Of course, each new variable will have to be approached cautiously.

In presenting our data, we believe that we have set an example of the importance of highlighting not only the exciting efficacy data but have also highlighted complications. It is from the complications that we can all learn how to develop the optimal treatment approaches and to avoid untoward events in the future. We have shared and continue to share our experiences on a wide range of topics, including surgical approaches, vector selection data, and issues relating to vulnerable subjects with the goal of helping to expand the opportunities to rescue vision in individuals of all ages. We strive to present our data and the most logical explanations without political agenda. Most importantly, we believe that we have set an example of how individuals with complementary talents and experience in a large team can work safely, efficiently, and persistently together toward the goal of generating not only a safe treatment for a blinding disease but also to create a path whereby treatments can be developed for other blinding diseases.

Compliance with Ethical Requirements

Conflict of Interest Jean Bennett and her husband and collaborator, Albert M. Maguire, are coinventors of a patent for a method to treat or slow the development of blindness (US Patent 8,147,823 B2; April 3, 2012), but both waived financial interest in this technology in 2002. JB serves on a data safety monitoring board for Sanofi and as a scientific advisor to Spark Therapeutics and Avalanche Biotechnologies, Inc. She is a cofounder of GenSight Biologics.

No animal or human studies were carried out by the author for this chapter.

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