JOURNAL OF OCULAR PHARMACOLOGY AND THERAPEUTICS
Volume 30, Numbers 2 and 3, 2014
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DOI: 10.1089/jop.2013.0220

The Role of TGF-β2 and Bone Morphogenetic Proteins in the Trabecular Meshwork and Glaucoma

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Abstract

Primary open-angle glaucoma (POAG) is the second leading cause of blindness worldwide. Elevated intraocular pressure (IOP) is a primary risk factor associated with POAG. Increased aqueous humor (AH) outflow resistance through the trabecular meshwork (TM) results in elevated IOP in POAG patients. Resistance to AH outflow is associated with increased accumulation of extracellular matrix (ECM) proteins in the TM. In addition, levels of transforming growth factor-beta2 (TGF-β2) are elevated in the AH and TM tissue of POAG patients. Elevated levels of TGF-β2 in other tissues have been associated with fibrosis and increased tissue stiffness. However, locally produced effectors that maintain homeostatic relationships must also be present. Bone morphogenetic proteins (BMPs) serve this purpose in the TM as they inhibit TGF-β2-induced ECM changes in TM cells. This review article first describes the TGF-β superfamily of growth factors including BMPs and their canonical and noncanonical signaling pathways. The article then addresses the role of TGF-β2 in the pathophysiology of POAG as related to the ECM and ECM crosslinking enzymes. This is followed by a discussion of potential homeostatic control mechanisms of TGF-β2 signaling in the TM including the inhibitory role of BMP-4 and BMP-7. We then describe the relationship of TGF-β2 and BMPs in TM fibrosis including the role of antagonists. Lastly, in future directions, we identify potential future studies that explore new and unique cellular interactions within the TM for potential therapeutic interventions.

The Transforming Growth Factor-β Superfamily of Growth Factors

There are over 40 members of the transforming growth factor (TGF)-β superfamily of growth factors, including TGF-β isoforms, bone morphogenetic proteins (BMPs), activins, inhibins, nodal, growth differentiation factors, myostatin, and anti-Mullerian hormone.[1] In general, members of the TGF-β superfamily share significant structural and functional homology. For example, members share 6 conserved cysteine residues that are required to form covalently linked dimers.[2] Dimers interact with specific receptor complexes to activate downstream signaling pathways with subsequent positive or negative regulation of specific genes.[3] The biological actions of TGF-β superfamily members are dynamic and contribute to a wide variety of cellular processes, including proliferation, differentiation, motility, adhesion, extracellular matrix (ECM) protein synthesis, cancer metastasis, and apoptosis.[4] The specific target cell and the cellular context of the target cell will largely determine the exact function of any member of the TGF-β superfamily at a specific time.

TGF-β isoforms

Within the TGF-β superfamily of growth factors, 3 specific genes encode 3 different TGF-β isoforms (*TGF-β1*, *TGF-β2*, and *TGF-β3*). These isoforms share 60%–80% homology in amino acid composition. Somewhat surprisingly, all 3 isoforms activate the same canonical signaling pathway. *In vitro* they also appear to have similar functions. However, knockout mice have been produced for all 3 isoforms and the isoforms can have different *in vivo* roles.[2,5] TGF-β2 has been reported to be the most abundant TGF-β isoform in the eye.[6–8] TGF-β2 influences many aspects of cellular behavior, including proliferation, differentiation, migration, and ECM synthesis and breakdown.[9,10]

To prevent uncontrolled activation of the TGF-β signaling pathway, TGF-β isoforms are secreted as part of a large latent complex (LLC).[11] In this complex, TGF-β isoforms are unable to bind to the corresponding type II receptor (TGF-βRII).[12] In the LLC, the TGF-β isoform is noncovalently associated with latency associated protein (LAP) and the latent TGF-β binding protein. The secreted LLC complex can be covalently linked to the ECM and serve as a

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local reservoir for the TGF-β isoforms. Enzymatic action on LLC via MMP-2, MMP-9, or plasmin can lead to local release of biologically active TGF-β isoforms.[13] Since enzyme activation is required before TGF-β dimers can bind TGF-βRII and activate downstream signaling pathways, the ability of the cells to enzymatically activate latent TGF-β is a critical regulatory step.

In addition, thrombospondin-1 (TSP-1) is a potent activator of latent TGF-β2.[14–16] TSP-1 is a member of the secreted matricellular protein family that influences local cell activity by modulating interactions between the cell and the ECM. In the human trabecular meshwork (TM), TSP-1 is constitutively present in the juxtacanalicular (JCT) region of the TM.[17] Importantly, the expression and secretion of TSP-1 in TM cells is induced by TGF-β2.[18] Thus, a local feed-forward autocrine loop may function in the TM in which elevated TGF-β2 production leads to increased TSP-1 secretion and subsequent activation of ECM-sequestered TGF-β2.

Bone morphogenetic proteins

With 20 members,[19] BMPs constitute the largest subfamily of the TGF-β superfamily and were originally identified as osteoinductive factors that promoted bone and cartilage formation.[20] However, it is now clear that BMPs are expressed in a number of other tissues and are involved in diverse cell functions, including development, morphogenesis, cell proliferation, fibrosis, and apoptosis.[19,21] A review of BMPs, BMP receptors, and BMP signaling in ocular tissues is available.[22]

Members of the BMP subfamily can be classified into several subclasses based on structural similarities.[23,24] For example, BMP-2 and BMP-4 have 80% amino acid homology and constitute class 1. Members of subclass 2 consist of BMP-5, BMP-6, and BMP-7 and have 78% amino acid homology. Class 3 consists of BMP-3 because its amino acid structure is significantly different from that of the other BMPs. It should also be noted that BMP-1 has been erroneously included as a member of the BMP subfamily. BMP-1 is actually a protease and has no homology to other BMP proteins.[25,26] The potential role of BMP-1 in glaucoma will be addressed later in the article.

The BMPs are synthesized as precursor proteins that contain (1) an N-terminal signal peptide that directs secretion, (2) a prodomain involved in proper folding, and (3) the C-terminal mature peptide.[27,28] However, unlike TGF-β isoforms, BMP proforms do not form latent complexes with their mature counterparts. The mature BMP peptide is derived following proteolytic cleavage of the C-terminal region by furin, proprotein convertase 6 or proprotein convertase 7.[29] This enzymatic action results in the generation of the biologically active dimeric mature BMP-4 protein. A review article should be consulted for a detailed discussion of BMP activation.[19]

TGF-β2 and BMP Signaling Pathways

TGF-β2 and BMP ligands have the ability to signal via canonical and noncanonical pathways. The target cell type and the cellular context of the target cell often determine which signaling pathway is utilized. For example, the specific cellular effect of any TGF-β isoform or BMP will depend on (1) the specific ligand, (2) the concentration of the ligand at the target cell, (3) the context of the target cell including expression of cofactors (eg, coactivators or corepressors), (4) the level/expression of TGF-β/BMPR receptors on the target cell, (5) the type and amount of receptor Smads (R-Smads) expressed by the target cells, (6) the degree of cross-talk between the canonical and noncanonical signaling pathways, and (7) the expression of phosphatases that reduces signaling. TM cells have the capacity to utilize both the canonical and noncanonical signaling pathways. It is not clear why one signaling pathway dominates or why both pathways can be simultaneously activated in the TM cell.

The canonical signaling pathway will be briefly presented first with differences between TGF-β and BMP signaling noted. The noncanonical signal pathway will then be reviewed. The reader is directed to several review articles that provide greater depth with respect to TGF-β and BMP signaling pathways.[3,19,30]

Canonical signal transduction

The canonical signaling pathway (Fig. 1) is utilized by both TGF-β2 and BMPs and is initiated with ligand binding to specific receptor complexes on the cell membrane. With respect to TGF-β2, the TGF-β dimer utilizes 2 specific serine-threonine trans-membrane receptors. The TGF-β dimer first binds the type II TGF-β receptor, which then transphosphorylates the type I TGF-β receptor. Phosphorylation of the receptor complex activates downstream signaling via receptor Smad (R-Smad) proteins.[31–33] The R-Smads make up a group of intracellular proteins/transcription factors that play a critical role in TGF-β signaling.[3] In the Smad canonical signaling pathway, binding of the TGF-β isoforms to the TGF-β receptor complex results in phosphorylation of R-Smad2 and/or R-Smad3. Phosphorylated Smad 2/3 then triggers heterodimerization with Co-Smad4 and translocation of the complex through the nuclear pore into the nucleus. Within the nucleus, the R-Smad2/3-Co-Smad4 complex, in cooperation with other transcription factors, coactivators and/or corepressors, induces or inhibits the transcription of specific target genes. Since several reports indicate that R-Smad3 is utilized predominately for fibrotic responses to TGF-β2,[34,35] R-Smad3 may be of greater significance to fibrotic changes within the ECM of the TM. This concept has not been explored in detail. An excellent review article on Smad protein/transcription factors is available and should be consulted for a detailed description of the Smad-dependent signaling pathway.[30]

Connective tissue growth factor (CTGF) has been identified as a downstream mediator of TGF-β2.[36,37] It is a member of the CCN family and is expressed in the TM[38] and is present in the aqueous humor (AH).[39] Of importance to this review was the report that exogenous TGF-β2 increased CTGF expression in human TM cells. In addition, Junglas et al.[40] reported that CTGF was a potent inducer of ECM proteins in the TM [e.g., fibronectin and selective collagens]. Inhibition of CTGF via siRNA blocked induction of ECM proteins.

Signaling by BMP ligands initially involves 2 types of transmembrane serine/threonine kinase receptors termed type I (BMPR-I) and type II (BMPR-II).[41] Both receptors are needed to form a functional complex and initiate

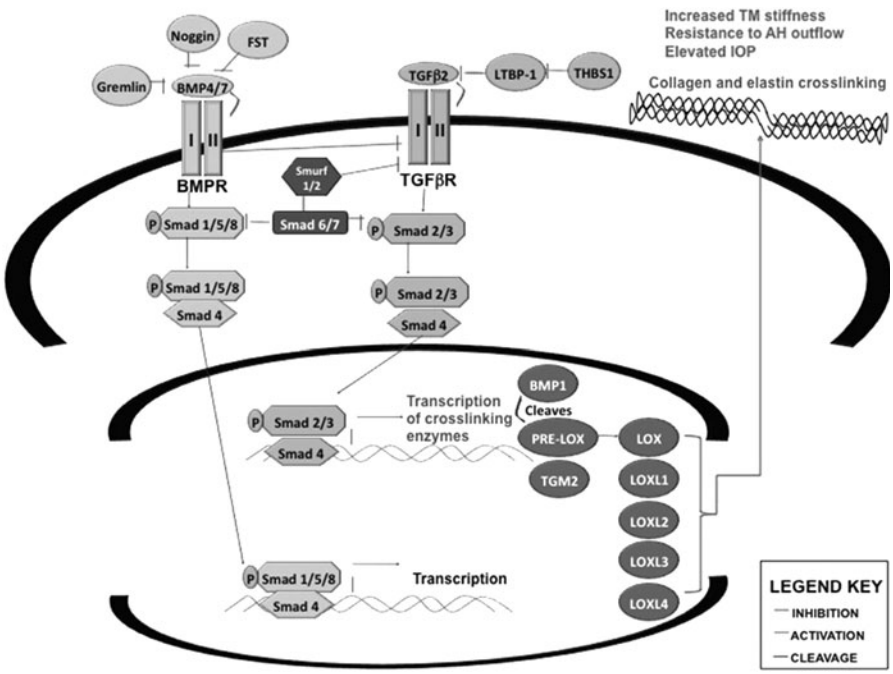

FIG. 1. A schematic representation of the TGF-β signaling pathway and regulation of ECM crosslinking enzymes in the TM. The TGF-β ligand first binds the TGFβ-RII receptor resulting in the phosphorylation of the TGFβ-RII receptor. Subsequently, transcription factors R-Smad2/3 are phosphorylated and bind to Co-Smad4. The R-Smad2/3-Co-Smad4 complex is translocated into the nucleus, and in the presence of coactivators, it binds specific genes to activate transcription of proforms of BMP-1 and LOX family members. The proform of BMP-1 is activated by procollagen C proteinase enhancer (PCOLCE) proteins 1 and 2. BMP-1 then cleaves pro-LOX and pro-LOXL1 into their active enzymes. Activated LOX and LOXL1 subsequently crosslink collagen and elastin fibers in the ECM of the TM. Activation of TGM2 is thought to be either by Ca^{2+}, guanidine nucleotides, or the redox potential.[82] BMP, bone morphogenetic protein; ECM, extracellular matrix; LOX, lysyl oxidase; TGF-β, transforming growth factor-beta; TM, trabecular meshwork.

downstream signaling (Fig. 1). BMPR-I and BMPR-II are expressed at the cell surface as homeric and/or heteromeric complexes.[42] Similar to TGF-β signaling, the serine/threonine kinase domains of the BMPR-II receptor are constitutively active, and upon BMP binding, phosphorylation of BMPR-I occurs. BMPs can interact with 2 distinct type I receptors termed BMPR-IA [also known as activin-like kinase-3 (ALK-3)] and BMPR-IB (ALK-6).[19] BMP-2 and BMP-4 preferentially bind to BMPR-IA and BMPR-IB, respectively. BMPs can interact with 3 distinct type II receptors (BMPR-II, ActR-II, and ActR-IIB). However, the majority of BMP signaling utilizes the BMPR-II receptor. BMPR-IA, BMPR-IB, and BMPR-II are expressed differentially in various cells, and the pattern of receptor expression can influence cellular responses to BMP.[43]

However, unlike TGF-β signaling, binding of BMP ligands to the BMP receptor complex results in phosphorylation of R-Smad1, R-Smad5, or R-Smad8.[44] Subsequently, phosphorylated R-Smads trigger heterodimerization with Co-Smad4 resulting in translocation of the complex to the nucleus to activate specific target genes (Fig. 1). While R-Smad1, R-Smad5, and R-Smad8 are structurally similar, there appears to be functional differences. For example, BMP-6 and BMP-7 can activate both R-Smad1 and R-Smad5 but have no effect on R-Smad8, whereas BMP-2 and BMP-4 can activate all 3 BMPR-Smad proteins.[44]

Because TGF-β/BMP ligands stimulate similar signaling pathways involving type I receptors, type II receptors, and R-Smads to effect a variety of cellular responses, it is most likely that a specific set of DNA-binding cofactors expressed in the target cell determines the response to a specific ligand. The expression of specific coactivators or corepressors is also most likely to be cell context dependent. A review of cofactors and transcription factors that play a role in TGF-β2 or BMP signaling is beyond the scope of this article. However, it should be noted that there are virtually no experiments in normal or glaucomatous TM cells that have examined cofactor interactions with either TGF-β or BMP-R-Smad/Co-Smad4 complexes.

Signaling by BMP ligands is exquisitely controlled at the cellular level by specific spatial and temporal control mechanisms. BMP signaling can be inhibited by extracellular control of ligand access to BMP receptors via secreted BMP antagonist proteins. These molecules bind to the BMP ligand or BMPR-II and prevent receptor activation.[45] Second, a pseudoreceptor (Bambi) can be present in the cell membrane. BMP ligands can bind to the pseudoreceptor but the receptor lacks kinase activity, hence a signal is not generated.[46] Third, cytoplasmic inhibitory Smad molecules (I-Smad6 and I-Smad7) can block R-Smads from binding to either the intracytoplasmic tail of the BMP receptor complex or directly to R-Smad or Co-Smad4.[21] In all tissues, a fine balance coexists between BMP activity and inhibition. It appears that this balance occurs through both temporal and spatial expression of BMPs, BMP receptors, and both extracellular and intracellular BMP inhibitors. Thus a priori, it can be assumed that this balance also exists in the human TM.

Noncanonical signal transduction

While many cell types utilize the canonical Smad pathway for TGF-β2-induced fibrosis, several use non-Smad pathways. In non-Smad signaling pathways, activated TGF-β receptors utilize MAP kinases, including extracellular signal-regulated kinases (ERK), p38 mitogen-activated protein kinases (p38MAPK), or c-Jun N-terminal kinases (JNK) signaling proteins, to activate gene targets.[47–49] Although TGF-β may utilize noncanonical signaling pathways in various cell types, R-Smad2 and R-Smad3 are thought to be primarily involved in TGF-β2-driven fibrosis in many cells, including mesangial cells, retinal pigment epithelial cells, and skin fibroblasts. This is also most likely the case with respect to human TM cells.

However, another layer of complexity exists. Although the R-Smad proteins are usually activated through C-terminal phosphorylation by type I receptors (eg, TβRI or BMPR-I), other intracellular kinases can also regulate R-Smad activity. The linker region on R-Smads contains several serine/threonine sites that can be phosphorylated by CDK2/4, JNK, (ERK)1/2, p38MAPK, and glycogen synthase kinase 3β (GSK3β).[1] The finding that R-Smads can be phosphorylated and regulated by kinases other than those activated by members of the TGF-β superfamily has expanded our understanding of both the significance and complexity of TGF-β/BMP signaling and opens the distinct possibility that cross-talk occurs with other key signaling pathways. These types of interactions have not been addressed adequately in the TM cell.

The Role of TGF-β2 in Primary Open-Angle Glaucoma

Background

Elevated intraocular pressure (IOP) is a major risk factor associated with development[50] and progression of primary open-angle glaucoma (POAG).[51] Increased AH outflow resistance through the TM has been associated with elevated IOP in POAG patients. Resistance to AH outflow is correlated with increased accumulation of ECM proteins in the TM. TM cells express an active TGF-β receptor complex (TGF-βR-I; TGF-βR-II)[52] and respond to exogenous TGF-β2.[52,53] TGF-β2 is known to increase ECM protein synthesis and inhibit ECM protein turnover in other tissues.[31]

A total of 10 studies during the past 15 years have reported significantly elevated TGF-β2 levels in the AH of POAG patients.[54] In addition, Min et al.[55] reported that both active and total TGF-β2 forms (eg, LAP-bound plus active TGF-β2) are elevated in the AH of POAG patients. Significantly, TGF-β2 levels are also elevated in cultured human glaucomatous cell strains and isolated human glaucomatous TM tissues.[56] The cause of elevated levels of TGF-β2 in glaucomatous AH is not clear. In addition, the source of TGF-β2 has not been definitively identified. Fuchshofer and Tamm[53] have suggested that because of the flow of AH, the ciliary body and lens are likely candidates. However, it is also clear that TM cells can secrete TGF-β2 into the local microenvironment.

Relationship of TGF-β2 and the TM ECM

In other fibrotic diseases, elevated TGF-β2 levels lead to pathological deposition of ECM proteins and increased tissue stiffness. Similar pathological changes occur in the glaucomatous TM and appear also to be related to elevated TGF-β2 levels in the AH and TM cells. In cultured human TM cells, exogenous TGF-β2 increases expression of a variety of ECM proteins, including fibronectin (FN), collagen (COL), elastin (ELN), and proteoglycans. In addition, exogenous TGF-β2 induces plasminogen activator inhibitor-1 (PAI1) and tissue inhibitor of metalloproteinase-1 (TIMP1). These inhibitors suppress proteolytic degradation of the ECM, and their heightened activity would lead to increased ECM accumulation.[53] It is also likely that there is a change in the composition and organization of the TM ECM, which can also alter TM function.

Perfusion of TGF-β2 in the perfused *ex-vivo* anterior eye segment organ culture model caused an accumulation of fibrillary material in the TM.[57] In addition, Fleenor et al.[58] reported that perfusion of TGF-β2 elevated IOP in a time-dependent manner, increased FN and PAI-I levels in the perfusate, and decreased AH outflow facility. Intraocular injection of bioactive TGF-β2 induced ocular hypertension in both rats and mice and significantly reduced AH outflow facility in the mouse.[59]

Thus, using cultured TM cells, perfused *ex vivo* anterior eye segment organ culture models, or animal models, exogenous TGF-β2 is able to mimic the pathophysiology of POAG. In toto, the preponderance of clinical and experimental evidence indicates that elevated TGF-β2 levels in the AH or TM tissue are highly correlated with POAG. Thus, it is reasonable to conclude that TGF-β2 has a direct role in the deposition and accumulation of ECM proteins in the TM, leading to increased AH outflow resistance and elevated IOP.

The relationship of TGF-β2 and ECM crosslinking enzymes

Another mechanism to account for outflow resistance in the glaucomatous TM is increased TM stiffness. Last and colleagues[60] reported that the mean elastic modulus (a measure of tissue stiffness) was increased significantly in the glaucomatous human TM when compared with age-matched controls.[60] They suggested that a change in the physical properties of the TM might directly modulate AH outflow resistance and IOP. We have reported that human TM cells synthesize and secrete ECM crosslinking enzymes, including tissue transglutaminase (TGM2),[56] lysyl oxidase (LOX), and lysyl oxidase-like proteins (eg, LOXL-l-4).[61] Significantly, treatment of human TM cells with TGF-β2 utilizes the Smad canonical signaling pathway (Fig. 1) to induce secretion of enzymatically active TGM2,[62] LOX, and LOXL proteins.[61] In addition, TGM2 protein levels are increased in glaucomatous TM tissues.[56] In other tissues, these enzymes are known to change the physical properties of the ECM and increase stiffness by covalently crosslinking ECM proteins (eg, collagens and elastin) and inhibiting ECM turnover. Thus, increased stiffness of the glaucomatous TM may be the result of localized, increased ECM protein crosslinking enzymes. Future studies using animals and/or *ex vivo* models are needed to verify that overexpression of crosslinking enzymes in the TM leads to increased TM stiffness, increased AH outflow resistance, and elevated IOP.

The relationship of TGF-β, LOXL-1, pseudoexfoliation syndrome, and BMP-1

An indication that an alteration in expression or function of crosslinking enzymes in the TM may be associated with POAG comes from studies of pseudoexfoliation syndrome (PXS) and pseudoexfoliation glaucoma (PXG). The systemic disease PXS presents with significant eye involvement.[63,64] Picht et al.[65] reported that TGF-β2 was not elevated in the AH of PXG patients. However, TGF-β1 and TGF-β3 levels are elevated in the AH of PXS and PXG patients.[66]

Single-nucleotide polymorphisms (SNPs) of the *LOXL-1* gene are strongly associated risk factors for PXS. Microfibers coated with amorphous material (eg, pseudoexfoliation deposits) coat various structures, including the TM.[64] The LOXL-1 enzyme is necessary for tropoelastin crosslinking and elastic fiber formation, maintenance, and remodeling.[67,68] At specific sites for elastic fiber formation, the inactive precursor form of LOXL-1 (eg, pro-LOXL-1) binds to both fibulin-5 and tropoelastin and targets the formation of elastic microfibrils. At this site, a scaffold is built using fibrillins and microfibril-associated glycoproteins that aids in the alignment of tropoelastin crosslinking domains.[69] Significantly, following LOXL-1 proform binding to the scaffold, BMP-1 (i.e. procollagen C-terminal proteinase) cleaves the proform resulting in LOXL-1 enzymatic activation.[70]

As noted earlier, BMP-1 was erroneously included as a member of the BMP subfamily and has no homology to other BMP proteins. In actuality, BMP-1 is a zinc protease that converts secreted precursor proproteins into mature, functional proteins. Both LOX and LOXL-1 are substrates for BMP-1.[70] As a result of LOXL-1 activation, lysine residues are covalently crosslinked and elastin fibers become highly resistant to degradation or turnover. The SNPs associated with PXS are located in exon 1 that codes for an N-terminal domain of the proform of LOXL-1. It is predicted that this domain is involved in LOXL-1 activation and binding to the scaffold. However, it is not clear how a polymorphism in this region may affect enzyme function and subsequent PXM production.[69]

Because BMP-1 is involved in the activation of ECM protein crosslinking enzymes, the regulation of BMP-1 expression and biological activity may be important in understanding TM stiffness and AH outflow resistance. We recently reported that TM cells and tissues express BMP-1 mRNA and protein and that BMP-1 protein is induced by TGF-β2.[71] Using a LOX activity assay, we also demonstrated that secreted BMP-1 from human TM cells was biologically active and increased secreted LOX enzyme activity. Significantly, we showed that glaucomatous TM cells secreted higher levels of BMP-1 and that BMP-1 secretion in glaucomatous TM cells was further induced by exogenous TGF-β2 treatment compared with normal TM cells.

Relationship of TGF-β2 and BMPs in TM Fibrosis

BMP-4 and BMP-7 inhibit TGF-β2 induction of ECM proteins by TM Cells

To maintain a homeostatic balance in any given tissue, mechanisms to control growth factor signaling must exist. Within the normal human TM, a balance exists with respect to ECM protein synthesis and turnover that aids in maintaining IOP within normal limits. Any imbalance of ECM protein synthesis and/or degradation would lead to ECM protein accumulation, AH outflow resistance, and elevated IOP. Our laboratory reported that BMP-4 significantly inhibited the ability of TGF-β2 to induce ECM protein synthesis and secretion by cultured human TM cells.[72] BMP-4 alone did not influence ECM synthesis or secretion by cultured TM cells. We had previously reported that human TM tissues and cultured TM cells expressed several BMPs (i.e. BMP-2, BMP-4, BMP-5, and BMP-7), the BMPR-I and BMPR-II receptor complex, and several BMP antagonist proteins.[73] Human TM cells are capable of responding to exogenous BMP-4 and BMP-7 via R-Smad phosphorylation. We suggested that BMP4 was an endogenous inhibitor of TGF-β2 action within the human TM and aids in controlling ECM deposition thus maintaining IOP within normal limits.[72] Similarly, BMP-7 has also been shown to antagonize the action of TGF-β2 in human TM cells.[74] Treatment of cultured human TM cells with TGF-β2 induced a number of ECM proteins and proteins associated with the ECM (e.g., CTGF, FN, collagens IV and VI, PAI-1, and TSP-1). BMP-7 significantly inhibited TGF-β2-induced expression of these proteins in cultured human TM cells.

Fuchshofer et al.[74] further examined the signaling pathway in human-cultured TM cells following exogenous BMP-7 treatment. Initially, microarray studies were performed to identify differentially regulated genes. They noted that both TGF-β2 and BMP-7 caused an induction of I-Smad7. I-Smad7 is an intracellular antagonist of TGF-β2 signaling.[75] Using siRNA specific to I-Smad7, they were able to inhibit the effects of BMP-7 on TGF-β2 signaling in TM cells. Thus, I-Smad-7 appears to be a significant molecule that prevents TGF-β2 induction of ECM proteins in the TM cell. In other cell types, the mechanism of action of I-Smad7 inhibition has been identified. One mechanism involves stable binding of I-Smad7 to the activated TGF-βRI thus preventing downstream signal processing.[76] A second mechanism involves competition of I-Smad7 with R-Smad molecules. Lastly, I-Smad7 can recruit Smurf1 and Smurf2 to TGF-βRI receptors. Smurf1 and Smurf2 are E3 ubiquitin ligases that can subsequently trigger receptor degradation via ubiquination.[77] How I-Smad7 specifically acts in the human TM cell is yet to be determined.

Previously we reported that human TM cells express several BMP signaling antagonist proteins [i.e. gremlin, follistatin, and chordin] and the pseudoreceptor Bambi.[74] Initially we wanted to determine whether differences in protein expression of the BMP signaling pathway existed in glaucoma. We compared the expression of BMP-associated genes in normal TM cells with gene expression in age-matched glaucomatous TM cells. The expression of gremlin, a BMP antagonist protein, was significantly increased in glaucomatous TM cells and tissues.[74] In addition, we reported that gremlin was able to significantly antagonize BMP-4 inhibition of TGF-β2-induced fibronectin and PAI-1 secretion and elevate IOP in an perfused *ex-vivo* anterior eye segment organ culture model.[74] Lastly, exogenous TGF-β2 significantly increased gremlin expression in TM cells. We have proposed that gremlin potentiates the profibrotic effects of TGF-β2 in the TM by blocking BMP-4 regulation of TGF-β2 activity (Fig. 2).[74]

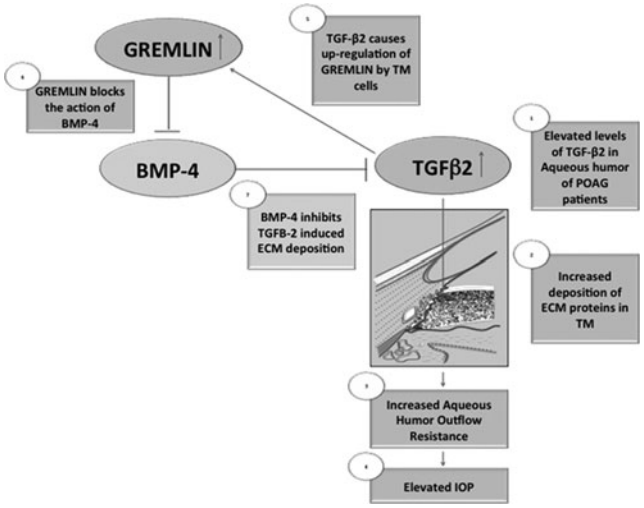

FIG. 2. A schematic representation of the interactions of BMP-4, TGF-β2, and gremlin within the TM. Elevated TGF-β2 in the aqueous humor and TM of glaucomatous patients leads (1) to increased ECM protein deposition in the TM (2), increased resistance of aqueous humor outflow from the TM (3), and elevated IOP (4). In addition, elevated TGF-β2 levels upregulate the expression of the BMP antagonist gremlin in TM cells (5). Gremlin directly binds to BMP-4 and prevents its biological activity (6). Thus, the ability of BMP-4 to counterbalance the effect of TGF-β2 is inhibited (7). BMP-7 acts similarly to BMP-4.[74] IOP, intraocular pressure.

It also appears that gremlin and TGF-β2 may be involved in a ''feed-forward'' pathogenic pathway. For example, gremlin increases TGF-β2 expression and TGF-β2 increases gremlin expression in TM cells.[78] In addition, gremlin utilizes canonical and noncanonical TGFβ signaling pathways to induce LOX genes in human TM cells.[78] Together these mechanisms would further exacerbate ECM deposition within the TM, potentially leading to increased AH outflow resistance and IOP elevation. Thus, it is plausible to hypothesize that elevated levels of gremlin in the glaucomatous TM might lead to elevated TGF-β2 levels in the glaucomatous TM.

Our original examination of BMP/BMPR/BMP antagonist mRNA in TM cells also identified the expression of follistatin (*FST*) mRNA. FST is also a secreted BMP antagonist protein. The primary *FST* transcript undergoes alternative splicing to produce mRNAs (*FST* 317/344) that encode for proteins that are proteolytically cleaved, yielding FST 288 and FST 315.[79,80] Both *FST* mRNA as well as FST 288 and FST 315 proteins were expressed in normal human TM cells, but there was significantly higher expression in glaucoma TM cells.[81] In addition, TGF-β2 induced FST mRNA and protein expression in a dose- and time-dependent manner in cultured TM cells.[81]

In summary, (1) BMP antagonist proteins gremlin and FST are expressed and secreted by human TM cells and tissues and (2) TGF-β2 significantly upregulates gremlin and FST mRNA and protein levels. The induction of BMP antagonist proteins by TGF-β2 may (3) amplify the action of TGF-β2 via inhibition of either BMP-4 and/or BMP-7. Under TGF-β2 induction, the TM cell is inhibiting an endogenous inhibitor of TGF-β2 signaling thus resulting in a cellular context where TGF-β2 activity is uncontrolled (Fig. 2). Taken together, these findings highlight the complex relationship of TGF-β2, BMP, and BMP antagonists in the human TM.

Future Directions

TGF-β isoforms

A comparison between the effects of different TGF-β isoforms in the human TM has not been extensively studied. However, some studies indicate that TGF-β isoforms have overlapping effects. Fleenor et al.[58] showed that all 3 isoforms induced both fibronectin and PAI-1 secretion by human TM cells. In addition, all 3 TGF-β isoforms induce the crosslinking enzymes TGM2,[56] LOX, and LOXL proteins[61] in cultured TM cells. All TGF-β isoforms inhibit human TM cell proliferation.[52] However, the physiological significance of all 3 TGF-β isoforms in the TM has not been addressed and may yield significant information related to TM cell function. The AH of patients with different types of glaucoma (POAG and PXG) appears to have different levels of specific TGF-β isoforms as well as other growth factors. The cellular source of these factors has not been identified nor an explanation as to why these factors are elevated. If numerous factors are elevated in the AH of glaucoma patients, then how does that translate into altered cellular responses and by what mechanisms? It is quite apparent that we really do not understand the relationships of various growth factors as related to various forms of glaucoma. Studies directed to understanding these relationships in more detail may identify new therapeutic targets not obvious to us at this time.

BMP signaling

BMPs represent a significant subfamily of the TGF-β superfamily of growth factors. It is clear from this review that there are significant gaps in our understanding of the role of BMPs and signaling in the TM. For example, BMPR-IA, BMPR-IB, and BMPR-II are expressed differentially in various cells, and the pattern of receptor expression can influence cellular responses to BMPs. Is this also true for the human TM cell? What specific cellular activities would be activated based on the differential expression of BMPR-IA and BMPR-IB in TM cells? Are there differences in receptor expression in normal versus glaucomatous TM cells? What are the downstream effects of BMP signaling in the TM? In addition to R-Smad7, are there other molecular mechanisms by which BMP block TGF-β signaling? Important advances have been made with respect to specific BMP signaling pathways. A more detailed understanding of the BMP signaling pathway in the TM will help us to further elucidate the role of BMPs in the pathogenesis of glaucoma.

Canonical and noncanonical signaling pathways

The canonical TGF-β and BMP signaling pathways use different R-Smads (Fig. 1). In response to TGF-β2, both R-Smad2 and R-Smad3 are phosphorylated. What is the significance at the cellular level? Are there differences in signaling pathways between normal and glaucomatous TM cells? Does phosphorylation of R-Smad3 lead to activation of fibrosis in the TM as reported in other tissues or are other factors at play? In response to BMP, R-Smad1, R-Smad5, and R-Smad8 are phosphorylated. Why is there such redundancy? Is there competition between TGF-β and BMP

canonical signaling via Co-Smad4 that is shared between these pathways? Detailed experiments in human TM cells with respect to TGF-β and BMP canonical signaling are needed to determine (1) why different receptor Smad proteins are selected and utilized, (2) the cellular context that drives this selection, (3) specific cellular responses that are triggered by R-Smad selection, and (4) whether differences exist between normal and glaucomatous TM cells? It should also be noted that there are virtually no experiments in normal or glaucomatous TM cells that have examined intranuclear expression of cofactor interactions with either TGF-β or BMP-R-Smad/Co-Smad4 complexes.

The finding that R-Smads can be phosphorylated and regulated by kinases other than those activated by members of the TGF-β superfamily has expanded our understanding of both the significance and complexity of TGF-β/BMP signaling and opens the distinct possibility of cross-talk with other key signaling pathways. TGF-β and other factors can also activate noncanonical signaling via ERK, p38MAPK, or JNK in the human TM cell. This type of signaling can occur simultaneously with activation of the canonical signaling pathway. What is the physiological significance of activating both pathways? What specific cellular mechanisms are inhibited or activated by each pathway? What significant degree of cross-talk occurs between the pathways? May the noncanonical signaling pathway be more significant depending on cellular context? Virtually no experiments have been done to specifically address these questions in normal or glaucomatous TM cells.

BMP antagonist proteins

It has become apparent that secreted BMP antagonist proteins (eg, gremlin) have the capability of inhibiting BMP antagonism of TGF-β2 induction of ECM proteins. However, more detailed studies are required to delineate additional molecular mechanisms that may be involved. I-Smad7 has been shown to block BMP-7 inhibition of TGF-β2 activity. The exact mechanism of this inhibition has not been examined. In addition, other intracellular controlled mechanisms may also be important. Is it possible that I-Smad7 can inhibit TGF-β2 signaling via E3 ubiquitin ligases that target TGFβ-RI degradation? Are other secreted BMP antagonist proteins also functioning in the human TM cell? The potential role(s) of FST 288 and FST 315 in the pathophysiology of glaucoma is currently not known and should form the basis of future studies. Thus, similar to gremlin, TGF-β2 upregulation of FST may block BMP-4 inhibition of TGF-β2 induction of ECM proteins in the TM. Does the pseudoreceptor BAMBI function in the TM cell? Do other growth factors control expression of different inhibitory molecules? It is possible that new therapies centered on BMP antagonism of TGF-β2 activity may have promise as a new treatment for POAG. All these areas are open for discussion and experimentation in normal and glaucomatous TM cells.

Cellular context

TGF-β isoforms, BMPs, and other growth factors can signal through closely related signaling pathways but stimulate different responses in the target cell. The concept of cellular context is now being addressed to explain these differences. Factors that should be considered include (1) the specific growth factor isoform, (2) the concentration of the ligand at the target cell, (3) the context of the target cell including expression of cofactors or corepressors, (4) the expression and diversity of receptors on the target cell, (5) the type and level of intracellular signaling molecules (e.g., R-Smads) expressed by the target cells, (6) the degree of cross-talk acting on the target cell from noncanonical signaling pathways, and (7) the level and expression of phosphatases in the target cell in order to reduce signaling. For example, in the TM cell, it is not clear why one signaling pathway may dominate or why several signaling pathways can be simultaneously activated? Also, there are few if any studies that examine specific phosphatases. Thus, more detailed studies are needed to define cellular context in normal and glaucomatous TM cells.

Role of TGF-β2 and crosslinking enzymes in POAG

Since exogenous TGF-β2 and gremlin increase crosslinking enzyme expression in cultured human TM cells, we have suggested that in POAG, elevated levels of TGF-β and gremlin in the TM increase expression and secretion of TGM2, LOX, and BMP-1. The presence of BMP-1 cleaves the precursor proforms of LOX and LOXL-1 to yield enzymatically active molecules. Subsequent covalent crosslinking of collagen/elastic fibers increases stiffness of the TM that may result in AH outflow resistance and elevated IOP. It should be noted that the molecular mechanism for activation of TGM2 has not been clearly defined and warrants examination in the human TM cell. Since all preliminary data have been obtained via cell culture experiments, future studies using animals and/or *ex vivo* models are needed to verify that overexpression of crosslinking enzymes in the TM leads to increased TM stiffness, increased AH outflow resistance, and elevated IOP.

Author Disclosure Statement

No competing financial interests exist.

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Received: November 4, 2013
Accepted: December 31, 2013

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