Evaluation of trabecular meshwork-specific promoters in vitro and in vivo using scAAV2 vectors expressing C3 transferase

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Abstract

- **AIM:** To evaluate the potential of two trabecular meshwork (TM)-specific promoters, Chitinase 3-like 1 (Ch3L1) and matrix gla protein (MGP), for improving specificity and safety in glaucoma gene therapy based on self-complementary AAV2 (scAAV2) vector technologies.

- **METHODS:** An scAAV2 vector with C3 transferase (C3) as the reporter gene (scAAV2-C3) was selected. The scAAV2-C3 vectors were driven by Ch3L1 (scAAV2-Ch3L1-C3), MGP (scAAV2-MGP-C3), enhanced MGP (scAAV2-eMGP-C3) and cytomegalovirus (scAAV2-CMV-C3), respectively. The cultured primary human TM cells were treated with each vector at different multiplicities of infections. Changes in cell morphology were observed by phase contrast microscopy. Actin stress fibers and Rho GTPases/Rho-associated protein kinase pathway-related molecules were assessed by immunofluorescence staining, real-time quantitative polymerase chain reaction and Western blot. Each vector was injected intracameraly into the one eye of each rat at low and high doses respectively. In vivo green fluorescence was visualized by a Micron III Retinal Imaging Microscope. Intraocular pressure (IOP) was monitored using a rebound tonometer. Ocular responses were evaluated by slit-lamp microscopy. Ocular histopathology analysis was examined by hematoxylin and eosin staining.

- **RESULTS:** In TM cell culture studies, the vector-mediated C3 expression induced morphologic changes, disruption of actin cytoskeleton and reduction of fibronectin expression in TM cells by inhibiting the Rho GTPases/Rho-associated protein kinase pathway-related molecules were assessed by immunofluorescence staining, real-time quantitative polymerase chain reaction and Western blot. Each vector was injected intracameraly into the one eye of each rat at low and high doses respectively. In vivo green fluorescence was visualized by a Micron III Retinal Imaging Microscope. Intraocular pressure (IOP) was monitored using a rebound tonometer. Ocular responses were evaluated by slit-lamp microscopy. Ocular histopathology analysis was examined by hematoxylin and eosin staining.

- **CONCLUSION:** In scAAV2-transduced TM cells, the promoter-driven efficiency of Ch3L1 is close to that of cytomegalovirus, but obviously higher than that of MGP. In the anterior chamber of rat eye, the transgene expression...
pattern of scAAV2 vector is presumably affected by MGP promoter, but not by Ch3L1 promoter. These findings would provide a useful reference for improvement of specificity and safety in glaucoma gene therapy using scAAV2 vector.

- **KEYWORDS:** self-complementary AAV2; chitinase 3-like 1; matrix gla protein; trabecular meshwork; C3 transferase

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**INTRODUCTION**

Elevated intraocular pressure (IOP) is considered as a major risk factor in the progression of glaucomatous optic nerve deterioration. Currently, proper IOP-lowering treatments remain the major therapeutic strategy to prevent or slow down the progressive vision loss for glaucoma[1-2,15-27]. The trabecular meshwork (TM) is one of the main structural components of the conventional outflow pathway, playing an important role in formation and modulation of aqueous humor outflow resistance and IOP of the eye[10]. Therefore, there is an increasing interest and need in developing the IOP-lowering gene therapy approaches with long-term TM-targeted effects. Previous studies have tested several therapeutic genes delivered intracameraly by different viral vectors in order to lower IOP in living animals[6,9,17]. In these studies, gene transfer into the anterior segment has been performed using expression vectors driven by the cytomegalovirus (CMV) promoter, which delivers high and broad-spectrum transgene expression in a different tissues of anterior segment of the eye. As shown in our previous studies, intracameral delivery of the CMV-driven vectors expressing C3 transferase (C3), a protein capable of disrupting actin cytoskeleton by inhibiting Rho GTPase (Rho)/Rho-associated kinase (ROCK) pathway and facilitating aqueous humor outflow via TM, transduced not only TM, but also corneal endothelium and/or iris in the monkey eyes[4,9]. Consequently, some undesirable side effects were found in the tissues other than TM to varying degrees. To avoid undesirable viral vector transduction of ocular tissue, it is critically necessary to identify specific promotes by which the transgene specifically expresses in TM.

Chitinase 3-like 1 (Ch3L1) and matrix gla protein (MGP) were previously confirmed as the tissue specific markers of human TM cells[13-15]. In these studies, the expression of β-galactosidase was specifically restricted in the TM of human perfused anterior segment organ culture after delivery of recombinant adenoviruses containing the LacZ gene driven by either MGP promoter or Ch3L1 promoter. This result indicates that the tissue-specific transgene expression can be controlled by use of different promoters.

In the present study, we evaluated the efficacy and specificity of Ch3L1 and MGP promoters, when compared to CMV, to promote C3 gene expression in the TM in vivo and in vitro using recombinant self-complementary AAV2 (scAAV2) vector. This study would provide a useful reference for improvement of specificity and safety in glaucoma gene therapy using scAAV2 vector.

**MATERIALS AND METHODS**

**Ethical Approval** All animals were maintained and handled in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. Study protocol was approved by Institutional Animal Care and Use Committee of Sichuan Provincial People’s Hospital (AE-14/01.01).

**Viral Vectors** Recombinant scAAV2 vectors expressing either enhanced green fluorescent protein (eGFP) or C3 protein, was prepared by the Beijing Five-plus Molecular Medicine Institute (Beijing, China). Expressions from these scAAV2 vectors were driven by CMV (scAAV2-CMV-C3; scAAV2-CMV-eGFP), MGP (Figure 1; scAAV2-MGP-C3), enhanced MGP (scAAV2-eMGP-C3), Ch3L1 (scAAV2-Ch3L1-C3; Figure 1) and CAG (a combination of chicken beta-actin promoter and CMV immediate-early enhancer; scAAV2-CAG-eGFP) promoter, respectively. Briefly, the pscAAV-CAM (a human CMV enhancer with a chicken beta-actin promoter and mouse parvovirus intron) plasmid was constructed using pAAV2neo as a precursor plasmid. The pscAAV-CAM was used as the basis for the construction of pscAAV-CAM-C3 plasmid. The human Ch3L1 gene with restriction sites and pscAAV-CAM-C3 were digested with XhoI/KpnI (New England Biolabs, Ipswich, MA, USA) and ligated together. The human MGP gene with restriction sites and pscAAV-CAM-C3 were also digested with XhoI/KpnI and then ligated. The eMGP promoter was a modified version of the MGP promoter and designed by the Beijing Five-plus Molecular Medicine Institute. Next, the ligation products were transformed into Escherichia coli JM109 (Takara Bio, Dalian, China) for further replication. The subsequent processes including preparation, identification, purification, and titer determination of recombinant viruses, were performed as previously described[6,9]. The concentration unit of scAAV2 vector is vg/mL (vector genome per milliliter).

Recombinant lentivirus (LV) vectors encoding either GFP alone or GFP and C3 together, were prepared by the Beijing LKL Gene Company (Beijing, China), as previously described[4]. Expressions from these vectors were driven by CMV promoter (LV-CMV-C3-GFP; LV-CMV-GFP). The
concentration unit of LV vector is TU/mL (transducing units per milliliter).

**Cell Culture and Treatments** Primary human TM cells (ScienCell Research Laboratories, Inc., Carlsbad, CA, USA) were cultured in TM cell medium (TMCM; ScienCell Research Laboratories, Inc.) at 37°C in 5% CO₂, as previously described [4,6,9]. Group assignments and summary of transducing conditions are described in Table 1. Cells of passages 4 to 5 were transduced with several multiplicities of infections (MOIs) of the scAAV2-CMV-C3, scAAV2-Ch3L1-C3, scAAV2-eMGP-C3 or scAAV2-MGP-C3 vectors. At 24, and 48h after treatment, cells were harvested and processed for subsequent analyses. Images of morphology and fluorescence were taken using an Olympus CKX53 inverted fluorescence microscope (Olympus, Tokyo, Japan).

**RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction Analysis** Real-time quantitative polymerase chain reaction assays were performed as described previously with some modifications[9]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. The relative expression for each mRNA was calculated using the expression 2^(-ΔΔCt) method. The specific gene products were amplified using the following primer pairs: C3-forward, 5’-TACAAATATCGACCAGGCAAAGGC-3’; C3-reverse, 5’-GCCCCCTTGGCCACCTTAAACCTT-3’; RhoA-forward, 5’-GGATCTTCGGAATGATGAC-3’; RhoA-reverse, 5’-TGTTTGCCATATCTCTGCCTT-3’; ROCK-2-forward, 5’-AAGTGGGTTAGTCGGTTG-3’; ROCK-2-reverse, 5’-GGCAGTTAGCTAGGTTTG-3’; GAPDH-forward, 5’-ACCACAGTCCATGCCATCAC-3’; GAPDH-reverse, 5’-TCCACCACCTGTTGCTGTA-3’.

**Western Blot Analysis** Transduced cells were rinsed with 1×Dulbecco’s phosphate-buffered saline (PBS) and incubated in a cold radio immunoprecipitation assay (RIPA) lysis buffer (CWBio, Beijing, China) containing protease/
phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA) on ice for 20min, and then clarified by centrifugation (16 000 g ×10min at 4°C). The protein concentrations were determined using the bicinchoninic acid (BCA) protein quantification kit (Yeasen Biotechnology Co., Ltd., Shanghai, China). A 4%-20% precast gel (Solarbio Science and Technology, Beijing, China) was loaded with 10 µg of protein lysate per lane, electrophoresed, and transferred to a commercial polyvinylidene fluoride membrane (Merck Millipore, Burlington, MA, USA). Membranes were blocked in a 5% non-fat dry milk in Tris-buffered saline and Tween-20 at room temperature for 1h. Membranes were incubated with primary antibodies against RhoA (#2117, rabbit monoclonal antibody, 1:1000; Cell Signaling Technology, Danvers, MA, USA), fibronectin (ab6328, Mouse monoclonal antibody, 1:1000; Abcam, Cambridge, MA, USA) or GAPDH (AF5718, goat polyclonal antibody, 1 µg/mL; R&D Systems, Minneapolis, MN, USA) at 4°C overnight. Immunostained membranes were then incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10 000, ZSGB-Bio, Beijing, China), HRP-conjugated goat anti-mouse IgG (1:20 000, ZSGB-Bio) or HRP-conjugated rabbit anti-goat IgG secondary antibodies (1:20 000, ZSGB-Bio) at room temperature for 1h. Immunostained proteins were exposed with a Fluor ChemE ZSGB-Bio) or HRP-conjugated rabbit anti-goat lgG secondary antibody, 1:10 000; ZSGB-Bio, Beijing, MN, USA) at 4℃ overnight. Immunostained membranes were then incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10 000, ZSGB-Bio, Beijing, China), HRP-conjugated goat anti-mouse IgG (1:20 000, ZSGB-Bio) or HRP-conjugated rabbit anti-goat IgG secondary antibodies (1:20 000, ZSGB-Bio) at room temperature for 1h. Immunostained proteins were exposed with a Fluor ChemE (Cat. No.92-14860-00, ProteinSimple, San Jose, CA, USA). The signal densitometry was carried out by Image J software (Cat. No.92-14860-00, ProteinSimple, San Jose, CA, USA). The signal densitometry was carried out by Image J software.

**Actin Labeling and Immunocytochemistry** The human TM cells grown on climbing slices were washed in 1× Dulbecco’s PBS and immersed in 4% paraformaldehyde (prepared from 8% paraformaldehyde stock solution in pure water; Solarbio Science and Technology) for 15min at 37°C with a water bath. Actin analyses were performed with Rhodamine-phalloidin (7635-01; Hamilton Corp.). Side of injection was randomized.

**In Vivo Imaging of Green Fluorescence in Anterior Segments** The fluorescent image system of a Micron IV Retinal Imaging Microscope (Phoenix Research Lab., Pleasanton, CA, USA) was used to examine the findings of green fluorescence in anterior segments as previously described.

**Clinical Examination** Eyes were examined using a slit lamp biomicroscope (S350, Shanghai MediWorks Precision Instruments Co., Ltd., Hangzhou, China) with an attached camera (EOS 600D, Canon, Inc., Tokyo, Japan).

**Viral Delivery to the Anterior Segment** Rats were anesthetized with 10% chloral hydrate (0.4 mL/100 g body weight; Sigma-Aldrich) given intraperitoneally. Intracameral injections were conducted as described previously, with some modifications. Viral suspensions were delivered to one eye of each rat using a Hamilton glass syringe (10 μL volume; 7803-05; Hamilton Corp., Reno, NV, USA) with a 33-gauge needle (7635-01; Hamilton Corp.). Side of injection was randomized. The noninjected rats (both eyes) were used as blank control group. The grouping details and volume, titer and others of injection were summarized in Table 2.

<table>
<thead>
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<th>Group (injected vector)</th>
<th>Titer, particles/mL</th>
<th>Volume, µL</th>
<th>Dose, particles</th>
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<tr>
<td>1</td>
<td>scAAV2-Ch3L1-C3</td>
<td>6×10⁵</td>
<td>5</td>
<td>3×10⁸</td>
</tr>
<tr>
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<td>scAAV2-CAG-eGFP</td>
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<td>5</td>
<td>3×10⁸</td>
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<td>scAAV2-eMGP-C3</td>
<td>1.26×10⁶</td>
<td>3.7</td>
<td>4.68×10¹¹</td>
</tr>
</tbody>
</table>

The units of vg/mL (vector genome per milliliter) in scAAV2 vector and TU/mL (transducing units per milliliter) in LV vector. scAAV2: Self-complementary AAV2; LV: Lentivirus vector; CMV: Cytomegalovirus promoter; CAG: A combination of chicken beta-actin promoter and cytomegalovirus immediate-early enhancer; MGP: Matrix gla protein promoter; eMGP: Enhanced MGP promoter; Ch3L1: Chitinase 3-like 1 promoter; C3: C3 transferase; eGFP: Enhanced green fluorescent protein.

**Intraocular Pressure Measurement** IOP readings of the conscious rats were measured using the TonoLab rebound tonometer (Icare, Finland, Espoo, Finland) as previously described. Measurements were taken at the same time of the day between 2–4 p.m. on day 0 (before injection), day 3, 7, 14 and 21.
Eye Extraction and Histopathological Analysis  Eyeballs were enucleated from rats following sacrifice and fixed in a formaldehyde, acetic acid, and saline fixative (Wuhan Servicebio Co., Ltd., Wuhan, China). They were imbedded with paraffin, cut in 4-μm sections, and stained with hematoxylin and eosin. Digital images were acquired by an Olympus CKX53 inverted fluorescence microscope (Olympus).

Statistical Analysis  Statistics was done using SPSS (statistical product and service solutions) 18 software (IBM-SPSS, Chicago, IL, USA). Comparisons between two groups were analyzed using two-tailed paired Student’s t-test. Comparisons of multiple groups were performed using one-way analysis of variance. P-values of <0.05 were considered statistically significant. Data are presented as mean±standard error (SE).

RESULTS

Dose-effect Relationship at 24h Post Vector Delivery in Cultured Human TM cells  Human TM cells were transduced with C3-expressing scAAV2 vectors to evaluate the effects of C3 expression on cell morphology, actin cytoskeleton and its associated cellular adhesions. The expression and effects of C3 can also be evaluated as a “reporter gene” since C3 induced those changes are very sensitive and obvious. These C3-expressing scAAV2 vectors were driven by CMV, Ch3L1, eMGP and MGP promoter, respectively. The scAAV2 encoding eGFP gene was driven by CMV and CAG promoter, respectively.

Figure 2 showed the effects of C3 expression on the human TM cells after 24h treatment with different scAAV2 vectors at MOI of 5×10^3, 5×10^4, 2.77×10^4 or 1.37×10^5. At the MOI of 5×10^3, human TM cells transduced with scAAV2-CMV-C3 or scAAV2-Ch3L1-C3 was either elongated or rounded up (Figure 2A). In these two groups, significant expression of C3 mRNA level was detected, a consequent decrease in ROCK-2 mRNA and a modest increase in RhoA protein expression of RhoA and fibronectin were significantly decreased in the scAAV2-CMV-C3-treated cells, and these changes were more significant than those in the scAAV2-eMGP-C3-treated cells (Figure 2C). In the scAAV2-MGP-C3-treated cells, although the transcript of C3 mRNA was present, the changes above were still not observed (Figure 2A-2C). Since the effects were very weak in the cells treated with scAAV2-eMGP-C3 at an MOI of 5×10^4, we selected a MOI of 1.37×10^5 for further transduction. As shown in Figure 2D, the C3 expression-induced effects became more obvious in the cells after treatment of scAAV2-eMGP-C3 at MOI of 1.37×10^5. When the MOI was increased to 1.37×10^6, contraction of cells treated with scAAV2-MGP-C3 started to occur at 24h (Figure 2D). Significant reductions of protein levels in RhoA and fibronectin were also found in the scAAV2-MGP-C3-treated cells, but weaker than that in scAAV2-eMGP-C3-treated cells (Figure 2E). Meanwhile, a large number of floating cells were observed in the human TM cells treated with a MOI of 2.77×10^5 of scAAV2-CMV-C3. This may due to the excessive cell contraction and substantial reduction of the extracellular matrix, and was rare in both groups of cells described above.

In Vitro Fluorescent Signal of the Reporter Protein in Human TM cells Following Vector Transduction  For the in vitro transduction experiments in human TM cells, the scAAV2-CAG-eGFP and scAAV2-CMV-eGFP vectors were used as controls (Figure 4). At 24h post-transduction, the scAAV2-CAG-eGFP-transduced cells revealed higher reporter gene signal (integrated optical density) than that in the scAAV2-CMV-eGFP-transduced cells. When compared to the blank control, no significant changes of cell morphology, C3 mRNA and ROCK-2 mRNA, were found in cells transduced with either scAAV2-CAG-eGFP or scAAV2-CMV-eGFP. This result confirmed that effects of Rho/ROCK pathway inhibition were not induced by scAAV2 itself or scAAV2-mediated promoter expression, but rather the scAAV2-mediated C3 expression.

In vivo Fluorescent Signal of the Reporter Protein in Anterior Segment Following Vector Delivery  Previous animal studies by ourselves and others have demonstrated that scAAV2 vectors containing the transgenes driven by CMV promoter had a high tropism for the cells of the TM, iris and corneal endothelium,[4,18] while lentiviral vectors almost exclusively on the TM.[9,19]

For low-dose experiments, rat eyes were injected intracameral with an equal dose (3×10^5 vg) of scAAV2-Ch3L1-C3, scAAV2-eMGP-C3 and scAAV2-CAG-eGFP, respectively.
Table 2). As controls, LV-CMV-C3-GFP and LV-CMV-GFP were injected separately into rat eyes at a same dose of $2.5 \times 10^6$ TU (Table 2). Meanwhile, a high-dose experiment was also performed to further confirm the initiation capacity of MGP promoter. Rat eyes were injected intracamerally with an equal dose ($4.68 \times 10^{11}$ vg) of scAAV2-MGP-C3 and scAAV2-eMGP-C3, respectively (Table 2). Given the high initiation capacity of CMV promoter in the TM cells, a dose ($9.48 \times 10^{10}$ vg) of scAAV2-CMV-C3 was administrated to the rat eyes as a control. As shown in Figure 4, the features of the fluorescence...
Figure 3 Actin labeling and fibronectin immunofluorescence staining at 24h post-transduction  The human trabecular meshwork cells were transduced with scAAV2-CMV-C3, scAAV2-Ch3L1-C3, scAAV2-eMGP-C3, and scAAV2-CMV-eGFP, respectively. All vectors were delivered to the cells at multiplicities of infection of 5×10^3. Typical cells were outlined and enlarged in dashed boxes. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole (DAPI). Arrows indicate the contracted cell bodies. scAAV2: Self-complementary AAV2; CMV: Cytomegalovirus promoter; MGP: Matrix gla protein promoter; eMGP: Enhanced MGP promoter; Ch3L1: Chitinase 3-like 1 promoter; C3: C3 transferase; eGFP: Enhanced green fluorescent protein. Scale bar =200 μm.

Figure 4 In vitro and in vivo analyses after treatments of fluorescent protein-expressing vectors  A-C: Changes in cell morphology, enhanced green fluorescent protein (eGFP) expression and mRNA levels of C3 transferase (C3) and Rho-associated kinase 2 (ROCK-2) in human trabecular meshwork (TM) cells. All vectors were delivered to the cells at multiplicities of infection of 1×10^4. A: Cell morphology and green fluorescence. Scale bar =200 μm. B: Intensity of green fluorescence was calculated and presented as integrated optical density (IOD). C: mRNA levels of C3 and ROCK-2. Error bars show standard error of mean, and the significance difference was calculated using one-way analysis of variance (ANOVA). *P<0.01. D: In vivo expression of green fluorescent protein in the anterior segment tissues of rats at 15d post-injection. The black dashed lines indicate the position of the TM. To examine corneal fluorescence, 0.5% tropicamide/phenylephrine (Mydrin P; Santen Pharmaceutical Co., Ltd., Osaka, Japan) was used to dilate the pupil for avoiding the interference from the iris fluorescence. ACA: Anterior chamber angle; NAS: Nasal side; TEMP: Temporal side; CE: Corneal endothelium; P: Pupil; scAAV2: Self-complementary AAV2; LV: Lentivirus vector; CMV: Cytomegalovirus promoter; CAG: A combination of chicken beta-actin promoter and cytomegalovirus immediate-early enhancer; eMGP: Enhanced matrix gla protein promoter; Ch3L1: Chitinase 3-like 1 promoter.
distributions in rat eyes were very similar to that described above. The scAAV2-CAG-eGFP-injected eyes also exhibited obvious signals of green fluorescence in TM, iris, and corneal endothelium.

Monitoring Reactions in the Anterior Segments In the low-dose experiment (Figure 5A), none of the groups showed signs of opacity or inflammation at day 3. Subsequently, only the scAAV2-Ch3L1-C3 group exhibited variable degree of corneal opacity.

Figure 5 Slit-lamp examination, intraocular pressure (IOP) response and histological analyses in rat eyes at different timepoints after low-dose vector injections A: Representative images of anterior segment of eyes on day 3 and 14. B: IOP changes on day 0 (baseline), 3 and 14. ΔIOP indicates the difference between IOP before and after transduction. Error bars show standard error of mean, and the significance difference was calculated using two-tailed paired t-test. *P<0.01. C: Representative images of hematoxylin and eosin-staining on anterior chamber angles (ACA) and central corneas on day 21. Trabecular meshwork (TM) and corneal endothelium were outlined and enlarged in dashed box. Hollow arrowheads indicate Schlemm’s canal. Arrows indicate the cell contraction. I: Iris; CB: Ciliary body; scAAV2: Self-complementary AAV2; LV: Lentivirus vector; CMV: Cytomegalovirus promoter; CAG: A combination of chicken beta-actin promoter and cytomegalovirus immediate-early enhancer; eMGP: Enhanced matrix gla protein promoter; Ch3L1: Chitinase 3-like 1 promoter; C3: C3 transferase; eGFP: Enhanced green fluorescent protein. Scale bar =200 μm.
edema at day 14 post-injection. In the high-dose experiment (Figure 6A), obvious corneal edema was found in the scAAV2-CMV-C3 group as early as day 3 (data not shown), and markedly aggravated at day 21 post-injection. However, no abnormalities were observed in scAAV2-MGP-C3 and scAAV2-eMGP-C3 groups. No inflammation was observed in rat eyes post-injection.

**Intraocular Pressure Changes in Rat Eyes**  In the low-dose experiment (Figure 5B), the pretreatment IOP (baseline) was 11.73±0.42, 11.96±0.55, and 11.96±0.37 mm Hg in eyes to be injected with scAAV2-Ch3L1-C3 (n=10), scAAV2-eMGP-C3 (n=9) and LV-CMV-C3-GFP (n=8), respectively. At day 3 post-injection, the eyes injected with scAAV2-Ch3L1-C3 or LV-CMV-C3-GFP showed a significant IOP decrease, when
post-injection, the ΔIOP of LV-CMV-C3-GFP-injected eyes was 2.17±0.41 mm Hg. At day 14 post-injection, the ΔIOP of LV-CMV-C3-GFP-injected eyes was 1.58±0.33 mm Hg. However, corneal edema in scAAV2-Ch3L1-C3-injected eyes caused a higher measured value of IOP (12.17±0.47 mm Hg) and a lower calculated value of ΔIOP (0.43±0.73 mm Hg). This was consistent with our and other previous observations that the corneal thickening can result in higher measured IOP values.[4,20-22]. The IOP did not present significant changes in the scAAV2-eMGP-C3-injected eyes at day 3 and 14 post-injection. The pretreatment IOP (baseline) was 11.79±0.78 and 11.75±0.44 mm Hg in eyes to be injected with scAAV2-CAG-eGFP (n=8) and LV-CMV-GFP (n=8), respectively. No significant IOP changes were observed in these two groups at day 3 and 14 post-injection.

In the high-dose experiment (Figure 6B), the pretreatment IOP (baseline) was 10.61±0.22, 10.54±0.19, and 11.00±0.31 mm Hg in eyes to be injected with scAAV2-CMV-C3 (n=12), scAAV2-MGP-C3 (n=13) and scAAV2-eMGP-C3 (n=12), respectively. At day 14 post-injection, a significant IOP change began to be detected in the scAAV2-eMGP-C3 group, when compared to the baseline, with a ΔIOP of 1.08±0.36 mm Hg. Since obvious corneal edema was occurred as early as day 3, a trend toward higher measured values of IOP was detected on the scAAV2-CMV-C3-injected eyes, and began to be statistically significant at day 14. In the scAAV2-MGP-C3 group, there were no significant IOP changes compared with the baseline.

**Hematoxylin and Eosin Staining of Rat Eye Anterior Segment** In the low-dose experiment (Figure 5C), little difference in the structure of the TM tissues was observed among the blank control eyes and the eyes after treatment of scAAV2-eMGP-C3, scAAV2-CAG-eGFP, and LV-CMV-GFP, respectively. In contrast, the anterior segments transduced with scAAV2-Ch3L1-C3 or LV-CMV-C3-GFP exhibited C3-induced morphological changes in the TM including loose stroma and reduced cellularity. In the high-dose experiment (Figure 6C), morphological differences in the TM were not apparent between the scAAV2-MGP-C3 group and blank control. C3-induced TM morphological characteristics were observed in scAAV2-CMV-C3 group, and began to be visualized in the scAAV2-eMGP-C3 groups. As a result of actin-associated endothelial barrier disruption induced by C3 expression, the scAAV2-Ch3L1-C3-transduced and scAAV2-CMV-C3-transduced eyes showed an edematous corneal stroma with blurring borders of collagen fiber, and the contracted endothelium was loosely connected to the base membrane (Figures 5C and 6C). No abnormalities were found in the other groups (Figures 5C and 6C), indicating the vectors themselves were not able to cause obvious changes in corneal endothelium.

**DISCUSSION**

Glaucoma remains the leading cause of irreversible blindness in the world. Currently, almost all the modalities of management are focused on reduction of IOP, the major risk factor of the disease. However, the current medical lowering IOP strategies exhibit a number of problems. As an example, patients usually require more than 1 medication on a daily basis over time in order to lower the IOP, and may have hard time with adherence to multiple topical drop regimens. In addition, there are a number of side effects related to these medications. Therefore, much attention has been given to development of gene therapy, with a hope to lower the IOP at a relatively long duration after single treatment.

For IOP-lowering gene therapy, it is critical to use vectors capable of targeting TM, the conventional aqueous humor drainage pathway which account for major outflow resistance in human, when administered to anterior chamber. However, other than effectiveness of transgene expression, the specificity of gene targeting remains the major challenge. Previous in vivo studies[7,11,18,23] showed that intracameral delivery of the scAAV2 vectors (containing CMV promoter in most of these studies) transduced not only the TM, but also the corneal endothelium and iris without inducing an inflammatory response and other abnormalities. Our current and previous results were in agreement with these findings, and confirmed the scAAV2 vector possessed a broad-spectrum transduction of anterior chamber tissues in mice, rats and monkeys[4]. Moreover, in comparison with LV vector-mediated C3 expression system, scAAV2 vector-mediated C3 expression yielded a longer-lasting IOP-lowering effect (at least 4mo in LV-C3 vector versus at least 10mo in scAAV2-C3 vector)[7,49]. Therefore, as long as the duration of effectiveness is considered, scAAV2 vector may hold greater potential in application of glaucoma gene therapy for glaucoma in the future.

One critical challenge for bringing scAAV2-mediated C3 expression system into clinical trials involves equipping both significant protein expression (efficacy) and high TM-specificity (safety). The choice of the proper or specific promoter to initiate transgene expression in the TM remains a key issue in glaucoma gene therapy. Previous studies using the recombinant adenoviruses containing the LacZ gene driven by either MGP promoter or Ch3L1 promoter resulted in high transgene expression levels and specificity in the TM of perfused human anterior segments[13-15]. In the current study, the Ch3L1 promoter was similar to the CMV promoter in its ability to drive C3 protein expression in the TM, both in vivo and in vitro, but was far superior to the eMGP and MGP promoters. Furthermore, the Ch3L1 promoter did not change the tissue tropism of scAAV2 vector, and initiated C3
gene expression not only in the TM but also in the corneal endothelium. The reason why our findings are different from previous studies is not clear, but several factors might be involved. First, different viral vectors were used. When the CMV promoter was employed, the adenoviral vector had high tropism in several types of the anterior chamber tissues similar to the scAAV2 vector.[24-25] Interestingly, substituting CMV promoter for Ch3L1 promoter resulted in a lack of adenovirus-mediated transgene expression in the corneal endothelium, but failed to affect scAAV2-mediated transgene expression in these cells, as described in the previous[15] and current studies. As shown in the Figure 4, the CAG promoter was also selected for driving reporter gene (eGFP) in the scAAV2 vector and had no effect on the distribution of the fluorescence (eGFP expression) in the anterior chamber post-injection. A possible explanation for these differences is that promoter such as CMV, Ch3L1 or CAG had different effects on the patterns of transgene expression mediated by different viral vector genes in the anterior chamber tissues. Second, different experimental animals were used. The differences in anatomical spaces of the anterior chamber and/or aqueous humor dynamics may affect the transduction pattern of viral vector. Since volume and outflow facility of anterior chamber in human were about ten times higher than that in rat, the deliveries of viral particles to the outflow pathways were more efficient.[25-28] In other words, longer residence times of viral particles in anterior chamber may result in greater uptake by corneal endothelial cells or other cells. Furthermore, in the anterior chamber tissues, the Ch3L1 promoter preferentially expresses in the TM,[13], and is also detected in corneal endothelium when stimulated by fungal infection. This expression is regulated via antifungal innate immune responses in the cornea[20] indicating that the Ch3L1 promoter may be active in corneal endothelial cells under some conditions. Further studies are needed to ascertain whether Ch3L1 was expressed in the viral vector-transduced cornea without inflammation. These results may explain why transgene expression was detected in the corneal endothelium of rat eyes injected with the scAAV2-mediated C3 expression driven by Ch3L1 promoter, but not in that of human eyes injected with the adenovirus-mediated β-galactosidase expression driven by same promoter.

Previous reports have demonstrated abundant MGP expression not only in the TM but also in the corneal endothelium, which in turn initiated adenovirus-mediated β-galactosidase expression efficiently in the TM cells[13,30]. However, our results showed that the transgene expression was very inefficient in the TM cells transduced with scAAV2 vectors controlled by either MGP promoter or eMGP promoter. The reason for this difference was unclear and may also be related to the different viral vectors used.

Fibronectin fibrils are a major component of the extracellular matrix in the TM, and its abnormal accumulation is thought to increase IOP through several complex pathways[31-32]. The Rho/ROCK pathway play an important role in the IOP modulation, as demonstrated by the significant increase of outflow facility and reduction of IOP after treatment of its inhibitors[4,6,9,33-34]. Fujimoto et al[35] showed that a selective ROCK inhibitor blocked the increased fibronectin expression induced by dexamethasone in TM cells. RhoA is a major upstream signaling molecule of ROCK, and our results revealed that the C3-induced RhoA inhibition disrupted the actin cytoskeleton and reduced the fibronectin expression in the human TM cells. Thus, the C3 gene therapy may be effective to reduce IOP in eyes with steroid-induced ocular hypertension and glaucoma. In contrast to decreased levels of RhoA protein, significant transcription levels of RhoA gene (increased mRNA levels) were detected. Correspondingly, one of its downstream effectors, ROCK-2, showed reductions of mRNA levels as well. Similar discrepancies have also been reported in other studies[36-37] and could be due to a feedback autoregulation of signaling pathway in cells. Although the specific mechanism was unknown, at least in TM cells, decreased levels of RhoA protein were observed. This was likely caused by C3-induced RhoA inactivation which was accompanied by increased mRNA expression.

Currently, classic pressure-dependent rat models generated by a series of methods, including intracamerlal injection of microbead[38-40] or viscous agent[41-42], laser photococagulation of outflow pathway[43-44], cautery of extraocular veins[45-46], glucocorticoid induction[47-48], transduction of the TM with glaucoma related genes[49-51], or in combination with each other[52-53], are widely used to understand the pathogenic mechanism of glaucoma, and regarded as relatively cost-efficient tools for developing potential glaucoma therapies. In this study, normal rats were used to evaluate initiation efficiencies of two TM-specific promoters in the scAAV2 vector. This was chosen because simpler methods may help to decrease some uncertainties during tissue-specific observations. Previous studies have reported that glaucoma with higher baseline IOP showed greater IOP reduction after IOP-lowering treatment[49-51]. Nonetheless, the initiation efficiency of scAAV2 vector-mediated gene expression initiated by MGP promoter in TM did exhibit a relatively weaker effect compared with the Ch3L1 promoter or the commonly used CMV promoter.

In summary, in scAAV2-transduced TM cells, the promoter-driven efficiency of Ch3L1 was close to that of CMV, but obviously higher than that of MGP. In the anterior chamber of rat eye, the transgene expression pattern of scAAV2 vector was presumably affected by MGP promoter, but not by Ch3L1 promoter. These findings would provide a useful reference.
for improvement of specificity and safety in glaucoma gene therapy using scAAV2 vector.

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REFERENCES


