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Clustered Regularly Interspaced Short Palindromic Repeats-Based Genome Surgery for the Treatment of Autosomal Dominant Retinitis Pigmentosa

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Purpose: To develop a universal gene therapy to overcome the genetic heterogeneity in retinitis pigmentosa (RP) resulting from mutations in rhodopsin (*RHO*).

Design: Experimental study for a combination gene therapy that uses both gene ablation and gene replacement.

Participants: This study included 2 kinds of human *RHO* 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 involved 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 clustered regularly interspaced short palindromic repeats-based gene deletion and (2) enable expression of wild-type protein via exogenous cDNA.

Main Outcome Measures: Electroretinographic and histologic analysis.

Results: The thickness of the outer nuclear layer (ONL) after the subretinal injection of combination ablate-and-replace gene therapy was approximately 17% to 36% more than the ONL thickness resulting from gene replacement-only therapy at 3 months after AAV injection. Furthermore, electroretinography results demonstrated that the a and b waves of both Rho^{P23H} and Rho^{D190N} disease models were preserved more significantly 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 treatment is mutation independent, this strategy may be used to treat a wide array of dominant diseases in ophthalmology and other fields. Clinical trials using ablate-and-replace gene therapy would allow researchers to determine if this strategy provides any benefits for patients with diseases of interest. *Ophthalmology 2018;■:1–10 © 2018 by the American Academy of Ophthalmology*

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Retinitis pigmentosa (RP) is an inherited disease characterized by bilateral degeneration of rod–cone photoreceptors that 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 autosomal dominant RP (adRP) cases and 15% of all inherited retinal dystrophies. Two strategies are applied most commonly to treat adRP: 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. Although gene replacement itself may offset partially 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 bane causing the disease phenotype. However, this method also presents 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

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because 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 collaboration between Cas9 and a single guide RNA (abbreviated henceforth as CRISPRs), has been proposed specifically to 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 concomitantly with nonhomologous 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 insertions or deletions.[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 the mutations reside in the same gene.[2]

To address these issues, we present a 2-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- and Cas9-based gene ablation technique and (2) enables expression of wild-type protein through exogenous cDNA. For gene ablation, we used Cas9 and double gRNAs (abbreviated henceforth as CRISPRd) to create 2 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.

Methods

Plasmids and Adeno-Associated Virus Production

All gRNAs used in this study were designed by Benchling (San Francisco, CA) (https://benchling.com/). When selecting gRNAs, only those with excellent off-targeting scores (i.e., >80) were considered. The 2 gRNAs, labeled as gRNA1 and gRNA2, with the highest on-targeting scores were chosen and tested for use in this study (gRNA1 sequence, ctgtctacgaagagcccgtg; gRNA2 sequence, cccacaggctgtaatctcga). For in vitro gRNA specificity testing, gRNA1+gRNA2- or gRNA2 alone-expressing cassettes were cloned into pX459 (Addgene, Cambridge, MA), which encodes SpCas9. For the production of the adeno-associated virus (AAV) GR, gRNA1+gRNA2-expressing cassettes and a 2.2-kb mouse *Rho* (*mRho*) promoter-driven human *RHO* (*hRHO*) cDNA-expressing cassette were cloned into pZac2.1 vector (PL-C-PV0100; The Penn Vector Core, University of Pennsylvania, Philadelphia, PA). 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 simian cytomegalovirus promoter and synthetic polyadenylation signal sequence. The AAV2/8 (Y733F) was generated by The Penn Vector Core.

In Vitro Clustered Regularly Interspaced Short Palindromic Repeats 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, New England BioLabs, Inc, Ipswich, MA) and template *mRho* DNA (20 ng/μl, 750 base pairs [bp]) covering both targeting sites of gRNA1 and gRNA2, and they were incubated subsequently at 37°C for 2 hours. After Cas9 and 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 (Bar Harbor, ME) to generate $Rho^{P23H/+}$ and $Rho^{P23H/P23H}$ mice. Another human mutation knock-in model, D190N, was established as described previously.[14] Animals were maintained on a 12-hour light−dark cycle. Before electroretinography, animals were anesthetized with a mixture of ketamine hydrochloride (10 mg/100 g; Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (1 mg/100 g; Anased, Lloyd Laboratory, Shenandoah, IA). As per regulations of the Institutional Animal Care and Use Committee (Columbia University Medical Center), animals killed for histologic analysis were placed in a carbon dioxide chamber for 3 minutes 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 Institutional Animal Care and Use Committee 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

Electroretinography was 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 then were anesthetized with ketamine, and electroretinography responses were obtained using pulses of 3 cd×s/m^2 (white 6500 K) light. Electroretinography a- and b-wave magnitudes and maximum scotopic and photopic recordings were collected at 21 postnatal days for $Rho^{P23H/P23H}$, at 40 postnatal days for $Rho^{P23H/+}$, and at 90 postnatal days for $Rho^{D190N/+}$ mice.

Subretinal Injection

AAV-Cas9 (1×10^{13} particles/ml) was premixed with AAV-GR or AAV-SR (1×10^{13} particles/ml). Mice at age P1 through P3 were anesthetized according to established Institutional Animal Care and Use Committee guidelines, and subretinal injections were performed with a single injection of 1.5 μl. The injection was carried out 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 electroretinography then were killed. The left eyes served as controls and remained uninjected.

Genomic DNA Extraction and Genomic Polymerase Chain Reaction Analysis

Genomic DNA from retinae was extracted using the Blood & Tissue kit (Qiagen Inc., Chatsworth, CA). Genomic polymerase chain reaction (PCR) analyses were performed using Phusion DNA polymerase (Fisher Scientific, Hampton, NH). Primers for the