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The use of canine models of inherited retinal degeneration to test novel therapeutic approaches

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Abstract

Inherited retinal degenerations (RDs) are a common cause of blindness in dogs and in humans. Over the past two decades numerous genes causally associated with these diseases have been identified and several canine models have been used to improve our understanding of the molecular mechanisms of RDs, as well as to test the proof of principle and safety of novel therapies. This review briefly summarizes the drug delivery approaches and therapeutic strategies that have been and are currently tested in dogs, with a particular emphasis on corrective gene therapy, and retinal neuroprotection.

Keywords

canine; degeneration; gene therapy; model; neuroprotection; retina

INTRODUCTION

Retinitis pigmentosa (RP) is a heterogeneous group of human inherited retinal degenerative diseases clinically characterized by progressive peripheral vision deficits and night vision difficulties that can progress to central vision loss. In RP, the primary defect occurs in the retinal pigment epithelium (RPE) or rods, and cones are usually lost later. There are currently approximately 40 known or predicted genes that cause RP (<http://www.retnet.org>), and the condition may be inherited as an autosomal recessive, autosomal dominant or as an X-linked trait. However, most human patients have simplex RP (= no familial history of RP). To this date there is no treatment available for RP, yet several clinical trials are underway.

The use of animal models of retinal degeneration (RD) has been of paramount importance to understand the function of some of the causative genes, decipher the cellular and molecular mechanisms of disease, and develop novel therapeutic strategies. While rodents are commonly used, 'large' animal models (i.e. dog, cat, pig) have become increasingly attractive to assess efficacy and safety of a variety of treatment modalities that are being considered for clinical trials in human patients.

In dogs, progressive retinal atrophy (PRA) has been described in > 100 breeds, and to this day, 12 causative mutant genes (*PDE6β*,¹ *RPE65*,² *PDE6α*,³ *RPGR*,⁴ *RHO*,⁵ *CNGB3*,⁶ *PRCD*,⁷ *RPGRIP1*,⁸ *VMD2*,⁹ *RD3*,¹⁰ *STK38L*¹¹ and *NPHP4*¹²) have been identified. These canine forms of RD share great genetic and phenotypic similarities with their human

counterparts, and as a result the dog is a unique animal model to: (i) improve our understanding of the pathogenic mechanisms of these diseases, and (ii) test the ‘proof of principle’ of novel therapeutic strategies that are being developed for treating human patients. A significant advantage in using the dog is that it enables intraocular drug delivery studies, surgical interventions, and *in vivo* imaging procedures that cannot always be done in the much smaller rodent eye. The axial globe length (AGL: 17.75–20.25 mm)¹³ and vitreous volume (VV: 1.7 ± 0.86 mL)¹⁴ of the adult dog are smaller than that reported in humans (AGL: approximately 23 mm; VV: approximately 3.8 mL). Yet, the dimensions of the canine eye enable intravitreal implantation of devices or injection of compounds in solution that have respectively, a size, or volume targeted to the human eye. The difference in vitreous volume between the two species needs to be taken into account in pharmacological studies as injection of a similar dose may induce drug concentrations in the canine vitreous that are approximately twofold higher than reached in that of the human. The lack of a foveo-macular region in the canine retina is a major anatomical difference with that of primates. This limits the use of the dog for studies aimed at understanding the pathogenesis of macular degeneration and its treatments. However, an increased density in cones in the dog’s *area centralis* suggest that this region may contribute to visual acuity in this species and be of interest when characterizing the topography of a retinal disease and its response to therapy.¹⁵ Finally, features of the canine reproductive cycle (age at puberty, gestation period, interstrous interval, litter size) that can vary between breeds, as well as the high financial cost necessary to maintain a dog colony, need to be taken into consideration when designing an experiment. Indeed, these constraints may limit the use of this model in studies that require a high number of individuals.

Several therapeutic approaches for the treatment of retinal degenerative diseases including corrective gene therapy, retinal neuroprotection, retinal transplantation, stem cell therapy, retinal prostheses, and dietary supplementation are being developed and tested in animal models and some of them are currently in human clinical trials.¹⁶ The increasing number of canine models of inherited RD that have been genetically and phenotypically characterized represent an invaluable resource to evaluate both the efficacy and safety of some of these approaches (Table 1). This article will review some of the past, present, and potentially most promising therapeutic strategies for this devastating group of disorders that are being tested in the dog.

CANINE MODELS OF RD USED FOR THERAPEUTIC ASSESSMENT

In the context of preclinical studies, a prerequisite for evaluating the efficacy of a new therapeutic approach is to: (i) select the most optimal animal model; (ii) identify the time-window for therapeutic intervention; (iii) establish outcome measures of positive or negative effects; and (iv) determine the route and modalities of administration.

Therefore, before planning such studies in a canine model of inherited RD it is crucial to determine the mode of inheritance and the causative mutant gene, identify which retinal cell(s) (RPE, rods, cones) is/are primarily affected and should be the target of the therapy; and establish metrics that allow to stage the disease.

Among the 10 canine models that are currently being used for therapy studies (see Table 1) the majority (7 out of 10) are inherited in an autosomal recessive manner and the mutations result in a loss of function of the gene.

Two forms of X-linked PRA have been identified and although they are both caused by a different mutation in the same gene and exon (*RPGR* exon ORF15) they show a different time of onset and course of progression. In XLPR1, photoreceptor cell death has been reported to begin at approximately 11 months of age and progression takes place over the

course of several years.³¹ Recent results from our laboratory suggest that rare events of rod degeneration occur in fact as early as 4–5 months of age (Beltran, unpublished observation). It is hypothesized that the five nucleotide deletion in *RPGR* exon ORF15 that results in a premature Stop codon and a protein truncated of its 230 C-terminal amino-acids causes disease through a loss of function mechanism.⁴ XLPA2, on the other hand, is a very early onset and rapidly progressive form of RD, for which rod cell death has been shown to begin as early as 4–5 weeks of age.³⁴ Thus, it has been suggested that the two nucleotide deletion in *RPGR* exon ORF15 that results in a frameshift and changes the deduced protein sequence is responsible for a toxic gain of function.⁴ In humans, more than 240 different mutations in the *RPGR* gene have been identified and account for approximately 70–80% of familial X-linked RP cases. The majority of these mutations have been found in exon ORF15.³⁵ *RPGR*-XLRP constitutes one of the most severe group of RDs in humans. Males affected with this condition usually exhibit night blindness in their first decade of life, followed by reduction of their visual field and loss of their central visual acuity. By the age of 40, most patients are legally blind.³⁶ Based on the age of onset (during childhood) and the relatively rapid progression of the disease, the human condition appears to be better modeled by XLPA1. However, in the context of time-constrained preclinical studies, there is an advantage in the use of the XLPA2 dog as it offers earlier time points to assess treatment efficacy and/or toxicity. Some mutations in exon ORF15 of *RPGR* have also been associated with forms of X-linked cone rod dystrophy and X linked atrophic macular degeneration. Despite the absence of a foveo-macular region in the dog, preliminary results from our lab have shown increased disease severity in the *area centralis* of the XLPA2 dog resulting in a focal thinning of the outer nuclear layer.³⁷

The only autosomal dominant form of PRA described so far has been reported in the Bull Mastiff and English Mastiff breeds. In this disease, a mutation in the rhodopsin (*RHO*) gene that alters a glycosylation site at the N terminal end of the protein causes a regional form of RD in the central fundus.⁵ The mutant allele is considered to be deleterious to rods through a toxic gain of function. Recently, it was shown that exposure to light intensities typically used for clinical eye examinations can trigger the onset of disease and result in a complete atrophy of the illuminated areas of the retina within 2–4 weeks.³² This illustrates how the contribution of an environmental factor (in this case: light exposure) to a genetic defect (T4R *RHO* mutation) can drastically alter the disease phenotype. Interestingly, among human patients with AdRP, this is the most commonly mutated gene (> 100 different mutations), and there is concern that a subset of these patients may show accelerated retinal damage following exposure to ambient light.^{38,39} Among all animal models of light-induced RD the T4R *RHO* dog is together with the T17 M *RHO* mouse at the high end of light sensitivity. Indeed, a series of fundus photographs is sufficient to trigger within 24 h massive cell death (Beltran, unpublished observation). Although this experimental paradigm may cause a disease much more severe than the human condition it offers the possibility of synchronizing the cell death events to examine the signaling pathways of cell death, and rapidly testing the efficacy of target-oriented therapies.

Characterization of the clinical, histological, cellular and molecular changes that occur throughout the course of the disease can, not only provide a better understanding of the pathogenic mechanisms of the condition, but also provide benchmarks to objectively assess the response to therapy. The most common methods used *in vivo* for assessing visual function include: electroretinography (ERG), pupillometry, and obstacle course performance. In rodents, psychophysical tests used to evaluate visual acuity include optomotor systems, and the Water Visual Task.^{40,41} Similar methods to evaluate visual acuity in dogs have yet to be developed. Over the past recent years non-invasive imaging techniques such as functional MRI (fMRI) and optical coherence tomography (OCT), have

been applied to canine models of RD to either follow the course of a disease or its response to therapy.

A major concern in those forms of RD that are either congenital or have an early onset during childhood is the absence of response to therapy due to the development of amblyopia. The use of fMRI enables imaging of the visual cortex and its metabolic activity and was used in the *RPE65* mutant dog, a model of Leber's congenital amaurosis (LCA) to assess visual cortical responses before and after corrective gene therapy.²² Results showed minimal, retinal, subcortical, and cortical activity prior to treatment, but a significant recovery within one month of treatment that persisted for at least 2.5 years. In this same study, human patients with *RPE65*-LCA had preserved visual cortical responses to bright light stimuli despite experiencing severe visual impairment. These findings suggest that retinal function recovery by means of corrective gene therapy is likely to translate into vision restoration since it appears that in dogs and in humans there is preservation of visual pathway anatomy and cortical activation.

OCT produces high resolution cross sectional images of the retina, and has been used in dogs to monitor the retinal structure following subretinal injection of AAV-RPE65 gene therapy constructs,²³ and implantation of retinal prostheses.⁴² Post acquisition analysis of the longitudinal reflectivity profiles obtained with OCT enables to generate topographical maps of the retinal thickness to follow non-invasively the course and distribution of the disease. This approach has been used in the T4R *RHO* mutant dog to demonstrate *in vivo* localized retinal thinning following focal light exposure.³² Recently, it has been recommended that mapping of the retinal thickness by OCT be done in human candidates for corrective gene therapy to identify those retinal regions that should be targeted.⁴³ The latest generation of OCT instruments now provides such high resolution that inner segments of individual cones can be visualized and counted.⁴⁴ There is no doubt that this will considerably improve early diagnosis, and follow-up of disease progression in human patients. Retinal research that uses animal models is likely to benefit also from these technological advancements as it can provide a way of performing longitudinal studies and reducing the number of individuals.

A key factor in the potential success of a new therapeutic compound resides in its adequate delivery to the retina. The anatomical and immunological specificities of the eye make it an ideal organ for the local delivery of compounds that cannot be administered systemically. However, achieving therapeutically active concentrations in the posterior segment cannot be obtained by topical application and require either intravitreal or subretinal delivery.⁴⁵ Proper visualization of the procedure necessitates the use of an operating microscope and a magnifying Machemer vitrectomy lens (for subretinal injections). Access to the vitreal cavity is usually done by penetrating the sclera 7 mm behind the limbus in the superotemporal quadrant of an adult dog's eye to insure that the needle enters the eye through the *pars plana*.⁴⁶ When delivering a solution into the vitreous it is recommended to avoid deviations from a central injection position as variations from this site may cause significant changes in drug distribution and elimination from the vitreous, ultimately leading to differences in drug concentration in the retina.⁴⁷ At least three type of devices have been used to perform subretinal injections in the dog: custom-made glass micropipettes,²⁴ custom-built injectors with either a 30- or 44-gauge blunt tipped cannula,^{25,48} and a commercially available injector (retinaJect™, Surmodics, MN) that moves a 39-gauge cannula within a 25-gauge needle.⁴⁹ Detailed description of the surgical procedure used with two of these devices has recently been published.⁴⁵ Successful injection into the subretinal space of volumes ranging from 30 to 500 μ L in dogs^{23,25,49,50} and 150–1000 μ L in humans have been described.^{51–53} Injection of volumes up to 200 μ L can lead to the formation of a subretinal bleb covering approximately one-third of the retinal surface without prior vitrectomy;²⁵ however, delivery

of larger volumes may require removal of the core and peripheral vitreous.²³ As intravitreal/subretinal injections require invasive surgery into the eye, transscleral delivery and iontophoresis are also being considered as alternative routes of administration that could avoid the potential complications associated with intraocular delivery.^{54,55}

The importance of the choice of the vector that will provide the therapeutic agent to the retina cannot be overemphasized. Due to the rapid clearance of molecules injected into the vitreous or subretinal space, a number of sustained drug-delivery strategies have been developed and tested in animal models. Over the past decade there has been considerable interest in the use of viral vectors as a means of delivering a gene to specific cells of the retina (RPE; photoreceptors; ganglion cells). For this purpose, adenoviruses, lentiviruses and recombinant adeno-associated viruses have been developed and tested. A significant advantage of the adenovirus vector is its large 'cargo' capacity (approximately 36 kb); however, the short duration of the expression of the transgene has limited its use primarily because of its immunogenicity. In the dog a single report has described its use to transduce the ciliary body and iris as an approach to target uveal melanomas. Successful delivery to the anterior uvea and into a canine iridal tumor was achieved; however, induction of a severe cellular and humoral immune response was observed in all treated animals.⁵⁶ Lentivirus vectors can package a genome up to 10 kb but their cell tropism is limited to the RPE. As a result, the most widely used viral vector for retinal gene therapy is currently the recombinant adeno-associated virus (rAAV). This is a replication-defective virus that has been genetically modified by removing all the native AAV coding sequences with the exception of the two short inverted terminal repeats (ITRs) that flank the promoter, cDNA and polyA-addition site of interest (Fig. 1). The rAAV vector has many advantages over other viral vectors for the purpose of retinal gene therapy. It has been shown to be safe,^{26,57} and can transduce terminally differentiated cells where it enables long-term expression of the transgene.^{25,58} Yet, a limitation of the rAAV resides in its small 'cargo' capacity. The maximal size of the gene construct it can carry is considered to be restricted to 4.7 kb, which prevents packaging large genes. However it has been recently suggested that up to 8.9 kb of genome may be incorporated in a particular rAAV.⁵⁹ Initially, the most commonly used rAAV was the rAAV-2 serotype however with the cloning of more than 10 new serotypes that have an affinity for different cell receptors, chimeric rAAV serotypes (also termed pseudotypes) are being tested for their cell specific tropisms and efficiencies. Currently most chimeric rAAV serotypes that are being tested for retinal gene therapy have the transgene flanked by the AAV-2 ITRs and contained in the capsid of either an AAV-1, AAV-2, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, or AAV-9, thus generating rAAV-2/1, rAAV-2/2, rAAV-2/4, rAAV-2/5, rAAV-2/6, rAAV-2/7, rAAV-2/8, or rAAV-2/9, respectively.^{60,61} In the dog, rAAV-2/1, rAAV-2/2, rAAV-2/4, rAAV-2/5, and rAAV-2/8 have been used and shown to elicit distinct retinal cell tropism (Fig. 2).^{23–25,27,62–64} For the purpose of studies on RPE65 gene therapy pseudotypes AAV-2/2, and AAV-2/4 have been tested in the dog and shown to transduce the RPE.^{23,27,50} In recent human clinical trials the AAV-2/2 was chosen.^{51–53} Currently, there is no AAV pseudotype known to target solely photoreceptor cells, and thus the AAV-2/5 known to transduce both RPE and photoreceptors (PRs) in rodents was chosen to transduce cones of both the normal and *CNGB3* mutant canine retina.^{30,49} Recently, the biodistribution of an AAV-2/8 carrying the reporter gene GFP was injected into the subretinal space of non-mutant dogs and found to transduce several retinal cell populations including RPE, PRs, INL cells, entire length of ganglion cells (GC), and the brain along the visual pathway [lateral geniculate nucleus (LGN), superior colliculus (SC)].⁶⁴ For obvious safety reasons, these findings have led to exclude the use of AAV-2/8 for local retinal gene therapy. They have also highlighted the importance of thorough characterization of the biodistribution of novel viral vectors in multiple animal species before considering their use in human trials.

Numerous studies have also confirmed the importance of the choice of the promoter that drives the transcription of the transgene. Indeed, although ubiquitous promoters are commonly used, incorporating cell-specific promoters further reduces the risk of off-target expression of the transduced gene. Finally, it has been shown that a same viral vector construct can transduce different cell populations solely on the basis of the route of administration that is used (i.e. subretinal vs. intravitreal).⁶⁰ In summary, selecting the optimal viral vector/pseudotype, the promoter, and the route of administration is necessary to drive the expression of the therapeutic gene specifically in the targeted cells.

A particularly elegant approach to deliver therapeutically active proteins to the retina has been achieved with the Encapsulated Cell Technology developed by Neurotech Inc. This intravitreal implantable device contains genetically modified cells that release small doses of a survival factor [ciliary neurotrophic factor (CNTF)] over a prolonged period of time.⁶⁵ Other groups have tested the use of biodegradable polymers (e.g. PLGA microspheres) for the controlled release of drugs into the vitreous.⁶⁶

CORRECTIVE SOMATIC GENE THERAPY IN CANINE MODELS OF RD

Gene replacement strategy

It has now been 7 years since the first report of gene therapy successfully restoring vision in the *RPE65* mutant dog, a large animal model of congenital Leber's amaurosis. In dogs and humans affected with this disease the null mutation in the *RPE65* gene causes a blockade in the retinoid visual cycle that results in a severe congenital visual deficit. The use of a rAAV vector to transduce the RPE with the wild type copy of the cDNA of the *RPE65* gene has now been demonstrated in the dog by four independent groups.^{23,27,28,50} These studies showed that an improvement of both scotopic and photopic ERGs, reduction in nystagmus, improved pupillary light responses and navigational skills through an obstacle course could be achieved in congenitally blind animals, and this functional rescue persisted over time.^{25,58} Retrospectively, this was an ideal disease to demonstrate in the dog the proof of principle of such a gene replacement strategy. Indeed, the absence of any early structural alterations of the photoreceptor cells enabled recovery of visual function by turning ON the retinoid cycle. Over the past years, efforts have been directed at optimizing viral constructs to improve their efficiencies and specificities for the RPE,²⁸ and assessing their safety.^{26,57} Another important question that has been addressed in the *RPE65* mutant dog is whether enough visual cortex activity persists over time to allow a recovery in cortical function after retinal gene therapy. Results showed that the visual cortices of dogs treated at 1–4 years of age remained receptive to increased visual input after gene therapy, and that human LCA patients with severe visual loss since childhood maintained some level of cortical activity.²² Less than 7 years after the breakthrough result in the *RPE65* mutant dog,²⁷ the initial findings of phase I clinical trials in human patients affected with this disorder have just been reported by three independent groups.^{51–53} All three studies used an rAAV-2/2 to transduce the RPE with a wild-type copy of the human *RPE65* cDNA in three young-adults with severe vision loss caused by mutations in *RPE65*. However, the choice of the promoter used to drive the expression of the transgene differed between trials. The gene therapy did not trigger any cell-mediated nor humoral immune responses, and no major clinical side effects were observed. This was in line with the results of preclinical safety studies conducted in dogs as well as nonhuman primates.^{26,28,57} All three trials reported some degree of visual function recovery under dim illumination in at least one of their patients. Visual acuity remained unchanged in two of the studies;^{51,53} in the third one, statistically significant improvement was observed in all three patients, however post-treatment values remained below 20/200, the legal definition of blindness.⁵² Other psychophysical assessments of vision function that were used included visual field tests (improved in all three trials) as well as navigation through an obstacle course (improved in two patients out of two studies), and

dark-adapted visual sensitivity with full field stimulus test (FST) (improved in two patients in one study).⁴³ Objective outcome measures of visual function included: electroretinography (which remained unchanged in one study⁵¹), nystagmus assessment (improved in one study⁵²), and pupillometry (improved in two studies).^{52,53} Future clinical trials are likely to address whether high resolution visual acuity can be substantially improved in LCA patients. In the context of these future studies, it has been shown recently that the foveo-macular region of primates is dependent on RPE65 activity, and that this area of the retina which is responsible for high resolution visual acuity should be targeted for therapy.⁶⁷ This will require improving the subretinal delivery approach in order to reliably and safely target the fovea, but also developing objective outcome measures of foveal function in these patients. In the absence of an animal that models the changes that occur in the foveomacular region of the LCA retina, safety and efficacy evaluations will likely be conducted directly in humans. The use of high-resolution *in vivo* optical coherence tomography will significantly improve the selection of optimal candidates by providing topographical maps of the retinal layers⁶⁸ and identifying whether the fovea-macular region is sufficiently preserved to respond to therapy. This will be particularly critical when clinical trials will start enrolling children, as recent findings demonstrate significant variation in the spectrum of disease in young *RPE65*-LCA patients. Indeed, some individuals show severe ONL loss in both the foveal and extrafoveal retina before 10 years of age, while others in their second and third decade of life show a better preserved fovea and macula.⁴³ In the *RPE65* mutant dog individual variation in visual function has also been shown by behavioral testing and electroretinography.⁶⁹ Results show in affected animals a consistent dysfunction in the rod system resulting in night blindness. The cone system on the other hand is variably impaired with day vision ranging from being normal to severely impaired. Overall the disease appears to be less severe in the young dog (< 1 year of age) than in humans.

The next challenge for somatic retinal gene therapy is to target diseases that affect primarily photoreceptors. This has been initiated in a variety of rodent models^{70–72} and is being tested now in the dog. Disorders that affect the function of the photoreceptors (rods and/or cones) without causing, at least during the early stages of the disease, any cell loss or structural alterations are potentially good candidates for developing a corrective gene therapy approach. One such form of RD is CD, a disease identified in the Malamute and German Short Hair pointer that causes day blindness,⁶ and is known as achromatopsia in humans. With the recent development of viral vectors capable of specifically targeting cones,^{49,72–74} and preliminary results showing successful cone vision recovery in the dog,³⁰ this could become the second form of RD in humans, which is to be treated by replacement of the mutant gene.

Gene knockdown

In dominant or X-linked retinal diseases that are caused by toxic gain of function mutations, strategies that can inhibit the expression of the causative gene need to be applied. Such approaches require either to suppress or to reduce substantially the mRNA or the protein products of the mutated gene. Three types of therapeutic molecules (aptamers, ribozymes, and siRNAs) are being widely developed and tested (Fig. 3).

Aptamers are DNA or RNA molecules that have been screened to bind other molecules (nucleic acids, proteins, small organic compounds). Of particular interest are those that can bind to the functional domains of the target protein. One example of an aptamer that is currently on the market for the treatment of the neovascular ('wet') form of age-related macular degeneration is pegaptanib sodium (Macugen®). This is a stabilized RNA aptamer that binds specifically and with high affinity to VEGF165, the vascular endothelial growth factor isoform responsible for pathological ocular neovascularization.

Ribozymes are small catalytic RNAs capable of cleaving other complementary RNA sequences. After binding to their target, ribozymes will cleave the RNA if they recognize specific sequence motifs. Ribozymes can be engineered to recognize and cleave only the mutant RNA molecule without affecting the wild-type copy.⁷⁵ Such approach has been demonstrated in the P23H *RHO* transgenic rat model of autosomal dominant RP (AdRP). Results confirmed that cleaving the mutant copy of the rhodopsin mRNA rescued photoreceptor cells.^{76,77}

A new strategy to knockdown gene expression posttranscriptionally is the use of RNA interference (RNAi). This technology uses short interfering RNAs (siRNAs), which are double-stranded nucleic acids made of 21–23 nucleotides, to degrade the target mRNA. Intracellularly, the siRNAs are incorporated into a multiprotein RNA-inducing silencing complex (RISC) that directs the unwinding of the two strands of the siRNA molecule, the binding of the antisense strand to its target, and the site-specific cleavage of the message in the region of the siRNA-mRNA duplex. Because of the transient gene silencing conferred by siRNAs, viral vectors that allow long-term expression of short hairpin RNAs (shRNA) are being used instead. Once transcribed, these shRNAs are cleaved in the cytoplasm by the DICER enzyme to produce siRNAs (Fig. 4).

Ribozymes and siRNAs are currently being developed for testing in the XLPRA2 dog. As the frameshift mutation in *RPGR* responsible for this disease is considered to cause a toxic gain of function that leads to an early death of photoreceptor cells,^{4,34} the hypothesis is that silencing the mutant gene will delay the onset of the disease.

Gene knockdown and replacement strategy

Developing therapies for dominant or X-linked disorders that target specifically the mutation (= allele-specific) has been shown to be both technically challenging and not economically realistic. This is particularly relevant for highly mutated genes such as *RHO* and *RPGR* in which, respectively, more than 100 and 240 different mutations have been identified in human patients. As an alternative to mutation specific gene knockdown (via ribozymes or siRNAs), researchers are now testing a gene knockdown and replacement approach. Such a strategy aims at suppressing both the mutant and normal copies of the gene (= allele-independent),^{78–80} and replacing them with a copy of the wild type gene that has been modified to be resistant to suppression.⁸¹ The overall goal is to be able to treat patients that carry different mutations in the same gene by correcting the genetic defect in a mutation-independent manner. The first report demonstrating in a mouse model of *RHO* AdRP the effectiveness of this gene knockdown and replacement approach was published last year.⁸² Now that the molecular tools (rAAV vectors targeting rods; ribozymes, siRNAs) are available, testing this strategy in a large animal to further evaluate its effectiveness and safety is necessary. The T4R *RHO* dog is an ideal model in which these approaches are currently being evaluated.

NEUROPROTECTIVE AND OTHER STRATEGIES TESTED IN CANINE MODELS OF RD

Although corrective gene therapy offers the potential for curing RD, it is a therapeutic approach that is gene-specific. Even with a gene knockdown strategy that would allow inhibition in an allele-independent manner of both the wild type and all mutant forms of a gene, followed by replacement with a resistant wild-type cDNA, identification of the deleterious gene is a pre-requisite. An approach that would circumvent this inherent limitation of corrective gene therapy is the use of neuroprotective agents or artificial retinas that could be applicable to a wide range of forms of RP, whether or not the disease-causing

mutation is known. Since the gene defect is not corrected, this strategy does not ‘cure’ *per se*, but intends to slowdown the progression of the disease (neuroprotective strategy), or replace the defective photoreceptors (retinal prosthesis). Numerous neuroprotective agents that include nutrients, ionic channel blockers, anti-apoptotic genes, and neurotrophic factors, have been evaluated over the past decades for their photoreceptor rescue properties in various animal models. Those tested in dogs models of inherited RD are listed below.

Dietary supplementation with DHA and vitamin A

Docosahexaenoic acid (DHA) is the major fatty acid constituent of photoreceptor rod outer segment membranes, and it has been shown that the plasma and ROS levels of DHA are reduced in some RP patients and animal models such as the progressive rod-cone degeneration (PRCD), and RPE65 mutant dogs as well as in the Abyssinian cat with hereditary rod-cone degeneration.^{83–86} Administration of DHA-enriched supplements in the PRCD dog increases the plasma and liver DHA levels, but ROS level remain low and no rescue of either the morphology or the function of the retina is achieved.²¹ Vitamin A supplementation (15 000 IU/day) was shown in a randomized, controlled, double-masked clinical trial to slow the rate of decline in 30 Hz-ERG amplitudes in patients with RP.⁸⁷ Recently, two more studies have shown that supplementation with docosahexaenoic acid (DHA) shortens the time for vitamin A to have a positive effect, but only in those patients who were newly started on vitamin A.^{88,89} The results of these studies need to be considered with some degree of caution as they were based on group averages, and did not show any improvement in visual acuity. The only outcome measures that defined a positive response to therapy were limited to a slower rate of decline in visual field sensitivity and 30-Hz ERG amplitude. Although the mechanism by which vitamin A may preserve retinal function is unknown, dietary recommendations for RP patients have been made that include the intake of vitamin A palmitate (15 000 IU/day), the supplementation with DHA capsules (600 mg twice/day for 2 years) if no prior intake of vitamin A, and the consumption of omega 3-rich fish at least once a week.⁸⁹ With the exception of the aforementioned DHA supplementation trial conducted in the PRCD model,²¹ no other studies have investigated in the dog the potential beneficial role of dietary supplementation with either DHA, vitamin A, or their combination.

Calcium channel blockers

The beneficial effect of calcium channel blockers as a potential treatment for RDs has been a matter of debate since the first report that *D-cis*-diltiazem (a blocker of L-type calcium channels and cGMP-gated channels) rescued rod photoreceptors in the RD mouse.⁹⁰ Indeed, while these results were further confirmed by the same group,⁹¹ as well as in a light-induced model of RD,⁹² others failed to demonstrate a similar rescue effect in the RD mouse,⁹³ RCS rat,⁹⁴ P23H *RHO* mutant rat,⁹⁵ and RCD1 dog.¹⁷ Among the other calcium channel blockers tested, a positive photoreceptor rescue effect was observed with nilvadipine in both the rd mouse,⁹⁶ RCS rat,⁹⁴ and a rat model of cancer-associated retinopathy.⁹⁷ These conflicting results about the neuroprotective effect of calcium channel blockers on photoreceptor cells are probably explained by differences in experimental conditions such as mouse strains, animal model studies, or type of calcium channel blocker used.

Neurotrophins/survival factors

Over the past decade, extensive research using different rodent models has shown that various survival factors can slow the course of RD. Work conducted in the early 1990s on basic fibroblast growth factor launched the concept of using growth factors/neurotrophic factors as a potential strategy for treating RD.⁹⁸ A survival factor that has attracted a lot of attention is CNTF, as it slows the course of disease in a variety of animal models of RD including the RCD1 dog (for review see: Ref. 99).⁹⁹ However, because intravitreal

injections of the recombinant CNTF protein have a short half-life and are associated with side effects such as uveitis and cataracts, a long-term delivery system was developed (the Encapsulated Cell Technology) that enables the prolonged release of small doses of CNTF that are still therapeutically active. This ECT device was tested in the RCD1 dog and shown to provide the same level of rescue as the intravitreal injections but without any clinical side effects;¹⁸ however, subsequent histopathological examination revealed alterations in ganglion cell morphology as well as increased thickness of the inner retina.¹⁰⁰ The ECT-CNTF device was then tested in 10 human patients with end stage RP enrolled in a Phase I clinical trial and the results of this study showed an absence of side effects and some degree of functional rescue in two patients.¹⁰¹ Neurotech Inc. has recently announced completion of enrollment of its phase II/III RP and phase II dry age related macular degeneration clinical studies (<http://www.neurotechusa.com>). As the ultimate goal of using a survival factor is to target as many forms of RD as possible regardless of the causative genetic defect, we recently evaluated whether intravitreal injections could rescue photoreceptors in the XLPRA2 model. Besides failing to protect rods, CNTF caused in the peripheral retina prominent remodeling events suggestive of dedifferentiation and proliferation of retinal cell.¹⁹ Transient down-regulation of cone arrestin and rhodopsin was also seen. Decreased expression of phototransduction proteins following CNTF intraocular delivery has also been reported in the RCD1 dog¹⁰⁰ as well as in rodents, leading to lower ERG amplitudes,^{102,103} and impaired visual acuity.¹⁰⁴ As similar alterations have been observed by multiple groups in a variety of animal models (for review see Ref. 99), further research into the mechanism of action of CNTF in the retina is warranted.

Retinal prostheses

In an attempt to bypass degenerated photoreceptors, several groups have been developing artificial retinal implants. These devices are designed to stimulate existing neural circuits in diseased retinas to create a visual signal. There are two kinds of retinal prostheses that have been tested: subretinal and epiretinal implants (for review see Ref. 105). The subretinal implant is composed of an array of microphotodiodes that are connected to microelectrodes. The device is surgically placed in the subretinal space between the RPE and the outer plexiform layer of the degenerated retina. Light that enters the eye is converted by the microphotodiodes into small currents that are released by the microelectrodes and stimulate second order neurons (horizontal cells, bipolar cells) in the host retina. The epiretinal implant, on the other hand, is essentially a readout chip that generates electrical impulses and stimulates directly the ganglion cells and their axons after receiving information from a camera and an image processor that are external to the eye. Surgical implantation and long-term biocompatibility of the subretinal prosthesis has been evaluated in cats and shown to be well-tolerated despite marked loss of photoreceptors and inner nuclear layer disorganization in the retina directly overlying the implant.¹⁰⁶ The epiretinal implant approach has been tested in dogs.^{42,107,108} Results show that these electrode arrays can be successfully implanted onto the retinal surface for extended periods without causing any structural or electrophysiological complications. In a subsequent study conducted in wild-type and RCD1 dogs it was proven that electrical stimulation of the retina via an active epiretinal implant was safe. Efficacy of the epiretinal prosthesis was not evaluated since the RCD1 dogs used in this study were 3–3.5-year-old and had already late stage PRA. The positive results of the preclinical safety studies conducted in large animal models enabled that further testing be conducted in blind human patients with advanced RP.^{109,110} Implantation of epiretinal prosthesis in individuals with RP has shown that light perception could be obtained, and that these patients were able to perform simple visual tasks.^{109–111}

Improved visual function has also been observed in RP patients that were implanted with a subretinal prosthesis, yet the improvements occurred in visual areas that were distant from

the implant.¹¹² It has been suggested that mechanical injury of the retina during the surgical procedure as well as low-level electrical stimulation by the implant may elicit the release of survival factors that provide a generalized neuroprotective effect to the retina.

CONCLUSION

In summary, the dog has proven its value over the past recent years as a powerful animal model to test new treatment strategies for retinal degenerative diseases. The two therapeutic approaches (ECT-CNTF and AAV-2/2-RPE65) that are now in human clinical trials were initially tested for efficacy and safety in the dog. With the expectation that new naturally occurring forms of RD in dogs will be characterized, there is hope that additional canine models of RD will be available to improve our understanding of the pathogenic mechanisms of these diseases, and to find cures for this devastating group of disorders.

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Figure 1.

Structure and production of a rAAV vector. A recombinant AAV vector is produced by deleting the *rep* and *cap* genes from the wild-type AAV genome which leaves approximately 4.7 kb to insert a promoter (Pro) and the cDNA of a therapeutic gene capped with a poly-adenylation site (PolyA) between the two inverted terminal repeats (ITR). This genome construct is then inserted into a plasmid and used with a helper plasmid containing the *rep* and *cap* genes to transfect cells in culture. These cells produce a replication-defective recombinant AAV. In this figure both the genome and the capsid originate from an AAV2 serotype thus generating a rAAV2/2 vector.

Figure 2.

Cell tropism of rAAV chimeric serotypes in the canine retina. Packaging the same rAAV2 genome into capsids of different serotypes produces chimeric vectors (also called pseudotypes) that transduce distinct retinal (and brain) cell populations. RPE: retinal pigment epithelium; PRs: photoreceptors; INL: inner nuclear layer; GC: ganglion cells; LGN: lateral geniculate nucleus; SC: superior colliculus.

Figure 3.

Technologies used to inhibit gene expression for the treatment of retinal degenerative diseases that result from a toxic gain of function. Aptamers, siRNAs, and ribozymes are nucleic acids used to knockdown gene expression at the protein (aptamer) or mRNA (siRNA, ribozyme) level (Adapted from Fattal *et al. Adv. Drug Deliv. Rev.*, 2008).

Figure 4.

Vector-based RNA interference. Sustained silencing of a gene can be achieved by transfecting the target cells with a shRNA construct delivered via a viral vector. Following transcription, the small interfering RNA hairpin is degraded by the RNase DICER into a 21–23 nucleotide siRNA duplex. This short double-stranded siRNA molecule recruits a multiprotein complex known as RISC (RNA-induced silencing complex). RISC causes the unwinding of the 2 strands, the complementary binding of the antisense strand to the target mRNA, and its degradation, resulting in significant reduction in the levels of protein translation.

Table 1

Therapeutic studies conducted in canine models of retinal degeneration

Canine model	Breed	Mode of inheritance	Causative gene	Therapeutic studies	References
RCD1 [*]	Irish Setter	A.R.	<i>PDE6β</i>	D-cis-Diltiazem, CNTF, ECT-CNTF (ongoing)	17–20
RCD3 [†]	Cardigan Welsh Corgi	A.R.	<i>PDE6a</i>		3
PRCD [*]	Multiple breeds (> 25)	A.R.	<i>PRCD</i>	DHA diet supplement, Gene therapy (ongoing)	7,21
RPE65 mutant (canine LCA) ^{*,‡,§}	Briard	A.R.	<i>RPE65</i>	AAV-RPE65	22–29
CMR1 [*]	Great Pyrenees English Mastiff, Bullmastiff	A.R.	<i>BEST1 = VMD2</i> (Stop mutation)		9
CMR2 [*]	Coton de Tulear	A.R.	<i>BEST1 = VMD2</i> (Missense mutation)		9
CD (achromatopsia) [*]	Alaskan Malamute	A.R.	<i>CNGB3</i>	Gene therapy (ongoing)	6,30
XLPR1 [*]	German Short-Hair pointer				
	Siberian Husky, Samoyed	X-linked	<i>RPGR</i> (premature stop mutation)	Gene therapy (ongoing)	4,31
XLPR2 [*]	Mongrel	X-linked	<i>RPGR</i> (frameshift mutation)	CNTF, gene therapy (ongoing)	4,19
T4R RHO [*]	English Mastiff, Bullmastiff	A.D.	<i>RHO</i>	Steroids	5,32, 33

Abbreviations: RCD1, rod cone dysplasia 1; RCD3, rod cone dysplasia 3; PRCD, progressive rod cone degeneration; LCA, Leber's congenital amaurosis; CMR, canine multifocal retinopathy; CD, cone degeneration; XLPR1, X-linked progressive retinal atrophy; A.R., autosomal recessive; A.D., autosomal dominant; PDE6β, Phosphodiesterase 6β; BEST1, Bestrophin; CNGB3, cyclic nucleotide-gated channel β subunit; RPGR, retinitis pigmentosa GTPase regulator; RHO, rhodopsin.

References describing therapeutic studies are listed in bold.

Model bred and housed at: ^{*}the Retinal Disease Studies Facility, School of Veterinary Medicine, University of Pennsylvania, PA, USA;

[†]College of Veterinary Medicine, Michigan State University, MI, USA;

[‡]College of Veterinary Medicine, University of Missouri, MO, USA;

[§]Boisbonne center, School of Veterinary Medicine, Nantes, France.