Gremlin Induces Ocular Hypertension in Mice Through Smad3-Dependent Signaling

Colleen M. McDowell, Humberto Hernandez, Weiming Mao, and Abbot F. Clark

North Texas Eye Research Institute, Department of Cell Biology and Immunology, University of North Texas Health Science Center, Fort Worth, Texas, United States

Correspondence: Abbot F. Clark, NTERI/CBH-441, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107, USA; abe.clark@unthsc.edu.

Submitted: March 30, 2015

Accepted: July 15, 2015

Citation: McDowell CM, Hernandez H, Mao W, Clark AF. Gremlin induces ocular hypertension in mice through Smad3-dependent signaling. *Invest Ophthalmol Vis Sci*. 2015;56:5485–5492. DOI:10.1167/iovs.15-16993

PURPOSE. Transforming growth factor- β 2 induces extracellular matrix (ECM) remodeling, which likely contributes to the defective function of the trabecular meshwork (TM) leading to glaucomatous ocular hypertension. Bone morphogenetic proteins (BMPs) inhibit these profibrotic effects of TGF β 2. The BMP antagonist gremlin is elevated in glaucomatous TM cells and increases IOP in an ex vivo perfusion culture model. The purpose of this study was to determine whether gremlin regulates ECM proteins in the TM, signals through the Smad3-dependent pathway, and induces ocular hypertension in mice.

METHODS. Ad5.Gremlin or Ad5.TGF β 2 was injected intravitreally into one eye of each mouse. Intraocular pressure measurements were taken using a TonoLab tonometer. Gremlin, TGF β 2, fibronectin (FN), and collagen-1 (Col-1) expression in the TM was determined by immunofluorescence, Western immunoblot, and quantitative (q)PCR analyses.

RESULTS. Ad5.Gremlin or Ad5.TGF β 2 each caused significant IOP elevation in mice. Immunofluorescence and Western blot analysis demonstrated that gremlin and TGF β 2 reciprocally increased the expression of each other, and both increased FN expression in the TM and surrounding tissues. Ad5.Gremlin elevated IOP and increased *Fn* and *Col-1* gene expression in the TM of *Smad3* wild-type (WT) mice, but had no effect in *Smad3* HET or *Smad3* KO mice.

CONCLUSIONS. Our results demonstrate that intravitreal injections of either Ad5.Gremlin or Ad5.TGF β 2 elevate IOP and upregulate the ECM protein FN in the TM of mice. These data show that gremlin signals through the Smad3-dependent pathway in the TM to elevate IOP. We determined for the first time gremlin's role in inducing ocular hypertension in an in vivo model system.

Keywords: gremlin, glaucoma, trabecular meshwork, TGF β 2, intraocular pressure

Elevated IOP is a well-known causative risk factor for both the development and progression of glaucoma. Intraocular pressure is regulated by aqueous humor (AH) production in the ciliary body and drainage from the eye via the trabecular meshwork (TM) and uveoscleral pathway. The role of the TM and the surrounding extracellular matrix (ECM) in IOP regulation has been extensively studied. Primary open-angle glaucoma (POAG) is associated with changes in the ECM composition within the TM, increased AH outflow resistance, and elevated IOP.^{1,2}

Transforming growth factor- β 2 signaling pathway has been shown to be an important regulator of ECM proteins in the TM,^{3–9} and TGF β 2 has been found to be elevated in the AH and TM of POAG patients.^{7,10–13} Transforming growth factor- β 2 has also been shown to cause ocular hypertension in both ex vivo anterior segment perfusion organ culture models,^{14,15} and by overexpression of a bioactivated form of TGF β 2 in mouse eyes.^{4,16} Transforming growth factor- β 2 is known to regulate the expression of ECM proteins through the canonical Smad pathway as well as noncanonical signaling pathways.^{17–20} We have previously demonstrated that TGF β 2 signals through the canonical Smad and non-Smad pathways and alters the ECM in human TM cells.^{5,7} We have also determined that TGF β 2 signaling through the canonical Smad pathway is essential for TGF β 2-induced ocular hypertension in mice.²¹

Transforming growth factor- β 2 signaling is regulated by bone morphogenetic proteins (BMPs) that control a variety of functions in different cell types, and are present in the TM.^{9,22} Bone morphogenetic proteins are regulated by BMP antagonists, such as gremlin, which bind BMP ligands and block BMP binding to their receptors.^{23,24} We have previously shown higher expression of gremlin in glaucomatous TM cells and tissues.⁹ In addition, we have shown that gremlin antagonizes BMP4 inhibition of TGF β 2-induced ECM proteins in TM cells. Gremlin and TGF β 2 are involved in a “feed-forward” pathogenic fibrotic pathway in the TM, where gremlin increases TGF β 2 expression and TGF β 2 increases gremlin expression. Gremlin also elevates IOP in perfusion-cultured human anterior segments.^{5,9} Here, we demonstrate that gremlin induces ocular hypertension and alters the ECM composition in the TM of mice. We also show that gremlin signaling through the canonical Smad pathway is essential for this gremlin-induced ocular hypertension.

METHODS

Animals

Both A/J and 129-*Smad3*^{tm1Par}/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and subsequently

bred and aged at University of North Texas Health Science Center (UNTHSC; Fort Worth, TX, USA). We used A/J mice to test the effect of TGF β 2 and gremlin on ocular hypertension. We used homozygous wild-type (WT), heterozygous (HET), and homozygous knockout (KO) 129-*Smad3*^{tm1Par}/J mice to test the influence of Smad3 on gremlin signaling and ocular hypertension. Both male and female mice were used, and all mice were 5 to 6 months old at the start of the experiments. All experiments were conducted in compliance with the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research and the UNTHSC Animal Care and Use Committee regulations.

Adenovirus Injections

We used adenovirus 5 (Ad5) viral vectors to overexpress gremlin and TGF β 2 in the TM of mouse eyes. We have previously reported the preparation and use of Ad5.hTGF β 2^{226/228} (hereafter referred to as Ad5.TGF β 2 throughout the manuscript) and Ad5.null vectors.¹⁶ The human gremlin gene was PCR-amplified from an expression vector (Origene, Rockville, MD, USA) with XhoI and BamHI restriction sites introduced into 5' and 3' ends, respectively. The PCR product was ligated with the pGEMT vector (Promega, Madison, WI, USA), transformed into *Escherichia coli*, screened, and amplified. The gremlin fragment was digested with XhoI and BamHI (Promega) and inserted into the pacAd5CMV shuttle vector (University of Iowa, Iowa City, IA, USA). After sequence verification (Genewiz, South Plainfield, NJ, USA), the pacAd5CMV-Gremlin vector was sent to University of Iowa Gene Transfer Core for adenovirus production. Two microliters of Ad5.TGF β 2, Ad5.Gremlin, or Ad5.null (2.5 $\times$ 10⁷ plaque-forming units [pfu]) were intravitreally injected in one eye of each animal, and the contralateral uninjected eyes were used as negative controls. Previously we have shown that intravitreal injections of Ad5 transgenes produce a more pronounced and consistent IOP response compared with intracameral injections in mouse eyes.^{16,21,25,26} We have also demonstrated that Ad5.null, Ad5.GFP, and transgenes not associated with glaucomatous phenotypes have no effect on ocular hypertension.^{16,21,25,26}

IOP Measurement

Intraocular pressure was measured as previously described.^{21,27} In all experiments involving A/J mice, IOP was measured using a noninvasive method in conscious mice with the TonoLab tonometer (Colonial Medical Supply, Franconia, NH, USA). In all experiments involving 129-*Smad3*^{tm1Par}/J mice, IOP was measured after anesthesia with 2.5% isoflurane + 100% oxygen with the TonoLab tonometer. All measurements were made during the same 3-hour period of the lights-on phase. This study included IOP measurements in A/J mice: Ad5.TGF β 2-injected animals ($n = 9$), Ad5.Gremlin-injected animals ($n = 9$), and Ad5.null-injected animals ($n = 5$); as well as Ad5.Gremlin-injected 129-*Smad3*^{tm1Par}/J mice: WT ($n = 5$), HET ($n = 8$), and KO mice ($n = 8$). Area under the curve (AUC) was calculated for each individual mouse and then averaged for each mouse strain. The IOP exposure was calculated by subtracting the AUC of uninjected control eyes from the AUC of the Ad5.TGF β 2^{226/228} and Ad5.Gremlin-injected eyes. Statistical significance was calculated by one-way ANOVA and Tukey post hoc analysis. All data are reported as mean $\pm$ SEM.

Immunohistochemistry

At the end of the 3-week time course, whole eyes were removed and processed for immunohistochemistry to detect

FIGURE 1. Ad5.Gremlin and Ad5.TGF β 2 induce ocular hypertension in mice. All mice were injected in one eye with 2.5×10^7 pfu Ad5.Gremlin, Ad5.hTGF β 2, or Ad5.null, and IOP was measured for 21 days post injection. The contralateral uninjected eye served as a control. (A) Ad5.Gremlin significantly increased IOP at day 11 (* $P < 0.05$), day 14 (** $P < 0.01$), and day 18 (* $P < 0.05$) post injection ($n = 9$ mice). Ad5.TGF β 2 significantly elevated IOP at days 3 to 18 (*** $P < 0.001$) and day 21 (* $P < 0.05$; $n = 9$ mice). (B) Ad5.null had no significant effect on IOP at any time point ($n = 5$ mice). Significance was calculated by one-way ANOVA with Tukey post hoc analysis at each time point and P values reported are comparison of injected eye with the corresponding uninjected control eye for each treatment.

TGF β 2, gremlin, and fibronectin (FN) expression in Ad5.TGF β 2- ($n = 4$) and Ad5.Gremlin- ($n = 4$) injected eyes. Eyes were fixed in 4% paraformaldehyde for 24 hours, processed, and embedded in paraffin. Five-micrometer sections were cut, and sections were transferred to glass slides. Paraffin sections were dewaxed two times in xylene, 100% ethanol, and 95% ethanol for 2 minutes each. Slides were then soaked in PBS for 5 minutes. Rabbit anti-TGF β 2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-gremlin (Abcam, Cambridge, MA, USA), and rabbit anti-FN (EMD Millipore; Billerica, MA, USA) antibodies were used at a 1:1000 dilution, followed by Alexa-Fluor-labeled anti-rabbit antibody. Slides were mounted with ProLong Gold mounting medium with DAPI (Life Technologies, Carlsbad, CA, USA) and imaged using a fluorescent microscope. Hematoxylin and eosin (H&E) staining was performed on sections from Ad5.Gremlin-injected eyes and is visible as pink and purple stain in the images. All images were taken at $\times 400$ magnification; scale bars represent 50 μ m.

Quantitative (q)PCR and Western Blot Analysis

In order to detect RNA and protein changes at the peak of the IOP increase, eyes were harvested at day 14 post injection. Whole eyes from each group of mice were collected for qPCR analysis ($n = 3$ –5 mice/genotype). The TM rings were carefully dissected from the whole eye. The TM rings contained mainly TM tissue and small amounts of sclera and cornea. Great effort was made to dissect away as much of the sclera and cornea as possible. Samples were homogenized and RNA extracted in Isol

FIGURE 2. Gremlin and TGFβ2 reciprocally induce expression of each other in the mouse TM. Mice were injected in one eye with 2.5×10^7 pfu Ad5.Gremlin or Ad5.hTGFβ2 and the contralateral uninjected eye served as controls. At 14 days post injection, the eyes were harvested, and the TM ring was carefully dissected, protein isolated, and Western blot analysis performed. (A) Representative Western blots of gremlin and TGFβ2 expression in the TM of Ad5.Gremlin and Ad5.TGFβ2-injected eyes. (B) Ad5.Gremlin significantly increased expression in gremlin protein ($1110.36 \pm 186.49\%$) compared with uninjected control ($100.00 \pm 13.48\%$; $P < 0.05$, $n = 4$). (C) Ad5.TGFβ2 significantly increased gremlin expression ($192.68\% \pm 13.92\%$) compared with uninjected controls ($100.00 \pm 22.09\%$; $P < 0.05$, $n = 4$). (D) Ad5.Gremlin appeared to increase expression of TGFβ2 ($348.79 \pm 130.11\%$) compared with uninjected controls ($100.00 \pm 92.84\%$; statistically not significant, $n = 4$). (E) Ad5.TGFβ2 significantly increased TGFβ2 expression ($180.35 \pm 69.38\%$) compared with uninjected control ($100.00 \pm 56.79\%$; $P < 0.05$, $n = 4$). All values are mean $\pm$ SEM, statistical significance determined by Student's *t*-test.

RNA Lysis Reagent (5PRIME, Gaithersburg, MD, USA) and reverse-transcribed to cDNA (Bio-Rad iScript cDNA synthesis Kit; Bio-Rad, Hercules, CA, USA). Each PCR reaction contained: 10 μ L 2X iQ SYBR Green Supermix (Bio-Rad), 0.25 μ L forward primer (100 μ M), 0.25 μ L reverse primer (100 μ M), 8.5 μ L dH₂O, and 1.0 μ L cDNA template (25 ng/ μ L). Primer pairs used in PCR reactions include FN (5'-GGTGACACTTATGAGCGCCCTA-3', 5'-AACATGTAGCCACCACTCTCAT-3'), COL1 (5'-GGAATGAAAGG GACACAGAGG-3', 5'-TAGCACCATCATTCCACGA-3'), and GAPDH (5'-ACTCCACTCACGGCAAATTC-3', 5'-TCTCCATGGT GGTGAAGAACA-3'). Polymerase chain reaction conditions were: 95°C for 1 minute and 40 $\times$ (95°C for 1 minute, 65°C for 45 seconds, 72°C for 45 seconds). Fold change was calculated using

the $\Delta\Delta C_t$ method comparing expression to glutaraldehyde-3-phosphate dehydrogenase (GAPDH) and the uninjected control eye. Statistical significance was calculated by one-way ANOVA and Tukey post hoc analysis. All data are reported as mean $\pm$ SEM.

Similarly, tissue was collected for Western blot analysis by dissecting the TM rings as described above ($n = 4$ mice/treatment). The tissue was placed in lysis buffer and manually homogenized using a small plastic tissue homogenizer in a 1.5-mL microcentrifuge tube. Protein concentration was determined using DC protein assay system (Bio-Rad). Proteins were separated on denaturing polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes

FIGURE 3. Immunohistochemical analysis of gremlin and TGF β 2 expression in the mouse TM. Mice were injected in one eye with 2.5×10^7 pfu Ad5.Gremlin or Ad5.hTGF β 2 and the contralateral uninjected eye served as a control. (A–C) Ad5.Gremlin and Ad5.TGF β 2 increased gremlin expression in the TM and closely surrounding tissues compared with the uninjected control eyes. (D–F) Ad5.Gremlin and Ad5.TGF β 2 increased expression of TGF β 2 in the TM and closely surrounding tissues compared with the uninjected control eyes. Scale bar: 50 μ m ($n = 4$).

by electrophoresis. Membranes were blocked with SuperBlock blocking buffer (Pierce Biotechnology, Rockford, IL, USA) for 1 hour and then incubated overnight with primary antibodies: rabbit anti-TGF β 2 (Santa Cruz Biotechnology) rabbit anti-gremlin (Abnova, Walnut, CA, USA), and rabbit anti-FN (EMD Millipore). The membranes were washed with TBST and processed with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The proteins were then visualized (Fluor Chem 8900 imager; Alpha Innotech, San Leandro, CA, USA) using ECL detection reagent (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology, Waltham, MA, USA). Beta-actin expression (mouse anti- β -actin; EMD Millipore) was used as a loading control for each blot.

RESULTS

Gremlin is a known regulator of the TGF β 2/BMP signaling pathway. Gremlin antagonizes BMP4, which in turn allows uninhibited TGF β 2-induction of ECM proteins such as FN in human TM cells.⁹ We have also shown that gremlin alone can induce these ECM proteins in TM cells.⁵ The TM is a key tissue in IOP regulation. Therefore, we determined the effect of gremlin overexpression on IOP in mice (Fig. 1). Ad5.Gremlin was injected intravitreally into one eye of each animal, with the contralateral eye as an uninjected control. Intraocular pressure was significantly increased at day 11 ($P < 0.05$), day 14 ($P < 0.01$), and day 18 ($P < 0.05$) post injection (Fig. 1A). Previously, we reported that TGF β 2 induces ocular hypertension in mice.^{4,16} Here, we confirm that Ad5.TGF β 2 does indeed increase IOP with significant IOP differences at all time points measured post injection (days 3–18, $P < 0.001$; day 21, $P < 0.05$; Fig. 1A). Significance was calculated by one-way ANOVA with Tukey post hoc analysis at each time point and P values reported are comparison of injected eye with the corresponding uninjected control eye for each treatment ($n = 9$ mice/

group). Ad5.null was tested as a negative control, showing no change in IOP at any time point post injection ($n = 5$; Fig. 1B).

In order to determine the expression levels of gremlin and TGF β 2 in the TM post injection, Western blot analysis was performed. Eyes were harvested 14 days post injection of Ad5.Gremlin or Ad5.TGF β 2. The TM ring was carefully dissected and protein extracted. As expected, in the Ad5.Gremlin-injected eyes there was a significant increase in gremlin protein expression (Figs. 2A, 2B). There was also a trend for increased TGF β 2 expression in the Ad5.Gremlin-injected eyes compared with the uninjected control eye (Figs. 2A, 2D). In the Ad5.TGF β 2-injected eyes, there was a significant increase in TGF β 2 protein expression and gremlin protein expression (Figs. 2A, 2C, 2E). We also analyzed the Ad5.Gremlin- and Ad5.TGF β 2-injected eyes by immunohistochemistry and showed a similar effect with overexpression of TGF β 2 inducing gremlin expression and overexpression of gremlin-inducing TGF β 2 expression in the TM and surrounding tissue (Fig. 3). These data suggest a feed-forward pathogenic pathway. This feed-forward pathway was previously demonstrated in primary TM cell cultures.⁵ Both gremlin and TGF β 2 have been associated with increased production of ECM proteins, and these data suggest that a feed-forward mechanism could further effect the ECM deposition within the TM, possibly leading to increased aqueous humor outflow resistance and IOP elevation.

In order to test whether there was increased ECM deposition in the TM and surrounding tissue in the Ad5.-Gremlin- and Ad5.TGF β 2-injected eyes, we performed Western blot and immunohistochemical analysis on FN expression (Fig. 4). In both Ad5.Gremlin- (Figs. 4A, 4C) and Ad5.TGF β 2- (Figs. 4B, 4C) injected eyes, there was a significant increase in FN expression in the TM analyzed by Western blot compared with the uninjected control eyes. These results were recapitulated in immunohistochemical analysis with an increase in FN expression in the TM and surrounding tissues of Ad5.Gremlin- and Ad5.TGF β 2-injected eyes compared with uninjected

FIGURE 4. Fibronectin expression is increased in the TM of Ad5.Gremlin- and Ad5.TGFβ2-injected eyes. Mice were injected in one eye with 2.5×10^7 pfu Ad5.Gremlin or Ad5.hTGFβ2 and the contralateral uninjected eyes served as a control. **(A)** Ad5.Gremlin significantly increased expression in FN protein ($175 \pm 17.41\%$) compared with uninjected control ($100.00 \pm 10.33\%$; $P < 0.05$, $n = 4$). **(B)** Ad5.TGFβ2 significantly increased FN expression ($142.38\% \pm 6.51\%$) compared with uninjected control ($100.00 \pm 9.35\%$; $P < 0.05$, $n = 4$). **(C)** Representative blot of FN expression in the TM and surrounding tissue of Ad5.Gremlin- and Ad5.TGFβ2-injected eyes ($n = 4$, quantified in [A, B]). All values are mean $\pm$ SEM, statistical significance determined by Student's *t*-test. **(D-F)** Fibronectin expression is increased in the TM and surrounding tissues of Ad5.Gremlin- and Ad5.TGFβ2-injected eyes analyzed by immunohistochemistry. Scale bar: 50 μ m ($n = 4$).

control eyes (Figs. 4D–F). These data show that both gremlin and TGFβ2 induce expression of the ECM protein FN, and overexpression of gremlin and TGFβ2 increase IOP in mice.

Previously, we reported that TGFβ2 alters the ECM composition^{5,7} and induces ocular hypertension through a Smad3-dependent pathway.⁴ Here, we used our *in vivo* mouse model system to test whether gremlin overexpression alters the ECM composition in the TM and elevates IOP via the Smad3-dependent signaling pathway. Homozygous WT, HET, and homozygous KO *Smad3*^{tm1Par/J} mice were used in each of our experiments. We have previously reported that there is no difference in the gross morphology of the TM, gross morphology of the anterior chamber, or FN protein expression in the TM between *Smad3* genotypes.⁴ We have also previously reported that adenoviral injection can cause mild inflammation in the anterior chamber, but this inflammation does not affect ocular hypertension phenotypes based on the lack of ocular hypertension in animals injected with Ad5.null, Ad5.GFP, Ad5.MYOC^{WT}, or Ad5hTGFβ2^{WT}.^{4,16,25,28} We recapitulate these data here by injecting Ad5.Gremlin into one eye of

each Smad3 genotype. Eyes were harvested 14 days post injection and analyzed by immunohistochemistry. Hematoxylin and eosin staining revealed similar TM morphology and organization in WT, HET, and KO mice injected with Ad5.Gremlin (Fig. 5). The iridocorneal angles were open in all eyes examined. We did observe mild inflammation in eyes of each genotype post injection of Ad5.Gremlin; however, this did not affect ocular hypertension as IOP remained normal in the HET and KO mice throughout an entire 28-day time course (Figs. 6A, 6B).

Ocular hypertension experiments were conducted by injecting Ad5.Gremlin into one eye of each animal, the contralateral uninjected eye serving as a control. Intraocular pressure was significantly elevated at day 13 ($P < 0.01$), day 17 ($P < 0.001$), and day 20 ($P < 0.001$) in WT mice, while no significant IOP elevation was observed at any time-point in the HET and KO mice, $n = 5$ to 8 mice/genotype (Fig. 6A). Significance was calculated by one-way ANOVA with Tukey post hoc analysis at each time point and *P* values reported are comparison of injected eye compared with the corresponding

FIGURE 5. Histologic analysis of anterior chambers after Ad5.Gremlin injection. All mice were injected in one eye with 2.5×10^7 pfu Ad5.Gremlin, and tissue was harvested 14 days post injection. (A–F) Hematoxylin and eosin staining revealed similar TM morphology and organization in WT, HET, and KO eyes post injection with Ad5.Gremlin ($n = 4$, WT; $n = 4$, HET, $n = 3$ KO). The iridocorneal angle was open in all eyes examined. Scale bars: 50 μ m.

uninjected control eye for each treatment. Intraocular pressure exposure was calculated as millimeters of mercury multiplied by the days and also showed a significant difference between WT and HET mice ($P < 0.05$) and WT and KO mice ($P < 0.01$), but no statistical difference between HET and KO mice, analyzed by one-way ANOVA with Tukey post hoc analysis (Fig. 6B). These data are similar to the IOP response shown in SMAD3 WT, HET, and KO mice injected with Ad5.TGF β 2⁴ and demonstrate that two copies of Smad3 are necessary for the induction of ocular hypertension. In order to demonstrate that the Ad5.Gremlin virus was overexpressing gremlin in the TM for each mouse genotype, Western blot analysis was performed (Fig. 6C). A trace amount of endogenous gremlin is evident in the uninjected control eyes. Because the peak IOP elevation occurred at approximately 14 days post injection, this time point was used to determine a change in ECM gene expression. Both fibronectin and collagen-1 gene expression was elevated in the WT animals compared with HET and KO animals (Fig. 6D). These data indicate that gremlin, like TGF β 2, signals

through a Smad3-dependent pathway in the TM, and overexpression of gremlin causes increased ECM gene expression.

DISCUSSION

Previous studies have shown that TGF β 2/BMP signaling regulates the ECM architecture in the TM. Recently, we identified that Smad3 is necessary for TGF β 2-induced ocular hypertension in mice.^{4,16} Gremlin is an antagonist of BMP4, and has also been shown to be an important regulator of ECM proteins in TM cells and glaucomatous tissues.^{5,9,29} Combined, these data suggest that gremlin may play a role in ocular hypertension and be an important regulator of ECM proteins in the glaucomatous TM.

To directly test the hypothesis that overexpression of gremlin will increase IOP, we used our mouse model system of chronically elevating IOP with an Ad5 expression vector for gremlin or a bioactivated form of TGF β 2. Both gremlin and TGF β 2 induced ocular hypertension in mice. Similar to the data

FIGURE 6. Smad3 is necessary for Ad5.Gremlin-induced ocular hypertension. Mice were injected in one eye with 2.5×10^7 pfu Ad5.Gremlin and the contralateral uninjected eye served as a control. (A) Intraocular pressure was significantly elevated at day 13 ($P < 0.01$), day 17 ($P < 0.001$), and day 20 ($P < 0.001$) in WT mice. No significant IOP elevation was observed at any time point in the *Smad3* HET and KO mice ($n = 5$ mice/genotype). (B) Intraocular pressure exposure was calculated as millimeters of mercury multiplied by the days and also showed a significant difference between WT and HET mice ($P < 0.05$) and WT and KO mice ($P < 0.01$). (C) Ad5.Gremlin transduction overexpressed gremlin in the TM of each mouse genotype. (D) Fibronectin and collagen-1 gene expression was elevated in Ad5.Gremlin WT animals ($P < 0.05$) compared with HET and KO animals 14 days post injection ($n = 3$ –5 mice/genotype). Significance was calculated by one-way ANOVA with Tukey post hoc analysis at each time point and P values reported are comparison of injected eye with the corresponding uninjected control eye for each treatment.

reported in TM cells, each of these molecules increased expression of the other protein, creating a feed-forward loop, where TGF β 2 increased expression of gremlin and gremlin increased expression of TGF β 2 in the TM. Both gremlin and TGF β 2 induced expression of FN in the TM. In addition to increasing expression of TGF β 2, gremlin, and FN in the TM, we also saw increased FN expression in the surrounding tissues. Given that both TGF β 2 and gremlin are secreted proteins, they could be affecting the expression levels in tissues surrounding the TM as well. Our results are similar to those previously reported using the Ad5.TGF β 2 virus.⁴ These data provide further support to our previously published data and demonstrate that gremlin is an important regulator of the ECM in the TM and IOP in vivo.

The signaling pathways involved in the TGF β 2 induced changes to the ECM in the TM and ocular hypertension are complex.^{4,5,30} Both Smad and non-Smad signaling pathways are activated by TGF β 2 in TM cells and increase expression of ECM proteins as well as ECM cross-linking enzymes.^{5,6,30} We recently found that Smad3 was necessary for TGF β 2-induced ocular hypertension in mice.⁴ In cell culture studies, we showed that gremlin activates only the canonical Smad2/3 pathway, and inhibition of Smad signaling blocked gremlin's effect on TM ECM expression.⁵ However, we also reported that gremlin's induction of ECM cross-linking lysyl oxidase genes uses both the canonical and noncanonical TGF β 2-signaling pathways.²⁹ Here, we tested the Smad3-signaling pathway involved in gremlin-induced ocular hypertension using *Smad3* KO mice. Although the WT animals developed increased IOP following Ad5.Gremlin injection, the *Smad3* KO and *Smad3* HET mice had no significant IOP elevation. These data show that Smad3 also is necessary for gremlin-induced ocular hypertension in vivo.

Gremlin and TGF β 2 are important regulators of the ECM architecture, and overexpression of these proteins increased IOP in vivo. Transforming growth factor- β 2 has been shown to be elevated in the aqueous humor and TM of POAG,^{7,10–13} and gremlin has been shown to be increased in both glaucomatous TM cells and tissues.⁹ However, the mechanistic pathway regulating increased expression of each of these proteins has not been elucidated. Given the feed-forward pathogenic pathway between TGF β 2 and gremlin in both TM cells and in our mouse model system, it is conceivable that this relationship is also occurring in human glaucoma. We tested the potential role of matrix metalloproteases (MMP), MMP2, MMP3, MMP9, and MMP14, in the TM of Ad5.Gremlin-injected eyes and found no statistical difference in expression compared with uninjected control eyes (data not shown). Additional studies are necessary to determine the precise insult that is producing the initial increased expression of these molecules. Our data demonstrate that these molecules are important regulators of the ECM in the TM and cause ocular hypertension in mice.

Acknowledgments

The authors thank Robert Wordinger for many helpful discussions. Supported by Grants R21EY019977 and R01EY017374 from the National Institutes of Health (Bethesda, MD, USA).

Disclosure: C.M. McDowell, None; H. Hernandez, None; W. Mao, None; A.F. Clark, None

References

1. Lutjen-Drecoll E. Functional morphology of the trabecular meshwork in primate eyes. *Prog Retin Eye Res.* 1999;18:91–119.

2. Rohen JW, Witmer R. Electron microscopic studies on the trabecular meshwork in glaucoma simplex. *Albrecht Von Graefes Arch Klin Exp Ophthalmol*. 1972;183:251-266.
3. Fuchshofer R, Yu AH, Welge-Lüssen U, Tamm ER. Bone morphogenetic protein-7 is an antagonist of transforming growth factor-beta2 in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2007;48:715-726.
4. McDowell CM, Tebow HE, Wordinger RJ, Clark AF. Smad3 is necessary for transforming growth factor-beta2 induced ocular hypertension in mice. *Exp Eye Res*. 2013;116:419-423.
5. Sethi A, Jain A, Zode GS, Wordinger RJ, Clark AF. Role of TGFbeta/Smad signaling in gremlin induction of human trabecular meshwork extracellular matrix proteins. *Invest Ophthalmol Vis Sci*. 2011;52:5251-5259.
6. Sethi A, Mao W, Wordinger RJ, Clark AF. Transforming growth factor-beta induces extracellular matrix protein cross-linking lysyl oxidase (LOX) genes in human trabecular meshwork cells. *Invest Ophthalmol Vis Sci*. 2011;52:5240-5250.
7. Tovar-Vidales T, Clark AF, Wordinger RJ. Transforming growth factor-beta2 utilizes the canonical Smad-signaling pathway to regulate tissue transglutaminase expression in human trabecular meshwork cells. *Exp Eye Res*. 2011;93:442-451.
8. Welge-Lüssen U, May CA, Eichhorn M, Bloemendal H, Lutjen-Drecoll E. AlphaB-crystallin in the trabecular meshwork is inducible by transforming growth factor-beta. *Invest Ophthalmol Vis Sci*. 1999;40:2235-2241.
9. Wordinger RJ, Fleenor DL, Hellberg PE, et al. Effects of TGF-beta2, BMP-4, and gremlin in the trabecular meshwork: implications for glaucoma. *Invest Ophthalmol Vis Sci*. 2007;48:1191-1200.
10. Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch Clin Exp Ophthalmol*. 2001;239:109-113.
11. Ochiai Y, Ochiai H. Higher concentration of transforming growth factor-beta in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn J Ophthalmol*. 2002;46:249-253.
12. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. *Int Ophthalmol*. 2004;25:19-22.
13. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp Eye Res*. 1994;59:723-727.
14. Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGFbeta2-induced changes in human trabecular meshwork: implications for intraocular pressure. *Invest Ophthalmol Vis Sci*. 2006;47:226-234.
15. Gottanka J, Chan D, Eichhorn M, Lutjen-Drecoll E, Ethier CR. Effects of TGF-beta2 in perfused human eyes. *Invest Ophthalmol Vis Sci*. 2004;45:153-158.
16. Shepard AR, Millar JC, Pang IH, Jacobson N, Wang WH, Clark AF. Adenoviral gene transfer of active human transforming growth factor-(beta)2 elevates intraocular pressure and reduces outflow facility in rodent eyes. *Invest Ophthalmol Vis Sci*. 2010;51:2067-2076.
17. Javelaud D, Mauviel A. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. *Oncogene*. 2005;24:5742-5750.
18. Javelaud D, Mauviel A. Transforming growth factor-betas: Smad signaling and roles in physiopathology [in French]. *Pathol Biol (Paris)*. 2004;52:50-54.
19. Javelaud D, Mauviel A. Mammalian transforming growth factor-betas: Smad signaling and physio-pathological roles. *Int J Biochem Cell Biol*. 2004;36:1161-1165.
20. Massague J, Chen YG. Controlling TGF-beta signaling. *Genes Dev*. 2000;14:627-644.
21. McDowell CM, Luan T, Zhang Z, et al. Mutant human myocilin induces strain specific differences in ocular hypertension and optic nerve damage in mice. *Exp Eye Res*. 2012;100:65-72.
22. Wordinger RJ, Agarwal R, Talati M, Fuller J, Lambert W, Clark AF. Expression of bone morphogenetic proteins (BMP), BMP receptors, and BMP associated proteins in human trabecular meshwork and optic nerve head cells and tissues. *Mol Vis*. 2002;8:241-250.
23. Hsu DR, Economides AN, Wang X, Eimon PM, Harland RM. The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Mol Cell*. 1998;1:673-683.
24. Shimasaki S, Moore RK, Otsuka F, Erickson GE. The bone morphogenetic protein system in mammalian reproduction. *Endocr Rev*. 2004;25:72-101.
25. Shepard AR, Jacobson N, Millar JC, et al. Glaucoma-causing myocilin mutants require the peroxisomal targeting signal-1 receptor (PTS1R) to elevate intraocular pressure. *Hum Mol Genet*. 2007;16:609-617.
26. Millar JC, Pang IH, Wang WH, Wang Y, Clark AF. Effect of immunomodulation with anti-CD40L antibody on adenoviral-mediated transgene expression in mouse anterior segment. *Mol Vis*. 2008;14:10-19.
27. Kim CY, Kuehn MH, Anderson MG, Kwon YH. Intraocular pressure measurement in mice: a comparison between Goldmann and rebound tonometry. *Eye (Lond)*. 2007;21:1202-1209.
28. Wang WH, McNatt LG, Pang IH, et al. Increased expression of the WNT antagonist sFRP-1 in glaucoma elevates intraocular pressure. *J Clin Invest*. 2008;118:1056-1064.
29. Sethi A, Wordinger RJ, Clark AF. Gremlin utilizes canonical and non-canonical TGFbeta signaling to induce lysyl oxidase (LOX) genes in human trabecular meshwork cells. *Exp Eye Res*. 2013;113:117-127.
30. Liton PB, Li G, Luna C, Gonzalez P, Epstein DL. Cross-talk between TGF-beta1 and IL-6 in human trabecular meshwork cells. *Mol Vis*. 2009;15:326-334.