Supplementary Data

Materials and Methods

Animals and ocular surgery

Thirty-nine New Zealand White male rabbits (age 6–7 months at injection; weight 2.4–3.8 kg) were used in the present study. All *in vivo* procedures were conducted in compliance with the *ARVO Statement for the Use of Animals in Ophthalmic and Vision Research* and were approved by the Animal Care and Use Committee of the National Eye Institute.

Vector solution or vehicle was administered in the right eye by intravitreal injection using ½ ml insulin syringes with permanently attached 28-gauge needle (Ultrafine U-100 syringe; BD Biosciences) in an injection volume of 50 μl. Syringes were loaded under sterile conditions in a laminar flow hood on the day of injection. Rabbits were anesthetized with intramuscular ketamine, 40 mg/kg, and xylazine, 3 mg/kg. Sterile surgical instruments were used and the animals were prepared aseptically before injection. Povidone iodine (5% povidone iodine, 95% BSS-irrigating solution) was used to disinfect the eyelid margins and the conjunctiva at the injection site. BSS solution was used to wash the eyelids and for eye irrigation as needed to minimize corneal drying. An eyelid speculum was applied. Fifty microliters of vector or vehicle solution was injected via the pars plana region in the superior temporal quadrant approximately 2 mm posterior to the limbus in each eye. The injection was performed with the needle tip in the center of the vitreous and the vector was delivered at a moderately slow rate. The needle was then carefully extracted from the eye and a sterile cotton-tip applicator was applied to prevent reflux of both the vector and vitreous. Triple antibiotic ophthalmic ointment (neomycin, polymyxin, and bacitracin) was applied to the injection site after injection, and the rabbits were returned to their cages.

Vector description and preparation

AAV8-scRS/IRBPhRS delivers a self-complementary vector genome that contains a modified human retinoschisin promoter, an interphotoreceptor retinoid-binding protein (IRBP) enhancer, an intact human retinoschisin cDNA with a truncated first intron located in its authentic position between the exon 1 and 2 sequences, and a human beta-globin 3′ untranslated region and polyadenylation site. The AAV8-null served as a control vector that did not express any protein. It has a similar structure with AAV8-scRS/IRBPhRS, but the human RS promoter and IRBP enhancer are replaced by a stuffer DNA without promoter activity. In addition, the start codon of the hRS1 coding sequence is mutated in the AAV8-null vector to ensure no protein expression. These two vectors are packaged into AAV type 8, a serotype that transduces retinal cells in the Rs1-KO mice very efficiently following intravitreal injection (Park *et al.*, 2009).

Recombinant AAV vectors were prepared as previously described (Grimm *et al.*, 2003) with some modifications. Briefly, 293 cells cultured in 850 cm^2 roller bottles in DME (high glucose) medium containing 10% fetal bovine serum (HyClone SH30070.03) and supplemented with penicillin, streptomycin, and glutamine were transiently transfected with the helper plasmids pLadeno5 (encodes adenovirus type 2 E2A, E4, and VA RNAs) and pHLP19-8 (encodes AAV2 rep and AAV8 cap), and the appropriate vector plasmid (below) using the calcium phosphate method. Eighteen hours after transfection, the medium was changed and replaced with the same medium lacking serum. Forty-eight hours later, the cells were collected by centrifugation and stored at −80°C. For purification, the cell pellets were thawed; suspended in 50 m*M* Tris-HCl, 150 m*M* NaCl, and 2 m*M* $MgCl_2$, pH 8.0; and disrupted by 2 rounds of microfluidization. The cell debris was removed by centrifugation and the supernatant was adjusted to 25 m*M* $CaCl_2$ and the resulting pellet was also removed by centrifugation. Benzonase nuclease (Novagen) was added to the supernatant to a final concentration of 100 units/ml and the mixture was incubated for 1 hr at 37°C. Forty percent polyethylene glycol 8000 (PEG)/2 *M* NaCl was then added to produce final concentrations of 8% PEG and 0.650 *M* NaCl and the vector fraction was precipitated and collected by centrifugation. The vector fraction was solubilized in 50 m*M* HEPES, 150 m*M* NaCl, 20 m*M* EDTA, 1% sodium lauroyl sarcosinate, and 10 μg/ml RNase A, pH 8.0. This solution applied to a CsCl step gradient and vector was separated from the bulk protein and nucleic acids by ultracentrifugation. The vector fraction was collected and applied to a linear CsCl gradient and repurified. The purified vector fraction was collected; dialyzed against 10 m*M* Tris-Cl and 180 m*M* NaCl pH 7.4; formulated in 10 m*M* Tris-Cl, 180 m*M* NaCl, and 0.001% Pluronic F-68 pH 7.4; filter sterilized; and stored at −80°C.

Ophthalmological examination

All rabbits were clinically examined before injection and at 2, 4, 8, and 12 weeks after injection. Each rabbit underwent external ocular inspection and full ocular examination of both eyes by slit lamp biomicroscopy and by indirect ophthalmoscopy after pupillary dilation with Atropine 1%. Clinical changes were graded using a five-step severity scale (none, trace, mild, moderate, severe) by two examiners.

Clinical pathology

In a group of 18 rabbits, serum samples were collected before treatment and 30 days after injection with AAV8 vector or vehicle, and were assayed for circulating antibodies to the AAV8 capsid.

In vitro *neutralization assay for AAV8 in rabbit serum*

293 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 10% CO_2. An amount of 8×10^3 cells/well were seeded into 96-well plates, with 100 μl/well medium containing full supplement. Three days later, the media were changed to 70 μl/well fresh DMEM with full supplement. In a separate 96-well plate, 2-fold serial dilutions of the rabbit serum samples were prepared, using DMEM supplemented with 1%

penicillin/streptomycin and 1% BSA. The dilutions ranged from 1:100 to 1:3200 with a volume of 15 μl in each well. An amount of 4×10^8/well viral genomes of AAV8-luc in an equal volume of media were added to the serum samples, incubated at 37°C for 1 hr, and then added to the 293 cells. The wells containing mixture of AAV8-luc and media without serum samples were served as positive controls. Etoposide (100 μM) was also added to each well to increase AAV transduction. The wells without AAV8-luc infection were served as background controls for luciferase activity. Twenty-four hours later, luciferase activity was quantified using the One-Glo Luciferase Assay System (cat. no. E6120; Promega). The percentage of inhibition was calculated relative to positive control wells. The neutralization titer for each serum sample was defined as the serum dilution at which the relative light unit (RLU) was reduced by 50% compared with positive control wells after subtraction of the background RLU in nonvirus control wells.

Histopathology

Animals were euthanized for histologic examination at 4 and 12 weeks after injection (Table 1). Each eye was marked for orientation before enucleation using a Vycril suture tied temporally. Enucleation was carried out using a standard technique. After enucleation, a slit was made at the corneal limbus using a scalpel blade, with care to avoid disruption of the iris and lens, and the whole eye immersed in Bouin's fixative (Polysciences, Inc.) for 24–36 hr. Eyes were rinsed in running distilled water for 2 hr until the runoff was clear before putting them in 70% ethanol. Once in 70% ethanol and clear, eyes were embedded in paraffin, cut at 5 μm, and stained with hematoxylin and eosin (H&E). For each eye, three H&E sections were made on the sagittal plane (one each from nasal, temporal, and central blocks per eye) (Manzano *et al.*, 2008), and all sections were examined for histopathological changes. Comparable anatomical structures for each section were examined using a Zeiss Axioskop with AxioVision LE software. These included choroid, retinal pigment epithelium, neuroretina (inferior and superior, central, mid, and peripheral retina), optic nerve, vitreous cavity, lens, posterior chamber, ciliary body, iris, anterior chamber, filtration angle, cornea, and extraocular structures. To quantify the extent of inflammation, images of the central section were taken using the 5× objective to deliver a consistent area of 2.35 mm^2 centered on the optic nerve head, and all the inflammatory cells within this area were counted. The pathologist was masked as to the treatment groups.