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Leber Congenital Amaurosis Caused by Mutations in *RPGRIP1*

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Recessive null mutations in retinitis pigmentosa GTPase regulator interacting protein 1 (RPGRIP1) gene are the cause of LCA6 and account for 5% to 6% of the total patient population. RPGRIP1 has an essential role in the photoreceptor connecting cilia, and photoreceptors lacking RPGRIP1 are unable to maintain the light sensing outer segments. As a result, patients lose retinal functions at an early age but retain photoreceptors in the central retina well into adulthood thus holding out the prospect for gene augmentation therapies. Laboratory studies in animal models have demonstrated efficacy of gene therapy in slowing disease progression. With further refinement in the design of the replacement gene construct, clinical trials for Leber congenital amaurosis (LCA) caused by *RPGRIP1* mutations could be in the offing in the near future.

Leber congenital amaurosis (LCA) is a more severe form of retinal degeneration than retinitis pigmentosa, with visual deficit and loss of vision in early childhood (Heher et al. 1992; Fulton et al. 1996; den Hollander et al. 2008). Clinical findings indicate that both rod and cone photoreceptors are affected early on in LCA patients. Mutations in 16 different genes are currently known to cause LCA (Koenekoop 2004; den Hollander et al. 2008; Wang et al. 2009), one of which is the gene encoding retinitis pigmentosa GTPase regulator interacting protein 1 (RPGRIP1) (Dryja et al. 2001; Gerber et al. 2001; Koenekoop 2005). The rapid disease progression in LCA presents a significant challenge to gene therapy because retention of sufficient photoreceptors in the retina is a prerequisite for a satisfactory therapeutic outcome. In this regard, it is encouraging that LCA6 associated with *RPGRIP1* gene mutations have been reported to present with a more stable and somewhat nonprogressive disease course after the initial rapid decline in visual function (Hanein et al. 2004). Photoreceptors in the central retina appear to persist for long periods of time after visual function becomes immeasurable (Jacobson et al. 2007). Thus LCA6 patients with underlying *RPGRIP1* mutations have treatment potential for a gene replacement strategy if targeted to central, but not peripheral, retina. Continued improvement in retinal gene transduction vectors, validation of replacement gene design incorporating appropriate regulatory element and protein coding sequence, and better understanding of the disease pathophysiology will set the stage for clinical

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trials of therapies targeting LCA6 in the near future.

GENETICS AND PROTEIN FUNCTION

RPGRIP1 was initially identified through protein interaction screens (Boylan and Wright 2000; Roepman et al. 2000; Hong et al. 2001) as a binding partner of retinitis pigmentosa GTPase regulator (RPGR), a protein whose defects underlie the major form of X-linked retinitis pigmentosa (RP). Recessive mutations in *RPGRIP1* cause LCA (Dryja et al. 2001; Gerber et al. 2001; Hanein et al. 2004) and have also been found to cause cone–rod dystrophy (CORD13) (Hameed et al. 2003). About 5%–6% of all cases of LCA are caused by mutations in *RPGRIP1* (Dryja et al. 2001; Gerber et al. 2001; den Hollander et al. 2008). *RPGRIP1* alleles that cause LCA mostly create premature termination codons and are therefore presumed to be "null". The less severe form of disease, cone–rod dystrophy, is linked to missense mutations that likely retain partial function and thus may be considered hypomorphic. LCA6 patients manifest loss-of-function of both rods and cones early in life and develop a severe loss of central acuity, which leads to nystagmus. Electroretinography is nonrecordable from these patients even at an early age (Dryja et al. 2001; Galvin et al. 2005; Walia et al. 2010; Khan et al. 2013). Despite the early onset nature, there is some evidence that the central retinal laminar architecture including the photoreceptor cell layer could survive until much later (Jacobson et al. 2007). The retained retinal structure corresponded to the region of visual sensitivity. Other investigators have also observed that some LCA patients with *RPGRIP1* mutations can retain a substantial number of photoreceptors even when visual function has been lost as measured by visual field and electroretinogram (ERG) (Pawlyk et al. 2010). These observations stand in contrast to LCA caused by *AIPL1* mutations, in which maculopathy or loss of photoreceptors in the center is a prominent feature (Dharmaraj et al. 2004; Jacobson et al. 2011). The residual photoreceptors could be suitable as a therapeutic target, thus raising hope for gene therapy as a means to preserve or restore some vision.

The human *RPGRIP1* gene is located on chromosome 14q11 and is expressed as multiple splice variants (Lu and Ferreira 2005). Its expression can be detected embryonically and also in tissues outside of the retina. A major retinal variant consists of 24 exons and encodes a protein of 1287 amino acid residues. This variant of RPGRIP1 contains several structurally conserved motifs. A coiled coil domain, frequently found in centrosome-associated proteins, is located within its amino-terminal half. RPGRIP1 binds to the RCC1 homology domain of RPGR via its carboxy-terminal portion, hence this region is also known as the RPGR interacting domain. A bipartite nuclear localization signal is also found in the latter domain (Arts et al. 2009). In the middle portion of the protein are two C2 domains that may bind Ca^{2+}. RPGRIP1 has no integral membrane-spanning region but C2 domains in the protein could target it to cell membranes. Its closest homolog is RPGRIP1L, a protein also implicated in certain syndromic forms of retinal degeneration, with an overall identity between the two proteins at ~34%.

RPGRIP1 expression is enriched in retinal photoreceptors where it is stably associated with the connecting cilia (structurally and functionally analogous to the transition zone of primary cilia) and is resistant to extraction with high salt and detergent (Hong et al. 2001), suggesting it is one of the core component of ciliary axoneme of photoreceptors. By immune electron microscopy, RPGRIP1 is seen between the axonemal microtubules and the plasma membrane surrounding the connecting cilia. Recombinant RPGRIP1 expressed in cultured cells spontaneously form extended filamentous structures (Zhao et al. 2003). In vivo, RPGRIP1 has been shown to be required for anchoring RPGR at the connecting cilia (Zhao et al. 2003). The connecting cilium provides the only link between the biosynthetic inner segment and the light sensing outer segment in a photoreceptor cell. As such, it is the conduit through which large amounts of nascent proteins must be trafficked in support of daily outer segment renewal. The

precise function of RPGRIP1 in photoreceptors is not fully understood, but loss of RPGRIP1 leads to loss of RPGR at the connecting cilia, but not vice versa, suggesting RPGRIP1 tethers RPGR to the connecting cilia. In addition, RPGRIP1 also interacts with NPHP4, another ciliary protein, via a C2 domain (Roepman et al. 2005). Gene mutations in *NPHP4* have been shown to cause syndromic forms of retinal degeneration that also involve the kidney and other organs, known as ciliopathy. These data highlight a specific role for RPGRIP1 in ciliary structure and function. RPGRIP1 likely serves as a scaffold that anchors RPGR and additional ciliary components at the transition zone, and may play a role in ciliary trafficking in support of outer segment morphogenesis. In cells other than photoreceptors, RPGRIP1 is also localized to the ciliary/centrosomal compartment. The role of RPGRIP1 in these cells, if any, is not known. Given the fact that defects in RPGRIP1 cause only retinal degeneration, one may conclude that its function is important or nonredundant primarily in photoreceptors.

ANIMAL MODELS

A number of animal models have become available that carry recessive *RPGRIP1* mutations and recapitulate an LCA-like retinal phenotype. The first one was produced by targeted disruption of the *RPGRIP1* gene in mice (Zhao et al. 2003). Normally, the full-length mouse RPGRIP1 protein migrates on SDS-PAGE at ~200 kDa, which corresponds to the long RPGRIP1 variant mentioned earlier, and this full-length protein is ablated in the *RPGRIP1* knockout mouse. Mice lacking RPGRIP1 develop a rapid course of retinal degeneration involving both rods and cones. At the age of weaning (postnatal day 20), *RPGRIP1* knockout mice already show substantial mislocalization of photopigment in both rods and cones. Rudimentary outer segments do form but they are short and highly disorganized. ERG amplitudes are a fraction of the controls. Cell loss is relatively fast, such that by 5 mo of age, most photoreceptor cells have degenerated. In *RPGRIP1* knockout mice, RPGR is detectable by immunoblotting but is no longer found at the connecting cilium. Thus loss of RPGRIP1 leads to ectopic distribution of RPGR and therefore should abolish RPGR function as well. RPGRIP1 also likely performs additional functions at the connecting cilium, because mice lacking RPGRIP1 have a much more severe retinal phenotype than mice lacking RPGR alone. It was hypothesized that RPGRIP1 may be involved in photoreceptor disc morphogenesis. The relatively fast rate of photoreceptor degeneration in the knockout mice makes it a useful model of LCA both for studying the disease mechanism and for testing new therapies.

A second mouse model carrying a recessive *RPGRIP1* mutation, designated *Rpgrip1*^{nmf247}, was identified in an ethyl nitrosourea (ENU) chemical mutagenesis screen at the Jackson laboratory (Won et al. 2009). The mutation alters a splice site at exon 7 and results in skipping of exons 7 and 8 in the mRNA and premature termination of the open reading frame. Interestingly, this model shows a more severe retinal phenotype compared with the *RPGRIP1* knockout mice. In the homozygous *Rpgrip1*^{nmf247} mice, disease onset is early and the rate of degeneration is faster. ERG is nonrecordable from an early age. In photoreceptors, connecting cilia emerge from the apical inner segment, but this is not followed by the elaboration of any outer segments. Most photoreceptors will degenerate by the age of postnatal day 21. These data were interpreted to indicate that the *Rpgrip1*^{nmf247} mutation ablates an additional short variant of RPGRIP1 that escaped inactivation in the knockout model or that a truncated version of RPGRIP1 is still expressed in the knockout model giving rise to a residual RPGRIP1 function. The more severe phenotype and the accompanying protein analyses in the study indicate that the *Rpgrip1*^{nmf247} is likely a true null and more closely recapitulates human LCA.

A canine model carrying a spontaneous mutation in *RPGRIP1* has also been reported. This mutation was originally discovered and designated as cone–rod dystrophy 1 (*cord1*) that occurs naturally in miniature longhaired dachshunds (Curtis and Barnett 1993). In the affected dogs, ERG and histological changes

consistent with retinal degeneration are detectable after 10 wk of age. There is evidence that cones are affected earlier than rods. The *cord1* locus was mapped to a region of canine chromosome 15 containing the *RPGRIP1* gene. Genome sequencing identified a 44-nucleotide insertion in exon 2 of *RPGRIP1* that alters the reading frame and introduces a premature stop codon (Mellersh et al. 2006). In the initial study that identified the *RPGRIP1* mutation, affected and carrier dogs within an extended inbred pedigree were homozygous and heterozygous, respectively, for the mutation (Mellersh et al. 2006). It was therefore concluded that the *RPGRIP1* ins44 mutation was responsible for *cord1*. Subsequent studies, however, found considerable phenotypic variation and genotype–phenotype discordance (Miyadera et al. 2009), raising the possibility that the *RPGRIP1* ins44 mutation may not be the primary cause of *cord1* (Kuznetsova et al. 2012a). It was suggested that because *RPGRIP1* undergoes multiple splicing, the exon containing the insertion could be spliced out while retaining partial protein function (Kuznetsova et al. 2012b). Indeed, a genome-wide association study identified a second locus also on canine chromosome 15 that strongly influenced the manifestation of disease (Miyadera et al. 2012). Homozygosity for both *RPGRIP1* ins44 and the risk haplotype at the newly mapped locus was necessary for development of retinal degeneration. To complicate matters further, some dogs that developed retinal degeneration were neither homozygous for *RPGRIP1* ins44 nor for the second risk haplotype indicating the presence of further independent modifier loci (Miyadera et al. 2012). If this proves correct, it would make retinal degeneration in this dog breed a polygenic trait. Given the clearly showed and critical role of RPGRIP1 in human and mouse photoreceptors, it would seem certain that an *RPGRIP1* null or substantially null allele would also be causal for photoreceptor degeneration in dogs. Thus the available evidence points to *RPGRIP1* ins44 being a moderately hypomorphic allele. It is a significant contributor to disease but is not a sole determinant. Direct examination of the protein from native retinal tissues using appropriate antibodies should help resolve this puzzling question.

THERAPEUTIC STUDIES IN ANIMAL MODELS

Replacement gene therapy studies have been performed in both murine and canine models. The first study was conducted in the *RPGRIP1* knockout mice (Pawlyk et al. 2005). In that study, a mouse *RPGRIP1* cDNA was packaged into an AAV2 vector under the control of a mouse opsin promoter and delivered subretinally. Mice were followed up to 5 mo after treatment by ERG and then analyzed by immunocytochemistry and histology. Delivery of recombinant *RPGRIP1* by AAV led to the RPGRIP1 protein expression that correctly localized to the connecting cilia. The thickness of the photoreceptor layer was significantly increased in the treated retina, and the morphology of the outer segments, almost completely absent in the untreated eyes, was partly restored. Partial functional preservation was also shown by ERG. Remarkably, RPGR again localized to the photoreceptor connecting cilium in treated eyes, indicating restoration of its function. Thus the efficacy of AAV-mediated replacement gene therapy was unambiguously shown in this first study in a mouse model. Despite its apparent success, this study has left much room for further improvement. First, RPGRIP1 is known to be required in both rods and cones, and the use of an opsin promoter would limit expression primarily to rods. Second, AAV2 vector has a relatively poor tropism for photoreceptors leading to lower transduction efficacy and is slow in turning on expression of the recombinant gene. Because the mouse LCA model has a fast course of disease, by the time replacement gene is fully expressed, which could take $>4-6$ wk, a substantial fraction of photoreceptors would have degenerated. Lastly, the important objective of validating a human *RPGRIP1* replacement gene suitable for clinical trials was not tackled in this study.

To address some of those issues a second therapeutic study was conducted in the *RPGRIP1* knockout mice that delivered the human instead of the mouse *RPGRIP1* replace-

ment gene (Pawlyk et al. 2010). The second study incorporated an improved version of AAV vector, AAV8. AAV8 is far more efficient at transducing photoreceptors than AAV2 and leads to a faster onset of transgene expression. This meant that in a rapidly degenerating retina, the replacement gene would be up and functional before too many photoreceptors had disappeared thus leading to a more favorable therapeutic outcome. A human rhodopsin kinase gene promoter (Khani et al. 2007) was used in place of the rhodopsin promoter to drive gene expression in both rods and cones. After subretinal delivery of the replacement gene in the mutant mice, human RPGRIP1 was expressed specifically in photoreceptors, localized correctly in the connecting cilia, and importantly restored the normal localization of RPGR. Histological examinations showed improved photoreceptor survival in the treated eyes. ERG measurements showed better preservation of rod and cone photoreceptor function: The mean rates of monthly decline without treatment were 37% for the rod a-wave, 22% for the rod b-wave, and 25% for the cone b-wave. With treatment, these rates of decline decreased to 8% for the rod a-wave and rod b-wave and to no detectable decline for the cone b-wave. This represents overall a fourfold slowing of retinal function loss (Pawlyk et al. 2010). Thus, this study showed the efficacy of a human replacement gene and validated a gene therapy design that could be adopted for future clinical trials in patients. Despite marked improvement of photoreceptor function and survival in the treated eyes compared with the control eyes, photoreceptor degeneration was slowed but not stopped. Among many possible factors that may have influenced the extent of rescue, the low degree of conservation between human and mouse genes is likely a major one. The human and mouse RPGRIP1 protein sequences are only ~60% identical, relatively low for mammalian orthologs. In fact, the conservation is so poor between the two species that an antibody raised against one species would not cross-react with the other. This means a human RPGRIP1 would not function in mouse photoreceptors as effectively as the cognate mouse RPGRIP1. If this hypothesis is correct, which seems likely, a better therapeutic outcome could reasonably be expected in future clinical studies in which a human *RPGRIP1* gene is delivered to human recipients. Other factors such as precise level of transgene expression and time of replacement gene delivery also likely play roles in determining an optical outcome.

For preclinical development of gene therapies that target a specific type of retinal degeneration, proof-of-concept studies in large animal models that share clinical features with their human counterparts would be highly desirable. A recent study evaluated gene therapy in the canine *cord1* model with homozygous *RPGRIP1* ins44 mutation (Lheriteau et al. 2014). Subretinal injection of a canine *RPGRIP1* cDNA driven by the same rhodopsin kinase promoter as that used in the mouse study and packaged into AAV vectors improved photoreceptor survival in treated retinas. The study found that photoreceptor function was significantly improved in all treated eyes for up to 24 mo postinjection. The rescue of cone function appeared to be more stable and long lasting. The treatment did not completely halt photoreceptor degeneration, similar to most gene therapy studies, as indicated by the continued thinning of the photoreceptor layer over time. It should be noted that, to achieve a measurable therapeutic benefit, the study had to use a canine RPGRIP1 cDNA in the replacement gene construct. This was again presumably because of the low degree of conservation between the human and dog RPGRIP1 primary sequences (at under 70%) such that a human RPGRIP1 would produce a weaker therapeutic effect in canine photoreceptors to the point of being difficult to show. Another issue that is relevant to assessing the outcome of the study is the findings by other investigators that the *cord1* phenotype may be inherited as a polygenic trait. Nevertheless, the demonstration of efficacy of gene therapy in this large animal model of cone–rod dystrophy is clear evidence that the *RPGRIP1* ins44 allele is a major pathogenic factor and provides promise that the model will be useful for further preclinical development of therapies for human treatment.

CONCLUDING REMARKS

Studies by many investigators in the past decade have built up a wealth of knowledge about *RPGRIP1*, from genetics, protein function, to somatic gene therapies in animal models. The remaining tasks before a clinical trial can be initiated are to finalize to choice of promoter and human RPGRIP1 coding sequence, and to carry out efficacy and toxicology validation. Although all evidence points to the long *RPGRIP1* transcript being the functionally important variant, the possibility that some shorter variants additionally contribute to photoreceptor function could bear on efforts on optimizing efficacy of therapies and should be further examined. The timing issues of gene therapy intervention also deserve some attention (Cideciyan et al. 2013). Despite potential difficulties going forward, gene therapy development for LCA caused by *RPGRIP1* mutations seems on track and the future looks promising for the emergence of a therapy that could alleviate this form of retinal degeneration.

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