RS-1 Gene Delivery to an Adult Rs1h Knockout Mouse Model Restores ERG b-Wave with Reversal of the Electronegative Waveform of X-Linked Retinoschisis

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Results

To create and evaluate a mouse model of human X-linked juvenile retinoschisis (XLRS) and then investigate whether supplementing with the retinoschisin protein by gene delivery can reverse the abnormal “electronegative” electroretinogram (ERG) retinal response.

Methhods. An X-linked retinoschisis mouse (Rs1h-KO) model was created by substituting a neomycin resistance cassette for exon 1 and 1.6 kb of intron 1 of Rs1h, the murine orthologue of the human RS-1 gene. RS protein was evaluated by immunohistochemistry and Western blot analysis with a polyclonal RS N-terminus antibody. Retinal function was evaluated by conventional, full-field flash ERG recordings. RS protein supplementation therapy was evaluated by gene transfer with an AAV(2/2)-CMV-Rs1h vector containing C57BL/6J Rs1h cDNA under the regulation of a CMV promoter, and ERG functional analysis was performed.

Results. No RS protein was detected by Western blot analysis or immunohistochemistry in the Rs1h-KO mouse. Dark-adapted ERG responses showed an electronegative configuration, with b-wave reduction in both Rs1h+/– and Rs1h−/− mice, typical of XLRS in humans. Histologic examination of Rs1h-KO mice showed disorganization of multiple retinal layers, including duplication and mislocalization of ganglion cells, laminar dissection through the inner plexiform layer, disorganization of the outer plexiform layer, loss of regularity of the outer nuclear layer, and shortening of the inner/outer segments with mislocalization of photoreceptor nuclei into this layer. After intraocular administration of AAV(2/2)-CMV-Rs1h, immunohistochemistry showed retinoschisin expression in all retinal layers of Rs1h−/− mice, and ERG recordings showed reversal of the electronegative waveform and restoration of the normal positive b-wave.

Conclusions. The RS-KO mouse mimics structural features of human X-linked juvenile retinoschisis with dissection through, and disorganization of, multiple retinal layers. The Rs1h-KO functional deficit results in an electronegative ERG waveform that is characteristic of human retinoschisis disease and that implicates a synaptic transmission deficit in the absence of retinoschisin protein. Replacement therapy by supplementing normal Rs1h protein in the adult Rs1h-KO mouse restored the normal ERG configuration. This indicates that gene therapy is a viable strategy of therapeutic intervention even in the post-developmental adult stage of XLRS disease.

Electronegative waveform configuration, indicating deficient responses from the inner retina. The clinical course generally causes a moderate decrease in visual acuity, but more advanced stages are complicated by vitreous hemorrhage, retinal detachment, and neovascular glaucoma, which may cause severe loss of vision. Severely affected male infants may be blind at birth from bilateral retinal detachments. As with most other retinal degenerations, there is currently no definitive therapy for XLRS.

The causative RS-1 gene was identified by positional cloning. The RS-1 gene contains six exons that encode a small, 224-amino-acid protein, with an N-terminal secretory leader peptide sequence and a discoidin domain in exons 4 to 6 that is highly conserved across species. Discoidin domains are found in a large family of secreted or membrane-bound proteins and have been implicated in cell adhesion and cell-cell interactions.

Initial studies showed heavy expression of retinoschisin protein (RS) in the photoreceptor inner segment layer, which was surprising because the then current clinical understanding of the disease implicated a structural abnormality in the most proximal retina, distant from the photoreceptors.

Subsequent investigation extended the sites of expression to include bipolar cells, but controversy remained as to whether the retinal Müller glial cells provide intracellular transport of RS from distal to proximal retina.

A recent study indicates that XLRS is a retinal developmental condition and that mRNA and protein are expressed locally. We found that essentially all retinal neurons express retinoschisin in a developmental wave from proximal to distal retina, beginning first with retinal ganglion cells (RGCs) by postnatal day (P)1, suggesting that RS protein is not transported from distal to proximal retina. Retinoschisin expression by RGCs diminished after P1, but it continued in RGCs, even in the adult retina to lesser degree and most other retinal neurons.

This developmental study raised several important issues to be considered for intervention strategies. First, as all retinal neurons express retinoschisin during development and as retinoschisin protein need not be transported from outer to inner retina, gene therapy should target multiple classes of neurons. Repair of synaptic integrity and restoration of efficient synaptic
signaling would probably require targeting the intermediate retinal bipolar neurons and possibly the amacrine and ganglion cells as well. Delivery may be accomplished through the vitreous.

Second, the continuing expression of RS mRNA in adult retinal neurons suggests ongoing synthesis of retinoschisin in the adult and provides a rationale for attempting intervention even in the postdevelopmental retina but before catastrophic structural failure might result in full-thickness detachment.18,19

Third, because retinoschisin protein is found associated with postsynaptic elements in the outer plexiform layer (OPL) between photoreceptors and bipolar cells,12 monitoring synaptic integrity by ERG b-wave recordings may provide a suitable marker to judge successful intervention.

To further our investigations toward eventual human therapy, we generated mice lacking retinoschisin protein by gene knockout technology which targeted Rs1b, the murine orthologue of RS-1.20 Intraocular injection into adult Rs1b<sup>−/−</sup> mice of an AAV(2/2)-CMV-Rs1h vector containing Rs1h cDNA resulted in considerable levels of RS protein in all retinal layers as seen with immunohistochemistry and rescue of synaptic function by ERG analysis. As this occurred in adult mice otherwise lacking the retinoschisin protein, these results suggest that therapeutic RS-I gene transfer into adult men with XLR might be helpful in restoring visual function and possibly in reducing the incidence of subsequent serious structural sequelae of retinoschisis disease.

MATERIALS AND METHODS

Experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with protocols approved by the National Eye Institute Animal Care and Use Committee. Normal C57BL/6J mice were purchased from Charles River Laboratory (Wilmington, MA) and housed under fluorescent lights in a 12-hour light–dark cycle.

Generation of Rs1b-Knockout Mice

A 17.5-kb mouse genomic DNA fragment was cloned from the mouse 129Sv/Ev α genomic library, which contained exon 1 and most of intron 1 of the mouse Rs1b orthologue of the human RS-1 gene. An 11-kb fragment cut by SmaI and NotI was used to make the targeting construct. All exon 1, including 9 bp upstream of the ATG start codon, and a 1650-bp fragment of intron 1 was replaced by the neomycin resistance (neor) gene cassette (Fig. 1). Before electroporation, the targeting plasmid was linearized with NotI. The Rs1b-knockout (Rs1b-KO) was generated with assistance from Ingenious Targeting Laboratory, Inc. (Stony Brook, NY).

129Sv/Ev mouse embryonic stem cells were electroporated with linearized target plasmid DNA and selected using G418 antibiotic. DNA targeting was confirmed by PCR analysis with the primer pair RSDA6 and PLA2 that had been used to identify the targeted allele. An additional primer (5-CCTTGTCCTTGGTCACAG-3) of each primer, 2 mM MgCl<sub>2</sub>, 400 μM dNTPs, and 25 μL of tail DNA as template, with two sets of oligonucleotide primers. One set amplified the wild-type allele with primer RSDA6. Long-range PCR was performed in a 25-μL reaction containing 200 ng of tail DNA, 10 μM of each primer, 2 mM MgCl<sub>2</sub>, 400 μM dNTPs, and 1.5 units of Taq DNA polymerase (Promega, Madison, WI).

These males were mated with heterozygous females to produce homozygous female offspring. Rs1b-KO mice were genotyped by PCR methods using tail DNA as the PCR template, with two sets of oligonucleotide primers. One set was the primer pair RSDA6 and PLA2 that had been used to identify the targeted allele. An additional primer (5-GTTCCTTGGAGGCTCCTGCTAC-3), located just before the ATG start codon, was designed to amplify the wild-type allele with primer RSDA6. Longer-range PCR was performed in a 25-μL reaction containing 200 ng of tail DNA, 10 μM of each primer, 2 mM MgCl<sub>2</sub>, 400 μM dNTPs, and 1.5 units of Taq DNA polymerase (Promega, Madison, WI). These conditions were used for amplification of both alleles: 3 minutes at 95°C for initial denaturation, followed by 40 cycles of 30 seconds at 95°C, 50 seconds at 60°C, 2 minutes 40 seconds at 72°C, and 10 minutes at 72°C, for the final extension. The annealing temperature

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Creation of the retinoschisin-knockout mouse. (A) Vector construct substituted a neomycin resistance expression cassette for exon 1 and 1.6 kb of intron 1 of Rs1b, the orthologue of the human Rs-1 gene. (B) Western blot of total retinal protein extract, probed with polyclonal RS antibody raised against RS amino acid residues 24-57 (left). A 24-kDa RS protein was detected in the WT C57BL/6 mouse but not in Rs1b<sup>−/−</sup> or Rs1b<sup>+/−</sup> mice. RS dimer formation has been observed in previous studies.17,20 A polyacrylamide gel demonstrated the similar loading of retinal protein extract from all samples (right). (C) Confocal immunohistochemistry using polyclonal RS antibody on retinal sections. Retinoschisin was evident in WT mouse photoreceptor inner segments, the OPL, and the INL (left). No RS expression was detected in the retina of Rs1b<sup>−/−</sup> mice (right). Scale bar, 50 μm.

**WT mouse (##551)**

- **IS**
- **ONL**
- **OPL**
- **INL**
- **IPL**
- **RGC**

**Rs1h<sup>−/−</sup> mouse (##553)**

- **IS**
- **ONL**
- **OPL**
- **INL**
- **IPL**
- **RGC**
was decreased 0.4°C per cycle after five cycles until it reached 62°C. The PCR products were 2.4 kb and were analyzed by agarose gel electrophoresis.

**Mouse Rs1b cDNA Clone for Therapy Vector Construct**

Rs1b cDNA was cloned from C57BL/6 mouse retina using RT-PCR methods. Mouse total retinal RNA was isolated, and single-stranded random hexamer-primed cDNA was generated by using Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen, Carlsbad, CA). A PCR primer set was designed to amplify the entire coding region of mouse Rs1b cDNA (forward, 5'-GGCACACATGGCAACAAGAATTGACACCTTGCCCCG-3'; reverse, 5'-CATACGGCACACCTTGCCCCG-3'). PCR was performed in a 50-µL reaction containing 2 µL of cDNA template, 12.5 pM of each primer, 1.5 mM MgCl₂, 50 µM deoxyribonucleotide triphosphates, 2.5 µM Taq DNA polymerase (Platinum; Invitrogen), and 5 µL of 10X reaction buffer supplied by the manufacturer. The PCR amplification condition was 3 minutes of initial denaturation at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 1 minute at 68°C, and 8 minutes of final extension at 68°C. The plasmid pCR-Rs1b was generated by inserting the RT-PCR product into the pCR II-TOPO vector (Invitrogen), and the insert sequence was confirmed on a gene analysis system (CEQ 8000 Genetic Analysis System; Beckman, Fullerton, CA).

**Generation of Recombinant AAV(2/2)-CMV-Rs1b Vector**

The Cis pAAV(2/2)-CMV-Rs1b vector, in which the Rs1b cDNA was driven by the CMV promoter, was made by inserting the 705-bp EcoRI fragment of the pCR-Rs1b plasmid into the EcoRI restriction sites of the pZac2.1 vector provided by the Vector Core, Medical Genetics Division, Department of Medicine, Medical School, University of Pennsylvania, using methods previously described. Brieﬂy, HEK-293 cells were triple transfected with three plasmids at the Vector Core Facilities. The first plasmid encoded the Rs1b expression cassette packaged between the AAV2 internal terminal repeats, the second encoded the rep and AAV2 cap genes, and the third encoded the adenoviral helper function genes. The AAV2/2-CMV-Rs1b construct was puriﬁed by heparin column chromatography. The virus titer was assessed by real-time PCR, and the infectivity was assessed by an infectious center assay, as described previously. The ratio of the genomic copy number (GC) to the infectious center assay for this AAV(2/2)-CMV-Rs1b construct was 59.

**Delivery of Vector Construct to Rs1b-KO Mouse Retina**

Animals were anesthetized, and intracocular injections were performed with a technique similar to that described earlier, by inserting a 33-gauge needle into the eye posterior to the limbus. Rs1b<sup>-/-</sup> mice were given 2 µL AAV(2/2)-CMV-Rs1b at a titer of 2.5 × 10<sup>11</sup> GC/µL into the right eye. The contralateral left eye served as the control and either received 2 µL of phosphate-buffered saline (PBS) or remained untouched.

**Tissue Collection and Histopathology**

Mice were euthanized with intraperitoneal ketamine (70 mg/kg) and xylazine (6 mg/kg), were placed on a warming pad to maintain body temperature near 38°C, and preparations were made under dim red light. Pupils were dilated with atropine 0.1% and phenylephrine HCl 0.1%. A gold wire loop electrode was placed on the cornea after topical tetracaine 1% to suppress background. Responses were amplified at 5000 gain at 0.1 Hz. Xenon photostrobe 50-µs full-field white ﬂash stimuli had 0.6 log cd·s/m² maximum intensity and were attenuated with neutral density ﬁlters. Photopic cone ERGs were elicited with 0.6 log cd·s/m² stimuli on a 42 cd·s/m² white rod-suppressing background.

**Human XLRM Evaluation**

Evaluation of human subjects with juvenile XLRM was performed under consenting approvals of the NEI Investigational Review Board and in accordance with the provisions of the Declaration of Helsinki. Standard clinical evaluations were performed, including clinical ERG recordings according to the standard International Society for Clinical Electrophysiology of Vision (ISCEV) protocols, and ocular coherence tomography (OCT; Model 3000; Carl Zeiss Meditec, Dublin, CA). A search for Rs1-gene mutations was performed by sequencing the exons serially using standard techniques, as reported previously.

**RESULTS**

**Rs1b-KO Mouse Model**

Disruption of the Rs1 gene was accomplished by replacing exon 1 and 1.6 kb of intron 1 sequence with a neomycin resistance expression cassette (Fig. 1A). Successful germline transmission of the correctly targeted allele was conﬁrmed in the F1 generation by PCR analysis of tail DNA with primers RSDA6 and PLA2. F1 heterozygous females (Rs1b<sup>+/-</sup>) were crossed with C57BL6 males to generate hemizygous male offspring (Rs1b<sup>-/-</sup>) which were inbred with Rs1b<sup>-/-</sup> females was used in the present study. Grayson et al. had generated a different polyclonal RS antibody against these same residues. Immunohistochemistry of mouse retinal specimens was performed as described previously, with this RS antibody. Light microscopy was performed on parafﬁn-embedded, 4-µm-thick sections that were treated with 100 µg/mL proteinase K at room temperature for 15 minutes for epitope unmasking. The avidin-biotinylated enzyme complex (ABC) method (Vector, Burlingame, CA), was used, and the antibody complexes were visualized with 3,3′-diaminobenzidine (DAB). Sections for confocal microscopy were processed as previously described and were visualized with a confocal microscope (TCS SP-2; Leica Microsystems, Exton, PA).

**Western Blot Analysis**

Wild-type mice and Rs1b-KO mice were euthanatized by carbon dioxide, and retinas were dissected. The retinal extract was isolated with a mammalian cell lysis kit (Sigma-Aldrich) following the manufacturer’s instructions. The protein concentration was determined with a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL). Thirty micrograms of protein from each sample were separated on duplicate 12% SDS PAGE gels, and one gel was transferred onto a nitrocellulose membrane for Western blot analysis; the duplicate gel was stained (Simply Blue SafeStain; Invitrogen) to demonstrate the equal loading of protein extract. After incubation with the same polyclonal RS antibody used for immunohistochemistry, RS was detected with a chemiluminescent Western blot detection reagent (ECL; Amer sham Biosciences, Piscataway, NJ).

**Mouse ERG**

Mice were dark adapted overnight for 12 hours. After anesthesia with intraperitoneal ketamine (70 mg/kg) and xylazine (6 mg/kg), mice were placed on a warming pad to maintain body temperature near 38°C, and preparations were made under dim red light. Pupils were dilated with atropine 0.1% and phenylephrine HCl 0.1%. A gold wire loop electrode was placed on the cornea after topical tetracaine 1% for anesthesia, and a gold wire differential electrode was placed on the sclera near the limbus. A neutral wire electrode was attached to the ear. Both eyes were recorded simultaneously. Responses were amplified at 5000 gain at 0.1 to 1000 Hz. Xenon photostrobe 50-µs full-field white ﬂash stimuli had 0.6 log cd·s/m² maximum intensity and were attenuated with neutral density ﬁlters. Photopic cone ERGs were elicited with 0.6 log cd·s/m² stimuli on a 42 cd·s/m² white rod-suppressing background.
layer (IPL). Laminations of both the inner nuclear layer (INL), and OPL, which contains the synaptic connections between photoreceptors and bipolar cells, were irregular. Structural gaps from intralamellar dissections, equivalent to “retinoschisis cavities,” were observed in the INL. The photoreceptor cell number in the ONL appeared to be slightly reduced in retinal sections taken from multiple Rs1b−/Y animals, and the length of the photoreceptor inner and outer segments was shortened. Clumps of photoreceptor nuclei were mislocalized into the inner and outer segment zones rather than lying exclusively within the ONL. Retinas of Rs1b−/Y mice up to 6 months of age appeared similar, without exhibiting progressive degeneration (e.g., see the 24 week Rs1b−/Y retina in Fig. 3). Previously, we had shown that RGCs express retinoschisin early during retinal development by postnatal day 117 which affords opportunity for the occurrence of structural developmental changes of the INL in Figure 2A.

ERG in Rs1b-KO Mice

ERGs were recorded from Rs1b−/− male and Rs1b−/− female mice, and both showed diminished dark-adapted b-wave responses across the stimulus intensity range. The ERG elicited by higher intensity stimuli had an abnormal waveform in which the b-wave amplitudes were smaller than the negative-going a-wave which is generated by rod photoreceptors (Fig. 2B). This resulted in an electronegative configuration that is characteristic of human XLRs-affected males with only rare exception.27 The V-log I curve of b-wave amplitudes versus log-stimulus intensity from three Rs1b−/− mice (Fig. 2C) shows a threethirds reduction of b-wave amplitude across the entire stimulus range, but with otherwise nearly normal b-wave threshold, which is consistent with findings in human XLRs-affected males.4,50

AAV-Mediated Rs1b Gene Delivery to the RS-KO Mouse

An AAV vector that contained the murine Rs1b cDNA driven by a CMV promoter was administered to the right eyes of Rs1b−/− mice by intraocular injection. The contralateral left eyes were injected with PBS. These injections were not com-

to produce homozygous females (Rs1b−−). Because gene translation should be trapped inside the neor cassette, expression of the downstream exons was not expected. Their absence was confirmed by Western blot analysis, which showed no detectable retinoschisin protein in total retinal protein extracts from Rs1b−/− and Rs1b−/− mice, using the polyclonal RS antibody against an epitope which lies in exon 2 and 3, downstream of the neor cassette that replaced exon 1 (Fig. 1B). Immunohistochemical analysis of retinal sections using the same antibody showed that no RS protein expression could be detected in the retinas of Rs1b−/− mice, whereas robust expression of retinoschisin was detected in multiple retinal layers in WT mice (Fig. 1C). The Western blot and immunohistochemical results both indicate that the Rs1b−/− mouse is an Rs-null model of XLRs.

Retinal Layer Disruption in Rs1b−/− Mice

Histologic examination of retinal sections from 1- to 6-month-old Rs1b−/− mice showed changes in multiple layers (Fig. 2A). The innermost RGC layer showed an excess of cells relative to normal. Some RGCs were mislocalized into the inner plexiform layer (IPL). Laminations of both the inner nuclear layer (INL), and OPL, which contains the synaptic connections between photoreceptors and bipolar cells, were irregular. Structural gaps from intralamellar dissections, equivalent to “retinoschisis cavities,” were observed in the INL. The photoreceptor cell number in the ONL appeared to be slightly reduced in retinal sections taken from multiple Rs1b−/− animals, and the length of the photoreceptor inner and outer segments was shortened. Clumps of photoreceptor nuclei were mislocalized into the inner and outer segment zones rather than lying exclusively within the ONL. Retinas of Rs1b−/− mice up to 6 months of age appeared similar, without exhibiting progressive degeneration (e.g., see the 24 week Rs1b−/− retina in Fig. 3). Previously, we had shown that RGCs express retinoschisin early during retinal development by postnatal day 117 which affords opportunity for the occurrence of structural developmental changes of the INL in Figure 2A.

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complicated by hemorrhage. Injections were made in adult mice at 13 weeks of age, and analysis by ERG and histology was done 9 to 11 weeks later. An example of the outcome for an Rs1b<sup>−/−</sup> mouse is shown in Figure 3. The ERG of the PBS-injected control eye showed the abnormal, dark-adapted electronegative waveform found for these Rs1b<sup>−/−</sup> mice (e.g., Fig. 2B), whereas the AAV-injected eye showed reversal of the DRG-electronegative abnormality to a normal waveform configuration in which the positive-going b-wave was larger than the a-wave that characterizes normal responses of WT mice. The ERG response threshold did not change after introduction of the Rs1b gene, which is as expected, since the ERG of untreated Rs1b<sup>−/−</sup> mice extended down to the WT threshold (e.g., Fig. 2B). Nor was the a-wave amplitude affected, indicating that the number of rod cells and the outer-segment length was not altered<sup>23</sup> by the AAV-Rs1b injection. The b-wave response amplitudes, however, were increased across the entire stimulus range, suggesting either that RS protein augmented signaling across the rod-to-bipolar cell synapses that lie within the OPL, or that RS protein somehow augmented the b-wave generator function that involves bipolar cell depolarization.<sup>5</sup> We are exploring whether either scenario might be the case.

Postmortem immunohistochemistry on the eyes of this Rs1b<sup>−/−</sup> mouse was performed using the polyclonal antibody against Rs amino acid residues 24 to 37 which lie downstream of the insertion sequence of the neoR cassette (Fig. 3B). Retinoschisin protein was found in all retinal layers after AAV(2/2)-CMV-Rs1b vector delivery to the right eye, whereas the contralateral left eye injected with PBS did not show evidence of Rs protein. Photoreceptor inner-segments showed intense Rs staining. This retinal distribution is associated with AAV(2/2) vector delivery into the subretinal space.<sup>32</sup> Further subretinal injections confirmed ERG rescue and the presence of retinoschisin throughout the retina of treated Rs1b-KO mice.

**Discussion**

This study demonstrated that RS protein delivery into the retina of retinoschisis Rs1b<sup>−/−</sup> mice can reverse the abnormal electronegative ERG waveform that characterizes the retinal functional changes in dystrophic Rs1b-KO mice and in human XLRS disease. Knocking out the Rs1b gene resulted in structural abnormalities in the mouse retina, including irregular formation of the neuronal and plexiform layers, and the mislocalization of ganglion cells and photoreceptor cells outside of their normal positions. Structural retinal consequences also involved schisis dissection through the INL. Although retinoschisin protein is expressed during retinal development and the lack of Rs causes congenital retinal structural changes, this therapy study demonstrated that treatment even of adult animals was successful in reversing the b-wave functional abnormality, presumably at the level of the photoreceptor-to-bipolar synapse.

The retinal abnormalities found in our retinoschisin Rs1b<sup>−/−</sup> mice overlapped those described previously by Weber et al.<sup>33</sup> in their Rs1b<sup>−/−</sup> mice, which they developed by introducing a lacZ reporter gene in-frame into exon 3 of Rs1b along with a Neo<sup>R</sup> expression cassette under the separate control of the mouse Pgi phosphoglycerate kinase gene promoter. Like our retinoschisin mice, their Rs1b-KO model showed the characteristic electronegative ERG waveform pattern and developed splitting through the INL. They also reported a generalized decrease in rod photoreceptor cell number and a considerable reduction in cone numbers. Our Rs1b-KO mice showed irregularity of the ONL but otherwise had essentially normal ONL cellular thickening, although the inner and outer segments were shorter. The scotopic a-wave amplitude in our Rs1b<sup>−/−</sup> mice was approximately one-half normal, consistent with shorter inner and outer segments.<sup>34</sup> This murine a-wave reduction, however, is at variance with our human ERG studies of 13 XLRS-affected men, most of whom showed normal rod a-wave maximal amplitudes.<sup>35</sup> RS protein replacement by introduction of the AAV(2/2)-CMV-Rs1b construct into Rs1b<sup>−/−</sup> mice did not repair the retinal structure in these adult animals and hence did not reverse the reduced a-wave amplitude. It is against the baseline of the a-wave that was reduced similarly before and after introducing the gene transfer construct that the b-wave improvement is judged.

**Comparison with Human XLRS Disease**

The retinal structural and functional changes observed with retinoschisin Rs1b<sup>−/−</sup> mice mimicked the findings typical for human XLRS-affected males (Fig. 4). Although classic clinical descriptions of human XLRS disease emphasize that structural retinal splitting occurs in the superficial retina through the nerve fiber layer,<sup>11,14,35</sup> recent histopathology of postmortem XLRS retinal tissue shows involvement of multiple layers including the inner nuclear and the inner and OPLs.<sup>36</sup> This can now be confirmed clinically using optical coherence tomography (OCT).<sup>37</sup> Figure 4 shows an example of a boy with XLRS from a 598 C→T mutation in exon 6 of Rs-I, which causes an amino acid (aa) substitution Arg200Cys resulting in a charge change of a highly conserved residue within the discoidin domain extending from aa 63 to 219.<sup>38</sup> His macula exhibited the characteristic spoked-wheel pattern of parafoveal intraretinal cysts (Fig. 4A). Analogous to the Rs1b<sup>−/−</sup> mice, his ERG showed the classic XLRS electronegative waveform (Fig. 4B). OCT of the left eye shows macular schisis dissection involving not only the superficial ganglion cell/inner plexiform layer but also the mid and deeper retinal layers (Fig. 4C), consistent with the structural abnormalities in multiple retinal layers, including OPL/INL schisis, that were found in the Rs1b<sup>−/−</sup> mice. We
previously suggested that the structural developmental dynamics involved in forming the human macula compared with a nonmacular murine retina may account for the additional retinal levels of schisis dissection in human versus mouse retinoschisis disease.

**Locus of ERG Functional Recovery**

The V-log I curve of ERG recordings of Rs1h mice shows that, even though the b-wave amplitudes are diminished, the b-wave responses can be tracked down nearly to the normal thresholds of WT mice (Fig. 2). This coincides with the clinical findings that dark-adapted absolute perceptual thresholds typically are elevated less than 1 log unit. This corresponds appropriately to b-wave response amplitudes of XLS rats that normally are reduced by less than 1 log unit, even though the overall b-wave/a-wave ratio is abnormal, particularly for the highest intensities. The current consensus is that this reflects deficient synaptic transfer between the photoreceptor and bipolar cells. We have observed RS protein in the rod spherule by electron microscopy using immuno-gold histochemistry (unpublished observation, 2004). The demonstration in the present paper that supplying retinoschisin protein to Rs1h mice increased the b-wave amplitudes suggests that RS protein is necessary for full synaptic function.

**RS Protein Distribution after AAV-Rs1b Vector Injection**

Retinal immunohistochemistry after intraocular injection of the recombinant AAV-CMV-Rs1b vector showed RS protein throughout all the retinal neuronal and plexiform layers of the Rs1h-KO mouse, including the photoreceptors of the outer retina. These results demonstrate that recombinant AAV virus-mediated gene delivery is effective for administering a normal RS gene into adult retinal cells. Although the supplementation of RS protein did not give apparent improvement of retinal morphology in the treated adult retinoschisin knockout mice, the intense RS protein staining in the inner segments suggests that introducing normal RS protein into XLS rats may help to stabilize the integrity of the retina and prevent detachment from occurring, even when the intervention is in the postdevelopmental adult stage of human XLS disease.

**Implications for Human Therapeutic Intervention**

Conditions in which physiological reversal of impaired function is possible are attractive targets for exploring human disease intervention. Unfortunately, most forms of retinal dystrophy result in vision loss from progressive degeneration for which the goal of therapy is to slow the natural course, such as by delivery of a trophic protective factor. Long-term protection of retinal structure has been found in animal models of retinits pigmentosa after AAV-mediated delivery of ciliary neurotrophic factor. Clinical trials based on retarding the rate of progression, however, may well require an extended period of observation over many months to years to demonstrate an effect in humans.

The retinoschisis Rs1b-KO mouse is one of very few animal models of retinal disease demonstrated to show reversal of functional loss and to actually regain physiological function after protein treatment by gene delivery. Leber congenital amaurosis from mutations in the RPES5 gene is another example. Retinal function in dogs with RPE65 disease improves acutely after treatment, as monitored by ERG recordings after gene delivery. Conditions such as RPE65 and retinoschisis afford an opportunity to consider intervention even in adult affected humans.

This Rs1b-KO murine model exhibits a complete absence of retinoschisin protein expression, which translates most directly to the circumstance of human XLS-affected men who do not express RS. Such individuals appear to exist. More than 100 mutations associated with human XLS disease have been identified in RS-I. Approximately 80% of these are missense mutations in the discoidin domain coding region of the gene (exons 4–6) and cause amino acid substitutions. The remaining 20% of mutations are in exons 1 to 3 and consist primarily of major deletions, stop or nonsense codons or splice site mutations that are expected to disrupt transcription and result in no RS protein product or to give a truncated RS protein that contains no discoidin domain.

In genotyping more than 50 XLS patients, we noted that some families transmitted severe XLS disease and have multiple male relatives with retinal detachments or extensive peripheral retinal inner leaf tears. Several of these families had mutations expected to cause truncated or null protein products, such as deletions of the entire exon 2, or on exons 2 and 3 plus the intervening intron 3, or from an exon 1 splice site mutation. In such families, one might anticipate a clinical benefit if RS protein delivery could stabilize the neural tissue structurally before a severe clinical event such as a retinal detachment. In addition, many RS mutations, irrespective of the site at which they occur, have been shown to cause RS protein misfolding or to impair a processing step in the ER/Golgi compartments and to affect severely or even eliminate the secretion and assembly of RS protein at the target sites. Our results in Rs1b-KO mice demonstrate a partial restoration of retinal physiologic function following AAV-mediated delivery of RS protein and may provide an approach to preventing retinal detachment in humans irrespective of the type of RS-1 mutation they harbor. These results also show that the ERG provides an effective noninvasive way to monitor therapy delivery.

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