Advances Toward a Dual AAV CRISPR-Cas9-based "Knockout and Replace" Strategy to Treat Rhodopsin-Associated Autosomal Dominant Retinitis Pigmentosa

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Mariacarmela Allocca, Chi-Hsiu Liu, Radhika Nayak, Andrea Pinilla, Abhishek Dass, Ben Diner, Thomas Tallo, Eugenio Marco, Georgia Giannoukos, Shengfang Jin, Kate Zhang, Charles Albright, Deepak Reyon

Editas Medicine, Inc., Cambridge, MA, USA

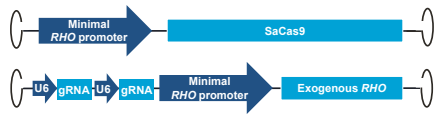
OBJECTIVE

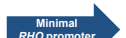
To identify a lead guide RNA (gRNA) and the optimal ratio of the dual adeno-associated virus (AAV) system in a CRISPR-Cas9-mediated therapeutic strategy to knock out the endogenous rhodopsin (*RHO*) gene in patients with RHO-associated autosomal dominant retinitis pigmentosa (RHO-adRP) and replace it with an exogenous functional *RHO* gene.

INTRODUCTION

- adRP, a retinal disease that results in blindness due to photoreceptor degeneration, is most commonly caused by mutations in the *RHO* gene, where over 150 autosomal dominant mutations have been identified.[1]
- CRISPR-Cas9 editing using a dual AAV system can simultaneously knock out the endogenous *RHO* gene (mutant or wild type), and replace it with an exogenous functional *RHO* gene.
- We have previously identified two potent, highly specific guide RNAs (gRNAs), gRNA 59 and gRNA 70, and an optimized *RHO* expression vector *in vitro*.[2]

CRISPR-Cas9-mediated "knockout and replace" dual AAV5 system





***RHO* promoter:**
Restricts both editing and *RHO* expression to rod photoreceptors

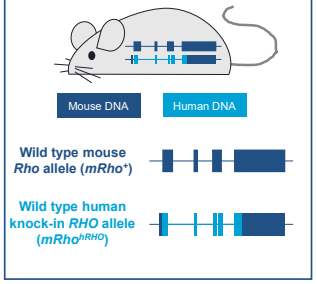
gRNA and SaCas9 on separate vectors:
Ensures editing only in the presence of replacement *RHO* expression

AAV5, adeno-associated virus 5; gRNA, guide RNA; *RHO*, rhodopsin; SaCas9, *Staphylococcus aureus* Caspase 9.

METHODS

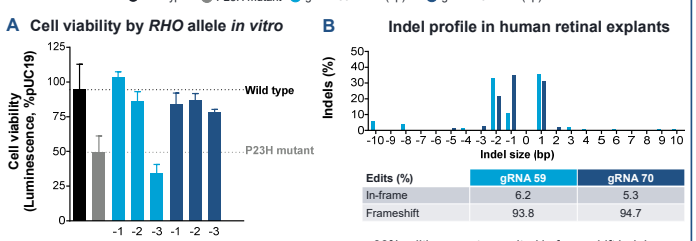
- The cytotoxicity of *RHO* alleles based on gRNA 59 and 70 indels in each open-reading-frame was assessed using an *in vitro* overexpression system.
- Next-generation sequencing (NGS) was used to quantify in-frame vs frameshift indels after transduction of human retinal explants with the dual AAV system.
- Humanized *mRho*$^{hRHO/+}$ mice were injected with the dual AAV system:
  - On-target editing and in-frame vs frameshift indels were quantified using NGS.
  - On-target editing (NGS), *Cas9*, gRNA, and *RHO* expression levels were quantified for four different AAV ratios.

Humanized *mRho*$^{hRHO/+}$ mice



RESULTS

Figure 1. Both gRNA candidates predominantly generated productive frameshifting indels *ex vivo*

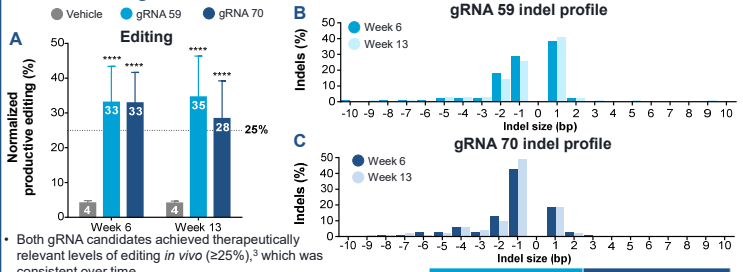


Edits (%)	gRNA 59	gRNA 70
In-frame	6.2	5.3
Frameshift	93.8	94.7

- Frameshift indels rescued P23H cytotoxicity *in vitro*.
- In-frame edits with gRNA 59 may generate a dominant-negative *RHO* allele.
- gRNA 59, but not gRNA 70, is NHP cross-reactive.
- >93% editing events resulted in frameshift indels *ex vivo*, suggesting minimal risk of generating a dominant-negative *RHO* allele through in-frame editing.

(A) Deletions (1, 2, 3 bp) engineered based on gRNA target sites within P23H *RHO* coding sequences and transfected into HEK293T cells. n=4. Mean (SD) is presented. Dotted lines indicate the mean cell viability (%) in cells with the wild type *RHO* allele (black) and the P23H *RHO* allele (gray).
(B) Retinal explants transduced with the dual AAV system; NGS performed at 4 weeks. n=36.
AAV, adeno-associated virus; bp, base pair; gRNA, guide RNA; NGS, next-generation sequencing; NHP, non-human primate; *RHO*, rhodopsin.

Figure 2. Both gRNA candidates achieved consistent and therapeutically relevant levels of editing *in vivo* with no deleterious effects observed

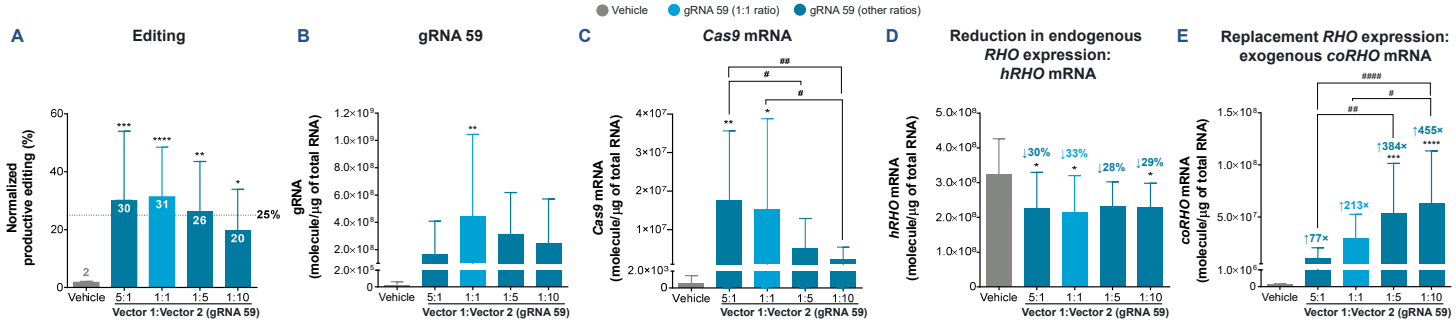


Indels (%)	gRNA 59		gRNA 70	
	Week 6	Week 13	Week 6	Week 13
3 bp	2.5	2.7	3.0	4.2
Total in-frame	4.9	5.6	8.8	7.1

- Both gRNA candidates achieved therapeutically relevant levels of editing *in vivo* (≥25%),[3] which was consistent over time.
- Both gRNA candidates produced <10% in-frame indels, which was consistent over time, suggesting that in-frame editing was unlikely and did not lead to deleterious effects *in vivo*.

mRho$^{hRHO/+}$ mice injected with the dual AAV system at a 1:1 ratio. NGS performed at 6- and 13-weeks post-injection (n=6 for vehicle; n=14 for gRNA 59 and gRNA 70).
(A) Mean (SD) is presented. Normalized to transduced area. ****p<0.0001 vs vehicle. Black dotted line indicates the threshold to achieve therapeutically relevant levels of editing (≥25%).[3]
AAV, adeno-associated virus; bp, base pair; gRNA, guide RNA; *hRHO*, human *RHO* allele; *mRho*, mouse *Rho* allele; NGS, next-generation sequencing; *RHO/Rho*, rhodopsin; SD, standard deviation.

Figure 3. Clinically relevant editing levels while maximizing *RHO* replacement expression levels were achieved by injecting the dual AAV vectors at a 1:1 ratio



- AAV vectors at a 1:1 ratio led to therapeutically relevant editing levels[3] and significant increases in gRNA and *Cas9* expression.
- gRNA and *Cas9* mRNA levels strongly correlated with editing at all ratios.
- Greatest reduction of endogenous *hRHO* expression with the 1:1 ratio of AAV vectors.
- Replacement *RHO* expression increased with increasing dose of Vector 2.

mRho$^{hRHO/+}$ mice injected with the dual AAV system with the identified lead gRNA 59 at various Vector 1:Vector 2 ratios and analyzed at 6 weeks post-injection (n=12 for vehicle; n=20–22 for gRNA 59). Mean (SD) is presented. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs vehicle. #p<0.05, ##p<0.01, ####p<0.0001 vs other vector ratios.
(A) Editing normalized to transduced area. Black dotted line indicates the threshold to achieve therapeutically relevant levels of editing (≥25%).[3] (B) gRNA levels. (C) *Cas9* mRNA levels. (D) Endogenous *hRHO* mRNA levels. Data labels indicate % decrease vs vehicle. (E) Exogenous replacement *coRHO* mRNA levels. Data labels indicate fold increase vs vehicle.
AAV, adeno-associated virus; bp, base pair; *coRHO*, codon optimized *RHO* allele; gRNA, guide RNA; *hRHO*, human *RHO* allele; *mRho*, mouse *Rho* allele; NGS, next-generation sequencing; *RHO/Rho*, rhodopsin; SD, standard deviation.

CONCLUSIONS

- Both gRNA 59 and 70 achieved clinically relevant editing levels with minimal risk of producing novel dominant-negative alleles. gRNA 59 has the additional benefit of being NHP cross-reactive.
- At a 1:1 ratio, the dual AAV system demonstrated clinically relevant editing levels, significant increases in gRNA and *Cas9* mRNA levels, the highest level of endogenous *hRHO* knockdown, and >200-fold higher levels of replacement *RHO* expression compared with the vehicle control.
- *In vivo* characterization of this CRISPR-Cas9 dual AAV system as a therapeutic strategy for RHO-adRP will proceed with gRNA 59 as the lead guide and 1:1 as the optimal ratio for the dual AAV system.

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2. Dass A, et al. ASGCT Annual Meeting 2020:Poster 226
3. Cideciyan AV, et al. PNAS 1998;95:7103–8

DISCLOSURES

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