Transforming Growth Factor- β 2 Induces Expression of Biologically Active Bone Morphogenetic Protein-1 in Human Trabecular Meshwork Cells

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PURPOSE. There are limited studies on the factors that regulate the processing of TGF- β 2 and extracellular matrix (ECM) proteins into their mature form. Bone morphogenetic protein 1 (BMP1) is an enzyme responsible for the cleavage and maturation of growth factors and ECM proteins. The purpose of our study was to determine whether cultured human trabecular meshwork (TM) cells express BMP1, BMP1 expression is regulated by TGF- β 2, BMP1 is biologically active, and BMP1 regulates LOX activity.

METHODS. Primary human TM cells were isolated and subjected to quantitative PCR (qPCR) and Western immunoblotting (WB) for BMP1. BMP1 immunolocalization was performed in TM tissues. qPCR was used to determine BMP1 mRNA expression and WB results were used to determine BMP1 protein expression. BMP1 activity was measured in TM cells treated with TGF- β 2 or with a combination of TGF- β 2/UK383367. Lysyl oxidase (LOX) enzyme activity was evaluated by WB in TM cells treated with BMP1 or with a combination of BMP1/ β -aminopropionitrile (BAPN).

RESULTS. Human TM cells expressed mRNA and protein for BMP1. Exogenous TGF- β 2 increased mRNA expression compared to their controls ($P < 0.05$). An ELISA showed TGF- β 2-induced BMP1 secretion compared to their controls in all cell strains ($P < 0.05$). Secreted BMP1 stimulated LOX enzymatic activity in TM cells.

CONCLUSIONS. BMP1 is expressed in the human TM. TGF- β 2 induction of BMP1 may be responsible for increased processing of growth factors and ECM proteins into their mature forms, resulting in TM stiffness and resistance to ECM degradation.

Keywords: trabecular meshwork, bone morphogenetic protein 1, transforming growth factor beta 2

Glaucoma is a major cause of irreversible blindness, affecting more than 70 million people worldwide.¹ Elevated IOP is a major risk factor in the development² and progression of glaucoma.^{3,4} Elevated IOP is due to increased aqueous humor (AH) outflow resistance, and is associated with morphologic and biochemical changes in the trabecular meshwork (TM).^{5,6} Our knowledge is limited with respect to the regulation of TM function and the pathophysiology of AH outflow resistance in the glaucomatous TM.

One mechanism to account for outflow resistance in the glaucomatous TM is increased TM stiffness. Last et al. reported that the mean elastic modulus (i.e., a measure of tissue stiffness) was increased significantly in the glaucomatous human TM when compared to age-matched controls.⁷ They suggested that a change in the physical properties of the TM might directly modulate AH outflow resistance and IOP.

Human TM cells synthesize and secrete enzymatically active lysyl oxidase (LOX) and lysyl oxidase like (LOXL) proteins.⁸ These enzymes are known to change the physical properties and increase stiffness of tissues by cross-linking extracellular matrix (ECM) proteins (e.g., collagens and elastin) and inhibiting ECM turnover. LOXL1 is an important risk factor for exfoliation syndrome and glaucoma.^{9,10} LOXL1 is induced by

TGF- β 1,⁸ and TGF- β 1 is elevated in the aqueous humor of exfoliation patients.¹¹ We have reported previously that treatment of human TM cells with exogenous TGF- β 2 caused increased expression and activity of LOX.⁸ These studies are significant since TGF- β 2 levels are elevated in the AH of primary open angle glaucoma (POAG) patients^{12–14} and in glaucoma TM cells.¹⁵

Bone-morphogenetic protein-1 (BMP1), also known as procollagen C-proteinase, is a zinc protease that converts secreted precursor pro-proteins into mature, functional proteins. LOX and LOXL proteins are secreted as inactive precursor pro-proteins. Importantly, LOX has been reported to be a substrate for BMP1. The pro-form of LOX is cleaved by secreted BMP1 to yield the active enzyme.^{16,17} Thus, the regulation of BMP1 expression and biological activity may be important in understanding AH dynamics, TM stiffness, and AH outflow resistance.

The purpose of our study was to determine if BMP1 is expressed in human TM cells and tissues, BMP1 expression is induced by exogenous TGF- β 2 in human TM cells, and TGF- β 2 stimulates secretion of biologically active BMP1 by human TM cells.

METHODS

Trabecular Meshwork Cell Culture

All experiments were performed with previously characterized primary human normal (NTM) and glaucoma (GTM) cell strains that were obtained from Alcon Research Ltd. (Fort Worth, TX).^{8,15,18–20} TM cells were grown in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO), L-glutamine (0.292 mg/mL), penicillin (100 units/mL), streptomycin (0.1 mg/mL), and amphotericin B (4 mg/mL). Antibiotics were purchased from HyClone (Logan, UT). Medium was changed every 2 to 3 days and cell cultures were maintained at 37°C with 5% CO₂. Confluent TM cell cultures were serum-deprived for 24 hours before treatment with 5 ng/mL TGF-β2 (R&D Systems, Minneapolis, MN) for 6 to 24 hours for mRNA and 48 hours for protein analyses.

RNA Isolation and Quantitative Real-Time (RT)-PCR (qPCR)

Total cellular RNA was prepared from normal TM cell strains ($n = 3$) using an RNeasy Lysis Kit (AM1912; Ambion, Austin, TX). Total RNA (1 μg) was used for cDNA synthesis with the iScript cDNA synthesis kit (Bio-Rad Laboratories; Hercules, CA) in a 20 μL reaction mix. qPCR was performed with 1 μL cDNA with a SSoAdvanced SYBR Green Supermix (Bio-Rad Laboratories) in a total volume of 20 μL. The thermoprofile parameters had an initial denaturation at 95°C for 30 seconds followed by 35 cycles of 95°C for 10 seconds; 65°C for 30 seconds followed by a melting curve step. PCR was performed on a real-time thermal cycler (model CFX96; Bio-Rad Laboratories). The expression of BMP1 was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $\Delta\Delta$ cycle thresholds (Ct) method. BMP1 primers were designed so that they flank exon-exon junctions, and GAPDH primers were taken from a previous publication²¹:

BMP1 forward: 5' CTGTGAGTGGGTCATTGTGG 3'
BMP1 reverse: 5' GGTGTCATCCGAGTGGAAC 3', giving an amplicon of 223 base pairs.
GAPDH forward: 5' GGTGAAGGTCGGAGTCAAC 3'
GAPDH reverse: 5' CCATGGGTGGAATCATATTG 3', giving an amplicon of 153 base pairs.

Each reaction for BMP1 and GAPDH was run in triplicate and Δ Ct relative expression values were normalized to GAPDH. The $\Delta\Delta$ Ct values were obtained by comparing the relative expression level of the Δ Ct treated sample to the Δ Ct control. The formula $2^{-\Delta\Delta Ct}$ was used to calculate the fold change of samples, and statistical analysis was performed on GraphPad Prism 5 (GraphPad, La Jolla, CA).

Protein Extraction, Conditioned Medium Collection, and Western Immunoblotting (WB)

Total cellular protein was isolated from cultured TM cells using mammalian protein extraction buffer (Pierce Biotech, Rockford, IL) and protease inhibitor cocktail (Pierce Biotech). Protein concentration was determined using the Bio-Rad Dc Protein Assay Systems as described by the manufacturer's instructions (Bio-Rad Laboratories). A standard curve was generated using bovine serum albumin and absorbance at 750 nm was read within 15 minutes. Conditioned medium (CM) was centrifuged at 68g then transferred to a new tube and stored at –80°C until used for WB, ELISA immunoassay, or analysis of BMP1 enzyme activity. Total cellular protein and

TABLE. List of Antibodies for Western Immunoblots/Immunolocalization

Antibody (Cat#)	Dilutions	Source
BMP1 (H00000649)-CM	1:500	Novus Biologicals, Littleton, CO
BMP1 (NBP1-02481) immunolocalization	1:100	Abcam, Cambridge, MA
Tropoelastin (Ab21600)	1:500	Abcam
Beta actin (1501)	1:1000	Millipore Corporation, Billerica, MA
Goat anti-rabbit (A11077)	1:100	Molecular Probes, Grand Island, NY
Goat anti-mouse (Sc2005)	1:10000	Santa Cruz Biotechnology, Inc., Santa Cruz, CA
Anti-rabbit (7074)	1:10000	Cell Signaling Technology, Inc., Danvers, MA

conditioned medium from each TM cell strain were run in parallel for WB analyses.

For WB, an equal volume of conditioned medium or 30 μg of total cellular protein from each sample was separated by SDS-PAGE, and separated proteins subsequently were transferred to PVDF membranes. The PVDF membranes were incubated in 5% nonfat milk in tris-buffered saline plus Tween (TBST) buffer for 60 minutes to block nonspecific binding. The polyvinylidene difluoride (PVDF) membranes were probed with primary antibodies followed by secondary antibodies (see Table). The Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotech) was used for signal development, and images were obtained using a Fluorchem 8900 imager (Alpha Innotech, San Leandro, CA).

Immunolocalization of BMP1 in Human Trabecular Meshwork Tissues

To document the presence of BMP1 in human TM tissues, 3 pairs of normal (82, 92, and 97 years) and 3 pairs of glaucomatous (87, 94, and 98 years) human eyes were used. Briefly, eyes were obtained from regional eye banks within 12 hours of death and fixed in 10% formalin. The eyes were obtained and managed in compliance with the tenets of the Declaration of Helsinki. For antigen retrieval, paraffin sections were deparaffinized, rehydrated, and placed in citrate buffer (pH 6) for 15 minutes at 95°C followed by 15 minutes at room temperature, and 0.5M glycine for 15 minutes. Paraffin sections were incubated in 5% goat serum in PBS for 30 minutes, washed, and incubated overnight at 4°C with BMP1 primary antibody (10 μg/ml, see Table). The primary antibody subsequently was detected by incubation with goat anti-rabbit Alexa 568 secondary antibody (Molecular Probes) for 1 hour. Cell nuclei were visualized by staining TM sections with 4',6-diamidino-2-phenylindole (DAPI, 300 nM) for 10 minutes at room temperature. Negative controls consisted of omission of the primary antibody or the addition of rabbit IgG (10 μg/ml, Vector Labs, Burlingame, MA) in place of the primary antibody. Images were captured using a Zeiss 510 confocal microscope (Carl Zeiss, Thornwood, NY).

BMP1 ELISA Immunoassay

BMP1 protein secretion was measured in CM of NTM ($n = 3$) and GTM ($n = 3$) cell strains using a commercially available BMP1 ELISA kit as described by the manufacturer's instructions (Cedarlane Laboratories, Burlington, NC). BMP1 assay results were obtained using a spectrophotometer plate reader

(Spectra max 340 PC; Molecular Devices, Sunnyvale, CA) at a wavelength of 450 nm. The amount of BMP1 protein secreted (ng/mL) was plotted for each sample using GraphPad Prism 5. The BMP1 ELISA assay has a sensitivity range between 0.156 and 10 ng/mL (Cedarlane Laboratories).

BMP1 Enzyme Activity Assay

BMP1 enzyme activity was measured using a fluorescent assay according to the manufacturer's instructions (R&D Systems). Briefly, cultured NTM cell strains ($n = 3$) were grown in a 6-well plate and maintained until 100% confluent. Subsequently, TM cells were serum deprived for 24 hours, and then pretreated with the potent and selective BMP1 inhibitor 3-(aminocarbonyl)- β -(3-cyclohexylpropyl)-N-hydroxy-1,2,4-oxadiazole-5-propanamide (UK383367; R&D Systems) at various concentrations (1, 3, or 5 μ M) for 1 hour. The medium was changed, and BMP1 inhibitor and TGF- β 2 (5 ng/mL) were added for an additional 24 hours. CM was collected and 25 μ L of CM were diluted to 50 μ L with buffer (25 mM HEPES, 0.1% Brij-35, pH 7.5). Then, 50 μ L of the fluorogenic substrate MCA-Tyr-Val-Asp-Ala-Pro-Lys (DNP)-OH (20 μ M; R&D Systems) were added and the solution subsequently was loaded into a black well plate. The enzymatic activity was measured using a fluorescent plate reader (M200; Tecan, Durham, CA) at an excitation wavelength of 320 nm and emission wavelength of 405 nm. A standard curve was derived using a standard compound (MCA-Pro-Leu-OH; Bachem, Torrance, CA) for BMP1 enzyme assays.

LOX Activity Assay

LOX enzyme activity was evaluated by WB as established previously in our laboratory.⁸ Briefly, cultured NTM cell strains ($n = 3$) were grown in 6-well plates and maintained until 100% confluent, and then serum-deprived for 24 hours. The cells were then pretreated with various concentrations of the LOX inhibitor β -aminopropionitrile (BAPN; #A-3134; Sigma-Aldrich, St. Louis, MO) for 6 hours. At the end of 6 hours, medium was changed, and selective concentrations of BMP1 and BAPN were added for an additional 48 hours. Negative controls consisted of no treatment, BMP1 alone, and BAPN alone. At the end of the experiment, protein lysates were collected for WB. The assay is based on the ability of LOX to catalyze elastin cross-linking and for BAPN to reverse the cross-linking, leading to higher levels of the substrate, tropoelastin.⁸

Statistical Analysis

Data were analyzed using Student's *t*-test or one-way ANOVA and Bonferroni correction for multiple comparison tests. Statistical analysis was performed for each experiment with the statistical test described in the respective figure legends. Where indicated, * indicates a statistical difference at $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, respectively.

RESULTS

BMP1 mRNA and Protein Expression in TM Cells

BMP1 mRNA was expressed in 3 NTM and 3 GTM cell strains (Fig. 1A). There was no statistical difference in mRNA expression between NTM and GTM cell strains as determined by qPCR. BMP1 protein was detected as a 75 kDa band in the CM of 3 NTM and 3 GTM cell strains (Fig. 1B). BMP1 protein levels were significantly increased ($P < 0.05$) in the CM of GTM cell strains compared to the CM of NTM cell strains (Fig. 1C).

FIGURE 1. Expression of mRNA and secreted BMP1 protein in NTM and GTM cells. (A) BMP1 mRNA expression was analyzed by qPCR for NTM ($n = 3$) and GTM ($n = 3$) cell strains. Data represent the Δ Ct expression values after normalization to GAPDH. (B) Representative WB for BMP1 in CM from NTM and GTM cell strains. (C) Densitometric readings of BMP1 in CM for NTM ($n = 5$) and GTM ($n = 5$) cell strains (mean $\pm$ SEM) * $P < 0.05$ (Student's unpaired two-tailed *t*-test).

BMP1 Immunolocalization in Human TM Tissues

Since human NTM and GTM cell strains express and secrete BMP1, we next wanted to verify endogenous expression of BMP1 in human TM tissues. Immunohistochemical localization of BMP1 was conducted using 3 NTM and 3 GTM tissues from human donor eyes. NTM and GTM tissues exhibited similar BMP1 localization and intensity levels, verifying BMP1 presence in TM tissues (Fig. 2). Negative controls consisted of omission of primary antibody (data not shown) or incubation with rabbit IgG antibody (Fig. 2A). Human TM cell nuclei were visualized with DAPI (Fig. 2B). BMP1 expression was located in all three layers of the TM (uveal, corneoscleral, and juxtacanalicular, Fig. 2C). A merged image of the TM for DAPI and BMP1 is shown in Figure 2D. No obvious differences were observed in BMP1 immunolocalization between NTM and GTM tissues.

TGF- β 2 Induction of BMP1 in TM Cells

We next determined if treatment with TGF- β 2 induced BMP1 mRNA and protein in TM cells. Normal TM cell strains ($n = 3$)

FIGURE 2. Immunohistochemical localization of BMP1 in the human TM. Human TM tissues ($n = 6$) were fixed, sectioned, and incubated with a rabbit antibody to BMP1 followed by goat anti-rabbit Alexa 568. Representative images of BMP1 expression in human TM tissue are shown. Negative controls consisted of tissue incubation in rabbit IgG (A) or omission of primary antibody (not shown). (A, B, D) DAPI nuclear staining. (C) BMP1 appeared to be uniformly localized throughout the TM tissue. (D) merged DAPI nuclear staining with BMP1 immunolocalization. Photomicrographs are 400 $\times$ magnification. Scale bar: = 20 μ m.

were treated with TGF- β 2 (5 ng/mL) for 6, 12, or 24 hours followed by RNA extraction and qPCR analysis (Fig. 3A). TGF- β 2 significantly increased BMP1 mRNA expression at all time points (Fig. 3A).

Our WB analyses also showed BMP1 protein in the CM of NTM and GTM cell strains (Fig. 3B). Immunoblots demonstrated that secreted BMP1 protein levels increased significantly ($P < 0.05$) following treatment with TGF- β 2 (5 ng/mL) for 48 hours (Fig. 3B, 3C). BMP1 protein levels appeared to be greater in GTM cell strains compared to NTM cell strains (Fig. 3B).

To verify induction of BMP1 protein secretion following TGF- β 2 treatment, we performed a quantitative BMP1 ELISA on CM samples of 3 NTM and 3 GTM cell strains. BMP1 was significantly increased in NTM and GTM cell strains by TGF- β 2 when compared to untreated control (Fig. 4A). GTM cell strains were significantly ($P < 0.0001$) more responsive to exogenous TGF- β 2 than NTM cell strains (Fig. 4B).

BMP1 Enzyme Activity in TM Cells

Having shown that BMP1 protein is present and induced by TGF- β 2 in the CM of TM cell strains, we then determined whether BMP1 enzyme activity also was increased in TM cells following TGF- β 2 treatment. BMP1 activity was analyzed using a specific fluorogenic substrate (MCA-Tyr-Val-Asp-Ala-Pro-Lys (DNP)-OH) enzyme assay. If BMP1 is present and active, the cleaved substrate is released and the quencher is excited at 320 nm. Increased fluorescence correlates to increased enzyme activity. We used the BMP1 inhibitor UK 383367 to determine if the enzyme assay is specific to BMP1 and if TGF- β 2 regulates BMP1 enzyme activity. Three TM cell strains were pretreated for 1 hour with the UK 383367 (1, 3, 5 μ M) before incubation with TGF- β 2 (5 ng/mL) for 24 hours. Dimethyl sulfoxide (DMSO) alone served as a negative control. TGF- β 2 significantly increased BMP1 enzyme activity ($P < 0.05$), and the BMP1

inhibitor blocked TGF- β 2 induction of BMP1 enzyme activity ($P < 0.05$, Fig. 5).

Lysyl Oxidase Enzyme Activity in TM Cells

Lastly, we determined whether BMP1 regulates LOX enzymatic activity in TM cells. We reported previously that the irreversible LOX inhibitor, BAPN, might be used to block elastin cross-linking in TM cells.⁸ Human TM cell strains were pretreated for 6 hours with BAPN before incubation with BMP1 for 48 hours, and cell lysates subsequently were analyzed for tropoelastin using WB. If LOX is enzymatically active, the LOX inhibitor (BAPN) will decrease cross-linking of elastin leading to higher levels of the substrate, tropoelastin. Human TM cells were treated with increasing concentrations of BAPN (1, 3, and 10 mM) along with BMP1 (15 ng/mL) for 48 hours. We observed that 1 mM of BAPN along with BMP1 (15 ng/mL) increased tropoelastin levels (Fig. 6A). However, there appeared to be decreased tropoelastin levels with higher concentrations of BAPN, perhaps due to cytotoxicity.⁸ Beta actin was used as an internal loading control. In addition, TM cells were treated with increasing concentrations of BMP1 (5, 15, 25, and 50 ng/mL) along with BAPN (1 mM) for 48 hours, and the levels of tropoelastin were determined by WB (Fig. 6B). Beta actin was used as an internal loading control. These data suggested that TGF- β 2 induces secretion of biologically active BMP1 in TM cells.

DISCUSSION

We have found that BMP1 is expressed, enzymatically active, and regulated by TGF- β 2 in human TM cells. We have demonstrated that TM cells and tissues express BMP1 mRNA and protein. Glaucomatous TM cells have higher levels of secreted BMP1 in the CM, which can be induced further by TGF- β 2 compared to NTM cells. BMP1 mRNA and protein are

FIGURE 3. TGF-β2-induced BMP1 mRNA and protein in TM cell cultures. (A) BMP1 mRNA induction following TGF-β2 (5 ng/mL) treatment for 6, 12, and 24 hours in NTM cell strains ($n = 3$). Data represent the fold levels of BMP1 mRNA in cell strains normalized to GAPDH (mean ± SEM). * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$. (B) Immunoblot results of secreted BMP1 protein for control (C) and treated (T; 5 ng/mL TGF-β2) NTM ($n = 3$) and GTM ($n = 2$) cell strains. PC, positive control. (C) Densitometric readings of BMP1 in CM following TGF-β2 treatment (±SEM). * $P < 0.05$.

induced upon exogenous treatment of TGF-β2. Also, TGF-β2 stimulates the secretion of biologically active BMP1 in TM cells. Finally, our lab has developed previously a novel LOX activity assay and this assay demonstrated that BMP1 increases LOX enzymatic activity in TM cells.

We were interested in the role of BMP1 due to its proteolytic activation, and processing of cross-linked enzymes and ECM proteins. BMP1 activates substrates, such as LOX, insulin-like growth factor-binding proteins, and TGF-β family members.^{16,17,22–25} For example, the activation of TGF-β requires cleavage from the latent TGF-β binding protein (LTBP) and latent associated peptide (LAP) before maturation. Interestingly, BMP1 can cleave the large LTBP from its smaller complex, LAP.²³ This cleavage would promote TGF-β release from LAP by metalloproteinases.^{23–25} It is reasonable to

suspect in the GTM, TGF-β2 induces BMP1, and the induction of BMP1 increases the cleavage of LTBP rendering TGF-β2 available for activation.

Our qPCR analysis showed that NTM and GTM cells express BMP1. These results support the previous study by Luna et al.,^{26,27} who first reported the mRNA presence of BMP1 in TM cells. Also, we demonstrated protein expression in NTM and GTM cells and tissues by WB and immunofluorescence analysis. Our results demonstrated that GTM cells have significantly higher levels of BMP1 in CM and BMP1 was induced further by TGF-β2. We also demonstrated by ELISA that TGF-β2 increases BMP1 secretion in NTM and GTM cell strains, and that GTM cells were more responsive to TGF-β2 treatment compared to NTM cells. Grgurevic et al.²⁸ isolated active BMP1 from the plasma samples of healthy and chronic kidney disease patients

FIGURE 4. TGF- β 2 induces secretion of BMP1 by NTM and GTM cells. (A) NTM ($n = 3$) and GTM ($n = 3$) cell strains were treated with or without TGF- β 2 (5 ng/mL) for 48 hours, and BMP1 levels in the CM were assayed by ELISA (each sample was run in triplicate). TGF- β 2 treatment significantly increased BMP1 secretion in all cell strains. Statistical differences were determined by Student t -test. * $P < 0.05$, ** $P < 0.001$, and *** $P < 0.0001$ ($\pm$ SEM). (B) BMP1 secretion levels were averaged from individual NTM and GTM cell strains. Statistical differences existed between control and TGF- β 2 treatment for NTM (* $P < 0.005$) and GTM (*** $P < 0.0001$) cell strains (paired Student's t -test).

and rats. The plasma samples of chronic kidney disease patients and rats had elevated levels of BMP1. With respect to rats, the addition of a neutralizing BMP1 antibody reduced the concentrations of circulating BMP1, TGF- β 1, and connective tissue growth factor. The authors concluded the BMP1 promotes ECM deposition leading to renal fibrosis.

However, the presence of BMP1 in TM cells or tissues does not necessarily mean that the enzyme is active biologically. To verify the activity of BMP1 in human TM cells we performed a BMP1 enzyme activity assay. BMP1 activity was analyzed using a fluorogenic substrate MCA-Tyr-Val-Asp-Ala-Pro-Lys (DNP)-OH. However, since other biologically active enzymes may cleave this substrate, we used a BMP1 inhibitor to confirm BMP1 specificity for this substrate. We demonstrated that BMP1

FIGURE 5. TGF- β 2 increases BMP1 activity in TM cells. TM cell strains ($n = 3$) were incubated with DMSO (control), TGF- β 2 (5 ng/mL), TGF- β 2, and increasing concentrations of BMP1 inhibitor (1.0, 3.0, 5.0 μ M), or BMP1 inhibitor alone (5.0 μ M). TGF- β 2 significantly (* $P < 0.05$) increased BMP1 enzyme activity compared to controls. TGF- β 2 induction of BMP1 enzyme activity was reduced significantly (# $P < 0.05$) by the BMP1 inhibitor (mean $\pm$ SEM, ANOVA and Bonferroni's multiple comparison test).

enzyme activity was induced by TGF- β 2, and the selective BMP1 inhibitor could reduce the action of TGF- β 2 in TM cells. Also, we used a LOX enzyme activity assay, since BMP1 can activate LOX in TM cells. These results demonstrated that the BMP1 enzyme is biologically active and is induced by TGF- β 2 in TM cells. We also have shown that BMP1 regulates the LOX enzymatic activity in TM cells.

If BMP1 were activated, then there would be an accumulation of ECM and cross-linked proteins in the TM, likely causing increased resistance of AH outflow and elevated IOP. In POAG patients, there is a build up of ECM proteins within the TM,^{5,29} which may result from enzymatically active proteins cross-linking ECM proteins. Tissue transglutaminase 2 and LOX are synthesized as pro-forms and require activation into their mature enzymatically forms. Once in their mature forms, they can cross-link ECM proteins allowing tissues to become stiffer and more resistant to degradation. In fact, a recent article reported that GTM tissues are significantly stiffer than NTM tissues demonstrated by atomic force microscopy.⁷ As stated previously, our data suggested that exogenous TGF- β 2 increases BMP1 and is significantly elevated in the GTM. Thus, this increase of BMP1 may be responsible for processing and cross-linking of ECM proteins leading to increased ECM stiffness in GTM tissues.

A recent study has suggested a role for microRNAs (miRNA) targeting BMP1 and ECM expression. Luna et al. reported that miRNA-29b negatively regulates multiple ECM genes and targets BMP1 in TM cells.²⁶ Using computational predictions, miRNA-29b targeted BMP1 3' untranslated regions, and this was confirmed with a psiCheck2 luciferase assay system. The authors also confirmed that the presence of miRNA-29b decreased BMP1 expression in TM cells using qPCR. Thus, they concluded that miRNA-29b downregulates BMP1, and the absence of miRNA-29b allows increased expression of ECM proteins and BMP1, increasing the processing and cross-linking of ECM in TM cells. A more recent article by the same group investigated the interactions of the miRNA-29 and TGF- β 1/TGF- β 2.²⁷ The effects of TGF- β 1 on the three miRNA-29s, was variable; however, TGF- β 2 decreased expression of all three miRNA-29 isoforms. The authors concluded that the interactions between TGF- β and miR-29 might contribute to ECM modulation in TM cells.

Our study expands the understanding of BMP1 expression in the TM; however, the biological and pathogenic roles BMP1

FIGURE 6. BMP1 increases LOX enzyme activity in TM cells. **(A)** Representative WB in cell lysates for tropoelastin (normalized to β -actin) in TM cells treated with BMP1 (15 ng/mL) along with increasing concentrations of LOX inhibitor, BAPN (1.0, 3.0, and 10.0 mM). Cells were cultured in serum-free medium (SFM). Tropoelastin levels increased with the combination of BMP1 and 1 mM BAPN compared to SFM alone. **(B)** Representative WB in cell lysates for tropoelastin (normalized to β -actin) in TM cells treated with BAPN (1.0 mM) along with increasing concentrations of BMP1 (5.0, 15.0, 25.0, and 50.0 ng/mL). Tropoelastin levels increased with the combination of BAPN with BMP1 at either 5 ng/mL or 15 ng/mL.

in the TM must be elucidated further. BMP1 has 11 alternatively spliced isoforms. Additional studies are needed to evaluate which BMP1 isoforms are present in the TM and better understand their function(s). If more than one isoform is present, then why are multiple isoforms needed in the TM? Also, further studies characterizing what other miRNAs are present in the TM, and their potential effects on BMP1 and the ECM must be conducted.

In conclusion, our results confirmed the expression of BMP1 mRNA and protein in human TM cells and tissues. Also, we demonstrated that cultured GTM cells secrete more BMP1 than NTM cells, and BMP1 expression is stimulated further by TGF- β 2 in NTM and GTM cells. We showed that BMP1 is biologically active and this activity is elevated further by TGF- β 2 in TM cells. Lastly, BMP1 increases LOX enzymatic activity in TM cells. We suggested that in the GTM, BMP1 expression is elevated by higher levels of TGF- β 2, thereby enhancing the processing and cross-linking of ECM proteins. This would lead to increased ECM deposition, increased TM tissue stiffness, decreased aqueous humor outflow and elevated IOP. Addition-

al research will determine the potential pathogenic role of BMP1 in glaucoma pathogenesis.

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