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# Human gene therapy for RPE65 isomerase deficiency activates the retinoid cycle of vision but with slow rod kinetics

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The *RPE65* gene encodes the isomerase of the retinoid cycle, the enzymatic pathway that underlies mammalian vision. Mutations in *RPE65* disrupt the retinoid cycle and cause a congenital human blindness known as Leber congenital amaurosis (LCA). We used adeno-associated virus-2-based *RPE65* gene replacement therapy to treat three young adults with *RPE65*-LCA and measured their vision before and up to 90 days after the intervention. All three patients showed a statistically significant increase in visual sensitivity at 30 days after treatment localized to retinal areas that had received the vector. There were no changes in the effect between 30 and 90 days. Both cone- and rod-photoreceptor-based vision could be demonstrated in treated areas. For cones, there were increases of up to 1.7 log units (i.e., 50 fold); and for rods, there were gains of up to 4.8 log units (i.e., 63,000 fold). To assess what fraction of full vision potential was restored by gene therapy, we related the degree of light sensitivity to the level of remaining photoreceptors within the treatment area. We found that the intervention could overcome nearly all of the loss of light sensitivity resulting from the biochemical blockade. However, this reconstituted retinoid cycle was not completely normal. Resensitization kinetics of the newly treated rods were remarkably slow and required 8 h or more for the attainment of full sensitivity, compared with <1 h in normal eyes. Cone-sensitivity recovery time was rapid. These results demonstrate dramatic, albeit imperfect, recovery of rod- and cone-photoreceptor-based vision after *RPE65* gene therapy.

dark adaptation | photoreceptor | retinal degeneration | retinoid cycle

The enzymatic pathway in the human eye that regenerates light-altered vitamin A molecules is known as the retinoid cycle of vision. Molecular defects in retinoid cycle genes can lead to inherited retinal diseases in man (1). The severity of visual disturbance in these diseases is thought to be related to how the mutation alters the biochemical activity and whether there is redundancy at the multiple biochemical steps of the cycle. A severe form of incurable childhood blindness, Leber congenital amaurosis (LCA), is caused by mutations in *RPE65* (retinal pigment epithelium-specific protein, 65 kDa), the gene in the retinal pigment epithelium (RPE) that encodes the isomerase. This is the only known enzyme that catalyzes isomerization of all-*trans*-retinyl esters to 11-*cis*-vitamin A. In *RPE65* deficiency, photoreceptor cells do not regenerate their visual pigment and vision is not sustained. Retinal anatomy also degenerates, but not entirely (2, 3).

*RPE65*-deficient animals have been characterized, and proof-of-principle studies using recombinant adeno-associated virus (AAV) vector delivery of *RPE65* to RPE cells have described restoration of vision (2, 4–14). These studies provided the impetus for human safety studies of *RPE65* gene replacement

(trials NCT00481546, NCT00643747, NCT00516477, and NCT00422721, www.clinicaltrials.gov). Early reports from human retinal gene replacement trials suggest there are no short-term safety concerns and that modest increases in visual function can be found in some of the subjects (15–17).

Key scientific questions were not answered in these recent human studies. It remains unclear whether rod or cone vision, or both, were restored. It is also unknown whether any vision improvement was complete and fully explained by the level of potentially rescuable photoreceptors remaining in the treatment area. To determine if the genetically flawed retinoid cycle could be restored, we used AAV-based *RPE65* gene replacement in three young adult subjects with severe visual loss caused by recessive *RPE65* mutations. Rod and cone visual parameters were studied before and after intervention. Rod photoreceptor-based vision was present in all patients after treatment. Unexpectedly, rod kinetics of dark adaptation, a surrogate for retinoid cycle activity (18), were remarkably slow. Increased cone photoreceptor-based vision was demonstrated in two of the patients. Comparison of photoreceptor nuclear layer structure and co-localized visual function indicated that the intervention had ameliorated the severe retinoid cycle blockade component of this complex disease and converted it to a simpler photoreceptor degeneration.

## Results

### Human Gene Therapy Surmounts Biochemical Blockade in *RPE65*-LCA.

The retinoid cycle enzymatic pathway allows continuous vision by regeneration of 11-*cis*- from all-*trans*-retinal (Fig. 1 *A* and *B*). AAV2-based *RPE65* gene replacement was delivered by injection ( $5.96 \times 10^{10}$  vg in 150  $\mu$ l) between the RPE cell layer and the photoreceptor layer (Fig. 1*C*) into one eye (i.e., study eye) of three *RPE65*-LCA subjects. Subjects' ages were 24 years (patient 1), 23

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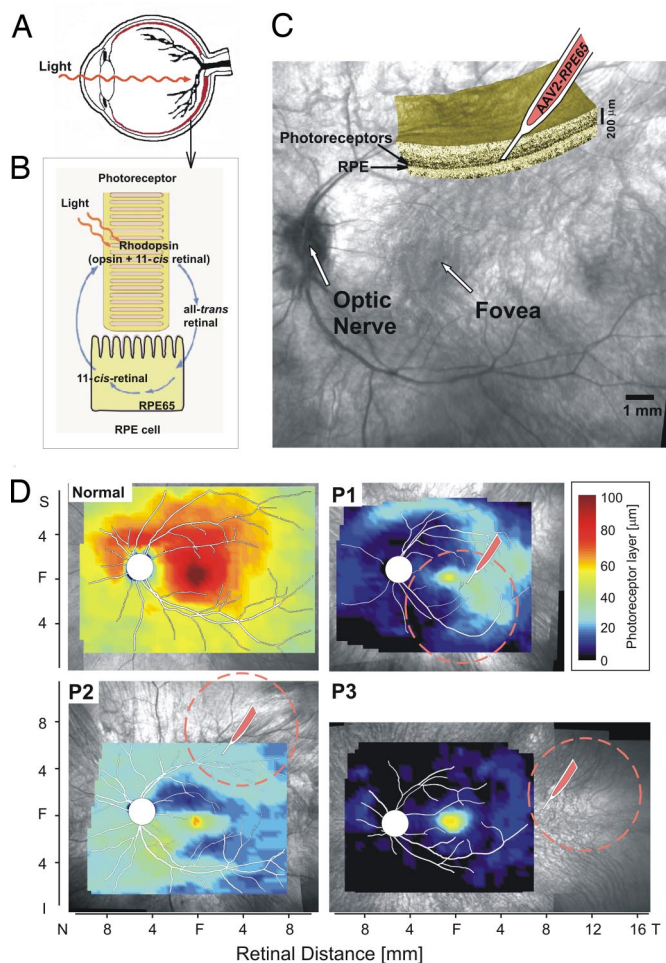
Conflict of interest statement: B.J.B., W.W.H., and the University of Florida have a financial interest in the use of AAV therapies and own equity in a company (AGTC Inc.) that might, in the future, commercialize some aspects of this work. J.M.W. is an inventor on patents related to gene therapy that have been licensed to a number of biopharmaceutical companies. University of Pennsylvania, University of Florida, and Cornell University hold a patent on the described gene therapy technology (United States Patent 20070077228, "Method for Treating or Retarding the Development of Blindness").

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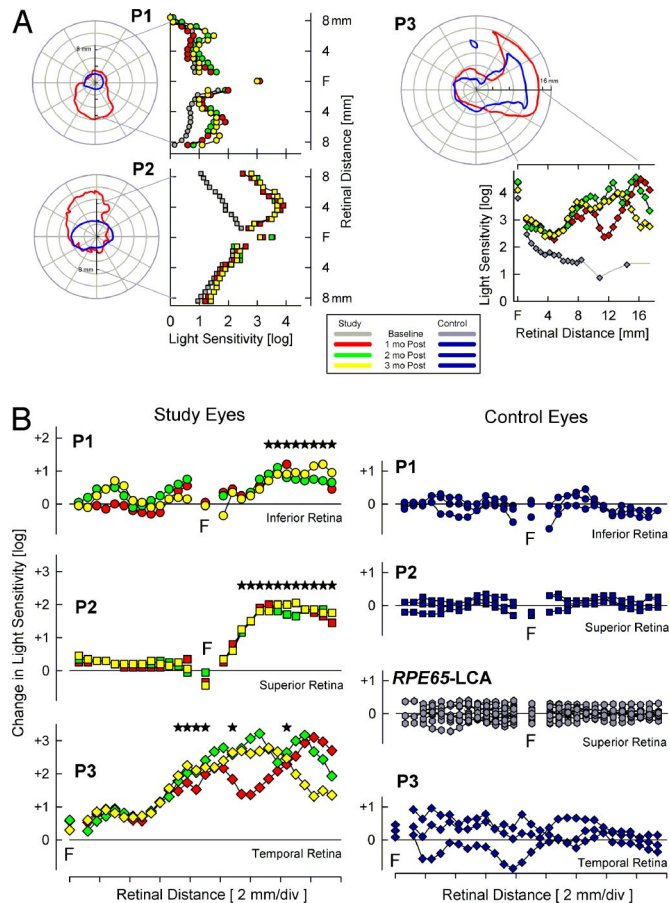
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**Fig. 1.** Schematic of the retina, retinoid cycle, and localized delivery of gene therapy in three patients with inherited blindness. (A) Light enters the eye and is absorbed by visual pigments (rhodopsin) in photoreceptors of the retina (red), the complex brain-like neural layer. (B) The retinoid cycle and its isomerase, RPE65, converts all-*trans*- to 11-*cis*-retinal to regenerate the visual pigment. (C) Series of retinal cross-sections from patient 2 rendered in three dimensions and overlaid onto the ocular fundus view illustrate the superior retinal site of subretinal vector injection. Fovea and optic nerve head locations are shown. (D) Photoreceptor cell layer (i.e., ONL) thickness topography (pseudocolor scale) in a normal subject and pretreatment in study eyes of patients 1, 2, and 3 (P1, P2, and P3). Site of injection (syringe tip) and the estimate of the bleb formed by the injection (dashed circle) are shown. Data in C and D are shown in equivalent left retina representation for clarity.

years (patient 2) and 21 years (patient 3) and all had severe vision disturbances from childhood. The disease-causing *RPE65* mutations (patient 1, E417Q/E417Q; patient 2, R91W/R44Q; patient 3, Y368H/Y368H) were reported *in vitro* to have less than 3% isomerization activity compared with WT (19, 20). The photoreceptor cell layer thickness in all three patients was reduced but sufficiently detectable to warrant treatment. Treatment involved the inferior retina in patient 1, superior retina in patient 2, and the far temporal retina in patient 3 (Fig. 1D). The biological activity of the human vector unused in the treatment syringe was proven with an *in vivo* bioassay in Rpe65-deficient *rd12* mice [supporting information (SI) Fig. S1A].

Patient 1 showed increased vertical extent of daylight visual field in the study eye versus the control eye when measured at 30 days after treatment (Fig. 2A). There was increased light sensitivity in the treated inferior retina compared with pretreatment results (Fig. 2A and B). The effect in patient 1 was present at 1 month as well as



**Fig. 2.** Biological activity resulting from localized gene therapy in three patients with inherited blindness. (A) Clinical kinetic visual field maps (represented as left retina for clarity and comparability) in control (blue) and study eyes at 1 month after treatment (red) are shown. Light sensitivity measures in study eyes along vertical (patients 1 and 2) and horizontal (patient 3) meridians at 1, 2, and 3 months after treatment compared with before treatment are also shown. The increased superior (patient 1), inferior (patient 2), and temporal (patient 3) retinal extent of vision boundary on visual field maps of study eyes corresponds to the region of vector injection. These regions show increases in light sensitivity after treatment compared with before treatment. (B) Retinal loci demonstrating significant change (stars) in light sensitivity at 1, 2, and 3 months after treatment compared with before treatment. All significant changes were increases in sensitivity (ranging from 1 to 3 log units) and correspond to regions of study eyes that received gene therapy; control eyes of the three patients did not show significant changes. Test-retest variability estimated from five additional patients with *RPE65*-LCA along the vertical meridian was smaller than the magnitude of sensitivity changes observed in study eyes. Biological activity in the study eye of patient 3 is assumed to cover a large contiguous region even though significance could be mathematically determined only at a subset of far temporal loci where pretreatment sensitivities were available. The baseline for each patient corresponds to the mean of two visits within 20 months of treatment. F, fovea.

2 and 3 months after treatment; there was no major change in the lateral extent or magnitude of the visual gain during this interval (Fig. 2A and B). Retinal loci corresponding to a contiguous region extending more than 4 mm showed statistically significant increases of sensitivity in the study eye; there were no significant changes in the control eye (Fig. 2B).

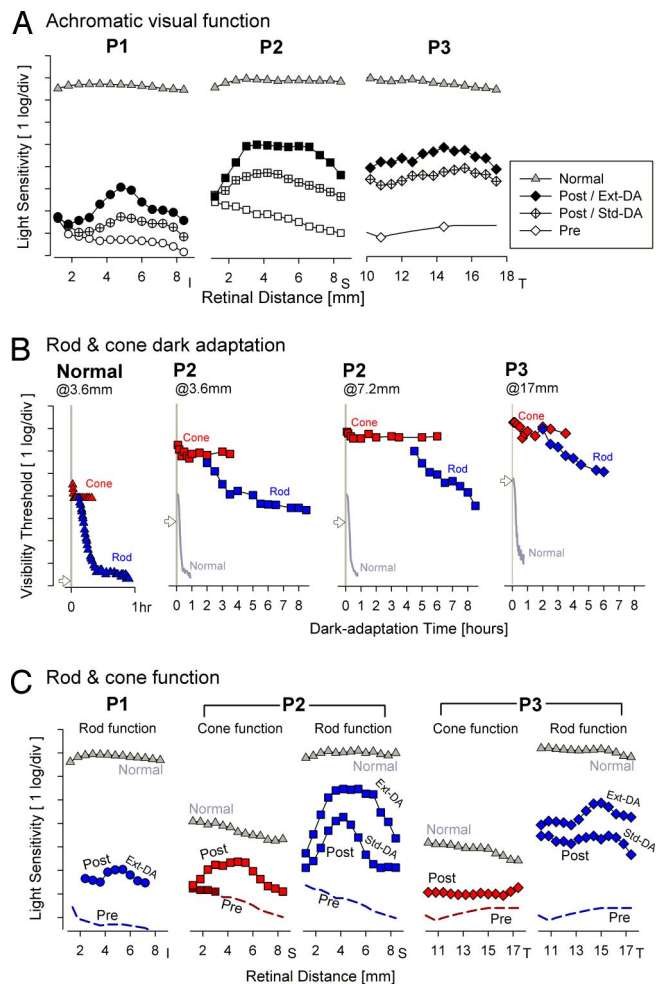
Patient 2 described a localized increase in brightness as early as 7 to 10 days after treatment. This self-reported increase in visual sensitivity posttreatment is earlier than times previously reported in most animal studies (2, 8, 9, 12, 14). Thus, biological activity was studied in *rd12* mice at 10 days after treatment with the clinical trial vector and compared with control injections. Even at this early

posttreatment time, there was a robust electroretinographic response to the vector and no such response from control injections with vehicle (Fig. S1B). Daylight visual fields in patient 2 at 30 days after treatment showed increased vertical extent in the study eye compared with the control eye (Fig. 2A). Visual thresholds measured in the dark revealed greater light sensitivity in the superior retina, a region included in the injection (Fig. 1D). No change in the lateral extent or magnitude of the visual gain was detectable between days 30 and 90 posttreatment (Fig. 2A and B). Statistically significant increases covered a contiguous retinal region of more than 6 mm; there were no significant changes in the control eye (Fig. 2B). Test–retest variability in other RPE65-LCA patients was smaller than the extent of the increases observed in the treated eyes of patients 1 and 2 (Fig. 2B).

Patient 3 also reported increased light sensitivity in the study eye at 7 to 10 days after treatment. Daylight visual fields were increased in horizontal extent compared with the control eye at 30 days after treatment (Fig. 2A). Visual testing in the dark at loci representing the far temporal retina revealed a wide region with increased sensitivity compared with pretreatment results. The effect was statistically significant, persistent upon re-tests at 60 and 90 days after treatment, and covered a retinal region greater than 10 mm in extent. There were no significant changes in sensitivity in the control eye. Pupillary reactions in the dark (21, 22) also indicated increased light sensitivity in the study eyes of patients 2 and 3; this method showed no detectable change in sensitivity in the study eye of patient 1 or the control eyes of all three patients (Fig. S2).

**Extended Dark Adaptation Reveals a Greater Magnitude of Visual Gain After Treatment.** Normal human vision becomes more sensitive to light after an instantaneous decrease in ambient illumination—the process is known as dark adaptation. Normally, full dark adaptation can require up to 1 hour for rod photoreceptor-based night vision (18, 23–27); further changes in light sensitivity after 1 hour are insignificant in normal eyes. Accordingly, initial testing in all patients was performed after a standard dark adaptation period of 1 to 2 h. Clues to the inadequacy of this period were suggested from reports by the subjects of noticeably increased brightness in their treated eye when they awoke from sleep in their darkened rooms. To understand the pathophysiology underlying these reports, testing was repeated after allowing adaptation of eyes to darkness for extended periods (3–8 h). Under these conditions, all three patients showed dramatic further gains in the magnitude of their visual sensitivity. Mean gains were 0.9, 1.3, and 0.7 log units within the treated regions of patients 1, 2, and 3, respectively (Fig. 3A); untreated retinal regions showed no significant sensitivity changes with extended adaptation (not shown). These results suggested that the kinetics of the reconstituted retinoid cycle in the study eyes may be abnormally slow. Further, spatially non-uniform improvements observed across the treated retina in all patients indicated large differences in the kinetics of dark adaptation may be present.

**Adaptation Kinetics for Treated Cones Are Rapid, but Treated Rod Recovery Takes Many Hours.** To define the kinetics of dark adaptation in treated retinas and to differentiate rod from cone kinetics, chromatic sensitivities were measured before and after a desensitizing light flash (Fig. 3B). In patient 2, two retinal loci within the treated superior retinal region were studied, whereas in patient 3 a single locus was tested in the far temporal retina; patient 1 did not have sufficient visual function to permit reliable testing. Within 1 minute after the flash, visual function was detectable, and it was mediated by the cone system (Fig. 3B). Cone-mediated function remained on a plateau (25), essentially unchanged for more than 120 min, compared with the same period lasting only 7 to 9 min in normal eyes. Emergence of rod function defined the end of the cone-plateau phase at  $\approx 2$  h for



**Fig. 3.** Rod- and cone-photoreceptor-mediated visual function across the retinal region of study eyes showing biological activity. (A) The magnitude of the biological activity can be improved by allowing for a period of extended dark adaptation (Ext DA) before testing. Changes in the shapes of the light sensitivity profiles in patient 1 and patient 2 between standard dark adaptation (StdDA) and extended dark adaptation conditions suggest local differences in adaptation rate. (B) Dark adaptation kinetics measured with chromatic stimuli after a 7 log scot-td.s yellow adapting flash (presented at time 0) in patient 2 (at 3.6 and 7.2 mm inferior loci) and patient 3 (at 17 mm temporal locus). Also shown are detailed results from one normal (N) subject at 3.6 mm inferior (Left) and mean results from normal subjects at each location (gray lines). Cone adaptation kinetics (red symbols) are fast and do not show a difference from healthy cones. Rod adaptation kinetics (blue symbols) are extremely slow compared with healthy rods, and in patient 2 there is evidence for a large interareal difference in recovery rate. Additionally, absolute thresholds of both rod and cone systems are abnormally elevated. Note, the vertical threshold scale is inverted compared to the sensitivity scale in A to be consistent with traditional presentations of such data sets. (C) Maximal improvement in rod-mediated function (blue) across the region of treated retina ranged from 2.3 log in patient 1, 4.8 log in patient 2, and 4.5 log in patient 3; cone vision improvements (red) range from 1.7 log in patient 2 to 1.2 log in patient 3. Note that pretreatment estimates for rod- or cone-mediated vision (dashed lines) are best-case extrapolations from achromatic sensitivity measures. Only in patient 2 were pretreatment chromatic sensitivities measurable at 1–3 mm superior retina, and these measures were consistent with cone mediation. For patient 1, after treatment, specialized testing with a 4° diameter stimulus centered at 4.8 mm inferior retina suggested rod mediation for the blue stimulus. Pretreatment (Pre) results are from  $\approx 18$  to  $\approx 3$  months before treatment; posttreatment (Post) results are from 1–3 months after treatment.

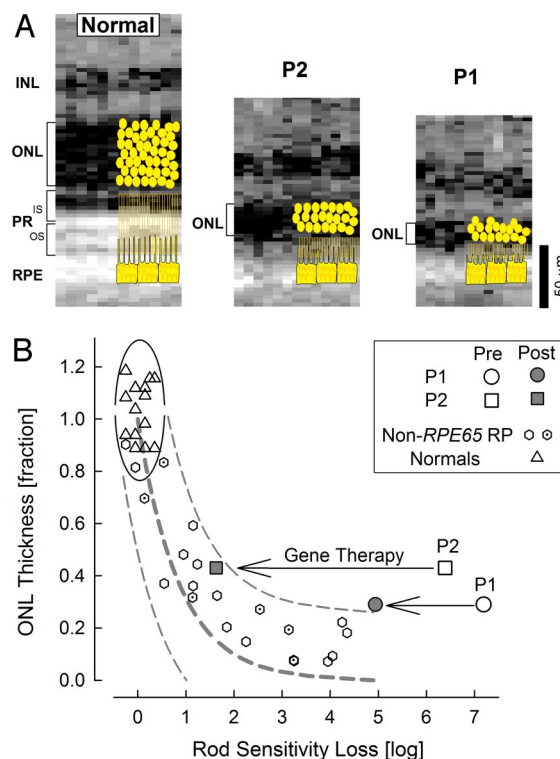
patient 2 at 3.6 mm superior retina and patient 3 at 17 mm temporal retina; patient 2 at 7.2 mm superior retina remained on the cone plateau for  $\approx 4$  h (Fig. 3B). Rod photoreceptor-

mediated recovery in both patients progressed slowly, lasting at least 8 h. The shape of the rod recovery function could be described with two log-linear segments in patient 2 at the 3.6 mm superior locus with slopes of  $-0.6 \text{ h}^{-1}$  and  $-0.18 \text{ h}^{-1}$  (corresponding to time constants of 43 and 145 min); the exact shapes of the other two recovery functions were less discernible but the major log-linear segments had slopes of  $-0.6 \text{ h}^{-1}$ . In normal eyes, rod recovery following a similar flash shows two log-linear segments with slopes of  $-0.25 \text{ min}^{-1}$  and  $-0.04 \text{ min}^{-1}$  (corresponding to time constants of 1.7 and 11 min).

**Rod- and Cone-Based Vision Increases After Gene Therapy.** Cone function was evaluated across the treated retinal regions of patients 2 and 3 during the extended cone-plateau phase of dark adaptation with chromatic stimuli (Fig. 3C). At retinal regions with peak biological activity, cone-mediated sensitivities increased by at least 1.7 log units in patient 2 and 1.2 log units in patient 3 compared with the most conservative (i.e., best-case) estimates of pretreatment cone vision (Fig. 3C, red dashed lines). In both patients, cone function posttreatment remained  $\approx 1.5$  log units less sensitive than normal. Cone vision in patient 1 was not detectable with the brightest available long-wavelength stimulus (implying a loss of greater than 2.6–3.4 log units, depending on retinal location) before or after treatment.

Under standard dark adaptation conditions, rod-mediated function was discernible after treatment in patients 2 and 3 (Fig. 3C) demonstrating 2.3 and 3.1 log units of increased sensitivity on average compared with conservative estimates of pretreatment rod vision (Fig. 3C). With an extended period of dark adaptation, rod-mediated function showed further gains of 1.7 log units in patient 2 and 1.0 log unit in patient 3 and became detectable in patient 1 (Fig. 3C). The rod function gain observed in patient 2 was not uniform (Fig. 3C), consistent with the large intraretinal difference in rate of dark adaptation in this subject (Fig. 3B). Posttreatment rod function reached within 1.5 log units of normal vision in patient 2 and within 2.2 log units in patient 3 but remained  $>4$  log units less sensitive than normal in patient 1.

**Gene Replacement Converts RPE65-LCA from a Complex to a Simpler Disease.** *RPE65-LCA* is a complex retinal disease in which visual loss is caused by a combination of a biochemical blockade of the retinoid cycle and degeneration of retinal photoreceptors. In human patients, as well as in canine and murine models, disease stages with partial degeneration of photoreceptors show a dissociation of retinal function from retinal structure whereby the loss of visual function can be orders of magnitude greater than expected from the partial loss of retinal photoreceptors alone (2). Gene replacement therapy would be hypothesized to ameliorate the functional blockade but not replace cells lost as a result of degeneration. To test this hypothesis, we determined the relationship between retinal structure and function in patients 1 and 2 over a  $\approx 2$ -mm expanse of retina corresponding to each individual's region of peak biological activity (Fig. 4); data on retinal structure could not be obtained within the far temporal locus of patient 3. Photoreceptor layer (outer nuclear layer, ONL) thickness was 29% of mean normal thickness for patient 1 and 43% for patient 2. Patients with retinitis pigmentosa with degenerative photoreceptor loss but without *RPE65* mutations who have ONL thickness values similar to those of patients 1 and 2 showed sensitivity losses ranging from 0.5 to 2.5 log units, consistent (to within measurement variability) with the predictions (0.7–1.1 log units) of a theoretical model (Fig. 4B). Pretreatment conservative estimates of the loss of rod function for patients 1 and 2 were 7.2 log and 6.4 log, respectively. After treatment, patient 1 showed a 2.3 log unit increase in rod sensitivity, which approached, but did not reach, the predictions of the simple photoreceptor degeneration (Fig. 4B). Patient 2, conversely, showed a posttreatment visual gain of 4.8 log units (i.e., 63,000 fold) and the result became no different from that expected of simpler



**Fig. 4.** Pathophysiology of complex visual loss resulting from photoreceptor degeneration and biochemical blockade, and the effect of gene therapy. (A) Retinal cross-sectional images at the region of maximal biological activity show the photoreceptor layer (ONL, brackets) to be abnormally thinned; greater degeneration of photoreceptors is evident in patient 1 compared with patient 2. Normal retina and patient 2 are at  $\approx 5$  mm superior retina, patient 1 is at  $\approx 5$  mm inferior retina. Schematics of photoreceptors and RPE are overlaid (yellow) and to the right of the actual optical images; these are derived from cynomolgus monkey histology (3, 28). INL, inner nuclear layer; PR-IS and PR-OS, photoreceptor inner and outer segments. (B) The relationship between photoreceptor layer thickness and rod sensitivity loss in patients 1 and 2 before and after therapy compared with normal subjects and patients with retinitis pigmentosa without mutations in the *RPE65* gene. Normal variability is described by the ellipse encircling the 95% C.I. of a bivariate Gaussian distribution. Dotted lines define the idealized model of the relationship between structure and function in pure photoreceptor degenerations and the region of uncertainty that results from translating the normal variability along the idealized model. Gene therapy (arrows) results in partial (patient 1) or a nearly complete (patient 2) amelioration of the biochemical blockade transforming the complex *RPE65-LCA* disease phenotype into a simpler retinal degeneration phenotype.

diseases with only a degenerative component contributing to the vision loss (Fig. 4B). Thus, gene therapy shows the potential to provide dramatic, albeit imperfect, restoration of the retinoid cycle disease component in *RPE65-LCA*.

## Discussion

Clinical trials of unioocular subretinal gene therapy in patients with *RPE65-LCA* were initiated after proof-of-concept studies using subretinal delivery of vector gene showed restored vision in *Rpe65*-deficient dogs (4, 5, 8, 11, 12) and mice (2, 6, 7, 9, 10, 13, 29, 30). Short-term safety results from three human clinical trials were recently reported: there were no serious adverse events in all subjects, and vision improved in some subjects (15–17). In the current study, we explored in detail the basis of increased vision resulting from the intervention.

Rod photoreceptor-based night vision increased in sensitivity in all three subjects who underwent gene therapy in this study. The increase in rod sensitivity was localized to the retinal regions exposed to the therapy. The magnitude of the increase differed

among subjects, and those with a better preserved photoreceptor layer in the treated region showed the greater increases in rod vision. These psychophysical results, complimented by objective evidence from dark-adapted pupillary reflexes, were consistent with our previous demonstration that visual pathways from retina to visual brain were intact and amenable to therapy despite the congenital defect of *RPE65-LCA* (22). The increased rod sensitivity is likely driven by an increased synthesis of 11-*cis*-retinal chromophore resulting from WT RPE65 protein introduced into the RPE by expression from the AAV2 gene therapy vector. These results in man add to the literature consensus that RPE65 is essential in the regeneration of rod visual pigment (i.e., rhodopsin) in vertebrates (1, 19, 31, 32). Both the magnitude of the increase observed (up to 4.8 log<sub>10</sub> units) in the human subjects and the lack of visual function increase found with sham subretinal injections in murine (Fig. S1) and canine (11) models with Rpe65 deficiency essentially rule out alternative hypotheses for visual gain involving the release of neurotrophic factors (33) secondary to subretinal surgery (34).

Extremely prolonged recovery of rod vision after light exposure, demonstrated by dark adaptation testing posttreatment in patients 2 and 3 (Fig. 3B), suggests a slowed delivery of 11-*cis*-retinal chromophore from the RPE to the rod photoreceptors and thus a protracted regeneration of light responsive rhodopsin. Two general hypotheses to entertain for a slowing of the chromophore delivery rate are (i) reduced rate of its synthesis or (ii) increased obstruction to its inter- or intracellular transport. Abnormally low expression of RPE65 (or other key retinoid cycle enzymes, ref. 35) could have reduced the rate of chromophore synthesis and resulted in an enzymatic limit to rhodopsin regeneration such as observed in mice that express very low levels of WT Rpe65 (36, 37) or a mutant Rpe65 protein (38). In previous studies, slowness of recovery of rod vision in human diseases of the RPE (23–27) has been ascribed to enzymatic limitation to the synthesis of chromophore (18). A limited expression of RPE65 through gene therapy could have caused such an enzymatic bottleneck. Unlike in disease states, however, the normal human rhodopsin regeneration rate is believed not to be enzymatically limited (18). Consistent with this hypothesis is the demonstration of normal rate of dark adaptation in heterozygotes for null mutations in *RPE65* (39) and *RDH5* (25) predicted to express half the normal amount for the two key retinoid cycle enzymes, the isomerase and the 11-*cis*-retinol dehydrogenase, respectively. As an alternative to an enzymatic limit, the major component of dark adaptation in normal human vision is hypothesized to be rate-limited by a “resistive barrier” to 11-*cis*-retinal diffusion or transport between RPE cells and rod photoreceptors (18). The identity of the resistive barrier is currently unknown, and it is possible that RPE65 disease exacerbates or adds to a natural barrier. The dramatic accumulation of all-*trans*-retinyl esters and lipid droplets observed in the RPE with RPE65 deficiency and/or disorganized rod outer segments may contribute to such a barrier (4, 9, 40, 41). Alternatively, the surgical detachment could have altered the RPE/photoreceptor interface (42). Follow-up studies in the current patients and other patients with similar or higher doses of vector-*RPE65* should help clarify the underlying cause of slowed rod kinetics by considering dose and disease stage dependence of the recovery rate as well as possible changes in dark adaptation rate with time after treatment.

Arguably, cone photoreceptor-based daylight vision is used more than night vision by people living in modern well-lit environments. The role(s) played by RPE65 in providing chromophore for cone function and/or survival remains unclear and controversial (1, 3, 40, 43–49). Restoration of cone- as well as rod photoreceptor-based visual function by subretinal AAV gene therapy in the canine model of *RPE65-LCA* (4, 8), together with the existence of remnant cone function in patients with untreated *RPE65-LCA* (3), suggested a potential to improve cone function in patients undergoing gene therapy. Indeed, in two of the patients in the current study (patients

2 and 3), robust improvement in cone-mediated visual function could be demonstrated (Fig. 3C). In the patient with the best treatment response (patient 2), both cones and rods reached a level of sensitivity within  $\approx 1.5$  log units of mean normal. Fast recovery rate of cone dark adaptation function could be attributed to the long-standing hypothesis of rod/cone competition, with cones extracting more of an extremely limited supply of 11-*cis*-retinal chromophore (50). However, alternative hypotheses involving a partial reconstitution of a cone-specific retinoid cycle by gene therapy cannot be ruled out.

How do the present results compare with other similarly conducted *RPE65-LCA* gene therapy trials? Maguire *et al.* (16) reported increased visual acuity after treatment, but these increases were from severely low levels of spatial vision to levels that were still low; extrafoveal rods or cones could have subserved the posttreatment levels of visual acuity reported. Improvement of vision in dim light was self-reported by all three subjects (16) but the photoreceptor cell type subserving this vision was not characterized. Bainbridge *et al.* (15) used dark-adapted thresholds and found as much as 2 log units of increased vision in one patient, but again the photoreceptor type mediating the improvement remained unclear (15). The need to experimentally dissect rod- from cone-mediated responses in our patients suggests that the source of visual improvement with therapy cannot be presumed but must be measured. Of note, none of our patients showed a decrease in nystagmus (Fig. S3), contrary to an observation made in another trial (16). Contributing to this discrepancy could be the large baseline differences in visual acuity between the treatment cohorts; we have previously shown fixation instability to be related to visual acuity in *RPE65-LCA* (3), and treatment of patients with severe loss of central vision as in Maguire *et al.* (16) could conceivably have different consequences on eye movement abnormalities.

There are some key practical implications of the finding of slow rod kinetics after treatment. Clinical trial protocols may need reconsideration as this therapy advances beyond early stages. It is evident that the maximum increase of vision after treatment cannot be measured unless patients undergo dark adaptation for extended periods of time. Comparisons of visual function improvement between patients within a trial or between trials cannot be made without rigorous attention to previous light exposure and length of dark adaptation. The order of testing protocols will need careful reconsideration. In particular, light exposures, such as fundus photography, preceding measures of dark-adapted sensitivities should be minimized. If monitoring the kinetics of adaptation becomes an assay for treatment efficacy, an efficient method should be devised, considering the lengthy time course we observed in two patients.

## Materials and Methods

**Human Studies.** Vision research studies were performed in eight patients with *LCA* caused by mutations in the *RPE65* gene and conducted according to guidelines of the Declaration of Helsinki after obtaining written consent. All studies were approved by the University of Pennsylvania Institutional Review Board (nos. 186900, 701705, 700942, and 804582). Three of the patients were assessed with these vision research studies before and after taking part in a phase I clinical trial (trial NCT00481546, www.clinicaltrials.gov) evaluating the safety of rAAV2-CB<sup>58</sup>-hRPE65 (IND Number, BB-IND 12824). Safety results of this clinical trial are published separately (17).

**Photoreceptor Layer Topography.** *In vivo* microscopy of the human retina was performed with optical coherence tomography (OCT) as published (2, 3, 51). A Fourier-domain OCT system (RTVue-100; Optovue) was used for data acquisition, and postacquisition processing of data was performed with custom programs (MatLab 6.5; MathWorks). Further details are described in the [S1 Text](#).

**Psychophysical Studies.** Visual field testing was performed with kinetic and static perimetry as published (3, 23–27, 52). For kinetic perimetry, white (318 cd·m<sup>-2</sup>) targets of Goldmann sizes III (patient 1) or V (patients 2 and 3) were used on a 10

cd-m<sup>-2</sup> achromatic background. Fixation location and stability were quantified (as described in *SI Text*) in all subjects studied. All subjects fixated at the anatomical fovea; variability of the excursions from the fovea resulting from abnormal eye movements was much smaller than the 0.6-mm sampling density used in static perimetry (Fig. S3). In all three patients, there were no consistent changes in fixation location or fixation instability before and after treatment (Fig. S3). Static perimetric sensitivities were determined with a modified computerized perimeter (Humphrey Field Analyzer; Zeiss) under dark-adapted conditions (see *SI Text* for details).

#### Modeling of the Relationship Between Retinal Structure and Visual Function.

The relationship between photoreceptor structure and co-localized visual function was defined in patients using ONL thickness and rod-mediated sensitivity. Patient results were compared with an idealized model of the expected relationship for “simple” photoreceptor degenerations in which vision loss is thought to be derived primarily from degenerative photoreceptor cell loss. The model assumes that absolute sensitivity of rod-mediated function near the visibility thresh-

old is limited by quantum catch and is thus proportional to the product of the number of surviving photoreceptor cells and the length of their outer segments; both of these parameters are proportional to ONL thickness (2, 53, 54). Thus, to a first approximation, loss of light sensitivity (in linear units) would be expected to be proportional to the square of ONL thinning.

**Animal Studies.** *Rpe65*-deficient *rd12* mice were injected subretinally with 4 × 10<sup>8</sup> vector genomes of rAAV2-CB<sup>58</sup>-hRPE65, which remained after each human surgery. Electroretinograms obtained in vector-injected and control eyes were used to determine the biological activity of the vector (13). Further details are described in the *SI Text*.

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- Travis GH, Golczak M, Moise AR, Palczewski K (2007) Diseases caused by defects in the visual cycle: retinoids as potential therapeutic agents. *Annu Rev Pharmacol Toxicol* 47:469–512.
- Jacobson SG, et al. (2005) Identifying photoreceptors in blind eyes caused by RPE65 mutations: prerequisite for human gene therapy success. *Proc Natl Acad Sci USA* 102:6177–6182.
- Jacobson SG, et al. (2007) Human cone photoreceptor dependence on RPE65 isomerase. *Proc Natl Acad Sci USA* 104:15123–15128.
- Acland GM, et al. (2001) Gene therapy restores vision in a canine model of childhood blindness. *Nat Genet* 28:92–95.
- Narfström K, et al. (2003) Functional and structural recovery of the retina after gene therapy in the *RPE65* null mutation dog. *Invest Ophthalmol Vis Sci* 44:1663–1672.
- Dejneka NS, et al. (2004) In utero gene therapy rescues vision in a murine model of congenital blindness. *Mol Ther* 9:182–188.
- Lai C, et al. (2004) Recombinant adeno-associated virus type 2-mediated gene delivery into the *Rpe65*<sup>-/-</sup> knockout mouse eye results in limited rescue. *Genet Vaccines Ther* 2:3.
- Acland GM, et al. (2005) Long-term restoration of rod and cone vision by single dose rAAV-mediated gene transfer to the retina in a canine model of childhood blindness. *Mol Ther* 12:1072–1082.
- Pang JJ, et al. (2006) Gene therapy restores vision-dependent behavior as well as retinal structure and function in a mouse model of RPE65 Leber congenital amaurosis. *Mol Ther* 13:565–572.
- Chen Y, Moiseyev G, Takahashi Y, Ma JX (2006) RPE65 gene delivery restores isomerohydrolase activity and prevents early cone loss in *Rpe65*<sup>-/-</sup> mice. *Invest Ophthalmol Vis Sci* 47:1177–1184.
- Jacobson SG, et al. (2006) Safety of recombinant adeno-associated virus type 2-RPE65 vector delivered by ocular subretinal injection. *Mol Ther* 13:1074–1084.
- Le Meur G, et al. (2007) Restoration of vision in *RPE65*-deficient Briard dogs using an AAV serotype 4 vector that specifically targets the retinal pigmented epithelium. *Gene Ther* 14:292–303.
- Roman AJ, et al. (2007) Electroretinographic analyses of *Rpe65*-mutant *rd12* mice: developing an in vivo bioassay for human gene therapy trials of Leber congenital amaurosis. *Mol Vis* 13:1701–1710.
- Bennicelli J, et al. (2008) Reversal of blindness in animal models of Leber congenital amaurosis using optimized AAV2-mediated gene transfer. *Mol Ther* 16:458–465.
- Bainbridge JW, et al. (2008) Effect of gene therapy on visual function in Leber's congenital amaurosis. *N Engl J Med* 358:2231–2239.
- Maguire AM, et al. (2008) Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med* 358:2240–2248.
- Hauswirth WW, et al. (2008) Phase I trial of Leber congenital amaurosis due to *RPE65* mutations by ocular subretinal injection of adeno-associated virus gene vector: Short-term results. *Hum Gene Ther*, in press.
- Lamb TD, Pugh EN Jr (2006) Phototransduction, dark adaptation, and rhodopsin regeneration. The Proctor lecture. *Invest Ophthalmol Vis Sci* 47:5137–5152.
- Redmond TM, et al. (2005) Mutation in key residues of RPE65 abolishes its enzymatic role as isomerohydrolase in the visual cycle. *Proc Natl Acad Sci USA* 102:13658–13663.
- Takahashi Y, Chen Y, Moiseyev G, Ma JX (2006) Two point mutations of RPE65 from patients with retinal dystrophies decrease the stability of RPE65 protein and abolish its isomerohydrolase activity. *J Biol Chem* 281:21820–21826.
- Aleman TS, et al. (2004) Impairment of the transient pupillary light reflex in *Rpe65*<sup>-/-</sup> mice and humans with Leber congenital amaurosis. *Invest Ophthalmol Vis Sci* 45:1259–1271.
- Aguirre GK, et al. (2007) Canine and human visual cortex intact and responsive despite early retinal blindness from RPE65 mutation. *PLoS Med* 4:e230.
- Jacobson SG, et al. (1995) Night blindness in Sorsby's fundus dystrophy reversed by vitamin A. *Nat Genet* 11:27–32.
- Cideciyan AV, Pugh EN Jr, Lamb TD, Huang Y, Jacobson SG (1997) Rod plateaus during dark adaptation in Sorsby's fundus dystrophy and vitamin A deficiency. *Invest Ophthalmol Vis Sci* 38:1786–1794.
- Cideciyan AV, et al. (2000) Rod and cone visual cycle consequences of a null mutation in the 11-cis-retinol dehydrogenase gene in man. *Vis Neurosci* 17:667–678.
- Jacobson SG, Cideciyan AV, Wright E, Wright AF (2001) Phenotypic marker for early disease detection in dominant late-onset retinal degeneration. *Invest Ophthalmol Vis Sci* 42:1882–1890.
- Cideciyan AV, et al. (2004) Mutations in ABCA4 result in accumulation of lipofuscin before slowing of the retinoid cycle: a reappraisal of the human disease sequence. *Hum Mol Genet* 13:525–534.
- Jacobson SG, et al. (2006) Safety in nonhuman primates of ocular AAV2-RPE65, a candidate treatment for blindness in Leber congenital amaurosis. *Hum Gene Ther* 17:845–858.
- Bemelmans AP, et al. (2006) Lentiviral gene transfer of RPE65 rescues survival and function of cones in a mouse model of Leber congenital amaurosis. *PLoS Med* 3:e347.
- Yanez-Munoz RJ, et al. (2006) Effective gene therapy with nonintegrating lentiviral vectors. *Nat Med* 12:348–353.
- Jin M, Li S, Moghrabi WN, Sun H, Travis GH (2005) Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. *Cell* 122:449–459.
- Moiseyev G, Chen Y, Takahashi Y, Wu BX, Ma JX (2005) RPE65 is the isomerohydrolase in the retinoid visual cycle. *Proc Natl Acad Sci USA* 102:12413–12418.
- Wen R, et al. (1995) Injury-induced upregulation of bFGF and CNTF mRNAs in the rat retina. *J Neurosci* 15:7377–7385.
- Del Priore LV (2005) Effect of sham surgery on retinal function after subretinal transplantation of the artificial silicone retina. *Arch Ophthalmol* 123:1156.
- Saari JC, et al. (2001) Visual cycle impairment in cellular retinaldehyde binding protein (CRALBP) knockout mice results in delayed dark adaptation. *Neuron* 29:739–748.
- Wenzel A, Reme CE, Williams TP, Hafezi F, Grimm C (2001) The Rpe65 Leu450Met variation increases retinal resistance against light-induced degeneration by slowing rhodopsin regeneration. *J Neurosci* 21:53–58.
- Lyubarsky AL, et al. (2005) Mole quantity of RPE65 and its productivity in the generation of 11-cis-retinal from retinyl esters in the living mouse eye. *Biochemistry* 44:9880–9888.
- Samardzija M, et al. (2008) R91W mutation in Rpe65 leads to milder early-onset retinal dystrophy due to the generation of low levels of 11-cis-retinal. *Hum Mol Genet* 17:281–292.
- Poehner WJ, et al. (2000) A homozygous deletion in RPE65 in a small Sardinian family with autosomal recessive retinal dystrophy. *Mol Vis* 6:192–198.
- Redmond TM, et al. (1998) Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nat Genet* 20:344–351.
- Porto FB, et al. (2002) Prenatal human ocular degeneration occurs in Leber's congenital amaurosis (LCA2). *J Gene Med* 4:390–396.
- Geller SF, Lewis GP, Fisher SK (2001) FGFR1, signaling, and AP-1 expression after retinal detachment: reactive Müller and RPE cells. *Invest Ophthalmol Vis Sci* 42:1363–1369.
- Seeliger MW, et al. (2001) New views on RPE65 deficiency: the rod system is the source of vision in a mouse model of Leber congenital amaurosis. *Nat Genet* 29:70–74.
- Znoiko SL, Crouch RK, Moiseyev G, Ma JX (2002) Identification of the RPE65 protein in mammalian cone photoreceptors. *Invest Ophthalmol Vis Sci* 43:1604–1609.
- Hemati N, et al. (2005) RPE65 surface epitopes, protein interactions, and expression in rod- and cone-dominant species. *Mol Vis* 11:1151–1165.
- Schonthaler HB, et al. (2007) Evidence for RPE65-independent vision in the cone-dominated zebrafish retina. *Eur J Neurosci* 26:1940–1949.
- Zhang H, et al. (2008) Trafficking of membrane-associated proteins to cone photoreceptor outer segments requires the chromophore 11-cis-retinal. *J Neurosci* 28:4008–4014.
- Feathers KL, et al. (2008) Nrl-knockout mice deficient in Rpe65 fail to synthesize 11-cis-retinal and cone outer segments. *Invest Ophthalmol Vis Sci* 49:1126–1135.
- Collyer R, et al. (2008) Duplication and divergence of zebrafish CRALBP genes uncovers a novel role for RPE- and Mueller-CRALBP in cone vision. *Invest Ophthalmol Vis Sci* 49:3812–3820.
- Rushton WA (1968) Rod/cone rivalry in pigment regeneration. *J Physiol* 198:219–236.
- Jacobson SG, et al. (2008) Photoreceptor layer topography in children with Leber congenital amaurosis caused by RPE65 mutations. *Invest Ophthalmol Vis Sci*, in press.
- Jacobson SG, Yagasaki K, Feuer W, Roman AJ (1989) Interocular asymmetry of visual function in heterozygotes of X-linked retinitis pigmentosa. *Exp Eye Res* 48:670–691.
- Rohrer B, et al. (2003) Correlation of regenerable opsin with rod ERG signal in *Rpe65*<sup>-/-</sup> mice during development and aging. *Invest Ophthalmol Vis Sci* 44:310–315.
- Jacobson SG, et al. (2007) RDH12 and RPE65, visual cycle genes causing Leber congenital amaurosis, differ in disease expression. *Invest Ophthalmol Vis Sci* 48:332–338.