Tissue Transglutaminase Elevates Intraocular Pressure in Mice

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PURPOSE. Tissue transglutaminase (TGM2) is elevated in glaucomatous trabecular meshwork (TM) tissues. We investigated whether increased expression of TGM2 increases extracellular matrix crosslinking in the TM, thereby increasing aqueous humor outflow resistance and elevating intraocular pressure (IOP) in mouse eyes.

METHODS. GTM3, primary human GTM 125-05, and cultured mouse TM cells were transduced with adenovirus serotype 5 expressing human transglutaminase 2 (Ad5.TGM2; multiplicity of infection [MOI]=75) and fixed for immunocytochemistry. To test the effect on IOP in living eyes, Ad5.TGM2 was injected intravitreally into one eye of BALB/cj (n = 18) or C57BL/6j mice (n = 9). The uninjected contralateral eye and Ad5.GFP served as controls. Daytime conscious IOPs were measured twice per week. Aqueous outflow facility (C) was measured by constant flow infusion on completion of IOP measurements. Immunohistochemistry was performed on BALB/cj mouse eyes to study TGM2 expression and activity.

RESULTS. The treatment of cultured TM cells with Ad5.TGM2 increased immunostaining of N-ε-(γ-glutamyl) lysine crosslinks. Ad5.TGM2 injection significantly increased IOP in BALB/cj (15.86 mm Hg [injected] vs. 10.70 mm Hg [control]) and in C57BL/6j mice (17.09 mm Hg [injected] vs. 12.01 mm Hg [control]). Mean aqueous outflow facility in the injected eyes of BALB/cj (0.013 μL/min/mm Hg) and C57BL/6j mice (0.012 μL/min/mm Hg) was significantly lower than in the uninjected control eyes (BALB/cj, 0.021 μL/min/mm Hg; C57BL/6j, 0.019 μL/min/mm Hg). The Ad5.TGM2 transduction of mouse eyes increased TGM2 expression in the TM region and increased N-ε-(γ-glutamyl) lysine crosslinks.

CONCLUSIONS. The increased expression of TGM2 in the TM increases N-ε-(γ-glutamyl) lysine crosslinking in the TM, increases aqueous outflow resistance, and elevates IOP in mice. TGM2 may be at least partially responsible for ocular hypertension in POAG.

Keywords: tissue transglutaminase, trabecular meshwork, fibrosis, IOP, glaucoma

Primary open angle glaucoma (POAG) is a multifactorial ocular disease characterized by optic neuropathy and is a leading cause of irreversible vision loss worldwide.1,2 One of the major risk factors associated with POAG is high intraocular pressure (IOP). Increased IOP is associated with changes in cellular and extracellular matrix (ECM) components of the trabecular meshwork (TM) that lead to increased aqueous humor (AH) outflow resistance and ocular hypertension.

Transforming growth factor-β2 (TGFβ2) is a profibrotic cytokine that is increased in the AH and TM of glaucomatous eyes when compared with controls.3–7 The ECM in TM tissues undergoes constant turnover, and TGFβ2 increases expression of ECM molecules such as collagen I, III, IV, and VI, elastin, fibronectin (FN), and laminin and the crosslinking enzymes tissue transglutaminase (TGM2) and lysyl oxidase (LOX).7–11 TGFβ2 plays a major role in ECM remodeling through secretion of ECM proteins along with plasminogen activator inhibitor-1, which inhibits matrix metalloproteinase (MMP) activity.12 The bone morphogenic protein antagonist gremlin is also elevated in glaucomatous trabecular meshwork (GTM) cells and causes ECM deposition by potentiating the fibrotic effects of TGFβ2.13,14 Because the major outflow pathway for AH is through the TM, modulation of ECM architecture can affect AH outflow through this tissue.15 Understandably, glaucoma research has focused on studying pathways that can modify the TM structure and thereby affect its filtration function.

Existing pharmacological treatments for glaucoma provide symptomatic relief and are primarily targeted to lower IOP.16 Surgical intervention by laser trabeculoplasty, trabeculectomy,17 or implantation of artificial drainage devices18 is used when medications no longer adequately control IOP. However, even with extensive research in this field, there presently is no cure for glaucoma. This is likely in part because of an inadequate understanding of the complete pathophysiology of the disease in the TM and a lack of treatments that directly inhibit glaucomatous damage to the TM. Active areas of research include genetic causes, for example, myocilin mutations that are responsible for a subset of POAG.19,20 Cytoskeletal rearrangements leading to cross-linked actin network formation are found in glaucomatous TM cells and tissues and may be responsible for increased cell stiffness and biomechanical alterations in the TM contributing to outflow resistance.21,22

One of the major causes for obstruction of outflow at the TM could be increased crosslinking and deposition of ECM proteins. There is increased production and deposition of ECM proteins such as collagen and FN in glaucoma.23,24 Normally, the endogenous MMPs and disintegrin-like and metalloprotei-
nases are capable of breaking down this ECM, thereby maintaining ECM homeostasis.25 However, in glaucoma, there seems to be an imbalance between ECM formation and degradation. The increased ECM deposition seen in glaucomatous TM may be attributed to irreversible covalent ECM protein crosslinking by the major crosslinking enzymes TGM2, LOX, and lysyl-oxidase like enzymes 1 to 4. These enzymes crosslink major ECM proteins, such as fibronectin, collagen, and elastin. The reactions mediated by these enzymes lead to irreversible covalent bond formation, which increases the stability of the crosslinked ECM.26 There are no known endogenous enzymes that can break these bonds. Chronic crosslinking decreases ECM turnover and can consequently lead to biophysical changes in the TM, and over a prolonged period, this crosslinking can cause the TM tissue to gain a fibrotic phenotype. In POAG, there is significant thickening of elastic fiber sheaths in the TM. For example, there is increased formation of sheath-derived plaques in the TM of POAG patients when compared with age-related controls.7,27 This can cause increased TM stiffness, thereby decreasing the ability of TM to facilitate AH outflow.28

Previously we have shown that glaucomatous TM cells and tissues have increased the expression of TGM2 when compared with age-matched controls.29 TGFβ2 induces TGM2 expression in TM cells using the canonical Smad-signaling pathway.7 TGM2 is involved in calcium-dependent covalent crosslinking of the ECM proteins collagen and FN through Nε-(γ-glutamly) lysine (GGEL) linkages.30 These cross-linked proteins are deposited in the TM and may reduce overall turnover of ECM proteins leading to a fibrotic phenotype. We therefore wanted to mimic the effects of TGM2 overexpression in the TM and study its effects on IOP and AH outflow resistance in mice.

**Materials and Methods**

Adenovirus Serotype 5 Expressing Human Transglutaminase 2 Viral Vectors and Cell Culture Transduction

Adenovirus serotype 5 expressing human transglutaminase 2 (Ad5.TGM2) using a cytomegalovirus (CMV) promoter was obtained from Vigene Biosciences (Ad5.TGM2 in PBS buffer; Rockville, MD, USA) and from Vector Biolaboratories (Ad5.TGM2 in Dulbecco's modified Eagle's medium with 2% BSA and 2.5% glycerol; Malvern, PA, USA). Basic Local Alignment Search Tool (BLAST) was performed for sequence homology in Uniprot (www.uniprot.org, in the public domain), and human and mouse TGM2 protein homology is 84.1%, Ad5.Null (Vector Biolaboratories) and Ad5.GFP (Vigene Biosciences) were used as experimental controls in cell culture and mouse work, respectively. A transformed human glaucomatous TM cell line (GTM3),31 a mouse primary TM (MTM) cell strain,32 and a primary glaucomatous TM (GTM 125-05) cell strain (isolated from a 78-year-old female donor eye obtained from Lions Eye Institute for Transplant and Research, Tampa, FL, USA) were cultured in Dulbecco’s modified Eagle’s medium (low glucose; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Atlas Biologics, Collins, CO, USA), L-glutamine (0.292 mg/mL; GE Healthcare Life Sciences, Logan, UT, USA) and penicillin (100 U/mL)-streptomycin (0.1 mg/mL; Sigma-Aldrich) as previously described.29,33 The medium was changed every 2 to 3 days, and the cells were grown to confluency in T-75 flasks. Approximately 50,000 GTM3, MTM, and GTM 125-05 cells were plated onto coverslips in 24 well plates. GTM 125-05, GTM3, and MTM cells were treated with Ad5.Null (Vector Biolaboratories) and Ad5.TGM2 (Vector Biolaboratories) at a multiplicity of infection (MOI) of 75, and the medium was changed after 24 hours. At 48 hours after the medium change, human TM cells were fixed for immunocytochemistry. MTM cells were grown for 5 days after the medium change and then fixed for immunocytochemistry.

**Immunocytochemistry for TGM2, FN, and GGEL Bonds**

At 2 days (GTM3 and GTM125-05 cells) or 5 days (MTM cells) after transduction, the cells were washed three times with PBS. The cells on the coverslips were then fixed in 4% paraformaldehyde (PFA) for 30 minutes and washed with PBS three times. The cells were blocked in PBS superblock (Thermo Fisher Scientific, Waltham, MA, USA) for 2 hours, followed by incubation with primary antibodies in GTM 125-05 cells, GTM3, and MTM cells (rabbit monoclonal [EP2957] to transglutaminase 2, diluted 1:500; Abcam [Cambridge, MA, USA]); GGEL in GTM 125-05, GTM3, and MTM cells (ab242, diluted 1:50; Abcam); and FN in GTM 125-05 cells (AB1945, diluted 1:500; Millipore, Temecula, CA, USA) in PBS superblock overnight at 4°C. The cells were then washed three times with PBS and incubated for 2 hours with the appropriate secondary antibodies (goat anti-rabbit Alexa Fluor 488 [Thermo Fisher Scientific] for TGM2, diluted 1:1000; goat anti-mouse IgM cross adsorbed secondary antibody, DyLight 594 [Thermo Fisher Scientific]) conjugate for GGEL, diluted 1:100; donkey anti-rabbit Alexa Fluor 488 for FN, diluted 1:1000) in PBS superblock. The cells were washed with PBS three times following by three quick washes with water (continuous washes without incubation) and mounted with ProLong Gold Antifade Mountant containing 4’,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific). The slides were allowed to dry overnight, and fluorescence imaging was performed at the end of the process using a Keyence (Itasca, IL, USA) all-in-one fluorescence microscope.

The fluorescence intensity of TGM2, GGEL, and FN were determined by ImageJ (version 1.50i; National Institutes of Health, Bethesda, MD, USA). To quantify, two locations in three coverslips per treatment group were imaged at low magnification (100×). Intensity was measured in ImageJ, and nuclei in the same area were counted in Adobe Photoshop (Adobe Systems, San Jose, CA, USA). The fluorescence intensity was normalized to DAPI-counted nuclei for all treatment groups in GTM 125-05 and MTM cells. In the GTM3 cells, the DAPI count was challenging at low magnification. However, because an equal number of cells were plated, the fluorescence intensity of the different treatment groups under 100× was compared.

**Decellularization**

The GTM 125-05 cells plated separately onto coverslips in a 24-well plate were decellularized prior to fixing.34,35 Briefly, 3 days after transduction with Ad5.TGM2, the cells were washed three times with PBS. The cells were incubated in 1 ml of 0.2% Triton X-100 in water at room temperature. Solubilized and detached cells were removed with three PBS washes. Subsequently, 1 ml of 5% ammonium hydroxide in water was slowly added to the wells and incubated in room temperature for 5 minutes, incubated at –80°C for 5 minutes followed by thawing in room temperature for 5 more minutes. The plates were checked under a phase contrast microscope to ensure decellularization and washed three times with PBS. Following decellularization, ECM was fixed with 4% PFA and blocking and staining for GGEL and DAPI was continued as described previously.
Mouse Studies

All experiments were conducted in compliance with the ARVO Statement of the Use of Animals in Ophthalmic and Vision Research and the UNTHSC Institutional Animal Care and Use Committee regulations. A total of 35 female BALB/cj retired breeder mice (17 for first study and 18 for the repeat study) and 9 female C57BL/6j mice aged 6 to 10 months obtained from The Jackson Laboratory (Bar Harbor, ME, USA) were used for our experiments. The animals were maintained on a 12-hour light/dark cycle (lights on at 0600 hours). Food and water were available ad libitum.

Adenoviral Injections

At day 0, the mice were anesthetized using inhalation anesthesia (isoflurane 2.5%; O2[g] 0.8 L/min) prior to intravitreal injection with Ad5.TGM2 or Ad5.GFP. The eyes were pretreated with one to two drops of Alcaine (Alcon Laboratories Inc., Fort Worth, TX, USA; proparacaine hydrochloride [HCl] 0.5%) prior to the injections. Ad5.GFP (1 × 10^6 plaque forming units) was injected intravitreally (left eye) in the BALB/cj retired breeder mice (n = 5) for the pilot study, and Ad5.CMV.TGM2 (1-50 × 10^6 plaque forming units) was injected intravitreally (left eye) in the BALB/cj retired breeder mice (n = 6 for pilot study and n = 18 for repeat) or C57BL/6j mice (n = 9) using a 10-μL glass microsyringe (Hamilton Company, Reno, NV, USA) fitted with a ½” 33G needle with a 12° bevel to deliver 2 μL, as previously described.35,36 The uninjected (right) eyes served as control. For our first study, we also used 6 naïve uninjected mice (female BALB/cj retired breeders) to establish that our experimental conditions did not affect IOP. The eyes were observed at each IOP measurement with a direct ophthalmoscope (Welch-Allyn, Skaneateles Falls, NY, USA) for signs of inflammation of the anterior segment (including iridal hyperemia, corneal edema/opacity, discharge, synchia(e), or lenticular cataract). The pupillary light reflex was also observed. The presence or absence of lenticular opacity observed ophthalmoscopically in each eye was scored (Supplementary Table S1).

Conscious IOP Measurements and AH Outflow Studies

The IOP measurements were taken using the Tonolab rebound tonometer (Icare Finland Oy; Vantaa, Finland) as previously described.36,37 The mice were handled and acclimatized for approximately 2 weeks. Conscious baseline IOPs were measured the day prior to intravitreal injection (day −1). Injections took place on day 0. Following injection, IOP was measured in conscious animals twice per week for up to 4 weeks. To circumvent issues associated with circadian variation in IOP, the measurements were taken in the afternoon at the same time of day commencing between 1:30 PM and 2:30 PM.

Upon completion of the IOP readings, 6 of the 18 BALB/cj mice and 5 of the 9 C57BL/6j mice were used for AH outflow experiments. AH outflow facility was measured using a constant flow infusion methodology as previously published.38,39 Briefly, the animals were anesthetized with an intraperitoneal injection of an anesthetic cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg), given in a volume of 10 mL/kg (for induction). During the experiment, maintenance doses (approximately ¼× to ½× the initial dose) were given as required, and the animals were placed on an electrically heated pad to maintain body temperature at 37°C. The anterior chambers of the mouse eyes were cannulated with a 30G needle. After allowing up to 30 minutes for stabilization, the pressure within the eye was determined at different flow rates (0.1 μL/min to 0.5 μL/min in 0.1 μL/min increments). Following conclusion of the experiment, the anesthetized animals were removed from the perfusion apparatus and humanely killed via exposure to 100% CO2 (g) in a chamber until breathing stopped. A thoracotomy was immediately performed to ensure death. Outflow facility (C) was computed from the reciprocal of the slope of the regression line drawn through the plot of pressure as ordinate and flow rate as abscissa.

Immunohistochemistry for TGM2, Alpha Smooth Muscle Actin (αSMA), GGEI, and FN in Mouse TM

At the end of the time course of IOP readings, the mice were humanely killed as noted previously, and the eyes were enucleated, fixed in 4% PFA, and paraffin-embedded for immunohistochemistry. The 5-μm sagittal sections were mounted on slides, and the slides were warmed and deparaffinized prior to staining (three sections per eye were stained and imaged for all staining; the number of pairs of eyes used for each staining differed). Costaining for αSMA and TGM2 was performed. The slides were stained for GGEI and costained for GGEI and FN.

Briefly, for αSMA and TGM2 costaining, the sections from 6 BALB/cj mouse eyes (n = 6 pairs) were used. The deparaffinized slides were placed in antigen retrieval buffer (citrate buffer, 0.01 M, pH 6) and placed in a water bath at 60°C for 2 hours for heat-induced epitope retrieval followed by incubation in blocking buffer (10% goat serum/0.1% Triton X-100 in PBS superblock) overnight at 4°C. The sections were then incubated with αSMA antibody (ab7817, mouse monoclonal [1A4], diluted 1:500) and with TGM2 primary antibody (rabbit monoclonal [EP29577], diluted 1:500; Abcam). The sections were then washed in PBS three times for 10 minutes each followed by washing with PBS-Tween-20 (0.05%) once and incubated for 10 minutes followed by two quick washes with water. The sections were then incubated in appropriate secondary antibodies (goat anti-mouse Alexa Fluor 594 [Thermo Fisher Scientific] for αSMA, diluted 1:1000 and goat anti-rabbit Alexa Fluor 488 [Thermo Fisher Scientific] for TGM2, diluted 1:1000) in PBS superblock for 2 hours. The sections were then washed in PBS three times for 10 minutes each followed by wash with PBS-Tween-20 (0.05%) once and incubated for 10 minutes followed by two quick washes with water. The tissue sections were stained with DAPI to detect nuclei and allowed to dry overnight prior to imaging using Keyence all-in-one fluorescence microscope.

For the GGEI staining (n = 7 pairs of BALB/cj mouse eyes), following deparaffinization, antigen retrieval was performed with 0.01 M citrate buffer (pH 6) followed by blocking with mouse on mouse blocking solution (M.O.M. Kit, Vector Biolaboratories, Inc.) for 2 hours, followed by incubation with GGEI primary antibody (ab124, diluted 1:100) in PBS superblock overnight at 4°C. The sections were then incubated with an appropriate secondary antibody (goat anti-mouse Immunoglobulin M [IgM] cross-adsorbed secondary antibody, Dylight 594 [Thermo Fisher Scientific], diluted 1:200) in PBS superblock for 2 hours and washed with PBS + Tween 20 (0.05%) followed by PBS washes. The tissue sections were stained with DAPI to detect nuclei and allowed to dry overnight prior to imaging using the Keyence all-in-one fluorescence microscope.

For GGEI and FN costaining (n = 3 pairs of eyes, 1 BALB/cj and 2 C57BL/6j), the slides were deparaffinized and directly blocked with PBS superblock overnight at 4°C. The slides were then coincubated with primary antibody to FN (AB1945; rabbit anti-human FN, diluted 1:500; Millipore) and GGEI primary
FIGURE 1. Ad5.TGM2 transduction of cultured primary cell strain GTM 125-05 increases TGM2 expression, increases GGEL crosslinks, and fibronectin expression. Increased TGM2 staining is observed in Ad5.TGM2-transduced GTM 125-05 cells (B, D) when compared with the Ad5.Null-treated control (A, C; quantification of TGM2 expression-I; \( P = 0.0035, n = 6 \)). A, B = 100×; C, D = 400×. Increased GGEL crosslinks and fibronectin costaining in GTM 125-05 cells transduced with Ad5.TGM2 (F, H) when compared with Ad5.Null-treated controls (E, G; quantification of GGEL-J; \( P = 0.1 \), nonsignificant; F, N–K, \( P = 0.5 \), nonsignificant; \( n = 6 \)). A, B, E, F = 100×; C, D, G, F = 400×. Green: TGM2; red: GGEL; blue (DAPI): nuclei.
FIGURE 2. GTM3 cells transduced with Ad5.TGM2 showed increased TGM2 expression and GGEL crosslinks. Increased TGM2 staining is observed in Ad5.TGM2-transduced GTM3 cells (B, F, H) when compared with Ad5.Null-treated controls (A, E, F; quantification of TGM2 expression-I; $P = 0.01; n = 6$). Increased GGEL crosslinking in GTM3 cells transduced with Ad5.TGM2 (D, F, H) when compared with untreated control (C, E, G; quantification of GGEL-J; $P = 0.09$, nonsignificant; $n = 6$). A–F 100×; G–H, 200×.
FIGURE 3. MTM cells transduced with Ad5.TGM2 showed increased TGM2 expression and GGEL crosslinks. Increased TGM2 staining is observed in Ad5.TGM2-transduced MTM cells (B, F, H) when compared with Ad5.Null-treated control (A, E, F; quantification of TGM2 expression-I; \( P = 0.08 \), nonsignificant; \( n = 6 \)). Increased GGEL crosslinking is observed in MTM cells transduced with Ad5.TGM2 (D, F, H) when compared with untreated control (C, E, G; quantification of GGEL-J; \( P = 0.1 \), nonsignificant; \( n = 6 \)). A to F: 100×; G to H: 200×.
antibody (ab424, diluted 1:100) in PBS superblock overnight at 4°C. Sections were then washed in PBS 3 times for 10 minutes each followed by incubation with PBS-Tween-20 (0.05%) for 10 minutes followed by 2 quick washes with water. Sections were incubated in appropriate secondary antibodies (goat anti-mouse IgM cross-adsorbed secondary antibody, DyLight 594 [Thermo Fisher Scientific] for GGEL, diluted 1:200, and goat anti-rabbit Alexa Fluor 488 [Thermo Fisher Scientific] for FN, diluted 1:1000) in PBS superblock for 2 hours. The sections were then washed in PBS three times for 10 minutes each followed incubation with PBS-Tween-20 (0.05%) once and incubated for 10 minutes followed by two quick washes with water. The tissue sections were stained with DAPI to detect nuclei and allowed to dry overnight prior to imaging using Keyence all-in-one fluorescence microscope.

Statistics
All statistical tests were conducted using the software provided by GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) and SigmaPlot 12.5 (Systat Software, Inc, San Jose, CA, USA). Multiple comparisons were conducted using ANOVA followed by Holm-Sidak post hoc testing. For immunocytochemistry quantification of the GTM 125-05 and MTM cells, fluorescence intensity was normalized to cell number (DAPI), and the Ad5.Null treated group was compared to the Ad5.TGM2 transduced group using paired Student’s t-test (two-tailed). In the GTM3 cells, total fluorescence intensity for the Ad5.Null treated group was compared to the Ad5.TGM2 transduced group using paired Student’s t-test (two-tailed). For aqueous outflow facility, the values for the injected eyes were compared with their fellow uninjected contralateral control eyes using paired Student’s t-test (two-tailed). In all cases, a P value of < 0.05 was considered to be significant.

RESULTS
TGM2 Crosslinking Activity in GTM 125-05, GTM3, and MTM Cells
Previously, we and others and reported that adenovirus serotype 5 efficiently transduces TM cells and expresses the transgene of interest.40,41 Prior to in vivo injections, we wanted to test and validate our viral TGM2 transgene in cultured TM cells.

To determine overexpression of TGM2, GTM 125-05, GTM3, and MTM cells were transduced with Ad5.TGM2 and with Ad5.Null as a treatment control. The GTM 125-05 and GTM3 cells treated with Ad5.TGM2 (Figs. 1B, 1D, 2B, 2F, 2H) showed a significant increase (Figs. 1I, 2I; P = 0.0035 and P = 0.01, n = 6) in TGM2 expression when compared with the cells treated with Ad5.Null (Figs. 1A, 1C, 2A, 2E, 2G). The MTM cells treated with Ad5.TGM2 (Figs. 3B, 3F, 3H) showed an apparent increase (Fig. 3I; P = 0.08, n = 6) in TGM2 expression when compared with the cells treated with Ad5.Null (Figs. 3A, 3E, 3G).

To validate the crosslinking activity of TGM2, transduced GTM 125-05, GTM, and MTM cells were fixed in 4% PFA and stained for GGEL bonds. The GGEL antibody detects covalent crosslinks mediated by TGM2 between glutamine and lysine side chains (GGEL) of ECM proteins. There appeared to be increased GGEL staining (Figs. 1F, 1H) when compared with the Ad5Null treated cells (Figs. 1E, 1G, 2C, 2E, 2G, 3C, 3E, 3G). This indicates that increased TGM2 crosslinking activity correlated with increased expression of TGM2 as seen in Ad5.TGM2 transduced cells when compared with controls. There was no apparent increase in FN in the Ad5.TGM2 transduced cells (Figs. 1F, 1H) when compared with the Ad5.Null treated cells (Figs. 1E, 1G, 2C, 2E, 2G, 3C, 3E, 3G).
FIGURE 5. Effect of Ad5.TGM2 transduction of mouse eyes on expression of TGM2 in the TM region. Ad5.TGM2 increased TGM2 expression in the iridocorneal angle including the TM of Ad5.TGM2-transduced eyes (B, D) when compared with control eyes (A, C) determined by immunofluorescent staining ($n = 6$ pairs of eyes). Costaining with α-SMA stained the TM, ciliary muscle, and endothelium of Schlemm’s canal (A, B; red: α-SMA; blue [DAPI]: nuclei). TGM2 staining only (C, D) without α-SMA staining of the same sections as A and B, respectively (blue [DAPI]: nuclei; green: TGM2). White boxes highlight TM region. H&E images (E, F) of sections from the same eyes represented above (A-D). Black boxes highlight TM region. TM, trabecular meshwork.

1G; Fig. 1K, $P = 0.5$, $n = 6$). This is likely a result of the early time point (48 hours), which was not sufficient time for accumulation of the crosslinked FN. There also appeared to be some colocalization between GGEL and FN.

To ensure that the antibody stains extracellular crosslinks, we decellularized cultured GTM 125-05 cells and stained the ECM with the GGEL antibody. The extracellular matrix derived from cells treated with Ad5.TGM2 showed higher crosslinking ($n = 3$; Fig. 4B) when compared with the ECM from untreated cells ($n = 3$; Fig. 4A). The coverslips were stained with DAPI to identify nuclei, and the lack of any visible nuclei ensured that all cells were removed (Figs. 4C, 4D).

**Immunofluorescence of TGM2 Transduced Mouse Eyes Showed Increased TGM2 Expression and Increased Crosslinking Following Intravitreal Injection of Ad5.TGM2**

After confirming the expression and activity of our Ad5.TGM2 transducing vector in vitro, one eye of each mouse (BALB/cJ and C57BL/6) was injected with Ad5.TGM2 to evaluate in vivo expression and activity. Six BALB/cJ mice were used for TGM2 immunofluorescence. The images were assessed by two independent masked observers, and each observer identified increased TGM2 staining at the iridocorneal angle, including the TM, in five of the six Ad5.TGM2 transduced eyes (Figs. 5B, 5D) when compared with the uninjected control (Figs. 5A, 5C). Increased TGM2 expression was observed in the TM. The sections were costained with a-SMA to identify TGM2 staining in the TM, and interestingly colocalization of TGM2 and a-SMA was observed in the TM. Positive staining of TGM2 was also seen in ciliary body as previously reported.42,43

To determine whether increased TGM2 expression led to increased ECM crosslinking activity, we performed GGEL immunofluorescent staining of mouse eye sections. The primary antibody identified GGEL isopeptide bonds. Three independent masked observers assessed GGEL staining between Ad5.TGM2 transduced (Figs. 6B, 6D) and their contralateral uninjected eyes (Figs. 6A, 6C) in BALB/cJ. Two of three masked observers identified increased GGEL crosslink staining in the TM region in seven of seven Ad5.TGM2 injected eyes when compared with their contralateral uninjected
controls, and the remaining masked observer identified increased crosslink staining in five of seven Ad5.TGM2 injected eyes when compared with their contralateral uninjected controls.

We also wanted to see if increased TGM2 expression and crosslinks affected FN expression. We tested for GGEL and FN coexpression in two C57Bl/6j and one BALB/cj mouse eyes. We found increased fibronectin staining in two of the three Ad5.TGM2-transduced eyes (Figs. 7B, 7D) when compared with their contralateral uninjected controls (Figs. 7A, 7C). However, we were unable to see colocalization because the optimal antigen retrieval and blocking methods for GGEL and FN staining varied (i.e., the method optimum for FN was unable to sufficiently stain GGEL at the TM).

**FIGURE 6.** Effect of Ad5.TGM2 transduction of mouse eyes on GGEL crosslinks in the TM region. Ad5.TGM2 increased TGM2 crosslinking activity (GGEL) in the TM region of Ad5.TGM2-transduced mouse eyes (B, D) when compared with respective contralateral controls (A, C) as determined by immunofluorescent staining (n = 7). Representative images from two pairs of eyes are shown. Scale 400X. White boxes highlight TM region. Inserts are brightfield images of the same sections. CB, ciliary body.

**FIGURE 7.** Effect of Ad5.TGM2 transduction of mouse eyes on FN in the TM region. Ad5.TGM2 transduction of mouse eyes shows increased FN staining (B, D) when compared with uninjected controls (A, C, respectively) as determined by immunofluorescent staining (n = 3). Costaining with GGEL (red). Representative images from two pairs of eyes are shown (blue [DAPI]: nuclei; green: FN).
Intravitreal Injection of Ad5.TGM2 Increases IOP in Mouse Eyes

To assess whether overexpression of TGM2 in the mouse TM could affect the IOP, we transduced living mouse eyes with Ad5.TGM2. We first did a pilot study with 17 BALB/cJ mice, 6 in the naïve group, 5 in the Ad5.GFP-transduced group, and 6 in the Ad5.TGM2-transduced group (Fig. 8A). In all mice, intravitreal injections were performed in the left eyes only, whereas the right eyes were used as a contralateral uninjected control. Ad5.GFP transduction was assessed 1 week following injection by examination of each eye in a darkened room under ultraviolet illumination (Illumatool Model LT-9900; Lightools Research, Encinatas, CA, USA) through a filter (excitation 470 nm, emission 515 nm) for fluorescence as an indication of presence of GFP in the anterior chamber. Green fluorescence was observed in the Ad5.GFP-transduced eyes in vivo at 7 days, which confirmed Ad5.GFP transduction. Ad5.TGM2-transduced eyes showed significantly increased IOP on day 9 that continued for 2 weeks. Differences between the left (injected) eye and right (uninjected contralateral control) eye Δ(OS-OD) show a significant difference in IOP (P < 0.0001) in Ad5.TGM2-transduced eyes when compared with the Δ(OS-OD) of the naïve or Ad5.GFP group (P < 0.0001; ****P < 0.0001; B, C).

Intravitreal Injection of Ad5.TGM2 Increases IOP in Mouse Eyes

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**Figure 8.** Effect of Ad5.GFP and Ad5.TGM2 transduction on mouse IOP. Ad5.TGM2 transduction of the left eyes of the BALB/cJ mice (ocular sinister; OS) significantly increased IOP when compared with uninjected right eyes (ocular dexter; OD; n = 6). Ad5.GFP transduction in the left eye (OS) did not affect the IOP of either eye (OD or OS) in the Ad5.GFP group (n = 5). The IOPs of the naïve mice (n = 6) were measured in same experimental conditions to ensure that the environmental conditions did not affect IOP readings (A). **P = 0.01; ***P < 0.001. Significance between injected TGM2 and noninjected groups was analyzed by two-factor ANOVA followed by Holm-Sidak post hoc analysis. The difference between the left (injected) eye and right (uninjected contralateral control) eye Δ(OS-OD) shows a significant difference in IOP (P < 0.0001) in Ad5.TGM2-transduced eyes when compared with the Δ(OS-OD) of the naïve or Ad5.GFP group (P < 0.0001; ****P < 0.0001; B, C).
next experiment using both BALB/c\(j\) \(n=18\) and C57BL/6\(j\) \(n=9\) mice.

In BALB/c\(j\) mice, the IOPs were increased in the transduced eyes (Fig. 9A) with significant increases on days 14, 19, and 22 and a maximum difference of 5.16 mm Hg on day 19. The mean IOP of injected eyes on day 19 was 15.86 ± 1.06 (mean ± SEM), whereas the mean IOP of uninjected eyes was 10.7 ± 0.48 (mean ± SEM), which was the day with a maximum difference in IOPs \(P < 0.001\). Similarly, in C57BL/6\(j\) mice, injection of Ad5.TGM2 increased IOP from day 13 with maximum difference at day 17 with mean IOP of 17.09 ± 2.03 mm Hg (injected) vs. 12.01 ± 0.47 mm Hg (control). There was little evidence of inflammation or altered IOP in any of the contralateral control eyes. In the injected eyes, we saw none to mild lenticular opacity (score = 0–1), which has previously shown to not affect IOP.\(^{50}\)

Statistical analysis was performed to ensure that there was no significant difference between eyes prior to injection. In the BALB/c\(j\) mice, baseline IOP readings were taken 2 days prior to injections (Fig. 9A). Holm-Sidak’s multiple comparisons test was performed, and the adjusted \(P\) value for day −2 (baseline) was greater than 0.9999. In the C57BL/6\(j\) mice, baseline IOP readings were taken 1 day prior to injections. Holm-Sidak’s multiple comparisons test was performed, and the adjusted \(P\) value for day −1 (baseline) was 0.5461 (Fig. 9B). For our \(P\) values less than 0.05 were considered significant; therefore, the difference between the baseline IOP of both eyes was not significant.

The baseline IOP dropped during the course of the study in the second BALB/c\(j\) group, by day 8 (adjusted \(P\) value was 0.7710) onward, and persisted until the end of the study day 22 (adjusted \(P\) value was 0.5091). A similar result was seen in the C57BL/6\(j\) group by day 9, although in this group the difference was lost by day 20. We feel that these drops over time were modest (≤ 2 mm Hg) and were likely a reflection of the animals’ settling into the procedure of conscious IOP measurement and becoming more comfortable with the procedure. These drops were also considerably smaller than the rise in IOP seen in the Ad5.TGM2-injected groups of between 3 and 5.5 mm Hg. However, we acknowledge that the drop in the vehicle-injected groups may have also reflected a masking effect on the rise in the Ad5.TGM2-injected groups.

**Overexpression of TGM2 in Mouse Eyes Decreases Aqueous Humor Outflow Facility**

Aqueous humor outflow facility (C) measurements were performed using a constant flow infusion method after final IOP readings. In the BALB/c\(j\) mice, the mean aqueous outflow facility of the TGM2-transduced eyes (0.015 ± 0.002 \(\mu\)l/min/mm Hg) was significantly lower when compared with the control eyes (0.021 ± 0.002 \(\mu\)l/min/mm Hg; \(P = 0.01\); Figs. 10A, 10B). In the C57BL/6\(j\) mice, the mean aqueous outflow facility of TGM2-transduced eyes (0.012 ± 0.0018 \(\mu\)l/min/mm Hg) was significantly lower when compared with control eyes (0.019 ± 0.0036 \(\mu\)l/min/mm Hg; \(P < 0.05\); Figs. 10C, 10D). Therefore, significantly increased outflow resistance was observed in the Ad5.TGM2-transduced eyes in both strains.

**DISCUSSION**

Our current study addresses the hypothesis that the increased expression of TGM2 in the TM may lead to physiological changes in the TM, causing increased outflow resistance and elevated IOP. We investigated the effects of using adenovirus serotype 5 to overexpress TGM2 in the TM of mouse eyes. The overexpression of TGM2 protein and activity (i.e., increased GGEL crosslinks) was confirmed by immunohistochemistry. With an increase in TGM2 expression and activity, we observed a significant decrease in aqueous outflow facility and a significant increase in IOP in TGM2 overexpressing eyes (Fig. 11).

The endogenous expression of TGM2 in TM tissues and cells has been previously reported.\(^{29,43,44}\) The overexpression of TGM2 in the mouse TM mimicked the increased expression of TGM2 in the TM of human glaucomatous eyes as reported previously.\(^{29}\) Our immunocytochemistry studies were performed at 48 hours or 5 days (in MTM cells), where Ad5.TGM2 transduction significantly elevated TGM2 expression and also showed a trend for increased GGEL immunostaining. Although increased TGM2 expression validates our adenovirus (Ad5.TGM2) and an increase in expression of TGM2 corresponds with an increase in its crosslinking activity, 48 hours or 5 days may not be sufficient to cause significantly increased crosslinking of FN, one of the main...
substrates for TGM2, because ECM turnover is a dynamic process. We believe that longer time points would eventually show significantly increased GGEL crosslinking and FN accumulation. Irreversible covalent bonds between FN molecules and other ECM proteins would lead to reduced turnover and greater ECM deposition.

Recently, Yang et al. have reported that inhibition or induction of LOX, another ECM crosslinking enzyme, affected outflow resistance by modulating ECM crosslinking in perfused human and porcine anterior segments. While adenoviral transduction of factors including bioactivated TGF-β2 have been previously shown to elevate IOP,46 in the present study, we determined that increased crosslinking by TGM2 alone can significantly elevate IOP in mouse eyes and can therefore be a potential therapeutic target. However, it will be interesting to see whether inhibition of TGM2 crosslinking activity can significantly reduce IOP or whether other crosslinking enzymes such as LOX and lysyl-oxidase like enzymes 1-4 compensate it.

TGF-β2 is elevated in the AH and TM of POAG patients.3-7 Excess TGF-β2 activates the profibrotic pathway. TGF-β2 increases production of ECM proteins along with ECM crosslinking enzymes (i.e., TGM2 and LOXs). A fibrotic phenotype is a result of dysregulation in the formation of ECM and/or its breakdown. TGM2, which is elevated in POAG eyes, is associated with other fibrotic diseases.29 Classic actions of TGM2 include crosslinking ECM proteins causing increased tissue stiffness and loss of homeostasis.47,48 The presence of increased GGEL crosslinks indicates that increased expression of TGM2 in the TM is associated with increased crosslinking activity.29 The present study suggests that an increase in crosslinking can decrease turnover of ECM in the TM thereby promoting ECM deposition that leads to increased outflow resistance through the TM. Although we believe that this is the most significant factor leading to ECM deposition and

![Figure 10](http://tvst.arvojournals.org/) Ad5.TGM2 decreases aqueous outflow facility in Ad5.TGM2-transduced eyes. (A) BALB/cJ mouse pressure-flow rate curves in the eyes of live animals (n = 6; anterior chamber perfusion). Error bars show SDM. Over the flow rate range 0.1 to 0.5 µL/min, pressure in Ad5.TGM2-transduced eyes (pressure range of 24.94 ± 3.39 to 60.36 ± 12.95 mm Hg) was significantly higher when compared with uninjected controls (pressure range of 14.58 ± 3.12 to 35.31 ± 10.19 mm Hg). (B) Corresponding mean C was significantly lower in TGM2-transduced BALB/cJ eyes when compared with uninjected controls (pressure range of 0.013 ± 0.002 µL/min/mm Hg vs. 0.021 ± 0.002 µL/min/mm Hg [mean ± SEM]; P = 0.01) and correlated closely with the associated increase in IOP. (C) C57BL/6J mouse pressure-flow rate curve in the eyes of live animals (n = 5; anterior chamber perfusion). Error bars show SDM. Over the flow rate range 0.1 to 0.5 µL/min, pressure in Ad5.TGM2-transduced eyes (pressure range of 24.02 ± 2.27 to 58.79 ± 13.44 mm Hg) was significantly higher when compared with uninjected controls (pressure range of 14.43 ± 2.22 to 36.75 ± 8.61 mm Hg). (D) Corresponding mean C was significantly lower in TGM2-transduced C57BL/6J eyes when compared with uninjected control eyes (0.012 ± 0.0018 µL/min/mm Hg vs. 0.019 ± 0.0036 µL/min/mm Hg [mean ± SEM]; P < 0.05) and correlated closely with the associated increase in IOP. SDM, standard deviation of mean.
increased outflow resistance, TGM2 is also known to activate TGFβ2 from its latent form.49 This pathway could therefore also be partially responsible for the downstream effects of TGFβ2 (including a TGFβ2-mediated increase in ECM production). Fibronectin is an ECM substrate for TGM2, and increased expression of FN has been shown in the TM of POAG eyes.24 Similarly, other ECM proteins can be crosslinked by TGM2, thereby contributing to ECM deposition. It would be interesting to further investigate which of the ECM proteins are crosslinked by TGM2 in the glaucomatous TM tissue. Intracellular TGM2 also has a variety of signaling functions,50 which may affect IOP or AH outflow facility and will require further study.

A remaining intriguing question in POAG is among all the pathways and genes activated by TGFβ2, which are capable of reducing aqueous outflow and significantly increasing IOP? In the past, ex vivo cultures perfused with MMPs and more recently wild-type mice infected intracamerally with adeno-associated virus metrix metalloproteinase 3 decreased IOP, suggesting that the TM ECM regulates IOP.15,51 In our present study, TGM2 overexpression decreased the AH outflow facility, suggesting that ECM remodeling in the TM significantly increased IOP. From the modified Goldmann equation,38,51 and assuming reported values for aqueous humor formation rate (Fin), uveoscleral outflow (Fu), and episcleral venous pressure (Pe), in BALB/cJ mice,52 the theoretical mean IOP of the injected eyes as calculated from the outflow facilities is 15.2 mm Hg (the measured mean value by rebound tonometer the day before was 14.70 ± 1.01 mm Hg), whereas the theoretical mean IOP of the un.injected eyes is 11.7 mm Hg (the measured mean value the day before by rebound tonometer was 10.69 ± 0.52 mm Hg). The modified Goldmann equation states that IOP = ((Fin – Fu)/C) + Pe. Thus, for naive (uninjected) BALB/cJ mouse eyes (assuming that $C = 0.021 \mu l/min/mm Hg$): IOP = $[(0.17 - 0.05)/0.013] + 6 = 15.2$ mm Hg (assuming Fu is 30% of Fin). For Ad5.TGM2-injected BALB/cJ mouse eyes (assuming C has dropped to 0.013 $\mu l/min/mm Hg$): IOP = $[(0.17 - 0.05)/0.013] + 6 = 15.2$ mm Hg (assuming Fu is 30% of Fin). These computed IOP values are very close to those we actually measured in the BALB/cJ mice. The aqueous humor dynamic parameters for BALB/cJ mice are from a previous publication.52 Therefore, the TGM2-induced IOP elevation corresponded closely with the TGM2-mediated reduction of the outflow facility. For our outflow studies on the C57BL/6J mice, we did not have a large enough "n" to obtain a better significance. In the future, it would be interesting to determine whether TGM2 leads to the dense fibrillar depositions that have been observed in POAG eyes.

In POAG, increased resistance to aqueous outflow elevates IOP, which likely contributes to the damage to the optic nerve. Consistently high IOP or IOP fluctuations in POAG could be the result of fibrotic changes in the TM.52,53 In normal conditions, the IOP is constantly maintained, but in pathological conditions there is a dysregulation in the TM, which affects the tissue dynamics and leads to a fibrotic phenotype. During the initial stages of disease progression, the tissue probably has higher compensation capability leading to the fluctuations. As the disease progresses, the ECM deposition increases to an extent that cannot be compensated any further leading over time to a high IOP. Our model has features of increased ECM crosslinking, increased outflow resistance, and elevated IOP, which phenocopies ocular hypertension in POAG. TGFβ2 has a number of effects on the TM, so in the future it will be interesting to determine whether TGFβ2-induced ocular hypertension is mediated at least partially by increased TGFβ2 expression and activity. Consequently, inhibition of TGM2-mediated ECM crosslinking by could be a potential therapeutic approach. However, the challenge would be to determine if TGM2 inhibition alone would suffice or whether TGM2 inhibition in combination with other IOP lowering agents would be more effective as a cure for glaucoma.

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