Lecture

Concepts and Strategies in Retinal Gene Therapy

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Genetic defects of the retina or retinal pigment epithelium (RPE) cause a substantial number of sight-impairing or blinding disorders, many of which eventually cause the degeneration and death of the visual cells.[1,2] Previously considered incurable, some of these retinal diseases can now be treated, at least experimentally, by gene therapy.

This new era of retinal therapeutics followed the successful restoration of retinal function in a canine model of *RPE65* Leber congenital amaurosis (LCA) through adeno-associated virus 2 (AAV2) vector-mediated gene augmentation targeting the RPE layer of the eye.[3] Restoring isomerohydrolase activity in the RPE corrected the retinoid visual cycle and vision defect. When treated at the predegenerate disease stage, treatment was both effective and permanent, and photoreceptor structure was preserved.[4,5] Validation studies by other groups in both large[6] and small[7,8] animal models, along with preclinical safety studies in nonhuman primates (NPHs) and dogs,[9,10] confirmed that the treatment was safe and effective. A further series of detailed studies in patients and animal models established the dependence of human cone photoreceptors on RPE65 isomerase,[11] determined that the remaining photoreceptors in blind eyes were amenable to treatment,[12–14] showed that the visual cortex in man and dog was intact and responsive in spite of early blindness,[15] and developed outcome measures that could be used readily to assess treatment outcomes.[16,17] These studies were followed by three independent clinical trials showing the treatment to be safe.[18–21] Since then, additional *RPE65*-LCA clinical trials have been initiated both in academic settings and through commercial entities in the United States and elsewhere (https://clinicaltrials.gov/ct2/results?cond=Leber+congenital+amaurosis&term=RPE6, in the public domain). To date, LCA remains the only blinding genetic disease to be successfully treated in humans.

While the early successes in the treatment of LCA were clearly encouraging, it appears that these gene therapy effects do not last "forever." Despite functional recovery in treated areas, two studies now have shown continual loss of photoreceptors[22] with the structural phenotype of treated areas eventually becoming comparable to untreated regions.[5,23] Similar results were obtained in the canine model when treatment was delayed until degeneration had begun, a situation comparable to what occurs in human patients.[5] This series of discoveries at the level of a human clinical trial indicates there can be unexpected pitfalls even in the most well thought through studies. The RPE65 gene therapy trials show that even when there is strong evidence of efficacy early after treatment, it cannot be assumed that it will be long lasting. The same care given to defining efficacy in the short term should be used to define the longevity of the treatment success. Thus it is important to emphasize the need to properly assess the treatment outcomes in relation to the natural history of the disease before claiming the success of a putative treatment.

In this overview, I will present concepts and strategies relevant to developing and translating retinal gene therapeutics. These range from selection of the animal model and the therapeutic vector/promoter combination to application of the model system to address translationally relevant questions.

Animal Models

In vivo studies in animal models are the essential proof-of-concept first step to establish efficacy of a treatment paradigm. In addition to being a bona fide disease homologue, that is, caused by the mutations in the same gene with expression in the same target cell(s), the models should have a proportionally comparable disease time course. Ideally, the model disease should be "fast enough" that the therapeutic outcome can be assessed in a reasonable time scale, but "not too fast and overwhelming" such that efficacy cannot be established and that the disease bears no resemblance to the human disorder. Naturally occurring or genetically engineered models have been the basic toolbox used for examining cellular and molecular mechanisms of gene function and disease, and for developing retinal therapeutics. These animal models cover the size spectrum from *Drosophila*[24] to cow[25] and horse,[26] and include all sizes and species in between. In biology and experimental medicine, the models have been arbitrarily divided into large (≥dog or cat) and small, with small almost exclusively referring to rodents. As a veterinarian, this division is somewhat ironic given that the model system for my studies is the dog and that in veterinary medicine dogs and cats are considered "small animals."

For retinal disease studies and for the development and testing of novel therapies, the dog is an ideal intermediate model between mouse and man, as it is well suited to facilitating translational studies. Indeed, in cases where the appropriate model exists, experimental studies in the dog have led the way to clinical trials (*RPE65*-LCA, *CNGB3*-ACHM, and *RPGR*-XLRP), or trials in the late stages for Food and Drug Administration pre-IND (investigational new drugs) application (*BEST1*-BVMD) (Table 1). Moreover, with the development and application of new genomic tools, there has been a marked acceleration of disease gene discovery, and a combination of genome-wide association studies (GWAS) along with next-generation sequencing of whole genomes or exomes has facilitated progress in identifying additional genetic models of disease (Fig. 1). The identified mutations affect both the retinal pigment epithelium (RPE) and the rod and/or cone photoreceptors, with defects involving members of the phototransduction cascade, integral outer segment disc proteins, and the

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Table 1. Proof-of-Concept Studies in Dogs for Translational Applications; Comparison With Similar Studies in Mice, and Dates the Studies Were Published

Species	Leber Cong Am, *RPE65*	Achromatopsia, *CNGB3*	X-Linked RP, *RPGR*	Best Disease, *BEST1*	Leber Cong Am, *NPHP5*
Dog	Acland et al., 2001[3]	Komaromy et al., 2007*, 2010[48]	Beltran et al., 2012, 2015[50,68]	Guziewicz et al., 2011†, 2013[44]	Aguirre et al., 2016‡
Mouse	Rakoczy et al., 2003[7]	Carvalho et al., 2011[86]	Wu et al., 2015[87]	NR	NR

Leber Cong Am, Leber congenital amaurosis; NR, not reported.
* Komaromy AM, et al. *IOVS* 2007;48:ARVO E-Abstract 4614.
† Guziewicz KE, et al. *IOVS* 2011;52:ARVO E-Abstract 4378.
‡ Aguirre GD, et al. *IOVS* 2016;57:ARVO E-Abstract 2293.

photoreceptor sensory cilium, as well as other structures (for review see Refs. 27, 28). These models represent bona fide human disease homologues where the disease phenotype in model and man are the same. Selected examples include *RPE65*-LCA,[3,5,29] *BEST1*-BVMD,[30–32] *CNGB3*- and *CNGA3*-achromatopsia,[33,34] *RHO*-ADRP,[35] *RPGR*-XLRP,[32,36–38] and *NPHP5*-LCA.[39]

Quite apart from the particular merits of any individual disease model, the dog and the canine eye offer advantages for a broad range of translational studies. Because of its life span and the time course of the diseases, disease progression in the dog more closely resembles that of humans than do similar smaller laboratory animal disease models. Furthermore, as the size of canine and human eyes is similar,[40] viral vectors or drugs can be injected using the same surgical approaches and dose volumes, and implantation of devices (e.g., retinal prostheses or for sustained delivery of therapeutic agents) is identical to those intended for human trials.[3,41,42] In addition, the instruments and methods for surgical intervention and in vivo outcome assessments are comparable. Lastly, the recently identified fovea-like region within the canine retina has a similar cone density to the human and nonhuman primate (NHP) fovea, and is equally susceptible to inherited macular diseases, making it an ideal model system to study macular degenerations and therapies.[32]

It is critical to emphasize, however, that regardless of their translational value, the canine models are not alternatives to other laboratory model systems such as rodents. Rather they are a complementary and synergistic model, serving as an intermediate between rodents and man that provides an excellent test bed to develop or test new therapies. The history of the field clearly demonstrates that progress toward therapy of human patients has been served best by judicious use of a comprehensive set of model systems among which are rodent, canine, and others.

Vectors, Promoters, and Translational Applications

A critical issue that must be addressed during development of proof-of-concept gene therapy studies in animal models is to determine whether the results obtained with a vector–promoter combination used in the animal can be directly applied to patients in subsequent clinical trials. While this has been possible in the case of the *RPE65*-LCA, in most cases the vector-

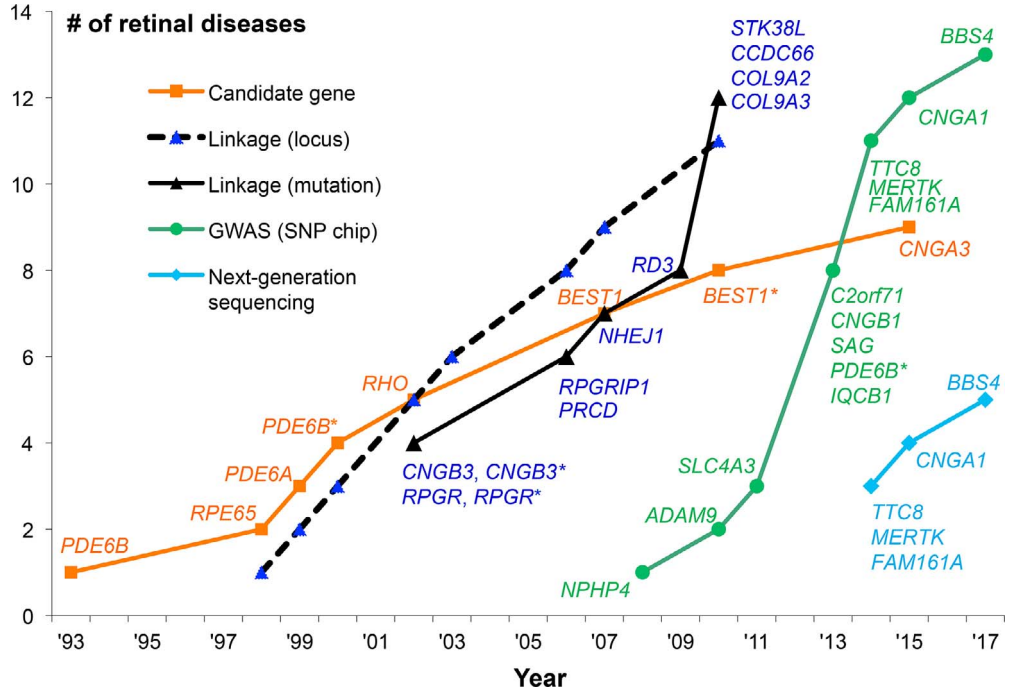

Figure 1. Discovery approaches for canine retinal degeneration loci and genes. The cumulative numbers of loci and causative mutations identified are shown, classified according to the discovery approach used. Note that the chromosomal location and the mutation were reported concurrently for cd, cord1, osd1, and osd2. *New mutation in a gene for which a mutation is already known. Figure courtesy of Keiko Miyadera.

promoter validated in proof-of-concept studies differs when optimized for patients (Supplementary Table S1). Thus the interplay between vector serotype tropism, promoter, and model species selected has to be considered before translation to the clinic is possible.

Promoters

Promoters are traditionally selected to limit transgene expression to the target cell population and minimize off-target expression, and are evaluated using reporter genes such as *GFP*. Obviously, this is most optimal when the promoter selected regulates the same therapeutic transgene, although that is not always possible. Generally, promoters are selected based on two criteria: (1) The endogenous gene regulated by the promoter is selectively expressed in the target cell(s); (2) there is robust expression of a reporter gene in the target cells when regulated by the chosen promoter. In general, the testing of target gene specificity and robustness of different AAV vector serotypes is done in normal retinas, as shown in our studies,[32,43,44] or in vitro[45] (Supplementary Table S2). Although there are a few notable exceptions,[46–48] these studies rarely assess expression of the endogenous gene targeted for promoter selection at the planned treatment stages, or use the promoter/reporter gene combination to confirm specific expression in the affected mutant cells. Thus the direct application of results obtained in normal retinas to mutants requires a cautious leap of faith. Indeed, we previously showed that the human G-coupled receptor kinase 1 (hGRK1) promoter directed expression of a green fluorescent protein (GFP) reporter to rods but not cones in normal canine retinas.[43] This observation confirmed earlier studies which clearly showed that dog cones expressed GRK7, but not GRK1.[49] However, in retinas affected by mutations in *RPGR*,[50,51] *RPGRIP1*,[52] and *NPHP5* (Aguirre GD, et al. *IOVS* 2016;57:ARVO E-Abstract 2293),[53] the hGRK1 promoter directs expression of the therapeutic transgene to mutant cones as well as rods, resulting in rescue of function and structure, even at quite advanced disease stages.

Studies from our lab and others have also shown that species-specific differences markedly influence the expression of reporter or therapeutic transgenes (Supplementary Table S2). In developing a therapeutic strategy for *CNGB3* achromatopsia, we tested a series of promoters based on the human red cone opsin locus control region.[54] In this dichromatic species with a single long wavelength–sensitive (L/M-) class of cones and short wavelength–sensitive S-cones,[55] the full-length promoter, PR2.1, directed GFP expression to all L/M-cones in normal and *CNGB3*-mutant retinas[48,56] (Fig. 2). In contrast, a chimeric interphotoreceptor retinoid binding protein/G protein subunit alpha transducin 2 (IRBP/GNAT2) promoter expressed GFP in both L/M- and S-cones in the dog (Fig. 2; Supplementary Table S2). However, when tested in murine and NHP (*Macaca fascicularis*) retinas, the PR2.1 promoter directed GFP expression to the RPE, cones, and rods in the mouse; expression was present but weak in the NHP L/M-cones, and the chimeric IRBP/GNAT2 promoter showed no photoreceptor specificity in NHP retinas.[57] The promoter selected for eventual therapeutic use was the PR1.7 modification of the human red cone opsin promoter, which showed robust GFP expression in L/M- and S-cones in NHP[57] as well as in dogs (Komaromy AM, unpublished observations, 2017).

An equally complex situation exists with cell-specific promoters targeting rods and cones. In our lab, we chose the IRBP promoter for *RPGR*-XLRP gene therapy studies, as it directed GFP expression specifically to rod and cone photoreceptors in normal dog[50] and mouse (Lewin AS, unpublished observations, 2011) retinas (Supplementary Table S2; Fig. 2). Unfortunately, this promoter was ineffective in directing GFP expression to foveal or peripheral cones after subretinal administration in two closely related NHP macaque species (*M. mulatta* and *M. fascicularis*).[51] This finding was surprising given that IRBP expression has been detected in rods and cones of the human retina by in situ hybridization.[58] A possible explanation for this discrepancy is that the small IRBP promoter contained only 235 bp of the full-length human IRBP promoter[59] and may not have all the regulatory elements needed for cone expression in NHP retina. It is likely that careful dissection of the human IRBP promoter will identify sequences that direct expression to NHP rods and cones. However, such studies appear less necessary now, as we have found that the hGRK1 promoter is highly effective in directing expression to rods and both peripheral and central cones in the NHP retina[51] (Fig. 2), and in mutant canine retinas[50–53] as well.

Vectors

The most widely used vectors for retinal gene transfer and therapy have been recombinant AAVs. Each AAV serotype shows tropism for distinct retinal cells in a species- and administration route–dependent manner. These vectors are considered safe and effective, with long-term stability of expression so that most experimental therapy studies require only a single vector administration. Their main limitation is their cargo-carrying capacity, maximal at ~4.7 kb, which makes them unsuitable for use with full-length, large-sized genes, for example, *CEP290*, *ABCA4*, and others.

Of the vectors used for therapy studies in dogs, AAV2 based, and to a lesser extent AAV1 and -4, are used for targeting the RPE, and AAV5, -8, and AAV_{2tYF} for photoreceptors (Supplementary Table S1). However, the vector serotype toolbox is large, and new versions are continually being developed.[60,61] Among the new vectors being developed for dog studies are those identified by directed evolution using the canine retina.[62] Some newer AAV vectors have single or multiple mutations that replace critical capsid tyrosine residues to enhance nuclear targeting by bypassing ubiquitination and proteasomal degradation.[63,64] These vector constructs also can be packaged as self-complementary vectors to avoid delays caused by DNA synthesis, as must occur to generate double-stranded DNA from the single-stranded genome of older AAV vectors; but such modifications further limit their cargo-carrying capacity.[65] Self-complementary AAV vectors with capsid modifications have been evaluated in dogs as a means of increasing transduction efficiency and onset of gene expression using GFP reporter or therapeutic genes[66] (see below), and are therapeutically very effective (Aguirre GD, et al. *IOVS* 2016;57:ARVO E-Abstract 2293).

As with promoters, AAV vector serotype selection for proof-of-concept and therapeutic applications is complex. Additionally, for translation to the clinic, experience with vector production protocols by the commercial entity, as well as intellectual property considerations, often directs serotype selection. The complexity in vector selection for experimental studies is illustrated by our own work in dogs using the X-linked retinal degeneration *RPGR*-XLRP and *NPHP5*-LCA models. Both diseases are characterized by abnormal photoreceptor development and early degeneration.[36,39,67] We have found that an AAV5-hIRBP-*hRPGRstb* vector (Supplementary Table S1) is effective in arresting the degeneration in *RPGR*-XLRP when treatment is initiated at 6 weeks of age, that is, early disease stage.[50] Delaying treatment until the mid and late stages of disease is equally effective and results in long-term preservation of structure and function[68] (see below). However, when the same vector/promoter combination with a *cNPHP5* therapeutic transgene is used at the same vector dose in the *NPHP5*-LCA

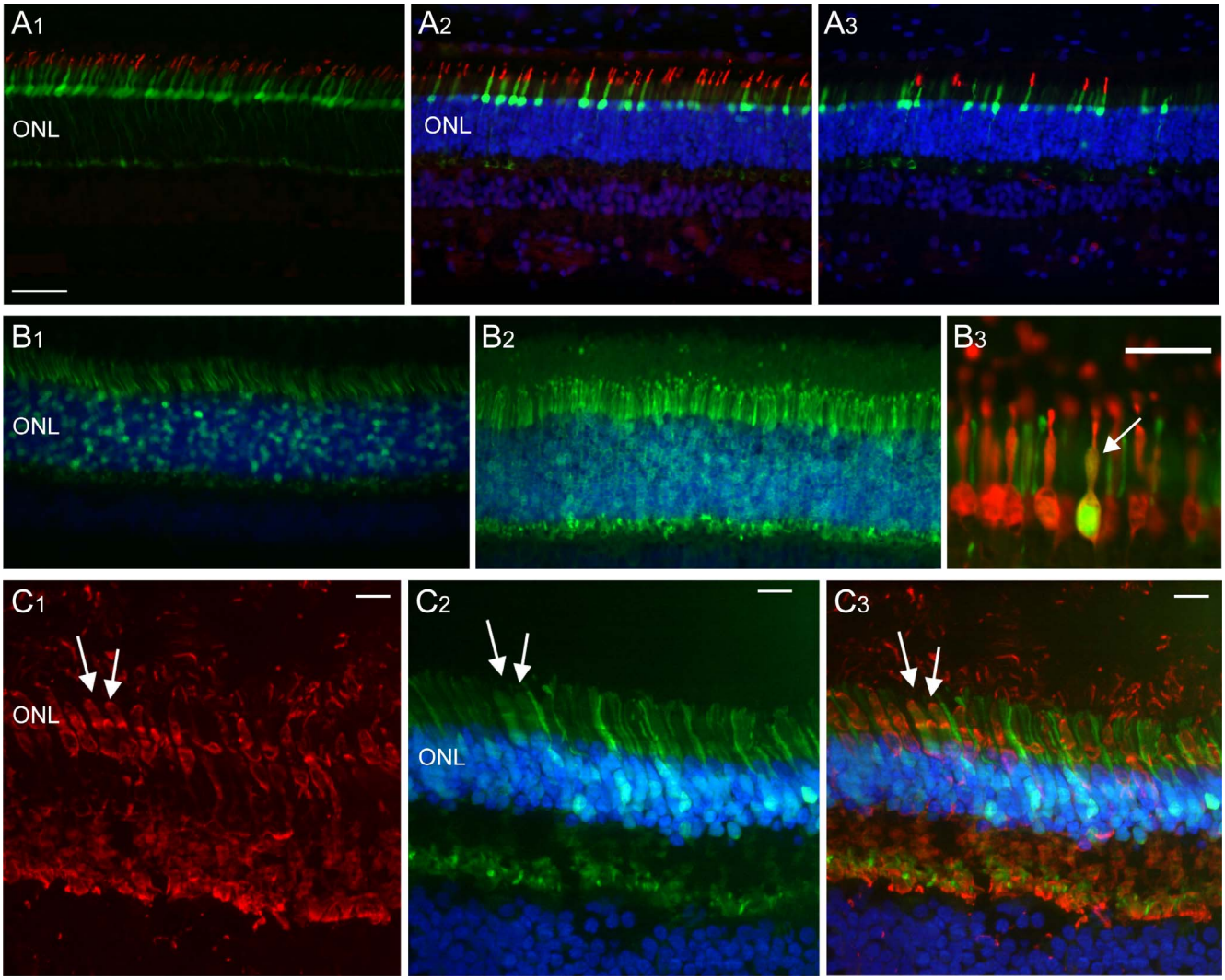

Figure 2. Targeting photoreceptors in the *CNGB3*$^{-/-}$ mutant (**A1–A3**) and normal (**B1–B3**) dog retina, and in normal nonhuman primate (NHP, *M. fascicularis*; **C1–C3**) using AAV5 vectors with different promoters. (**A1**) Four weeks after subretinal injection using PR2.1 promoter, there is robust native GFP expression (*green*) in L/M-cones (*red*), which is the predominant cone class in the canine retina (*scale bar*: 40 μm). Figure from Komaromy AM, Alexander JJ, Rowlan JS, et al. Gene therapy rescues cone function in congenital achromatopsia. *Hum Mol Genet*. 2010;19:2581–2593. © The Author 2010. Reprinted with permission from Oxford University Press. (**A2, A3**) The hybrid GNAT2/IRBP promoter results in robust GFP expression in both L/M- (**A2**, *red*) and S- (**A3**, *red*) cones (Figs. A2, A3 courtesy of András Komaromy). (**B1–B3**) The hIRBP promoter targets native GFP expression (*green*) after injection of a 1.5×10^{11} μg/mL titer. GFP expression is low after 2 weeks (**B1**), and increases by 8 weeks post injection (**B2**); at 8 weeks, hCAR labeling (*red*) confirms expression in cones (**B3**). *Scale bar*: 20 μm. Figures B1–B3 reprinted from Beltran WA, Cideciyan AV, Lewin AS, et al. Gene therapy rescues photoreceptor blindness in dogs and paves the way for treating human X-linked retinitis pigmentosa. *Proc Natl Acad Sci U S A*. 2012;109:2132–2137. © 2012 The Authors. (**C1–C3**) Peripheral retina from NHP after subretinal injection using hRGK1 promoter to target eGFP (*green*) expression to rods and cones. hCAR antibody labels cones (*red*; **C1**), and colocalizes with eGFP in cones (**C3**). *White arrows* identify the same cone cells in the three images; *scale bar*: 17 μm. ONL, outer nuclear layer. Figures C1–C3 reprinted with permission from Beltran WA, Cideciyan AV, Boye SE, et al. Optimization of retinal gene therapy for X-linked retinitis pigmentosa due to RPGR mutations. *Mol Ther*. 2017;25:1866–1880. © 2017 The American Society of Gene and Cell Therapy.

model at 7 weeks of age, the treatment is ineffective; efficacy, however, is obtained by a 10-fold increase in dose if administered at 5.7 weeks of age (Aguirre GD, et al. *IOVS* 2016;57:ARVO E-Abstract 2293). Preliminary studies have shown that switching the vector/promoter combination from AAV5-hIRBP- to scAAV8-hGRK1- or scAAV8$_{C\&G+T449V}$-hGRK1-results in recovery of cone function, and long-term preservation of structure and function when treatment is initiated at early and at mid/late stages of disease (Beltran WA and Aguirre GD, unpublished observations, 2017). These results suggest that for diseases that are genetically distinct but phenotypically similar, the vector/promoter used for the experimental studies may have to be disease specific, and that a hoped-for "universal" vector/promoter useful for a large class of similar diseases is not possible at this time. This complicates further translational applications, at least in the near term, until a sufficient database resource is obtained from animal studies and human clinical trials that will inform on vector/promoter selection.

Concepts and Strategies in Retinal Gene Therapy

Critically, translating findings from the cage to the bedside requires careful interpretation of the preclinical data based on the experimental studies, and a precise determination of how

closely the model disease parallels the human clinical phenotype. This information, along with a careful assessment of the natural history of the patient's disease, will determine when to treat, where to treat, how to treat, and how and when to evaluate the therapeutic outcome. The studies William Beltran and I have carried out with Samuel G. Jacobson and Artur V. Cideciyan are a valuable illustration of how model systems can be maximized to inform on clinical applications. Examples are studies done in *RPE65*-LCA,[5,23,69] *NPHP5*-LCA,[39,70] *RPGR*-XLRP,[50,71] and *RHO*-ADRP.[35,72–74] In this section, I will discuss three issues of relevance to translational applications.

Is Treatment Forever?

The proof-of-concept studies in both dog and mouse models of *RPE65*-LCA by several groups using different AAV vectors and promoters provided an impetus to finalize all the steps needed for clinical trials (see Supplementary Table S1). In addition to the product being safe[9,10] and effective, the treatment outcomes all showed stability of functional rescue, and three independent clinical trials were initiated and reported in 2008.[18–20]

The RPE was a very compelling cellular target for gene therapy, and the *RPE65*-LCA model is an ideal test bed for the first venture into this therapeutic modality. Firstly, the RPE is a homogeneous monolayer with an extensive apical microvillar network. Administration of vector by subretinal injection brings the vector into close proximity to the extensive RPE cellular processes without the need of crossing additional cellular barriers or the external limiting membrane. Secondly, AAV2 vectors readily target the RPE cells. Thirdly, tissue-specific promoters, for example, VMD2 and RPE65, limit expression to this cell layer, as does the constitutive hybrid CMV/CBA promoter, at least in the dog.[4,44] Of greatest significance, however, is the dramatic phenotypic change that occurs within a matter of a few weeks following treatment. Before therapy, the animal has searching nystagmus, has incomplete and delayed pupillary responses, and is functionally blind with only limited and poor vision at very high photopic luminances, and the ERG shows absent rod-mediated responses and absent or very low-amplitude and abnormal cone signals. Following treatment, all of these clinical signs are reversed, and functional vision is restored.[3,15,75] Thus an efficacy readout is obtained almost immediately with direct measurements and without the need for waiting months to years to assess outcomes, as would be the case in slowly progressive diseases. Even though the initial studies in the dog did not assess the stability of the treatment, this was demonstrated subsequently in longitudinal studies of a subset of the initially treated dogs and additional animals.[3–5]

While the proof-of-concept studies in *RPE65*-LCA dog and mouse models showed great efficacy, treatment was done when retinas were primarily dysfunctional with little or no degeneration. In contrast, the planned clinical trials treated patients after degeneration had already commenced. Indeed, a study has shown that of a group of clinically well-characterized young *RPE65*-LCA patients (6–17 years) all exhibited abnormal photoreceptor layer topography, and most had reductions in foveal and extrafoveal outer nuclear layers.[13] This is not surprising given that the only histopathology assessment of human retinas with *RPE65*-LCA showed prenatal degeneration.[76]

The initial clinical trials indicated that the treatment was safe and effective in the short term.[18–20,77] For example, in one study patients were followed for up to 36 months, and all showed improved visual function as measured with full-field stimulus testing.[21] However, long-term observation of the clinical trial patients in two studies showed continual loss of photoreceptors,[22] which, in treated areas, became comparable to untreated regions.[5,23] Based on the long-term efficacy of treatment in the dog model, the question arose as to whether the discrepant results between man and model result from a unique susceptibility of the human retina associated with the disease or its treatment, or if efficacy depends on the extent of degeneration at the time of treatment.

We have examined this question in a cohort of mutant dogs treated unilaterally at the stage of disease when only dysfunction is present (ages: 0.3–2.4 years), and followed noninvasively by ERG and optical coherence tomography (OCT) and terminally by histopathology (ages: 6.9–11.2 years). In parallel, a second cohort of unilaterally treated dogs was examined noninvasively by OCT and ERG after treatment at the dysfunction/degeneration stage of disease (ages: 4.9–6.6 years).[5] Early-treated dogs show recovery of rod and cone function that is sustained and preservation of outer nuclear layer integrity in the treated regions, both by OCT and by histopathology; the treated areas show RPE65 expression and preservation of rod outer segments[5] (Figs. 3IA–D, 3IIA$_{1\text{-}5}$, 3III). The late-treated dogs show recovery of rod and cone ERG function in the treated eyes, an indication that the therapy was successful, but noninvasive assessment of outer nuclear layer (ONL) structure showed degeneration that is comparable to that untreated regions[5] (Figs. 3ID, 3IIB$_{1\text{-}5}$). This is similar to the situation occurring in patients treated at the dysfunction/degeneration stage of disease.

The reason(s) for the short-lived positive treatment effect in patients, and in dogs treated at the dysfunction/degeneration stage of disease, is unknown. One group posits that their vector had insufficient potency to provide the required RPE65 enzymatic activity needed for long-term sustained gains in function and preservation of structure. Consequently an optimized AAV5-OPTIRPE65 vector has been developed that reportedly has 300-fold or greater RPE65 enzymatic activity,[78] and now is in clinical trials (NCT02781480) in the United Kingdom. A second group has proposed that the ongoing degeneration in the presence of rescued function emphasizes the need for combinatorial therapies that combine one of several neuroprotective, antiapoptotic, or other agent(s) as adjunct to the specific gene augmentation therapy,[5] and these studies are ongoing. Yet another group questions the findings of the latter study,[79] but have not provided details yet that the cohort of patients treated in their initial clinical trial fail to show progressive degeneration and dysfunction when measured with the same quantitative retinal structure and visual function methods used in the other two trials.[18] What is clear is that in at least two clinical trials, progressive degeneration continues in spite of initial positive treatment effects. The ongoing studies to determine the cause and prevention of this unanticipated finding will be important for managing patients with this disease after treatments are commercialized, as well as informing on the basic biology of retinal diseases in general and the development of future treatments.

What Happens When Treatment Is a Success but the Patient Is Blind: CNTF-Mediated Photoreceptor Deconstruction in *CNGB3* Achromatopsia

Two mutations in canine *CNGB3* result in very severe loss of cone ERG function and photopic vision. These mutations, a ~500-kb genomic deletion and a missense change, result in an identical clinical phenotype.[33] The disease locus name, *cd* for cone degeneration, was based on the marked decrease in the number of cones at very late stages of the disease, but does not truly reflect the status of the cone photoreceptor mosaic in the

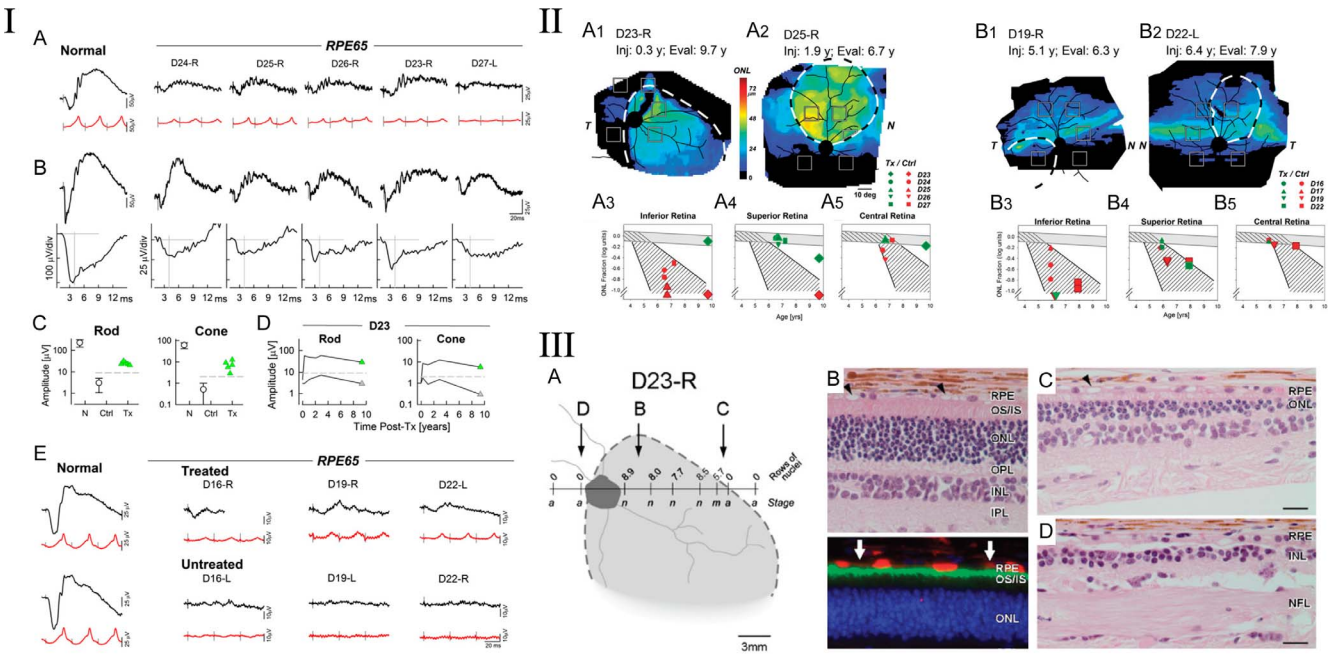

Figure 3. **I**. Long-term efficacy of rescued rod and cone retinal function in RPE65 mutants treated at an early disease stage and evaluated up to a decade later (**A–D**), or treated late and evaluated 1 month after the treatment (**E**). (**A**) Dark-adapted (DA) ERGs evoked by standard white flashes (*black traces*), or under light-adapted (LA) (*red traces*) conditions. *Black vertical lines* show the timing of the flashes. (**B**) ERG photoresponses evoked by white flashes of high energy under dark-adapted conditions; same data are shown on slow (*upper*) and fast (*lower*) time scales. *Gray lines* show the baseline and the 4-ms time point where rod photoreceptor responses were measured. (**C**) Comparison of rod and cone function in the treated RPE65-mutant dogs (Tx; *green triangles*) compared with previously published[4] normal (N) and untreated control (Ctrl) eyes. Rod function shown refers to the DA ERG photoresponse amplitude at 4 ms, and cone function refers to the peak amplitude of the LA 29-Hz waveform. *Horizontal dashed lines* represent the upper limit (mean + 3 SD) of the respective measurement in the group of untreated control eyes. (**D**) Durability of the rod and cone ERG amplitudes in dog D23 treated a decade earlier. Subretinally treated right eye is shown with *green symbols*, and the intravitreally treated left eye is shown with *gray symbols*. *Lines* show previously published data extending to age 3 years.[4] (**E**) Bilateral ERGs recorded with similar methods and stimuli as shown in (**A**) in a normal dog and three unilaterally treated older RPE65-mutant dogs (age range from 4.9 to 6.6 years). ERG recordings performed 1 month later show definite treatment effects in the eyes with gene therapy. **II**. Gene therapy outcomes in *RPE65*-mutant dogs treated before (**A1–A5**) and after (**B1–B5**) the onset of retinal degeneration. (**A1**, **A2**) Photoreceptor (ONL) thickness topography in two dogs treated before the onset of the degeneration and evaluated ~5 to 9 years later. There is retention of ONL thickness within the treatment region (*dashed lines*). (**A3–A5**) ONL thickness quantified as a function of age at five retinal locations in five dogs treated before the initiation of degeneration. *Red symbols* correspond to retinal locations outside the treatment region, and *green symbols* correspond to locations within the treatment region. (**B1, B2**) ONL thickness topography in two dogs treated after the onset of degeneration. There is no evidence for thicker ONL within the treatment regions compared with outside the treatment regions. (**B3–B5**) ONL thickness quantified as a function of age at five retinal locations in treated eyes. Both untreated control (*red symbols*) and treated (*green symbols*) regions are not substantially different compared with the natural history of disease. **III**. Retina of dog D23 treated at 0.3 year before the onset of retinal degeneration shows remarkable rescue of photoreceptors from degeneration when assessed over a decade later. (**A**) Schematic representation of the en face image showing the treatment area (*dashed lines*), on which is superimposed the ONL rows (mean of three values in each area sampled) and disease staging (a, advanced atrophy with gliosis and loss of retinal layer organization; m, moderate photoreceptor loss with 1/3 to 1/2 of ONL remaining; n, normal) assessed at 11.2 years. (**B–D**) Representative images taken from areas identified in (**A**). In the treatment area, there is normal retinal preservation (**B**), although the RPE shows vacuolated inclusions typical of the disease (*arrowheads*). At the edge of the treatment border (**C**), the photoreceptor layer becomes markedly attenuated and is absent (**D**) outside of the treatment region. Double immunolabeling with RPE65 (*red*) and rod opsin (*green*) taken from region corresponding to (**B**). RPE labeling: RPE is present inside the treatment region, and rod outer segment labeling is distinct. RPE, retinal pigment epithelium; GCL, ganglion cell layer; NFL, nerve fiber layer; OPL, outer plexiform layer; OS/IS, outer and inner segment layer; INL, inner nuclear layer; IPL, inner plexiform layer; *scale bar*: 20 μm. Figures and legends modified from Cideciyan AV, Jacobson SG, Beltran WA, et al. Human retinal gene therapy for Leber congenital amaurosis shows advancing retinal degeneration despite enduring visual improvement. *Proc Natl Acad Sci U S A*. 2013;110:E517–E525. © 2013 The Authors.

first 3 to 4 years of life. Affected dogs have small, abnormal cone ERG responses until ~8 to 10 weeks of age, which then disappear. The absence of cone function persists for the rest of the dog's life. Presumably, the presence of intact cyclic nucleotide gated channel alpha 3 (CNGA3) protein in these mutant retinas allows for transient formation of functional CNGA3 homotetramer channels, and cone function, albeit abnormal, is present early during development.[48,80]

Subretinal injections of a therapeutic transgene (AAV5-PR2.1-*hCNGB3*) restored ERG cone function and photopic vision in *CNGB3* mutants regardless of the mutation class. Long-term assessment in a subset of treated dogs showed that cone flicker was preserved stably for more than 2.5 years following treatment[48] (Komaromy AM and Aguirre GD, unpublished observations, 2017; Figs. 4A, 4B). Recovery of cone function following gene therapy was accompanied by the restoration of normal cone phototransduction protein localization to the cone outer segments in treated regions. Specifically, while the cone phototransduction proteins, GNAT2 and CNGA3, were mislocalized from the outer segment to elsewhere in the cone cell in the untreated mutant retinas, *hCNGB3* augmentation resulted in the proper localization of these proteins in the L/M-cone outer segments[48] (Fig. 4E).

These initial studies established treatment efficacy, and, in concordance with the promoter assessment[56] (Supplementary Table S2), confirmed that the PR2.1 promoter was the most

Table 2. Cone Function Rescue in *CNGB3* Mutants After Gene Augmentation Therapy; Effect of Age and Treatment With CNTF Prior to Gene Therapy With AAV5-PR2.1-*hCNGB3*

Studies	No. Eyes	Age, y	*CNGB3* Genotype		Sustained Cone Function Rescue	
			CNGB3$^{-/-}$	CNGB3$^{m/m}$	Yes	No
Study 1[48]						
Vector	14	≤0.54	11	3	11	3, CNGB3$^{-/-}$
Vector	3	≥1	0	3	1	2
Study 2[81]						
CNTF+vector	7	1.2–3.5	4	3	7	0
PBS+vector	7	1.2–3.5	4	3	0	7

Eyes treated with intravitreal CNTF (12 μg in 30 μL PBS) or PBS (30 μL) 1 week prior to subretinal injection of AAV5-PR2.1-*hCNGB3* (injection volumes 140–200 μL; dose = 7.96×10^{11}–4.02×10^{13} vg/ml; the same vector dose was used in pairs of eyes pretreated with intravitreal CNTF or PBS). For additional details, see Table 1 in Ref. 81.

effective in producing a sustained recovery of cone function. The shorter versions of the human red cone opsin promoter, PR0.5 and 3LCR-PR0.5, were not effective in treating young animals; and recovery of cone function either did not occur or was transient, and *hCNGB3* transgene expression, in general, was low[48] (Fig. 4C). However, studies using the AAV5-PR2.1-*hCNGB3* therapeutic vector did reveal an apparent age-dependent effect in the rescue of cone function. While 11 of 14 eyes recovered cone function when treated at less than 0.5 years of age, only 1 of 3 did so when treatment was initiated after 1 year of age. This absence of functional rescue was not due to cone loss in older retinas as cone loss is gradual, and at 1 year of age the superior central region of the retina, the region targeted for therapy, still retains ∼80% and 97%, respectively, of the L/M- and S-cone numbers when compared to control.[81] Similarly, treatment failure was not due to inefficient targeting of mutant cones, as *hCNGB3* mRNA expression in the "nonrescued" retinas was comparable to or only slightly lower than in successfully treated eyes (Fig. 4C). In addition, untreated mutant retinas had levels of cone gene expression (*CNGA3*, *CNGB3* [present only in missense mutants], *L/M-* and *S*-cone opsins) that were comparable to wild type, an indication that the principal components underlying cone function are not compromised.[48] Based on these findings, we posited that treatment failure in these eyes resulted from the inability of the structurally stable mutant cone outer segment to assemble functional CNG channels, despite the expression of both channel subunits after treatment. We further reasoned that if cones could reform an outer segment at the time of treatment, functional channels would be assembled. Such an approach would have required the transient elimination of the cone outer segment structure without permanently impairing their long-term viability and function. This effect can be mediated by ciliary neurotrophic factor (CNTF), and we have used it as a therapeutic adjunct to gene therapy.[81]

Intravitreal injection of CNTF in the rat retina leads to a marked shortening of the photoreceptor outer segments and decrease in photoreceptor gene expression; maximal effects occur within 3 to 6 days after injection, and are fully reversible within 3 weeks.[82] Similarly, intravitreal CNTF in the normal dog retina has a maximal effect by 1 week in terms of decreased rod and cone ERG amplitudes, shortening of rod, S- and L/M-cone outer segments, and rod and cone gene expression. By 5 weeks after treatment the retina returns to normal. As the changes are reversible and photoreceptors transiently become more immature immediately following CNTF, we have termed this process transient photoreceptor deconstruction.[81] Although the effects are panretinal and affect rods and cones equally, for the purpose of the *CNGB3* gene therapy work, the cell of interest for the effect is the cone.

To determine if CNTF-mediated transient photoreceptor deconstruction would enhance cone functional rescue in older *CNGB3* mutant retinas, we injected eyes from older (age range, 1.2–3.5 years) mutant dogs with either 30 μL CNTF (∼4–5 μg/mL vitreous) or PBS 7 days prior to a subretinal injection of AAV5-PR2.1-*hCNGB3*. Significantly, all seven mutant eyes pretreated with CNTF had sustained recovery of cone function following *hCNGB3* gene augmentation, an effect that was not found in any of the seven eyes pretreated with PBS[81] (Table 2). Quantitative RT-PCR assessment of *hCNGB3* therapeutic transgene levels indicated comparable expression levels between PBS- and CNTF-pretreated retinas (Fig. 4D). However, only the CNTF-pretreated retinas showed the proper localization of GNAT2 and CNGA3, two cone phototransduction proteins required for normal function, in the L/M-cone outer segments (Fig. 4E); as a specific CNGB3 antibody was not available, the expression of this critical protein and its localization could not be determined.

The achromatopsia gene therapy studies in the canine model raise important translational issues. First, will patients have cones present at the age of treatment? Recent studies combining high-resolution OCT and adaptive optics scanning light ophthalmoscopy have shown that while patients have lower than normal numbers of foveal cones, those remaining likely provide suitable therapeutic targets for gene augmentation.[83] Furthermore, a 6- to 26-month short-term longitudinal study of *CNGB3*-achromatopsia patients reported that the fovea remained structurally stable.[83] Secondly, it is still an open question whether the need for CNTF-mediated photoreceptor deconstruction at later stages of the disease is a canine-specific effect or may be required as an adjunct to gene augmentation in human patients. In studies of gene augmentation in sheep with *CNGA3*-achromatopsia, successful cone functional rescue resulted regardless of the animal's age at the time of treatment.[84] This difference can possibly be explained by the ability of CNGA3, but not CNGB3 subunits, to form functional channels on their own.[80]

The issue of pretreatment with CNTF prior to *CNGB3* augmentation in ongoing clinical trials is not possible or practical, due in part to regulatory issues, but also because one cannot predict a priori which patients, if any, will require such treatment. Pretreatment, however, may not be necessary, as preliminary studies have shown that intravitreal CNTF administered after unsuccessful gene therapy rescues cone function in the mutant dog, and CNTF-Encapsulated Cell Therapy devices are able to effectively deconstruct cone photoreceptors in mutant dogs (Komaromy AM, unpublished observations, 2013). The CNTF ECT device (NT-501 ECT) from

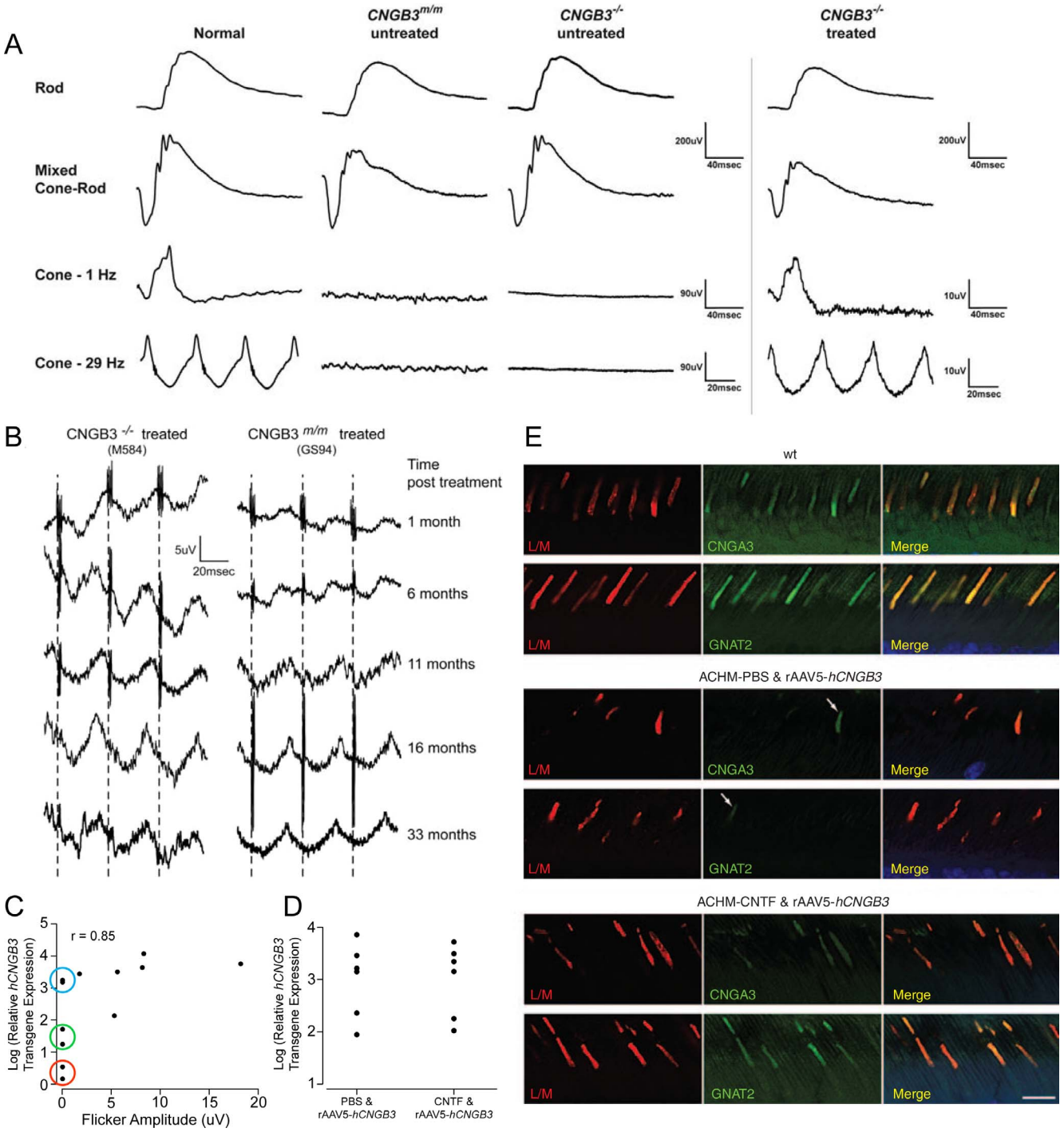

Figure 4. Gene therapy outcomes in *CNGB3*-achromatopsia. (**A**) *CNGB3* mutants with either a missense mutation ($^{m/m}$) or genomic deletion ($^{-/-}$) show normal rod ERG responses, but absent cone responses. Gene therapy restores the cone ERG responses (*far right column*), and the effect is sustained for at least 2.5 years (**B**). (**C**) Cone ERG flicker amplitude increased with higher *hCNGB3* transgene expression. Dogs with no recovery of cone function had low levels of transgene expression and were treated with the less robust 3LCR-PR0.5 promoter (*red circle*, treatment age 8, 23, 28 weeks; *green circle*, treatment age 60–81 weeks). The optimal PR2.1 promoter resulted in high levels of transgene expression in one dog (*blue circle*), but no cone function rescue when treatment was done at 54 weeks. Figures 4A–C reprinted from Komaromy AM, Alexander JJ, Rowlan JS, et al. Gene therapy rescues cone function in congenital achromatopsia. *Hum Mol Genet*. 2010;19:2581–2593. © 2010 The Author. Reprinted with permission from Oxford University Press. (**D**) Photoreceptor deconstruction with CNTF. The relative amounts of retinal *hCNGB3* mRNA expression were comparable and not significantly different when subretinal AAV injections were preceded by either intravitreal PBS (no cone function recovery) or CNTF (cone function recovery). (**E**) In the wild-type retina, CNGA3 and GNAT2 colocalize with L/M opsin in the cone outer segment (*top*). Gene therapy following intravitreal PBS (*middle*) fails to correct the mislocalization of CNGA3 and GNAT2 from the outer segment (*middle*). However, pretreatment with CNTF 1 week prior to gene therapy corrects the mislocalization in the now functional L/M cones (*middle*). *Scale bar*: 10 μm. Figures 4D, 4E reprinted with permission from Komaromy AM, Rowlan JS, Corr AT, et al. Transient photoreceptor deconstruction by CNTF enhances rAAV-mediated cone functional rescue in late stage *CNGB3*-achromatopsia. *Mol Ther*. 2013;21:1131–1141. © 2013 The American Society of Gene & Cell Therapy.

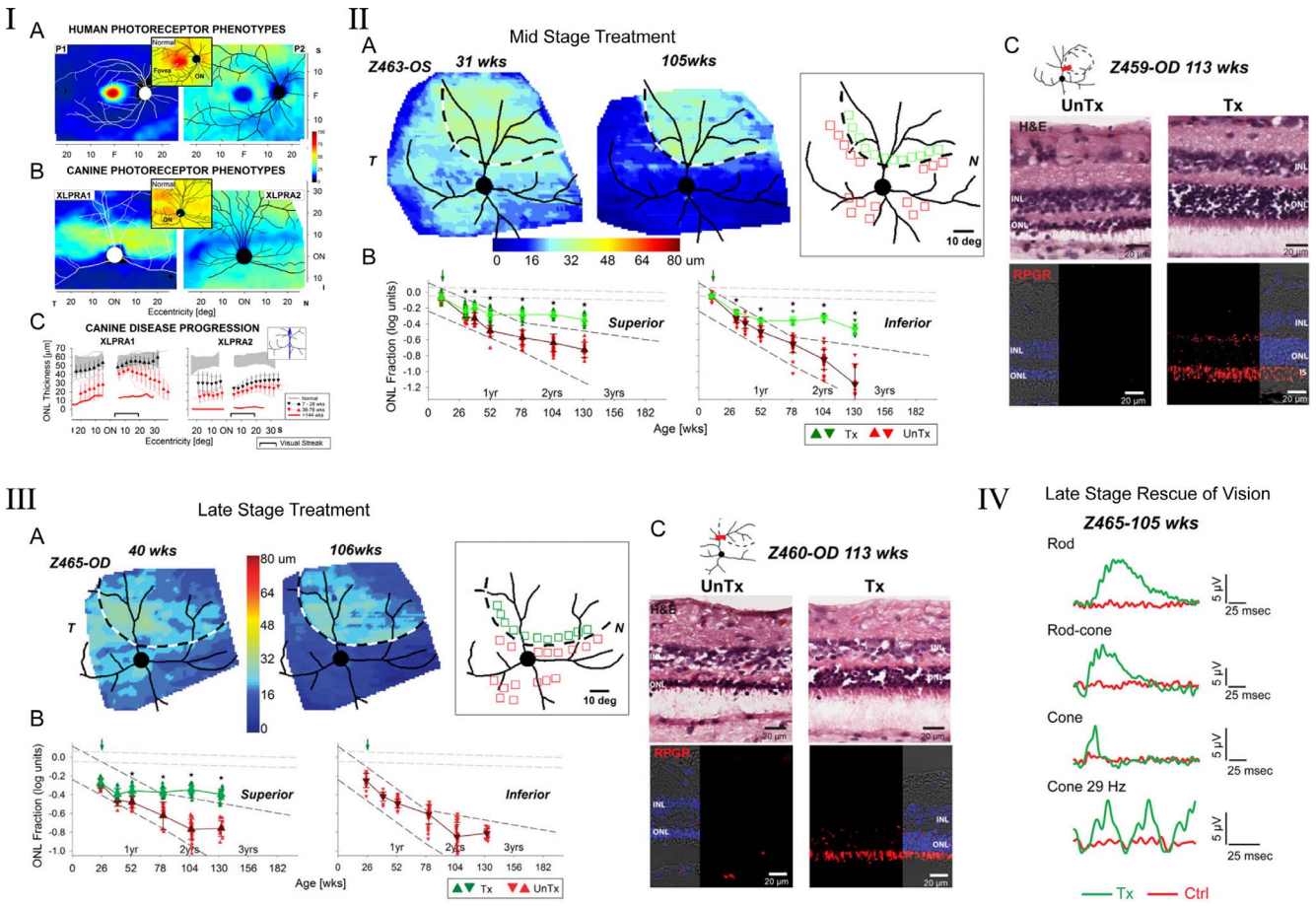

Figure 5. **I**. Retinal disease phenotypes caused by RPGR-ORF15 mutations in human patients and in dogs. (**A**) Different patterns of photoreceptor topography in two XLRP patients with RPGR mutations. ONL thickness topography is mapped to a pseudocolor scale. (*Inset*) Representative normal subject. Locations of fovea and optic nerve (ON) are shown. (**B**) Different patterns of photoreceptor topography in the canine models of RPGR-ORF15; mapping as performed with the human data. (*Inset*) Map of a representative wild-type dog with location of ON labeled. (**C**) ONL thickness profile along the vertical meridian (*Inset*) comparing XLPRA1 and XLPRA2 of different ages (*thin traces*) versus normal results (*gray band*). Mean (±SD) results are from groups of younger (7–28 weeks) and older (36–76 weeks) dogs. The *thicker red line* represents the data from the oldest dogs examined (>144 weeks old). *Brackets* mark the location of the high photoreceptor density corresponding to the canine visual streak. Figures and legends in **I** modified from Beltran WA, Cideciyan AV, Lewin AS, et al. Gene therapy rescues photoreceptor blindness in dogs and paves the way for treating human X-linked retinitis pigmentosa. *Proc Natl Acad Sci U S A*. 2012;109:2132–2137. © 2012 The Authors. **II, III**. Efficacy and long-term stability of gene therapy intervention at (**II**) mid-stage and (**III**) late-stage disease. (**A**) Pseudocolor maps of ONL thickness topography in XLPRA2 dogs treated at 12 (mid-stage) and 26 (late-stage) weeks of age. *Dashed outline* is the retinal region corresponding to the subretinal vector bleb at treatment. Schematic, *right*, paired loci across the treatment boundary and in the inferior retina chosen for quantitative evaluation. Eyes are shown as equivalent right eyes with optic nerve and major blood vessels overlaid for ease of comparability. T, temporal; N, nasal retina. (**B**) Progressive changes in ONL fraction recorded serially between 11 (mid-stage) and 25 (late-stage) weeks through to 130 weeks of age in treated (*green*) and untreated (*red*) loci in the superior and inferior retinas of three XLPRA2 dogs treated for each disease stage. None of the three late-stage treated eyes received injection in the inferior retina; thus, only untreated loci are shown in inferior retina. *Vertical green arrows* depict the timing of treatment. *Dashed lines* show the range of ONL fraction expected in wild-type eyes or natural history of progression in untreated XLPRA2 eyes. *Smaller symbols* represent the individual data and *larger symbols with error bars* represent mean ± SD; $^*P < 0.01$ for paired *t*-tests between treated and untreated loci. (C) Retinal morphology at 113 weeks of age in the untreated (UnTx) and treated (Tx) areas of a dog injected at mid- and late-stage disease and immunohistochemistry labeling of stable human RPGR transgene product, which is present only in treated areas. **IV**. Long-term durability of retinal function after gene therapy intervention at late-stage disease. Representative ERG traces of rod and mixed rod–cone responses recorded dark-adapted and cone responses to single stimuli, or 29-Hz cone flicker recorded light-adapted. Figures and legends in **II**, **III**, and **IV** modified from Beltran WA, Cideciyan AV, Iwabe S, et al. Successful arrest of photoreceptor and vision loss expands the therapeutic window of retinal gene therapy to later stages of disease. *Proc Natl Acad Sci U S A*. 2015;112:E5844–E5853. © 2015 The Authors.

Neurotech (Cumberland, RI, USA) is commercially available and approved for the treatment of macular telangiectasia.

Developing Treatments at Patient-Relevant Disease Stages

Proof-of-principle studies optimize successful outcomes by using animals prior to or during the early disease stages to eliminate confounding disease variables, and determine the optimal vector, promoter, transgene, and dose needed for effective therapy. If treatment fails under these ideal conditions, further preclinical and clinical development of the therapy usually is not warranted unless alternative data from other model systems, for example, cell culture, human induced pluripotent stem cells (IPS cells), are available. Once treatment success is established, optimizing the treatment at patient-relevant disease stages is critical to inform and direct the translational studies that develop the actual treatments. It is at this stage that treatments often fail, either because the model does not recapitulate the essential features of the human

disease, or because the disease is so aggressive and rapidly progressive that treatments are not effective. The lack of sustained efficacy in the initial *RPE65*-LCA clinical trials serves as an important lesson[5,22,23] to emphasize that translation to the clinic following successful proof-of-concept results[3] should be based on studies in which efficacious treatments are done at the patient-relevant disease stages, and in which detailed information is generated a priori on the natural history of the disease in the model and man. Such information will determine when to treat, where to treat, how to treat, and how and when to evaluate the therapeutic outcomes.[85] The *RPGR*-XLRP studies in the canine model illustrate this optimal approach.

In the dog, two naturally occurring distinct microdeletions in ORF15 result in different disease phenotypes referred to as X-linked progressive retinal atrophy 1 (XLPRA1; del 1028-1032) and XLPRA2 (del 1084-1085). XLPRA1 is juvenile but postdevelopmental in onset, and progresses over several years; XLPRA2 is early onset and rapidly progressive.[36] Both models correspond to the disease spectrum of human X-linked retinitis pigmentosa (XLRP), and, although differing in relative severity, they would be equivalent to human disease occurring within the first decade of life. XLPRA1, as in many *RPGR*-XLRP patients, shows dramatic photoreceptor loss peripherally, with relatively greater retention of ONL thickness at and near the central visual streak region. In contrast, XLPRA2 is characterized by loss of central photoreceptors and diseased, yet better preserved, peripheral photoreceptors[50] (Figs. 5IA–5IC).

Based on the disease topography in the XLPRA2 model, we directed treatments to the superior nasal quadrant to avoid issues concerning greater central versus peripheral loss of photoreceptors.[50] Gene augmentation with *AAV5*-hIRBP-*bRPGR* vectors showed that XLPRA1 disease was prevented when treatment was initiated in the predegenerate stage (treatment: 28 weeks; termination: 77 weeks), when photoreceptor structure and function remained normal. Treatment of XLPRA2 retinas at 5 weeks, just before the peak of cell death,[67] showed rescue of rod and cone function along with structural preservation of photoreceptors and ONL by 33 weeks of age. At this age, the bipolar dendritic arbors had reformed and inner retinal remodeling was abrogated in treated areas.[50] Treatment at this age shows ~3 year stability of rescued rod and cone function, vision, and structure.[68]

To determine if treatment is still successful if delayed until more advanced disease stages, we carried out studies in older animals. Two disease stages were selected as these represent intermediate time points in the degenerative process that are representative of disease stages in the patient population. At the mid-stage and late-stage disease, the mutant retina had lost ~40% and 60% of the photoreceptors and corresponding ONL. Unilateral treatment of affected dogs showed a remarkable arrest of further disease progression. This could be monitored over time noninvasively by mapping ONL topography, and by objectively assessing ERG function and vision using an obstacle-avoidance course and a forced two-choice Y maze (Figs. 5IIA–5IIC; 5IIIA–C, 5IV). Not only was the treatment successful, but it also was stable, and there was no further progression in disease over the 2.5+ years of posttreatment assessment.[68] Such efficacy and stability, regardless of the disease stage at the time of treatment, holds promise for forthcoming clinical trials.

Summary

Gene therapy as a therapeutic modality for treating previously incurable forms of retinal blindness is making great advances since the successful proof-of-concept studies of canine *RPE65*-LCA in 2001.[3] The field is still young, but the excitement in both the scientific community and patient advocacy groups has been energizing. I feel fortunate to be part of this therapeutic adventure, and to have collaborated with superb colleagues who continually make this work enjoyable and exciting. Of equal importance, I am proud to have conveyed to the scientific community the importance of the canine model of inherited retinal degeneration as a model for disease gene discovery, for investigating molecular mechanisms of disease, and, most important, for developing therapies to treat human and canine retinal blindness. Such studies truly confirm that dogs are man's best friend.

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