**HEALTH AND MEDICINE**

**CRISPR-Cas9–mediated therapeutic editing of Rpe65 ameliorates the disease phenotypes in a mouse model of Leber congenital amaurosis**

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Leber congenital amaurosis (LCA), one of the leading causes of childhood-onset blindness, is caused by autosomal recessive mutations in several genes including RPE65. In this study, we performed CRISPR-Cas9–mediated therapeutic correction of a disease-associated nonsense mutation in Rpe65 in rd12 mice, a model of human LCA. Subretinal injection of adeno-associated virus carrying CRISPR-Cas9 and donor DNA resulted in >1% homology-directed repair and ~1.6% deletion of the pathogenic stop codon in Rpe65 in retinal pigment epithelial tissues of rd12 mice. The a- and b-waves of electroretinograms were recovered to levels up to 21.2 ± 4.1% and 39.8 ± 3.2% of their wild-type mice counterparts upon bright stimuli after dark adaptation 7 months after injection. There was no definitive evidence of histologic perturbation or tumorigenesis during 7 months of observation. Collectively, we present the first therapeutic correction of an Rpe65 nonsense mutation using CRISPR-Cas9, providing new insight for developing therapeutics for LCA.

**INTRODUCTION**

Leber congenital amaurosis (LCA) is a hereditary retinal degenerative disease that leads to childhood-onset blindness (1). Among the genes causing LCA, CEP290, GUCY2D, CRB1, and RPE65 are those most frequently mutated. In particular, about 6% of LCA cases are caused by mutations in RPE65 (2). To date, no substantial treatments or cures for LCA have been developed, except for voretigene neparvovec (Luxturna, Spark Therapeutics), which is approved by the U.S. Food and Drug Administration (FDA). This approach is based on gene delivery by adeno-associated virus (AAV) to the retina in patients who lack the functional RPE65 protein (3–5). On the basis of preclinical studies performed in RPE65 null dogs (6, 7) and mice (8, 9), initial clinical trials tested the adverse events and therapeutic outcomes of AAV containing the human RPE65 complementary DNA (cDNA) (3–5). Although normal vision was not achieved, these trials showed that subretinal injection of AAV had an acceptable local and systemic adverse event profile and improved visual function (10). Nevertheless, because gene therapy cannot totally correct the mutated sequence and has a limitation in the cargo size of the AAV (~4.8 kb) when AAV is used, there are still unmet needs in the gene therapy area for broader clinical application.

CRISPR-Cas9 is a genome editing tool that can lead to the insertion of genes or the deletion of mutations (11–15) by homology-directed repair (HDR) or by nonhomologous end joining (NHEJ), respectively. Although the frequency of HDR is generally lower than that of NHEJ, the ability of HDR to correct pathogenic mutations permanently has great potential for curing various genetic disorders (16–18). For example, intravenously infused AAV expressing the Cas9 machinery and the donor DNA reversed the disease-causing mutation and rescued the disease phenotypes in two different mouse models of metabolic diseases (16–18).

In this context, we hypothesized that CRISPR-Cas9 could represent another therapy for LCA and tested the therapeutic outcomes of CRISPR-Cas9–mediated HDR in rd12 mice, a model of human LCA that bears a disease-associated premature stop codon in Rpe65. We used CRISPR-Cas9 technology to correct the nonsense mutation in this model and found that HDR and in-frame NHEJ in response to CRISPR-Cas9–mediated DNA cleavage led to the recovery of retinal functions and protection from retinal degeneration. The results from this proof-of-concept study demonstrate the feasibility of our approach and imply that CRISPR-Cas9–mediated HDR could be expanded to other ophthalmologic diseases in which HDR might be needed.

**RESULTS**

**CRISPR-Cas9–mediated editing of Rpe65 in vitro**

To use CRISPR-Cas9 technology to treat LCA, we first screened single guide RNA (sgRNA) sequences targeting Rpe65 exon 3 in the region that corresponds to the C-to-T nonsense mutation locus using mouse embryonic fibroblasts (MEFs) from rd12 mice (fig. S1A). Of nine potential candidates tested, the TS4 sgRNA was selected for further studies because it generated double-strand break (DSB) at the nearest locus from the premature stop codon and resulted in highly efficient insertion or deletion (indel) rates, as determined by targeted deep sequencing when transfected with SpCas9 protein as ribonucleoprotein (RNP) complex (fig. S1B). Next, we examined the correction frequencies by HDR induced by the TS4 sgRNA and Rpe65 donor in rd12 MEFs. Synonymous mutations were introduced into the donor sequence...
to prevent cleavage of the donor itself or recleavage of the repaired Rpe65 locus after correction (Fig. 1, A and F). Single-stranded oligodeoxynucleotide (ssODN) was used as an Rpe65 donor and induced correction in a range of 3 to 6% (Fig. 1, B, C, and F). When NHEJ was analyzed by deep sequencing, TS4 sgRNA–mediated indels were dominated by deletions, and approximately one-fourth of the mutations corresponded to in-frame indels (Fig. 1, D and E). Among the in-frame indels, one-codon deletions accounted for 5.6% of the total reads (Fig. 1F). The in-frame indels could result in the removal of the premature Rpe65 stop codon from rd12 mice, we suggest that NHEJ-mediated editing might also contribute to the therapeutic effects of CRISPR-Cas9. These data indicate that we identified an optimized sgRNA sequence for therapeutic, CRISPR-Cas9–mediated genome editing to treat LCA.

**In vivo genome editing of Rpe65 using the dual AAV system**

To evaluate the therapeutic efficacy of CRISPR-Cas9–mediated editing of the Rpe65 nonsense mutation in a disease model, we changed one nucleotide in TS4 to design the TS4rd12 sgRNA, which perfectly matches the sequence of the Rpe65 exon 3 mutation locus in rd12 mice. All required editing components were incorporated into two separate AAVs (AAV-SpCas9 and AAV-TS4rd12 sgRNA-Rpe65-donor, hereafter referred to as AAV-TS4rd12 donor), and in vivo delivery was carried out using the dual AAV system (Fig. 2A). We optimized the ratio of AAV components by varying their concentrations to obtain maximized genome editing results. A low dose (a total of $2 \times 10^{10}$ vector genomes (vg) per eye) or high dose (a total of $2 \times 10^{11}$ vg per eye) of AAVs was administered via subretinal injection into 3-week-old rd12 mice. After 4 weeks of AAV treatment,

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**Fig. 1. Genome editing of Rpe65 by CRISPR-Cas9 in vitro.** (A) A schematic drawing of Rpe65 in rd12 MEF. Red text, sequence of the premature stop codon; orange arrow, TS4 sgRNA target sequence; blue text, protospacer adjacent motif (PAM). (B) TS4 sgRNA–induced indel frequencies measured by targeted deep sequencing ($n = 4$). ssODN dose (in microgram scale) are indicated in parenthesis. (C) Correction frequencies in the rd12 mutation measured by targeted deep sequencing ($n = 4$). Of the total reads, about 61% contain deletions. Ins, insertion; Del, deletion; WT, wild type. (D) The mean values of the number of deep sequencing reads in different categories show the pattern of indels induced by the TS4 sgRNA ($n = 4$). Of the total reads, about 61% contain deletions. Ins, insertion; Del, deletion; WT, wild type. (E) Mean percent values of different types of in-frame and out-of-frame indels induced by the TS4 sgRNA ($n = 4$). 3N, one codon; 3N + 1, one codon + 1 nucleotide; 3N + 2, one codon + 2 nucleotides. (F) Nucleotide sequences showing types of editing induced by the TS4 sgRNA and 1.5 μg of ssODN in rd12 MEF ($n = 4$). Violet triangle, position of the DSB induced by the TS4 sgRNA; red text, sequences of the stop codon in rd12 MEF; blue text, PAM sequences; green text, synonymous mutations in the donor template; underlined sequences, nucleotides encompassing the premature stop codon and their corresponding amino acid sequences. Error bars indicate SEM. **P < 0.01; ***P < 0.001 by Kruskal-Wallis test with Dunn’s multiple comparison test.
the targeted region of \textit{Rpe65} was analyzed by deep sequencing using cells from the retina and retinal pigment epithelium (RPE). Subretinal injection resulted in indel rates in the retina and RPE that reached \textasciitilde 20\% but varied depending on various parameters (Fig. 2, B and C). Low-dose subretinal injection induced low and high indel rates in the retina and RPE, respectively. This result indicates that subretinal AAV administration is more efficient in the RPE versus the retina; this conclusion was further verified by the finding of a higher AAV copy number per diploid cell (Fig. 2, G).
In vivo correction of editing.

Subretinal delivery is an efficient method of inducing in vivo administration (Fig. 2, B and C). Collectively, these data suggest that subretinal delivery is an efficient method of inducing in vivo editing and that the Cas9-to-sgRNA ratio is critical for optimized editing.

In vivo correction of the Rpe65 mutation in rd12 mice

Subsequently, we analyzed HDR events near the pathogenic C-to-T mutation in Rpe65 in the retina and RPE after AAV treatment in rd12 mice. HDR events were observed in the RPE but not in the retina (Fig. 2E). Although indel frequencies were comparable between low-dose AAV- and high-dose AAV–treated RPE when a 1:1 ratio of AAV-SpCas9 to AAV-TS4-donor was used (Fig. 2C), HDR events were more frequent in the high-dose AAV–treated group (high dose, 1.17 ± 0.31%; low dose, 0.59 ± 0.22%) (Fig. 2E), as similarly observed in the CRISPR-mediated correction of the ornithine transcarbamylase gene in hepatocytes (18). Moreover, analysis of the HDR-to-indel ratio revealed that HDR events were approximately three times more frequent in the high dose–treated group (Fig. 2F). Successful HDR (1.01 ± 0.38%) was also induced by the application of a 0.5:1.5 ratio of AAV-SpCas9 to AAV-TS4-donor (Fig. 2E). It should be noted that most HDR events resulted in a precise correction of the T-to-C mutation, so that the resulting protein sequences were identical to that of wild-type Rpe65 (Fig. 2H). We additionally characterized NHEJ-mediated editing in the RPE and found that in-frame deletions occurred at a frequency of 3.83%; 1.61% were one-codon deletions that resulted in the removal of the premature stop codon in rd12 mice (Fig. 2, G and H). The 1-codon-Del-2 type (CGT-deletion) was also predicted as one of the top three end-joining categories (table S1) by inDelphi, the machine learning model that predicts SpCas9-mediated template-free genome editing (19). In addition, substantial in-frame editing was achieved in human RPE65 with the use of various sgRNAs in human embryonic kidney (HEK) 293 cells, suggesting the clinical potential of our approach (fig. S3, A to C).

Recovery of retinal function induced by Rpe65 correction in rd12 mice

To investigate the therapeutic effects of HDR-mediated Rpe65 gene correction in rd12 mice, we performed electroretinography after dark adaptation at 6 weeks and 7 months after subretinal AAV injection (Fig. 3A). AAV treatment led to the recovery of Rpe65 expression in RPE cells at 6 weeks after injection (Fig. 3B). Furthermore, AAV treatment resulted in increased Rpe65 gene expression in RPE cells at 7 months after injection (Fig. 3C). In electroretinography, rd12 mice demonstrated severely attenuated dark-adapted light responses compared with age-matched normal C57BL/6 mice (20). In contrast, there were definite responses to bright stimuli (0 dB) by which both rod and cone responses are provoked after dark adaptation in the AAV–treated rd12 mice at 6 weeks after the subretinal injection (Fig. 3, D to F, and fig. S4), whereas there were no responses to dim stimuli (~25 dB) (fig. S5). The electrical responses were maintained up to 7 months after the initial injection (Fig. 3, G and H). The estimated a- and b-wave amplitudes were 21.2 ± 4.1% and 39.8 ± 3.2% of those of normal C57BL/6 mice. These functional recoveries were accompanied by anatomical changes in which HDR-mediated Rpe65 gene correction prevented the loss of neuronal cells in the outer nuclear layer of the retina in rd12 mice (Fig. 3, I and J).

DISCUSSION

In this study, we deployed CRISPR-Cas9–mediated HDR as a new treatment for LCA. We showed that dual AAV–mediated delivery of CRISPR-Cas9 and an Rpe65 donor sequence can lead to the correction of a disease-causing mutation in Rpe65 and an improvement in retinal function in a mouse model of LCA. In addition to this improvement, our study showed a level of functional recovery that exceeds the measured gene correction level. The results are provocative because in postmitotic cells, HDR events are generally known as rare. However, our observations are supported by recent studies showing CRISPR-Cas9–mediated HDR events in postmitotic neurons (22) and muscles (23). Our results are also supported by previous studies in which CRISPR-Cas9 treatment for certain autosomal recessive diseases, such as Duchenne muscular dystrophy (23) and retinitis pigmentosa (24), resulted in functional improvement beyond the level expected from the gene correction frequency. Together, these results suggest that HDR can be activated in these postmitotic cells and that therapeutic outcomes exceeding that expected from the editing level could be achieved in some cases.

Gene therapy works by using viruses as a means of overexpressing wild-type copies of defective, disease-associated genes. For instance, Luxturna, the FDA-approved gene therapy for LCA, uses AAV2 to express functional RPE65 to replace the mutant, LCA-associated RPE65 protein. Different types of viruses, such as lentivirus and retrovirus, can also be used for gene therapy, but AAV is clinically validated (3–5, 25, 26). Although AAV is very attractive for gene therapy because it does not integrate into the host genome, its limited cargo packaging capability hinders its application in many cases. Therefore, for some ocular diseases such as Stargardt disease or Usher syndrome, which require the delivery of large DNA fragments, our approach using CRISPR-Cas9–mediated HDR could be suitable therapeutically. In addition, because the desired wild-type sequence can be introduced at the genomic site where the gene should exist, replacing the mutant sequence by HDR, the HDR-corrected cells
can express the introduced genes at the endogenous level. In this study, CRISPR-Cas9 was randomly introduced into the eye by subretinal injection in rd12 mice because mice lack a macula, the center and core of vision in humans. The injection resulted in a frequency of gene correction of nearly 3% by HDR and in-frame NHEJ (Fig. 2, E, F, and H) and led to substantial improvement in electroretinography measurements (Fig. 3, D to H). We expect that these results in mice can be further improved in human patients with LCA because ophthalmologists can selectively perform subretinal injection near the macular area, enabling focused editing around this structure to achieve even more restoration of vision.

The deep sequencing in our study provides a detailed description of the types of indels generated and the extent of off-target mutations produced, which was extensive. Although the active off-target sites that we detected were within intronic or intergenic regions, this level of off-target effects could undermine the therapeutic potential of our approach using CRISPR-Cas9–mediated genome editing. As the first study to use CRISPR-Cas9 to treat LCA, we used wild-type SpCas9,
the conventional and most commonly used form of Cas9. To reduce the frequency of off-target mutations, high-fidelity versions of Cas9 [HFCas9, eCas9, HypaCas9 (27), SniperCas9 (28), or self-targeting AAV (29)] or inducible-editing (30) could be tried as an alternative. We tested SniperCas9 (28), a high-fidelity version of SpCas9 that was developed by our group in the C2C12 cell line. We found that SniperCas9 was associated with a significant reduction in indel frequencies at the off-target sites recognized by SpCas9 (fig. S7). In addition, SniperCas9 showed comparable activity against the mRosa26 target, indicating that its low level of off-target effects does not come at the expense of on-target activity. These results suggest that various approaches might be tried to reduce off-target mutations in further studies. In addition, the off-target mutations detected in this study might not be found in the clinic because of differences between the human and mouse genomes. Moreover, it is noteworthy that there were no definite morphological changes in the RPE at 7 months after the subretinal injection, suggesting that the off-target mutations do not exert definite harmful effects (Fig. 4D).

In conclusion, we provide a new, CRISPR-Cas9–based approach for treating LCA. To our knowledge, no previous reports have shown in vivo HDR-mediated correction in animal models of ophthalmologic diseases. Our permanent therapeutic genome editing approach could

Fig. 4. Analysis of potential off-target sites. (A) Genome-wide Circos plot showing in vitro cleavage sites in the rd12 mouse genome in the absence (gray) or in the presence (blue) of TS4 sgRNA. On-target cleavage is indicated by the red arrow. (B) Targeted deep sequencing results for the top 29 Digenome-seq candidates in AAV-treated retina (n = 3). Nucleotides in red and orange indicate sequences that are mismatched relative to the on-target site, and nucleotides in blue indicate PAM sequences. (C) Reanalysis of the active off-targets by targeted deep sequencing in AAV-treated RPE (n = 3). (D) Representative whole mount of RPE tissues from rd12 mice at 7 months after treatment with or without the dual AAV system encoding SpCas9 and the donor template. Scale bar, 10 μm.
be used alone, or together with gene therapy or protein therapy, to treat LCA.

**MATERIALS AND METHODS**

**Cell culture and transfection**

MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with glucose (4.5 g/liter), 4 mM glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). RNPs and 120-mer ssODN were transfected using Neon Transfection following the manufacturer’s procedures (Life Technologies). Briefly, $2.5 \times 10^5$ cells were mixed with materials for transfection and were subjected to electrical shock using 10-µl tips on the Neon system for 30 ms at 1350 V for one pulse. Five days after transfection, the cells were harvested, and genomic DNA was extracted using a DNasey Blood and Tissue kit (Qiagen). For transfections of HEK293 cells, cells were seeded at $2 \times 10^5$ cell per well into 24-well plates the day before transfection. One microgram of plasmids encoding human optimized SpCas9 and sgRNA was mixed with 2 µl of Lipofectamine 2000 (Invitrogen) in 200 µl of Opti-MEM, left for 20 min, and then added to cells that had reached 70% confluence in 500 µl of DMEM. After overnight incubation, the medium was replaced with 500 µl of fresh medium, and genomic DNA was extracted after 5 days of transfection.

**Animals**

Eight-week-old male C57BL/6 mice and mating pairs of rd12 mice (stock no. 005379, the Jackson laboratory) were purchased from Central Laboratory Animal and maintained under a 12-hour dark-light cycle. rd12 mice were mated with each other to produce offspring with a homozygous mutation in the Rpe65 gene. All animal experiments were performed following guidelines from the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research and approved by Institutional Animal Care and Use Committees of both Seoul National University and Seoul National University Hospital.

**Targeted deep sequencing**

To quantify indel frequency, on-target and off-target regions were amplified by polymerase chain reaction (PCR) from genomic DNA extracted from transfected cells, retina, or RPE using the primer sets summarized in table S2. To quantify HDR frequencies, PCRs were first performed from genomic DNA (50 ng) using primers outside of the Rpe65-homology arm, after which products were further amplified to generate amplicons for deep sequencing. The produced amplicons were barcoded during subsequent PCR with Illumina TrueSeq adaptors. The products were purified with a PCR purification kit (Geneall) and were then pooled in equimolar ratio. The final libraries were paired-end sequenced using the Illumina Miseq platform according to the manufacturer’s protocol. Indel frequencies were quantified using Cas-Analyzer (www.rgenome.net) (31). Quantification of specific patterns such as in-frame or out-of-frame indels, one-codon deletions, and precise HDR were determined by manual counting.

**In silico identification of off-target sites**

Potential off-target sites were identified using an in silico tool, CasOFFinder (32). Mouse genomic sites containing up to 3-base pair (bp) mismatches were considered as off-target sites and further confirmed by targeted deep sequencing using the primers shown in table S3.

**In vitro cleavage of genomic DNA and Digenome-seq**

Genomic DNA was purified from the retina of rd12 mice with a DNasey Tissue kit (Qiagen) according to the manufacturer’s instructions. Genomic DNA (8 µg) was incubated with purified Cas9 protein (100 nM) and sgRNA (300 nM) in a reaction volume of 400 µl of buffer at 87°C in a buffer [100 mM NaCl, 40 mM tris-HCl, 10 mM MgCl2, and bovine serum albumin (100 µg/ml; pH 7.9)]. Digenetic genomic DNA was purified again with a DNasey Tissue kit (Qiagen) after RNase A (50 µg/ml) was added to remove sgRNA. To check targeted in vitro DNA cleavage, digested genomic DNA was mixed with 2× SYBR Green Master Mix and analyzed by real-time quantitative PCR (qPCR) using forward primer 5′-GGACATTCTACATATGAATC-CAGG-3′ and reverse primer 5′-TAACATTCTCAGGGTCTGTC-CAA-3′. The fraction of target sites that were cleaved was measured using the comparative C_T method (33).

For Digenome-seq, genomic DNA (1 µg) was sonicated to produce fragments in the 400- to 500-bp range using the Covaris system (Life Technologies) and blunt ended using End Repair Mix (Thermo Fisher). Fragmented DNA was ligated with adapters to produce libraries, which were then subjected to whole-genome sequencing (WGS) using a HiSeq X Ten Sequencer (Illumina) at Macrogen. WGS was performed at a sequencing depth of 30× to 40×, and the DNA cleavage score was calculated using a previously published scoring system (34).

**AAV vector construction and virus production**

Sequences of the elongation factor 1α short (EFS) promoter and human optimized SpCas9 with a nuclear localization signal, hemagglutinin (HA) tag, and bovine growth hormone poly-A tail were synthesized and subcloned into an AAV2 inverted terminal repeat (ITR)–based plasmid using Not I restriction sites. For the other AAV vector, sequences of the U6 promoter, TS4 sgRNA, and Rpe65 donor were also synthesized and subcloned into the AAV2 ITR–based plasmid using the same restriction sites. In the Rpe65 donor sequences, five nucleotides were substituted without causing codon changes to prevent possible TS4 sgRNA–mediated cleavage and to precisely distinguish knocked-in sequences from endogenous genomic sequences. Recombinant AAV particles were produced using a helper adenovirus-free packaging system. Briefly, HEK293 cells were cotransfected with pAAV-ITR-EFS-SpCas9 or pAAV-ITR-TS4-SpCas9 and AAV9-sgRNA- Rpe65-donor, AAV9-capsid plasmid, and helper plasmid. After 3 days of transfection, the cells were lysed and the virus particles were purified by iodixanol gradient ultracentrifugation and concentrated to obtain titers greater than $10^{13}$ vg/ml.

**Subretinal injection of AAV**

After deep anesthesia, AAV9-SpCas9 and AAV9-TS4 sgRNA-Rpe65-donor (a total of $2 \times 10^{10}$ or $2 \times 10^{11}$ vg in 1 µl of phosphate-buffered saline) were mixed and injected into the subretinal space of the mouse eye through the vitreous cavity using a customized Nanofil syringe with a 33-gauge blunt needle (World Precision Instrument) under an operating microscope (Leica), as previously described (35).

**qPCR for AAV genome detection in tissues**

qPCR to determine the AAV genome copy number was performed using genomic DNA extracted from the retina or RPE. After measuring the genomic DNA concentration using a Quatus fluorometer together with the Quantifluor Dye system (Promega), 50 ng of genomic DNA was subjected to qPCR analysis using an AAVpro titration kit (Takara). Thermocycling conditions were as follows:
95°C, 2 min followed by 35 cycles of 95°C, 5 s; 60°C, 30 s. The number of AAV genome copies was calculated against a standard curve. The number of diploid mouse cells was calculated using a conversion factor of ~1.6 x 10^6 diploid cells per 100 µg of genomic DNA. 

Electroretinography 
Mice were dark adapted for over 16 hours. After deep anesthesia, pupils were dilated with an eye drop containing phenylephrine hydrochloride (5 mg/ml) and tropicamide (5 mg/ml). Full-field electroretinography was performed using the universal testing and electrophysiologic system 2000 (UTAS-E-2000, LKC Technologies). The scotopic responses were recorded with a flash of 0 dB at a gain of 2 k using a notch filter at 60 Hz and were bandpass filtered between 0.1 and 1500 Hz. The amplitudes of the a-wave were measured from the baseline to the lowest negative-going voltage, whereas the peak b-wave amplitudes were estimated from the trough of the a-wave to the highest peak of the positive b-wave.

Immunofluorescence (paraffin sections) 
At 6 weeks after AAV-mediated delivery of SpCas9 and TS4 sgRNA-Rpe65-donor, paraffin blocks were prepared from enucleated eyes. Thin sections were immunostained with anti-HA antibody (1:1,000; cat. no. 3F10, Roche) and anti-Rpe65 antibody (1:1,000; cat. no. sc-73616, Santa Cruz Biotehtologies), followed by treatment with Alexa Fluor 488 or 594 immunoglobulin G (IgG) (1:500; Thermo Fisher). Nuclear staining was performed using 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich). The slides then were observed under a fluorescence microscope (Leica).

Real-time qPCR 
Total RNA was isolated using TRI Reagent (Molecular Research Center) from RPE cells of rd12 mice at 7 months after AAV-mediated delivery of SpCas9 and TS4 sgRNA-Rpe65-donor. cDNAs were prepared with a High-Capacity RNA-to- cDNA Kit (Thermo Fisher). Real-time PCR was performed with the StepOnePlus Real-Time PCR System (Thermo Fisher) using TaqMan Fast Advanced Master Mix (Thermo Fisher) with the StepOnePlus Real-Time PCR System (Thermo Fisher) using TaqMan Fast Advanced Master Mix (Thermo Fisher) with SpCas9 and TS4 sgRNA-Rpe65-donor. cDNAs were prepared with a High-Capacity RNA-to-cDNA Kit (Thermo Fisher). Real-time PCR was performed with the StepOnePlus Real-Time PCR System (Thermo Fisher) using TaqMan Fast Advanced Master Mix (Thermo Fisher) with Gene Expression Assays (Thermo Fisher) for gene expression analyses. Product IDs of the gene expression assays for genes are as follows: for Rpe65, Mm00504133_m1; for Gapdh, Mm99999915_g1; and for Rn18s, Mm03928990_g1. The relative expression levels of the Rpe65 gene were normalized to those of Gapdh and Rn18s. All procedures were performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines.

Histologic evaluation 
At 7 months after AAV-mediated delivery of SpCas9 and TS4 sgRNA-Rpe65-donor, paraffin blocks were prepared from enucleated eyes. Thin sections were then prepared for hematoxylin and eosin (H&E) staining.

Immunofluorescence (whole-mount preparation of RPE-choroid-scleral complexes) 
At 7 months after AAV-mediated delivery of SpCas9 and TS4 sgRNA-Rpe65-donor, RPE-choroid-scleral complexes were prepared from enucleated eyes. Then, the complexes were immunostained with anti-ZO-1 antibody (5 µg/ml; cat. no. 61-7300, Thermo Fisher), followed by treatment with Alexa Fluor 594 IgG (1:500; Thermo Fisher). Nuclear staining was performed using 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich). The slides then were observed under a fluorescence microscope (Leica).

Statistical analysis 
All group results are expressed as means ± SEM, if not stated otherwise. Statistical significance as compared to untreated controls is denoted with * (P < 0.05), ** (P < 0.01), and *** (P < 0.001) in the figures and figure legends. Statistical analyses were performed using GraphPad PRISM 5, and the specific tests are mentioned in the figure legends.

SUPPLEMENTARY MATERIALS 
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/10/eaa1210/DC1

Figure S1. Screening of sgRNAs targeting the Rpe65 gene in rd12 MEFs.
Figure S2. Delivered AAV copies between retina and RPE.
Figure S3. Analysis of human RPE65 editing.
Figure S4. Electroretinographic responses to bright stimuli (0 dB) after dark adaptation from normal C57BL/6 and rd12 mice at 6 weeks after the injection (n = 4).
Figure S5. Electroretinographic responses to bright stimuli (−25 dB) after dark adaptation from normal C57BL/6 and rd12 mice at 6 weeks after the injection.
Figure S6. Analysis of T54 sgRNA off-target effects.
Figure S7. Comparison of off-target frequency between wild-type SpCas9 and SniperCas9.
Table S1. Summary of predictions at the target site with sgRNA by the inDelphi approach.
Table S2. Active off-target sites.
Table S3. Primers used in this study.

View request a protocol for this paper from Bio-protocol.

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CRISPR-Cas9–mediated therapeutic editing of Rpe65 ameliorates the disease phenotypes in a mouse model of Leber congenital amaurosis

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