Gene Augmentation for X-Linked Retinitis Pigmentosa Caused by Mutations in *RPGR*

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X-linked retinitis pigmentosa (XLRP) caused by mutations in the *RPGR* gene is a severe and early onset form of retinal degeneration, and no treatment is currently available. Recent evidence in two clinically relevant canine models shows that adeno-associated viral (AAV)-mediated *RPGR* gene transfer to rods and cones can prevent disease onset and rescue photoreceptors at early- and mid-stages of degeneration. There is thus a strong incentive for conducting long-term, preclinical efficacy and safety studies, while concomitantly pursuing the detailed phenotypic characterization of XLRP disease in patients that may benefit from such corrective therapy.

X-linked retinitis pigmentosa (XLRP) includes some of the earliest onset and rapidly progressive forms of inherited retinal degenerations, and accounts for 6%–20% of all cases of retinitis pigmentosa (Fishman 1978; Jay 1982; Breuer et al. 2002). XLRP has been genetically mapped to six loci (RP2, RP3, RP6, RP23, RP24, and RP34); of these, RP3, which is associated with mutations in the retinitis pigmentosa GTPase regulator (*RPGR*) gene, is the predominant subtype (Meindl et al. 1996; Roepman et al. 1996). Indeed, mutations in *RPGR* are found in >70% of families affected by XLRP and ~15% of simplex retinitis pig-

mentosa cases (Bader et al. 2003; Sharon et al. 2003; Pelletier et al. 2007; Branham et al. 2012). Over the past decade, substantial effort has been dedicated to unveiling *RPGR*'s function in photoreceptors, identifying novel mutations and the spectrum of disease phenotypes in humans, and characterizing small and large animal models of *RPGR*-XLRP that could be used to test novel therapeutic approaches. This review will primarily focus on the results of the preclinical studies conducted in canine models, as these provide strong evidence that a corrective gene therapy for XLRP may become a reality in the not-so-distant future.

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RPGR AND ITS ROLES IN THE PHOTORECEPTOR CONNECTING CILIUM

The *RPGR* gene (172 kb) was initially described as containing 19 exons, and mutations in *RPGR*^{ex1-19} accounted for only 15%–20% of XLRP patients (Meindl et al. 1996). Subsequently, an alternatively spliced carboxy-terminal exon called ORF15 was identified and found to be a hot spot for microdeletions, frameshift mutations, and premature stop mutations (Vervoort et al. 2000). Of the more than 350 *RPGR* mutations reported to date, >55% are found in exon ORF15 (see <http://rpgr.hgu.mrc.ac.uk>).

Among the different splice variants (Vervoort et al. 2000; Neidhardt et al. 2007; Schmid et al. 2010), *RPGR*^{ex1-ORF15} and constitutive *RPGR*^{ex1-19} are the most abundantly expressed *RPGR* isoforms in the retina. Both proteins have been immunolocalized to photoreceptor connecting cilium of both rods and cones (Hong et al. 2003; Wright et al. 2011). One group found *RPGR*^{ex1-ORF15} in bovine and human photoreceptor outer segments (Roepman et al. 2000; Mavlyutov et al. 2002), although this was later disputed (Hong et al. 2003). The different temporal pattern of retinal expression of these two isoforms suggests that *RPGR*^{ex1-19} may play a role in photoreceptor development whereas *RPGR*^{ex1-ORF15} may be necessary for maintaining the integrity of mature photoreceptors (Wright et al. 2011). The constitutive human *RPGR*^{ex1-19} isoform encodes an 815 amino acid protein (MW: 90–100 kDa) that is widely expressed, whereas the full-length human *RPGR*^{ex1-ORF15} isoform, which encodes a 1152 amino acid, soluble protein (MW: 200–220 kDa), has been found predominantly in photoreceptors.

Both isoforms contain a common amino-terminal RCC1-like domain (Meindl et al. 1996) (encoded by exons 2 to 11), which has recently been shown to play the role of a guanine nucleotide exchange factor (GEF) by directly interacting with the GDP-bound form of the small GTPase RAB8A (Murga-Zamalloa et al. 2010a). *RPGR* has also been shown to interact directly or via molecular complexes with the following proteins: PDE6d (Linari et al. 1999);

a number of ciliary proteins, including *RPGR*-interacting protein 1 (*RPGRIP1*) (Boylan and Wright 2000; Roepman et al. 2000; Hong et al. 2001); the nephrocystins *NPHP1* (Murga-Zamalloa et al. 2010b), *NPHP4* (Murga-Zamalloa et al. 2010b), *NPHP5/IQCB1* (Otto et al. 2005), *NPHP6/CEP290* (Chang et al. 2006), and *NPHP8/RPGRIP1L* (Khanna et al. 2009); the chromosome-associated proteins *SMC1* and *SMC3* (Khanna et al. 2005); the centrosomal protein *NPM1* (Shu et al. 2005); and proteins associated with intraflagellar transport (*Tg737/Polaris/IFT88*) and microtubule motors (dynein subunits, kinesin-II) (Khanna et al. 2005). Exon ORF15 encodes for a highly repetitive, glutamic acid-rich region whose function is currently unknown, yet its carboxyl terminus has been recently shown to interact with whirlin, which is a member of the Usher interactome (Wright et al. 2012). Localization of *RPGR* to the sensory cilia of photoreceptors (Hong et al. 2003) and its interaction with numerous complexes involved in protein transportation suggest that *RPGR* plays a role in regulating protein trafficking between the inner and outer segments at the level of the connecting cilium; however, its exact molecular function is still poorly understood.

Recent reports have suggested that *RPGR* may contribute to the biogenesis and maintenance of photoreceptor cilia and the transitional zone of motile cilia (Murga-Zamalloa et al. 2010a; Gakovic et al. 2011; Ghosh et al. 2011). This role is consistent with the occurrence of hearing dysfunction, respiratory infections, and cilia dyskinesia in some rare cases of syndromic *RPGR*-XLRP (van Dorp et al. 1992; Iannaccone et al. 2003; Koenekoop et al. 2003; Zito et al. 2003; Iannaccone et al. 2004; Moore et al. 2006; Bukowy-Bieryllo et al. 2013).

RETINAL DEGENERATION PHENOTYPE OF XLRP PATIENTS WITH *RPGR* MUTATIONS

A range of clinical diagnoses has been associated with *RPGR* mutations that cause progressive retinal degeneration of photoreceptors. *RPGR*-associated retinal degeneration most commonly presents in males of XLRP families or in simplex

males as early loss of night vision and peripheral vision, abnormal electroretinograms, and relatively better retention of central vision in younger patients (Meindl et al. 1996; Roepman et al. 1996; Vervoort et al. 2000; Sandberg et al. 2007; Zahid et al. 2013). Less commonly, there is early involvement of the fovea and loss of visual acuity. Depending on the degree of abnormality in retina-wide measurements of cone versus rod function, clinical diagnoses have included X-linked cone-rod dystrophy, cone dystrophy, or macular degeneration (Mears et al. 2000; Ayyagari et al. 2002; Demirci et al. 2002; Yang et al. 2002; Ebenezer et al. 2005; Pelletier et al. 2007; Thiadens et al. 2011; Beltran et al. 2012). Retinally colocalized measures of function have often shown both rod and cone loss (Jacobson et al. 1997; Lorenz et al. 2003; Huang et al. 2012) consistent with expression of the *RPGR* gene product in rod and cone cilia (Hong et al. 2000, 2003; Roepman et al. 2000; Khanna et al. 2005; Shu et al. 2005). High allelic heterogeneity has been implied as cause for the spectrum of severity and phenotypes encountered between families carrying different *RPGR* mutations, and there is evidence that genetic modifiers may contribute to intra-familial phenotypic divergence (Keith et al. 1991; Walia et al. 2008; Ruddle et al. 2009; Fahim et al. 2011).

Carrier females with *RPGR* mutations also show a wide spectrum of retinal disease expression ranging from asymptomatic with normal electrophysiological and psychophysical test results to very severe retina-wide disease (Andreasson et al. 1997; Jacobson et al. 1997; Weleber et al. 1997; Bauer et al. 1998; Fishman et al. 1998; Flaxel et al. 1999; Ayyagari et al. 2002; Lorenz et al. 2003; Ebenezer et al. 2005; Jin et al. 2006; Ruddle et al. 2009; Thiadens et al. 2011). There tends to be significant interocular asymmetry of disease severity in most carriers (Jacobson et al. 1989; Weleber et al. 1997; Fishman et al. 1998; Lorenz et al. 2003; Ebenezer et al. 2005). A distinct golden tapetal-like reflex, hypothesized to be located at cone inner segments (Cideciyan and Jacobson 1994), or peripheral photoreceptor outer segments (Berendschot et al. 1996), has been variably described in a subset of female carriers of *RPGR* mutations (Andreasson et al. 1997; Jacobson et al. 1997; Weleber et al. 1997; Bauer et al. 1998; Fishman et al. 1998; Flaxel et al. 1999; Ayyagari et al. 2002; Lorenz et al. 2003; Ebenezer et al. 2005; Jin et al. 2006; Ruddle et al. 2009; Thiadens et al. 2011).

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ANIMAL MODELS OF *RPGR*-XLRP

Murine Models

The description of the phenotypes of several mouse models of *RPGR* disease, including XLRP (Hong et al. 2000, 2004; Brunner et al. 2010; Wright et al. 2011; Huang et al. 2012; Thompson et al. 2012) and cone-rod dystrophy (Brunner et al. 2010), have been published. Evidence that mice sharing the same *RPGR* mutation but on a different genetic background can express a different disease phenotype further highlights the potential role of genetic modifiers (Brunner et al. 2010). Overexpression of the *RPGR*^{ex1-19} isoform in photoreceptor cells of transgenic mice leads to early onset (2 months of age) degeneration, whereas no retinal alterations are associated with overexpression of the *RPGR*^{ex1-ORF15} isoform (Wright et al. 2011). This finding further supports the claim that the severe phenotype described in a transgenic mouse expressing a truncated *RPGR*^{ex1-ORF15} isoform is caused by a toxic gain-of-function and not to the overexpression of the transgene (Hong et al. 2004). With the exception of this transgenic model that shows rapid kinetics of photoreceptor loss, the other *RPGR* mutant mice, including the naturally occurring *Rd9* mouse (Thompson et al. 2012) that could be used to test a gene augmentation therapy strategy, show a slowly progressive photoreceptor degeneration resulting in retention of >50% outer nuclear layer thickness by 2 years of age (summarized in Huang et al. 2012, Supplementary Table S1, see <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10070/-/DCSupplemental>).

Canine Models

To date, the XLPRA1 and XLPRA2 dogs are the only identified large animal models of

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RPGR-XLRP. Although both diseases are caused by nearby microdeletions in the same exon ORF15, they show distinct ages of onset, rates of progression, and spatiotemporal distribution of retinal degeneration.

In XLPRA1, a five nucleotide deletion in exon ORF15 (c.1028–1032delGAGAA; GeneBank accession no. AF385621) introduces an immediate premature stop codon, and the putative protein has 230 amino acids truncated at its carboxyl-terminal end (Zhang et al. 2002). This deletion causes a rod–cone degeneration detectable by histology and electroretinogram in juvenile hemizygote male dogs (approximately one year of age) that progresses from the peripheral to the central retina (Zeiss et al. 1999; Beltran et al. 2012). Even though the research colony has been established from a single affected male, the extent of severity of the retinal changes, and their rate of progression varies from one individual to another even in animals of the same age (Zeiss et al. 1999; Guyon et al. 2007; Beltran et al. 2012), suggesting that genetic modifiers which have been implicated in humans with *RPGR*-XLRP (Fahim et al. 2011) may also be responsible for this variability in disease phenotype. As a result of random X-inactivation (also called lyonization), heterozygous female dogs undergo a patchy form of retinal degeneration (Zeiss et al. 1999; Beltran et al. 2009) that is the result of loss of photoreceptors that are clonally derived from a progenitor cell expressing the mutant XLPRA1 allele. This mosaic pattern of retinal degeneration, which is histologically detectable at 1.4 yr and is still apparent in 7.8-yr-old dogs, resembles the phenotype reported in human XLRP carriers (Szamier and Berson 1985; Jacobson et al. 1997; Aguirre et al. 2002; Vajaranant et al. 2002).

A second form of canine *RPGR*-XLRP, termed XLPRA2, is caused by a two nucleotide deletion in ORF15 (c.1084–1085delGA) (Zhang et al. 2002). This microdeletion introduces a frameshift followed by a premature stop codon. The putative protein includes 34 additional basic residues and an increased isoelectric point (4.30 vs. 4.01). XLPRA2 retinas undergo early (4 weeks of age) disorganization of photoreceptor outer segments, which is followed by

increased cell death that peaks at about 7 weeks of age before returning, at 12 weeks, to a reduced yet constant rate. These photoreceptor cell death kinetics result in rapid outer nuclear layer thinning, that leads to an average thickness of three rows of nuclei by 41 weeks of age. Early cone disease has been shown to occur in the newly discovered canine fovea-like area (Beltran et al. 2014a). Mislocalization of rod opsin, short-wavelength (blue) cone opsin, and middle/long-wavelength (red/green) opsin are other early events that suggest that this canine ciliopathy affects all populations of photoreceptors. Similar evidence for altered protein trafficking through the connecting cilium has been reported in murine models (Hong et al. 2000, 2005; Brunner et al. 2010; Wright et al. 2011; Thompson et al. 2012), and in a carrier patient of XLRP (Adamian et al. 2006). Initiation of photoreceptor loss is accompanied by early retinal remodeling events such as rod neurite sprouting, retraction of rod bipolar dendrites, and Müller cell gliosis. Subsequently, other inner retinal remodeling events, including altered labeling of cell-specific markers of horizontal and amacrine cells, and loss of normal inner plexiform lamination follows (Beltran et al. 2006). Despite the severe progression of the disease, particularly in the central (visual streak) area (Beltran et al. 2012), dogs at 41 weeks of age retain sufficient rod- and cone-mediated visual function to navigate without difficulties in an obstacle avoidance course (WA Beltran, unpubl.). Similarly to XLPRA1 carriers, XLPRA2 heterozygous mutant dogs undergo random X-inactivation during embryogenesis that results in a patchy form of retinal degeneration (Fig. 1). However, unlike XLPRA1, the focal areas of cell loss do not persist. Indeed, improved outer nuclear layer lamination, loss of opsin mislocalization, and improved morphology of the outer plexiform layer and rod bipolar cell dendrites in older carriers suggests that retinal plasticity in the young XLPRA2 carriers allows tangential displacement of remaining (nonmutant) photoreceptors in the depleted foci, and rewiring with postreceptoral neurons (Beltran et al. 2009). Similar lateral dispersion of photoreceptors following focal laser damage has been

Figure 1. Mosaic pattern of retinal degeneration in XLPRA2 female carriers. (A) Focal patches of photoreceptor degeneration surrounded by normally laminated retina in a 24-wk-old carrier. (B) Numerous patches of rod opsin mislocalization to the inner segments (IS) and outer nuclear layer (ONL), and features of rod neurite sprouting in the outer plexiform layer (OPL) in a 7-wk-old carrier. (Modified from data in Figure 1 from Beltran et al. 2009).

shown in the nonhuman primate retina (Strazzeri et al. 2014).

Earlier on, we speculated that the more severe disease phenotype observed in the XLPRA2 model could be caused by a toxic gain-of-function, whereas the slower progression and delayed onset seen in XLPRA1 might be explained by a loss-of-function mechanism. These hypotheses led us to initially develop a gene augmentation strategy for the XLPRA1 disease, while preparing for the potential need to combine an allele-independent gene knockdown and a hardened (resistant) gene replacement approach to correct the XLPRA2 phenotype (see below).

OUTCOME MEASURES OF PRECLINICAL EFFICACY

Detailed analysis of the spatiotemporal features of the two canine diseases and development of staging classifications allowed establishment of outcome measures of therapeutic intervention. These are listed in Table 1.

THERAPEUTIC INTERVENTIONS: PROOF OF CONCEPT STUDIES IN CANINE MODELS OF RPGR-XLRP

Note: All studies reported below were carried out after approval by the University of Pennsyl-

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Table 1. Outcome measures of therapeutic intervention for XLRP

Outcome measures of photoreceptor rescue	Methods	Expected positive therapeutic effect
ONL thickness	In vivo OCT, histology	Retention
IS and OS structure	In vivo OCT, histology	Preservation
Opsin mislocalization	IHC (rod, cone opsins)	Absence
Rod neurite sprouting	IHC (rod opsin, synaptophysin)	Absence
Rod function	Scotopic ERG (a wave amplitude)	Retention
Cone function	Photopic ERG (30 Hz flicker)	Retention
Bipolar cell dendrite length	Histology	Retention
IPL thickness and lamination	IHC (GABA)	Retention
Müller cell gliosis	IHC (GFAP)	Absence
Postreceptoral function	Scotopic ERG (b wave amplitude)	Retention

ERG, electroretinogram; GFAP, glial fibrillary acidic protein; IHC, immunohistochemistry; IPL, inner plexiform layer; IS, inner segment; OCT, optical coherence tomography; ONL, outer nuclear layer; OS, outer segment.

vania IACUC, and conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, the USDA's Animal Welfare Act and Animal Welfare Regulations, and in compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Neuroprotection Fails to Rescue Photoreceptors in the XLPRA2 Dog

Following successful demonstration of neuroprotection therapy in the *rcd1* dog model of autosomal recessive retinitis pigmentosa (*PDE6B* mutation) (Tao et al. 2002), we examined whether single or repeated intravitreal injections of recombinant human ciliary neurotrophic factor (CNTF) would also modify the course of retinal degeneration in the XLPRA2 dog. Our results (Beltran et al. 2007) failed to demonstrate any positive outcome even when the compound was delivered before the peak of rod loss. However, we observed an increased number of rod opsin-positive cells in the peripheral retina, suggesting the potential for CNTF-mediated photoreceptor cell proliferation in the early postnatal XLPRA2 retina.

Gene Transfer Considerations: Choice of Viral Vector, Promoters, Route of Delivery

Among the various chimeric serotypes of recombinant adeno-associated viral (AAV) vec-

tors previously used for gene transfer to the canine retina (Beltran 2009), we selected the AAV2/5 serotype, as it had been shown in dogs to infect both rods and cones (Weber et al. 2003; Komaromy et al. 2008; Petersen-Jones et al. 2009; Beltran et al. 2010). Photoreceptor-specific promoters that would restrict transgene expression to either rods (mOP, a 476 nt segment of the proximal mouse opsin promoter), or rods and cones (hGRK1, a 292 nt segment of the human G-protein-coupled receptor kinase 1 promoter; hIRBP, a 235 nt segment of the human interphotoreceptor binding protein promoter) were used (al-Ubaidi et al. 1992; Beltran et al. 2010, 2012). Subretinal injections were performed with a subretinal cannula as previously reported (Komaromy et al. 2006; Beltran et al. 2010), and volumes injected (70–150 μ L) were adjusted to the dog's age and globe size, aiming to produce a bleb that covered $\sim 1/5$ – $1/4$ of the retinal surface.

Initial RPGR Gene Therapy Failures and Complications

The first viral vector constructs that were developed and tested in XLPRA1 dogs included a shortened version of the canine RPGR^{ex1-ORF15} cDNA (cshort RPGR^{ex1-ORF15}). The decision to use this shortened RPGR transgene, which had 708 bp removed in frame from the purine-rich region of exon ORF15, was made to circumvent the DNA packaging restric-

tion of the AAV capsid (<4.7 kb) in the event that a knockdown construct (small hairpin RNA or ribozyme) should need to be added to correct the XLPRA2 disease. This strategy was based on prior evidence that similar in-frame truncation of murine exon ORF15 did not eliminate its function (Hong et al. 2005). Five XLPRA1-affected dogs (four hemizygous males and homozygous mutant female) received, at 26–28 weeks of age (before the onset of retinal degeneration), a 150 μ L subretinal injection of either an AAV2/5-mOP-cshortRPGR^{ex1-ORF15} or AAV2/5-hIRBP-cshortRPGR^{ex1-ORF15} vector preparation (viral titer: 1.5×10^{11} vg/mL), or balanced salt solution. At 5–7 wk postinjection retinal examination revealed multifocal hyper-reflective lesions in the treated area of five out of the six eyes that received either of the two vectors. Noninvasive imaging by optical coherence tomography showed disrupted lamination, which represented photoreceptor rosettes on histologic examination (Fig. 2) (Beltran et al. 2011, 2012). The mechanism by which transduction of photoreceptors with a shortened canine RPGR^{ex1-ORF15} cDNA led to these complications was not further investigated, and it was decided instead to focus only on the gene augmentation strategy by developing and testing a viral vector that would carry instead the full-length RPGR^{ex1-ORF15} transgene.

Gene Augmentation with Full-Length Human RPGR^{ex1-ORF15} Prevents Disease Onset in the XLPRA1 Model

With the goal of accelerating translation of gene therapy to clinical trials, we decided to include the human rather than the canine full-length RPGR^{ex1-ORF15} in the new viral constructs. In addition, availability of antibodies directed against human RPGR (that do not cross-react with the canine isoform) would allow monitoring of therapeutic transgene expression. An AAV2/5 construct including the hRPGR^{ex1-ORF15} transgene under the control of the hIRBP promoter was subretinally injected in two XLPRA1 dogs at 28 weeks of age (before the onset of photoreceptor loss). Dogs were monitored by noninvasive confocal scanning

laser ophthalmoscopy and spectral-domain optical coherence tomography until 77 weeks of age (well after the start of retinal degeneration). Topographical mapping of the outer nuclear layer showed increased thickness in the treated region that remained within the range seen in wild-type dogs, while significant thinning was seen in the untreated region of the same eye or in the fellow, balanced salt solution-injected control eye (Fig. 3A,B). In addition, improved backscatter signal originating from the inner/outer segment layer was also detectable in the treated area (Beltran et al. 2012). Histological analysis confirmed the rescue of photoreceptors within the bleb area where RPGR transgene was expressed, as evidenced by an increased number of photoreceptor cells, preserved inner and outer segment structure, and absence of rod opsin and R/G opsin mislocalization (Fig. 4A–C). This positive effect on the outer retina was also associated with improved structure of the outer plexiform layer, bipolar cells, and a lack of reactivity of Müller cells (the major glial cell population in the retina) (Fig. 4D–F). These results suggest that early intervention with corrective gene augmentation can prevent the onset of photoreceptor loss and impede deleterious secondary remodeling events in the inner retina.

Gene Augmentation with Full-Length Human RPGR^{ex1-ORF15} Rescues Photoreceptors in the XLPRA2 Model When Delivered at Early and Mid-Stages of Disease

The successful outcome in the XLPRA1 model led us to test whether RPGR gene augmentation alone could also positively alter the course of photoreceptor degeneration in the early-onset and more rapidly progressive XLPRA2 disease. An AAV2/5 construct including the hRPGR^{ex1-ORF15} transgene under the control of the hIRBP promoter, and one with the hGRK1 promoter, were each subretinally injected in one XLPRA2 dog at 5 weeks of age (after disease onset, but before the peak of cell death; outer nuclear layer near normal thickness). Fellow eyes were subretinally injected with balanced salt solution. Similar longitudinal analysis (up to 36 weeks of age) of outer nuclear

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Figure 2. Retinal toxicity induced by early viral vector construct. Retinal complications in an affected XLPRA1 dog 7 wk following subretinal injection of an AAV2/5 carrying a shortened version of canine RPGR^{ex1-ORF15} cDNA under the control of hIRBP promoter. (A) Multifocal hyperreflective lesions (red arrows) seen by ophthalmoscopy within the treated region. (B) Similar lesions observed by confocal scanning laser ophthalmoscopy (cSLO) and optical coherence tomography (OCT). (C) Histological features of photoreceptor rosettes, using hematoxylin and eosin (H&E). (D) Immunohistochemical characterization of the photoreceptor rosettes lesions. (D₁–D₃) Immunolabeling (green) of the retinal pigment epithelium (RPE) is lost (white arrows) at close proximity (D₁) and within (D₂–D₃) the lesions. (D₄–D₆) Normal lamination (D₄) and structure of rods (rod opsin, green) and cones (cone arrestin, red) is disrupted within the lesions (D₅–D₆). (D₇–D₉) The normal architecture of Müller cells (D₇, vimentin, green) is altered within the lesions (D₈–D₉) with prominent extension of radial processes into the vestigial subretinal space. Limited reactivity of Müller cells (GFAP, red) is seen.

layer thickness from spectral-domain optical coherence tomography-derived topographical maps illustrated a potent rescue of photoreceptors in the treated region with the AAV2/5-hIRBP-hRPGR^{ex1-ORF15} vector (Fig. 3C,D), which was confirmed histologically (Fig. 5).

Treatment corrected the mislocalization of op-
sins, improved expression of presynaptic markers, resulted in normal length and arborization of bipolar cell dendrites, and prevented Müller cell gliosis. Although structural preservation appeared to be more pronounced with the con-

Figure 3. In vivo retinal imaging of photoreceptor rescue following gene therapy in XLPR1 and XLPR2 dogs. Longitudinal follow-up of outer nuclear layer (ONL) thickness changes in eyes of XLPR1 (H484) and XLPR2 (Z412) dogs treated with a subretinal injection of AAV2/5-hIRBP-*hRPGR*^{ex1-ORF15} vector. (A) ONL topography of H484 measured at 28 weeks of age, immediately before the injection, and at 43 and 76 weeks of age corresponding to 15 and 48 wk after the injection, respectively. (B) Progression of ONL thickness at regions of interest within the injection bleb (green symbols) compared with those outside the bleb (red symbols); results from wild-type animals measured at the same retinal locations are also shown (gray symbols). (C) ONL topography of Z412 measured at 21 and 36 weeks of age; injection was performed at 5 weeks of age. (B,D) Progression of ONL thickness at regions of interest within the injection bleb (green symbols) compared with those outside the bleb (red symbols); results from wild-type animals measured at the same retinal locations are also shown (gray symbols). (D) Progression of ONL thickness at regions of interest within the injection bleb (green symbols) compared with those outside the bleb (red symbols); results from wild-type animals measured at the same retinal locations are also shown (gray symbols). Dashed lines in (A) and (C) demarcate the transient blebs created by the subretinal injections; retinal blood vessels and optic nerve are superimposed on the pseudo-color ONL data. Squares in (A) and (C) denote the two regions of interest chosen within the bleb boundary and two regions chosen outside the bleb boundary for the quantitative measures shown in (B) and (C). (Modified from data in Figures 2 and S1 from Beltran et al. 2012).

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Figure 4. Gene augmentation therapy with AAV2/5-hIRBP-hRPGR^{ex1-ORF15} vector prevents retinal degeneration onset in XLPR1 disease. *Upper right* diagram shows the area of subretinal injection performed at 28 weeks of age (green dotted line), and the site of the histological sections shown below at 77 weeks of age (red line) in XLPR1 dog H484. (A) H&E stained cryosection shows preserved outer nuclear layer (ONL) thickness and inner (IS) and outer segment (OS) structure in the treated region. Dotted gray line shows the abrupt demarcation between the treated and untreated regions. (B) RPGR immunolabeling confirms expression of the transgene exclusively in the photoreceptors of the treated area. (C) Mislocalization of rod opsin (RHO, green) and red/green cone opsin (R/G, red) as well as cone morphology (cone arrestin, red) is corrected in the treated area. (D) Preserved density of CtBP2/RIBEYE immunolabeled (red) synaptic ribbons in photoreceptor terminals and normal thickness of the outer plexiform layer (OPL) in the treated area. (E) Preserved dendritic arborization in rod bipolar cells immunolabeled with PKCα (green) and Goα (red) in the treated area. (F) Absence of Müller cell reactivity assessed by GFAP (red) immunolabeling in the treated area. (Modified from data in Figures 3 and S4 from Beltran et al. 2012).

Figure 5. Gene augmentation therapy with AAV2/5-hIRBP-hRPGR^{ex1-ORF15} vector rescues photoreceptors when delivered at early-stage of XLPR2 disease. *Upper right* diagram shows the area of subretinal injection performed at 5 weeks of age (green dotted line), and the site of the histological sections shown below at 38 weeks of age (red line) in XLPR2 dog Z412. (A) H&E stained cryosection shows increased outer nuclear layer (ONL) thickness and inner (IS) and outer segment (OS) structure in the treated region. Dotted gray line shows the abrupt demarcation between the treated and untreated regions. (B) RPGR immunolabeling confirms expression of the transgene exclusively in the photoreceptors of the treated area and is seen throughout the cells with the exception of the outer segments. (C) Mislocalization of rod opsin (RHO, green) and red/green cone opsin (R/G, red) as well as cone morphology (cone arrestin, red) is corrected in the treated area. (D) Increased density of CtBP2/RIBEYE immunolabeled (red) synaptic ribbons in photoreceptor terminals and increased thickness of the outer plexiform layer (OPL) in the treated area. (E) Preserved dendritic arborization in rod bipolar cells immunolabeled with PKCα (green) and Goα (red) in the treated area. (F) Absence of Müller cell reactivity assessed by GFAP (red) immunolabeling in the treated area. (Modified from data in Figures S3 and 4 from Beltran et al. 2012).

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struct that included the hIRBP rather than the hGRK1 promoter, both viral vectors rescued photoreceptor- and postreceptor electroretinogram function (Beltran et al. 2012). Comparative studies including higher numbers of animals and dose groups are warranted to adequately establish which of these two promoters is optimal.

AAV2/5-hIRBP-hRPGR^{ex1-ORF15} was also delivered to three 12-wk-old XLPRA2 dogs at mid-stage disease. At this age, the outer nuclear layer thickness is ~70% of normal, retraction of rod bipolar cells has begun, and there is increased reactivity of Müller cells (Beltran et al. 2006). This is right after the peak of photoreceptor cell death and just before the beginning of the “chronic” phase of cell death, during which numerous proapoptotic and prosurvival genes are differentially expressed (Genini et al. 2013). Dogs were regularly evaluated by noninvasive retinal imaging (scanning confocal laser ophthalmoscopy and spectral-domain optical coherence tomography) and electroretinography at 39 and 42 weeks of age, respectively. Although better rescue was achieved in an age-matched XLPRA2 dog treated at five weeks of age (early disease stage), both increased outer nuclear layer thickness in the bleb area and improved electroretinogram function in the treated eye of all three dogs suggest that gene therapy intervention may be beneficial even in retinal areas that have lost >30% of their photoreceptors (Beltran et al. 2013). Ongoing studies are assessing the efficacy of therapeutic intervention at more advanced stages of disease, and whether a long-term beneficial effect can be sustained (Beltran et al. 2014b).

Positive response to gene augmentation (alone) in the early onset and rapidly progressive XLPRA2 disease challenges the original assumption that the causative frameshift mutation leads to photoreceptor cell loss through toxic gain-of-function of the mutant RPGR^{ex1-ORF15} isoform. A more plausible hypothesis might be that the XLPRA2 mutation results in a loss-of-function that severely impairs photoreceptor maturation and survival, whereas the XLPRA1 disease may be caused by a hypomorphic mutation that impairs long-

term rod and cone viability. While these preclinical findings in two naturally occurring large animal models of RPGR-XLRP provide confidence that a similar RPGR gene augmentation strategy may be effective and sufficient in treating patients with a wide variety of RPGR mutations, one must remain open to the possibility that a subset of mutations may cause photoreceptor death via a toxic gain-of-function mechanism, and would require instead a combined gene knockdown and gene replacement strategy.

CONCLUDING REMARKS

Availability of two complementary canine models that recapitulate distinct spatiotemporal features of the phenotypes reported in human RPGR-XLRP patients has enabled us to establish initial proof of concept that a corrective gene augmentation approach may be sufficient to both prevent and stop the degenerative process of rods and cones. These results have provided invaluable information about the viral constructs currently being considered. Indeed, there was initially concern that fine-tuning of the levels of RPGR transgene expression in photoreceptors may be required via supposedly less “active” promoters (e.g., the RGPR promoter [Shu et al. 2012]) than the ones currently used. Yet, the potent hRPGR immunolabeling that was observed with hIRBP and hGRK1 promoters was not limited to the connecting cilium region of transduced canine rods and cones, thus suggesting that some degree of hRPGR^{ex1-ORF15} overexpression is tolerable and does not induce measurable cytotoxicity. A similar conclusion can be inferred on the basis of the normal retinal phenotype reported in transgenic mouse overexpressing RPGR^{ORF15} (Wright et al. 2011).

These initial proof-of-concept studies in large animals are very encouraging but will need to be monitored over a longer time period. Indeed, recent evidence in patients as well as in dogs treated by gene augmentation for the RPE65 form of Leber congenital amaurosis showed that, despite persistent functional rescue, ongoing photoreceptor loss continues (Cideciyan et al. 2013). Ongoing studies in mu-

tant dogs should also identify the latest stage of disease that can still respond to gene therapy. Careful extrapolation of these stages from dogs to humans could provide valuable inclusion criteria for the recruitment of patients to future clinical trials. Assessing the effects of *RPGR* gene augmentation in wild-type dogs also needs to be investigated in preparation for investigational new drug-enabling preclinical safety studies. *RPGR* overexpression in the wild-type retina will also inform as to whether similar gene therapy intervention could be safely considered for some carrier female patients. Finally, pursuing detailed phenotypic characterization of XLRP patients (Jacobson et al. 1997; Lorenz et al. 2003; Aleman et al. 2007; Huang et al. 2012) is necessary to establish valid outcome measures of therapeutic efficacy for future human clinical trials.

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A patent application on AAV-mediated gene therapy for *RPGR* X-linked retinal degeneration has been filed (PCT/US2013/022628). W.W.H. and the University of Florida have a financial interest in the use of AAV therapies, and own equity in a company (AGTC Inc.) that might, in the future, commercialize some aspects of this work.

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