TGF- β 2–Mediated Ocular Hypertension Is Attenuated in SPARC-Null Mice

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Submitted: May 22, 2013
Accepted: May 21, 2014

Citation: Swaminathan SS, Oh D-J, Kang MK, Shepard AR, Pang I-H, Rhee DJ. TGF- β 2-mediated ocular hypertension is attenuated in SPARC-null mice. *Invest Ophthalmol Vis Sci*. 2014;55:4084–4097. DOI:10.1167/iops.13-12463

PURPOSE. Transforming growth factor- β 2 (TGF- β 2) has been implicated in the pathogenesis of primary open-angle glaucoma through extracellular matrix (ECM) alteration among various mechanisms. Secreted protein acidic and rich in cysteine (SPARC) is a matricellular protein that regulates ECM within the trabecular meshwork (TM), and is highly upregulated by TGF- β 2. We hypothesized that, in vivo, SPARC is a critical regulatory node in TGF- β 2-mediated ocular hypertension.

METHODS. Empty (Ad.empty) or TGF- β 2-containing adenovirus (Ad.TGF- β 2) was injected intravitreally into C57BL/6-SV129 WT and SPARC-null mice. An initial study was performed to identify a stable period for IOP measurement under isoflurane. The IOP was measured before injection and every other day for two weeks using rebound tonometry. Additional mice were euthanized at peak IOP for immunohistochemistry.

RESULTS. The IOP was stable under isoflurane during minutes 5 to 8. The IOP was significantly elevated in Ad.TGF- β 2-injected ($n = 8$) versus Ad.empty-injected WT ($n = 8$) mice and contralateral uninjected eyes during days 4 to 11 ($P < 0.03$). The IOPs were not significantly elevated in Ad.TGF- β 2-injected versus Ad.empty-injected SPARC-null mice. However, on day 8, the IOP of Ad.TGF- β 2-injected SPARC-null eyes was elevated compared to that of contralateral uninjected eyes ($P = 0.0385$). Immunohistochemistry demonstrated that TGF- β 2 stimulated increases in collagen IV, fibronectin, plasminogen activator inhibitor-1 (PAI-1), connective tissue growth factor (CTGF), and SPARC in WT mice, but only PAI-1 and CTGF in SPARC-null mice ($P < 0.05$).

CONCLUSIONS. SPARC is essential to the regulation of TGF- β 2-mediated ocular hypertension. Deletion of SPARC significantly attenuates the effects of TGF- β 2 by restricting collagen IV and fibronectin expression. These data provide further evidence that SPARC may have an important role in IOP regulation and possibly glaucoma pathogenesis.

Keywords: SPARC, matricellular proteins, TGF- β 2, intraocular pressure

Primary open-angle glaucoma (POAG) is a disease characterized by painless vision loss due to retinal ganglion cell death, affecting over 70 million individuals worldwide.^{1,2} Elevated IOP is the only modifiable risk factor,^{3,4} and is due to poor outflow through the trabecular meshwork (TM), known as the conventional outflow pathway.⁵ Approximately 85% of aqueous humor exits the anterior chamber through the TM, while the remaining 15% exits through the uveoscleral pathway.⁶ Reducing IOP has been shown in numerous studies to delay or prevent the progression of glaucoma.^{7,8} In conjunction with the inner wall endothelium of Schlemm's canal, the juxtacanalicular connective tissue (JCT) region of the TM has been shown to be essential in modulating aqueous humor outflow resistance and IOP.⁹ However, the exact mechanisms by which the TM regulates IOP remain unknown.

Extracellular matrix (ECM) proteins are thought to be involved, and have been shown to affect IOP when altered within the JCT.^{10–14} Matricellular proteins are secreted glyco-

proteins that modulate ECM deposition in many tissues throughout the body, including the TM.¹⁵ The family of matricellular proteins includes the thrombospondins-1 and -2, tenascins-C and -X, hevin, and Secreted Protein Acidic and Rich in Cysteine (SPARC).^{16–18} SPARC is expressed strongly in the TM, especially within the JCT.¹⁹ SPARC knockout (KO) mice have a 15% to 20% lower IOP and a significant increase in the amount of TM used for outflow.^{20,21} SPARC KO eyes also have a decreased collagen fibril diameter within the JCT.²¹ Overexpression of SPARC increases IOP in perfused human cadaveric anterior segments, which correlates with an increase in fibronectin, and collagens I and IV in the JCT.²² Among other factors, TGF- β 2 regulates SPARC via Smad 2/3, JNK, and p38 pathways in human TM cells.^{23,24}

A critical cytokine in development and tissue growth, TGF- β 2 has been shown to modulate multiple ECM proteins.^{25–30} Clinically, TGF- β 2 is elevated approximately 2- to 3-fold in the aqueous humor of POAG patients.^{31–34} In vitro, TGF- β 2 is

significantly elevated in cultured glaucomatous TM cell lines compared to normal TM cell lines.³⁵ In perfused human cadaveric anterior segments, treatment with TGF- β 2 in the perfusion medium elevates IOP and decreases outflow facility.^{28,36} Additionally, in mouse and rat models, injection of TGF- β 2-containing adenovirus leads to a significant increase of IOP.²⁹ The increases in IOP are likely related to changes in ECM, as fibronectin and plasminogen activator inhibitor-1 (PAI-1) are upregulated by TGF- β 2 in the TM of perfused cadaveric anterior segments and mouse eyes.^{29,30,36–41} Thus, TGF- β 2 and ECM alteration appear to have a critical role in POAG development.

In TM cells stimulated with TGF- β 2, SPARC is the most highly upregulated protein.²⁷ Thus, SPARC may be a critical regulatory node in TGF- β 2-mediated ocular hypertension. We hypothesized that TGF- β 2-mediated ocular hypertension would be attenuated in the SPARC-null mouse, and that corresponding morphologic changes would occur within the iridocorneal angle and TM.

METHODS

Animal Husbandry

All experiments were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of Animals in Ophthalmic and Vision Research. Local Institutional Animal Care and Use Committee (IACUC) approval was obtained. The SPARC-null and corresponding C57BL6-SV129 wild type (WT) mice were originally obtained as a generous donation from E. Helene Sage of the Benaroya Research Institute at Virginia Mason (Seattle, WA, USA). Heterozygotes were bred together, and their offspring subsequently were genotyped to identify homozygous WT and SPARC KO mice. In addition, WT and KO breeding pairs were used to increase offspring yield for no more than 1 to 2 generations to limit genetic drift. Animals used in these experiments were between 5 and 8 weeks in age, and were housed in the animal biosafety level-2 suite of the Schepens Eye Research Institute animal facility.

IOP Measurement Under Anesthesia

To demonstrate that IOP remains stable under an inhaled anesthetic, measurements were taken using isoflurane. Age- and temporally-matched WT and KO mice were used. Each mouse was anesthetized with 2% to 4% isoflurane (IsoSol; Vedco, St. Joseph, MO, USA) in 1 to 2 L/min 100% oxygen in a closed chamber; a timer was started when isoflurane was released into the chamber. After exactly 70 seconds, the sedated mouse was moved to a facemask, through which it received 1% to 1.5% isoflurane mixed with the same concentration of oxygen. The IOP was measured every minute between 4 and 15 minutes on either the left or right eye (randomly chosen for each mouse) without adjusting the stand or tonometer. The IOP was not measured before 4 minutes because the mouse was not sufficiently anesthetized before this time to allow corneal contact. Artificial tears were not administered, as a previous trial demonstrated that it caused aberrations in IOP measurement (data not shown). The cornea appeared to be normal under the study conditions.

IOP Measurement

Rebound tonometry was used to measure IOP. Our measurement technique and its accuracy have been described in detail previously.^{20,21,42} Briefly, the anesthetized mouse was placed on a movable stand (BrandTech Support Jack; BrandTech

Scientific, Essex, CT, USA) with its nose inside the facemask. The TonoLab tonometer (Colonial Medical Supply, Franconia, NH, USA) was fixed horizontally and a remote pedal was used to actuate measurements to eliminate potential artifact caused by manual handling of the device. The mouse was positioned to ensure the probe contacted the central cornea perpendicularly. A set of 6 measurements was repeated three times, and the modes of each set were averaged; this value was recorded as the IOP. All measurements and injection experiments were conducted between 11 AM and 3 PM to minimize any potential artifact from circadian variability.^{19,20} For eyes that received an injection, IOP of both eyes were measured the day before injections and every other day after intravitreal injection starting on the fourth day and ending on the 13th day.

Intravitreal Injection

Adenoviruses containing either an empty vector or an hTGF- β 2 mutant constructs were generated by Allan Shepard (Ad5.CMV.hTGF- β 2mut and Ad5.CMV.empty).²⁹ The hTGF- β 2 construct was mutated at amino acids 226 and 228 to create constitutively active hTGF- β 2 in the tissue; additional details on engineering of the adenoviruses have been reported previously.²⁹ The mouse was anesthetized with an intraperitoneal injection of 5 μ L/g ketamine/xylazine mixture (90 mg/kg ketamine + 9 mg/kg xylazine; Webster Veterinary Supply, Devens, MA, USA). After reaching a suitable level of anesthesia (confirmed by the absence of toe-pinch reflex), the mouse was positioned on a mounting stage (Mouse and Neonatal Rat Adaptor; Stoelting Co., Wood Dale, IL, USA). Using a 10 μ L Hamilton microsyringe (Nanofil; World Precision Instruments, Sarasota, FL, USA) connected to a 35-gauge needle (NF35BL-2, World Precision Instruments), empty (hereby referred to as Ad.empty) or TGF- β 2-containing adenovirus (hereby referred to as Ad.TGF- β 2) was injected into the intravitreal space of the right eye, just posterior to the limbus, for a final viral titer of 6×10^6 plaque-forming units (pfu; 1.5 μ L of 4×10^9 pfu/mL). The volume was delivered at 4 nL/s by a microprocessor-based microsyringe pump controller (Micro4; World Precision Instruments). Lubricating eye drops (Nature's Tears; Rugby Laboratories, Duluth, GA, USA) were applied to the cornea to prevent dehydration during the injection. After completion of the injection, the needle was rapidly withdrawn, and the presence of the needle in the desired space was confirmed with observation of vitreous on the needle tip. One drop each of proparacaine 0.5% (Alcaine; Alcon, Fort Worth, TX, USA), ofloxacin 0.3% (Akorn, Lake Forest, IL, USA), and prednisolone acetate 1% (Pacific Pharma, Irvine, CA, USA) was applied to the injected eye to minimize pain, risk of infection, and inflammation respectively, in accordance with IACUC policy. Prednisolone was necessary to prevent the formation of corneal edema; a pilot study demonstrated that without an anti-inflammatory, injection of either Ad.TGF- β 2 or Ad.empty led to significant corneal edema, thereby confounding IOP measurements using rebound tonometry (data not shown).

Tissue Processing and Histology

After determining the overall IOP trend in a cohort of WT and KO mice injected with TGF- β 2 over approximately 2 weeks, the peak IOP was noted to be 8 days after injection. A smaller cohort of mice subsequently was injected with Ad.empty and Ad.TGF- β 2 for morphologic analysis specifically at this time point of 8 days. At that time, the mice were euthanatized with CO₂, and their eyes were enucleated immediately for fixation in 10% formalin (VWR International, Radnor, PA, USA) for 48 hours at room temperature, followed by 70% ethanol at 4°C. Eyes then were processed and embedded in paraffin wax for

FIGURE 1. Measurements of IOP under isoflurane. The IOP is relatively stable after the fifth minute. The difference in IOP between WT and KO is statistically significant at all time points ($n = 8$). All IOPs in further experiments were measured between minutes 5 and 8 (red). Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

serial sectioning of 5- μ m thick sections, which were stained with hematoxylin and eosin.

Immunohistochemistry

The following antibodies were used in this study: collagen I (600-401-103-0.1; Rockland, Gilbertsville, PA, USA), collagen IV (AB756P; EMD Millipore, Billerica, MA, USA), collagen VI (SAB4500387; Sigma-Aldrich, St. Louis, MO, USA), fibronectin (ab23750; Abcam, Cambridge, MA, USA), laminin (AB2034; EMD Millipore), PAI-1 (ab28207; Abcam), connective tissue growth factor (sc-14939; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), SPARC (AF942; R&D Systems, Minneapolis, MN, USA), TGF- β 2 (ab36495; Abcam). Sections were washed with xylene and subsequently hydrated with ethanol dilutions (100%, 95%, 70%). Tissue then was blocked in 5% bovine serum albumin (when probing for fibronectin) or 10% donkey serum (all other antibodies) for 1 hour at room temperature. Animal serum contained fibronectin epitopes, making it incompatible with the fibronectin antibody. Tissue then was permeabilized using 0.2% Triton X-100 and primary antibody was applied at 1:100 concentration overnight at 4°C. Slides subsequently were washed with $\times 1$ PBS-T and 1:200 secondary antibody was applied for 1 hour at room temperature. Slides were washed once again, and the tissue was imaged using the Zeiss Axiovert 200M inverted fluorescent microscope (Carl Zeiss Meditec; Heidelberg, Germany) attached to a digital camera (AxioCam MR3; Carl Zeiss Meditec). Images were obtained using the associated software (Axiovision 4.8.2; Carl

Zeiss Meditec). Slides of tissue treated with Ad.empty and Ad.TGF- β 2 were imaged at the same time using the same control slide to minimize background.

Fluorescence Intensity Measurements

Quantification of fluorescence was completed using previously established methodology.^{22,43–45} Briefly, the section stained with secondary antibody only was used to identify optimal exposure times for target ECM proteins (Alexa Fluor 488; Life Technologies, Grand Island, NY, USA) and TGF- β 2 (Alexa Fluor 594; Life Technologies) that would eliminate signal due to nonspecific binding. These exposure times were used to image fluorescence from the target protein and TGF- β 2 expression in all other sections. Three areas within the TM were selected at random for each section, and ImageJ (National Institutes of Health [NIH], Bethesda, MD, USA) was used to calculate the average fluorescence in the 488 and 594 channels using a rectangular area of interest with consistent dimensions ($w0.40 \times h1.20 \mu$ m).

Immunoblotting

Additional intravitreal injections with Ad.empty or Ad.TGF- β 2 were completed, and the mice were euthanized 8 days after injection (at the time of peak IOP). Eyes were enucleated and placed in 100 μ L $\times 1$ Dulbecco's modified Eagle medium. Each globe was dissected, with the anterior segment isolated from the lens and posterior segment. Anterior segments then were placed in 50 μ L $\times 1$ radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors and homogenized. The solution was centrifuged at room temperature for 5 minutes at 13,500g. Supernatant was isolated and equal amounts of total protein were mixed with $\times 2$ SDS reducing buffer (125 mM of Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue). Using 8% polyacrylamide gels, SDS-PAGE followed by immunoblotting were performed as described previously.²³

Statistical Analyses

All data were analyzed in Microsoft Excel (Microsoft, Redmond, WA, USA) or Prism 6 (GraphPad, La Jolla, CA, USA). Two-way ANOVA with multiple comparisons followed by post hoc Bonferroni correction were completed to compare the three treatment groups to obtain the adjusted P value. Percentage of IOP change in WT compared to KO was calculated and assessed for statistical significance using the 2-tailed unpaired Student's t -test. Statistical analyses for immunohistochemistry data were completed using paired Student's t -tests, as each

FIGURE 2. Immunoblot analysis of anterior segment tissue for TGF- β 2 and SPARC. (A) Representative immunoblot bands demonstrating a significant increase in TGF- β 2 with Ad.TGF- β 2 infection in WT and KO tissue. SPARC also was significantly increased by approximately 56% with Ad.TGF- β 2 infection. (B) Densitometric quantification of immunoblot bands for TGF- β 2 ($n = 4$ /group). TGF- β 2 was increased significantly in WT tissue ($P = 0.0023$) and KO tissue ($P = 0.0141$).

FIGURE 3. (A) Effects of Ad.TGF- β 2 injection on WT mice. The IOPs of uninjected mice and mice injected with Ad.empty also are shown. The contralateral eye in the two treatment groups served as uninjected controls ("uninj."). When compared to the IOPs of Ad.empty-injected WT mice, the IOP increase of Ad.TGF- β 2-injected WT mice is significant during days 4 to 11. (B) Effects of Ad.TGF- β 2 injection on KO mice. Compared to the IOPs of Ad.empty-injected KO mice, the IOP increase of Ad.TGF- β 2-injected KO mice is not statistically significant at any time point. Data are presented as mean $\pm$ SEM. * P < 0.05, *** P < 0.001.

individual group of tissue (i.e., WT Ad.empty, WT Ad.TGF- β 2, KO Ad.empty, KO Ad.TGF- β 2) was treated together, but at a separate time from other groups comprising the total sample size. Paired t -tests were used to allow for appropriate comparison.

RESULTS

Under isoflurane anesthesia, IOP measurements were consistent and stable between minutes 5 and 8 (Fig. 1). The

FIGURE 4. Comparison of percentage increase in IOP of WT and KO mice injected with Ad.TGF- β 2 (n = 8). The difference was statistically significant at days 4, 6, and 8. Data are presented as mean $\pm$ SEM. * P < 0.05.

difference of IOP between WT and SPARC KO mice was reproducible at all time points (P < 0.05 at all points, n = 8). Mean WT IOP during minutes 5 to 8 was 16.5 ± 2.5 mm Hg, while mean KO IOP was 12.4 ± 1.1 mm Hg (P = 0.0057). The mean percentage IOP decrease in KO eyes was $24.6\% \pm 0.7\%$.

Infection of the TM by TGF- β 2 was verified with immunoblotting (Fig. 2). TGF- β 2 was increased significantly in WT and KO eyes treated with Ad.TGF- β 2 compared to eyes treated with Ad.empty (n = 4/group, P = 0.0023 and P = 0.0141, respectively). There was no significant difference in TGF- β 2 expression levels between WT and KO Ad.empty-injected eyes (P = 0.7293). SPARC also was noted to increase significantly in WT tissue by 56.2% with Ad.TGF- β 2 infection (P = 0.0425).

In WT mice, a significant difference in IOPs between the Ad.TGF- β 2-injected and Ad.empty-injected eyes, and between the Ad.TGF- β 2-injected and uninjected contralateral eyes was detected (P < 0.0001, Fig. 3). Ad.TGF- β 2-injected WT mice had an increase of IOP at the fourth day after injection (day 4), day 6, day 8, and day 11 when compared to the Ad.empty-injected WT mice (P = 0.0166, 0.0230, <0.0001, 0.0230, respectively; Fig. 3). When compared to the IOPs of uninjected contralateral eyes, Ad.TGF- β 2-injected eyes had a significant increase during the same period. The Ad.empty-injected WT eyes had no significant increase compared to the uninjected contralateral eyes (Table 1).

A difference in IOP also was observed among the treatment groups in KO mice (P = 0.0023). In contrast to WT mice, Ad.TGF- β 2-injected KO mice had a mild elevation in IOP that was not significant compared to Ad.empty-injected KO mice (Fig. 3; Table 1). If Ad.TGF- β 2-injected KO eyes were compared to uninjected contralateral eyes, the change in IOP became statistically significant only at day 8 (P = 0.0385).

TABLE 1. P Values From Two-Way ANOVA With Bonferroni Correction Between the Three Treatment Groups in WT and KO Mice

Day	-1	4	6	8	11	13
WT						
Ad.TGF- β 2 vs. Ad.empty	>0.9999	0.0166	0.0230	<0.0001	0.0230	0.3522
Ad.TGF- β 2 vs. uninj.	>0.9999	0.0003	0.0004	<0.0001	0.0007	0.2800
Ad.empty vs. uninj.	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
KO						
Ad.TGF- β 2 vs. Ad.empty	>0.9999	>0.9999	>0.9999	0.7518	0.7517	>0.9999
Ad.TGF- β 2 vs. uninj.	>0.9999	0.7150	0.1684	0.0385	0.1196	>0.9999
Ad.empty vs. uninj.	0.9059	>0.9999	0.9059	0.7150	>0.9999	>0.9999

FIGURE 5. Representative iridocorneal angle morphology of WT and KO mice 8 days after injection with either Ad.empty or Ad.TGF- β 2. The iridocorneal angle remained open in all 4 groups. Scale bar: 50 μ m.

The peak of IOP increase was noted to be at day 8 for WT and KO eyes. At this time, the average IOP of Ad.empty-injected WT IOP was 14.3 mm Hg, Ad.TGF- β 2-injected WT IOP 18.3 mm Hg ($P < 0.0001$), Ad.empty-injected SPARC-null IOP 12.1 mm Hg, and Ad.TGF- β 2-injected SPARC-null IOP 13.0 mm Hg ($P = 0.7518$).

The difference in percentage of IOP increase between WT and SPARC-null eyes was significant at days 4, 6, and 8. At day 8, the percentage increase of IOP from baseline was $28.5\% \pm 4.2\%$ in WT mice and $14.4\% \pm 4.9\%$ in KO mice ($P = 0.0444$, $n = 8$; Fig. 4).

No significant difference was observed among all 4 experimental groups using light microscopy (Fig. 5). In contrast to previous findings regarding TGF- β 2 injection in mice,²⁹ the iridocorneal angle remained open in all groups. No synechiae were observed in the anterior chamber.

Immunohistochemistry was performed on whole eyes to demonstrate tissues infected by Ad.TGF- β 2. Injected mice were sacrificed at peak IOP, 8 days after adenoviral injection. The WT and KO eyes that were uninjected, injected with Ad.empty, or injected with Ad.TGF- β 2 were sectioned and stained for TGF- β 2 ($n = 3$ for each condition; Fig. 6). Qualitatively, TGF- β 2 staining was similar between uninjected and Ad.empty-injected

FIGURE 6. Sagittal sections of representative WT and KO tissue stained for TGF- β 2 ($n = 3$ for each condition). In WT and KO mice, Ad.empty-injected eyes had a similar staining pattern to uninjected eyes. Ad.TGF- β 2-injected eyes demonstrated a marked increase in staining at day 8 within the retina, vitreous humor, and TM. However, this effect was nullified at day 13. Scale bar: 800 μ m.

FIGURE 7. Changes in fluorescence intensities of select ECM proteins in (A) WT mice (B) KO mice 8 days after treatment with Ad.empty or Ad.TGF- β 2 ($n = 3$). In WT tissue, the intensities of collagen IV, fibronectin, PAI-1, CTGF, and SPARC were significantly increased following TGF- β 2 stimulation (asterisks, $P < 0.05$). In KO tissue, only PAI-1 and CTGF fluorescence increases were significant with TGF- β 2 stimulation (asterisks, $P < 0.05$). Changes in collagen IV and fibronectin levels were nonsignificant. SPARC was not found in SPARC KO mice (B).

eyes in WT and KO tissue. Staining was increased in Ad.TGF- β 2-injected eyes on day 8 within the retina, vitreous humor, and TM. Additional Ad.TGF- β 2-injected mice were sacrificed on day 13 and their eyes were stained for TGF- β 2 to demonstrate a reduction in TGF- β 2 expression to levels similar to Ad.empty-injected eyes ($n = 3$ for each condition; Fig. 6).

Following TGF- β 2 stimulation in WT mice, the fluorescence intensities of collagen IV, fibronectin, PAI-1, connective tissue growth factor (CTGF), and SPARC were noted to increase significantly 8 days after injection compared to WT mice injected with Ad.empty ($P < 0.05$, $n = 3$; Figs. 7–9). Collagen I and laminin intensities also were noted to increase, but were not significant. No change was noted in collagen VI levels. When assessing the fluorescence of the same proteins in KO mice 8 days after injection, only the intensities of PAI-1 and CTGF increased significantly with injection of Ad.TGF- β 2 versus Ad.empty ($P < 0.05$, $n = 3$; Figs. 7–9). The collagen IV and fibronectin increases observed in WT mice were attenuated in KO mice and did not achieve statistical significance (Table 2).

DISCUSSION

SPARC appears to have a key role in TGF- β 2-mediated ocular hypertension in mice. When compared to eyes injected with Ad.empty, Ad.TGF- β 2-injected KO eyes demonstrated no significant elevation in IOP. In contrast, the IOP of WT eyes injected with Ad.TGF- β 2 increased significantly. When compared to the contralateral uninjected eyes, Ad.TGF- β 2-injected KO eyes had a substantial delay of IOP elevation (significant at only one time point, day 8), a blunting of the magnitude of increase, and shortening of the increase to only one time point. The failure to completely inhibit the IOP response in Ad.TGF- β 2-injected KO mice likely represents redundancy among extracellular matrix proteins. SPARC, thrombospondins-1 and -2, tenascins-C and -X, hevin, and osteopontin all have been shown to have important roles in affecting ECM assembly, matrix metalloproteinase (MMP) activation, and collagen fibrillogenesis.^{15,46,47} In addition, TGF- β 2 also is thought to act through other pathways, such as inducing TM cell senescence or altering the actin cytoskeleton.^{24,48,49} It is likely that these pathways still are active with TGF- β 2 injection in the SPARC KO mouse.

Our results seem to confirm the previously reported increases in PAI-1 and fibronectin in Ad.TGF- β 2-injected mice.²⁹ We also observed an increase of CTGF and collagen IV fluorescence. Shepard et al.²⁹ found an increase, albeit nonsignificant, in the mRNA levels of these proteins. However, mRNA levels do not necessarily correlate with protein expression, as changes in protein levels could result from mechanisms other than an increase in transcription and translation, such as decreased turnover. We previously have observed this phenomenon with SPARC-induced changes in JCT ECM.²² Increases of PAI-1 and CTGF, which have been observed in perfused human anterior segments,³⁶ appear to be mediated by mechanisms other than SPARC. CTGF and PAI-1 often are regulated in a similar fashion; both have been shown to increase in TM cells after TGF- β 2 stimulation.^{27,50} The elevated levels of PAI-1 in POAG patients and its function in decreasing ECM turnover by inhibiting MMPs underscore the role of ECM dysregulation in glaucoma pathogenesis.^{51,52}

Immunohistochemistry and immunoblot demonstrated an increase of SPARC following TGF- β 2 stimulation. In perfused human anterior segments, SPARC overexpression increased collagen IV and fibronectin within the JCT TM,²² illustrating conservation of function between humans and mice. TGF- β 2 overexpression in mice caused increases in collagen I and laminin that did not reach statistical significance, which also was observed in perfused human anterior segments.²² However, it appears as though some effects are species-specific, as collagen VI was noted to increase with SPARC overexpression in human tissue, but not in TGF- β 2-stimulated mice.

Collagen IV and fibronectin are significant components of the JCT basal laminae. SPARC stimulates the partial unfolding of fibronectin, which is crucial to overall ECM assembly,^{53,54} and is essential to collagen IV deposition.^{55,56} In *Drosophila*, the inhibition of SPARC leads to the complete absence of collagen IV in the basal laminae, potentially due to improper folding or secretion of collagen IV from the endoplasmic reticulum.^{55,57} Thus, the deletion of SPARC would be expected to impact basal lamina homeostasis. It is possible that without SPARC, the attenuation of the collagen IV and fibronectin upregulation is significant enough to reduce outflow resistance, and blunt the TGF- β 2-mediated IOP increase. In addition, we have shown previously that in perfused human anterior segments, SPARC overexpression decreases MMP-9

activity, thereby reducing ECM degradation.²² SPARC moderates MMP and tissue inhibitor of metalloproteinase (TIMP) levels with the effect of altering activity.²² It is possible that MMP activity, and thus ECM turnover, is higher in the SPARC-null mouse, leading to greater ECM degradation and a diminished IOP increase. While CTGF and PAI-1 have been shown to be important contributors to ECM synthesis and degradation, respectively, they have not been shown to interact with SPARC. The lack of change in their levels in TGF- β 2-injected SPARC-null mice seems to suggest that they may work through SPARC-independent pathways to modulate ECM protein expression and MMP activity.

Aside from probing for SPARC, immunohistochemistry findings were not corroborated with other forms of molecular analysis due to the difficulty of observing subtle changes in ECM protein expression specifically within the mouse TM. Isolation of mouse TM is technically challenging, and invariably involves contamination by adjacent tissues, such as the iris and choroid. We believe that the elevation in SPARC appreciated by immunoblot was possible due to SPARC being the most highly upregulated protein with TGF- β 2 stimulation.²⁷ Other ECM proteins are not nearly as highly upregulated as SPARC, making any increase in expression within the TM difficult to observe by molecular analyses.

FIGURE 8. Representative immunolabeling of (A) collagen IV and (B) fibronectin in WT and KO mice 8 days after treatment with Ad.empty or Ad.TGF- β 2. Collagen IV and fibronectin were significantly increased in Ad.TGF- β 2-injected WT mice, but not in Ad.TGF- β 2-injected KO mice compared to respective Ad.empty groups. Mild fluorescence appeared in the TGF- β 2 of the Ad.empty-injected tissue, as the primary antibody was bound to endogenous mouse TGF- β 2. (C) Secondary antibody-only staining is shown as a negative control. Scale bars: 10 μ m.

FIGURE 8. Continued.

There were differences in methodology and results with TGF- β 2 overexpression compared to a previous study.²⁹ We used a lower viral titer of 6×10^6 pfu instead of 6×10^7 pfu. In preliminary studies, a higher titer caused corneal edema as early as one day after injection that could not be prevented with steroid treatment. At 6×10^6 pfu, we applied one drop of prednisolone after injection to prevent corneal edema. It is unlikely that the IOP elevations observed in our study were a corticosteroid-mediated response from the single topically-applied drop containing approximately 30 μ g of prednisolone acetate—even assuming intraocular penetration of 100% of the available drug contained within a 30 μ L drop. In humans, the tear film and conjunctival cul-de-sac can hold only approximately 30 μ L from a topically applied drop.⁵⁸ Although the equivalent volumes are unknown in a mouse, it can be safely assumed to be much smaller given the size

difference between the eyes of humans and mice. Corticosteroids require chronic treatment to raise IOP.⁵⁹ In mice, there is only a minimal change in IOP even with a subconjunctival dose of 800 μ g of triamcinolone.⁶⁰

The difference in mouse strain and age may explain the lower magnitude of IOP increase in our Ad.TGF- β 2-injected mice, the mild variation of IOP observed in uninjected eyes, and the shorter duration of IOP elevation compared to Shepard et al.²⁹ The duration of IOP elevation that we observed is consistent with the natural decay of foreign genetic material within a mammalian cell of 5 to 12 days.^{61,62} Adenoviral delivered plasmids remain for approximately 14 days in murine eye tissue,^{63,64} although other studies have demonstrated that adenovirus can last up to 4 weeks.^{29,65–67} Animals used in those studies often were older and of different strains; viral titers differed as well. Interestingly, certain mouse strains have

TABLE 2. Fluorescence Intensity Measurements of ECM Proteins and TGF-β2 From Immunostained Images

ECM Protein	WT Change, %	KO Change, %	P Value
Collagen I	32.07 ± 13.57	−11.12 ± 2.91	0.0965
Collagen IV	55.13 ± 9.50	14.77 ± 12.17	0.0227
Collagen VI	0.95 ± 1.34	13.23 ± 13.40	0.4605
Laminin	26.38 ± 14.94	13.57 ± 15.92	0.4233
Fibronectin	40.93 ± 6.54	−2.37 ± 9.31	0.0190
PAI-1	64.64 ± 10.54	39.87 ± 6.42	0.2720
CTGF	57.36 ± 14.41	64.75 ± 17.28	0.7040
SPARC	48.64 ± 9.71	0	<0.001
TGF-β2	108.74 ± 12.16	78.38 ± 22.71	0.2582

P values were derived from comparing the percentage increase in WT and KO tissues using paired Student's *t*-tests.

FIGURE 9. Representative immunolabeling of (A) PAI-1, (B) CTGF, and (C) SPARC in WT and KO mice 8 days after treatment with Ad.empty or Ad.TGF-β2. PAI-1 and CTGF were significantly increased in Ad.TGF-β2-injected WT mice and Ad.TGF-β2-injected KO mice compared to respective Ad.empty groups. SPARC was upregulated in Ad.TGF-β2-injected WT mice, and was absent in KO mice as expected. The fluorescent artifact in the SPARC panel of Ad.TGF-β2 WT was not included in calculations, as it lies outside the TM. Mild fluorescence appeared in the TGF-β2 of the Ad.empty-injected tissue, as the primary antibody was bound to endogenous mouse TGF-β2. (D) Secondary antibody-only staining is shown as a negative control. Scale bars: 10 μm.

FIGURE 9. Continued.

been shown not to respond to adenoviral gene delivery,⁶⁶ demonstrating the variability in susceptibility of different mouse strains to various adenoviruses. Immunohistochemistry completed in our study showed that TGF- β 2 synthesis was attenuated at day 13 (Fig. 6), corroborating our IOP data and illustrating the time course of infection using this viral titer in this particular mouse strain. In contrast to the study of Shepard et al.,²⁹ our Ad.TGF- β 2-injected eyes did not have angle closure. The lower viral titer and one-time application of prednisolone could have mitigated the formation of synechiae and subsequent angle closure. Empty adenovirus can cause modest anterior chamber inflammation²⁹; the prednisolone may have sufficiently reduced such inflammation to prevent angle closure.

We found that IOP remains stable in mice between minutes 5 and 8 with isoflurane. To our knowledge, this is the first time-course study of IOP in mice using an inhaled anesthetic. We chose the 5- to 8-minute period because it was early enough to ensure that corneal dehydration would not become an active issue and the earliest point at which IOP reached a stable plateau. Similar to our previous report, SPARC KO mice had a lower IOP than their corresponding WT mice in this study.²⁰ Inhaled anesthetics generally are known to reduce IOP.⁶⁸⁻⁷⁰ A decrease in IOP absolute values with isoflurane compared to those obtained with ketamine was expected and observed. The greater decrease in percentage of baseline IOP (approximately 24%) is likely normal variability, as we found a 22.7% IOP decrease in SPARC KO mice in another of our studies.²¹

FIGURE 9. Continued.

We have demonstrated that IOP can be measured accurately in mice with rebound tonometry under isoflurane. SPARC is essential to modulating TGF- β 2-mediated ocular hypertension in mice, given the minimal redundancy with other matricellular proteins as demonstrated by this study. This finding is consistent with SPARC being the most upregulated protein following TGF- β 2 stimulation.²⁷ Immunohistochemistry suggests that collagen IV and fibronectin levels, which appear to be SPARC-dependent, are vital to the TGF- β 2-mediated IOP increase. Since TGF- β 2 has been implicated strongly in the pathogenesis of POAG, SPARC itself may have a role in glaucoma pathogenesis. Future work includes molecular analysis to understand the mechanisms by which the ocular hypertensive phenotype is inhibited in the SPARC-null mouse, and how SPARC mediates ECM levels.

Acknowledgments

Supported by National Eye Institute Grants EY 019654-01 (DJR) and EY14104 (MEEI Vision-Core Grant), Howard Hughes Medical Institute Medical Research Fellowship (SSS), and Alcon Research.

Disclosure: **S.S. Swaminathan**, None; **D.-J. Oh**, None; **M.H. Kang**, None; **A.R. Shepard**, Alcon (F, E); **I.-H. Pang**, None; **D.J. Rhee**, Alcon (F, C), Aquesys (F, C), Merck (F, C), Aerie (C), Allergan (C), Glaukos (C), Santen (C)

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