

THE USE OF CANINE MODELS TO DEVELOP TRANSLATIONAL GENE THERAPIES FOR THE TREATMENT OF SIX FORMS OF INHERITED RETINAL DEGENERATIONS (WORK CONDUCTED AT THE UNIVERSITY OF PENNSYLVANIA-USA)

UTILISATION DE MODÈLES CANINS POUR DES APPROCHES DE THÉRAPIES GÉNIQUES DÉVELOPPÉES À L'UNIVERSITÉ DE PENNSYLVANIE (USA) DANS LE TRAITEMENT DE SIX MALADIES HÉRÉDO-DÉGÉNÉRATIVES DE LA RÉTINE EN VUE D'APPLICATIONS TRANSLATIONNELLES

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ABSTRACT

Identification of genetic mutations responsible for inherited retinal degenerations in a variety of breeds of dogs, coupled with phenotypic characterization of the natural history of disease has provided large animal models that have had a significant impact in translating preclinical discoveries in retinal gene therapies to humans. More than 20 years ago, proof of concept studies conducted in a dog model of childhood blindness led to the first retinal gene therapy to receive market approval in the US and EU. Since this first breakthrough success, vision scientists from the Schools of Veterinary Medicine and Medicine of the University of Pennsylvania have pursued the use of patient-relevant canine models to test and move to the clinic novel retinal gene therapies for five additional diseases.

Key-Words: dog, canine model, retina, degeneration, dystrophy, gene therapy

RÉSUMÉ

L'identification de mutations génétiques responsables de dégénérescences rétinienne héréditaires dans plusieurs races canines, couplée au phénotypage de l'évolution naturelle de ces maladies, a fourni des modèles « grands animaux » qui ont largement contribué à l'application clinique de découvertes en thérapies géniques chez l'homme. Il y a plus de 20 ans, des études preuve-de-concept réalisées dans un modèle canin de cécité infantile ont permis l'obtention d'autorisations de mise sur le marché aux États-Unis et dans l'Union Européenne pour la première thérapie génique rétinienne. À la suite de ce premier succès, des chercheurs en sciences de la vision des Écoles Vétérinaire et de Médecine de l'Université de Pennsylvanie ont continué d'utiliser des modèles canins afin de tester de nouvelles thérapies géniques pour cinq autres maladies en vue d'essais cliniques chez l'homme.

Mots-Clés : chien, modèle canin, rétine, dégénérescence, dystrophie, thérapie génique

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INTRODUCTION

The explosion of genetic/genomic information has enabled studies identifying genetic traits and diseases in dogs; the lack of genomic resources before 1990 prevented such studies. The first linkage map of the dog genome published in 1997 (Mellersh *et al.* 1997) led to the rapid mapping and subsequent identification of the first autosomal retinal disorder of dogs, progressive rod-cone degeneration (prcd). Now, high density SNP chips as well as targeted, exome, whole genome, or RNA sequencing, etc. are possible due to the sequencing of the canine genome (Kirkness *et al.* 2003, Lindblad-Toh *et al.* 2005). Leading the way in developing the needed tools and resources has been the study of inherited retinal diseases, and there are now more identified genes/mutations in canine retina than for any other organ system (Miyadera *et al.* 2012, Winkler *et al.* 2020).

Identification of new genes/mutations causing inherited retinal diseases has had a major impact in reducing the incidence of blindness in canine populations through implementation of DNA testing by commercial resources. For autosomal recessive diseases which comprise the majority of canine inherited retinopathies, this has allowed the breeding of affected or carrier dogs of superior quality to genetically normal dogs. The strategy avoids producing clinically affected dogs while retaining important genetic qualities, and slowly decreasing the frequency of mutant alleles in the population. A previous study (Aguirre *et al.* 1999) and yearly summaries provided by OptiGen, LLC to breed clubs for specific breeds/diseases clearly showed the decrease in mutant allele frequencies over time by implementing this strategy.

In parallel, the gene discovery and phenotype characterization studies have emphasized the close similarity in disease expression between human patients and dogs when affected by homologous gene defects (Miyadera *et al.* 2012, Winkler *et al.* 2020). In contrast, mouse models have disease phenotypes that poorly if at all recapitulate the spectrum of clinical abnormalities in patients, e.g. no phenotype compared to the dog and human disease (Best vitelliform macular degeneration (BVMD)) (Guziewicz *et al.* 2007, Marmorstein & Kinnick 2007, Guziewicz *et al.* 2017), or very mild/slowly progressive phenotype (RPGR-X-linked retinitis pigmentosa (XLRP) (Beltran *et al.* 2006, Beltran *et al.* 2009, Thompson *et al.* 2012) or most aggressive and severe phenotype (NPHP5-Leber congenital amaurosis (NPHP5-LCA)) (Downs *et al.* 2016, Ronquillo *et al.* 2016). The canine retinal degenerations are bona fide models of the human diseases.

The close similarity of the homologous canine and human diseases is one of several factors that have contributed to the dog's preeminence as a translational model to develop, treat and establish outcome measures (Beltran *et al.* 2015) that are equally applicable to patients. Among the advantages of the dog over laboratory rodents (mice, rats) is its eye size (Gilger *et al.* 2005, Tuntivanich *et al.* 2007) that is comparable to the human so that issues of vector delivery and dosing can be assessed more accurately than in smaller animal species. Also, its diurnal behavior enables to easily assess visual performance under both scotopic (rod-mediated), mesopic (mixed rod-cone mediated) but also photopic (cone-mediated) conditions of illumination (Gearhart

et al. 2008, Garcia *et al.* 2010). The latter is specifically supported by a higher density of cones that are spatially distributed in central retinal areas of higher specialization. Indeed, unlike rodents, the dog has a structurally well-defined cone-enriched fovea-like region (Beltran *et al.* 2014) within the center of the *area centralis* (Mowat *et al.* 2008) which is comparable to the foveo-macular area in man, albeit with no macular pigment nor a foveal pit. This area, which is affected early and very severely in several human and canine diseases, can be readily targeted by delivering therapeutic vectors subretinally under the fovea-like region or intravitreally over the area when this route of administration is optimized. Finally, with the exception of visual acuity charts and perimetry which require a subjective response by patients, the same instruments used in a research environment, ERG, sdOCT/cSLO fundus imaging among others, are used in the clinic without the need of modification. Although the cost for maintaining canine research colonies, and conducting translational investigations with this species is significantly higher than that required when working with rodents, it is fair to say that it is the success of the preclinical work conducted more than 20 years ago in a canine model of childhood blindness that led the way to Phase 1-3 clinical trials, and the commercialization after approval by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) of the first retinal gene therapy, voretigene neparvovec-rzyl (Luxturna™), for bi-allelic mutation in RPE65.

In this manuscript we provide a brief overview of six corrective retinal gene therapies that our Division (www.vet.upenn.edu/ExpeRTs) at the School of Veterinary Medicine of the University of Pennsylvania has progressed sufficiently to be either commercialized, in Phase 1-3 clinical trials, in the investigational new drug (IND) enabling phase, or in the late proof of concept (POC) stage (Figure 1). These are primary diseases of the retinal pigment epithelium (RPE) or photoreceptors; in the latter case they affect the cones, rods, or both photoreceptor classes. A limitation of corrective gene therapies currently under investigation, including the six described in this review, is that they are by definition gene-specific. Since there are more than 250 genes identified in man to be causative of a form of inherited retinal disease (<https://sph.uth.edu/retnet/>), this would in theory require developing as many gene therapies to provide a treatment for each of these different conditions. As this may be both technically and economically challenging, other approaches including the delivery of neurotrophic factors, antiapoptotic agents, or gene-agnostic gene therapies that can rescue photoreceptors, or replace them (optogenetic strategies, cell therapies) are currently being investigated (Ku & Pennesi 2020). Recombinant adeno-associated viruses (rAAVs) are the most commonly used vector in retinal gene therapies and have currently been used in more than 30 clinical trials (Buck & Wijnholds 2020). It is non-integrating (its gene cassette remains in the nucleus as episomal concatemers), its transgene expression is stable for years in terminally differentiated non-dividing cells like photoreceptors and RPE, and it is generally considered as safe at therapeutic doses (Nuzbrokh *et al.* 2020), although there is increasing concern that it may be associated with the triggering of ocular immune responses (Bucher *et al.* 2021,

Mehta *et al.* 2021). Its packaging capacity is however limited to 4.7 kb. Delivery of larger genes (e.g. ABCA4, MYO7A) has been attempted with the equine infectious anemia virus (EIAV) (Trapani *et al.* 2014). EIAV is an integrating lentivirus capable of packaging transgenes up to 8 kb and was being used in clinical trials for Stargardt disease and Usher1B until studies were stopped because of lack of efficacy. AAV-based retinal gene therapies aimed at targeting photoreceptors and/or RPE are quasi-exclusively being delivered by subretinal injections, although efforts at finding potentially easier, and safer routes (e.g. intravitreal, suprachoroidal) are actively being investigated.

Development of retinal gene therapies in dogs often take advantage of the protracted course of disease to determine whether intervention can first prevent or stop the onset of disease when delivered at early stages (Acland *et al.* 2001, Beltran *et al.* 2012, Aguirre *et al.* 2021), and if successful, subsequent studies are then focused at determining the latest stage of disease that is still responsive to therapeutic intervention (Beltran *et al.* 2015, Gardiner *et al.* 2020). Such approach has translational value by helping predict the response to therapy when intervening at patient-relevant stages of disease.

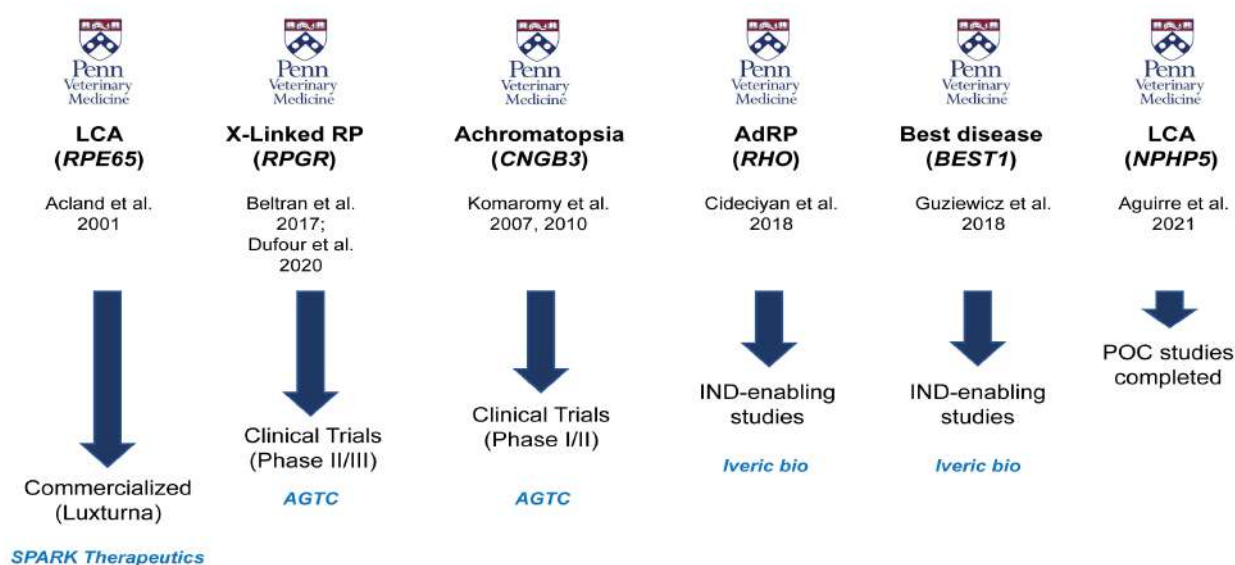


Figure 1: Stage of development of retinal gene therapy programs initiated at the School of Veterinary Medicine of the University of Pennsylvania using naturally occurring canine models. LCA: Leber congenital amaurosis; X-linked RP: X-linked retinitis pigmentosa; adRP: autosomal dominant retinitis pigmentosa; IND: investigational new drug (application); POC: proof of concept.

DISEASES OF THE RETINAL PIGMENT EPITHELIUM

Canine RPE65-Leber Congenital Amaurosis (LCA)

Leber congenital amaurosis is a rare genetic retinal disorder that affects photoreceptors (rods and cones) and/or the RPE and causes decreased visual responsiveness at birth or early infancy. LCA is a monogenic disease most often inherited in a recessive manner. Mutations in at least 26 genes have been implicated in LCA. Of these, mutations in the RPE65 gene account for approximately 10% of LCA.

In dogs, the disease originally was termed congenital stationary night blindness because of profound night vision defects and Riggs type ERG abnormalities, and later was called retinal dystrophy because day and night vision were found in affected Briards and fundus changes showed progressive degeneration with time (Narfström 1999). The early fundus changes reported by Narfström, however, have not been found by other investigators who have studied the disease in outcrossed breeding from

Briards or purebred dogs (Aguirre *et al.*, unpublished). Because of homology to the human disease caused by mutations in the same gene, RPE65, the appropriate disease designation is canine RPE65-LCA.

RPE65 is localized in the RPE, functions as an isomerohydrolase in the retinoid cycle, and converts all-trans retinyl esters to 11-cis retinol which is then changed to the aldehyde for eventual transport to the photoreceptor opsins to form the functional visual pigments in rods and cones. Absence of RPE65 results in profound visual function defects, and, depending on the species, causes early or late retinal degeneration. In the absence of the chromophore (11-cis retinal) constitutive activity of the opsin apoprotein causes continuous activation of transducing-mediated phototransduction (Woodruff *et al.* 2003). Through a mechanism still unclear this persistent activation of the visual cascade leads to progressive loss of photoreceptors (Lem & Fain 2004). Treatment of affected dogs by a single subretinal injection of the therapeutic vector targeting the RPE early in life resulted in a remarkable recovery of ERG function (Figure 2) and vision (Acland *et al.* 2001, Acland *et al.* 2005). Other investigative groups later showed similar functional restoration using different AAV constructs (Le Meur *et al.* 2007, Annear *et al.*

2011). The successful treatment of the previously incurable RPE65-LCA in a dog was followed by a series of clinical (Jacobson *et al.* 2005) and safety (Jacobson *et al.* 2006a, Jacobson *et al.* 2006b) studies that led to 3 independent Phase I clinical trials (Bainbridge *et al.* 2008, Cideciyan *et al.* 2008, Hauswirth *et al.* 2008, Maguire *et al.* 2008), and a Phase III trial (Russell *et al.* 2017). The results of the trials showed that the treatment was safe and effective and were followed by the approval of the therapy for marketing (Luxturna™, voretigene neparvovec-rzyl) in the US (2017) and Europe (2018).

of degeneration (Cideciyan *et al.* 2013). The area to be treated was analyzed by optical coherence tomography (OCT) to determine the photoreceptor integrity as assessed by outer nuclear layer (ONL) thickness maps and following treatment the dogs were followed for an additional 4-4.6 years. We found that treatment was successful long-term if the ONL was equal to or better than 63% of the normal thickness; only then treatment stopped the degeneration. However, if less than 63% of normal ONL, the degeneration continued (Gardiner *et al.* 2020). This information will be critical in selecting treatment areas in patients.

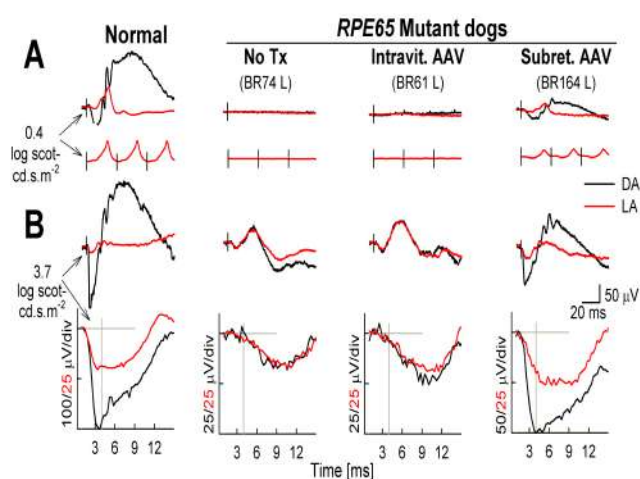


Figure 2: short term restoration of rod and cone function after a single subretinal injection of AAV-RPE65. (A) Representative ERGs evoked by standard white flashes (0.4 log scot cd.s.m⁻²) presented under dark-adapted (DA) and light-adapted (LA) conditions. (B) ERG photoresponses evoked by white flashes of high energy (3.7 log scot cd.s.m⁻²) under DA and LA conditions, same data are shown on slow (top) and fast (bottom) time scales to allow interpretation of late and early components, respectively. AAV: Adeno-associated virus. Reprinted with permission from (Acland *et al.* 2005).

But, is the treatment a cure forever? Once again, the canine model provided critical insights into the limitations of the therapy. The long-term consequences of treatment on visual function and arrest of retinal degeneration have been controversial. Data from some investigators are consistent with the hypothesis that the natural rate of photoreceptor loss due to RPE65 mutations is not modified by the gene therapy when treatments are initiated after the onset of degeneration in patients (Cideciyan *et al.* 2013), and the short term improvements in visual function start waning in the long term (Bainbridge *et al.* 2015, Jacobson *et al.* 2015). Data from others suggest that improved visual function is stable over the long term (Ashtari *et al.* 2017), and imply that retinal degeneration has been stabilized even though supporting details have not been provided. To address this clinically important question we carried out studies in dogs aged 4.9-6.3 years, well after the onset

Canine Best disease

The disease was initially described as Canine Multifocal Retinopathy (cmr) by Grahn in the Great Pyrenees breed (Grahn *et al.* 1998). Subsequent studies at the University of Pennsylvania School of Veterinary Medicine identified BEST1 as the gene responsible for cmr in this breed as well as in the Coton de Tulear; different mutations in the same gene are responsible for the disease, and we refer to them as cmr1 and cmr2 (Guziewicz *et al.* 2007). Lastly, studies of a phenotypically similar disease in the Lapponian Herder at the University of Pennsylvania identified a different causative mutation in BEST1 responsible for cmr3 (Zangerl *et al.* 2010). Of the 3 known cmr mutations, the most widespread one is cmr1 in which the English Mastiff breed is the founder breed, and is present in large breeds that are mastiff derived, e.g. Great Pyrenees (Zangerl *et al.* 2010). The one exception being the Australian Shepherd breed where the cmr1 mutation has been identified in one dog (Hoffmann *et al.* 2012). As a group, cmr is one of several diseases classed as canine bestrophinopathies.

Regardless of the mutation, homozygous affected dogs show focal to multifocal fundus lesions which develop after ~3-4 months of age. The multifocal lesions are primarily in the tapetal region although non-tapetal lesions also occur. These appear as serous retinal detachments with contents that appear gray with less fluid (Figure 3 B1, B2), brown, or serous with red/brown contents that settle to the lower aspects of the bullae (Figure 3C, black arrows) (Guziewicz *et al.* 2007). Focal lesions begin in the fovea-like region of the *area centralis* (Beltran *et al.* 2014) thus modeling the site of disease in human Best vitelliform macular dystrophy (BVMD). Initially, the bullae appear as clear (= vitelliform stage), but with disease progression the bullae expands and the dependent portions accumulate an autofluorescent brown precipitate suggestive of lipofuscin (pseudohypopyon stage) (Figure 4). With time the lesion expands, and satellite lesions similar to what occurs in humans with autosomal recessive bestrophinopathy (ARB) develop and eventually the large foveo-macular lesion atrophies (= atrophic stage).

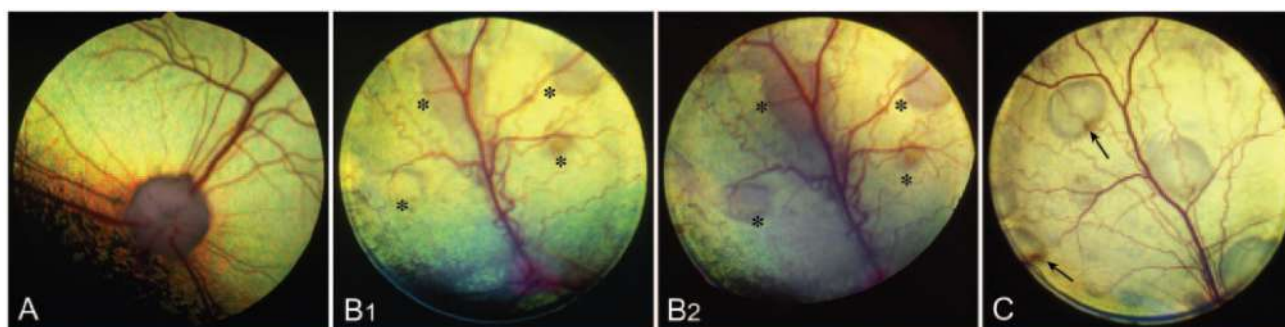


Figure 3: Fundusoscopic lesions seen in dogs affected with canine multifocal retinopathy (*cmr*). (A) Fundus of a normal dog. (B1-B2) multifocal tan-colored lesions (*) in a Great Pyrenees affected with *cmr1* at 5 (B1) and 8 (B2) months of age. (C) Multifocal retinal detachments (arrows) in a 18 month-old Coton de Tulear affected with *cmr2*. Reprinted with permission from (Guziewicz et al. 2007).

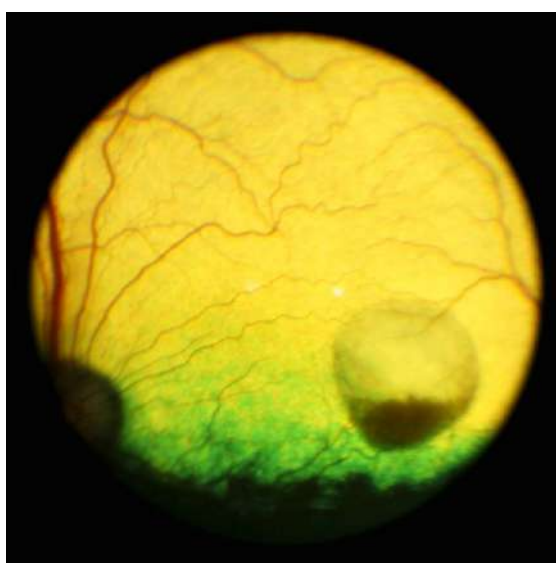


Figure 4: Fundus photograph of a classic *cmr* lesion at the pseudohypopyon stage in the area centralis of the left eye of a 21-month-old dog. (Image courtesy of Dr. K. Guziewicz, School of Veterinary Medicine, University of Pennsylvania).

The BEST1 protein is located in the baso-lateral aspect of the RPE cell membrane where it functions as a calcium-mediated chloride channel. With *BEST1* mutations, the apical microvilli of the rods and cones are underdeveloped which results in retina-wide microdetachments with separation of the retina from the RPE. Areas with ophthalmoscopically visible lesions represent actual focal detachments with accumulated photoreceptor and RPE debris (Guziewicz et al. 2017). A single subretinal injection targeting the RPE with a serotype 2 adeno-associated viral (AAV2) vector that has tropism for this cell layer along with a *BEST1*-specific promoter and a canine or human transgene reverses the microdetachments and focal/multifocal bullae (Guziewicz et al. 2018). The treatment is effective and long lasting. These impressive results are now being extended through the investigational new drug (IND) translational pathway in order to treat the first human patients in late 2023/2024.

DISEASE OF THE ROD PHOTORECEPTORS

Rhodopsin autosomal dominant progressive retinal atrophy

A form of progressive retinal atrophy (PRA) had been identified in the English Mastiff breed, but never formally reported in a publication as a clinical entity. Dr. Gregory Acland, who was in contact with the breed club and developed an interest in the disease, obtained several dogs to start a breeding colony at the Retinal Disease Studies Facility (RDSF) of the University of Pennsylvania. Dr. Acland noted that while disease was usually diagnosed in dogs ~3-4 years of age, dogs that had frequent ophthalmic examinations and fundus photography appeared to develop an earlier disease onset and a faster progression. Outcross breeding to WT Beagles showed that the progeny was also affected indicating that, unlike most forms of PRA which are autosomal recessive in inheritance, the disease in the English Mastiff was autosomal dominant (Kijas et al. 2002). A mutation in Rhodopsin was found as a single nonsynonymous C 3 G transversion at nucleotide 11 which changed Thr-4 to Arg (T4R) and co-segregated with the disease (Kijas et al. 2002). The mutation abrogates the first consensus glycosylation site on Asn2 in the protein; while it does not alter trafficking of newly synthesized opsin to the outer segment, it does affect the stability of the protein especially when the protein lacks the 11-cis retinaldehyde chromophore such as occurs after bleaching the dark-adapted retina with light (Zhu et al. 2004). Mutations that affect the second consensus glycosylation site at Asn15, e.g. T17M, also affect Rhodopsin stability and is a common cause of autosomal dominant Retinitis Pigmentosa (adRP) (John et al. 2000, White et al. 2007).

The T4R mutation renders the retina highly susceptible to light, and rod degeneration occurs following light exposures used under routine ophthalmic examination conditions (Cideciyan et al. 2005). Rod photoreceptor degeneration begins within 6 hours after acute light exposure and progresses for ~2 weeks; the extent and rapidity of degeneration, whether partial or complete, depends on the light intensity used (Cideciyan et al. 2005, Marsili et al. 2015, Iwabe et al. 2016). For example, light

intensities used in conventional fundus photography result in degeneration of the exposed regions and sparing of the unexposed regions (Figure 5). Similar susceptibility to light also is found in mice with T17M Rhodopsin mutations that affect the second consensus glycosylation site (White *et al.* 2007).

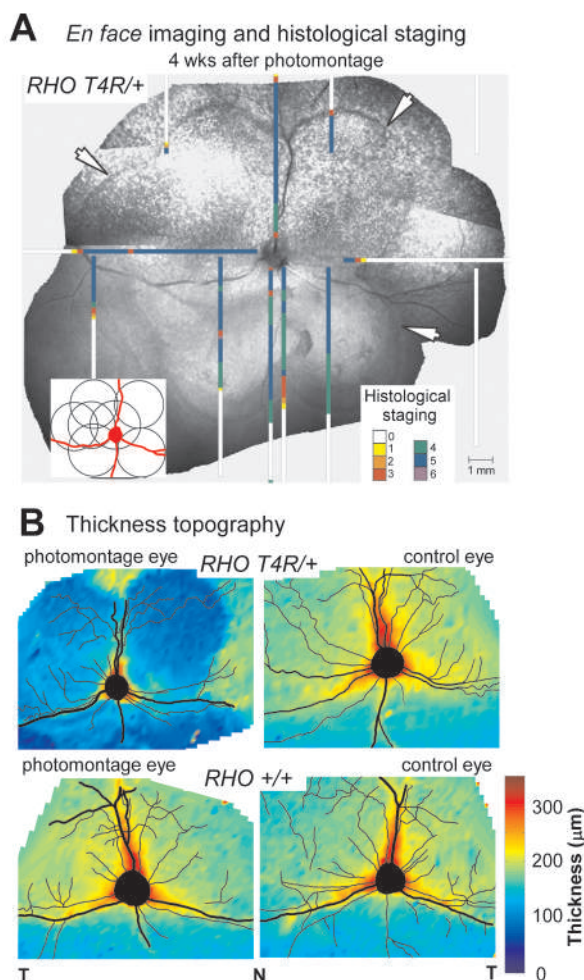


Figure 5: Light-induced retinal degeneration caused by fundus photography in a mutant RHO-T4R dog. (A) Digital montage of en face infrared images 4 weeks after fundus photography using seven standard overlapping fields (Inset). Superimposed on the retinal photograph are maps of retinal disease staging by light microscopy in this dog. Higher numbers represent more severe disease. (B) Topographical maps of full retinal thickness in a heterozygous mutant (RHO T4R/+) dog (top left panel) 4 weeks after a series of seven overlapping fundus photographs, and of its contralateral eye (top right panel) that was not exposed to flashes of light. Two bottom panels show retinal thickness maps of a wildtype dog (RHO +/+) after fundus photography (bottom left) and in the contralateral unexposed eye (bottom right). Modified and reprinted with permission from (Cideciyan *et al.* 2005).

Two issues with mutations in Rhodopsin have slowed progress to treatment. The first is that the mutant allele in this dominant disease is generally a gain of function rather than a null allele. That requires that the mutant allele be 'neutralized' (knockdown, KD) preferably in an agnostic manner so that the

same approach can be used in all patients with mutations in this gene. As this will also eliminate the wildtype allele, it is necessary to combine the KD with replacement of a normal copy of Rhodopsin that has been 'hardened', i.e. made resistant to the KD reagent used. Because it is important to deliver both the KD and replacement constructs to the same cell, it is essential that both are packaged in the same AAV vector. This has been accomplished using a short hairpin RNA (shRNA) and a KD-resistant version of human Rhodopsin. The dual function vector construct, scAAV2/5-hOP-RHO820-H1-shRNA820, has been shown to be safe and effective (Cideciyan *et al.* 2018).

The second issue is the exquisite light sensitivity of the T4R mutant retina which creates difficulties for clinical assessment, but also opportunities. To maintain the retina structurally normal long-term, dogs need to be housed under dim red-light conditions, and all clinical examinations and imaging must be carried out with near infrared/infrared illumination. As well, surgical procedures to administer vectors for gene therapy also has to be done under infrared illumination (Komaromy *et al.* 2008a). On the other hand, an advantage of the mutation is for assessing effective therapies as light exposure can be used to trigger degeneration and rapidly determine if the treatment is preventive. The combined KD-replacement vector injected subretinally has been highly effective in preserving the structure and function of the treated areas of the retina (Figure 6 A-B).

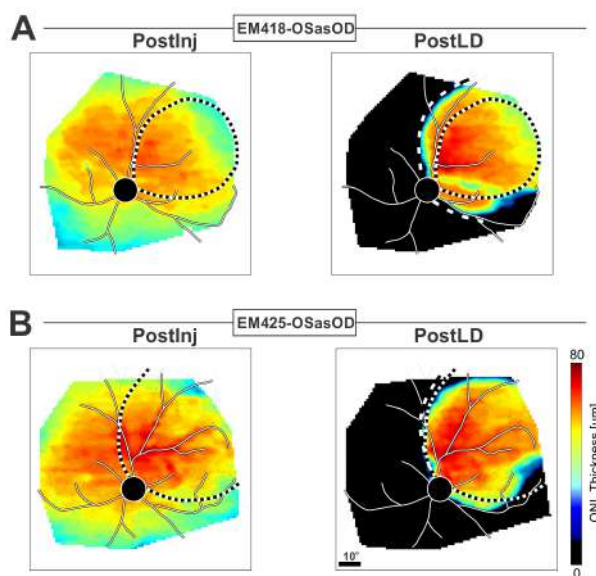


Figure 6: Use of the light sensitivity in the T4R RHO mutant dog for validation of a RHO gene knockdown and replacement strategy as a mutation-independent gene therapy for RHO-adRP. (A-B) topographical maps of ONL thickness in two T4R RHO mutant dogs subretinally-injected (dotted lines show the bleb boundaries) with a scAAV2/5-hOP-RHO820-H1-shRNA820 viral vector (PostInj), and 2 weeks post light exposure (PostLE). The treated areas show preservation of ONL structure (warm colors within the dashed lines, right figures) while the untreated areas of treated eyes show complete degeneration (black areas, right figures). Modified and reprinted with permission from (Cideciyan *et al.* 2018).

DISEASE OF THE CONE PHOTORECEPTORS

Cone Degeneration

A recessively inherited form of day blindness was reported by Rubin in the Alaskan Malamute breed (Rubin *et al.* 1967, Rubin 1971). The disease was previously known as hemeralopia. However, because of the discrepant definition of hemeralopia between French/Italian/Spanish (night blindness) and German/English (day blindness), this term is no longer in use (Long & Aguirre 1991). Cone degeneration in dogs also is referred to as achromatopsia which is the clinical term used in medical ophthalmology.

Affected dogs are profoundly day blind but have normal scotopic

visual function. ERG studies confirm the loss of cone function (Figure 7). Fundus examination shows no abnormalities at any age, and lesions in the fovea-like region of the visual streak (Beltran *et al.* 2014) are not present, at least in dogs less than 4 years of age. Retinal development in the mutant retina is generally normal, but cone outer segments are disorganized and disoriented, and neurofilament bundles accumulate in the perinuclear cytoplasm and the inner segments (Aguirre & Rubin 1974). With time, cone nuclei move into the inner segment which becomes elongated and extends to the retinal pigment epithelium (Long & Aguirre 1991). On histologic sections, the cone nuclei appear to rest on the apical surface of this cell layer (Gropp *et al.* 1996).

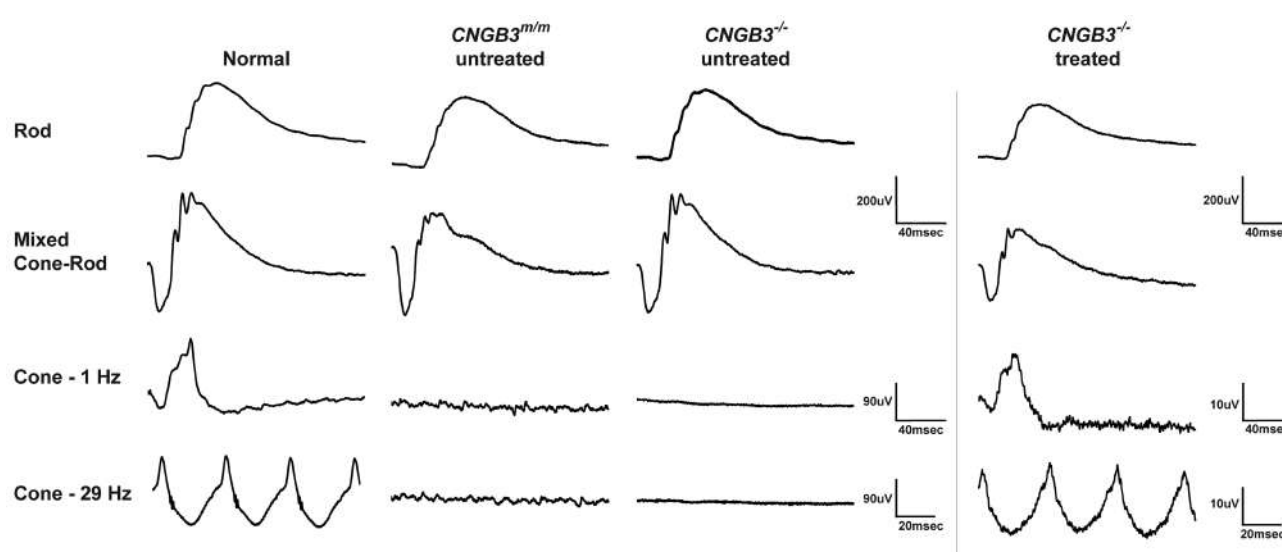


Figure 7: Normal rod function and absent of cone function in dogs with inherited cone degeneration caused by a missense mutation or a genomic deletion of CNGB3 (CNGB3^{m/m} or CNGB3^{-/-} respectively). Affected dogs have absence of cone function as illustrated using 1 or 29 Hz flickering light stimuli under light adapted conditions. The treated eye of the null CNGB3^{-/-} dog showed restoration of cone function 7 weeks after subretinal injection. Reprinted with permission from (Komaromy *et al.* 2010).

Linkage mapping of a cone-degeneration informative pedigree localized the candidate region to canine chromosome 29 (CFA29) in a region corresponding to the distal q arm of human chromosome 8. This region, in both man and dogs, has the cyclic nucleotide gated channel beta subunit (CNGB3), a cone specific gene involved in the last steps of cone phototransduction. A large genomic deletion encompassing the entire CNGB3 gene was found in Malamute dogs with cone degeneration. At the same time, a clinically identical disorder, but caused by a missense mutation in CNGB3, was identified in the German Shorthair Pointer breed, and interbreed crosses resulted in affected progeny (Sidjanin *et al.* 2002). We refer to the two allelic mutations a CNGB3^{-/-} and CNGB3^{m/m} to indicate, respectively, the complete deletion of the gene and the missense mutation. More recently, scientists at the School of Veterinary Medicine of the University of Pennsylvania have identified two different mutations in the cyclic nucleotide gated channel alpha subunit (CNGA3) in German Shepherd and

Labrador Retriever dogs (Tanaka *et al.* 2015). These two breeds have the same clinical phenotype resulting from the mutation, and, as well, to the dogs with CNGB3 mutations.

To develop a gene therapy that specifically targets cones, we initially tested recombinant AAV5 serotype with different versions of the human red cone opsin promoter and the human blue cone opsin promoter. The PR2.1 promoter gave robust and specific expression of a GFP reporter gene in M/L cones (Komaromy *et al.* 2008b). The AAV5-PR2.1 construct was used with the human CNGB3 cDNA as a therapeutic vector. When injected subretinally, treated dogs showed a recovery of cone function and photopic vision that was long-lasting provided dogs were less than 28 weeks at the time of treatment. Older dogs showed no functional recovery even though the expression of the therapeutic transgene, i.e. transduction efficiency, was comparable between older and younger treated eyes (Komaromy *et al.* 2010).

To address the difference in outcomes between younger and older treated eyes in eyes that had comparable numbers of cone cells remaining, we tested whether we could improve the assembly of the heterodimeric cGMP gated channel by treating older mutant eyes with the therapeutic vector ~1 week after 'deconstructing' the outer segments by prior intravitreal administration of ciliary neurotrophic factor (CNTF). CNTF results in marked but transient shortening or disappearance of outer segments which regrow and function normally (Figure 8) (Wen *et al.* 2006). Following subretinal gene therapy 1 week after intravitreal CNTF administration, 8 dogs ranging in age from 14.3-42 months of age at the time of treatment recovered cone function, and this recovery was sustained for the duration

of the study. In contrast, none of the fellow eyes of these dogs that were pre-treated with PBS recovered cone function. Immunohistochemistry of the CNTF-vector treated eyes shows that other cone phototransduction proteins, e.g. CNGA3 and GNAT2, localized to the cone outer segment in the now successfully treated eyes. In contrast, these proteins did not localize to the cone outer segments in PBS-vector treated eyes that failed to recover cone function (Komaromy *et al.* 2013). Photoreceptor deconstruction by CNTF also is effective in older treated eyes that failed to regain cone function following gene therapy. Such post-gene therapy deconstruction has potential to be used in patients that fail to regain function following initial therapeutic intervention.

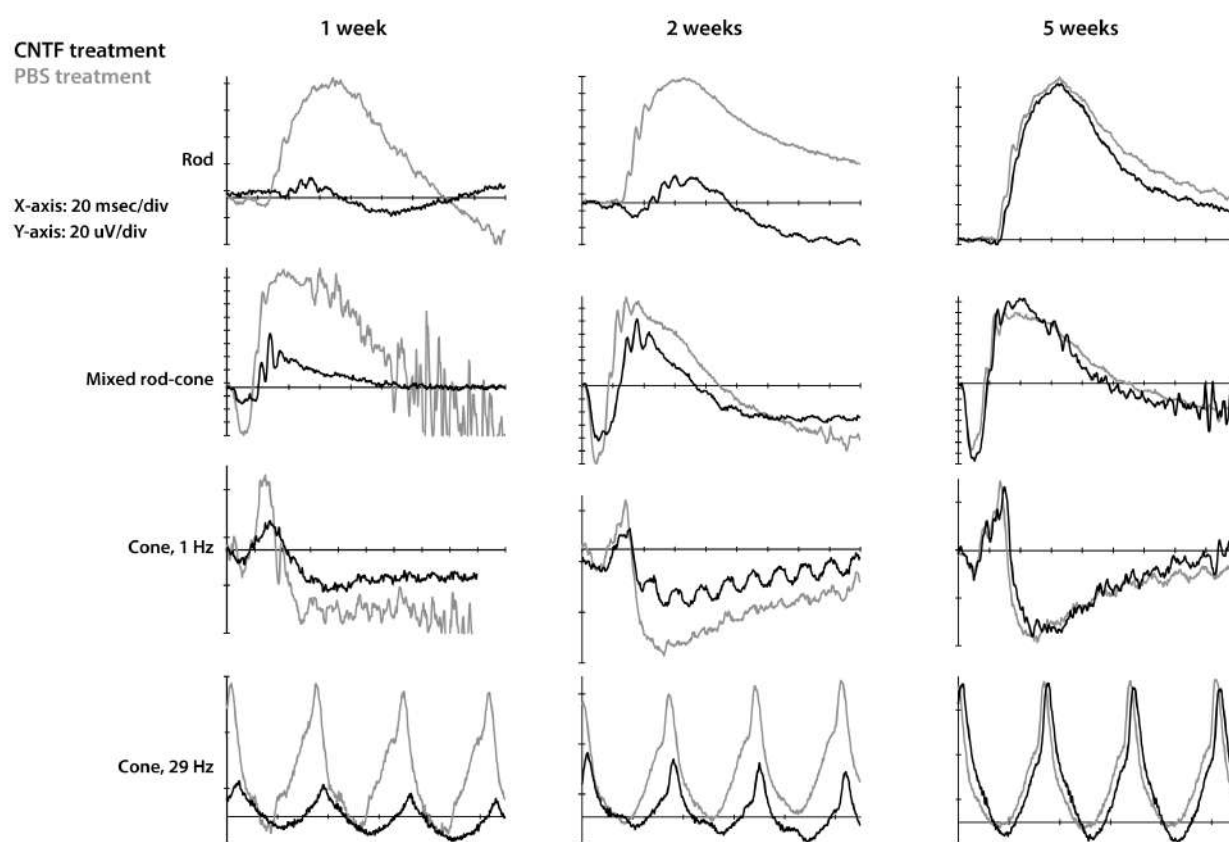


Figure 8: Intravitreal CNTF results in a transient decrease in rod- and cone-mediated ERG responses in normal retina. In comparison with intravitreal PBS that had no effect on the ERG (gray traces), intravitreal CNTF (black traces) markedly reduced rod and cone amplitudes. Retinal function was most severely affected at 1 week, partially restored at 2 weeks, and returned to normal at 5 weeks after injection. Reprinted with permission from (Komaromy *et al.* 2013)

DISEASES OF THE ROD AND CONE PHOTORECEPTOR CELLS

NPHP5-Leber Congenital Amaurosis

An autosomal recessive retinal disorder was reported in the American Pit Bull terrier and characterized by severely compromised vision, abnormal ERGs with markedly reduced or absent rod responses and non-recordable cone signals, and early onset retinal degeneration. The disease was termed cone-rod degene-

tion 2 (crd2) because of the cone predominant nature of the disease (Kijas *et al.* 2004). Genetic linkage and fine mapping of the disease locus identified a C insertion in exon 10 of NPHP5 (IQCB1) that results in a 12 amino acid frame-shift and truncates ~45% of the C-terminal amino acids (Goldstein *et al.* 2013). In the normal retina, the protein is localized to the photoreceptor ciliary transition zone, aka connecting cilium, and together with other proteins located in this narrow structure, mediates transport between the inner to the outer segment (den Hollander *et al.* 2008). The NPHP5 ciliary protein

also functions in the kidney, and mutations cause the retinal-retinal disorder Senior Loken syndrome, one of several nephronophthisis disorders (Hildebrandt & Otto 2005). Unlike humans where the renal component is a frequent and severe associated problem, dogs with NPHP5 mutations do not have kidney disease (Downs *et al.* 2016), and thus do not model the syndromic condition described in people. So far, all retinal ciliopathies identified in dogs (NPHP5-LCA, RPGR-XLPRA, NPHP4-CRD) are non-syndromic and show only an ocular phenotype (Zhang *et al.* 2002, Wiik *et al.* 2008, Downs *et al.* 2016).

Retinal structural abnormalities are present during postnatal retinal development. As photoreceptors are differentiating, cell death as assessed by TUNEL labeling is beginning and reaches a peak at postnatal week 5; thereafter cell death continues, albeit at a much-reduced rate. The developmental abnormality is characterized by rod outer segments which are disoriented and misaligned. In contrast, cone outer segments fail to develop even though the population number of cones in the retina is the same as in normal dogs. Lack of cone outer segments explains why cones fail to function even though their numbers are unchanged (Downs *et al.* 2016) (Figure 9).

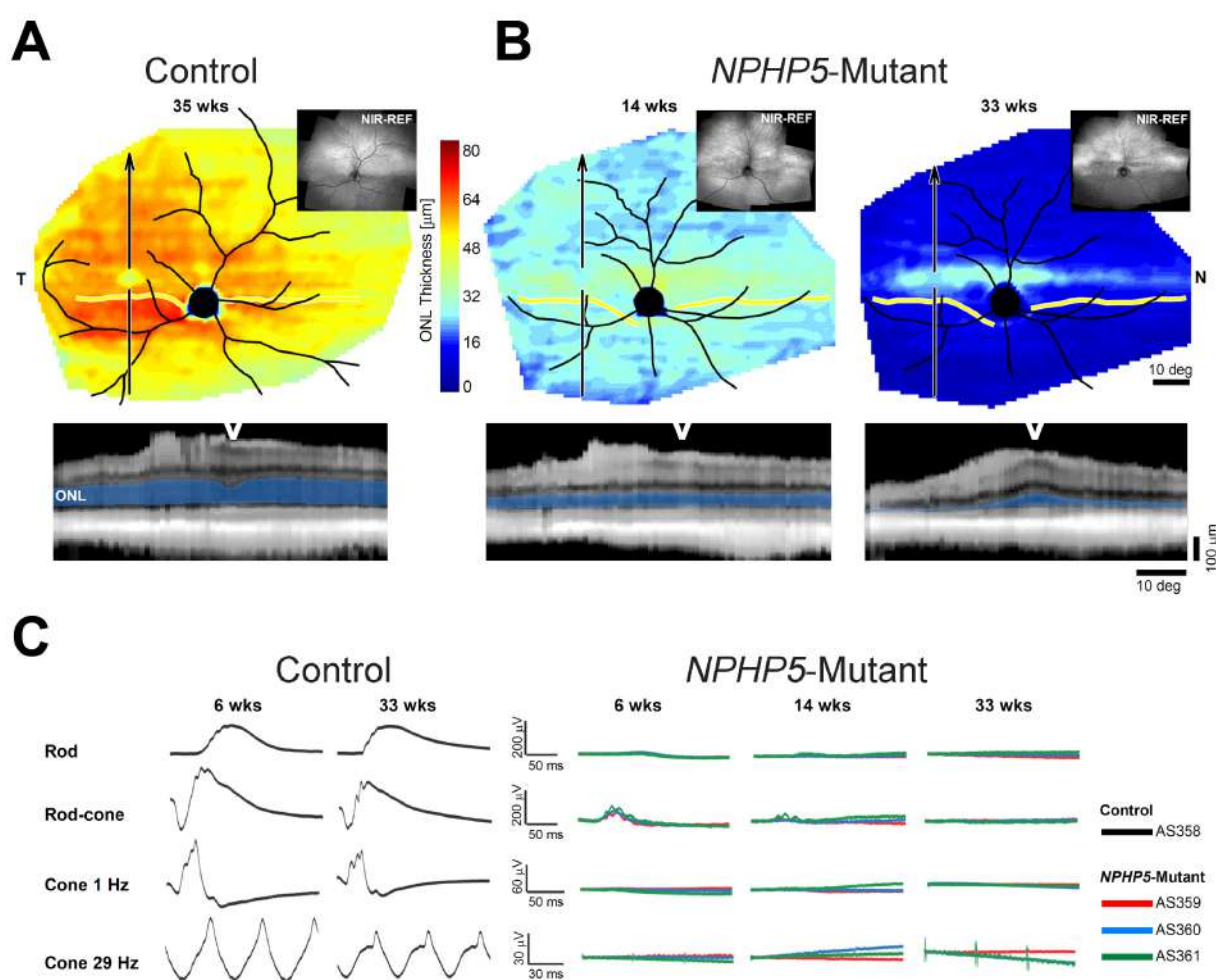


Figure 9: Natural history of disease progression in NPHP5-mutant dogs evaluated with imaging and electrophysiology. (A, B) Pseudocolor maps of ONL thickness topography (upper) in a 35-week-old control dog (A) and a NPHP5-mutant dog (B) at 14 and 33 weeks of age. Insets, near-infrared reflectance images. Arrows on the maps localize the reconstituted OCT scans (lower) along a superior–inferior meridian crossing the central visual streak at the gaps of the lines. ONL on reconstituted scans is highlighted in blue and location of the visual streak is shown with a white wedge. All eyes shown as equivalent right eyes and optic nerve, major blood vessels (black) and tapetum boundary (yellow) are overlaid for ease of orientation. T, temporal; N, nasal retina. This pattern of generalized cell degeneration with preservation of non-functional cones is identical to the pattern of disease expression and progression in human patients (Downs *et al.* 2016). (C) Representative ERGs from control (6 and 33 weeks of age), and mutants (6, 14 and 33 weeks of age) in response to mixed rod-cone, or isolated rod or cone (1 Hz or 29 Hz) stimuli. In mutants, dark-adapted rod responses were barely recordable at 6 and 14 weeks, and absent thereafter. They showed an altered waveform, and amplitudes that were <10% of normal using maximal white light stimuli ($1.01 \log \text{cd}\cdot\text{s}\cdot\text{m}^{-2}$); cone responses were not recordable. Reprinted in part with permission from (Downs *et al.* 2016).

Because of the early and severe structural abnormalities, we designed several vectors that were self-complementary and did not require double-stranded DNA synthesis prior to expression, an important feature for treating rapidly progressive diseases. However, single stranded and self-complementary vectors were equally effective using the rod and cone specific GRK1 promoter regulating either the human or canine therapeutic NPHP5 transgene. Treatment of eyes at 5 weeks of age, when rod- and cone-mediated ERG responses were non-recordable resulted in recovery of function in both photoreceptor classes (Figure 10), and cone responses that were comparable to normal. The recove-

ry of function results from improved rod outer segment structural organization, and growth of cone outer segments in photoreceptor cells that lacked this structure (Aguirre *et al.* 2021). The structural and functional outcomes of treatment were effective for more than 6 months. Current studies are now directed at treating more advanced patient-relevant disease stages prior to moving forward with an IND application to the US Food & Drug Administration in order to beginning Phase I/II clinical trials. Preliminary results from those studies indicate that the late-stage treatments also are effective.

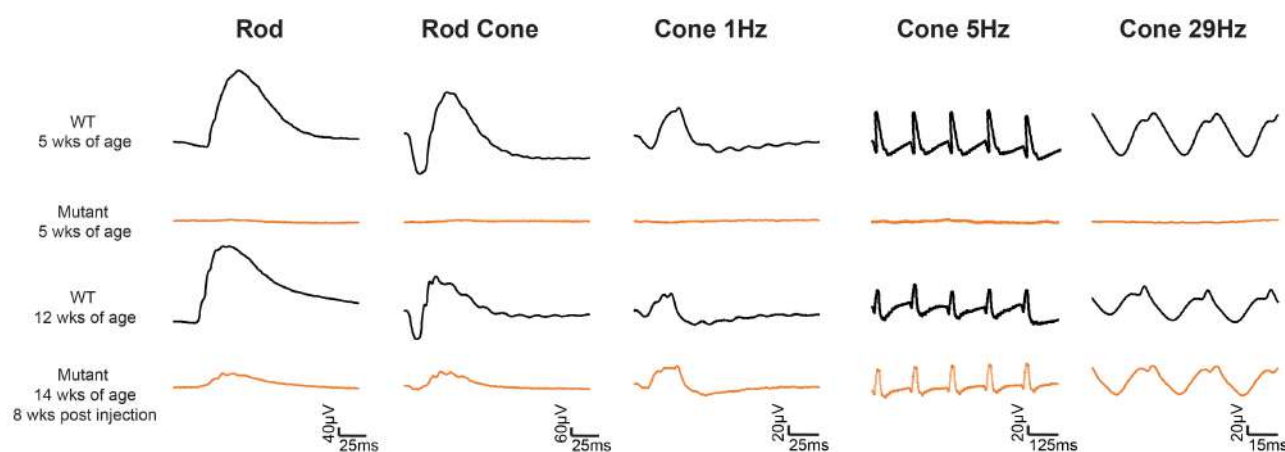


Figure 10: Electoretinograms of the same WT control (black tracing) and mutant (WM46-right eye; orange tracing) dogs before and 8 weeks after gene augmentation therapy in the mutant. There is recovery of rod- and cone-mediated ERG responses, with cone responses similar in waveforms and amplitudes to WT control. Light intensities used to elicit the illustrated responses are Rod = 1.7 log cd-s-m⁻²; Rod Cone = 0.5 log cd-s-m⁻²; Cone 1Hz = 0.5 log cd-s-m⁻²; Cone 5Hz = 0.25 log cd-s-m⁻²; Cone 29Hz = 0.25 log cd-s-m⁻². Reprinted with permission from (Aguirre *et al.* 2021).

RPGR-X-linked progressive retinal atrophy

An X-linked form of PRA (XLPR) was identified in the Siberian Husky breed (Acland *et al.* 1994). In the process of mapping the disease and identifying the disease-causing gene/mutation, a breeding colony of dogs with the disease was established at the School of Veterinary Medicine of the University of Pennsylvania, and the functional and pathologic features of the disease characterized. The disease is a post-developmental defect of rods and cones that begin to degenerate after having developed and functioned normally up to ~ 1 year of age (Zeiss *et al.* 1999). Fine mapping of the disease confirmed that it was the locus homolog to RP3, the locus where a form of X-linked retinitis pigmentosa (XLRP) resided but the gene had not been identified (Zeiss *et al.* 2000, Zhang *et al.* 2001). Further fine mapping of this interval identified the mutation in open reading frame 15 (ORF15) which was initially suspected as being an intron but subsequently was found to be a protein coding continuation of RPGR exon 15. In the Siberian Husky the mutation is a 5 nucleotide deletion in the C-terminal domain of ORF15 that truncates the protein (Zhang *et al.* 2002). At the time, we had identified another X-linked PRA in a

mongrel-derived pedigree, and in these dogs a 2-nucleotide deletion in RPGR-ORF15 was found. This deletion causes a frameshift which changes the protein charge and truncates part of the C-terminal region (Zhang *et al.* 2002). We subsequently named these diseases/mutations as XLPR1 for the one in the Siberian Husky, and XLPR2 for the one in the mongrel-derived dogs. Recent studies have now established that XLPR2 originated in the Miniature Schnauzer (Murgiano *et al.* 2019). Unlike XLPR1, the disease in XLPR2 is a developmental abnormality where rod photoreceptors mature and function abnormally before degenerating (Beltran *et al.* 2006).

The two forms of XLPR are bona fide models of human XLRP. In man, the predominant XLRP phenotype is a peripheral disease with better preservation of the fovea and macular areas (Figure 11 A, P1). This is a pattern of disease we observe in XLPR1 (Beltran *et al.* 2012) (Figure 11 B, Left). However, some patients with RPGR mutations have a predominant central disease with sparing of the periphery (Ayyagari *et al.* 2002) (Figure 11 A, P2). That is the pattern of disease we observe in XLPR2 (Beltran *et al.* 2012) (Figure 11 B, Right).

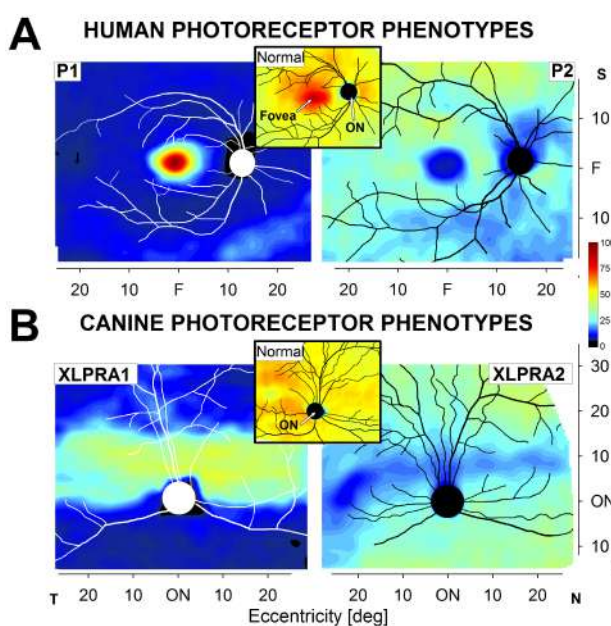


Figure 11: Retinal disease phenotypes caused by RPGR-ORF15 mutations in human patients and in dogs. (A) Different patterns of

photoreceptor topography in two XLRP patients with RPGR mutations (P1: c.ORF15+483_484delGA, p.E746fs; P2: c.ORF15+ 652_653-delAG, p.E802fs). ONL thickness topography is mapped to a pseudocolor scale. (Inset) Representative normal subject. Location of fovea and optic nerve (ON) are shown. (B) Different patterns of photoreceptor topography in the two canine models of XLRP with RPGR-ORF15 mutations; mapping as performed with the human data. (Inset) Map of a representative WT dog with location of the optic nerve (ON) labeled. Reprinted with permission from (Beltran et al. 2012).

To develop a relatively "rapid" outcome assessment of efficacy of treatment, we selected timepoints whereby we could assess function based on ERG, and structural integrity based on OCT (Beltran et al. 2015). As disease progression and severity, at least at the initial stages, varies between XLPRA1 and XLPRA2, we selected as outcome measure the prevention of disease onset in XLPRA1; in this case, dogs were treated at 28 weeks of age, and efficacy established at 77 weeks of age, almost 1 year following treatment (Beltran et al. 2012). In contrast, for XLPRA2 we chose to determine if treatment arrested disease progression while at the same time restoring function. In this case, treatment was carried out at 6 weeks of age and the study ended ~6 months later, at 33 weeks of age (Beltran et al. 2012).

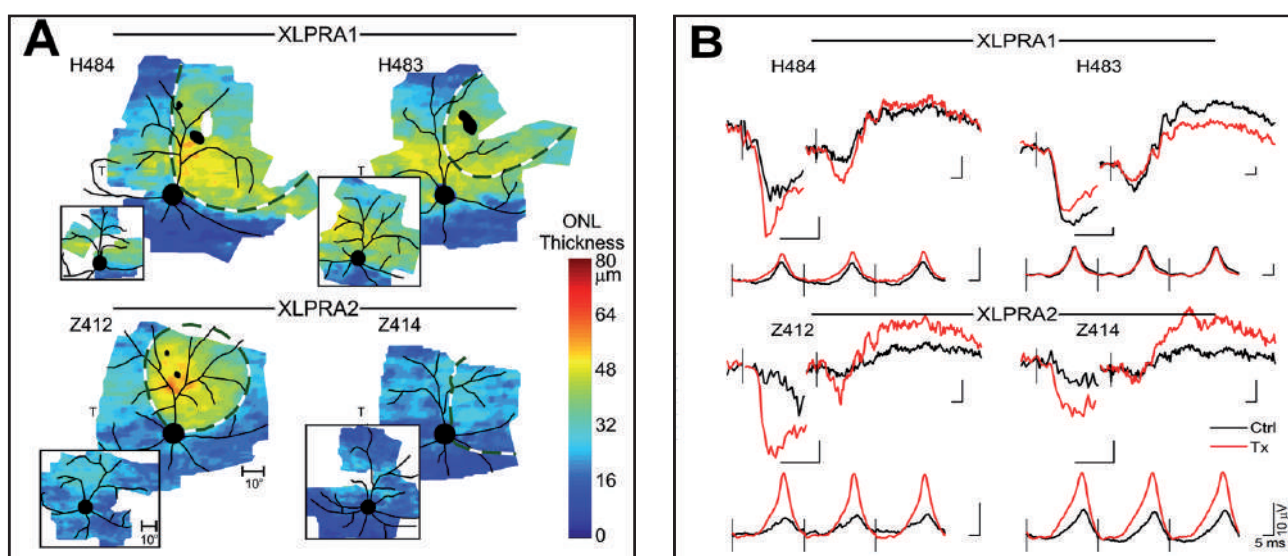


Figure 12: In vivo evidence of gene augmentation therapy success in XLPRA dogs. (A) Topography of ONL thickness in treated eyes shown on a pseudocolor scale with superimposed retinal blood vessels and optic nerve. White represents no data; irregularly shaped black foci indicate retinotomy sites. Bleb boundaries are outlined with black-and-white dashed lines. Small inset figures are BSS-treated control fellow eyes. (B) ERGs in treated (red traces) and BSS-injected control fellow eyes (black traces). For each panel in B, the upper-left waveforms are the leading edges of the photoreponses driven by rod photoreceptor activation, and the upper-right waveforms are the b-waves dominated by rod bipolar cells, both recorded under dark-adapted conditions. Lower waveforms are 29-Hz flicker responses dominated by cone function recorded under light-adapted conditions. Black vertical lines show the timing of flash onset. Calibrations are 5 ms (abscissa) and 10 μ V (ordinate); note the ~3 larger waveforms of H483. Reprinted with permission from (Beltran et al. 2012).

Treatment of XLPRA1 dogs (Figure 12; dog IDs: H483, H484) confirms that structure is preserved when treatment is initiated before the disease onset, yet functional rescue was not obvious because it was assessed before sufficient degeneration in the untreated eye had occurred. In contrast, treatment at the early

stages of degeneration in XLPRA2 dogs (Figure 12; dog IDs: Z412, Z414) restores ERG rod and cone function, and there is no further degeneration in the treated areas. These studies have been expanded to assess treatment at patient-relevant disease stages. In all cases, treatments initiated in the XLPRA2 model at

initial, mid- and late-stage disease are effective in arresting progression of the disease, preserving ERG function and vision (Beltran *et al.* 2015) (Figure 13).

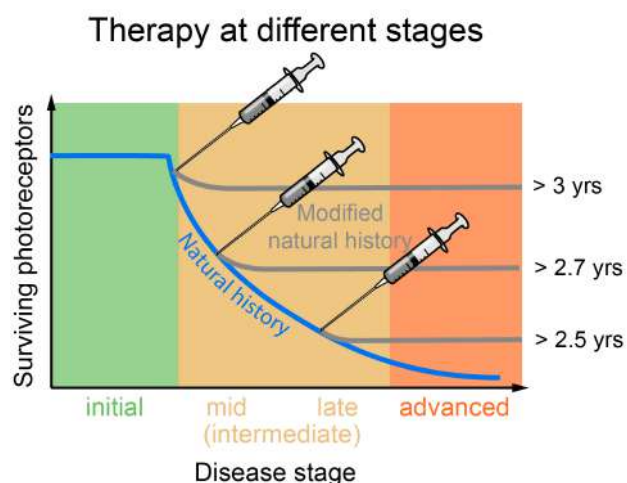


Figure 13: Summary of long-term follow-up of RPGR gene augmentation in the XLPRA2 model following intervention at initial, mid, and late stages of disease. Modified and reprinted with permission from (Beltran *et al.* 2015).

SUMMARY AND CONCLUSIONS

In this review we have aimed to illustrate the advantageous qualities of the canine retina as a model system to develop therapies that can be transitioned to the clinic. For the mutations so far studied, the canine diseases recapitulate the

human conditions and are truly disease homologues. The canine and human retinal dystrophies have a comparable phenotype and the cell classes affected are the same. As more and more diseases are identified in canine populations (Miyadera *et al.* 2012, Winkler *et al.* 2020), the value of this model system will increase. These studies will be important, not only as they will help veterinarians and veterinary ophthalmologists to better understand the disease mechanisms and how they affect their patients, but as a means of developing therapies that will be important for human patients. The hope is that these treatments after being developed for people will then come back to help our four-legged friends.

CONFLICT OF INTEREST

Gustavo D. Aguirre and William A. Beltran are co-inventors on patents and patent applications related to gene therapies for RPE65-LCA2 (GDA); RPGR-XLRP (GDA & WAB); RHO-adRP (GDA & WAB); BEST1 (GDA & WAB), and NPHP5-LCA (GDA & WAB).

ETHICAL COMMITTEE

All studies that involved the use of animals were conducted in full compliance with the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) approval, adhered to the Association for Research in Vision and Ophthalmology (ARVO) Resolution for the Use of Animals in Ophthalmic and Vision Research, and followed the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health of the United States of America.

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