Role of TGF β /Smad Signaling in Gremlin Induction of Human Trabecular Meshwork Extracellular Matrix Proteins

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PURPOSE. The bone morphogenic protein (BMP) antagonist gremlin is elevated in glaucomatous trabecular meshwork (TM) cells and tissues and elevates intraocular pressure (IOP). Gremlin also blocks BMP4 inhibition of transforming growth factor (TGF)- β 2 induction of TM extracellular matrix (ECM) proteins. The purpose of this study was to determine whether Gremlin regulates ECM proteins in cultured human TM cells.

METHODS. Human TM cells were treated with recombinant gremlin to determine the effects on ECM gene and protein expression. Expression of the ECM genes *FN*, *COL1*, *PAII*, and *ELN* was examined in cultured human TM cells by quantitative RT-PCR and Western immunoblot analysis. TM cells were pre-treated with TGFBR inhibitors (LY364947, SB431542 or TGFBR1/TGFBR2 siRNAs), inhibitors of the Smad signaling pathway (SIS3 or Smad2/3/4 siRNAs), or CTGF siRNA to identify the signaling pathway(s) involved in gremlin induction of ECM gene and protein expression.

RESULTS. All ECM genes analyzed (*FN*, *COL1*, *PAII*, and *ELN*) were induced by gremlin. This gremlin induction of ECM genes and protein expression was blocked by inhibitors of TGFBR and the canonical Smad2/3/4 and CTGF signaling pathways.

CONCLUSIONS. Gremlin employs canonical TGF β 2/Smad signaling to induce ECM genes and proteins in cultured human TM cells. Gremlin also induces both TGF β 2 and CTGF, which can act downstream to mediate some of these ECM changes in TM cells. (*Invest Ophthalmol Vis Sci.* 2011;52:5251-5259) DOI: 10.1167/iops.11-7587

Glaucoma is a leading cause of irreversible visual impairment and blindness in the world, with primary open-angle glaucoma (POAG) being the major form of glaucoma.^{1,2} Elevated intraocular pressure (IOP) is a major risk factor for the development and progression of glaucoma.^{3,4} Ocular hypertension is due to increased aqueous humor outflow resistance in the trabecular meshwork (TM) and is associated with increased deposition of extracellular matrix (ECM) material within the TM. Transforming growth factor (TGF)- β 2 levels are elevated in the aqueous humor⁵⁻⁷ and TM (Tovar-Vidales T et al. submitted

for publication) of POAG patients. Trabecular meshwork cells express TGF β 2 receptors, and TGF β 2 has several effects on the TM.⁸ It has been shown to increase aqueous outflow resistance and elevate IOP in perfusion-cultured human and porcine eyes,⁹⁻¹¹ as well as in rodent eyes.¹²

TGF β 2 modulates ECM metabolism in TM cells and tissues. This cytokine increases expression of a variety of ECM proteins, including fibronectin (FN), collagen (COL), elastin (ELN), and proteoglycans, as well as levels of plasminogen activator inhibitor-1 (PAI1) and tissue inhibitor of metalloproteinase-1 (TIMP1), inhibitors that suppress proteolytic degradation of the ECM.¹³ In addition, TGF β 2 increased expression of the ECM cross-linking enzymes transglutaminase-2 (TGM2)¹⁴ and the lysyl oxidases (LOXs).¹⁵

We have previously reported that TM cells express several members of the bone morphogenic protein (BMP) family, including BMP ligands (BMP2, BMP4, BMP5, and BMP7), receptors (BMPRIa, BMPRIb, and BMPRII), and the antagonists gremlin, follistatin, and noggin.^{16,17} BMPs are members of the TGF β superfamily of proteins that control multiple functions in a variety of cell types.^{18,19} BMP4 and BMP7 block the TGF β 2-induction of a variety of ECM proteins, including fibronectin-1, collagen IV and VI, TSP-1, and PAI1.^{19,20}

Several structurally distinct BMP antagonists tightly regulate BMP cellular activity. BMP antagonists like gremlin directly bind BMP ligands and block BMP binding to their receptors.^{21,22} We have reported that there are greater levels of gremlin in glaucomatous TM cells and tissues.¹⁹ Gremlin antagonizes BMP4 inhibition of TGF β 2-induced ECM proteins like FN and PAI1 in TM cells and elevates IOP in perfusion-cultured human anterior segments.¹⁹ It is a highly conserved 20.7-kDa glycoprotein that heterodimerizes with BMP-2, -4, and -7 and plays a key role in regulating multiple cellular functions both during early development and in adult tissue homeostasis.^{22,23}

Gremlin may potentiate the profibrotic effects of TGF β 2 by blocking the BMP4 regulation of TGF β 2 activity. However, whether gremlin alone can induce fibrosis-like activities in cultured TM cells is currently unknown, and the potential signaling mechanisms involved have not been characterized. The purpose of the present study was to determine (1) whether gremlin induces ECM genes and proteins in cultured TM cells and (2) what signaling mechanisms are involved in gremlin induction of ECM genes and proteins.

METHODS

TM Cell Culture

Human TM cells were isolated from carefully dissected human TM tissue explants derived from patients with glaucoma or from normal donors and characterized as previously described.^{10,16,19,24,25} All donor tissues were obtained from regional eye banks and managed according to the guidelines in the Declaration of Helsinki for research involving human tissue. Isolated TM cells were grown in Dulbecco's modified

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Eagle's medium (DMEM) containing L-glutamine (0.292 mg/mL; Invitrogen-Gibco), penicillin (100 units/mL)/streptomycin (0.1 mg/mL; Invitrogen-Gibco), and 10% fetal bovine serum (all from Invitrogen-Gibco, Grand Island, NY).

TM Cell Treatments

TM cells were grown to 100% confluence and then kept in serum-free medium for 24 hours before treatments, to avoid the effect of serum proteins. TM cells were incubated with fresh medium containing specific signaling inhibitors for 1 to 6 hours before the addition of various concentrations of recombinant gremlin protein (R&D System, Minneapolis, MN). The small molecule inhibitors LY364947 (5 μ M; Tocris Biosciences, Ellisville, MO) and SB431542 (5 μ M; Sigma-Aldrich, St. Louis, MO) were used to examine the effects of inhibition of TGF β receptor-1/2. The Smad3 phosphorylation inhibitor SIS3 (10 μ M; Sigma-Aldrich), the JNK inhibitor SP600125 (10 μ M; Sigma-Aldrich), and the P-38 inhibitor SB203580 (5 μ M; Tocris Biosciences) were used to examine effects of inhibition on canonical Smad, JNK, and P-38 signaling pathways.

Small Interfering RNA and Transfection

siRNAs for Smad2, -3, and -4; TGFBR1; TGFB2; and CTGF as well as nontargeting control siRNAs (SMARTpool) were purchased from Dharmacon (Lafayette, CO). siRNA transfection was performed as described previously.^{26,27} Three different TM cell strains were grown in 12-well plates containing DMEM with 10% FBS. In one tube, 4 μ L of transfection reagent (DharmaFECT 1; Dharmacon) was mixed gently with 200 μ L of reduced-serum medium (Opti-MEM Invitrogen, Carlsbad, CA) and incubated for 5 minutes at room temperature. In separate tubes, siRNAs were mixed gently with 200 μ L of the serum-reduced medium. These two tubes were combined, gently mixed, and incubated for 20 minutes at room temperature. After incubation, DMEM without FBS and antibiotics was added to obtain a final volume of 2 mL for each well (10 nM of test and control siRNAs). The cells were washed with sterile PBS, incubated with siRNA transfection solution for 24 hours at 37°C, washed with sterile PBS, and incubated with 10% FBS containing DMEM for 24 hours at 37°C. They were then washed with serum-free DMEM for 24 hours and treated with TGF β 2 in serum-free DMEM for an additional 48 hours. Cell lysates and conditioned medium were analyzed for various proteins by Western blot analysis (see Table 1 for list of the antibodies used).

TABLE 1. List of Various Antibodies Used for Western Immunoblot Studies

Antibody	Dilution	Source
Rabbit Anti-FN	1:1000	Chemicon, Temecula CA
Rabbit Anti-PAI1	1:500	Novus Biologicals, Littleton, CO
Rabbit Anti-COL1	1:500	Abcam, Cambridge, MA
Mouse Anti-ELN	1:250	Abcam
Mouse Anti-ACTB	1:1000	Millipore, Billerica, MA
Rabbit Anti-TGF β 2	1:500	Santa Cruz Biotechnology, Santa Cruz, CA
Rabbit Anti-Gremlin	1:250	Abcam
Rabbit Anti-TGFBR1	1:250	Abcam
Rabbit Anti-CTGF	1:500	Novus Biologicals
Rabbit Anti-SMAD2	1:1000	Cell Signaling, Danvers, MA
Rabbit Anti-Phos-SMAD2	1:1000	Cell Signaling
Rabbit Anti-SMAD3	1:1000	Cell Signaling
Rabbit Anti-Phos-SMAD3	1:1000	Cell Signaling
Rabbit Anti-JNK1/2	1:1000	Cell Signaling
Rabbit Anti-Phos-JNK1/2	1:1000	Cell Signaling
Rabbit Anti-SMAD4	1:1000	Cell Signaling
Rabbit Anti-P38 MAPK	1:1000	Cell Signaling
Rabbit Anti-Phos-P38	1:1000	Cell Signaling
Donkey anti-mouse IgG	1:10,000	Santa Cruz Biotechnology
Goat anti-rabbit IgG	1:10,000	Santa Cruz Biotechnology

TABLE 2. Primers Used for PCR Studies

Gene		Primer (5'→3')
FN	Left	AGCGGACCTACCTAGGCAAT
	Right	GTTTTCGGATGGTACAGCTT
COL1a	Left	GGAATGAAGGACACAGAGG
	Right	TAGCACCATCATTTCCACGA
PAI1	Left	CCACTCTTCAGGCTGTTCC
	Right	CCGTTGAAGTAGAGGGCATT
ELN	Left	AGCCAGTATGGAGCTGCTG
	Right	GCTGCTTCTGGTACACAAC
TGFB2	Left	CCGGAGGTGATTTCCATCTA
	Right	CTCCATTGCTGAGACGTCAG
GREM1	Left	AAGCGAGACTGGTGCAAAAC
	Right	CTTGCGAAGGAGCAGGACT
ACTB	Left	GTCCACCTTCAGCAGATGT
	Right	AAAGCCATGCCAATCTCATC

RNA Isolation and RT-PCR

Total cellular RNA was extracted from cultured TM cells (TRI Reagent RT extraction; MRC Inc., Cincinnati, OH), and a cDNA synthesis kit (SuperScript VILO; Invitrogen) was used for first-strand cDNA synthesis. Primers for the various *LOX* genes were designed by using Primer3 software (<http://frodo.wi.mit.edu/primer3/>; Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA). The primer pairs are listed in Table 2.

Quantitative Real-Time PCR

Real-time PCR was performed as described previously.¹⁹ Briefly, 2.5 μ L of cDNA was used in a reaction consisting of 1.5 units per reaction of antibody-bound *Taq* enzyme (Jump Start; Sigma-Aldrich), 10 $\times$ PCR buffer, 1.5 mM MgCl₂, 200 nM dNTP mix, 100 nM PCR primers (Table 2), 2.5 μ L green nucleic acid dye (EvaGreen; Biotium, Hayward, CA), as well as 30 nM passive reference dye (Rox; USB, Cleveland, OH) per 50- μ L reaction. PCR was performed on a real-time thermal cycler (model Mx3000p; Stratagene, La Jolla, CA), with cycling parameters of initial denaturation at 95°C; 40 cycles of 95°C 30 seconds, 60°C 30 seconds, and 72°C 60 seconds, and a denaturation cycle for creation of a dissociation curves. Reactions for each sample were run in duplicate, cycle thresholds (Ct) were normalized to either β -actin or GAPDH expression as housekeeping genes, and comparative quantitation was performed (MxPro ver. 4.0 software, Stratagene). Only individual PCR samples with single-peak dissociation curves were selected for data analysis.

Protein Extraction and Western Blot Analysis

Secreted Proteins. ECM proteins secreted by TM cells were determined by Western immunoblot analysis. Conditioned medium was collected from human TM cells after 24-hour treatment with gremlin in serum-free medium containing 0.5 mg/mL BSA. Proteins were separated on a 10% denaturing polyacrylamide gel and transferred by electrophoresis to a PVDF membrane. Blots were blocked with 5% fat-free dry milk in Tris-buffered saline tween buffer (TBST) for 1 hour and then incubated overnight with primary antibodies (Table 1). The membranes were washed with TBST and processed with corresponding horseradish peroxidase-conjugated secondary antibodies (Table 1). The proteins were then visualized (Fluor Chem 8900 imager; Alpha Innotech, San Leandro, CA) using ECL detection reagent (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology Rockford, IL).

Cell-Associated Proteins. Total cellular protein was extracted from the TM cells using mammalian protein extraction buffer (MPER; Pierce Biotechnology), containing protease inhibitor (Pierce Biotechnology) and phosphatase inhibitor (Pierce Biotechnology) cocktails. Protein concentration was determined using a Dc protein assay system (Bio-Rad Laboratories, Hercules, CA). The cellular proteins were sepa-

rated on denaturing polyacrylamide gels and then transferred to PVDF membranes by electrophoresis. Blots were blocked with 5% fat-free dry milk in TBST for 1 hour and then incubated overnight with primary antibodies (Table 1). The membranes were washed with TBST and processed with corresponding horseradish peroxidase-conjugated secondary antibodies (Table 1). The proteins were then visualized (Fluor Chem 8900 imager; Alpha Innotech) using ECL detection reagent (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology). To ensure equal protein loading, the same blot was subsequently developed for β -actin expression.

Fibronectin ELISA

Conditioned medium of human TM cells was collected and evaluated for gremlin effects on fibronectin production using a commercially available ELISA kit (Quantimatrix Human Fibronectin; Chemicon International, Billerica, MA). We previously demonstrated that treatment of cultured human TM cells with TGF β 2 significantly increases fibronectin levels in the culture medium.^{10,19}

Statistical Analysis

For comparing results between two groups, the Student's *t*-test was performed. One-way ANOVA was used for comparison of results between more than two groups.

RESULTS

Gremlin Induces ECM mRNA and Proteins in TM Cells

Gremlin has been previously reported to antagonize BMP4 inhibition of TGF β 2-induction of ECM proteins like FN and PAI1 in human TM cells.¹⁹ However, it is not known whether gremlin alone can induce these ECM proteins in TM cells. Therefore, we determined the effect of gremlin on FN, COL1a, PAI1, and ELN expression in cultured human TM cells. Treatment with gremlin (1 μ g/mL) for 24 hours significantly induced FN, COL1a, PAI1, and ELN mRNA expression ($n = 3$; $P < 0.05$; Fig. 1A), as well as ECM protein expression in cell lysates and conditioned medium (Fig. 1B). We also performed quantitative FN ELISA on the conditioned medium samples of the four TM strains used for the Western immunoblot assay (Fig. 1B), and gremlin significantly elevated the amount of secreted FN in the treated TM cells compared with the controls ($n < 0.001$; Fig. 1C).

Gremlin Induces ECM Genes and Proteins in a Concentration- and Time-Dependent Fashion

TM cell strains ($n = 3$) were treated with increasing concentrations of gremlin (0–5 μ g/mL) for 24 hours. The mRNA and protein expression of FN, COL1, PAI1, and ELN were determined using qRT-PCR and Western immunoblot, respectively. Gremlin induced expression of ECM mRNA (Fig. 2A), cell-associated (Fig. 2B), and secreted ECM proteins (Fig. 2C) in a concentration-dependent manner. Gremlin also significantly elevated the amount of secreted FN assessed by ELISA in a concentration-dependent manner (Fig. 2D). TM cells were treated with gremlin for 6, 12, and 24 hours to determine the time dependence of ECM mRNA induction. Gremlin significantly ($P < 0.01$) induced FN, PAI1, COL1, and ELN mRNA expression, although the time course of induction varied slightly for each gene (Fig. 3A). Similarly, TM cell strains ($n = 3$) were treated with gremlin (1 μ g/mL) for 3, 12, 24, 48, and 72 hours to evaluate effects on ECM protein expression. Gremlin induced both cell-associated and secreted ECM proteins as early as 12 hours and maintained this induction for up to 72 hours (Figs. 3B, 3C). The gremlin induction of elastin mRNA peaked and then decreased after 24 hours of treatment,

FIGURE 1. Gremlin induced ECM mRNA and proteins in TM cells. (A) Induction of FN, COL1a, PAI1, and ELN mRNA in three TM cell strains treated with gremlin (T) (1 μ g/mL) for 24 hours versus their untreated control samples (C). Data represent the ratio of induction of ECM genes normalized to GAPDH. Three replicates of each sample were used. Gremlin significantly induced ECM genes in all the three cell lines: **0.0001 < P < 0.01 and *** P < 0.0001. (B) Western immunoblots of cell-associated and secreted ECM proteins in two TM cell strains treated with gremlin (1 μ g/mL) for 24 hours. Gremlin induced cell-associated ECM proteins were normalized compared with ACTB, with equal volumes of conditioned medium for secreted proteins. Similar results were observed in two additional TM cell strains. These Western blot images are representative of three independent experiments. (C) Induction of secreted FN in four TM cell strains treated with gremlin (1 μ g/mL) for 24 hours. FN ELISA values represent the ng/mL of FN. Three replicates of each sample were used. *** P < 0.0001.

whereas levels of elastin protein were still increased after 72 hours. This apparent discrepancy may be due to the inherent stability and slow turnover of the elastin protein, which would persist even after levels of elastin mRNA have decreased. Gremlin also significantly elevated the amount of secreted FN in a time-dependent manner (Fig. 3D). Therefore, gremlin induction of ECM mRNA and proteins was both time and dose dependent.

TGF β Signaling in Gremlin Induction of ECM Proteins

Gremlin was previously reported to antagonize the BMP4 inhibition of TGF β 2-induced ECM proteins in human TM cells,¹⁹ but the signaling mechanism(s) involved was not determined. We used various small molecule inhibitors to explore the involvement of TGF β signaling pathway(s) in gremlin-mediated ECM induction. SB431542 is a selective TGFBR1 and TGFBR2 receptor inhibitor,²⁸ whereas LY364947 is a relatively selective inhibitor for the TGFBR2 receptor.²⁹ We pretreated TM cell strains ($n = 3$) for 1 hour, with or without 5 μ M SB431542 or LY364947, followed by treatment with recombinant gremlin (1 μ g/mL) for 24 hours. Gremlin elevated FN, COL1, PAI1, and ELN mRNA expression compared to untreated or inhibitor only-treated samples ($P < 0.001$). Pretreatment with either of the two inhibitors, LY364947 or SB431542, completely blocked gremlin-mediated mRNA induction in all the cell strains ($P < 0.001$; Fig. 4A).

We used the same strategy to evaluate the effects of these TGFBR inhibitors on gremlin induction of ECM proteins. Gremlin elevated cell-associated as well as secreted FN, COL1, PAI1, and ELN protein levels compared with untreated or vehicle-

FIGURE 2. Concentration-dependent gremlin induction of ECM mRNA and proteins. Concentration-dependent induction of ECM gene mRNA (A), cell-associated protein (B), and secreted proteins (C) by 0 to 5 μ g/mL gremlin in cultured TM cell strains ($n = 3$). qRT-PCR values (A) represent gremlin induction compared to controls and normalized to *ACTB* as the housekeeping gene. Three replicates of each sample were used. Concentration-dependent induction of cell-associated (B) and secreted FN proteins (C, D) by 0 to 5 μ g/mL gremlin in cultured TM cell strains ($n = 3$). Western immunoblots (B, C) are representative of data obtained in three TM cell strains. Three replicates of each sample were used for the FN ELISA (D). One-way ANOVA was used for statistical analyses. * $0.01 < P < 0.05$, ** $0.0001 < P < 0.01$, and *** $P < 0.0001$.

treated samples. Each of the two inhibitors, LY364947 (Fig. 4B) and SB431542 (Fig. 4C), completely inhibited the gremlin-mediated ECM protein induction. Treatment with the inhibitors alone did not have any effect on the ECM proteins' expression. We also analyzed the conditioned medium samples using FN ELISA. Gremlin treatment significantly elevated the amount of secreted FN, which was blocked by pretreatment with the two TGFBR inhibitors (Fig. 4D). The FN ELISA data agreed with our Western immunoblot data (Figs. 4B, 4C).

In addition to TGFBR1/2 inhibitors, we used siRNA-mediated TGFBR1 knockdown to confirm the role of TGF β receptor signaling in gremlin induction of ECM proteins. Gremlin-treated TM cells were untransfected or transfected with a nontargeting siRNA control or TGFBR1 siRNA. As previously shown, gremlin induced ECM protein expression. Control siRNA did not affect endogenous TGFBR1 levels and did not affect gremlin induction of ECM protein expression. Consistent with the data with small molecule TGFBR1 or TGFBR2 inhibi-

tion, TGFBR1 siRNA knockdown inhibited gremlin induction of ECM proteins (Fig. 4E).

TM cells endogenously express TGF β 2^{6,30} and gremlin^{16,19} proteins, and both TGF β 2^{10,31} and gremlin (Figs. 1–3) induce ECM proteins in TM cells. We wanted to determine whether gremlin treatment alters endogenous levels of TGF β 2 and vice versa, which in turn may regulate gremlin's ECM induction profile. TM cell strains ($n = 3$) were treated with increasing concentrations of gremlin (0–5 μ g/mL) or TGF β 2 (0–10 ng/mL) for 24 hours. The mRNA and protein expression of TGF β 2 and gremlin were determined using qRT-PCR and Western immunoblot, respectively. Gremlin induced TGF β 2, and TGF β 2 induced gremlin mRNA (Fig. 5A) and protein (Fig. 5B) expression in a concentration-dependent manner.

We used siRNA-mediated TGF β 2 knockdown to confirm the role of TGF β 2 in gremlin induction of ECM proteins. Gremlin-treated TM cells were either untransfected or transfected with a nontargeting siRNA control or TGF β 2 siRNA. Gremlin in-

FIGURE 3. Time-dependent gremlin induction of ECM proteins. Time course induction (0–72 hours) of ECM mRNA (A), cell-associated proteins (B), secreted ECM proteins (C), and secreted FN proteins (D) after treatment of cultured TM cell strains with gremlin (1 μ g/mL; $n = 3$). qRT-PCR values (A) represent the ratio of gremlin induction to controls and normalized to *ACTB*. Three replicates of each sample were used for qRT-PCR (A) and FN ELISA (D). Western immunoblots (B, C) are representative of data obtained in three TM cell strains. One-way ANOVA was used for statistical analyses. * $0.01 < P < 0.05$, ** $0.0001 < P < 0.01$, and *** $P < 0.0001$.

FIGURE 4. TGF β receptor inhibition blocked gremlin induction of ECM mRNA and proteins. Effect of the TGFBR inhibitors LY364947 and SB431542 on gremlin induction of ECM mRNA (A) and protein expression (B–D). qRT-PCR values (A) represent the ratio of induction normalized to *ACTB* in treated samples compared with controls (triplicates of three TM strains). Cell-associated and secreted proteins were analyzed with Western immunoblot (B, C) and with FN ELISA (D). (B, C) Western immunoblots of TM cells treated with 1 μ g/mL of gremlin for 24 hours along with 5 μ M of LY364947 (B) or SB431542 (C). Untreated and inhibitor-treated cells served as negative controls. *ACTB* was used as the loading control. Blots shown are representative of data from three different TM cell strains. (D) FN ELISA of conditioned medium. (E) Western immunoblots of ECM proteins after siRNA mediated TGFBR1 knockdown followed by gremlin treatment. TM cells were treated with TGFBR1 or control siRNA, followed by treatment with 1 μ g/mL of gremlin for 24 hours. *ACTB* was used as the loading control. Blots are representative data from three different TM cell strains. One-way ANOVA was used for statistical analyses (A, D). *#0.01 < P < 0.05, **##0.0001 < P < 0.01, ***### P < 0.0001. #, differences between gremlin samples versus gremlin + inhibitor samples; *, differences between gremlin-treated and untreated cells.

duced both ECM proteins and TGF β 2. Control siRNA did not affect endogenous TGF β 2 levels and did not alter gremlin induction of ECM protein expression. However, TGF β 2 knockdown blocked gremlin induction of ECM proteins (Fig. 5C). Taken together, our results (Figs. 4, 5) strongly support the roles of TGF β 2 and TGFBR in gremlin induction of ECM protein expression.

Role of CTGF in Gremlin Induction of ECM Proteins

Connective tissue growth factor (CTGF) regulates several ECM proteins in cultured human TM cells and mediates TGF β 2 induction of FN; collagens I, II, and IV; and integrins.³² We wanted to determine whether CTGF is involved in gremlin induction of ECM proteins in TM cells. TM cells were either untransfected or transfected with a nontargeting siRNA control or CTGF siRNA before treatment with gremlin. Gremlin alone induced both ECM proteins and CTGF. Control siRNA did not affect endogenous CTGF levels and did not affect gremlin induction of ECM proteins expression. However, CTGF knockdown blocked gremlin induction of cell-associated and secreted ECM proteins FN and COL1 but not ELN or PAI1 (Fig. 6).

Gremlin Induces ECM Proteins Using Smad Signaling Pathway

The profibrotic cytokine TGF β 2 can activate both canonical Smad and noncanonical signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway in various cells and tissues.^{33–35} TGF β signaling is complex because these different signal transduction pathways can interact with each other.^{36,37} We wanted to determine whether gremlin treatment activates any of the TGF β signaling pathways and whether any of the TGF β signaling mechanism(s) are involved in gremlin induction of ECM proteins in TM cells.

A previous study showed that TGF β 2 activates both canonical Smad and MAPK signaling in TM cells.³⁸ We treated four primary TM cell strains with TGF β 2 (5 ng/mL) or gremlin (1 μ g/mL) for 15, 30, 60, 120, and 240 minutes, and total and phosphorylated Smad2 and Smad3, as well as P38 and JNK1/2 MAPK proteins were evaluated by Western immunoblot. TGF β 2 phosphorylated both canonical (Smad2 and Smad3) and noncanonical (P38 and JNK1/2) pathways (Figs. 7A, 7B). In contrast, gremlin activated only the Smad pathway (Fig. 7A) but not the P38 or JNK1/2 pathways (Fig. 7B). There were no changes in total Smad2, Smad3, P38, and JNK1/2 levels.

FIGURE 5. TGFβ2 knockdown inhibited gremlin induction of ECM proteins. TGFβ2 and gremlin reciprocally induce the expression of each other's mRNA (A) and protein (B). TGFβ2 treatment induced expression of gremlin mRNA (A) and protein (B) expression, while gremlin treatment induced TGFβ2 mRNA (A) and protein (B) expression. Three replicates of each sample were used. One-way ANOVA was used for statistical analyses $^{**}0.0001 < P < 0.01$; $^{***}P < 0.0001$. (C) Western immunoblots of ECM proteins after siRNA mediated TGFβ2 knockdown followed by gremlin treatment. TM cells were treated with TGFβ2 or control siRNA, followed by treatment with 1 μg/mL of gremlin for 24 hours. ACTB was used as the loading control. Blots are representative data from three different TM cell strains.

Phosphorylated Smad2 and -3, together or individually, form a complex with co-Smad4 to regulate transcription of their target genes.^{39,40} To determine whether Smad3 transcriptionally regulates the ECM proteins, we used SIS3, a selective small molecule inhibitor of Smad3. Three TM cell strains were treated with SIS3 (10 μM) 6 hours before treating with recombinant gremlin for an additional 24 hours, to study mRNA and protein expression of FN, COL1, PAI1, and ELN. Untreated cells and SIS3-alone-treated cells served as negative controls. Gremlin induction of ECM mRNA and cell-associated protein expression was inhibited by SIS3 pretreatment ($P < 0.01$; Figs. 8A, 8B). SIS3 treatment also blocked gremlin induction of cell-associated and secreted ECM proteins as well as soluble FN

in conditioned medium analyzed by Western immunoblot (Fig. 8B) and ELISA (Fig. 8C), respectively. These results concurred with our mRNA results (Fig. 8A). Therefore, gremlin induction of ECM mRNA and proteins is mediated by Smad3 signaling.

To confirm the role of Smad signaling in gremlin regulation of ECM protein expression, we used siRNA-mediated knock-

FIGURE 6. CTGF knockdown inhibited gremlin induction of ECM proteins. Western immunoblots of ECM proteins after siRNA mediated CTGF knockdown followed by gremlin treatment. TM cells were treated with CTGF or control siRNA, followed by treatment with 1 μg/mL of gremlin for 24 hours. ACTB was used as the loading control. Blots are representative data from three different TM cell strains. CTGF knockdown blocked gremlin induction of FN and COL1, but had no effect on gremlin induction of PAI1 and ELN.

FIGURE 7. TGFβ2, but not gremlin, activated both canonical and non-canonical signaling pathways in TM cells. Representative Western immunoblots of canonical Smad2/pSmad2 and Smad3/pSmad3 signaling (A), as well as noncanonical JNK1/2/pJNK1/2 and P38/pP38 signaling (B) in four TM cell strains treated for 0 to 240 minutes with TGFβ2 and gremlin. TGFβ2 and gremlin treatment caused a time-dependent increase in pSmad2 and pSmad3 expression. TGFβ2 treatment caused a time-dependent increase in pJNK1/2 and pP38 expression, whereas gremlin did not affect pJNK1/2 or pP38 levels.

FIGURE 8. Smad2/3/4 inhibition blocks gremlin induction of ECM proteins. Treatment of TM cells with Smad3 inhibitor SIS3 blocks gremlin induction of ECM mRNA (A), cell-associated protein (B), and secreted protein expression (B, C). (A) qRT-PCR analysis of the gremlin induction of ECM genes mRNA in the presence of specific inhibitor of Smad3 (SIS3). qRT-PCR values represent the ratio of induction of ECM mRNA normalized to *ACTB* in gremlin-treated samples compared with the controls (triplicates of three TM cell strains). One-way ANOVA was used for statistical analyses of qRT-PCR (A) and FN ELISA (C) results. #, differences between gremlin samples versus gremlin+inhibitor samples; *, differences between gremlin treated versus the untreated cells; *** and ### $P < 0.0001$. (B) Western immunoblots of cell-associated and secreted ECM proteins after pretreatment with SIS3 (10 μ M) followed by gremlin (1 μ g/mL) treatment for 24 hours. Immunoblots are representative of three different TM cell strains. *ACTB* was used as a loading control. Untreated and SIS3-only-treated cells served as negative controls. (C) FN ELISA of secreted FN from gremlin $\pm$ SIS3 treated TM cells ($n = 3$ strains). (D–F) Western immunoblots of ECM proteins in TM cells pretreated with Smad3 (D), Smad2 (E), or Smad4 (F) siRNAs followed by gremlin treatment. Control cells were transfected with nontargeting siRNA. Immunoblots are representative of results from three TM cell lines. Each Smad siRNA knocked down its target protein. SIS3 siRNA suppressed the gremlin induction of all four ECM proteins. Smad2 and -4 siRNAs consistently suppressed gremlin induction of FN and PAI1, but variably suppressed gremlin induction of ELN and COL1 proteins.

down of Smad2, -3, and -4. Nontargeting siRNA served as the negative control. Cells transfected with Smad3 (Fig. 8D), -2 (Fig. 8E), or -4 (Fig. 8F) siRNAs were followed by treatment with or without gremlin. Untransfected and untreated cells served as negative controls, while untransfected cells treated with gremlin served as the positive control. Gremlin increased the expression of ECM proteins. Control siRNAs neither affected gremlin induction of ECM proteins nor did they affect the endogenous Smad2/3/4 levels. As expected, knockdown of Smad3 completely inhibited gremlin induction of the cell-associated and secreted ECM proteins (Fig. 8D). Smad2 and -4 knockdown also completely inhibited gremlin induction of the cell-associated and secreted FN and PAI1 proteins. However, Smad2 or -4 knockdown did not consistently inhibit gremlin induction of COL1 and ELN even though levels of each Smad were sufficiently reduced (Figs. 8E, 8F). This variable effect was seen within the same cell strain as well as between different cell strains. Taken together, these results (Fig. 8) strongly support Smad3-dependent regulation of gremlin induction of all ECM protein expression.

DISCUSSION

Interactive TGF β /BMP signaling plays an important role in ECM homeostasis, and perturbation in the balance of this signaling is associated with fibrotic diseases, including glaucoma. TGF β 2

plays an important role in glaucoma pathogenesis.⁵ Aqueous humor levels of TGF β 2 are significantly elevated in POAG patients,^{5–7} and TGF β 2 is also elevated in glaucomatous TM cells and tissues (Tovar-Vidales, submitted for publication). TGF β 2 increases the expression of several ECM proteins in the TM and also elevates IOP in perfusion cultured anterior segments and rodent eyes.^{9–12} Trabecular meshwork cells and tissues express BMPs, BMP receptors, and BMP antagonists, and BMP4 and -7 inhibit TGF β 2 induction of ECM proteins.^{19,20} Inhibition of BMP signaling exacerbates the TGF β 2 effect on the TM ECM. Gremlin protein levels are higher in GTM cells, and gremlin blocks BMP suppression of TGF β 2 mediated effects on the TM ECM.¹⁹ In addition, gremlin treatment alone elevates IOP in perfusion cultured anterior segments,¹⁹ suggesting that perturbation of normal TGF β 2/BMP homeostasis can play a role in ocular hypertension.

To directly test this latter hypothesis, we examined the effect of gremlin on TM ECM expression. We found that gremlin increased ECM mRNA and protein expression. However, in contrast to TGF β 2, which activates both the Smad and non-Smad MAPK signaling pathways, gremlin activated only the canonical Smad2/3 pathway. Inhibition of Smad signaling blocked gremlin's effect on TM ECM expression. Connective tissue growth factor is induced by TGF β 2 and acts as a downstream mediator of TGF β signaling, regulating the induction of multiple ECM proteins including FN and collagen types I, II, IV,

FIGURE 9. Proposed mechanism of gremlin regulation of ECM proteins in TM and implications in glaucoma. Gremlin inhibits BMP ligand binding to the BMP receptor complex, in turn blocking the ability of BMP to inhibit TGFβ2's profibrotic response. TGFβ ligands bind to the TGFβR1-2 receptor heterotetramer complex on the cell surface and activate the Smad2/Smad3 complex, which utilizes Smad4 to translocate to nucleus. This complex binds by itself to the upstream promoter regions of ECM genes like *FN*, *PAI1*, *COL1*, and *ELN*, to regulate their transcription. ECM mRNA transcribes to proteins and deposit in the TM. This may lead to increased resistance to aqueous humor outflow and may lead to elevated IOP.

and VI.³² Interestingly, our results show that gremlin induced CTGF and that the gremlin induction of FN and COL1 was dependent on CTGF. In contrast, gremlin induction of PAI1 and ELN were not dependent on CTGF. Others have also reported that CTGF does not induce PAI1 expression in human TM cells.³² We did not examine whether CTGF can also induce gremlin in a feed-forward loop and whether gremlin can act also as a mediator of CTGF signaling. These experiments are currently under investigation.

We suggest that gremlin increases TM cell ECM expression by inhibiting the balance between BMP and TGFβ2 in the regulation of ECM metabolism (Fig. 9). Gremlin binds to BMP and inhibits BMP's modulatory effect on TGFβ2 induction of ECM proteins. Pretreatment of TM cells with specific siRNAs effectively knocked down endogenous TGFβ2 and CTGF expression before treatment with gremlin. Therefore, the inhibition of BMP by gremlin has no effect on ECM expression because there is no longer endogenous TGFβ2 or CTGF to enhance ECM expression.

Most studies of gremlin have been focused on its role in development of fibrotic diseases. It is not uncommon to find developmental genes re-expressed in several diseased conditions including several kinds of cancer. However, additional studies are needed to address this hypothesis in glaucoma.

It also appears that gremlin and TGFβ2 are involved in a "feed-forward" pathogenic pathway. We have shown that gremlin increases TGFβ2 expression, and TGFβ2 increases gremlin expression in TM cells. This process would further exacerbate ECM deposition within the TM, potentially leading to increased aqueous humor outflow resistance and IOP elevation. Levels of both TGFβ2 and gremlin are elevated in the anterior segment in glaucoma, but the primary cause of increased expression of these signaling molecules in glaucomatous eyes is currently unknown. Mechanical stress (i.e., cyclic stretch) and substrate elasticity have been shown to increase TGFβ expression in the TM.^{41,42} The effects of these perturbations on gremlin expression have not been evaluated. It is plausible to hypothesize that elevated levels of gremlin in glaucoma patients leads to higher TGFβ2 levels in the TM.

TGFβ and gremlin also play a role in other fibrotic diseases. Gremlin has been associated with several fibrotic diseases of lungs⁴³ and kidneys^{44,45} and in osteoarthritis.⁴⁶ Gremlin was shown to induce expression of FN²⁷ and several types of collagens.⁴⁷ Gremlin has also been reported as a downstream mediator of TGFβ's fibrotic effects in the kidney.⁴⁵ Several growth factors like CTGF have been reported to induce ECM proteins like TGFβ2 in several kinds of cells.⁴⁸

The potential relationship between the ECM proteins in regulating aqueous outflow in gremlin-induced ocular hypertension and POAG warrants further study. Do any of these ECM proteins play a direct role in gremlin-induced ocular hypertension? Which ECM proteins are more essential for normal TM homeostasis, and are any of these proteins directly involved in glaucoma pathogenesis? Do different gremlin signaling mechanisms regulate glaucoma-like changes in the TM and directly cause IOP elevation? Our current results provide a foundation to address these questions in future studies.

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