

## Supplementary Online Content

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**eMethods.** Supplemental Methods

**eData.** Supplemental Data

This supplementary material has been provided by the authors to give readers additional information about their work.

## eMethods. Supplemental Methods

### Construction of the viral vector scAAV2.dnRhoA

The three recombinant plasmids needed for the generation of the scAAV2.dnRhoA virus were as follows. Plasmid pXX6-80, which carries the adenovirus helper functions genes.<sup>1</sup> Plasmid pXX2, which carries the AAV virus rep and cap genes that confer the serotype of the virus.<sup>1</sup> Plasmid pMG27, containing the AAV terminal repeats and the transgene expression cassette encoding a dominant-negative *RhoA* gene (dnRhoA). Plasmids pXX6-80 and pXX2 were obtained from the University of North Carolina (UNC) Vector Core facility (<http://genetherapy.unc.edu/services.htm>) and pMG27 was generated in our laboratory.

The plasmid vector backbone used for the construction of pMG27 was pHpa-trs-SK,<sup>2</sup> which contains one wild-type and one-deleted AAV terminal repeat to allow the generation of a double stranded AAV (scAAV) upon co-infection of HEK293 cells with pXX2 and pXX6-80. This plasmid was provided by the UNC Gene Therapy center.

For the transgene pMG27 expression cassette, the *dnRhoA* cDNA (273-859 in NM\_001664.2) was amplified from our plasmid pRG1<sup>3</sup> using primers 5'-GGT ACC CGC GTT ACA TAA CTT ACG GT-3' (forward) and 5'-GAA TTC TTG GAC CTG GGA GTG GAC AC-3' (reverse). A series of subcloning steps include the generation of plasmids pMG22, a TOPO plasmid containing the *RhoA-19N* DNA fragment from pRG1, and pMG23, a TOPO plasmid containing in addition the  $\alpha$ B-crystallin promoter (-700/+45). To improve the efficacy of the vector we introduced an optimized cytomegalovirus immediate-early promoter enhancer (CMV5).<sup>4</sup> This enhancer-promoter differs from the commonly used CMV in that it contains the adenovirus tripartite leader<sup>5</sup> followed by the adenovirus major late promoter and an intron flanked by splice donor and acceptor sites. This modified CMV5 has been shown to significantly increase the transprotein production in transduced cells. The CMV5 element (1,049 bp) was isolated from plasmid pQBI-AdCMV5 (719-1767 nt Quantum Biotechnologies) using primers 5'-GAA TTC GAG CAA TGG CTG CCA TCC GGA AGA AAC-3' (forward) and 5'-GCG GCC GCT CAC AAG ACA AGG CAA CCT C-3' (reverse) (pMG25). The designed construction also includes the own human *RhoA* Kozack sequences for further translation efficiency. Amplified fragments from pMG23 and pMG25 were joined in a TOPO cloning plasmid flanked by *KpnI-NotI* for confirmation of sequence (pMG26). The CMV5.dnRhoA expression cassette (1,649 bp) was then isolated, gel purified and inserted into *KpnI-NotI* predigested plasmid pHpa-trs-SK.<sup>2</sup> The resulting plasmid, pMG27, thus contains one wild-type and one deleted AAV terminal repeats and the CMV5.dnRhoA expression cassette.

Triple transfection with pXX6-80, pXX2 and pMG27 for the generation of the recombinant virus serotype 2, scAAV2.dnRhoA, was conducted at the UNC Vector Core as previously described.<sup>1,6</sup> Viral preparations used in this study were control virus: scAAV2.GFP: lots AV3221 ( $1 \times 10^{12}$  viral genomes (vg)/mL), AV2795b ( $1 \times 10^{12}$  vg/mL) and AV4067 ( $2 \times 10^{12}$  vg/mL) and transgene: Ad5.dnRhoA lot 072811 ( $2.8 \times 10^{12}$  vg/mL) and scAAV2.dnRhoA lot AV4709 ( $7.5 \times 10^{11}$  vg/mL).

### **Culture of Outflow Pathway Cells**

To prepare primary human trabecular meshwork (TM) cells, the TM tissue is dissected out, cut into small pieces, carefully attached to the bottom of a 2% gelatin-coated 35-mm dish, and covered with a drop of HyClone Minimum Essential Medium (Richter's modification) (IMEM) (Thermo Fisher Scientific) / 20% FBS/ 50 µg/mL gentamicin media, and a coverslip. Cells were not treated with enzymes and allowed to just grow out from the explants for a period of 4 weeks, after which they were passed twice for expansion, and frozen in liquid nitrogen labeled as passage-3. This primary, nontransformed cells subsist for five to six passages. These outflow pathway cultures comprise all cell types involved in maintaining resistance to flow. That includes cells from the three distinct regions of the TM plus cells lining the Schlemm Canal. Because most of the cells in these cultures come from the TM, they are commonly referred to as "trabecular meshwork cells".

### **Administration of Recombinant Viruses to Primary HTM cells and Actin Staining**

For viral infection, primary human TM cells seeded on 6-well dishes and maintained in IMEM supplemented with 10% FBS, 50 µg/mL gentamicin were allowed to reach 70% to 80% confluency. For actin fluorescence studies, cells were grown on glass coverslips pre-coated with poly-D-lysine (Sigma). Before infection, cells were extensively washed with phosphate-buffered saline (PBS), and then incubated for 1 hour with  $1.8 \times 10^{10}$  vg of scAAV2.*dnRhoA* or vehicle in 1 mL serum-free IMEM (multiplicity of infection (moi)  $6.2-19 \times 10^3$  vg/cell). Viral absorption continued for 18 hours in the presence of 2% FBS. After viral exposure, media was changed and cultures evaluated 72 hours post-infection.

For the evaluation of actin stress fibers, cells were washed 2X with PBS, fixed with fresh paraformaldehyde (PFA), 4%, for 10 minutes, and washed three more times with PBS (5 minutes each wash). Then, cells were permeabilized with 0.1% TritonX-100 in PBS for 5 minutes, washed, blocked in 10% goat serum for 20 minutes, washed, and stained with rhodamine-conjugated phalloidin (1:500; Sigma) for 1 hour. After washing, cells were mounted with Fluoromount-G (Southern Biotech), and the actin visualized in a fluorescence microscope (Olympus, IX71) equipped with a DP-70 digital camera and software.

### **Experimental Animals, Intraocular Pressure (IOP) Measurements and Intraocular Injections**

Rats were purchased from Charles River Laboratories and provided with food and water ad libitum. Intraocular pressure (IOP) measurements were taken once or twice a week and dark and light IOPs from a single animal were taken on consecutive days. For the measurement, rats were lightly anesthetized with isoflurane followed by an intraperitoneal cocktail at half-dose (25 mg/kg ketamine, 2.5 mg/kg xylazine, 0.5 mg/kg acepromazine) (Butler Schein) as described.<sup>7</sup> At 5 minutes of recumbence corneas were numbed with an eye drop of 0.5% tetracaine (Bausch & Lomb) and eyes positioned with the visual axis horizontal to the TonoLab probe.<sup>7</sup> At each time, three consecutive TonoLab readings (total 15) were

conducted according to manufacturer's recommendations. Baseline values were calculated as the average of two measurements prior injection treatment. Integral IOP (cumulative pressure received by each rat on the entire duration of the post-injection experiment) was calculated using the Area Under the Curve (AUC) tool of the GraphPad Prism 5 software (GraphPad Software Inc.) and written as mm Hg-days.

Intraocular injections were performed when nocturnal IOP increase appeared in two consecutive measurements. After a brief isoflurane inhalation, rats were anesthetized by intraperitoneal injection at the full dose (50 mg/kg ketamine, 5 mg/kg xylazine and 1 mg/kg acepromazine). While resting on its side, rats were placed under a surgical microscope with the head propped up in a holder and eyes directed upward. Corneas received an additional 1 drop of 0.5% tetracaine prior to injection. For the intracameral vector delivery, a 30-gauge needle was inserted through the superior cornea at the limbus with the bevel up, to gently reach the center of the anterior chamber. The needle device consisted of the tip of a 30-gauge needle, broken at the middle, connected to one end of a long intramedic-polyethylene tubing (PE-10; BD/Clay Adams). The remaining half of the needle, plus the hub, was attached at the other end of the tubing and then connected to a dispenser (Gilmont micrometer syringe, Thermo Fisher Scientific). The syringe and part of the tubing were filled with a colored glycerol driving solution to allow aspiration of 4-5  $\mu$ L of the viral sample. Inside the tubing, the driving solution was kept separated by 3 to 4 cm with air from the viral sample at all times. When the needle was inside the anterior chamber, the sample was delivered by the assistant over 30 seconds and fluid entry monitored by direct visualization through the operating microscope. The needle was left in place for 1 to 2 minutes and withdrawn gradually to minimize leaking. Topical ophthalmic ointment (neomycin 3.5 mg, polymyxin B 10,000 units) was applied to the eyes and the animals were returned to their cages, resting on absorbent paper for recovery.

## Histology

Morphology of the TM region was examined on both eyes of 4 rats from a group subjected to reverse cycle model 2. The treated eye (left eye) was injected with  $3.7 \times 10^9$  vg of scAAV2.*dnRhoA* while the control eye (right eye) was injected with  $5 \times 10^9$  vg of scAAV2.*GFP*.

For paraffin, tissue wedges were immersed in 4% PFA/ 2.5% glutaraldehyde/0.1 M cacodylic acid, pH 7.35 at 4°C overnight. Specimens were then rinsed in distilled water for 10 minutes and transferred to 70% ethanol for delivery to the UNC Histology Core facility for paraffin embedding. Meridional 5  $\mu$ m sections were mounted on microscope slides (Superfrost Plus; Thermo Fisher Scientific) and stained with hematoxylin and eosin. For plastic, tissue wedges were immersed in 4% PFA/ 2.5% glutaraldehyde overnight. Next day, tissues were dehydrated in two changes 1 hour each of 50% ethanol and transferred to 70% ethanol at 4°C overnight. On day three, tissues were further dehydrated in two changes 1 hour each of 80%, 95%, and 100% ethanol followed by two changes 1 hour each of acetone and 100% ethanol. Tissue wedges were then infiltrated for 48 hours consisting of 3 changes 16 hours

each in Technovit 7100 2-hydroxyethyl-Methacrylate (GMA) plus Technovit GMA 7100 Hardener I (1g per 100 ml of resin) at 4°C (Heraeus Kulzer GmbH). Following infiltration, the globes were polymerized and embedded in the same Technovit GMA resin with Technovit GMA 7100 Hardener II (0.5 ml) added at room temperature for 2 hours then overnight at 4°C. Blocks were sectioned at 5 microns on a Microm HM 355S Microtome (Thermo Fisher Scientific). Sections were placed onto Superfrost Plus slides (Thermo Fisher Scientific), dried for 5 minutes on a 55°C hotplate (C&A Scientific), stained with toluidine blue and coverslipped with Permount Mounting Medium (Thermo Fisher Scientific).<sup>8</sup>

To evaluate GFP fluorescence, tissue wedges were post immersed in fresh 4% PFA for 4-18 hours, then consecutively washed in 10% sucrose (6 hours) and 30% sucrose (overnight) and embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek; Sakura Finetek) in liquid nitrogen. Frozen meridional 10 µm sections were mounted on Superfrost Plus microscope slides with Fluoromount G and GFP visualized on a fluorescence microscope (model IX71; Olympus). Images were captured with a digital camera (DP70; Olympus) and accompanying software. Digital images were arranged with image-analysis software (Photoshop CS; Adobe Photoshop).

### **RNA Extraction, Reverse Transcription and Real-Time PCR**

After RNAlater immersion, anterior segments were cleaned of the iris and lens, ciliary body carefully folded back and parallel cuts made at both sides of the TM using an Optimal Microsurgery blade (Wilson Ophthalmic). Homogenization of the isolated TM tissue strips was conducted in 350 µL guanidine thiocyanate buffer and loaded onto a QIAshredder column (Qiagen). The tissue strips, from pooled or single TMs were placed in a sterile glass micro-tissue grinder or disposable plastic-tube/pestle (Kimble-Kontes) for RNA extraction. Extraction continued using the RNeasy kit with on-column RNase-free DNase digestion (Qiagen) according to manufacturer's protocol and purified RNA was eluted with 30 µL RNase-free water. Concentrations of RNAs were measured on a spectrophotometer (NanoDrop ND-100; Thermo Fisher Scientific).

Reverse transcription (RT) reactions were conducted in a 25 µL total volume of proprietary RT buffer containing random primers, dNTPs, and 62.5 U of enzyme with RNase inhibitor (MultiScribe MuLV RT; High Capacity cDNA kit; Applied Biosystems, ABI), according to manufacturer's recommendations (25°C for 10 minutes, 37°C for 2 hours, 85°C for 5 min). The total amount of rat angle tissue RNA used in each of the RTs was 0.5 µg.

Fluorescently labeled TaqMan probe and primers sets for the human *RhoA* carried in the recombinant virus were purchased from the TaqMan Gene Expression Assays collection (Applied Biosystems). The human *RhoA* (Hs00357608\_m1) corresponded to sequences from exons 4 and 5. Reactions were performed in triplicate 20 µL aliquots using TaqMan Universal PCR Master mix No AmpErase UNG, run on an Applied Biosystems 7500 Real-Time PCR System, and analyzed by 7500 System SDS v.2.0.4 software. Relative Quantification (RQ) values between treated and control samples were expressed in Fold Change and calculated by the formula  $2^{-\Delta\Delta C_T}$  where  $C_T$  is the cycle at threshold and  $\Delta C_T$  is  $C_T$  of the

assayed gene minus  $C_T$  of the endogenous control (18S).  $\Delta\Delta C_T$  is the  $\Delta C_T$  of the normalized assayed gene in the treated sample minus the  $\Delta C_T$  of the same gene in the untreated one (calibrator). Because of the high abundance of the 18S rRNA used as the endogenous control, and in order to get a linear amplification, RT reactions from treated and untreated samples were diluted  $10^4$  times prior to their hybridization to the 18S TaqMan probe.

## eData. Supplemental Data

### **Intraocular pressure details on the effect of scAAV2.*dnRhoA* on nocturnal IOP elevation on models 1 and 2**

Model 1 was used in two groups (n=8 each). In the first group, dark IOPs at baseline had mean (SE) values of 17.7 (0.4) mm Hg in controls (naive right eye CI 0.8) and 17.9 (0.5) mm Hg in scAAV2.*dnRhoA* pre-injected eyes (treated left eye CI 1.1). Mean (SE) integral nocturnal IOPs at the end of the experiment (31 days) were 557.8 (9.2) mm Hg-days (control) and 547.4 (7.9) mm Hg-days (treated) ( $P=0.04$ ). In a second group, dark IOPs at baseline had mean (SE) values of 16.6 (0.4) mm Hg in control (naive right eye CI 0.7) and 16.8 (0.4) mm Hg in Ad5.*dnRhoA* pre-injected eyes (left eye CI 0.8). The mean (SE) difference between the integrals IOP of treated and untreated eyes in this group, 300.2 (5.5) mm Hg-days in controls and 298.9 (5.0) mm Hg-days in Ad5.*dnRhoA* eyes, was not significant probably due to the short period available for measurement. However, mean (SE) IOPs at the end of the experiment (18 days), 16.8 (0.2) mm Hg (controls CI 0.5) and 15.1 (0.7) mm Hg (Ad5.*dnRhoA* CI 1.7), were significant ( $P=0.02$ ).

Model 2 was used also in two groups (n=7 each). In the first group, dark IOPs at baseline had mean (SE) values of 13.1 (0.4) mm Hg in control (pre-injected scAAV2.*GFP*, right eye CI 1.1) and 11.5 (0.6) mm Hg in scAAV2.*dnRhoA* pre-injected eyes (treated left eye CI 1.5). Integral nocturnal IOPs at the end of the experiment (54 days) had mean (SE) values of 1,073.6 (51.7) mm Hg-days (control) and 659.4 (30.4) mm Hg-days (treated) ( $P<0.001$ ). In the second group, dark IOPs at baseline had mean (SE) values of 15.0 (0.6) mm Hg in control (pre-injected scAAV2.*GFP*, right eye CI 1.5) and 15.0 (0.5) mm Hg in scAAV2.*dnRhoA* pre-injected eyes (treated left eye CI 1.1) (treated). Integral nocturnal IOPs at the end of the experiment (42 days) had mean (SE) values of 698.8 (11.7) mm Hg-days (control) and 686.2 (9.1) mm Hg-days (treated) ( $P=0.08$ ).

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