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Co-Delivery of a Short-Hairpin RNA and a shRNA-Resistant Replacement Gene with Adeno-Associated Virus: An Allele-Independent Strategy for Autosomal-Dominant Retinal Disorders

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Abstract

Recombinant adeno-associated virus (rAAV) has become an important gene delivery vector for the treatment of inherited retinal degenerative diseases. Many of the mutations leading to retinal degeneration are inherited in an autosomal-dominant pattern and can produce toxic gain-of-function and/or dominant-negative effects. Here we describe an allele-independent gene therapy strategy with rAAV to treat autosomal-dominant retinal degenerative diseases. In this methodology, we co-deliver a short-hairpin RNA (shRNA) to inhibit expression of both the toxic and (WT) copies of the gene as well as an shRNA-resistant cDNA for functional gene replacement with a rAAV.

Keywords

Gene therapy; Recombinant adeno-associated virus; Autosomal-dominant; Retinal degeneration; Allele-independent; Short-hairpin RNA; shRNA-resistant cDNA

1 Introduction

Mutations in over 250 genes can cause retinal degeneration [1]. In the case of autosomal-recessive or X-linked mutations, delivery of a wild-type (WT) copy of the gene with a rAAV should be sufficient to restore retinal physiology, which has been demonstrated in Leber Congenital Amaurosis 2 (LCA2) [2–4]. However, many of these mutations, such as those seen in rhodopsin (RHO) [5] and bestrophin (BEST1) [6], are inherited in an autosomal-dominant pattern and may demonstrate toxic gain-of-function and/or dominant-negative effects. Treatment of autosomal-dominant retinal degenerative disease entails removal of the expression of the mutated gene and replacing its lost expression to restore homeostasis.

The RNA interference (RNAi) pathway in eukaryotic cells represents a powerful tool to inhibit the expression of specific genes [7]. The effector of the RNAi pathway is the RNA-induced silencing complex (RISC) composed of Argonaute proteins bound to an approximately 22 nucleotide single-stranded RNA (ssRNA): either a microRNA (miRNA) or a small-interfering RNA (siRNA). The RISC-bound ssRNA, also known as the guide strand

of the siRNA or miRNA, can base-pair with a complimentary target site on an mRNA in order to inhibit expression of that mRNA (Fig. 1a).

The extent of base-pairing between the guide strand in RISC and its binding site on the mRNA (the target site) determines the efficiency of inhibition. Guide strands of miRNAs typically base-pair with their “seed sequence,” which leads to modest inhibitory effects (Fig. 1b) while those associated with siRNAs can exhibit extensive base-pairing with their target mRNA, resulting in mRNA cleavage and robust inhibition (Fig. 1c). A detailed discussion of the RNAi pathway is out of the scope of this chapter; however, this pathway has been reviewed elsewhere [8, 9].

By delivering a carefully designed short-hairpin RNA that shares important features with miRNAs and siRNAs with a rAAV to a retinal cell, the expression of disease-associated proteins can be blocked to treat autosomal-dominant retinal disorders. These features include (reviewed Fakhr et al. [10]):

1. A dsRNA stem and loop structure of approximately 20 nucleotides forming a hairpin and a 3' dinucleotide overhang. Since these structural features are similar to miRNA precursors (pre-miRNAs) (Fig. 2a), an shRNA can enter the RNAi path-way after its association with and processing by Dicer into a dsRNA duplex (Fig. 1a).
2. A guide strand with low 5' thermodynamic stability. The strand of the dsRNA duplex that is typically selected for loading into RISC has lower thermodynamic stability at its 5' end (due to a A-T rich sequence) (Fig. 2d).
3. A guide strand with complete complementarity to the target site of an mRNA. This will enable robust, siRNA-like inhibition of the target mRNA.
4. A guide strand whose “seed sequence” does not base-pair with mRNAs of important genes. Although the shRNA is like a siRNA in terms of base-pairing, a “seed sequence” will always be present (Fig. 1c).

In theory, the shRNA can be carefully designed to base-pair with the mutation site of the mRNA of the disease-causing gene, in effect preserving expression of the WT gene. However, many mutations within the same gene can cause retinal degeneration. For example, more than 100 mutations have been identified in RHO that can lead to autosomal-dominant Retinitis Pigmentosa (adRP) [1, 5]. Generating shRNAs to target each mutation individually would not be labor or cost efficient. Therefore, the ideal shRNA should be allele-independent, capable of targeting any mutant form of the gene. This allele-independent shRNA, however, would result in loss of expression of the associated endogenous WT gene, thus requiring the co-design of an shRNA-resistant cDNA.

Here, we will first describe how to design an shRNA against a mutant gene of interest (GOI). We will then explore how to generate a functional and shRNA-resistant cDNA of the GOI. Co-delivery of the shRNA and the shRNA-resistant cDNA to a diseased cell represents a methodology that could potentially treat any autosomal-dominant disorder of the retina [11–13]. This methodology has proved successful in the mutant RHO mouse model of autosomal-dominant Retinitis Pigmentosa (adRP) [14, 15].

2 Materials

2.1 Materials and Reagents

1. H1-shRNA Cassette Plasmids.
2. Mammalian Expression plasmid.
3. Pipettes and tips.
4. 1.5 mL tubes.
5. 12 well cell culture plates.
6. Polyethylenimine ~25,000 MW (PEI; 1 mg/mL).
7. Human Embryonic Kidney Cells 293 T (HEK293T) cells.
8. DMEM Cell Culture Media.
9. Fetal Bovine Serum.
10. Penicillin and Streptomycin.
11. NucBlue™ Live Cell Stain.
12. Mouse anti-turboGFP antibody (Origene).
13. Rabbit anti- β -Tubulin antibody.
14. Protease Inhibitor Cocktail (100 $\times$; Thermo-Halt).
15. Phosphate-buffered saline.
16. Cell scraper.
17. Sucrose.
18. 4 $\times$ Loading dye buffer: 200 mM Tris-Cl (pH 6.8) + 400 mM DTT + 8% SDS + 40% glycerol + bromophenol blue.
19. 25-gauge insulin syringe.
20. 660 nm Protein Assay Reagent (Pierce™).
21. Ionic Detergent Compatibility Reagent.
22. Bovine Serum Albumin (BSA).
23. 10% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio Rad).
24. Chameleon ladder (Li-COR).
25. iBlot PVDF Transfer Stacks.
26. Methanol.
27. Odyssey blocking buffer (LiCor).
28. IRDye 800CW Donkey-anti-rabbit (LiCor).
29. IRDye 680RD Goat-anti-mouse (LiCor).

30. Tween.
31. Q5® Site-Directed Mutagenesis Kit (NEB).
32. Primer Oligonucleotides.
33. rAAV Plasmid.
34. Restriction Enzymes: KpnI-HF (20 units/ μ L), XbaI (20 units/ μ L), NotI (10 units/ μ L), SalI (20 units/ μ L), and XmaI (10 units/ μ L).
35. Phusion polymerase (2 units/ μ L; NEB).
36. dNTPs (10 mM).
37. Agarose.
38. Scalpel.
39. Monarch® DNA Gel Extraction Kit (NEB).
40. Tris-Borate-EDTA (TBE) buffer.
41. Ethidium Bromide (10 mg/mL).
42. Calf-Intestinal Alkaline Phosphatase (CIAP, 1 unit/ μ L; Promega).
43. T4 Ligase (400 units/ μ L).
44. 10 $\times$ Ligase Buffer.
45. Nuclease-free H₂O.
46. Monarch PCR Cleanup and Purification kit (NEB).
47. SURE2 Cells (Agilent).
48. Luria Broth.
49. Agar plates (50 μ g/mL).
50. Ampicillin (100 mg/mL in 1:1 H₂O: isopropanol).
51. GeneJET Plasmid Miniprep kit (ThermoFisher).
52. Vetropolycin HC.
53. Proparacaine HCl Ophthalmic Solution (0.5%).
54. Antisedan (5 mg/mL).
55. Hypromellose 2.5%.
56. Phenylephrine HCl Ophthalmic Solution (2.5%).
57. Atropine sulfate Ophthalmic Solution (1%).
58. KetVed (100 mg/mL).
59. Xylazine (100 mg/mL).
60. Sterile Normal Saline.

2.2 Equipment

1. Computer with internet access.
2. ApE plasmid editor software (<http://biologylabs.utah.edu/jorgensen/wayned/apE/>).
3. ImageJ software.
4. Water bath.
5. Incubator at 37 °C with 5% CO₂.
6. Fluorescent Microscope.
7. Micro-centrifuge.
8. iBlot system (Invitrogen).
9. Electrophoresis apparatus (Bio Rad).
10. Odyssey CLx system (LiCor).
11. UV-transilluminator.
12. Heating block.
13. Electroporation Machine.
14. Hamilton syringe (85RN, 26S G).

3 Methods

3.1 Finding the shRNA Target Sites on Your GOI

1. Free online software is available for determination of potential shRNA target sequences within your GOI. We have constructed successful shRNAs based on the target sequences generated from siRNA design software as well, such as the Whitehead Institute siRNA Design Program (<https://www.nature.com/cgt/journal/vaop/ncurrent/pdf/cgt20164a.pdf?origin=ppub>): Select three versions of siRNA or shRNA design software such that the output can be compared.
2. If your GOI has been annotated, the sequence can be found by visiting www.pubmed.gov, selecting the “nucleotide” tab on the search bar dropdown menu, and searching for your GOI. On the results page, filter the results by selecting “mRNA” under “Molecule Types” and your target organism under “Top Organisms.”
3. Input the coding sequence and 3′ untranslated region (UTR) into the three versions of siRNA or shRNA software. Do not input intronic sequences of genomic DNA.
4. Compare the output of the programs and select between four and ten 19-nt target sequences to be tested (see **Note 1**). Base the selection on the method parameters described by Fakhr et al. [10]:

- a. Target sequences that appear in more than one program output.
- b. Target sequences with an A-T rich 3' end, such that the proper guide strand is loaded into the RISC complex.
- c. Target sequences with limited potential off-target effects. Perform two BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), for each target sequence. First, input the entire target sequence and exclude those target sequences that share extensive homology (>13 nucleotides (nt) in a row) with other genes. Second, input nt 13–18 of the target sequence (complimentary to the seed sequence of the guide strand, nt 2–7 (Fig. 1b)) and ensure that none of the hits are highly expressed in your cell type of interest.
- d. The shRNAs to be tested should target different areas (i.e., 5' vs. 3') of the GOI's mRNA.

3.2 In Silico Design and Cloning of the H1- shRNA Cassette

1. shRNAs are efficiently expressed from the H1-promoter [16, 17]. The transcription start site for the H1 promoter is the 26th nucleotide downstream of the TATA box (TATAA). The H1 promoter has low preference for the +1 nucleotide, although the naturally occurring +1 nucleotide is adenosine and transcription initiation may be variable [18, 19] (Table 1 Panel A) (*see Note 2*).
2. The elements of the H1-shRNA cassette are: (a) the modified H1-promoter containing a BamHI site at positions –6 to –1 (Table 1 Panel B), (b) the shRNA containing the Target Sequence, the Loop sequence [17], and the Guide Sequence (Table 1 Panel C), and (c) a modified transcription termination signal following the shRNA sequence.
3. The Final H1-shRNA Cassette (Table 1 Panel G) can be modified to contain the target and guide sequences for the GOI with the free ApE plasmid editor software (<http://biologylabs.utah.edu/jorgensen/wayned/ap/>). Paste the 19-nt target sequences from the shRNA design software output in place of the example target sequence (CTGCATGGATACTTTGTCT). Copy the reverse complement of the 19-nt target sequence and paste it in the place of the example guide sequence (AGACAAAGTATCCATGCAG).
4. The completed gene for each of the potential target sites can be ordered directly from a manufacturer, making sure to include restriction sites at either side of the H1-shRNA cassette (here the SalI site: GTCGAC) for downstream cloning into the rAAV plasmid.
5. A scrambled negative control shRNA should also be designed. Online tools are available to scramble one of the target sequences, such as <http://www.invivogen.com/sirnavizard/scrambled.php>. For example, the example target sequence can be scrambled to produce GTCATTTCGTTCTGGTCATA.

3.3 Finding the H1- shRNA Cassettes with the Highest Knockdown Efficiency of the WT GOI

1. Knockdown efficiency can be measured by performing co-transfections of the control and on-target shRNAs and a plasmid expressing the GOI.
2. Create an empty DNA control that does not express an shRNA.
3. The sequence of the WT GOI can be found as described in Subheading 3.1, **step 2**. Order or clone the GOI, into an expression plasmid with a highly expressed, ubiquitous promoter, such as the cytomegalovirus (CMV) promoter. Ensure that the GOI contains a segment of the 5' untranslated region that contains the gene's endogenous Kozak sequence for efficient translation. Alternatively, a consensus Kozak sequence can be included—GCCACC(ATG) before the gene's open reading frame. If possible, add a green-fluorescent protein (GFP) tag, such as turboGFP.
4. The following protocol was used for measuring shRNA-mediated knockdown of turboGFP-tagged RHO by Western Blot. Optional companion fluorescent microscopy images can be taken. The protocol can be modified as needed for the GOI.
5. Transfect 500 ng of the CMV-GOI-GFP plasmid and 1 µg of each shRNA plasmid, including the scrambled shRNA and empty DNA controls, into separate wells of a 12 well plate containing HEK293T cells, or another cell line with a high transfection efficiency. Include additional wells for a non-transfected control. Perform the transfection in triplicate at room temperature. Briefly:
 - a. Plate 1×10^5 HEK293T cells in 1 mL of complete DMEM containing 10% FBS, 1% Penicillin/Streptomycin, and 0.1% Amphotericin B in each well of a 12 well plate. Incubate overnight at 37 °C with 5% CO₂.
 - b. The next day, the cells should be >70% confluent. Two hours prior to transfection, change the media of the HEK293T cells to pre-warmed DMEM containing only 10% FBS.
 - c. For each shRNA, including the scrambled shRNA and empty DNA controls, mix 1 µg of the shRNA plasmid, 500 ng of the CMV-GOI-GFP plasmid, and 50 µL Opti-MEM I reduced serum medium media. For each shRNA and control plasmids, to a second tube, add 4.5 µL PEI to 50 µL Opti-MEM I reduced serum medium media (1 µg DNA: 3 µL PEI). Incubate for 5 min (*see Note 3*).
 - d. Transfer the 50 µL of Opti-MEM I reduced serum medium containing the PEI to the corresponding 1.5 mL tube containing the shRNA or control plasmid, and incubate at room temperature for 15 min.
 - e. Add the 100 µL PEI: plasmid mixture dropwise to the separate wells of the 12 well plate.
 - f. Incubate at 37 °C with 5% CO₂ for 48 h.

6. *Optional.* Perform fluorescent microscopy to visualize the extent of GOI-turboGFP expression.
 - a. Place a drop of NucBlue™ Live Cell Stain into each well of the 12 well plate, cover in tin foil, and incubate at 37 °C with 5% CO₂ for 15 min.
 - b. Image each well at 4 × and 10 × magnification using the DAPI and Green fluorescent filters of a fluorescent microscope.
 - c. Ensure that the exposure time and other settings are the same for each well.
7. Isolate protein and perform a Western Blot to measure the abundance of the GOI-GFP using the mouse anti-turboGFP antibody relative to the rabbit anti-β-Tubulin antibody loading control.
 - a. Aspirate the media from each well and pipette 1 mL of ice-cold PBS 1 × into each well.
 - b. HEK293T cells are weakly adherent and can be removed from the plate by pipetting up and down. Alternatively, a cell scraper can be used. Transfer the 1 mL PBS containing the cells to fresh 1.5 mL tubes.
 - c. Pellet the cells by centrifuging at 3000 × *g* for 10 min at 4 °C.
 - d. Aspirate the PBS, leaving the cell pellet in the 1.5 mL tube.
 - e. Resuspend the pellet in 0.23 M Sucrose with 1 × Thermo Halt Protease Inhibitor Cocktail (100 ×) in PBS (typically 150–200 µL) and sonicate for 10 s.
 - f. Add 4 × loading dye buffer to each sample so that its final concentration is 1 ×.
 - g. Since RHO-GFP is prone to aggregation, the samples were incubated at room temperature for >10 min and returned to ice. Other proteins can be boiled for 10 min to denature then returned to ice.
 - h. Pass each sample through a 25-gauge insulin syringe five times to shear the DNA to decrease clogging of pipette tips when loading the protein gel.
 - i. Centrifuge the sample at max speed for 10 min at 4 °C and transfer 75% of the upper sample to a new 1.5 mL tube, leaving the cell debris behind.
 - j. Quantify the concentration of each sample using the Pierce™ 660 nm Protein Assay Reagent and the Ionic Detergent Compatibility Reagent relative to a twofold standard curve of BSA over a range of 1.5 to 0.094 µg per sample.
 - k. Load 20 µg of sample into each well of a 10% Mini-PROTEAN® TGX™ Precast Protein Gels. Load one well with 5 µL the Li-COR Chameleon ladder.

- l.** Run the Mini-PROTEAN gel at 100 V until the bromophenol blue of the loading dye has reached the bottom of the gel.
- m.** Transfer the protein from the gel to a PVDF membrane using Invitrogen's iBlot system.
- n.** Incubate the PVDF in methanol while shaking at room temperature for 5 min.
- o.** Wash the membrane with diH₂O five times.
- p.** Block the PVDF membrane in Odyssey blocking buffer for 1 h while shaking at room temperature.
- q.** Wash three times with 0.1% Tween in PBS 1 × while shaking for 5 min each.
- r.** Dilute the mouse anti-turboGFP (1:2000) and rabbit anti-β-Tubulin (1:5000) in Odyssey blocking buffer. Apply to the PVDF membrane and incubate while shaking at room temperature for 2 h or 4 °C overnight.
- s.** Wash three times with 0.1% Tween in PBS while shaking for 5 min each.
- t.** Dilute IRDye 800CW Donkey-anti-rabbit (1:5000) and IRDye 680RD Goat-anti-mouse (1:5000) in Odyssey blocking buffer. Apply to the PVDF membrane and incubate while shaking at room temperature for 45 min.
- u.** Wash three times with 0.1% Tween in PBS while shaking for 5 min each.
- v.** Image gel with an Odyssey CLx Imaging system.
- w.** Quantify the intensity of the band corresponding to GOI-GFP relative to the band corresponding to β-Tubulin for each sample using ImageJ software [20].

3.4 Validate that the H1-shRNA Cassettes Are Allele-Independent

- 1.** Find disease-causing mutations associated with the GOI. Select at least one mutation to validate that the shRNAs are allele-independent (*see Note 4*). The mutation can be selected based on its prevalence or severity. For example, we chose to study the P23H mutation in RHO that causes adRP due to its high prevalence in the United States.
- 2.** The mutation can be induced with the Q5® Site-Directed Mutagenesis Kit using the CMV-GOI-GFP plasmid.
- 3.** Primers for this kit can be designed using the NEBasechanger tool (<http://nebasechanger.neb.com/>), which will also provide the T_m for the PCR reaction.

4. Generate a CMV-mutant GOI-GFP plasmid as per the manufacturer's instructions. For example, the CMV-P23H RHO-GFP plasmid was created by altering codon 23 of the open reading frame from CCC to CAC.
5. Repeat the experiments in Subheading 3.3, except using the CMV-mutant GOI-GFP plasmid in the place of the CMV-GOI-GFP plasmid.
6. Select the two shRNAs, herein named shRNA A and shRNA B, with the highest knockdown efficiency of both the WT and mutant GOI-GFP for further analysis. This is important in case shRNA A or B has unforeseen off-target effects in future experiments.

3.5 Generating shRNA-Resistant cDNAs of the GOI for Functional Gene Replacement

1. Create two shRNA-resistant cDNAs of the GOI (shr-GOI) corresponding to the shRNAs A and B.
2. shRNA resistance can be conferred to the replacement GOI by inducing silent mutations in the shRNA's target sequence by altering the codon wobble base positions.
3. If possible, four silent mutations should be induced, particularly in the wobble bases around nucleotides 9–11 (RISC-cleavage site) and 13–18 (seed sequence) in the target sequence of the WT GOI's mRNA (Fig. 2a).
4. Each shRNA's target sequence can be input into a codon optimization software, such as JCat (<http://www.jcat.de/>) [21]. Ensure that the target sequence is pasted "in-frame," otherwise the mutations will not be silent, and that the correct target organism is selected. For the example target sequence, CTGCATGGATACTTTGTCT, which is in-frame (i.e., the 5' CTG is the first codon), the jcat software produced five silent mutations: two appear in the "seed sequence," one in the "cleavage site," and two in the 3' end of the shRNA (Fig. 2b). These alterations should be sufficient to render the codon-optimized version resistant to the shRNA (see Note 5).
5. If method 1 fails, then codons can be selected by hand as a secondary method. To select appropriate codons, reference a codon usage table for your organism of interest. Choose the silent mutation associated with the highest possible codon usage proportion of the same amino acid.
6. When using either method 1 or 2, A to G and C to T mutations should be avoided because guanidine in the mRNA or shRNA guide strand can wobble base-pair with uracil, resulting in less than maximal resistance (Fig. 3c). An example can be seen for valine in Fig. 3d. Consider using a lesser used codon in this circumstance.
7. The silent mutations can be introduced with the Q5® Site-Directed Mutagenesis Kit.

3.6 Validating that the shRNA-Resistant cDNA Will Co-express with the shRNA

1. Repeat the experiments in Subheading 3.3, except using the CMV-shr-GOI-GFP plasmids in the place of the CMV-GOI-GFP plasmid. Perform the experiment with both shRNA A and B versions of the shr-GOI-GFP.
2. At least five conditions should be studied for each CMV-shr-GOI-GFP plasmid. Into a 12 well plate, make triplicate co-transfections of CMV-shr-GOI-GFP plasmid and the following:
 - a. Negative scrambled control shRNA.
 - b. shRNA A.
 - c. shRNA B.
 - d. Empty DNA.
 - e. Include a no transfection control as well.
3. Measure the expression of the shr-GOI-GFP via Western blot and ImageJ analysis as described in Subheading 3.3. A shRNA A-resistant GOI-GFP should show minimal to no loss of expression when co-expressed with shRNA A, but should be reduced in expression when co-expressed with a separate validated shRNA B. The converse should be true for the shRNA B-resistant GOI.

3.7 Cloning the rAAV Plasmid to Contain the shRNA and the Corresponding shRNA-Resistant GOI

1. Using the ApE plasmid editor software, construct a map of the rAAV plasmids, which will contain standard plasmid elements (i.e., origin of replication, antibiotic resistance) as well as AAV2 inverted-terminal repeats (ITRs) flanking [the plasmid in Fig. 4 will be used as an example for cloning the plasmid]:
 - a. A cell-type-specific promoter with 5' and 3' restriction sites, here the human-opsin promoter (HOP) for rod photoreceptors with a 5' KpnI site and 3' XbaI restriction sites (RSs).
 - b. The shr-GOI open reading frame, containing the endogenous or consensus Kozak sequence, with 5' and 3' restriction sites, here NotI RSs.
 - c. A suitable polyA signal, here the SV40 PolyA signal.
 - d. The corresponding H1-shRNA cassette with surrounding restriction sites, here SalI RSs.
2. If the proposed rAAV genome is <2.4 kb, then a self-complimentary (sc) rAAV can be used. The use of sc rAAV would result in faster transgene expression [22, 23]. Otherwise, use a single-stranded (ss) rAAV with WT ITRs.
3. Clone the two shr-GOIs into the NotI RS of the rAAV plasmid.
 - a. Perform PCR with NEB Phusion polymerase using the CMV-shRNA-resistant GOI-GFP plasmids as templates.

- b. Design primers that anneal to the 5' and 3' end of the GOI with a T_m between 60 and 72 °C calculated with the NEB T_m Calculator (<https://tmcalsculator.neb.com/#/>). Add TAT-GCGGCCGC to the 5' terminus of the for-ward primer. The additional TAT nucleotides allow efficient cutting at the NotI RS (GCGGCCGC). Again, ensure that either the endogenous or consensus Kozak sequence is included in the shr-GOI. The primer should begin to anneal either at the 5' end of the 5' UTR or the consensus Kozak sequence of the GOI. Add TAT-GCGGCCGC to the annealing sequence of the reverse primer, ensuring that the annealing sequence is antisense and begins with the antisense stop codon (ACT, ATT, or ATC) (Table 2). The T_m should be calculated for only the portion of the primer that anneals to the GOI.
- c. Create a PCR mixture according to Table 3.
- d. Perform a PCR reaction with the parameters in Table 4.
- e. Run the PCR reaction with loading dye next to NEB's TriDye-2-log ladder on a 1% agarose gel in TBE at 120 V for 30 min. Excise the band corresponding to the anticipated size with a scalpel under a UV-transilluminator and extract the band with the Monarch® DNA Gel Extraction Kit according to the manufacturer's instructions, eluting in 50 µL of nuclease-free H₂O.
- f. To restriction digest the gel extracted PCR product and the rAAV plasmid for cloning, set up the reactions in Table 5. Incubate at 37 °C for >2 h. To the rAAV plasmid only, after 2 h, add 10 µL of Promega's CIAP and incubate at 37 °C for an additional >1 h to prevent self-ligation. Heat-inactivate both reactions at 65 °C for 20 min.
- g. Purify the NotI-digested PCR fragment of the shr-GOI with NEB's Monarch PCR Cleanup and Purification kit as per the manufacturer's instructions, eluting in 30 µL H₂O.
- h. Isolate the NotI-digested rAAV plasmid by gel extraction according to Subheading 3.6, **step 2(e)**, and elute in 30 µL H₂O.
- i. Make ligation reactions using NEB's T4 Ligase at different molar ratios of digested rAAV plasmid to shr-GOI PCR fragment according to Table 6.
- j. Incubate at 16 °C overnight.
- k. Transform ligation reaction into Agilent SURE2 cells and grow at 30 °C for approximately 16 h on 50 µg/mL Ampicillin Agar Plates.
- l. The next day, pick colonies and inoculate a 5 mL culture containing Luria Broth and 5 µL of 100 mg/mL Ampicillin stock solution and incubate for approximately 16 h at 30 °C in a water bath while shaking.

- m.** Perform minipreps using Thermo's GeneJET Plasmid Miniprep kit on the resulting cultures. Perform two separate restriction digestions for each Miniprep using:
- NotI: to check that an insert of the appropriate size was ligated.
 - XmaI: to check that at least 50% of the ITRs were retained. Retained ITRs will appear as two intense bands situated above and below 3 kB. Lost ITRs will appear as a single band around 6 kB, the size of the linearized rAAV plasmid. >90% Retained ITRs can be seen in Fig. 5.

Restriction digestions of the Miniprep DNA should be performed for >2

h. Run the samples in loading dye next to NEB's TriDye-2-log ladder on a 0.7% agarose gel in TBE at 120 V until the dye has reached the bottom of the gel. Image with a UV camera.

- n.** For colonies containing an insert and retained ITRs, Sanger sequence with the appropriate primers to verify that the GOI has been inserted properly. For the example plasmid, primers that anneal within the SD/SA element in the forward direction (AAAGCTGCGGAATTG-TACCC) and SV40 PolyA element in the reverse direction (GCATTCTAGTTGTGGTTTGTCC) were used.
- o.** For correct colonies containing intact ITRs, perform an XmaI.
- p.** In the next step, the shRNAs will be cloned into the plasmids isolated from the correct bacterial colonies. Double verify that these rAAV plasmids have >50% ITRs before continuing.
- 4.** Clone the shRNAs into the SalI RS of the corresponding rAAV plasmids containing the shr-GOI obtained from Subheading 3.6, **step 3**.
- a.** Digest the shRNA and the rAAV shr-GOI plasmids using the restriction enzymes and the reactions conditions out-lined in Table 7. Incubate at 37 °C for >2 h. To the rAAV shr-GOI plasmid backbones only, after 2 h, add 10 µL of Promega's CIAP and incubate at 37 °C for an additional >1 h to prevent self-ligation. Heat-inactivate both reactions by incubating at 65 °C for 15 min.
- b.** Isolate the shRNAs and rAAV shr-GOI plasmid back-bones as described in Subheading 3.6, step 3(e), except elute in 40 µL H₂O.
- c.** Make ligation reactions at different molar ratios of digested rAAV shr-GOI plasmid backbones and the corresponding shRNAs according to Table 8.
- d.** Transform, inoculate, miniprep, and sequence as described in Subheading 3.6, **step 3(l, m)**, except using a primer that anneals within the SV40 PolyA sequence in the forward direction

(GGACAAACCACAACCTA-GAATGC). Notify the sequencing service that the template is a shRNA construct with a strong hairpin structure so that the correct reaction conditions can be used.

- e. For correct colonies, perform an XmaI restriction digestion to ensure that the ITRs of the rAAV plasmid have not recombined. If <80% of the ITRs are intact, simply retransform in SURE2 cells and screen for >80% ITR retention by XmaI digestion. The rAAV is ready for packaging and testing (*see Note 6*).

3.8 Selection of rAAV Capsid for Ocular Delivery

1. Successful gene delivery to the retina requires the careful selection of a rAAV capsid together with the appropriate selection of delivery route. The most commonly used rAAV capsid serotypes used in ocular gene therapy are AAV1, AAV2, AAV5, AAV6, AAV8, and AAV9. Versions of these capsid serotypes have been engineered to improve the transduction efficiency or to modify the cell tropism within the retina. Below there is a list of rAAV capsid serotypes and their target cells within the retina:

- a. AAV1—RPE transduction after subretinal delivery [24].
- b. AAV2—RPE (subretinal), and RGC (Intravitreal) transduction [25].
- c. AAV5—Photoreceptors and RPE transduction after subretinal delivery [26].
- d. AAV6—RPE transduction after subretinal delivery [27].
- e. AAV8—Photoreceptors after subretinal delivery [28].
- f. AAV9—Photoreceptors after subretinal delivery [29].
- g. AAV2(quadY-F) + T491 V—RGC, Bipolar cells, and VEC after intravitreal delivery [30, 31].

Direct evolution has allowed the production of engineered capsid types leading to improved transduction of retinal cells [32]. Further capsid variants of the abovementioned are currently available and have been demonstrated to have improved transduction profiles. Further information can be found at the UF Ocular Gene Therapy Core [33]. It should be noted that the choice of capsid type depends on the species of experimental animal.

3.9 Ocular Injections in Mice

1. Mouse injections.
 - a. Mice should receive 2 drops of phenylephrine HCl ophthalmic solution (2.5%), atropine sulfate ophthalmic solution (1%), and proparacaine HCl ophthalmic solution (0.5%). This will ensure proper dilation and will provide topical anesthesia before the procedure.

- b.** Mice are anesthetized with an intraperitoneal injection (5 mL/kg) of a ketamine/xylazine mixture (20 mg/mL ketamine, 0.79 mg/mL xylazine, in normal saline).
- c.** The plane of anesthesia is assessed by tail-pinching reflex until the mouse is completely anesthetized.
- d.** Use a Hamilton syringe with a 26 G point/blunt needle and load it with 1 μ L of diluted vector containing Akfluor® (Fluorescein). This reagent will allow visual assessment of the injection quality.
- e.** Use an alcohol pad to clean the needle before proceeding with the injection.
- f.** Place the anesthetized mouse under stereoscope with the desired eye facing the lens.
- g.** Overlay a drop of Gonak (2.5% hypromellose ophthalmic solution) on the eye to protect it from dehydration.
- h.** With the bevel of a 25G needle facing upward, an incision is made within the limbus zone carefully avoiding blood vessels surrounding the area and retracting the needle as soon as the bevel is seen through the pupil.
- i.** Retrieve the Hamilton syringe and pass the needle through the incision carefully avoiding the lens.
- j.** *For an intravitreal injection:* As soon as the needle is seen through the pupil, ask an assistant to push the needle plunger with a slow and steady rate. The injection should take between 15 and 20 s to complete. A bright green color, due to the fluorescein, should be seen inside the eye. Once completed hold the needle in place for 15 seconds to avoid immediate outflow of the vector injected.
- k.** *For a subretinal injection:* As soon as the needle is seen through the pupil, incline the syringe 10–15% toward the posterior pole of the retina and introduce it further into until the globe seems to retract into the orbit. At this point, retract the needle until the globe seem to move forward to its original position. Then ask an assistant to push the plunger slow and steady. This injection should take between 1 and 2 min to avoid causing a retinal tear. The retina should acquire a pale green color as the injection proceeds. Once done carefully retract the needle.
- l.** After the injection has been completed, apply vetropolycin HCl on the incision to avoid infections.
- m.** Administer Antisedan (0.25 mg/kg) intraperitoneally followed by 0.5 mL of warm normal saline to help hydrate the mouse.

- n. Place the mouse in a warm pad and wait until the mouse is ambulatory before returning it to its housing location.

4 Notes

1. Slightly longer target sites of 20 or 21-nt target sequences can be used as well.
2. The U6-promoter can also be used to express small RNA molecules, such as shRNAs [18, 19]. The BamHI and HindIII sites allow cloning of oligonucleotides into the target-loop-guide shRNA-TTTTT site of the H1-shRNA cassette. We chose to order the completed H1-shRNA genes as cloning can be expensive in terms of labor and cost. Finally, other loop sequences are available, such as AAGTTCTCT (Promega) and TTTGTGTAG [34, 35].
3. We recommend including the endogenous Kozak sequence in the 5' UTR of the GOI over the consensus Kozak sequence.
4. The ratio of plasmid DNA to PEI can be altered to optimize the experiment; ratios up to 1 µg:1 µL are acceptable. The ratio of the shRNA plasmid to the GOI-GFP plasmid can be increased such that better knockdown can be achieved. If the GOI-GFP is not prone to aggregation, then the samples can be boiled for 15 min instead of incubating at room temperature. As an alternative approach, cells can be submitted for flow cytometry to measure mean fluorescence across a sample of cells for each condition.
5. Less than four silent mutations may be sufficient to render the GOI shRNA-resistant, particularly if they lie within the cleavage site and “seed sequence.” On the other hand, too many silent mutations may destabilize the mRNA and lead to less efficient translation of the shr-GOI mRNA.
6. Since the XmaI digestions assess a population of bacterial colonies, the ITRs can be salvaged even when less than 10% of the colonies retain their ITRs by retransforming in SURE2 cells and rescreening colonies. In our hands, ITRs are typically lost on the Agar plate, not during growth in larger liquid cultures. Picking small colonies on the Agar plate may facilitate with maintaining ITRs. Using Terrific broth rather than Luria broth may also be helpful to maintain ITRs.

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Fig. 1.

The RISC complex and mRNA silencing. **(a)** siRNAs and miRNAs are generated from longer RNA precursors molecules that are processed by Dicer, an RNaseIII, into short ~20-nt dsRNA duplexes. shRNAs share a common structure with pre-miRNAs, allowing them to be processed by Dicer and enter the RNAi pathway. Only one strand of the RNA duplex is incorporated into RISC. The ssRNA in RISC can base-pair with complementary mRNAs to induce their inhibition. **(b)** The magnitude of mRNA inhibition is dependent on the extent of mRNA binding. If the shRNA guide strand binds like a miRNA only with a seed sequence, then inhibition is weaker. **(c)** However, if the shRNA binds to its mRNA extensively, like an siRNA, then cleavage of that mRNA and robust inhibition can occur. Even with extensive binding, the seed sequence can still bind to other mRNAs, producing off-target effects

Fig. 2.

Important features of shRNAs. **(a)** shRNA's have a hairpin structure and 3' dinucleotide overhang that enables their processing by dicer. When expressed from a H1-shRNA cassette, transcription initiates at the first nucleotide of the 5' arm of the shRNA and terminates with a string of five Ts (TTTTT). It is predicted that the UU overhang originates from the TTTTT termination signal. **(b)** The strand of the RNA duplex with the lesser stability at its 5' end is typically loaded into RISC. When designing shRNAs, the guide strand is encoded on the 3' arm

Fig. 3.

Generating the shRNA-resistant GOI. **(a)** The example target sequence is bound extensively by the guide strand of the example shRNA. Alterations to the underlined wobble bases in the “seed sequence” and “cleavage site” are particularly important to mutate to prevent base-pairing between the guide shRNA and the mutagenized mRNA. **(b)** Codon optimization of the example target sequence yielded five silent mutations, situated both in the “seed sequence” and “cleavage site,” which should provide shRNA resistance. **(c)** Mutations that create G::U wobble base-pairing should be avoided. **(d)** Algorithm for selecting the wobble base-pair by hand. Here, the WT codon is GUA and needs to be altered. Look for a codon of the same amino acid (valine) with the highest usage proportion. If the silent mutation leads to a change from A to G or C to T, then select the next available codon with the highest usage to avoid G:U wobble base-pairing between the guide strand and the shRNA-resistant target sequence

Fig. 4.

Example of rAAV genome designed to treat adRP caused by mutant RHO [36]. The rAAV genome is situated between ITRs, with the self-complementary ITR oriented at the 3' terminus. The HOPs promoter (flanked by KpnI and XbaI sites) drives the expression of shRNA-resistant RHO mRNA (flanked by NotI sites), which includes the 5' UTR with the endogenous Kozak sequence, with a SV40 PolyA signal. The H1-shRNA (flanked by SalI sites) is located near and transcribes toward the mutant ITR

Fig. 5. XmaI digestion of a rAAV plasmid that retained >90% of its ITRs, suitable for capsid packaging. A plasmid that has lost ~90% of its ITRs can be recovered by retransforming and rescreening for ITRs with XmaI

Table 1

Designing the H1-shRNA cassette

Panel A. Minimal H1 Promoter, Wild-type DNA Sequence ATATTGCCATGTCGCTATGTGTCTCGGGAAATCACCATAAACGTGAAATGTCTTTGGATTTCGGGAATCTATAAAGTCTCTGTGTGTGAGACCACTCTTCCCT / A + 1
Panel B. Modified H1 Promoter DNA Sequence TAAACGACGCGCCAGTGAATCTCAATATTGTCATGTCGCTATGTGTTCGGGAAATCACCATAAACGTGAAATGTCTTTGGATTTGTGTGTGAGACCACTCTGGATCC / +1 Modifications are underlined.
Panel C. shRNA DNA Sequence 19-nt Target Sequence – TTCAAAGAGA (Loop sequence) – 19-nt Guide Sequence
Panel D. Example shRNA DNA Sequence with target sequence: CTGCATGGATACTTTGTCT CTGCATGGATACTTTGTCTTCAAGAGAGACAAAGATCCATGCAG
Panel E. Termination signal TTTTT
Panel F. Modified Termination Signal TTTTTAAGGCTTTTGGCGTAATCATG HindIII Restriction is underlined.
Panel G. Final H1-shRNA Cassette GTGCACTTAAACGACGCGCCAGTGAATTCATATTTCGATGTGTGTCTCGGGAATCACCATAAACGTGAAATGTCTTTGGATTTGTGTGTGAGACCACTCTGGATCC Sub site for cloning of 5'AV flanking Target and guide sequences need to be replaced.

Table 2

Primer design for cloning of the shRNA-resistant GOI

Forward Primer	TAT-GCGGCCGC-GCCACC-ATGNNNNNNNNNNNN
Reverse Primer	TAT-GCGGCCGC-TTANNNNNNNNNNNNN

Ns in the forward primer should begin with the start codon and be sense, whereas those in the reverse primer should begin with the stop codon and be antisense

Table 3

PCR mixture for cloning of the shRNA-resistant GOI

Nuclease-free H ₂ O	Up to 50 µL
HF-Phusion Buffer	10 µL
10 mM dNTPs	1 µL
10 µM Forward Primer	2.5 µL
10 µM Reverse Primer	2.5 µL
shRNA-resistant GOI Plasmid	1 ng
Phusion Polymerase	0.5 µL

Table 4

PCR conditions for amplification of the shRNA-resistant GOI

30–35 cycles	Initial denaturation	98 °C for 30 s
	Denaturation	98 °C for 10 s
	Annealing	T _m for 30 s
	Extension	72 °C for 30 s per kB of GOI
	Final Extension	98 °C for 7 min

Table 5

Restriction digestions for shRNA-resistant GOI cloning

	Gel-purified PCR fragment of shRNA-resistant GOI	rAAV Plasmid
H ₂ O	0 µL	Up to 50 µL
Buffer 3.1	5 µL	5 µL
Template	44 µL of the Gel-Purified Product	2 µg of plasmid
NotI	1 µL	1 µL

Table 6

Ligation reactions of the shRNA-resistant GOI into the rAAV plasmid

	1:1 Ratio (μL)	1:2 Ratio (μL)	1:3 Ratio (μL)	Negative control (μL)
rAAV Backbone	3	3	3	3
GOI PCR Fragment	3	6	9	0
Ligase Buffer	2	2	2	2
T4 Ligase	1	1	1	1
H ₂ O	11	8	5	14

Table 7

Restriction digestions for shRNA cloning into the rAAV shRNA-resistant GOI plasmid

	shRNA	rAAV containing resistant GOI
H ₂ O	Up to 50 µL	Up to 50 µL
Buffer 3.1	5 µL	5 µL
Template	10 µg of plasmid	2 µg of plasmid
SalI	1 µL	1 µL

Table 8

Ligation reactions of the shRNA and rAAV shRNA-resistant GOI plasmid

	1:2.5 Ratio (μL)	1:5 Ratio (μL)	1:7.5 Ratio (μL)	Negative control (μL)
rAAV Backbone	2	2	2	3
GOI PCR fragment	5	10	15	0
Ligase buffer	2	2	2	2
T4 ligase	1	1	1	1
H ₂ O	10	5	0	14