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

ABSTRACT 576

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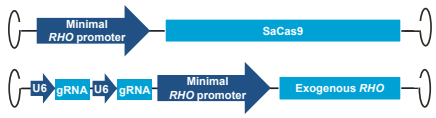
OBJECTIVE

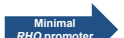
To identify a lead guide RNA (gRNA) and the optimal ratio of the dual adeno-associated virus (AAV) system in a CRISPR-Cas9-mediated therapeutic strategy to knock out the endogenous rhodopsin (*RHO*) gene in patients with RHO-associated autosomal dominant retinitis pigmentosa (RHO-adRP) and replace it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

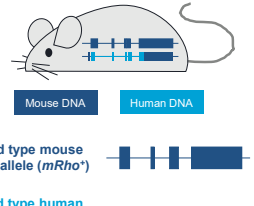
gRNA and SaCas9 on separate vectors:
Ensures editing only in the presence of replacement *RHO* expression

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

METHODS

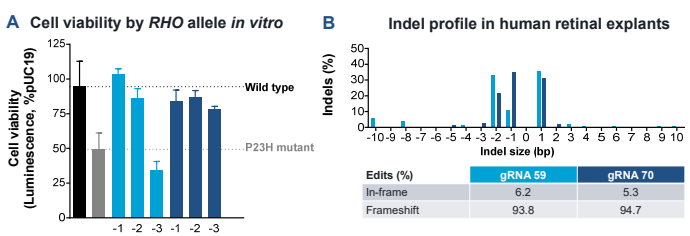
- The cytotoxicity of *RHO* alleles based on gRNA 59 and 70 indels in each open-reading-frame was assessed using an *in vitro* overexpression system.
- Next-generation sequencing (NGS) was used to quantify in-frame vs frameshift indels after transduction of human retinal explants with the dual AAV system.
- 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*

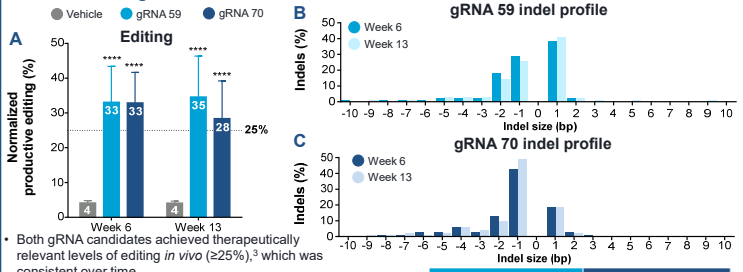


Edits (%)	gRNA 59	gRNA 70
In-frame	6.2	5.3
Frameshift	93.8	94.7

- 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

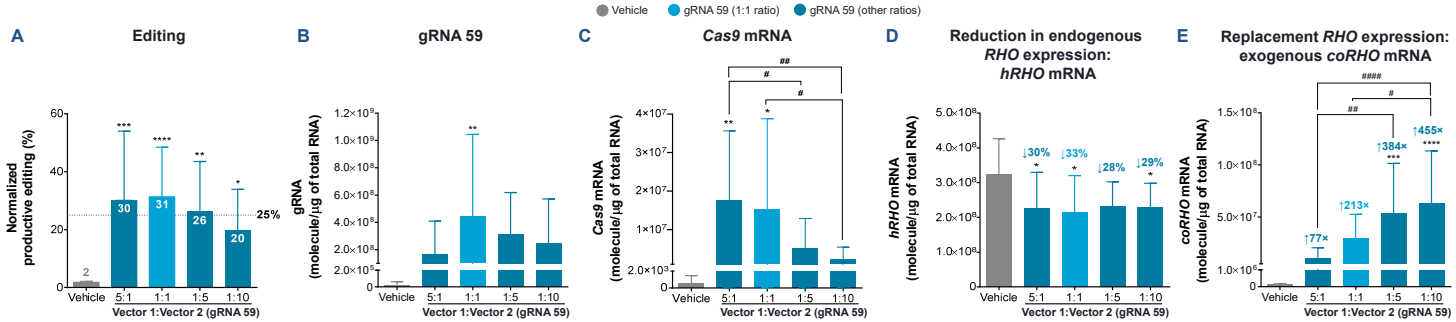


Indels (%)	gRNA 59		gRNA 70	
	Week 6	Week 13	Week 6	Week 13
3 bp	2.5	2.7	3.0	4.2
Total in-frame	4.9	5.6	8.8	7.1

- 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.
- Significant 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, significant levels 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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DISCLOSURES

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