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

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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 visual 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.

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 × 10^10 vg in 150 µl) between the RPE cell layer and the photoreceptor layer (Fig. 1C) into one eye (i.e., study eye) of three RPE65-LCA subjects. Subjects’ ages were 24 years (patient 1), 23


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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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).

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 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).

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 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).

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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 greater than 10 mm in extent. There were no significant changes with extended dark adaptation conditions suggest local differences in adaptation rate. (6) Dark adaptation kinetics measured with chromatic stimuli after a 7 log scot-t-d-5 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 loci). 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 intraretinal 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 -18 to -3 months before treatment; posttreatment (Post) results are from 1–3 months after treatment.

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 h for rod photoreceptor-based night vision (18, 23–27); further changes in light sensitivity after 1 h 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 ~2 h for 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 ~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 h⁻¹ and −0.18 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 h⁻¹. In normal eyes, rod recovery following a similar flash shows two log-linear segments with slopes of −0.25 min⁻¹ and −0.04 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 ~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 blockage 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 ~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 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 uniocular 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...
therapy. Indeed, in two of the patients in the current study (patients of RPE65-LCA (4, 8), together with the existence of remnant cone visual function by subretinal AAV gene therapy in the canine model (39) and RPE65 (16) 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 RDHS (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 ~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 AA2V-2-BH4-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 SI 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⁻²) targets of Goldmann sizes III (patient 1) or V (patients 2 and 3) were used on a 10

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 threshold 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 × 10^6 vector genomes of rAAV2-CB-hRPE65, which remained after each human surgery. Electrotetrograms 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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