**Introduction**

The RHO gene encodes for rhodopsin, a G protein coupled receptor primarily linked to visual function, involved in the purine segment of retinal photoreceptors. Rhodopsin is essential for normal vision as it is the last protein in the visual cycle and photoreceptor survival. Dominant negative mutations in RHO result in a progressive loss of rod, and subsequently, cone photoreceptor function. Promoter expression in photoreceptors is critical for both rod and cone photoreceptors, and therefore, requires maintenance of both promoters. The intron 2 promoter maintains function in a recent animal retinal pigmentosa (RHO adRP) mouse model, potentially present with loss of night vision as young adults, followed by loss of peripheral vision, and eventually significant decline in central vision. There are currently no approved treatments.

**Results**

Among the ~20,000 RHO adRP patients worldwide, over 150 RHO mutations have been identified that span the length of the entire gene. Given the lack of available treatments, and limited RHO promoter expression, we sought to develop a murine independent CRISPR-Cas9-based strategy to introduce AAVs into intron 1 (as plasmid) and replace it with exogenous functional RHO, using a dual AAV system.

1. **Identification of the top 3 RNP candidates for potent RHO knockdown**

   - Lead RNPs can be ranked ordered in human T cells based on their concentration-dependent RHO editing

2. **Simulating gRNA editing to characterize novel and potentially deleterious RHO alleles**

   - 2,465 RNPs were generated targeting 100 RHO WT and P23H human retinal cell lines

3. **Optimization of co-delivery elements for promoting RHO transgene expression**

   - Varying gRNA-ORF cassette orientation affects RHO transgene expression in HEK293T

4. **Lead gRNA candidates for expression in retinal explants**

   - The top 3 RNP candidates robustly express RHO in multiple culture systems

5. **Top ‘replace’ vector configuration robustly expresses RHO**

   - P23H RHO background

6. **Optimized delivery and expression in human retinal explants**

   - HEK293T cells

7. **Evaluate CRISPR-Cas9 editing in human retinal explants**

   - Knockdown of RHO

8. **Vector delivery in retinal explants**

   - Knockdown of RHO

9. **In vivo delivery in retinal explants**

   - Knockdown of RHO

**Conclusions**

- Of 460 RNPs targeting the g1 genic elements of the RHO gene sequence, 11 robustly edit the RHO coding sequence.
- AAVs silence endogenous RHO and promote homogenous protein in HEK293T, in a plasmid-concentration dependent manner.
- gRNA are highly specific to RHO by multiple unbiased specificity analyses (Eisenstat and GuoSeq, in this protocol). Predicted off-target sites require verification in future work.
- Optimized expression vector with minimal RHO promoter sequence, optimized coding sequence, and a heterologous 3’ UTR element expresses RHO transgene higher than the industry control vector
- AAVs-based delivery of optimized expression vector promotes exogenous RHO nRNA levels comparable to endogenous RHO in human retinal explants.
- These data suggest that it is feasible to develop a dual AAV CRISPR-Cas9 based system to knockdown and replace recombinant in non-photorceptors.

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