Published in final edited form as:

Ophthalmology. 2018 September ; 125(9): 1421–1430. doi:10.1016/j.ophtha.2018.04.001.

CRISPR-based genome surgery for the treatment of autosomal dominant retinitis pigmentosa

Yi-Ting Tsai^{1,2}, Wen-Hsuan Wu¹, Ting-Ting Lee¹, Wei-Pu Wu¹, Christine L. Xu¹, Karen S. Park¹, Xuan Cui¹, Sally Justus¹, Chyuan-Sheng Lin³, Ruben Jauregui^{1,4}, Pei-Yin Su¹, and Stephen H. Tsang^{1,2,5}

¹Jonas Children's Vision Care and Bernard & Shirlee Brown Glaucoma Laboratory, Departments of Ophthalmology, Pathology and Cell Biology, Columbia University, New York, NY 10032, USA

²Institute of Human Nutrition, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

³Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, New York, NY 10032, USA

⁴Weill Cornell Medical College, NY 10065, USA

⁵Edward S. Harkness Eye Institute, New York Presbyterian Hospital, New York, NY 10032, USA

Abstract

Objective—Develop a universal gene therapy to overcome the genetic heterogeneity in retinitis pigmentosa (RP) due to mutations in rhodopsin (*RHO*).

Design—Experimental study for a combination gene therapy that utilizes both gene ablation and gene replacement.

Participants—This study includes two kinds of human rhodopsin mutation knock-in mouse models, *Rho*^{P23H} and *Rho*^{D190N}. In total, 23 *Rho*^{P23H/P23H}, 43 *Rho*^{P23H/+} and 31 *Rho*^{D190N/+} mice were used for analysis.

Methods—This study involves gene therapy using dual adeno-associated viruses (AAVs) that 1) destroy expression of the endogenous *Rho* gene in a mutation-independent manner via an improved CRISPR-based gene deletion, and 2) enable expression of wild-type protein via exogenous cDNA.

Main outcome measures—Electroretinography and histology analysis.

Address correspondence to: Stephen H. Tsang, 635 W. 165th Street, Columbia U College of Physicians & Surgeons, Box 112, New York, NY 10032, USA. Phone: 001.212.342.1189; sht2@columbia.edu.

Address for reprints: 635 W. 165th Street, Columbia U College of Physicians & Surgeons, Box 112, New York, NY 10032, USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest: No conflicting relationship exists for any author

Results—The thickness of the outer nuclear layer (ONL) after the subretinal injection of combination ablate-and-replace gene therapy was around 17%~36% greater than the ONL thickness resulting from gene replacement-only therapy at 3 months after AAVs injection. Furthermore, results from electroretinography (ERG) demonstrated that the a and b waves of both *Rho*^{P23H} and *Rho*^{D190N} disease models were more significantly preserved using ablate-and-replace gene therapy ($p < 0.001$) but not by gene replacement monotherapy.

Conclusions—As a proof-of-concept, our results suggest that the “ablate-and-replace” strategy can ameliorate disease progression as measured by photoreceptor structure and function for both of the human mutation knock-in models. These results demonstrate the potency of the ablate-and-replace strategy to treat RP caused by different *RHO* mutations. Furthermore, because ablate-and-replace is mutation-independent, this strategy may be used to treat a wide array of dominant diseases in ophthalmology and other fields. Clinical trials utilizing ablate-and-replace gene therapy would allow researchers to determine if this strategy provides any benefits for patients with diseases of interest.

Keywords

CRISPR; gene therapy; neurodegeneration; retinitis pigmentosa; autosomal dominant disorders

Introduction

Retinitis pigmentosa is an inherited disease characterized by bilateral degeneration of rod-cone photoreceptors, which ultimately leads to night blindness and progressive visual impairment.¹ The rod-specific light-sensitive pigment, rhodopsin (RHO), is a specialized G-protein coupled receptor that initiates phototransduction. Thus far, approximately 150 different mutations have been found in *RHO*, which account for 30% of dominant RP cases and 15% of all inherited retinal dystrophies. Two strategies are most commonly applied to treat autosomal dominant (ad) retinitis pigmentosa: expression of the wild-type RHO protein and elimination of the mutant protein.²⁻⁴ The former strategy can be achieved by gene replacement, a well-established technology that uses viral vectors to introduce wild-type protein into cells of interest. While gene replacement itself may partially offset the adverse effects of dominant-negative proteins, it is powerless when used to counteract gain-of-function mutants.^{2, 3} The latter strategy, elimination of the mutant protein, could eradicate the bent causing the disease phenotype. However, this method also presents with its own set of challenging issues. For example, mRNA knockdown of pathologically mutant genes using either short interfering RNAs (siRNAs) or ribozymes only partially and transiently decreases mutant protein levels.⁵⁻⁷ Moreover, these tools often exhibit poor specificity when distinguishing between mutant versus wild-type alleles due to the fact that most of the mutations in *RHO* are single-nucleotide mutations.

The new emerging gene ablation tool, clustered regularly interspaced short palindromic repeats (CRISPR), which involves the collaboration between Cas9 and a *single* guide RNA (abbreviated henceforth as “CRISPRs”), has been proposed to specifically destroy the mutant gene by targeting the unique mutation.^{8, 9} Traditionally, this gene ablation is performed by introducing a frameshifting nucleotide insertion or deletion (InDel) concomitantly with non-homologous end joining (NHEJ) at the CRISPRs-targeted site.^{10, 11}

However, the drawbacks of CRISPRs are significant. For one, not every mutation is unique enough for CRISPRs, which involves highly allele-specific designs. Moreover, efficiency is compromised by the fact that most NHEJ results in precise ligation rather than the desired frameshifting InDels.^{12, 13} Last but not least, the costs of CRISPRs drug development are prohibitive given that the specificity of the guide RNA (gRNA) mandates separate clinical trials for each mutation, regardless of whether or not the mutations reside in the same gene.²

To address these issues, we present a two-pronged “ablate-and-replace” strategy that (1) destroys the expression of all endogenous chromosomal *Rho* genes in a mutation-independent manner using an improved, mutation-independent CRISPR/Cas9-based gene ablation technique and (2) enables expression of wild-type protein through exogenous cDNA. For gene ablation, we utilize Cas9 and *double* gRNAs (abbreviated henceforth as “CRISPR_d”) to create two double-strand breaks and, therefore, a large deletion that permanently destroys the targeted gene on both of the alleles. We combine this gene-ablation tool with gene replacement to deliver wild-type cDNA that compensates for the lost endogenous *RHO* protein. We hypothesize that this toolset can be used to treat adRP caused by different types of *Rho* mutations.

Material and Methods

Plasmids and AAV production

All gRNAs used in this study were designed by Benchling (<https://benchling.com/>). When selecting gRNAs, only those with excellent off-targeting scores (i.e. >80) were considered. The two gRNAs, labeled as gRNA1 and gRNA2, with the highest on-targeting scores were chosen and tested for use in this study (gRNA1 sequence: ctgtctacgaagagcccggtg; gRNA2 sequence: cccacaggctgtaatctga). For *in vitro* gRNA specificity test, gRNA1+gRNA2 or gRNA2 alone expressing cassettes were cloned into pX459 (Addgene), which encodes SpCas9. For the production of the AAV-GR, gRNA1+gRNA2-expressing cassettes and a 2.2 kb *mRho* promoter-driven *hRHO* cDNA-expressing cassette were cloned into pZac2.1 vector (PL-C-PV0100, The Penn Vector Core, University of Pennsylvania). For the AAV-SR, gRNA sequences were replaced with scrambled sequences that do not exist in the mouse genome. For the AAV-Cas9, codon-optimized SpCas9 was cloned into pZac2.1 between the sCMV promoter and SPA sequence. The AAV2/8 (Y733F) was generated by The Penn Vector Core, University of Pennsylvania.

In vitro CRISPR digestion assay

To validate the targeting efficiency of our system, gRNA (25 ng/μl) was added to the reaction mixture alongside Cas9 protein (30 ng/μl, NEB) and template *mRho* DNA (20 ng/μl, 750 bp) covering both targeting sites of gRNA1 and gRNA2, and they were subsequently incubated at 37° C for 2 hr. After Cas9/gRNA digestion, the mixture was analyzed by agarose gel electrophoresis.

Animals

Human mutation P23H knock-in and C57BL/6J mice were purchased from Jackson Labs to generate *Rho*^{P23H/+} and *Rho*^{P23H/P23H} mice. Another human mutation knock-in model,

D190N, was established as described before.¹⁴ Animals were maintained on a 12-h light-dark cycle. Before ERG, animals were anesthetized with a mixture of ketamine hydrochloride (10 mg/100 g; Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA, USA) and xylazine (1 mg/100 g, Anased®; Lloyd Laboratory, Shenandoah, IA, USA). As per regulations of the Institutional Animal Care and Use Committee (IACUC), animals sacrificed for histology were euthanized by placement in a CO₂ chamber for 3 min followed by cervical dislocation. All efforts were made to minimize the number of animals used and their suffering. All mouse experiments were approved by the IACUC and conform to regulatory standards. All mice were used in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology, as well as the Policy for the Use of Animals in Neuroscience Research established by the Society for Neuroscience.

Electroretinography

ERGs were performed at indicated time points as previously described.¹⁴ Briefly, animals were dark-adapted overnight, and their pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine. Animals were then anesthetized with ketamine, and ERG responses were obtained using pulses of 3 cd × s/m² (white 6,500 K) light. ERG a- and b-wave magnitudes and maximal scotopic and photopic recordings were collected at P21 for *Rho*^{P23H/P23H}, P40 for *Rho*^{P23H/+}, and P90 for *Rho*^{D190N/+} mice.

Subretinal injection

AAV-Cas9 (1 × 10¹³ particles/ml) was premixed with AAV-GR or AAV-SR (1 × 10¹³ particles/ml). Mice at age P1–P3 were anesthetized according to established IACUC guidelines, and subretinal injections were performed with a single injection of 1.5 µl. The injection was done from the posterior part of the eye. All mice included for analysis had ideal bleb detachments at the retinal site of the injection as judged by postsurgical fundus examination. Mice with complete retinal detachment confirmed by both postsurgical fundus examination and ERG were then euthanized. The left eyes served as controls and remained uninjected.

Genomic DNA extraction and genomic PCR

Genomic DNA from retinae was extracted using the Blood & Tissue kit (QIAGEN). Genomic PCRs were performed using Phusion DNA polymerase (Fisher Scientific). Primers for the detection of gene truncation and NHEJ were as follows: Forward: tacctaaggcctccaccg; Reverse: ttgccaatgaataagctggg. PCR amplicons generated from 3T3 cell culture or gross retinal DNA samples were further subcloned by the TOPO-TA cloning kit (Invitrogen) and analyzed by Sanger sequencing.

Cell culture and plasmid transfection

Mouse fibroblast 3T3 cell line was purchased from ATCC. The mycoplasma contamination test was performed every month. The cells were seeded in a 6-well plate at 1 × 10⁶ cells/well. When the cells reached 75% confluency, the pX459 plasmids (2.5 µg) of CRISPRd or CRISPRs were transfected into 3T3 cells by Lipofectamine 2000 (Invitrogen). The cell

culture was further purified by puromycin selection at 2µg/ml starting at 48 hours after transfection. DNA extraction was carried out after two weeks of selection.

Immunostaining

Mice were euthanized, and eyes were enucleated and placed in 4% paraformaldehyde for 1 h at room temperature. The optic nerve, cornea, and lens were removed. The whole eyecup was then flattened by means of four radial cuts extending out from the optic nerve and mounted with mounting medium (Vectashield, Vector Laboratories). Anti-Cas9 primary antibody (1:200 Abcam ab191468) and secondary antibody-conjugated Alexa 488 (Invitrogen, A11017) staining were performed according to the manufacturer's instruction. Visualization was achieved by fluorescence microscopy, and bright-field imaging was used to visualize the whole retina (Leica DM 5000B microscope). Pictures were taken using the Leica Application Suite Software (Leica Microsystems).

Histology

Mice were euthanized, and eyes were enucleated and fixed. H&E histology was carried out as previously described.¹⁴

Real-time PCR and relative mRNA quantification

Retinas were harvested at the indicated time and lysed with TRIZOL reagent (Invitrogen). Total RNA was isolated according to the manufacturer's instructions. DNase I (Invitrogen) treatment was then performed to prevent genomic DNA contamination. The reverse transcription reaction was conducted by Superscript III Reverse Transcription kit, and a random hexamer (Invitrogen) was used to generate cDNA. Real-time PCR method was performed using Maxima SYBR Green/ROX qPCR Master Mix (Fisher Scientific) with StepOne Real-time PCR System (Invitrogen) to quantify gene expression levels. The *mRho* and *hRHO* mRNA expression level was determined and normalized with the rod photoreceptor cell housekeeping gene, *Pde6g*. The PCR products were validated by melting curve and agarose gel electrophoresis. The following primers were used: *mRho* forward: 5'-TGGGCCCACAGGCTGTAATCTC-3'; *mRho* reverse: 5'-GAAGACCACACCCATGATAGCGTGA-3'; *hRHO* forward: 5'-CTTTGCCAAGAGCGCCG-3'; *hRHO* reverse: 5'-AGCAGAGGCCTCATCGTCA-3'. *Pde6g* forward: 5'-ACCACCTAAGTTTAAGCAGCGGCA-3'; *Pde6g* reverse: 5'-CGTGCAGCTCTAGGTGATTGAAG-3'.

Statistics

An unpaired, two-sided t-test was used for the comparisons of mRNA levels and ERG response.

Results

To develop our CRISPRd gene excision tools, we first designed two gRNAs to target sequences in exon 1 of mouse *Rho*, leading to double-strand breaks 27 base pairs (bp) upstream (gRNA1) and 336 bp downstream (gRNA2) of the start codon (Fig. 1A). Of note, both sites are relatively void of pathogenic mutations and single nucleotide polymorphisms.

To compare the gene-ablating efficiency of CRISPRd vs CRISPRs *in vitro*, we cloned gRNA1+2 or gRNA2 into respective modified pX459 vectors (Fig. 1B) and transfected each respective plasmid into 3T3 fibroblasts. After two weeks of puromycin selection, the total genomic DNA was extracted for PCR. We expected that the CRISPRd and CRISPRs plasmids would respectively yield 5 and 2 gene editing scenarios (Fig. 1C, type 1~5 and type 1~2), which would generate three gene expression outcomes: (1) normal/unaffected mouse *Rho* (*mRho*) expression resulting from either no editing or non-destructive NHEJ; (2) no *mRho* expression resulting from gene truncation or destructive NHEJ (frameshift); and (3) compromised expression resulting from non-destructive NHEJ at the 5'-untranslated site targeted by gRNA1. We first examined genomic PCR products to grossly confirm genetic outcomes (Fig. 1D). CRISPRd (gRNA1+2) yielded a prominent 400-bp band that represents truncated *mRho* exon 1 (Fig. 1C, **row 5**) and a minor 750-bp band that represents parental, full-length exon 1 (Fig. 1C, **row 1**). In contrast, CRISPRs (using only gRNA2) generated just the 750-bp band. To more precisely gauge genetic outcomes and quantify their respective frequencies, PCR amplicons were subcloned using TA cloning for Sanger sequencing. With CRISPRs, only $60.0 \pm 9.9\%$ of the *mRho* gene was ablated due to destructive NHEJ resulting in frameshift mutations; in the remaining $40.0 \pm 9.9\%$ of the gene that was not ablated, exon 1 was either intact (24.4%) or had in-frame mutations (15.6%) (Fig. 1C, E). With CRISPRd, more than $90 \pm 7.8\%$ of the *mRho* gene was ablated due to truncation (62.3%) or NHEJ-induced frameshift at the gRNA2 targeting site (28.3%) (Fig. 1C, E). Similarly, with CRISPRd, only 5.7% of amplicons contained intact *mRho* sequence compared to 24% with CRISPRs, even after two weeks of puromycin selection (Fig. 1C).

To test our combination CRISPRd gene ablate-and-replace strategy *in vivo*, all components were cloned into two AAV 2/8 vectors (Fig. 2A). Codon-optimized Cas9 cDNA driven by the sCMV promoter was packaged into one vector (AAV-Cas9), while the dual gRNA expression cassettes and human *RHO* (*hRHO*) cDNA (for xenogeneic gene replacement) driven by a 2.2 kb *mRho* promoter were cloned into another (AAV-GR, stands for gRNA and *hRHO*). Thus, gene ablation could only occur in cells that took up both vectors (AAV-Cas9 + AAV-GR) while gene replacement could occur in any rod photoreceptors that took up just the *hRHO* cDNA-containing vector (AAV-GR). Since overexpression of wild-type *RHO* can, by itself, might slightly improve vision in *hRHO*^{P23H} transgenic mice,⁴ we designed a control AAV vector (AAV-SR, stands for scrambled gRNA and *hRHO*), in which both gRNAs are replaced with scrambled sequences (Fig. 2A); thus, in rods transfected with the AAVs-Cas9+SR vector pair, CRISPRd does not function, but xenogeneic gene replacement does (Fig. 2B). Both gRNA1 and 2 were designed to specifically target *mRho*, not *hRHO* (Fig. 2C). Indeed, in an *in vitro*, cell-free assay, gRNA1 or gRNA2 each facilitated Cas9-mediated cleavage of *mRho*, but not *hRHO* (Fig. 2D and Fig. S1). To validate that AAVs-Cas9+GR can mediate CRISPRd gene ablation *in vivo*, right eyes of wild-type C57BL/6J adult mice received a single, 1.5-microliter subretinal injection of AAVs-Cas9+GR (Fig. 2E); as required by animal protocol, left eyes were uninjected, negative controls. Two weeks after the injection, retinas were collected for analysis. Cas9 immunostaining of whole, flat-mount retinas revealed AAV-Cas9 transduction and expression in ~30% retinal cells (Fig. 2F and Fig. S2A). Genomic PCR analysis revealed the

400-bp truncated and 750-bp parental *mRho* fragments; uninjected, fellow retinæ exhibited only the 750-bp fragment (Fig. 2G and Fig. S2B). Dideoxy-Sanger sequencing analysis of the 400-bp PCR amplicon confirmed that it was, in fact, truncated *mRho* exon 1, and that the large deletion had removed the start codon (Fig. 2H). These data suggest that our CRISPRd dual-vector tool truncates *mRho* exon1 *in vivo*.

We further determined if this *in vivo* AAVs-Cas9+GR-mediated gene ablation leads to decreased endogenous *mRho* levels in photoreceptors. Since there does not exist an antibody that can distinguish mRHO from hRHO protein, we extracted total retinal mRNA and used qPCR to evaluate the change in mRNA as a reflection of the change in protein. Retinæ treated with AAVs-Cas9+GR exhibited a 25% decrease in *mRho* mRNA compared to AAVs-Cas9+SR (Fig. 3A), thus demonstrating that gene ablation is occurring in rod cells transduced with AAVs-Cas9+GR. In addition, xenogeneic *hRHO* mRNA expression was clearly detectable in the injected eyes, indicating the transduction of AAV-GR into rod cells (Fig. 3B). We also found that *mRho* mRNA levels decreased as the *hRHO* mRNA increased based on a scatter plot of qPCR Ct values (Fig. 3C). This negative correlation (coefficient $r = -0.69$) between *mRho* and *hRHO* levels indicates that gene ablation consistently occurs in proportion to gene replacement, as our dual vector system was designed to achieve.

Next, we tested if our dual AAV, ablate-and-replace combination system has therapeutic efficacy in dominant retinal degenerative disorders, and also whether our dual vectors act in a mutation-independent fashion. To do this, we chose two knock-in mouse models of human RP caused *Rho* mutations: P23H on exon 1 and D190N on exon 3, which both localize to extracellular loops (Fig. 4A). The P23H mutation, which causes dominant-negative and gain-of-function phenotypes such as protein retention in the Golgi apparatus and the unfolded protein response,^{2, 15} is the most prevalent *hRHO* mutation in North America. The D190N mutation compromises *RHO* thermal stability and, therefore, leads to a gain-of-function increase in dark noise and slow yet progressive retinal degeneration.^{14, 16–18} For these two mutations, we tested one homozygote (*Rho*^{P23H/P23H}) and two heterozygote (*Rho*^{P23H/+} and *Rho*^{D190N/+}) models, since nearly all *RHO* mutant patients are heterozygous. Single subretinal injections of AAVs-Cas9+GR, AAVs-Cas9+SR or PBS were performed between postnatal days 1 and 3 (Fig. 4B); left eyes were not injected. At P21 or later, retinal function was assessed by electroretinography (ERG), and then eyes were dissected and processed for histological analysis (Fig. 4B). ERG responses are characterized by a photoreceptor-mediated a-wave (negative deflection) followed by an inner retina-mediated b-wave (positive deflection). Importantly, we controlled for mouse-to-mouse variation by dividing a- and b-wave amplitudes for the injected (right) eyes by their respective amplitude for the uninjected fellow (left) eyes. To compare the number of surviving photoreceptors, thickness of the photoreceptor nuclei-containing outer nuclear layer (ONL) was approximated in histological images by counting the layers of photoreceptor nuclei (in wild-type mature retinæ, ONL is 10–12 layers thick).¹⁴

Rho^{P23H/P23H} homozygous mutant mice exhibit rapid and severe rod-driven retinal degeneration wherein rod cell death begins shortly after birth and results in complete loss of rods by P30.¹⁹ In fact, in control retinæ injected with PBS, the ONL was only 0 to 1 layer thick at P21 (Fig. 4C). In contrast, retinæ transduced with AAVs-Cas9+GR or AAVs-

Cas9+SR typically had ONLs that were 2–3 and 1–2 layers thick, respectively (Fig. 4C). Such improvement is also reflected in the outer segment (OS) thickness (Fig. S3). Compared to the PBS control group, the OS length is 142% and 85% longer in the AAVs-Cas9+GR and AAVs-Cas9+SR group, respectively. These data are consistent with the notion that rescue is more efficacious with our AAVs-Cas9+GR ablate-and-replace combination therapy compared to AAVs-Cas9+SR gene replacement. These qualitative structural data were validated by our quantitative ERG functional analysis (Fig. 4D). Specifically, the mean b-wave amplitude for the AAVs-Cas9+GR group was 130% (relative to uninjected fellow eyes) versus 80% and 30% for the AAVs-Cas9+SR and PBS groups, respectively; these differences were highly significant (Fig. 4E). In addition, the peaks of oscillatory potentials of the AAVs-Cas9+GR group were more pronounced compared to either of the controls (Fig. 4D, arrows). These functional data suggest that neural signaling is significantly more robust in AAVs-Cas9+GR-treated retinæ. The fact that all of the individual b-wave amplitudes in the PBS group and most of those in the AAVs-Cas9+SR group were less than 100% (i.e., less than uninjected left eyes) (Fig. 4E) is likely due to surgical trauma, which is inevitable in early postnatal mouse eyes. That the AAVs-Cas9+GR group showed greater b-wave amplitudes than the uninjected eye despite surgical trauma is a reflection of the robustness of the intervention. Since subretinal injections induce only insignificant damage in human retinæ, efficacy could be even more robust in patients.

In *Rho*^{P23H/+} and *Rho*^{D190N/+} mice, retinæ were analyzed later than the *Rho*^{P23H/P23H} mutant since rod-driven retinal degeneration is dramatically slower in these heterozygotes: rod death is complete by about 6 and 10 months, respectively (Fig. 4B). In P90 *Rho*^{P23H/+} retinæ, AAVs-Cas9+GR treatment yielded 6–8 layer thick ONLs compared to 4–5 layers for AAVs-Cas9+SR treatment and 3–4 layers for PBS (Fig. 4C). The increase in OS thickness is more significant after AAVs-Cas9+GR treatment but is less efficacious in the AAVs-Cas9+SR group (Fig. S3). In addition, AAVs-Cas9+GR-treatment significantly increased a- and b-wave amplitudes in *Rho*^{P23H/+} retinæ compared to the AAVs-Cas9+SR and PBS controls (Fig. 4D, E). Similarly, in P90 *Rho*^{D190N/+} mice, rescue by AAVs-Cas9+GR treatment was again statistically superior for both a- and b-waves (Fig. 4D, E). These structural and functional efficacy data suggest that our ablate-and-replace combination, AAVs-Cas9+GR-treatment leads to significantly greater survival of functioning photoreceptors compared to AAVs-Cas9+SR-transduced retinæ. The data also suggest that the treatment is mutation-independent. These results are consistent with our dual AAV vector design (Fig. 2A) and analysis (Fig. 3C).

Discussion

The premise of our experiment is that ad mutations in the *RHO* gene cannot be ameliorated solely by conventional gene replacement/augmentation.^{2, 20} For ad diseases, a cure can only be achieved when the mutant allele is corrected or destroyed while leaving the wild-type allele intact. Currently, many studies utilize gene editing tools such as CRISPR directed by gRNA, Transcription Activator-like Effector Nucleases or Zinc Finger Nucleases to target the unique mutation.^{8, 9, 21–31} However, these methods would be too cumbersome and expensive for treating heterogeneous genetic disorders. A more cost-effective and efficient strategy is to ablate the mutated alleles and deliver an exogenous healthy copy of the gene.

In this present study, we provide evidence demonstrating the feasibility of ablate-and-replace strategy on treating adRP caused by different *RHO* mutations found in humans. By slight modification, the dual AAV toolset used in this study can be readily converted into a human version for clinical trials; gRNAs can be redesigned to target *hRHO*, while *hRHO* cDNA can be re-made to be Cas9/gRNA-resistant by codon-modification.^{32, 33} Furthermore, gene replacement driven by a native *Rho* promoter should exhibit higher safety and durability in patients when compared to gene replacement strategies involving a ubiquitous promoter (e.g. CMV).^{34, 35}

Our experiments provide evidence for the superiority of CRISPRd over CRISPRs in many regards. Consistent with previous findings,^{29–31, 36} our Dideoxy-Sanger sequencing results showed that the induction rate of CRISPRd is higher than that of CRISPRs. These differences in outcome are most likely due to the fact that less than 0.1% of the NHEJ generates InDel.³⁷ The production of destructive NHEJ may largely rely on repeated cutting. CRISPRd-induced truncation, on the other hand, is more independent of the error rate and only depends on the occurrence rate of NHEJ. Also, unlike homology-dependent repair, NHEJ is active in both dividing cells and non-dividing cells; thus, there is no limitation on cell cycle for our strategy.³⁸ In addition, our results also demonstrate that CRISPRd can minimize the creation of secondary, in-frame mutations induced by NHEJ (CRISPRd 1.9% vs. CRISPRs 15.6%), which is essential for preventing the creation of more toxic *Rho* mutants via therapeutic genome editing.

Current attempts to treat ad disorders by posttranscriptional perturbation, including interfering RNAs (siRNAs) and ribozymes, are less desirable than therapeutic editing for a multitude of reasons. For example, though siRNAs have entered human clinical trials, there are still many issues to be addressed. Both siRNAs and ribozymes use dosage dependent post-transcriptional perturbations to interfere with or suppress the expression of RNA, and both methods have been reported to have unpredictable off-target effects and Toll-like receptor activation.^{39, 40} Indeed, the dosage dependent nature of siRNA and ribozymes is a double-edged sword; while high dosages result in higher on-target efficiency rates, they may likewise be counterbalanced by unwanted higher immunogenic and off-targeting effects.^{39, 41–43} Pre-transcriptional perturbation via CRISPR, however, requires only a transient low level of Cas9/gRNA expression. Accordingly, many studies have demonstrated the lowered off-target effect of CRISPR compared to RNAi.^{44, 45} Furthermore, the transient effect of siRNA requires periodical supplementation, which may lead to nanotoxicity and immunogenicity in systemic treatments.^{39, 40, 46}

Finding that gene replacement alone can only rescue the vision in homozygous *mRho*^{P23H/P23H} model but not the models of heterozygous P23H and D190N was unanticipated. This result is inconsistent with the previous findings on P23H and P347S mutation models.^{3, 47} However, it is worth noting that all of the previous animal models were established by transgenic expression of mutant *hRHO* in *mRHO*^{-/-}, *mRHO*^{+/+} or *mRHO*^{+/-} backgrounds instead of the mutation knock-in method. Our findings suggest that selecting genetic models to simulate gene therapy of adRP in mice is pivotal for therapeutic development.

Though AAV-delivered gene replacement is not integrating to the host genome, based on recent findings and clinical trials, the AAV-delivered gene replacement therapy of *RPE65* has allowed the stable expression of retinal pigment epithelium (RPE) cells for 3+ years in humans and 9+ years in canine models.^{48–50} We expect that this gene replacement effect can last even longer in photoreceptor cells, because—unlike RPE cells—photoreceptor cells do not divide. However, further experimentation is required to understand the persistence of the AAV transgene and the need for additional supplementation of gene replacement in the long run.

Our ablate-and-replace system has potential for other autosomal dominant diseases beyond retinitis pigmentosa. For example, Vitelliform Macular Dystrophy is a blinding macular disorder that may be caused by any one from more than 200 autosomal-dominant point mutations in the gene *BESTROPHIN 1 (BEST 1)*, which encodes a calcium-dependent chloride channel^{51–53}; Transthyretin amyloidosis is associated with 83 ad mutations in the *TTR* gene⁵⁴; and amyotrophic lateral sclerosis has more than 80 mutations on the *SOD1* gene.²⁵ Instead of mutation-specific targeting, our system circumvents such heterogeneity.

In summary, our ablate-and-replace strategy provides the first electrophysiological evidence that efficacy can be achieved with CRISPR-based therapy for post-mitotic neurons. Because this technique can be applied in a mutation-independent manner, it represents a more fiscally practical strategy for overcoming the hurdle of allelic heterogeneity in different dominant disorders, especially in the field of ophthalmology. Thus, it will enable universal treatment of patients, regardless of their allelic heterogeneity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support: This study is supported by NIH (R01EY024698, R01EY026682 and R01 EY018213). The funding organization had no role in the design or conduct of this research.

References

1. Hartong DT, Berson EL, Dryja TP. Retinitis pigmentosa. *Lancet*. 2006; 368(9549):1795–809. [PubMed: 17113430]
2. Lewin AS, Rossmiller B, Mao H. Gene augmentation for adRP mutations in RHO. *Cold Spring Harb Perspect Med*. 2014; 4(9):a017400. [PubMed: 25037104]
3. Millington-Ward S, Chadderton N, O'Reilly M, et al. Suppression and replacement gene therapy for autosomal dominant disease in a murine model of dominant retinitis pigmentosa. *Mol Ther*. 2011; 19(4):642–9. [PubMed: 21224835]
4. Gorbatyuk MS, Gorbatyuk OS, LaVail MM, et al. Functional rescue of P23H rhodopsin photoreceptors by gene delivery. *Adv Exp Med Biol*. 2012; 723:191–7. [PubMed: 22183333]
5. Gorbatyuk M, Justilien V, Liu J, et al. Preservation of photoreceptor morphology and function in P23H rats using an allele independent ribozyme. *Exp Eye Res*. 2007; 84(1):44–52. [PubMed: 17083931]
6. Gorbatyuk MS, Pang JJ, Thomas J Jr, et al. Knockdown of wild-type mouse rhodopsin using an AAV vectored ribozyme as part of an RNA replacement approach. *Mol Vis*. 2005; 11:648–56. [PubMed: 16145542]

7. Mao H, Gorbatyuk MS, Rossmiller B, et al. Long-term rescue of retinal structure and function by rhodopsin RNA replacement with a single adeno-associated viral vector in P23H RHO transgenic mice. *Hum Gene Ther.* 2012; 23(4):356–66. [PubMed: 22289036]
8. Bakondi B, Lv W, Lu B, et al. In Vivo CRISPR/Cas9 Gene Editing Corrects Retinal Dystrophy in the S334ter-3 Rat Model of Autosomal Dominant Retinitis Pigmentosa. *Mol Ther.* 2016; 24(3):556–63. [PubMed: 26666451]
9. Latella MC, Di Salvo MT, Cocchiarella F, et al. In vivo Editing of the Human Mutant Rhodopsin Gene by Electroporation of Plasmid-based CRISPR/Cas9 in the Mouse Retina. *Mol Ther Nucleic Acids.* 2016; 5(11):e389. [PubMed: 27874856]
10. Jinek M, Chylinski K, Fonfara I, et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science.* 2012; 337(6096):816–21. [PubMed: 22745249]
11. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. *Science.* 2013; 339(6121):819–23. [PubMed: 23287718]
12. Ran FA, Hsu PD, Wright J, et al. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc.* 2013; 8(11):2281–308. [PubMed: 24157548]
13. Lin WY, Wilson JH, Lin Y. Repair of chromosomal double-strand breaks by precise ligation in human cells. *DNA Repair (Amst).* 2013; 12(7):480–7. [PubMed: 23707303]
14. Sancho-Pelluz J, Tosi J, Hsu CW, et al. Mice with a D190N mutation in the gene encoding rhodopsin: a model for human autosomal-dominant retinitis pigmentosa. *Mol Med.* 2012; 18:549–55. [PubMed: 22252712]
15. Mendes HF, van der Spuy J, Chapple JP, Cheetham ME. Mechanisms of cell death in rhodopsin retinitis pigmentosa: implications for therapy. *Trends Mol Med.* 2005; 11(4):177–85. [PubMed: 15823756]
16. Tsui I, Chou CL, Palmer N, et al. Phenotype-genotype correlations in autosomal dominant retinitis pigmentosa caused by RHO, D190N. *Curr Eye Res.* 2008; 33(11):1014–22. [PubMed: 19085385]
17. Liu MY, Liu J, Mehrotra D, et al. Thermal stability of rhodopsin and progression of retinitis pigmentosa: comparison of S186W and D190N rhodopsin mutants. *J Biol Chem.* 2013; 288(24):17698–712. [PubMed: 23625926]
18. Park SP, Lee W, Bae EJ, et al. Early structural anomalies observed by high-resolution imaging in two related cases of autosomal-dominant retinitis pigmentosa. *Ophthalmic Surg Lasers Imaging Retina.* 2014; 45(5):469–73. [PubMed: 25215869]
19. Sakami S, Maeda T, Bereta G, et al. Probing mechanisms of photoreceptor degeneration in a new mouse model of the common form of autosomal dominant retinitis pigmentosa due to P23H opsin mutations. *J Biol Chem.* 2011; 286(12):10551–67. [PubMed: 21224384]
20. Dalkara D, Goureau O, Marazova K, Sahel JA. Let There Be Light: Gene and Cell Therapy for Blindness. *Hum Gene Ther.* 2016; 27(2):134–47. [PubMed: 26751519]
21. Fink KD, Deng P, Gutierrez J, et al. Allele-Specific Reduction of the Mutant Huntingtin Allele Using Transcription Activator-Like Effectors in Human Huntington's Disease Fibroblasts. *Cell Transplant.* 2016; 25(4):677–86. [PubMed: 26850319]
22. Park CY, Kim J, Kweon J, et al. Targeted inversion and reversion of the blood coagulation factor 8 gene in human iPS cells using TALENs. *Proc Natl Acad Sci U S A.* 2014; 111(25):9253–8. [PubMed: 24927536]
23. Anguela XM, Sharma R, Doyon Y, et al. Robust ZFN-mediated genome editing in adult hemophilic mice. *Blood.* 2013; 122(19):3283–7. [PubMed: 24085764]
24. Hoban MD, Cost GJ, Mendel MC, et al. Correction of the sickle cell disease mutation in human hematopoietic stem/progenitor cells. *Blood.* 2015; 125(17):2597–604. [PubMed: 25733580]
25. Wang L, Yi F, Fu L, et al. CRISPR/Cas9-mediated targeted gene correction in amyotrophic lateral sclerosis patient iPSCs. *Protein Cell.* 2017; 8(5):365–78. [PubMed: 28401346]
26. Bengtsson NE, Hall JK, Odom GL, et al. Muscle-specific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. *Nat Commun.* 2017; 8:14454. [PubMed: 28195574]
27. Long C, McAnally JR, Shelton JM, et al. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science.* 2014; 345(6201):1184–8. [PubMed: 25123483]

28. Kocher T, Peking P, Klausegger A, et al. Cut and Paste: Efficient Homology-Directed Repair of a Dominant Negative KRT14 Mutation via CRISPR/Cas9 Nickases. *Mol Ther*. 2017; 25(11):2585–98. [PubMed: 28888469]
29. Nelson CE, Hakim CH, Ousterout DG, et al. In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science*. 2016; 351(6271):403–7. [PubMed: 26721684]
30. Tabebordbar M, Zhu K, Cheng JKW, et al. In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science*. 2016; 351(6271):407–11. [PubMed: 26721686]
31. Long C, Amoasii L, Mireault AA, et al. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science*. 2016; 351(6271):400–3. [PubMed: 26721683]
32. O'Reilly M, Palfi A, Chadderton N, et al. RNA interference-mediated suppression and replacement of human rhodopsin in vivo. *Am J Hum Genet*. 2007; 81(1):127–35. [PubMed: 17564969]
33. O'Reilly M, Millington-Ward S, Palfi A, et al. A transgenic mouse model for gene therapy of rhodopsin-linked Retinitis Pigmentosa. *Vision Res*. 2008; 48(3):386–91. [PubMed: 17920651]
34. Chira S, Jackson CS, Oprea I, et al. Progresses towards safe and efficient gene therapy vectors. *Oncotarget*. 2015; 6(31):30675–703. [PubMed: 26362400]
35. Wang W, Jia YL, Li YC, et al. Impact of different promoters, promoter mutation, and an enhancer on recombinant protein expression in CHO cells. *Sci Rep*. 2017; 7(1):10416. [PubMed: 28874794]
36. Mandal PK, Ferreira LM, Collins R, et al. Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. *Cell Stem Cell*. 2014; 15(5):643–52. [PubMed: 25517468]
37. Betermier M, Bertrand P, Lopez BS. Is non-homologous end-joining really an inherently error-prone process? *PLoS Genet*. 2014; 10(1):e1004086. [PubMed: 24453986]
38. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, et al. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature*. 2016; 540(7631):144–9. [PubMed: 27851729]
39. Jackson AL, Linsley PS. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. *Nat Rev Drug Discov*. 2010; 9(1):57–67. [PubMed: 20043028]
40. Lam JK, Chow MY, Zhang Y, Leung SW. siRNA Versus miRNA as Therapeutics for Gene Silencing. *Mol Ther Nucleic Acids*. 2015; 4:e252. [PubMed: 26372022]
41. Brown K, Samarsky D. RNAi off-targeting: Light at the end of the tunnel. *J RNAi Gene Silencing*. 2006; 2(2):175–7. [PubMed: 19771224]
42. Pecot CV, Calin GA, Coleman RL, et al. RNA interference in the clinic: challenges and future directions. *Nat Rev Cancer*. 2011; 11(1):59–67. [PubMed: 21160526]
43. Gavrillov K, Saltzman WM. Therapeutic siRNA: principles, challenges, and strategies. *Yale J Biol Med*. 2012; 85(2):187–200. [PubMed: 22737048]
44. Evers B, Jastrzebski K, Heijmans JP, et al. CRISPR knockout screening outperforms shRNA and CRISPRi in identifying essential genes. *Nat Biotechnol*. 2016; 34(6):631–3. [PubMed: 27111720]
45. Shalem O, Sanjana NE, Hartenian E, et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*. 2014; 343(6166):84–7. [PubMed: 24336571]
46. Xue HY, Liu S, Wong HL. Nanotoxicity: a key obstacle to clinical translation of siRNA-based nanomedicine. *Nanomedicine (Lond)*. 2014; 9(2):295–312. [PubMed: 24552562]
47. Mao H, James T Jr, Schwein A, et al. AAV delivery of wild-type rhodopsin preserves retinal function in a mouse model of autosomal dominant retinitis pigmentosa. *Hum Gene Ther*. 2011; 22(5):567–75. [PubMed: 21126223]
48. Acland GM, Aguirre GD, Bennett J, et al. 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*. 2005; 12(6):1072–82. [PubMed: 16226919]
49. Jacobson SG, Cideciyan AV, Roman AJ, et al. Improvement and decline in vision with gene therapy in childhood blindness. *N Engl J Med*. 2015; 372(20):1920–6. [PubMed: 25936984]
50. Swain GP, Prociuk M, Bagel JH, et al. Adeno-associated virus serotypes 9 and rh10 mediate strong neuronal transduction of the dog brain. *Gene Ther*. 2014; 21(1):28–36. [PubMed: 24131981]

51. Yang T, Justus S, Li Y, Tsang SH. BEST1: the Best Target for Gene and Cell Therapies. *Mol Ther*. 2015; 23(12):1805–9. [PubMed: 26388462]
52. Johnson AA, Guziewicz KE, Lee CJ, et al. Bestrophin 1 and retinal disease. *Prog Retin Eye Res*. 2017; 58:45–69. [PubMed: 28153808]
53. Marmorstein LY, Wu J, McLaughlin P, et al. The light peak of the electroretinogram is dependent on voltage-gated calcium channels and antagonized by bestrophin (best-1). *J Gen Physiol*. 2006; 127(5):577–89. [PubMed: 16636205]
54. Chakraborty R, Muchtar E, Gertz MA. Newer Therapies for Amyloid Cardiomyopathy. *Curr Heart Fail Rep*. 2016; 13(5):237–46. [PubMed: 27497715]

Strategy “ablate-and-replace”, combining therapeutic gene editing and gene replacement for the treatment of autosomal dominant retinitis pigmentosa.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. CRISPRd ablates *mRho* gene more efficiently than CRISPRs in 3T3 cells
A, Double (CRISPRd) or single (CRISPRs) gRNA strategies to specifically ablate mouse *Rho* exon 1. **B**, Modified pX459 vectors for CRISPRd and CRISPRs. **C**, Genetic and expression outcomes following CRISPRd- vs CRISPRs-mediated gene editing. **D**, Validation of *mRho* exon 1 truncation. 3T3 cells were transfected with CRISPRd or CRISPRs plasmid (Fig. 1B), and then submitted to 2 weeks of puromycin selection followed by genomic PCR (primers indicated in Fig. 2A). **E**, Schematic summary of the basic outcomes (Fig. 1C) produced by CRISPRd- and CRISPRs-mediated gene editing (n=2).
Valued of CRISPRd group: Gene ablation:

Figure 2. Validation of *mRho*-specific CRISPRd-mediated gene deletion *in vivo*

A, Schematic of experimental (AAVs-Cas9+GR) and control (AAVs-Cas9+SR) AAV2/8 vector pairs. **B**, Conventional gene replacement therapy vs CRISPRd plus gene replacement, compound therapy for a heterozygous loci. **C**, gRNA1 and gRNA2 sequences, their targeting sites on *mRho*, and the corresponding sites on *hRHO* – showing the 12 and 4 mismatches, respectively. **D**, Representative data of *in vitro* SpCas9/gRNA cutting. In a cell-free assay, *mRho* vs *hRHO* DNA template was mixed with recombinant SpCas9 protein and a single gRNA: gRNA1, gRNA2, or control (scrambled gRNA). (n=3) **E**, Experimental scheme and timeline for subretinal injection of dual AAV vectors in right eyes of wild-type C57BL/6J

mice (using a unique, posterior approach); respecting IACUC protocols, left eyes were uninjected. **F**, Representative data of Cas9-immunostaining in retinal flat mount. 14 days after Cas9+GR subretinal injection, the retinas were collected and stained for anti-Cas9 antibody (n=3). Image was assembled from multiple pictures. **G**, Representative data of PCR analysis of retinal genomic DNA from AAVs-Cas9+GR-injected right eyes and uninjected, fellow (left) eyes. (n=4, two mice data are shown). **H**, Representative data of *mRho* gene ablation validated by Sanger sequencing of PCR amplicon from Fig. 2G.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. Gene ablation and replacement are co-localized in rod photoreceptors *in vivo*

A, Total retinal mRNA from injected eyes was analyzed by qPCR for *mRho* expression; values were normalized to *Pde6g*, a rod photoreceptor housekeeping gene, and control, AAVs-Cas9+SR were defined as 100%. Values are presented as mean $\pm$ s.d. Unpaired two-sided t-test was used for the statistics (*, $p < 0.05$). **B**, RT-PCR of total mRNA isolated from whole retinas from two right eyes injected with AAVs-Cas9+GR and two uninjected fellow (left) eyes. **C**, Scatter plot of qPCR-derived ΔCt values of *mRho* and *hRHO* mRNA isolated from 11 whole retinas from 11 left eyes injected with AAVs-Cas9+GR. The trend line is presented with 95% confidence interval.

Figure 4. Ablate-and-replace gene therapy leads to functional and structural rescue in *RP* mice with distinct mutations on different exons

A, Genetic and structural position of P23H and D190N mutations; orange rectangles, translational start and stop codons. **B**, Experimental and disease progression timelines. Right eyes received a single 1.5-microliter subretinal injection of AAVs-Cas9+GR, AAVs-Cas9+SR or PBS between P1 and P3; left, fellow eyes were uninjected. On the indicated days, ERGs were performed and tissue prepared for histology. Approximate time windows for rod-driven photoreceptor degeneration in untreated mice are indicated for three genotypes. **C**, H&E stained retinal sections of right (injected) eyes from P21 *Rho*^{P23H/P23H} mice and P90 *Rho*^{P23H/+} and *Rho*^{D190N/+} mice, which were injected with either AAVs-Cas9+GR, AAVs-Cas9+SR or PBS; images were taken about 200 micrometer from the optic nerve. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, optic nuclear layer; RPE, retinal pigment epithelium. **D**, Representative ERG traces of injected right eyes (green traces) and uninjected left, fellow eyes (red traces). Arrows, peaks of oscillatory potentials. **E**, Percent change in ERG amplitudes. Each dot represents an a- or b-wave amplitude for an injected, right eye that was normalized to the respective amplitude value of the uninjected

fellow (left) eye. N values indicated on x axis; horizontal black lines, group means. Unpaired two-sided t-test was used for the statistics (*, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$). In *Rho*^{P23H/P23H} mice, only b-wave data are shown, because a-waves are no longer detectable at P21 (Fig. 4 B, D).