Effects of TGFβ2 and BMP4 Downstream Targets ID1 and ID3 in Trabecular Meshwork: Implications In Lowering IOP

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INTRODUCTION

Glaucoma is a chronic multifactorial eye disease which silently progresses into irreversible vision loss, affecting more than 70 million people worldwide[1]. In the United States alone, over 3 million individuals suffer from primary open angle glaucoma (POAG)[2]. Open angle glaucoma (OAG) comprises of primary and secondary OAG. The risk factors associated with POAG is age (40 and above), ethnicity, genetic inheritance, and elevated intraocular pressure (IOP).[3, 4] It is projected that by 2020, people wit OAG will increase to 5.9 million of which 4.5 million people will suffer bilateral blindness thus rendering OAG second leading cause of irreversible blindness.[1, 3, 5] All forms of glaucoma are characterized by loss of vision due to optic nerve head cupping and retinal ganglion cell death. Although we lack comprehensive understanding of the pathogenesis of POAG, increased resistance to aqueous humor (AH) outflow through the trabecular meshwork (TM),a shift in equilibrium between secretion of aqueous humor by the ciliary body and its drainage through the TM, increased ECM production and deposition in the TM region, and disruption in ECM turnover lead in many cases to elevation in IOP. While lowering of IOP, is currently the only known clinical intervention, which slows disease progression.

Aqueous Humor, Trabecular Meshwork and the Aqueous Outflow Pathway

Numerous studies have contributed to our current understanding of AH composition and its outflow pathway in normal and glaucomatous conditions.[6-10] The AH is clear, colorless solution which is secreted by the non-pigmented epithelium of ciliary body and nourishes various tissues in anterior chamber of the eye. AH is secreted at a flow rate of ~2.5 μl min^{-1} in humans. Following secretion into the posterior chamber, AH then flows which into the anterior chamber. The major site of aqueous outflow is through the TM into Schlemm's Canal (SC), than exiting the eye via the episcleral veins (EPV).[11]

The TM has been recognized as site for resistance to the AH outflow since 1892-1893. Since then many studies have contributed to our current understanding of TM tissue and the site of AH outflow resistance. The TM and the endothelial cells of SC are now believed to primary site for AH resistance.[12-15] Recent fluorescent microsphere perfusion studies in humans eyes show that the outflow pathway to be segmental, and the highest effective filtration area is located in TM compared to inner wall of SC or the EPV.[12] Morphologically, the TM is a fenestrated tissue made of three layers; the inner uveal TM, the middle corneoscleral TM, and juxtacanulicular TM (JCT) region.[16, 17] The outer JCT region is localized adjoining to the SC inner wall endothelium. These three layers of the TM are unique in their structure and porousness, which ensure unidirectional flow of AH. The uveal layer is made up of one to three trabecular layers, while corneoscleral is thicker with 5-15 layers of trabecular beams, and the third JCT layer is the thinnest but also most dense region. The stroma of trabecular beams is made of elastin and

collagen (I and III) fibers, TM cells rest on a basal lamina composed of the trabecular beam. The trabecular beams attach to each other forming fenestrated region with different pore sizes, ranging between 5-27μm and offering the least resistance to AH outflow. In contrast, the JCT layer is composed of unstructured ECM fibrils such as fibronectin, collagen IV and hyaluronans. TM cells are scattered within this amorphous ECM.[18, 19] The uveal and corneoscleral TM cells have an endothelial cell like phenotype, while JCT cells have phenotype more like that of a fibroblast.[20] The JCT region is considered as the site that offers the highest resistance AH outflow due to the dense nature and control of extracellular matrix (ECM) turnover[19]. Normal human AH fluid composition contains several growth factors including transforming growth factor superfamily members including transforming growth factor β (TGFβ) and bone morphogenetic proteins (BMPs). ECM homeostasis (production, turnover, and remodeling) plays crucial role in maintaining normal AH outflow resistance and IOP. Growth factors TGFβ1, TGFβ2, BMP4, and BMP7 play critical roles in maintaining ECM turnover equilibrium and TM homeostasis.[7, 10, 11, 21-25]

Transforming growth factor β expression and Glaucoma:

TGFβ is a versatile cytokine required for maintaining various cellular functions such as wound healing, apoptosis, ECM production, cell proliferation and migration, and fibrosis.[26, 27] In healthy anterior segments, TGFβ is involved in corneal wound repair and maintaining ocular immune privilege.[28-31] Three TGFβ isoforms ligands (TGFβ1, TGFβ2, TGFβ3) are found in mammals. Each isoform is encoded by a separate gene; however, they share 60-80 % homology and function through activating the same cell

surface receptor.[27, 32] TGFβ is secreted as an inactive 442 amino acid precursor molecule with hydrophobic N-terminal, latency associated peptide, and the bioactive C-terminal.[31] During maturation of TGFβ, the inactive form is cleaved at Arg-XX-Arg site leading to formation of latent complex, where the TGFβ homodimer is non-covalently bound to two latency associated peptides (LAP). This complex anchors to the ECM. For the active TGFβ (25kDa) molecule to be released from its inactive LAP complex, proteolytic cleavage occurs by plasmin, thrombospondin-1 or plasma transglutaminase (Figure1a). Stretching between integrins and ECM can also release the active TGFβ isoform.[33] TGFβ2 and TGFβ1 is expressed in various eye tissues, including limbus epithelial cells, ciliary body, AH, cornea, and the TM, but ocular tissues lack endogenous expression of TGFβ3. [27, 34] TGFβ ligand binding to TβRI and TβRII receptors activates intrinsic receptor serine-threonine kinase activity, which further activates the Smad dependent pathway. Phosphorylation of tyrosine residues on TβRI and TβRII facilitates activation of non-canonical pathway.[27, 32, 35, 36] However, the non-canonical pathways can be activated by Rsmad3, so that there is cross-talk between these 2 major signaling pathways.[27, 37] Recent studies have shown that TβRI and TβRII are present as monomers in absence of TGFβ binding ligand. Upon binding of TGFβ (dimeric ligand) to TβRII, TβRII forms homodimer, which enables recruitment and dimerization of TβRI forming active heterotetrameric complex and activation of downstream pathway.[36, 38-40] Expression of three TGFβ receptors (TβRI, TβRII and TβRIII) have be demonstrated in TM cells.[21, 41] It is largely unknown how cells quantitates the bioavailability of TGFβ but different levels of molecular availability of TGFβ will elicit different cellular responses.[38] In glaucomatous AH, there is often

increase in expression of both latent TGFβ2 and active TGFβ2 compared to normal AH.[42, 43] Studies have shown that ECM elasticity and biomechanical changes or activation of the Rho/GTPase pathway in the TM may contribute to increased TGFβ2 mRNA, signaling activity and expression of down-stream targets genes.[44-47] However, the understating on factors contributing to activation of Rho/GTPase in glaucomatous TM are not very well understood.[47]

Transforming growth factorβ2 induce ECM changes in TM

In TM cells, TGFβ2 is known to alter the cytoskeleton, ECM protein expression along with ECM regulatory enzymes. It also decrease TM cellularity via phosphorylation of canonical regulatory canonical R-smad 2/3 and non-canonical (MAP kinase and downstream regulators p38, JNK, AP-1, as well as Rho/GTPase signaling) pathways.[46, 48-51]Several in vitro experiments in TGFβ2 treated TM cells have shown upregulation and deposition of ECM proteins, including FN, collagen I and IV, laminin , tenascin C, versican and elastin.[48, 52-55] Elevated FN along with specific integrin receptors plays important role in additional deposition of other ECM by forming FN scaffolds.[56, 57] Further, TGFβ2 induces cross-linked actin network (CLAN) formation in TM cells.[58] Several groups have reported that TGFβ2 increases PAI-1 protein expression in TM cells, and ECM turnover is attenuated by PAI disrupting MMPs activity. TM cells treated with PAI-1 neutralizing antibody in the presence of TGFβ2 showed an increase in MMP2 and MMP9 activity.[50, 59, 60] Further induction of crosslinking enzymes like lysyl oxidase (LOX) and transglutaminase along with cytoskeleton changes may

increase TM stiffness and increase resistance to AH outflow.[52, 61, 62] This overall increase in ECM components, ECM crosslinking, and disruption of the ECM turnover machinery contributes to POAG pathologies and IOP elevation.

Transforming growth factor β2 increases IOP

Elevated IOP is a major risk factor associated with POAG development and progression. Loss of equilibrium between expression of ECM components and ECM turnover in the TM leads to increased AH outflow resistance, thereby elevating IOP. As mentioned above, TGFβ2 plays an important role in increased deposition of ECM in the TM (Figure 1b). Several clinical studies have demonstrated significant increases in biologically active TGFβ2 in POAG patients compared to other forms of glaucoma such as primary angle closure glaucoma (PACG), exfoliating glaucoma (EXG) and secondary glaucoma (SG), suggesting an important role for TGFβ2 in POAG progression.[10] The elevation in IOP and changes in the TM by activated TGFβ2 is also observed in exvivo perfusion cultured anterior segment model.[59] Interestingly, wild type TGFβ2 failed to elevate IOP in rodents; However, a genetically engineered active form of TGFβ2, which was generated by mutating 2 cysteine associated with LAP binding protein (C226S/C288S)(Figure1a), increased IOP in rats and mice. This TGFβ2 induced ocular hypertension model mimics many POAG changes in the TM.[63, 64]

Roles of BMPs in development, expression in ocular tissue and regulation of IOP

Bone morphogenetic proteins (BMPs) were first identified as important growth factors required for bone formation.[65-68] Advances in development and cytokine biology

recognized the crucial role of BMPs in adding complexity and regulating various stages of development and other cellular processes in other tissues.[69-71] Several researchers have proposed changing the nomenclature of bone morphogenetic proteins to body morphogenetic proteins based on their function in regulating development and body morphogenesis and in maintaining tissue homeostasis of various tissues including kidney, heart, brain, eye, and lungs.[68, 72]

BMPs as mentioned belong to the TGFβ super family and similar to TGFβ, undergoes maturation by proteolytic cleavage and dimerization; however, BMP dimers form disulfide bonds and proteolytic cleavage at Arg-X-X-Arg located at the C-terminal of the precursor molecule to release the active BMP.[71, 73] More than 30 BMPs are known, and each has their unique function but sometimes work in synergism. BMPs also activate downstream signaling pathways by binding to serine-threonine kinase receptors.[73] Binding of BMP to BMPRII phosphorylates Gly-Ser domain on BMPRIA (ALK3), BMPRIB (ALK6), which activates BMPRI kinase activity to signal via smad-dependent and smad-independent pathways activating different sets of downstream target genes.[73]

Expression of BMP2, BMP4, BMP5, BMP7 has been studied in the development of various ocular tissues including the cornea, optic never head (ONH), neural retina, optic cup, and lens.[71, 74, 75] Wordinger et. al. (2002) and others have shown mRNA and protein expression of BMPs (BMP 2,4,5,7) and BMP receptors along with the BMP antagonists gremlin, chordin, BAMBI, follistatin in TM cells and tissues, ONH astrocytes, lamina cribrosa cells, ciliary body, iris, retina cells, and cornea.[76-79] During development, BMP4 is essential for mesodermal differentiation and a homozygous inactivating mutation

leads to delayed lens induction during development.[71, 80] Recent reports suggest positive and negative autoregulation of BMPs and a BMP gradient is needed in lens and optic vesicle formation.[74] Furthermore, heterozygote-deficient BMP4 mice develop microphthalmia, anterior segment dysgenesis including malformation of Trabecular outflow pathway and elevated IOP.[71, 76] Over expression of BMP2 in eye of rat develop elevated IOP.[81] These data suggest that expression and function of each BMP is unique and important in the development of ocular tissue and is time and dose-dependent.

BMP signaling, regulators, down-stream targets, and regulation of the TGFβ2 pathway in TM

The bone morphogenetic protein signaling pathway has several layers of complexity and is positively and negatively regulated at several stages of signal transduction. BMPs form heterodimers (e.g., BMP2/BMP4 or BMP4/7) or homodimers (e.g., BMP4/BMP4), which play important roles in initiating and regulating signaling pathways.[77, 82] Binding of BMPs to their receptors can activate R-smads (1/5/8) and promote complex formation with co-smad smad4 in the nucleus, thereby activating various BMP target genes such as inhibitor of DNA binding protein (Id1-4), inhibitory smads (I-smad6,7), TIEG, and Snail. During osteoblast differentiation transcription factors like Hey-1 are upregulated by BMPs, which regulate Notch signaling. Activated Notch regulates BMP expression in the ciliary body, suggesting crosstalk between BMP and Notch signaling pathways.[73, 77, 83] BMPs also transduce signals via smad-independent pathways by phosphorylating the MAPK family members MAPK, ERK, p38, Jun, and JNK at their Thr-Gly-Try motif. Activation of MAPK, ERK, p38 is known to regulate various cellular functions

including cell survival, apoptosis, regulation of RNA splicing, and growth factor production.[73]

Positive regulators, as well as antagonists, add further complexity to the BMP signal transduction pathway. These regulators are involved at various stages of BMP signaling. The BMP antagonists gremlin, noggin, chordin, follistatin and DAN form BMP-BMP antagonist complexes, thereby inhibiting binding of BMP to the receptor. Similarly, BAMBI, a BMP pseudoreceptor, lacks the receptor intracellular signaling domain and inhibits or regulates the intensity of BMP signaling. Also the small molecule LDN 193189 and Dorsomorphin act as BMP signaling antagonists by inhibiting ALK2 and ALK3 receptors.[84]

The BMP signaling co-receptor enhancers Dragon and CD44 (the full length hyaluronan receptor) enhance BMP signaling by recruiting BMP to BMP receptors and forming tetramer complexes or activating R-smad 1 and smad complex formation, respectively.[73, 77, 85, 86] The inhibitory Smads I-Smad6 and I-Smad7 regulate BMP signaling intracellularly; I-Smad6 preferentially inhibits the BMP pathway, while I-Smad7 can inhibit both the BMP and TGFβ pathways.[73, 87] Similarly E3 ubiquitin ligases such as smad specific E3 ubiquitin ligases (smurf1/2) regulate the BMP signaling pathway intracellularly by proteasomal degradation of smads 1 and 5.[77, 88]

In the TM, TGFβ2 upregulates the extracellular and intracellular BMP antagonists gremlin, noggin, chordin, follistatin, BAMBI, BMPER, and Smurf 2, which negatively regulate BMP pathways.[50, 88] Further, Fuchshofer et. al. (2007) demonstrated that BMP7 negatively regulates the TGFβ2 pathway and the pathophysiological changes by induction of I-smad7.[24, 89] In contrast, Wordinger et al. (2007) showed that BMP4

activates the canonical pathway in TM cells and inhibits TGFβ2 mediated induction of FN, which is upregulated in the TM of glaucomatous eyes.[25] Further, they also demonstrated that gremlin, which is increased in the glaucomatous TM, blocked BMP4 inhibition of FN expression. In an ex vivo perfusion anterior segment perfusion culture model, gremlin reversibly increased IOP thereby suggesting that inhibition of BMP would elevate pressure by increased ECM deposition.[25, 50] However, the mechanism(s) by which BMP4 negatively regulates TGFβ2 induced fibrosis in the TM remained unexplored. We hypothesized that ID proteins may be the downstream targets of BMP4, and therefore IDs may be crucial in inhibiting TGFβ2 induced fibrosis.

Inhibitor of DNA binding protein (ID1-4), the down-stream targets of BMP4

BMPs are potent inducers of inhibitor of DNA binding proteins (IDs) in other tissues. The ID promoter region contains a smad binding response element and GC rich consensus sequence, which is essential for BMP induced gene expression (Figure 2) .[73]

Further ID proteins are very important BMP targets, as IDs are involved in regulating various important cellular process such as differentiation, proliferation, senescence, neurogenesis, angiogenesis, regulating fibrosis, development, cell cycle progression, proteasomal degradation, and the circadian clock [90-97]

ID proteins belong to the superfamily of basic helix loop helix (bHLH) transcription factors, which encompasses about 300 transcription factors.[98] Each contains an approximately 60 amino acid region that is highly conserved yet functionally distinct, and the C-terminal ends of the proteins contain a basic amino acid domain, which binds to the DNA hexanucleotide consensus sequence also known as E-box. IDs contain four family members ID1-4, which are highly conserved among species, from Drosophila to

human. Of the four IDs, ID4 has a highly distinct function from the other three members, while ID1-3 exhibit certain overlapping functions that varies from cell type.[99, 100]

While IDs belong to the bHLH family, they lack the basic DNA binding domain unlike other members of the bHLH super family; However, IDs contain the conserved HLH domain (Figure 3), which allows them to bind to other E-box proteins, thereby inhibiting transcription (Figure 5). The differences in their function is determined by the differences in their -C and -N terminal protein sequences.[92, 98, 101, 102] Along with E-box protein, IDs can regulate other transcription factors such as NFκB, pRB, smads, and Hes1 as well as regulate other protein functions such as cav-1, by direct binding.[103-107] The IntAct molecular interaction database (http://www.ebi.ac.uk/intact/) shows 63 binary interactions for ID1 and 65 binary interactions for ID3, some of which are common between the two groups, while others are unique to ID1 and ID3. Some of the common transcription factors that interact with both ID1 and ID3 are illustrated (Figure 4). These data suggest that there may be possible mechanisms by which IDs regulate various cellular functions.

IDs play a crucial role in deciding the fate of various retinal cells. During early development in mice, expression of IDs (1-3) is observed in both the inner and outer neuroblastic layers, and from E18.5 through adulthood, all four IDs have been observed in the ganglion cell layer (GCL) and the inner nuclear layer (INL). [108] However, the expression patterns of each ID are distinct and overlapping in various cells of GCL and INL. For example, ID1 is expressed in RGC, amacrine cells, bipolar cells and horizontal

cells, while expression of ID2 was localized to amacrine and horizontal cells. Furthermore, unlike ID1, ID3 expression was absent in bipolar cells. ID4 was only present in RGC and amacrine cells. Müller cells lacked ID expression, but miss-expression of ID1 and ID3 are sufficient to drive the production of Mueller and retinal progenitor cells (RPC).[97, 108] IDs also restrict RPC cells from developing into photoreceptor cells. ID1and ID3 are crucial regulators in development of the eye. Double mutant *Id1*$^{-/-}$ and *Id3*$^{-/-}$ mice exhibit smaller lenses and retina and overall small eye size.[97] In diabetic retinopathy, the retinal vascular endothelial cell layer thickens due ECM deposition. TGFβ1 induces ID1 or PAI-1 expression, which depends on the doses and types of receptor (ALK1 or ALK5) TGFβ1 activates.[109] In vascular endothelial cells, lower concentrations of TGFβ1 induce smad1/5/8 and Id1 expression. Inhibition of ALK5 negatively regulates FN expression by TGFβ1, and this phenomenon is cell type dependent. In cornea wound healing, upregulation of ID1-3 expression by BMP7 treatment downregulates α-smooth muscle actin (αSMA) expression, indicating that IDs play an important role in regulating fibrosis.[110] Additionally, ID1 promotes cell invasion by upregulating MMP2 and MMP9 leading to ECM degradation in cancer cells.[111] Moreover, lack of ID1 in mouse embryo fibroblasts increased thrombospondin-1, LOX, βV-integrin, inhibin-beta A, and FN expression.[107]

The overall rationale of BMP mediates induction of IDs and the role of IDs as negative regulators of fibrosis led us to our **hypothesis:** BMP induction of ID1 and ID3 inhibit TGFβ2 fibrosis in the TM and inhibits TGFβ2-induced ocular hypertension in mice. Since very little is known about the expression and physiological effects of IDs in TM

cells and in ocular hypertension, this study will explore the expression and function of ID1/ID3 and their roles in inhibiting TGFβ2 fibrosis. This will have important implications in better understanding the pathogenesis and potential treatment of POAG.

Study Overview:

The overall goals of this study are to: (1) determine the expression of IDs (ID1 and ID3) in the TM, (2) discover the downstream targets of BMP4 in TM cells, and (3) evaluate the potential roles of ID1 and ID3 as therapeutic candidates for TGFβ2 induced ocular hypertension and POAG. We have shown that ID1 and ID3 blocks TGFβ2 induced FN and PAI-1 expression in cultured human TM cells. Currently, we are evaluating the inhibitory effects of ID1 and ID3 on TGFβ2: (1) induced expression of ECM proteins in the mouse TM tissue, (2) induced ocular hypertension, and (3) reduction in AH outflow facility. In the future, it will be interesting to observe which promoter activity ID1 and ID3 regulates to attenuate ECM turnover, thereby aiding our discovery of new disease modifying therapeutic targets specific for regulating TGFβ2 pathways involved in fibrosis.

Hypothesis:

The major risk factor of POAG is increased intraocular pressure (IOP). Elevated IOP results from increased resistance to aqueous humor (AH) outflow through the TM, which is associated with an excessive accumulation of extracellular matrix (ECM) in the TM. The pro-fibrotic growth factor TGFβ2 is elevated in glaucomatous AH and TM cells and is known to increase the synthesis and secretion of ECM related proteins including fibronectin (FN) and plasminogen activator inhibitor-1 (PAI-1) in TM cells[27,

[52, 112]. Overexpression of TGFβ2 in the eye increases ECM molecules in the TM and elevates IOP. Interestingly, BMP4 attenuates TGFβ2 induced FN and PAI-1 expression in the TM. However, a gap in our knowledge exists in complete understanding of the mechanism by which BMP4 attenuates TGFβ2 effects.

BMP4 binds to BMPRI and RII, which phosphorylates R-Smads 1/5/8, thereby upregulating ID gene expression.[113] ID1 and ID3 are dominant negative transcription regulators that inhibit TGFβ2 induced ECM expression in cardiovascular and pulmonary fibrotic diseases. We ***hypothesized*** that: (1) BMP4 will temporally and spatially increase ID1 and ID3 expression in TM cells and (2) overexpression of ID1 and ID3 will attenuate profibrotic activity of TGFβ2 in TM cells.

Furthermore, TGFβ2 induces ocular hypertension in human anterior segment ex vivo perfusion culture and in vivo in mouse eyes, and this increased IOP is due to decreased AH outflow facility as a result of increased ECM deposition. In addition to increased ECM synthesis, TGFβ2 increased PAI-1 expression in TM cells, thereby inhibiting the activation of ECM proteolysis (via MMPs, uPA, tPA) resulting in reduction of ECM turnover.[114-116] Treating TM cells with recombinant PAI-1 also leads to increased fibronectin expression. Increased fibronectin expression and secretion leads to formation of insoluble extracellular fibrils, which may have contributed to IOP elevation. We also ***hypothesized*** that (3) increased ID1 and ID3 expression in the mouse TM will suppress TGFβ2-induced ocular hypertension and and elevated AH outflow resistance.

Specific Aims

Our central hypothesis is BMP4 will temporally and spatially increase ID1 and ID3 expression in TM cells and expression of ID1 and ID3 are BMP pathway dependent. Furthermore, overexpression of ID1 and ID3 will attenuate profibrotic activity of TGFβ2 and reduce AH outflow resistance, thus lowering IOP. We propose the following specific aims and experiments.

Specific Aim1: To determine if ID1 and ID3 are downstream signaling mediators of the BMP4 pathway and to elucidate their role in negative regulation of fibrotic TGFβ2 signaling in TM cells.

Specific Aim 1a. Determine the time dependence of BMP4 induced expression of ID1 and ID3 and their nuclear translocation.

In our preliminary study, we tested induction of ID1 and ID3 mRNA by two different doses (5ng/ml and10ng/ml) of recombinant human BMP4 (R&D Systems) in transformed human glaucomatous TM cells (GTM3). We found significant induction of ID1 and ID3 gene expression when treated with 10ng/ml of BMP4. In this experiment, we determined the time dependent induction of ID1 and ID3 gene and proteins following BMP4 treatment in primary TM cell cultures. We employed a minimum of three primary human TM cell strains. The cells were grown to 100% confluency and then serum starved (i.e. culturing in serum free medium) for 24 hours prior to the growth factor treatment. One set of non-treated cells served the negative control for the experiment, while the rest were treated with 10ng/ml of BMP4.

To determine the time dependent induction of ID1 and ID3 genes post BMP4 treatment, we treated the confluent serum starved cells with BMP4 for 7 different time points; 30 min, 1 hr, 2 hr, 6 hr, 12 hr, 24 hr, 48 hr. The control employed was serum starved untreated (0hr) group of cultured TM cells. Similarly, to determine time dependent ID1 and ID3 protein expression, we treated the confluent serum starved cells with BMP4 for 6 different time points; 1 hr, 2 hr, 6 hr, 12 hr, 24 hr, 48 hr and untreated serum starved cells were used as a baseline control.

The cells were processed with Isol-RNA lysis reagent for mRNA isolation, and quantitative Real Time QPCR (qRT-PCR) was performed to determine the changes in gene expression, using GAPDH as the internal control. Another set of treated cells were lysed using MPER buffer for isolation of proteins. The ID1and ID3 protein expression was studied by SDS-PAGE followed by western immunoblotting. β-Actin was used as a loading control. The BMP induced nuclear translocation of ID1 and ID3 proteins in TM cells was carried out by immunocytochemistry. DAPI was used as a nuclear stain.

Specific Aim 1b. Determine whether expression of ID1 and ID3 is BMP signaling pathway dependent

BMP4 signaling occurs through binding of BMPRI and BMPRII and activation of smad or non-smad signaling pathways. To determine the role of BMP signaling in induction of ID1 and ID3, we treated primary TM cells with the potent BMP receptor inhibitor LDN-193189 (Stemgent): a selective BMPRI inhibitor. We performed this experiment in three cultured primary human TM cell strains. The confluent serum starved cells were

pretreated with LDN-193189 (10nM and 100nM) for 6 hrs followed by BMP4 (10ng/ml) treatment for 12 hr. Two different controls were included in this study.

Following are the treatment groups for primary human TM cell cultures:

1. Negative control: No treatment.
2. Positive control: BMP4 (10ng/ml)
3. LDN-193189 (10nM) + BMP4 (10ng/ml)
4. LDN-193189 (100nM) + BMP4 (10ng/ml)

Protein was isolated from cultured TM cells after treatment using MPER buffer. ID1 and ID3 expression was analyzed by SDS-PAGE followed by western immunoblotting.

Specific Aim 1c. To determine whether ID1 and ID3 inhibit TGFβ2 induced FN and PAI-1 expression

TGFβ2 is known to increase FN and PAI-1 expression in TM tissue and cultured human TM cells. To determine the effect of ID1 and ID3 on TGFβ2 induced FN and PAI-1 in human TM cells, GTM3 cells were transfected with the plasmid expression vectors pCMV-hID1 and pCMV-hID3. To determine the efficiency of ID1 and ID3 plasmid transfection, we compared the expression of ID1 and ID3 between test and control groups. The test group consisted of cultured GTM3 cells transfected with either an ID1 or ID3 plasmid. As a control, we transfected GTM3 cells with empty plasmid vector (pCDNA). Following transfection, the cells were serum starved for 24 hrs. The serum starved cells

were then treated with or without TGFβ2 for 48 hr. Protein was isolated 48 post treatment, and ID1, ID3, FN, and PAI-1 expression was analyzed by western immunoblotting.

Experimental groups:

Experiment Group no	Controls/ Test group	Plasmid transfection in GTM3 cells	TGFβ2 (5ng/ml) Treatment
1	Baseline control	pCDNA	—
2	Negative control	pCDNA	+
3	Positive control	pCMV-ID1	—
4	Positive control	pCMV-ID3	—
5	Test group	pCMV-ID1	+
6	Test group	pCMV-ID3	+

Aim 2. To determine the roles of ID1 and ID3 in lowering of IOP and increasing aqueous outflow facility in the TGFβ2 induced hypertension mouse model .

Specific Aim 2a. To determine whether ID1 and ID3 lower elevated IOP in TGFβ2 induced hypertension mouse model.

Shepard and colleagues have previously shown that adenoviral vectors bearing bioactivated TGFβ2 (Ad5-CMV-hTGFβ2$^{C226S/C288S}$) significantly elevates IOP along with increased ECM expression and decreased AH outflow facility in rodent eyes. We

determined whether ID1 and ID3 lowers TGFβ2 induced ocular hypertension in mice. We anesthetized mice using isoflurane (2-2.5 %) in presence of oxygen, and performed intravitreal injections of either Ad5-CMV-hID1 (AdID1) or Ad5-CMV-hID3 (AdId3) vectors (Vector Biolab) along with Ad5-CMV-hTGFβ2$^{C226S/C288S}$ (Ad.TGFβ2) and Ad5 empty (Ad null) vector. For our preliminary study, we injected vector Ad.TGFβ2 intravitreally and measured conscious IOP to observe physiological differences between the control group (Ad null) and AdTGFβ2 injected groups.

To determine the IOP lowering effects of ID1 and ID3 expression on TGFβ2-induced ocular hypertension, we divided the mice into six groups. Each mouse received intravitreal injection of viral vector in the left eye. Right eyes served as uninjected controls. Total injected volume was 2-3µl, and the titer of each vector was approximately 5 x 10^{-7} pfu/ml.

The six groups of mice that received intravitreal injection are as follows:

Injection Day	Day -2	Day 0	Number of mice
Group of animals	First injection received	Second injection received	
1	Ad-null-vector	Ad-null-vector	5
2	Ad-null-vector	Ad-TGFβ2$^{226/228}$	5
3	AdID1	Ad-null-vector	5
4	AdID1	Ad-TGFβ2$^{226/228}$	5
5	AdID3	Ad-null-vector	5
6	AdID3	Ad-TGFβ2$^{226/228}$	5

Conscious IOPs were measured 3 days per week for 21 days after which animals were euthanized and ocular tissues were harvested for mRNA and immunohistochemistry.

Specific Aim 2b. To determine whether ID1 and ID3 lower increased AH resistance in the TGFβ2 induced hypertension mice model.

In POAG, experimental evidence indicates impaired AH outflow facility is responsible for IOP elevation. Shepard and colleagues have shown that TGFβ2-induced ocular hypertension in mice is due to decreased AH outflow facility, which correlates with increased PAI-1 mRNA expression in anterior chamber tissues. To determine whether ID1 and ID3 can restore the AH outflow facility to normal levels, we studied the outflow facility in the three groups of animals (2,3,4) listed in specific aim 2a, at the maximum IOP increase. The mice were anesthetized with intraperitoneal (IP) injections of anesthetic (ketamine and xylazine) cocktail. Depth of anesthesia was assessed by toe pinching 30 min after of IP injection. Subsequent 1/4 - 1/2 doses of induction dose were given intramuscularly to maintain surgical plain of anesthesia. We further treated each eye with the eye drop proparacaine–HCl (0.5%) for tropical anesthetic and then inserted the 30gauge steel needle intracamerally. The needle was inserted through the periphery of cornea, 1-2 mm from the limbus and pushed towards the opposing region of anterior chamber angle. The needle was connected to a flow-through pressure transducer (ELPRZ, world precision instruments), while the other end of pressure transducer was connected to 50-µl syringe containing filtered sterile PBS (PBS) loaded into a micro dialysis infusion pump with half stepping capacity. The mice were infused with PBS at

steadily increasing flow rates (0.1,0.2,0.3,0.4 and 0.5 µl/min) and pressure values were recorded at each flow rate. We then calculated the outflow facility. After assessing outflow facility, the eyes were harvested, stained with H&E to assess morphological changes. Sectioned eyes were also used for immunohistochemistry studies.

Significance of the propose study

Elevated IOP is a major risk factor associated with POAG. Lowering IOP using drugs or surgery is the only current line of treatment available which shows clinical efficiency, this slows disease progression for limited period, but eventually combinatorial therapies are required to maintain lower IOP. Along with multiple therapies, but increased side effects of multiple therapy and poor patient compliance tend to lead to the failure of the treatment.[117] Therefore, there is need for developing disease-modifying therapies. This study is aimed at exploring the roles of ID1 and ID3, the down-stream targets of BMP4, in preventing or reversing TGFβ2 induction of ECM components in the TM which lead to elevated IOP.

Figure and Figure legend

Figure 1a.

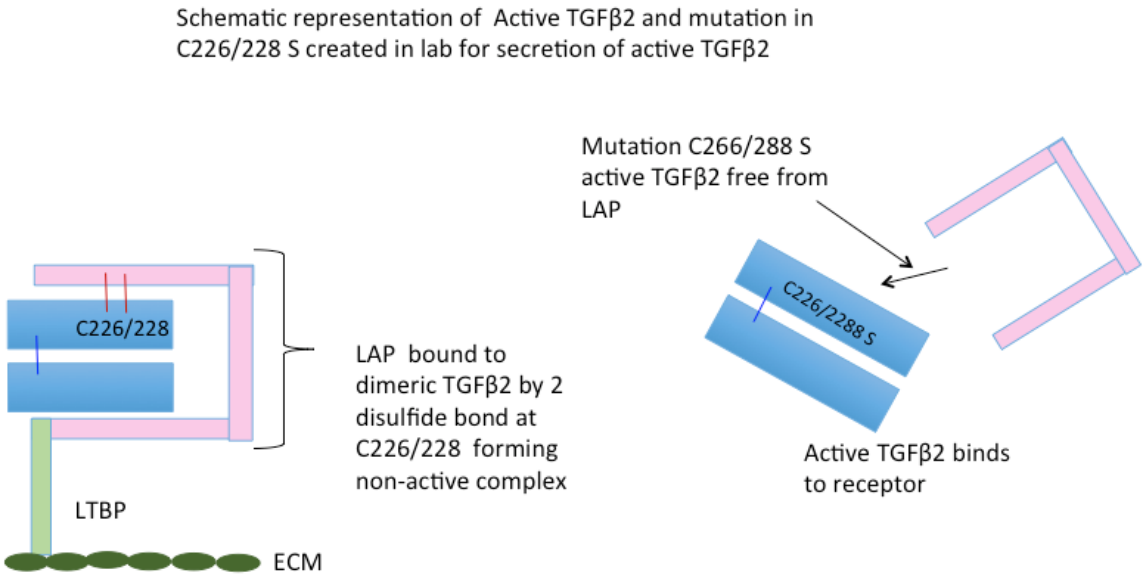

Figure 1a. Secreted Latent Binding Protein LAP attached to TGFβ forms an inactive complex. Mutation at C226S/C228S block the disulfide bond formation and secrets active TGFβ2

Figure 1b

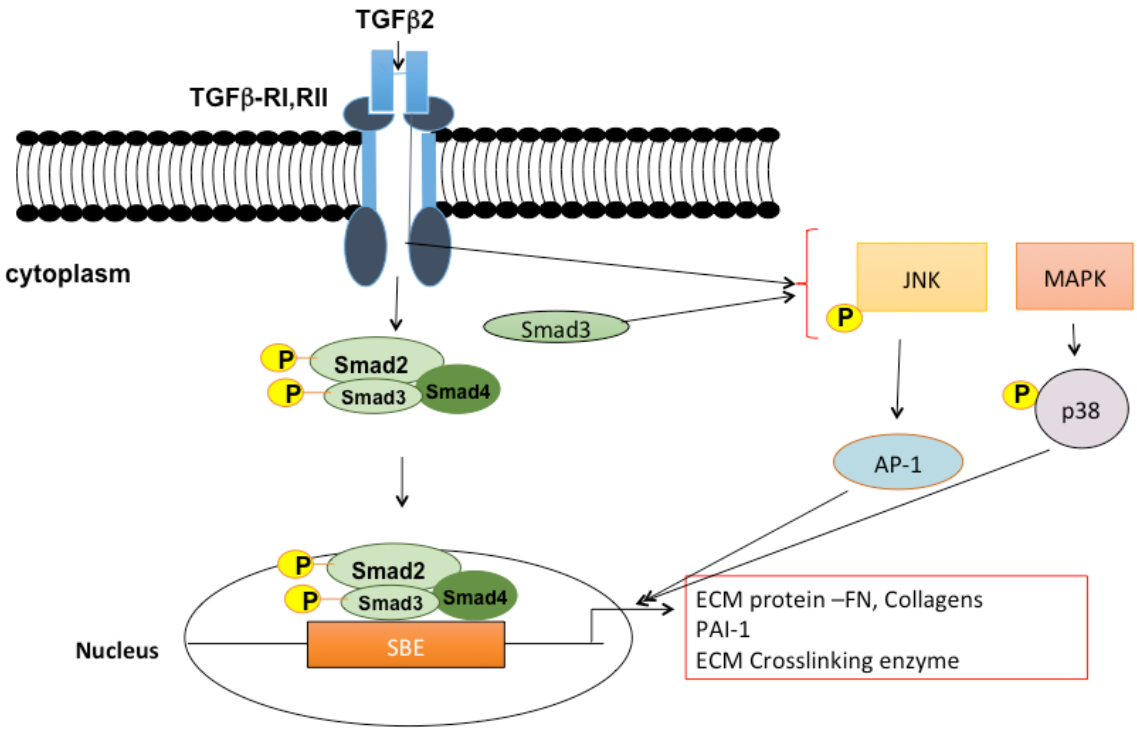

Figure 1b- TGFβ2 signals through canonical and non-canonical pathways, increasing ECM and ECM modifying component in TM.

Figure 2

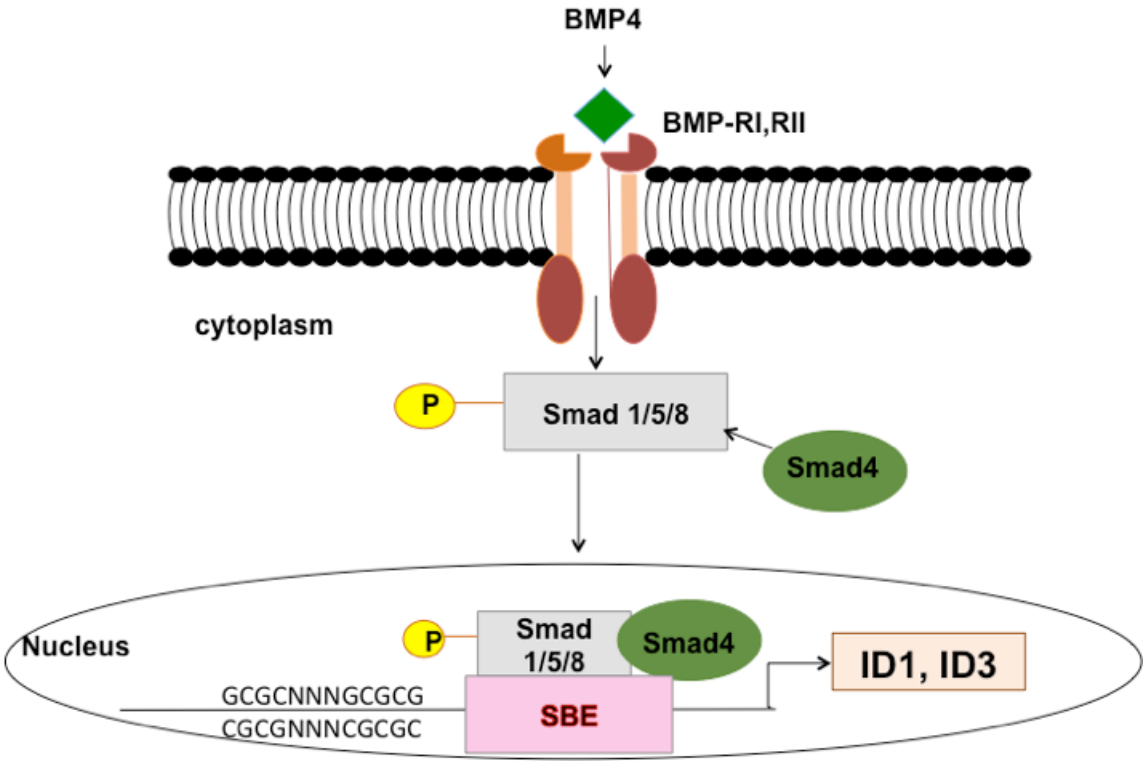

Figure 2. BMP4 upregulates ID1 and ID3 expression by activating the canonical pathway. The phosphorylated Smad1/5/8 complex binds to the Smad binding element to increase ID expression.

Figure 3

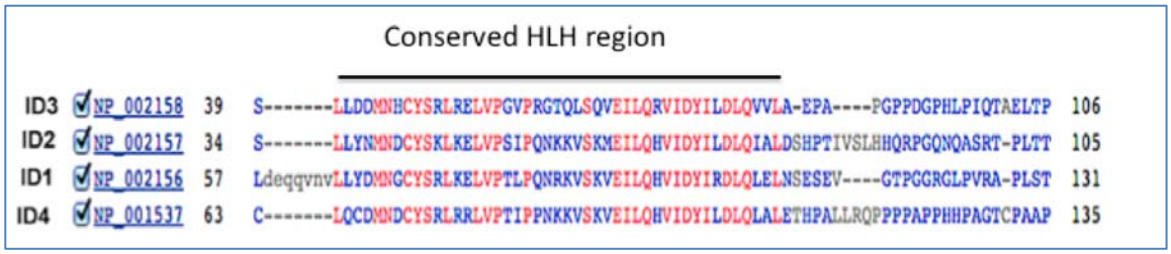

Figure 3. Protein sequence alignment demonstrates the conserved HLH region of ID1-4.

The figure demonstrate the conserved HLH domain in four ID proteins with some variation in amino acid sequence in the loop region. This variation in sequence and difference in N- and C- terminal determines unique function of each ID protein.

Figure 4

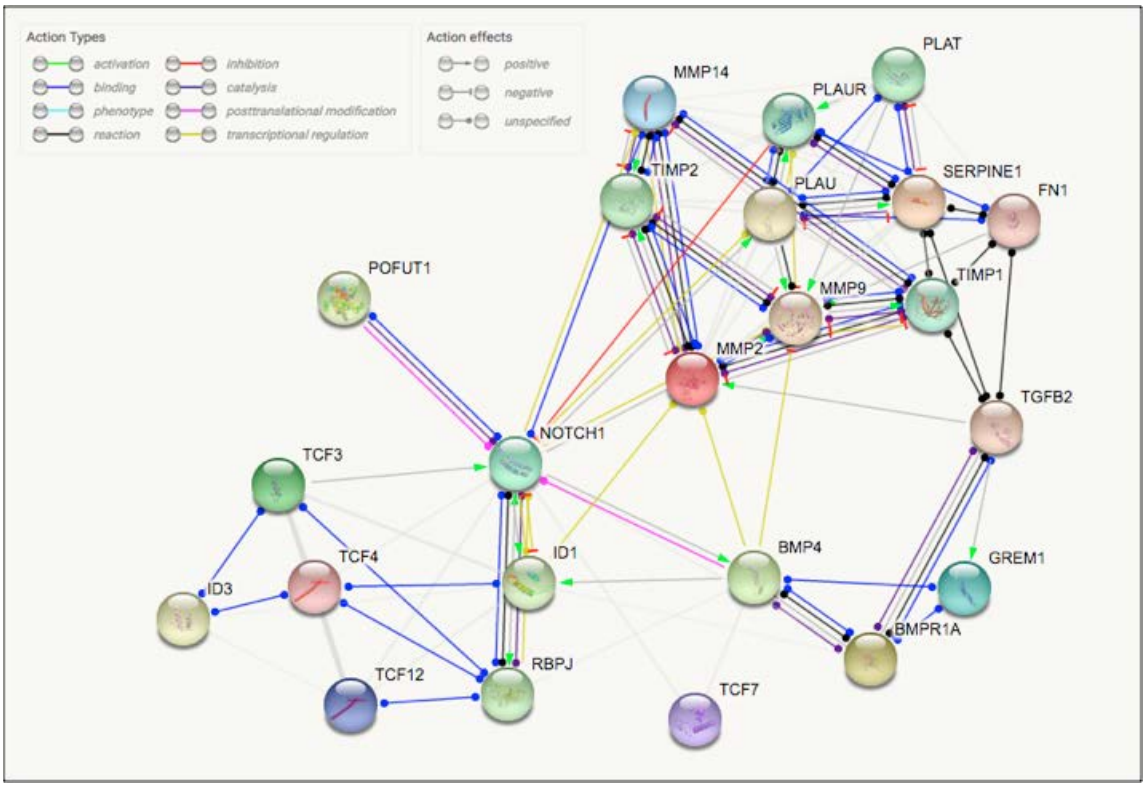

Figure 4. String diagram generated using string software and database.

The above figure was generated using string software, which considers available information about protein-protein interaction, protein expression and association. The inset in above corner of diagram suggests different associations by which these proteins may be related. Further ID1 and ID3 both are associated with TCF4, while BMP4 induces ID1 expression. Interestingly ID1 regulates NOTCH1 and MMP2 expression. This string data was generated keeping basic settings at 5 or more interaction and molecular interactions, while considering some other extra parameters, would generate additional information about the protein in interest.

Figure 5

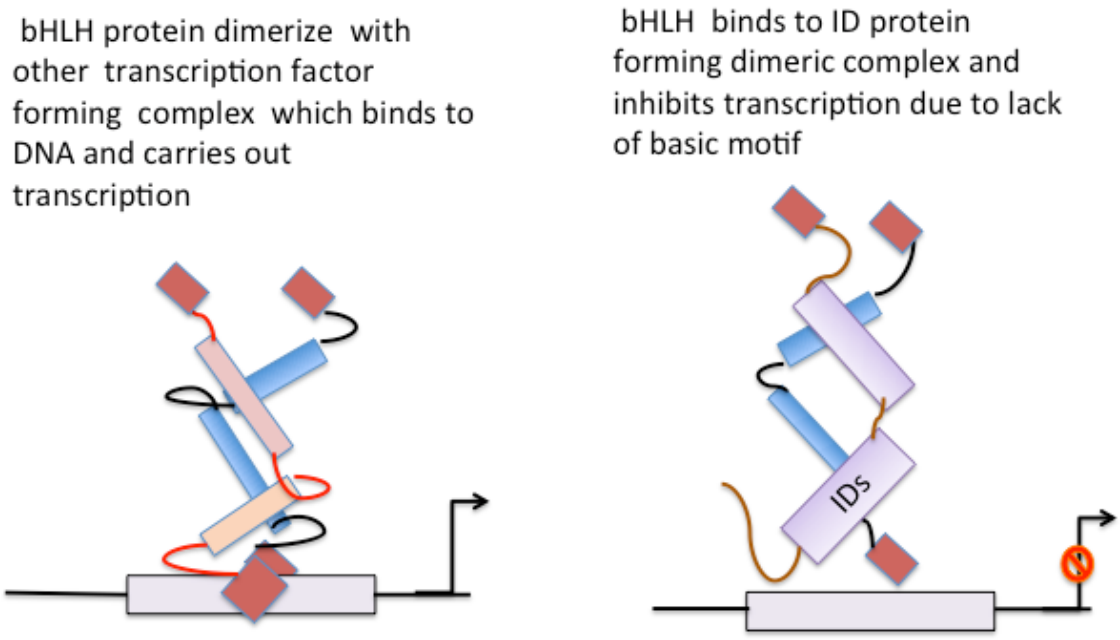

Figure 5 Inhibitor of DNA binding protein (ID) negatively regulates bHLH transcription factors. IDs represses bHLH transcription factor by forming a dimer complex. Since IDs lack a basic DNA binding motif the dimer complex cannot bind to promoter region and represses gene expression regulated by bHLH protein.

Figure 6

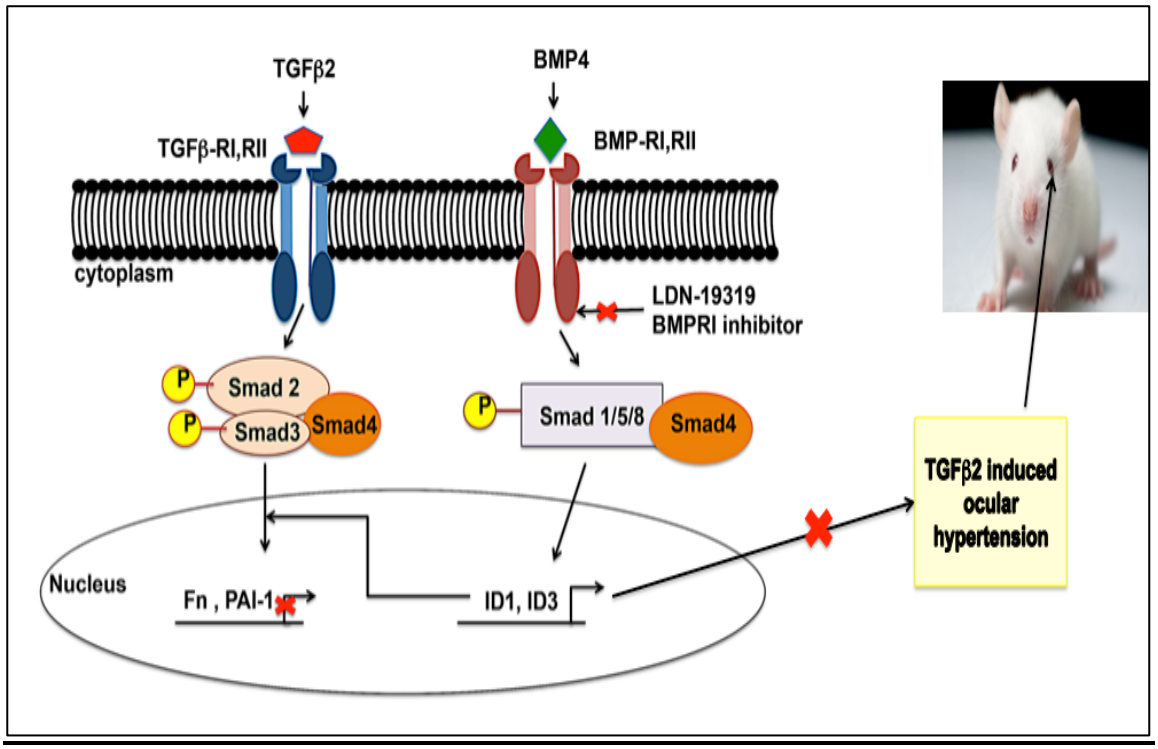

Figure 6: CENTRAL HYPOTHESIS

This diagram depicts our central hypothesis. We seek to explore the inhibitory role of the BMP4 downstream targets ID1 and ID3 on TGFβ2 induced FN and PAI-1 expression, ocular hypertension and AH outflow facility.

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CHAPTER II

ROLE OF ID PROTEINS IN BMP4 INHIBITION OF PROFIBROTIC EFFCTS OF TGFβ2 IN HUMAN TM CELLS

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Abstract

Purpose: Increased expression of transforming growth factor β2 (TGFβ2) in POAG aqueous humor (AH) and trabecular meshwork(TM) causes deposition of extracellular matrix (ECM) in the TM and elevated IOP. Bone morphogenetic proteins (BMPs) regulate TGFβ2 induced ECM production. The underlying mechanism for BMP4 inhibition of TGFβ2 induced fibrosis remains undetermined. BMP4 induces inhibitor of DNA binding proteins (ID1, ID3), which suppress transcription factor activities to regulate gene expression. Our study will determine whether ID1and ID3 proteins are downstream targets of BMP4, which attenuates TGFβ2 induction of ECM proteins in TM cells.

Methods: Primary human TM cells were treated with BMP4, and ID1 and ID3 mRNA and protein expression was determined by Q-PCR and western immunoblotting. Intracellular ID1 and ID3 protein localization was studied by immunocytochemistry. GTM3 cells were transfected with ID1 or ID3 expression vectors to determine their potential inhibitory effects on TGFβ2 induced fibronectin and plasminogen activator inhibitor-I (PAI-1) protein expression.

Results: Basal expression of ID1-3 was detected in primary human TM cells. BMP4 significantly induced early expression of ID1 and ID3 mRNA ($p<0.05$) and protein in primary TM cells, and a BMP receptor inhibitor blocked this induction. Overexpression of ID1 and ID3

significantly inhibited TGFβ2 induced expression of fibronectin and PAI-1 in TM cells ($p<0.01$).

Conclusion: BMP4 induced ID1 and ID3 expression suppresses TGFβ2 profibrotic activity in human TM cells. In the future, targeting specific regulators may control the TGFβ2 profibrotic effects on the TM, leading to disease modifying IOP lowering therapies.

Key Words: TGFβ2, BMP4, ID1, ID3, Fibronectin, TM cells

INTRODUCTION

Glaucoma is a chronic multifactorial neurodegenerative eye disease, affecting 70 million people worldwide[1-3]. An early sign of the glaucoma is loss of peripheral vision, and further disease progression leads to permanent vision loss. The major risk factor associated with primary open-angle glaucoma (POAG) is elevated intraocular pressure (IOP)[4, 5]. Increased resistance to aqueous humor (AH) outflow, due to defective trabecular meshwork (TM) function leads to ocular hypertension[6-9]. Several investigations suggest that disruption of extracellular matrix (ECM) homeostasis and increased deposition of ECM in the TM are responsible for this elevated IOP. In addition, changes in the TM cytoskeleton and deposition of ECM plaque material increase the stiffness of TM tissue, thereby increasing the AH resistance[9-12].

While ECM turnover and remodeling maintains normal IOP, growth factors such as transforming growth factor β isoforms (TGFβ1, TGFβ2) and bone morphogenetic proteins (BMP4, BMP7) play a critical role in maintaining ECM equilibrium[13]. Numerous studies have shown increased TGFβ2 levels, including the active form, in the AH of POAG patients[14-16]. Several in vitro studies in cultured TM cells showed that activation of TGFβ2 canonical pathway elevates expression of collagens, fibronectin, actin stress fibers, thrombospondin-1, lysyl oxidase (Lox), transglutaminase and plasminogen activator inhibitor-1 (PAI-1) [12, 17-20]. TGFβ2 increases ECM deposition in TM tissues and elevates IOP in ex vivo organ culture and in-vivo in rodent eyes[12, 21] . These studies link the increased levels of active TGFβ2 to upregulation of ECM deposition in TM tissue, decreased outflow facility and increased IOP.

BMPs, BMP receptors and BMP antagonists are expressed in TM cells and tissues[22]. Interestingly, BMPs, especially BMP4 and BMP7, antagonize the TGFβ2 effects in TM cells[23, 24]. BMP7 inhibits the fibrotic effects of TGFβ2 in cultured TM cells by inducing I-smad7, an inhibitory smad. Our group has demonstrated that BMP4 blocks TGFβ2 induced ECM production in cultured human TM cells[23]. However, the signaling mechanism for the BMP4 inhibition of TGFβ2 profibrotic effects remains unknown.

BMP4 induces early expression of Inhibitor of DNA binding proteins (IDs) dominant negative of HLH protein in various cell types and regulates cellular functions including angiogenesis, neurogenesis, and embryogenesis [25-28]. IDs play a critical role in regulating tissue specific cell proliferation, differentiation, apoptosis, and fibrotic processes[29]. IDs are distinct from other bHLH transcription factors as they lack basic amino acid DNA binding domain. There are four evolutionarily conserved family members of IDs (ID1, ID2, ID3 and ID4) and highly similar HLH domain sequence which are ubiquitously expressed in mammals[30]. While main difference in ID protein sequence lies outside the HLH domain and the different function due to these difference among ID protein is not completely understood.[31] The HLH domain in IDs heterodimerize with other transcription factors (especially bHLH group proteins) forming non-functional transcription complexes and prevents the complex from binding to DNA[31, 32]. Fibrotic pulmonary, dermal and corneal diseases studies have shown antifibrotic effects of BMPs via ID proteins suggesting ID's role as antifibrotic regulator.[33-35] In variety of cells, ID1 and ID3 downregulate extracellular components induced by TGFβ including fibronectin (FN), plasminogen activator inhibitor (PAI-1), collagen, and thrombospondin-1[34, 36-38].Hence

elucidating the downstream pathway of BMP4 in the TM will give us a better understanding and insight into potential disease modifying IOP therapies.

We therefore hypothesizes that downstream targets of BMP4,ID1 and ID3 will attenuate the pathogenic effects of TGFβ-2 in cultured TM cells. In this study, we demonstrate basal level expression of IDs (ID1-ID3) in primary human TM cells. We further show expression and localization of ID1 and ID3 proteins after BMP4 treatment. We also demonstrate the expression of ID1 and ID3 in primary TM cells is BMP pathway dependent and the crucial role ID1 and ID3 in blocking TGFβ2 induced ECM protein expression in cultured TM cells.

METHODS AND MATERIALS

Cell Culture:

Human primary TM cells were isolated and characterized from dissected explants of human TM tissues as previously described[12, 39]. Donor eyes were obtained from the Lions Eye Institute for Tissue and Research (Tampa, Florida) and were managed according to Helsinki research guidelines for human tissues. The pre-characterized primary human TM cells and the stable transformed cell line GTM3 were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Gibco BRL Life Technologies), L-glutamine (0.292 mg/ml; Gibco BRL Life Technologies, Grand Island, NY) and penicillin (100U/ml)/streptomycin (0.1 mg/ml)(Gibco BRL Life Technologies).[40-42] The pre-characterized primary human cell strains used are listed in (Table 1)

TM Cell Treatment:

Primary TM cells were grown to 100 % confluency. The cells were then serum starved for 24 hrs prior to growth factor or inhibitor treatment. GTM3 cells were treated with two different doses of BMP4 (5ng/ml and 10ng/ml; R&D system; Minneapolis, MN) for 12 hr to study does dependent mRNA expression of ID1 and ID3. Primary human TM cells were treated with BMP4 (10ng/ml) for 0-48 hr. Cell lysates were collected to study ID1 and ID3 mRNA and protein expression. Primary TM cells were treated with BMPRI inhibitor LDN-193189 (10nM and 100nM, Stemgent; Cambridge, MA) for 6 hr followed by BMP4 (10ng/ml) 12 hr treatment to determine BMP dependent expression of ID1 and ID3. To test transfection efficiency, ID1 and ID3 expression was studied 48 hr after transfection. To test the effect of ID1 and ID3 on

TGFβ2 induced expression of FN and PAI-1, the transfected GTM3 cells were serum starved and treated with TGFβ2 (5ng/ml) for 48hr. The experiments in primary human TM cells were repeated at least once in the same cell strain and each cell strain was considered as n=1. While, in GTM3 cells the experiment were performed in technical replicate (i.e. each treatment/well) of 3, n=3 and while each experiment was performed several time before reporting.

Transfection with Expression Plasmids:

Plasmid expression vectors for human ID1variant1 (pCMV6-XL5-ID1(SC125462), ID3 (pCMV6-AC-ID3(SC319486) and control (pCDNA3.1) were purchased from Origene (Rockville, MD). TM cell transfection was performed as described in the Origene Protocol for transient transfection of plasmid vectors. In brief, transfection reagent Attractene (Qiagen,US.) was used for transfection in serum free medium (Opti-MEM; Invitrogen; Grand Island, NY.). Plasmid vectors mixed with serum free medium were incubated for 10 min. Then plasmid and transfecting reagent were combined and incubated for 20 min at RT. GTM3 cells ($1.5X10^5$ per ml) are plated into each well of 12 well plates. Cells were then incubated with transfection reagent for 24 hr, washed with PBS and further incubated in DMEM without serum for the subsequent experiments. GTM3 cells were used for better transfection efficiency with the plasmid vectors.

Reverse Transcription and Quantitative Real Time PCR

Total cellular RNA was extracted from TM cells using Trizol (Invitrogen, CA), and 1μg of RNA was used for cDNA synthesis. Transcription Super mix (iScript Reverse; Bio-Rad Labs Inc., Richmond, CA.) was used for cDNA synthesis. To determine ID mRNA expression,

50ng/µl of cDNA was used for each reaction. The cDNA was amplified using 10µl Sso Advance SYBR Super Mix (Bio-Rad lab, Richmond, CA.) and 100nM primers sets (Table1) for each 20µl of reaction. The RT-PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide to detect DNA bands under UV exposure. Quantitative real-time PCR was performed as described previously[22, 43]. The quantitative real time PCR reaction was performed using the Bio-RadCFX96 Real Time system. Each reaction was repeated in triplicates and cycle thresholds (Ct) were normalized to housekeeping gene GAPDH. GAPDH was selected as housekeeping gene since GAPDH expression showed no significant change in the microarray data obtained from the TM cells treated with BMP4/ TGFβ2. The delta Ct method was used for quantitative analysis. The PCR primers were designed by Prime3 software (Table2) and were validated by sequencing the PCR product and BLASTing the sequence against the human genome.

Protein Extraction and Western Blot analysis:

Total cellular protein was extracted from TM cells using Mammalian Protein Extraction Buffer (Pierce Bio; Thermo Scientific, US), containing a protease and phosphatase inhibitor cocktail (Pierce Bio; Thermo Scientific, US). The Bio-Rad Dc protein assay system (Bio-Rad Lab, Richmond, CA.) was used to determined protein concentrations. The cellular proteins were separated by denaturing 10-15% SDS- PAGE and were electrophoretically transferred to PVDF membranes. Membranes were blocked in 10% fat free dry milk in Tris-buffered saline Tween buffer (TBST) 2 hr at RT. The blocked membranes were incubated overnight with primary antibodies (Table 3) at 4ºC. The blots were washed in TBST and incubated with corresponding

secondary horseshoe peroxidase conjugated antibody diluted (1:10,000) in 5% fat free milk at RT for an hour. The blots were developed using enhanced chemiluminescence (ECL) detection reagent (Pierce Biotechnology; Thermo scientific, US). The protein images were developed and analyzed using Fluor ChemTM8900 imager (Alpha Innotech, San Leandro; CA). The same blots were re-probed with an antibody for the housekeeping protein β-actin to ensure equal loading. β-actin was selected as loading control since no change was observed in β-actin expression in our microarray data from the TM cells post BMP4/ TGFβ2 treatment. For densitometry analysis, the relative density of each protein band was normalized to β-actin. AlphaEaseFC software was used for analyzing image (Alpha Innotech, San Leandro; CA.

Immunocytochemistry:

Primary human TM cells (n=4 different strains) were treated with or without BMP4 (10 ng/mL) for 12 hr as previously published [44]. In brief, coverslips of each primary human TM cell strain were immunolabeled overnight at 4°C with primary antibodies (Table 2). The primary antibody used was selected according to previous publication[26, 45, 46]. No primary antibody was used as a negative control (secondary antibody control). Coverslips were incubated for an hour with secondary antibody donkey anti-rabbit-Alexa Fluor 488 (1:1000; Invitrogen, Carlsbad; CA), diluted in Triton X-100/PBS. Coverslips were mounted using mounting medium containing DAPI (Prolong with DAPI; Invitrogen-Molecular Probes) for nuclear staining. Image acquisition was performed using a Nikon Eclipse Ti inverted fluorescence microscope (Nikon, Inc., Melville, NY) equipped with the Cri Nuance FX imaging System (Perkin-Elmer, Inc., Waltham, MA).

Statistical Analysis:

Results between two groups were compared using paired student's t-test. Comparison between 3 or more groups was performed using one way- ANOVA. Statistical test for each experiment is stated in their respective figure legend. Statistical analysis was performed using Graph Pad Prism 6 software (Graph Pad Prism, Inc. San Diego; CA.). The average value for each group was presented as mean ±S.E.M and $p<0.05$ was considered to be statistically significant.

RESULTS

Endogenous expression of ID mRNA in human TM cells

Transcription factors play a key role in maintaining the cellular homeostasis and regulatory functions. IDs (ID1-ID4) are essential dominant negative, tissue specific transcription regulators. In order to study the presence of basal expression of ID (ID1-ID4), we isolated mRNA from the serum starved normal primary human TM cell cultures (n=3) and performed reverse transcription PCR using ID1-ID4 primers. We were able to detect basal mRNA expression of ID1, ID2 and ID3 in TM cells (Figure 1).

BMP4 treatment induced early expression of ID1 and ID3 mRNA and protein in primary human TM cells

TM cells and tissues are known to express BMPs (BMP2, BMP4, BMP5, BMP7) and BMP receptors [22, 47]. BMP4 binds to BMP-RI/RII and phosphorylates Smad 1/5/8[23]. While it is well

established that ID1 and ID3 are the downstream targets of canonical BMP4 signaling pathway in other tissues, the downstream targets of BMP4 pathway in TM cells remained undetermined. Therefore, we studied the BMP4 induced mRNA of ID1 and ID3 in different primary human TM cell strains. We treated confluent and serum starved GTM3 cells with different doses of recombinant BMP4 (5ng/ml and 10ng/ml). We observed significant mRNA expression of ID1 and ID3 in TM cells (Figure 2A and 2B) induced by of BMP4 (10ng/ml) treatment. Further, we treated primary human TM cell strains with BMP4 (10ng/ml) for 0-48 hr to study mRNA and protein expression. We observed early induction of mRNA expression of both ID1 and ID3 (n=4) at 1 hr with a significant increase in mRNA expression of ID1 at 2, 12 and 24 hr (Figure 2C; $p<0.05$). ID3 mRNA expression significantly increased at 12 and 24 hr of treatment (Figures 2D; $p<0.05$). ID1 protein induction was observed from 2 to 24 hr after BMP4 treatment in primary human TM cells (Figure 3A) in 3 different primary human TM cell strain. Basal levels of ID3 expression was observed in untreated cells, which increased from 1hr to 24 hr post BMP4 treatment (Figure 3C). Our densitometry analysis of ID1 and ID3 protein expression suggest increase of protein expression from basal levels (Figure 3B and 3D), while ID1 expression increase significantly from 2-24hr. Hence, our data show that BMP4 treatment induced both ID1 and ID3 mRNA and protein expression in primary human TM cells.

Exogenous BMP4 increased the nuclear localization of ID1 and ID3 in primary human TM cells

ID1 and ID3 are transcription regulator proteins; therefore, the intracellular localization of ID1 and ID3 plays an important role in determining their function. Previous studies have shown that ID1 and ID3 are localized in both the cytoplasmic and nuclear regions[48]. We used four different

primary human TM cell strains to study ID1 and ID3 protein expression and localization after BMP-4 treatment. Our results show increased ID1 (Figure 4A) expression in cytoplasm and nucleus, while ID3 (Figure 4B) localized mainly in the nucleus after BMP-4 (10ng/ml) treatment, compared to untreated control cells. There was no ID1 and ID3 immunostaining with omission of the primary antibodies (negative control) (Figure 4C and 4D). The detailed expression of ID1 and ID3 is demonstrated in our 40 X images (Supplementary Figure 2).

Effect of BMPRI inhibitor on BMP4 regulation of ID1 and ID3 in primary human TM cells

Having shown that BMP4 induces expression of ID1 and ID3 in TM cells, we wanted to confirm that the expression of IDs (ID1 and ID3) is dependent on BMP signaling. LDN-193189 is BMPRI inhibitor that prevents phosphorylation of Smads1/5/8 and therefore inhibits the BMP canonical signaling pathway[49, 50]. We pretreated the three different serum-starved primary human TM cell strains with LDN-193189 for 6 hrs and then added recombinant BMP4 (10ng/ml) for an additional 12 hr. Protein lysates were isolated and analyzed by western blotting. We observed inhibition of ID1 and ID3 protein expression at 100nM and partial inhibition of IDs (ID1/ID3) at 10nM concentration of LDN-193189 (Figures 5A and 5C). Further the densitometry analysis demonstrated reduction of ID1 and ID3 protein expression after the LDN-193189 (100nM) treatment (Figure 5B and 5D). While ID3 expression significantly decreased ($p<0.05$) post LDN-193189 and BMP4 treatment when compared to BMP4 treatment alone. This study indicates that expression of ID1 and ID3 are regulated by BMP signaling in TM cells.

ID1 and ID3 overexpression in TM cells attenuates TGFβ2 effects

TGFβ2 induces fibronectin and PAI-1 expression along with other ECM components in primary TM cells, which can be blocked by the canonical BMP pathway [12, 23, 51-53]. To determine whether overexpression of ID1 and ID3 in TM cells attenuates the TGFβ2 effects in TM cells, we transfected GTM3 cells with ID1 and ID3 expression vectors (Figure 6A and 7A) the densitometry analysis demonstrate significant increase in ID1 and ID3 protein expression (Figure 6B and 7B) in ID1 and ID3 transfected cells. Cells were then treated with TGFβ2, and expression of fibronectin (FN) and PAI-1 was studied by western blot analysis (Figures 6A and 7A). The FN and PAI-1 expression was normalized to β-actin loading control. Overexpression of ID1 and ID3 significantly inhibited the TGFβ2 induction of FN and PAI-1 expression (Figures 6C-D and 7C-D; $p<0.01$). The results demonstrate that ID1 and ID3 significantly blocks TGFβ2 induced FN and PAI-1 expression in TM cells.

Discussion:

In POAG, elevated IOP remains the major risk factor for the development and progression of the disease. The ocular hypertension caused by a decreased AH outflow facility is the result of molecular and morphological changes in the TM. TGFβ plays a critical role in maintaining the homeostasis of various tissues of the anterior segment, including regulating ECM turnover. Increased amounts of profibrotic TGFβ2 in the AH and TM of POAG patients is believed to contribute in increased production and crosslinking of ECM in TM cells leading to the rise in IOP. Interestingly, BMP4 and BMP7 have been shown to block the TGFβ2 induction of ECM

and ECM regulatory proteins such as fibronectin, PAI-1, and thrombospondin-1 in TM cells. Heterozygous BMP4 deficient mice have severe anterior chamber deformities and elevated IOP, suggesting important role of BMP4 in developing the AH outflow pathway in mice[54, 55]. TM cells and tissues express BMPs (BMP2, BMP4, BMP5, BMP7) mRNA and protein as well as BMP receptors (BMPIa, BMPIb and BMPII) suggesting BMPs are essential for maintaining the homeostasis of TM tissue[22]. Fuchshofer and colleagues demonstrated that BMP7 blocked the TGFβ2 effects in TM cells by upregulation of inhibitory Smad7[24]. However, the blocking mechanism of BMP4 on profibrotic TGFβ2 signaling in TM cells remained unknown. In our study we have shown that ID1 and ID3 are key downstream regulators of BMP4 signaling pathway and expression of ID1 and ID3 proteins are BMP pathway dependent, inhibiting TGFβ2 induced fibronectin and PAI-1 expression in human TM cells. These further suggest that BMP4 regulated inhibition of TGFβ2 induced FN and PAI-1 may be mediated by ID1 and ID3 (Figure 9).

IDs (ID1-ID4) are dominant negative transcriptional regulators expressed in different tissues including lungs, kidney, cardiovascular tissue, reproductive organs and neuronal tissues [56-58]. IDs play critical roles in early embryonic development and demonstrate overlapping function during cell cycle progression.[31, 32, 59] However IDs promote cell proliferation and regulate cell differentiation depending on cell type and cellular function. For example, IDs inhibit cell differentiation in neural progenitor cells to maintain a neural cell population, while promoting natural killer (NK) cell differentiation [58, 60]. Hence, expression and function of IDs are cell and tissue specific. Knock down of both ID1 and ID3 allele is lethal for mouse embryos during development due to impaired angiogenesis and neurogenesis [25, 61]. During development the

expression of ID1, ID2 and ID3 are predominant in neural crest and neural cells[31, 58]. While ID4 expression has distinctively selective functions in neuronal proliferation and differentiation. [30, 62, 63]. Recent reports suggest that IDs play important roles in retinal development, bipolar cell lineage commitment and differentiation and fibrotic corneal disease [35, 64, 65]. However very little was known about ID expression and their role in human TM cells. We for first time demonstrate basal expression of ID1, ID2 and ID3 in primary TM cells. Our published microarray data of human TM tissues suggest expression of all four IDs (ID1-4)[66]. However, we failed to detect the expression of ID4 in our primary TM cell cultures. In our unpublished data in GTM3 cells the knockdown of ID1 by siRNA upregulated ID3 expression and vice versa. In addition to our observation, several published studies have demonstrated that expression of ID1 and ID3 are highly correlated.[45, 67] Whereas ID1/ID3 heterozygous knockout mice demonstrated suppression of BMP induced bone formation.[68] Additionally, most of the reports suggest ID1 to be involved in regulation of fibrosis by inhibiting FN and PAI-1 in various cells types induced by TGFβ, we therefore selected ID1 and ID3 to investigate their role in BMP4 signaling pathway. Further while we demonstrate the expression of ID2 in primary human TM cell strains and it's role in TM cells should be studied further.

Bone morphogenetic proteins (BMP2, BMP4, BMP5) are known as potent inducers of ID1 and ID3 expression in various other tissues. BMP4 induces expression of IDs in different cells including mesenchymal cells, endothelial cells, and mesengial cells.[63, 69-71] BMP4 binds to BMPRI and RII, which phosphorylates Smads 1/5/8, resulting in activation of the BMP response element on ID genes thereby upregulating their expression [28 27] .In our studies, we report significant upregulation of ID1 and ID3 mRNA expression after BMP4 treatment in the

TM cells. Several groups have reported that BMP induction of ID1 and ID3 mRNA expression is biphasic and cell dependent, which peaks from 30 min to 48 hr depending on the cell type[35, 72, 73]. Similar to these previous findings, our study indicates a rapid induction within 1hr of both ID1 and ID3 mRNA after BMP4 treatment. While ID1 mRNA expression significantly increased at 2, 12 and 24 hr, ID3 mRNA expression was significantly increased at 2 and 24 hr post BMP4 treatment in different primary TM cell cultures. While TGFβ2(5ng/ml) treatment on primary human TM cell strains (n=5) (supplementary Figure 1), exhibited no induction of ID2 and ID3 mRNA expression, ID1 mRNA expression showed high variability among cell strains. Suggesting ID1 and ID3 maybe downstream of BMP4 pathway. Further, we also report significant induction of ID1 protein from 2- 24 hr and ID3 protein induction from 1-48 hr in BMP4 treated primary human TM cells. The basic understanding of ID regulation of gene expression is through repression E-box promoter region, as IDs bind to E-box promoter regulators specially E2A gene products (E12 and E47)[31]. However, advances in ID studies have demonstrated diverse role of ID proteins. Some studies have shown that ID1 may regulate antifibrotic effect independent of E-box promoter regulators [34, 48, 74].Apart from their role of inhibiting and regulating gene transcription, ID1 binds to estrogen receptor β (ERβ) and inhibits the breast cancer cell proliferation.[46] Due to their varied cellular function in different cells type, ID1 and ID3 protein are localized in the nucleus as well as cytoplasm of cells[48, 75]. Our studies demonstrate a similar pattern of expression of ID1 and ID3 proteins in the primary human TM cells. However, post BMP4 treatment, expression of ID1 protein increase in cytoplasm as well as nucleus, while ID3 expression increased in nucleus of primary human TM cells.Therefore these studies suggest BMP4 significantly induces ID1 and ID3 mRNA and protein expression and controls their localization in primary TM cells.

To further confirm our hypothesis that is ID1 and ID3 expression is BMP4 pathway dependent, we pretreated primary human TM cells with BMPRI inhibitor LDN193189. This inhibitor inhibits BMPRI activity and therefor inhibits downstream signaling of BMP4 pathway by preventing phosphorylation of Smad 1/5/8. The complete inhibition of pSmad (1/5/8) at higher concentrations of LDN193189 has been reported[49, 50]. In our study, we demonstrate pretreatment with LDN193189 similarly reduces BMP4 induction of ID1 and ID3($p<0.05$) protein expression. However we did not observe statistically significant reduction of ID1 protein expression compared to BMP4 treated positive control due to the variation in primary human TM cell strain unlike we observed significant increase of ID1 protein expression by BMP4 earlier (Figure 3A and 3B). This study may suggest that expression of ID1 and ID3 in TM cells are BMP pathway dependent.

TGFβ2 increases the expression of various ECM components such as fibronectin, collagen IV, and thrombospondin-1 thereby increasing ECM levels in TM. TGFβ2 also increases expression of the ECM crosslinking enzymes LOX, LOXL1-4, TGM2 and BMP1 as well as inhibitors of ECM degradation by increasing expression of PAI-1 and TIMP. This decreases ECM degradation and increases outflow resistance in TM. [18, 52, 76-78] TGFβ2 in human and porcine anterior segment perfusion organ cultures and in rodent eyes increases IOP and decreases outflow facility as a result of increased in ECM deposition[12, 79, 80]. TGFβ2-induced PAI-1 expression in TM cells inhibits the activation of proteolytic system (MMPs) resulting in reduction of ECM turnover[12]. Treating TM cells with recombinant PAI-1 also increases fibronectin expression[12]. Similarly, increased expression and secretion of fibronectin (isoform

ED-A) leads to formation of insoluble extracellular fibrils[18, 44]. These increased fibronectin extracellular fibrils binds integrins, which increase actin stress fiber formation and may lead to IOP elevation[81]. Interestingly, we demonstrate IDs (ID1/ID3) can significantly downregulate the expression of FN and PAI-1 induced by TGFβ2 in human TM cells. Further studies in dermal fibroblast demonstrate ID1 to inhibit TGFβ induced fibrosis through reducing the expression of phosphorylated smad2 and smad3.[37] While ID1 is also known to bind to caveolin-1, may mediate internalization of TGFβRI receptor, as seen in alveolar epithelial cells.[82] Further the mechanism by which ID1 and ID3 inhibit the Fn and PAI-1 in TM cells remains under investigation.

In summary, this is the first report to demonstrate expression of ID proteins and their inhibitory effects on TGFβ2-induced FN and PAI-1 expression in primary TM cells. Our study shows basal expression of ID1, ID2 and ID3 in primary human TM cells. Primary human TM cells treated with BMP4 significantly induce ID1 and ID3 mRNA expression. Furthermore, BMP4 induces early expression of ID1 and ID3 mRNA and proteins through 1 hr-24 hr. ID1 and ID3 expression in primary human TM cells are BMP pathway dependent. Expression of ID1 and ID3 can suppress the TGFβ2 effects in TM cells. Therefore, this study suggests that BMP4 blocking of TGFβ2 induced FN and PAI-1 appears to be mediated by ID1 and ID3. This study also suggests that these novel regulators can inhibit the profibrotic TGFβ2 signaling pathway and may therapeutically prevent disease progression in the glaucomatous TM.

Figure Legends

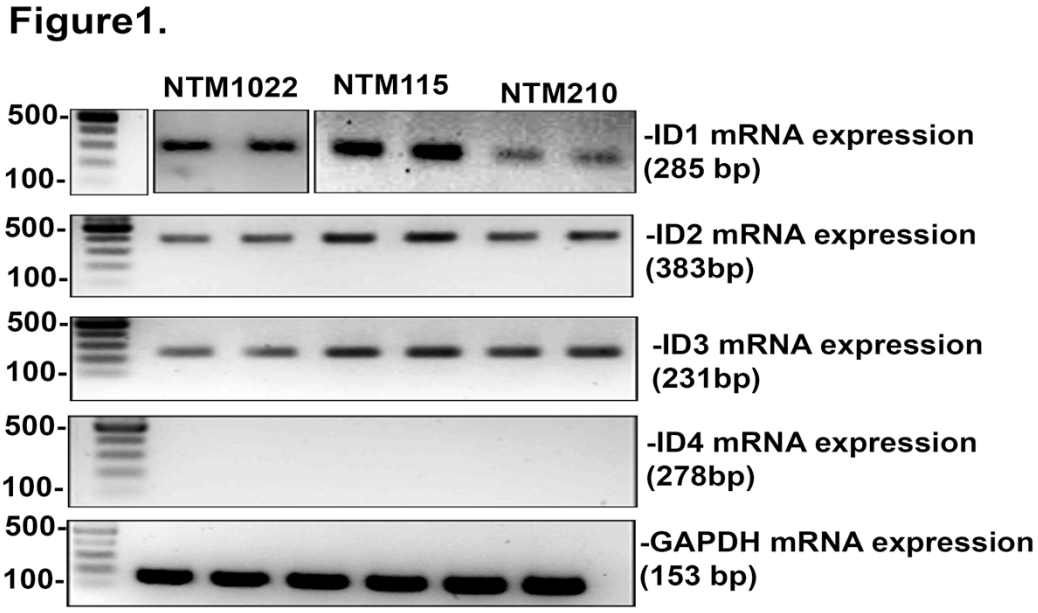


Figure 1. Expression of ID1-4 mRNA in primary human TM cells

RT-PCR was conducted using total RNA extracted from three primary human TM cell strains and the PCR primers listed in Table 1. The amplified products of ID1, ID2 and ID3 were detected in agarose DNA gels at their expected product size. GAPDH was used as internal control for every PCR reaction.

Figure 2

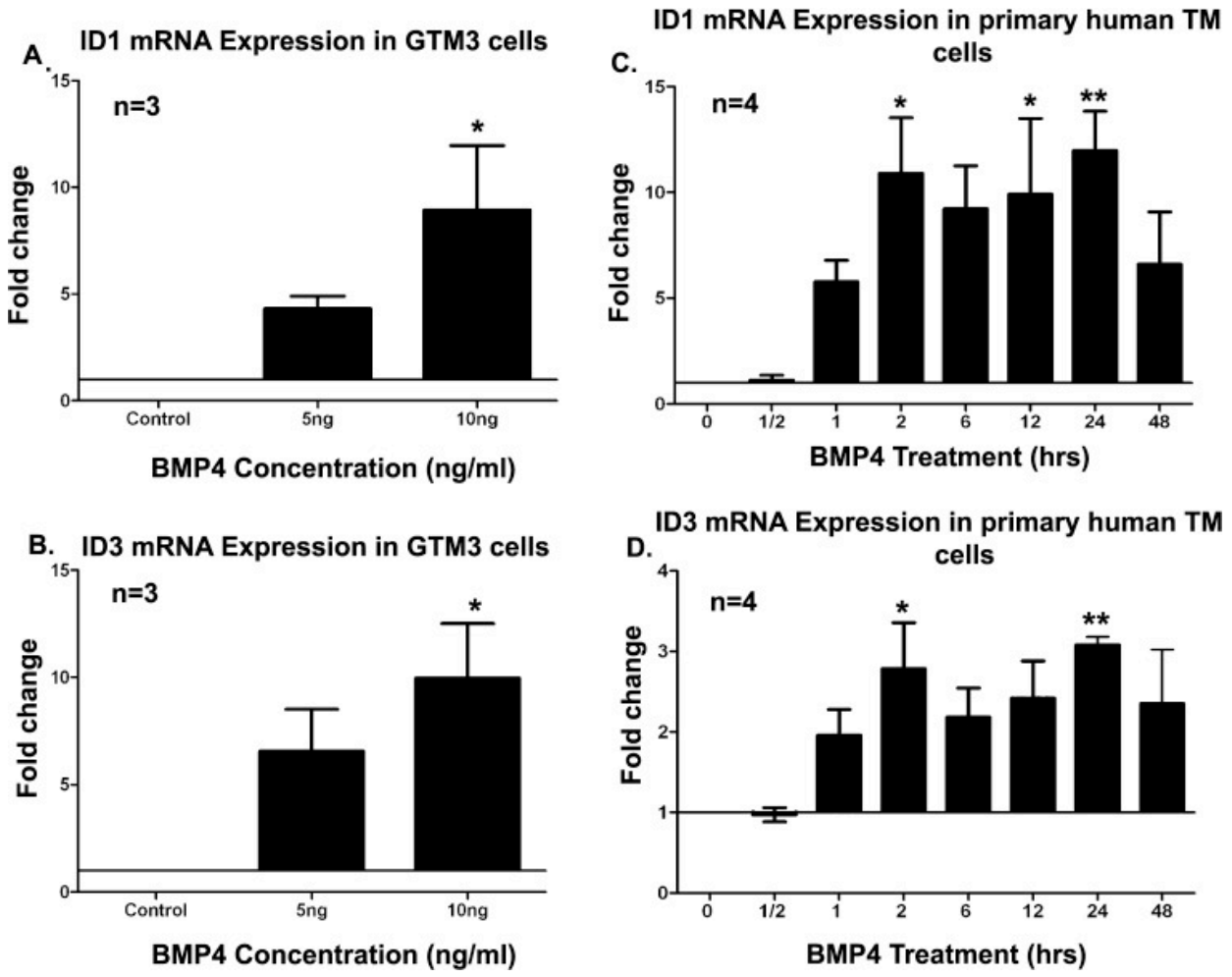


Figure 2. BMP4 dose and time dependent induction of ID1 and ID3 mRNA expression in human TM cell

GTM3 cells were treated with two different doses of BMP4 (5 and 10 ng/ml) for 12hr, and gene expression of ID1 (A) and ID3 (B) was quantified using Q-PCR. Significant induction of ID1 and ID3 was observed after 10ng/ml of BMP4 treatment. Following BMP4 (10ng/ml) treatment from 0-48hr, early induction of ID1(C) mRNA expression was observed within 1hour with significant fold changes observed at 2, 12 and 24 hr in primary TM cells (n=4). Significant ID3 mRNA expression (D) was observed at 2 and 24 hr in primary TM cell strains (n=4). Mean ± SEM, *P<0.05, ** P <0.004 when compared by one way ANOVA with the Dunnett test. BMP4

induced ID1 and ID3 gene expression were normalized to GAPDH and control group expression (0hr, no treatment).

Figure 3.

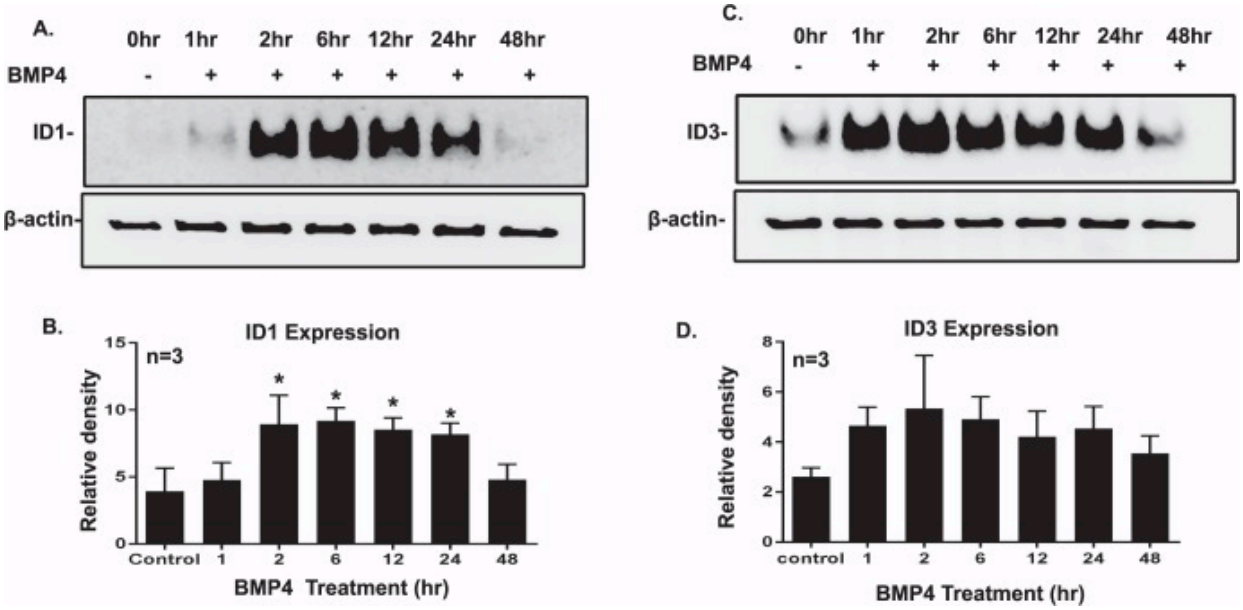


Figure 3. BMP4 induced time dependent expression of ID1 and ID3 proteins in TM cell

Primary human TM cell strains (n=3) were treated with BMP4 (10ng/ml) for 0- 48 hr. Protein expression was detected by western immunobloting. The image shown represents data for time dependent induction of ID1 (A) and ID3 (C) protein expression. ID1 protein expression was detected from 2-24 hr of BMP4 treatment. Basal expression of ID3 was seen at 0hr (control), while early induction in ID3 expression after BMP4 treatment was detected from 1 hr-48 hr. β-actin was used as a loading control. The densitometry analysis of ID1 (B) and ID3(D) protein expression. Mean ± SEM, *$p<0.05$ when compared by one way ANOVA with Dunnett Test.

Figure 4.

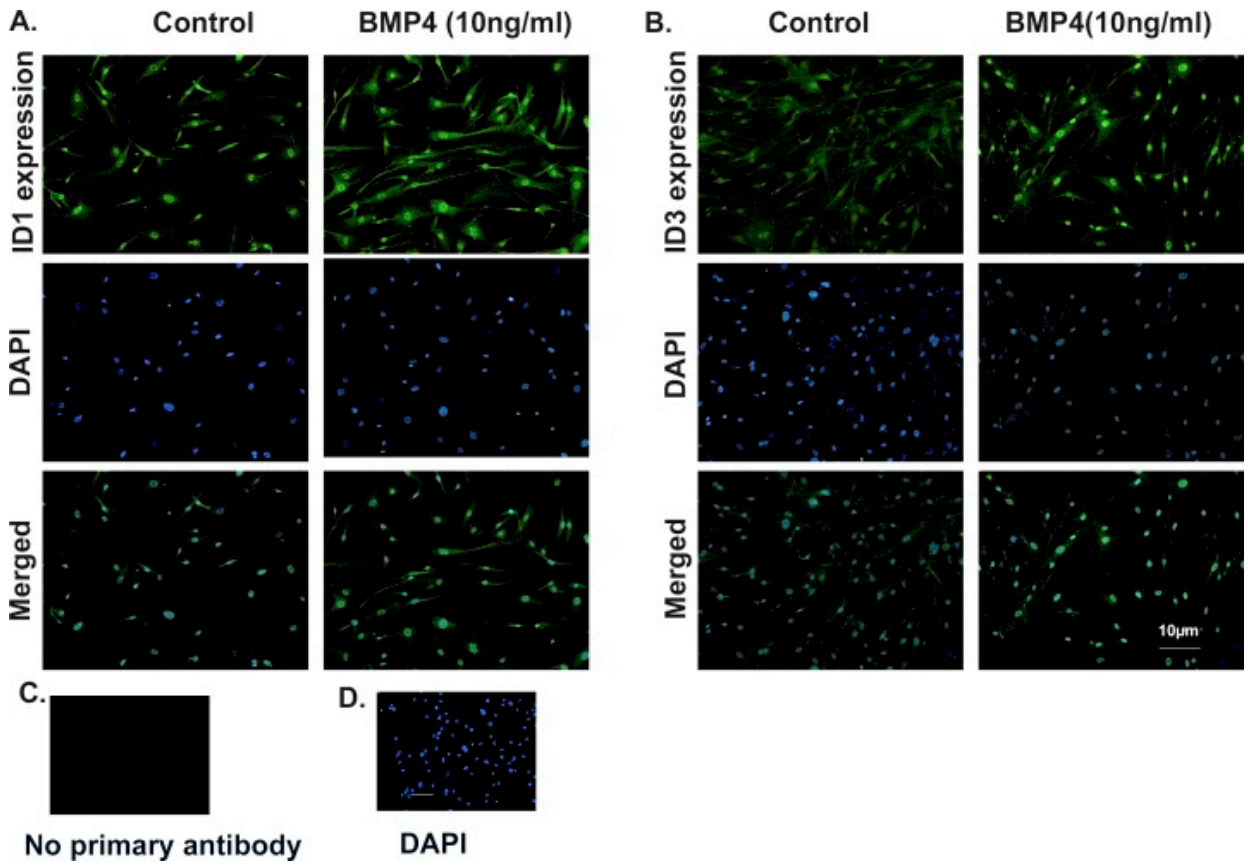


Figure 4. ID1 and ID3 expression is increased in the cytoplasm and nucleus after BMP4 treatment in primary human TM cells

Primary human TM cell strains (n=3) were treated with BMP4 (10ng/ml) for 12hr. Expression of ID1 (A)(20X) and ID3 (B)(20X) protein was compared between control and BMP4 treatment groups by immunocytochemistry. The slides were co-stained with DAPI to differentiate nuclei in TM cells. Following BMP4 treatment, ID1 cytoplasmic and nuclear expression increased in the TM cells, while ID3 expression and localization increased in nuclei in the TM cells. Primary antibody was omitted as a negative control.

Figure 5.

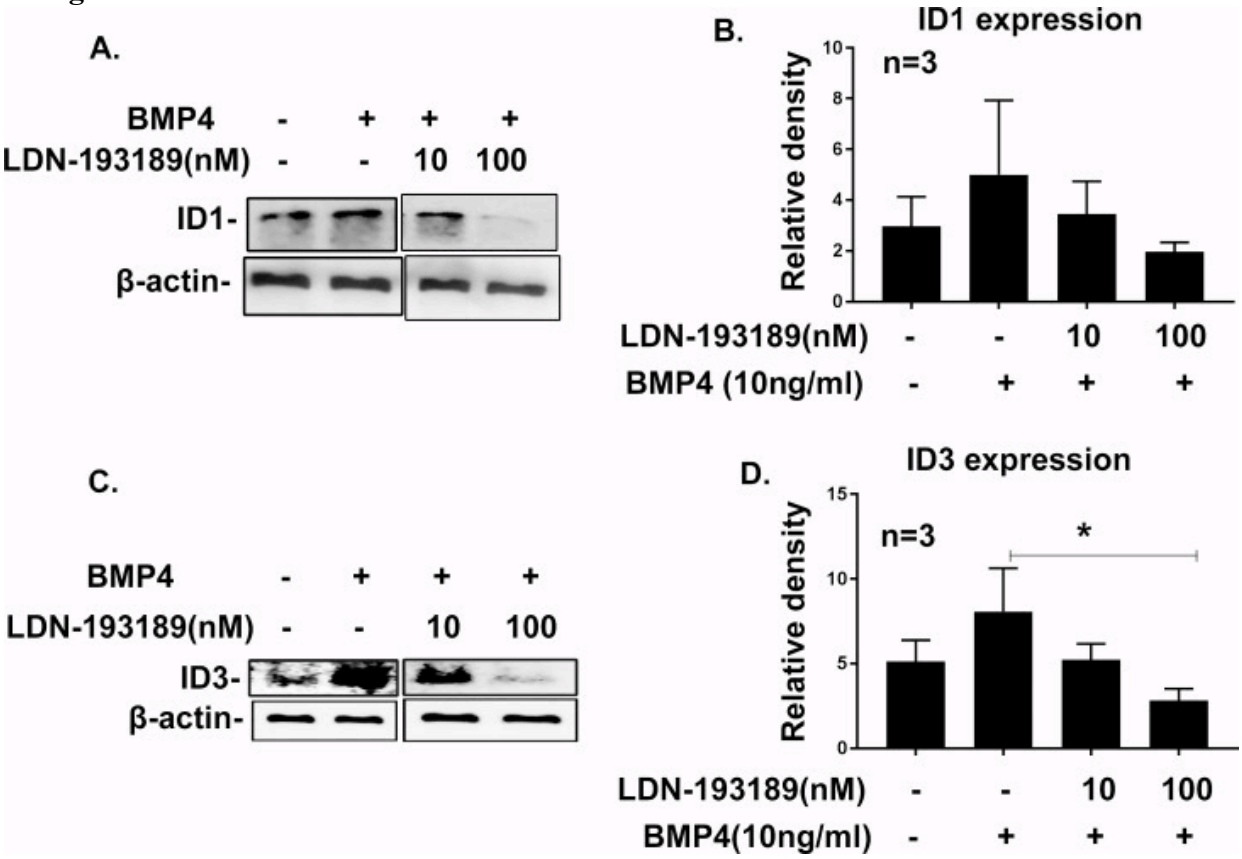


Figure 5. BMPRI inhibitor LDN-193189 blocks BMP4 induced ID1 and ID3 expression in TM cells

Primary TM cell strains (n=3) were treated with BMP4 with or without BMPRI inhibitor LDN-193189. The inhibitor blocked ID1 and ID3 expression, confirming ID1 and ID3 are downstream targets of BMP4 signaling pathway in TM cells. Representative western immunoblots for expression of ID1 (A) and ID3 (C) are shown. β-actin was used as a loading control. The densitometry analysis of ID1 (B) and ID3 (D) protein expression was normalized with corresponding β-actin expression. Expression of ID3 was significantly reduces post LDN-193189 treatment. Mean ± SEM (n=3), *$p<0.05$ determine using one-way ANOVA with Dunnett Test.

Figure 6.

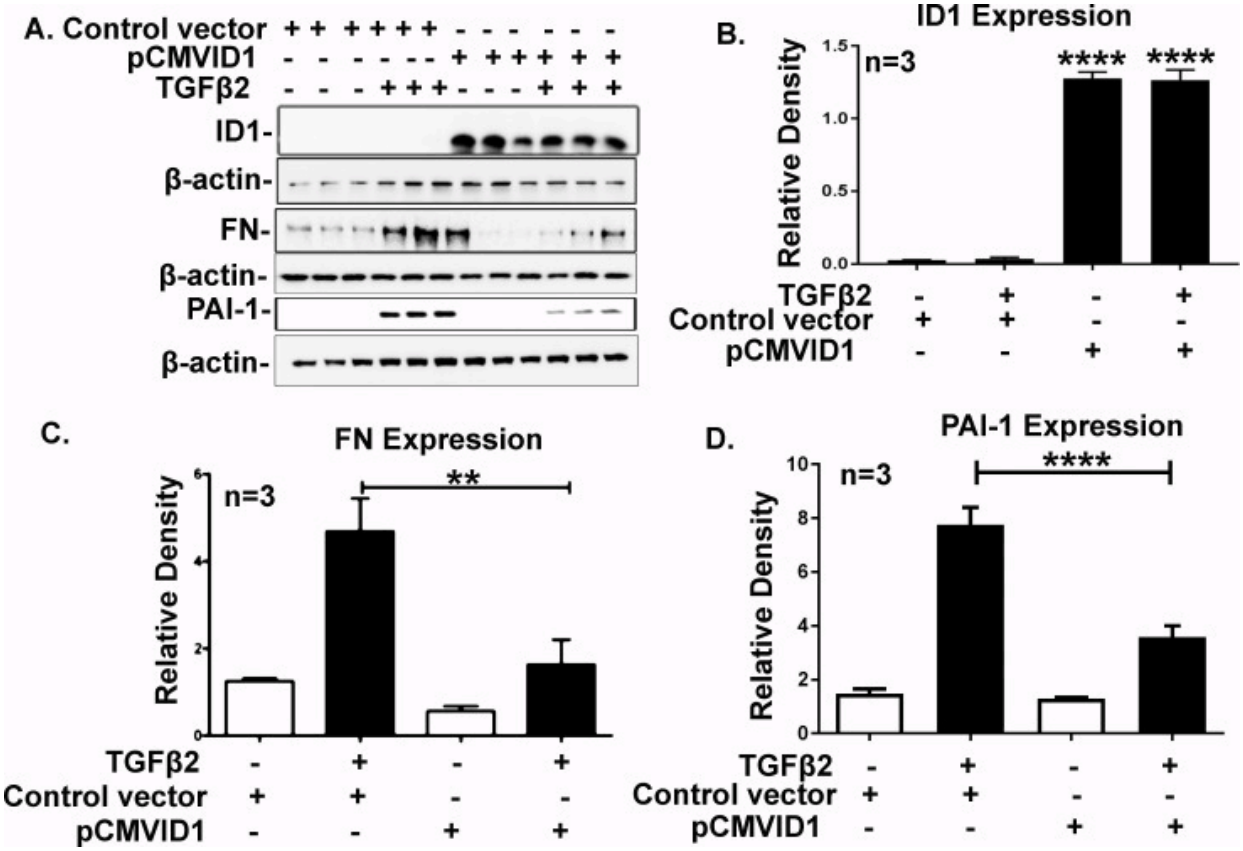


Figure 6. Overexpression of ID1 inhibits TGFβ2 induction of fibronectin and PAI-1 expression.

GTM3 cells transfected with the ID1 expression vector inhibited TGFβ2 (5ng/ml) induced FN and PAI-1 expression. Western immunoblots of TM cell lysate for ID1, FN and PAI-1 expression, after transfection of the ID1 plasmid and TGFβ2 treatment for 48 hr (A). Densitometric analysis of ID1(B), FN (C) and PAI-1 (D) expression. Increased ID1 expression significantly reduced TGFβ2 induction of FN and PAI-1 expression. Mean ± SEM,(n=3) **** $p<0.0001$, ** $p<0.01$ determined using one-way ANOVA with Tukeys test. β-actin was used as loading and normalizing control.

Figure 7.

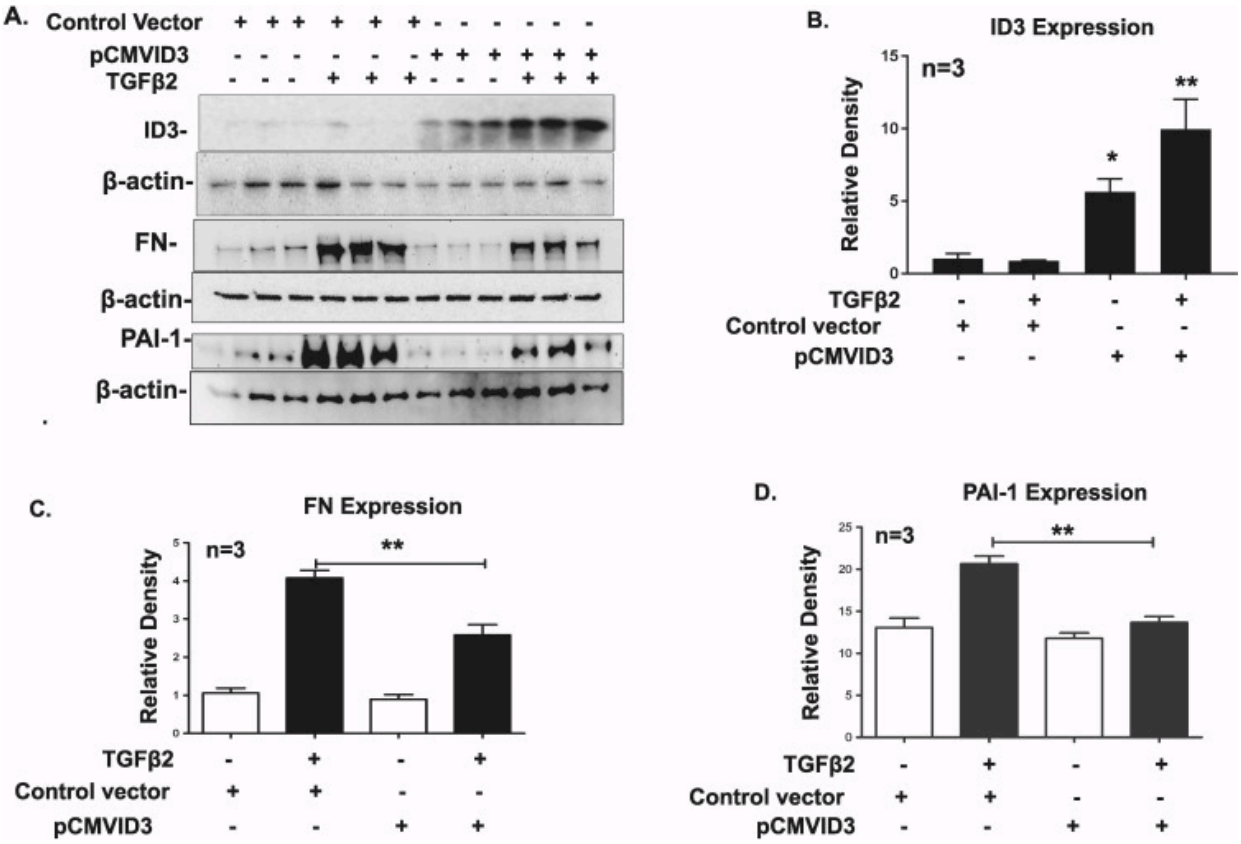


Figure 7. Overexpression of ID3 also inhibits TGFβ2 induction of fibronectin and PAI-1 expression. GTM3 cells were transfected with the ID3 expression vector and treated with TGFβ2 (5ng/ml) for 48 hr. ID3, FN and PAI-1 expression was observed by western immunoblotting (A). Densitometric analysis of ID3 (B), FN (C) PAI-1 (D) expression was normalized with it β-actin expression. Overexpression of ID3 significantly lowered FN and PAI-1 expression induced by TGFβ2. Mean ± SEM, (n=3), *$p<0.04$, **$p<0.01$ determined using one-way ANOVA with Tukeys test.

Figure 8

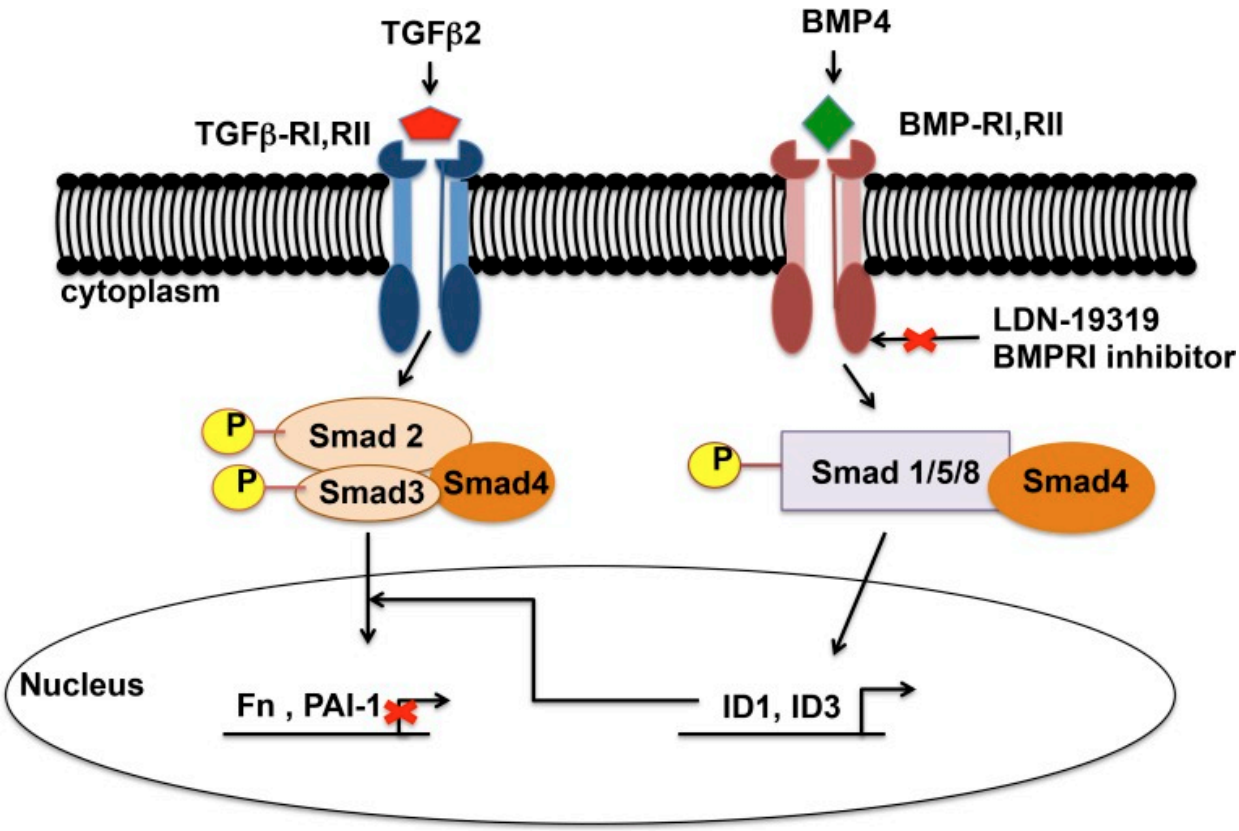
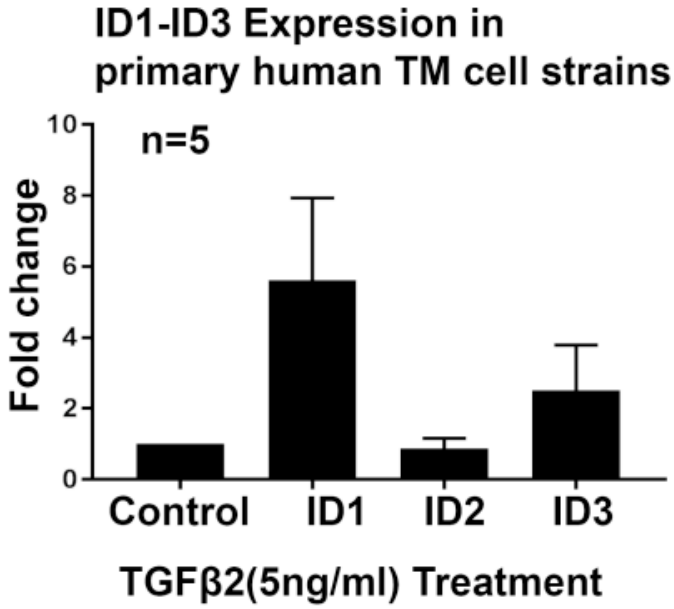
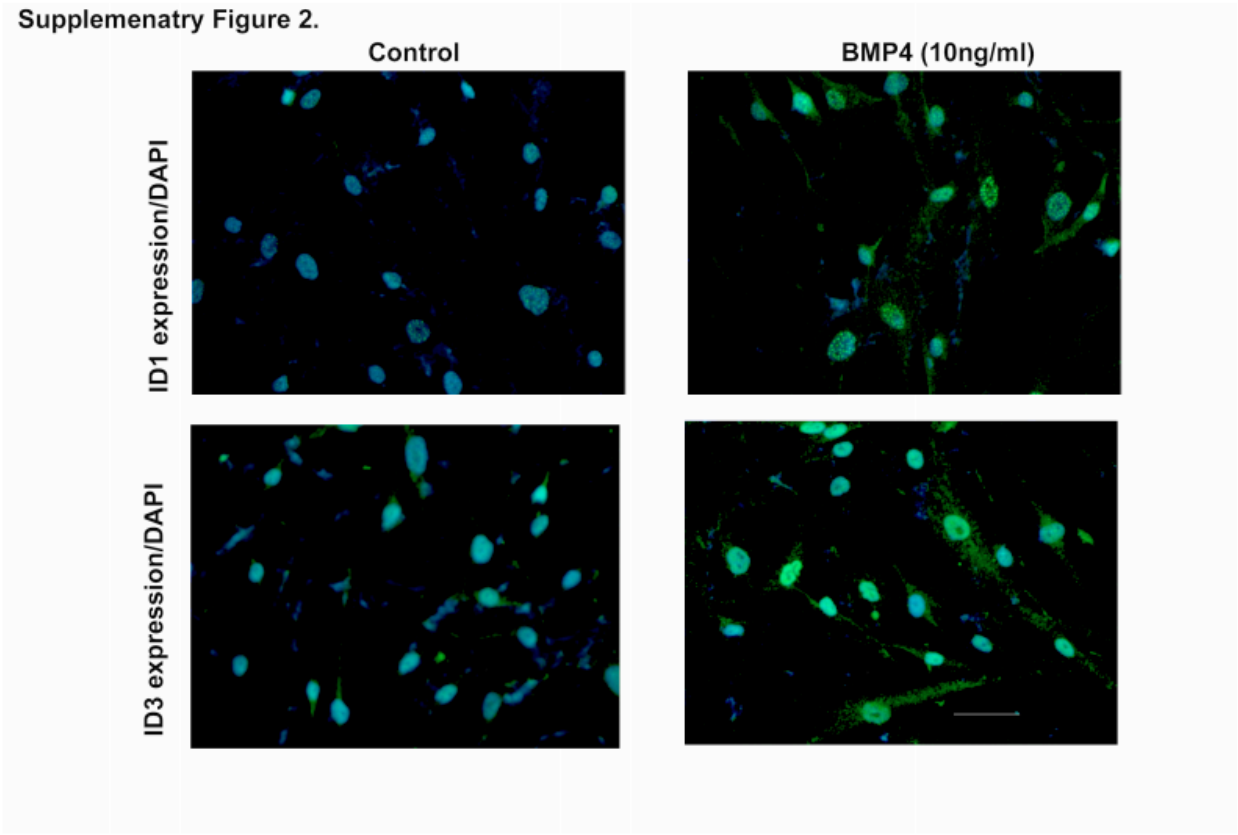


Figure 8. Schematic representation demonstrating BMP4 induced ID1 and ID3 inhibit TGFβ2 mediated FN and PAI-1 expression in TM cells. TM cells treated with TGFβ2 increase expression of ECM components such as FN and PAI-1. Active TGFβ2 binds to TGFβ-RI/RII to activate canonical signaling which upregulates FN and PAI-1 expression in TM cells. BMP4 binds to BMP-RI/RII and activates Smad signaling resulting in increased ID1 and ID3 expression. Increased ID1 and ID3 proteins block the TGFβ2 induced FN and PAI-1 expression. Further, treatment of TM cells with BMPRI inhibitor LDN-193189 abolish BMP4 induced ID1 and ID3 expression, suggesting that BMP4 inhibition of TGFβ2 induced expression of FN and PAI-1 may be mediated by ID1 and ID3.

Supplementary Figure 1



Supplementary Figure 1, TGFβ2 induce expression of ID1,ID2 and ID3 proteins in TM cell . Five human primary TM cells were treated with TGFβ2 (5ng/ml) and mRNA was isolated 6 hr of post TGFβ2 treatment. No significant expression of ID1, ID2 and ID3 was observed. There was no fold change in expression of ID2 and ID3 while ID1 although showed increase in expression but high variability was observed among the cell lines.



Supplementary Figure 2, ID1 and ID3 expression increased in nucleus and cytoplasm post BMP4 (10ng/ml) treatment (12hr). Higher magnified 40X images suggest increase in nucleus staining along with cytoplasm when compared to the untreated primary human TM cells.

TABLES

Table 1 : Primary cells used in experiments.

No.	Primary Human TM Cell Strains	Donors Age, yr
1	NTM210-05	0–1
2	NTM115	72
3	NTM176-04	72
4	NTM 1022-02	67
5	NTM340-07	80
6	NTM3681-14	65
7	NTM895-03	65

Table 2: Primer sequence generated using Prime3 software

Gene	Primer Sequence 5'–3'	Product Size	TM
ID1 (Total ID1)	FP: AAACGTGCTGCTCTACGACA RP: GCTTCAGCGACACAAGATGC	285bp	60
ID2	FP: CGTGAGGTCCGTTAGGAAAA RP: CACACAGTGCTTTGCTGTCA	383bp	60
ID3	FP: GCTGGACGACATGAACCAC RP: AAGCTCCTTTTGTCGTTGGA	231bp	60
ID4	FP: TGCAGTGCGATATGAACGAC RP: GTCGCCCTGCTTGTTCAC	278bp	60
GAPDH	FP: GGTGAAGGTCGGAGTCAAC RP: CCATGGGTGGAATCATATTG	153bp	60

Table 3: List of Antibodies used

Antibody	Sources	Dilution
Rabbit anti-ID1	Biocheck; BCH2/5-3	1:2500; WB
Rabbit anti-ID3	Biocheck; BCH4/6-1	1:1000; WB
Rabbit anti-ID1	Santa Cruz Biotechnology; C20; sc 488	1:500; ICC
Rabbit anti-ID3	Santa Cruz Biotechnology; C20; sc 490	1:500; ICC
Rabbit anti-fibronectin	Millipore; 14C10	1:1000; WB
Goat anti-PAI-1	Santa Cruz Biotechnology; C20; sc 661	1:500; WB
Mouse anti-βactin	Millipore; 1501	1:1000; WB

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CHAPTER III

ID1 AND ID3 BLOCK TGFβ2-INDUCED OCULAR HYPERTENSION AND DECREASED AQUEOUS HUMOR OUTFLOW FACILITY IN MICE

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To be submitted.

Abstract

Purpose: Primary open angle glaucoma (POAG) is the most prevalent form of glaucoma. The major risk factor associated with POAG is increased intraocular pressure (IOP). Elevated transforming growth factor β2 (TGFβ2) expression in the trabecular meshwork (TM) increases the deposition of extracellular matrix (ECM) and prevents ECM turnover by increasing expression of plasminogen activator inhibitor-I (PAI-1) leading to increased IOP. In many fibrotic diseases, the transcription regulators inhibitor of DNA binding proteins (ID1, ID3) are known to inhibit TGFβ induced ECM production. Our study examines the potential inhibitory effects of ID1 and ID3 proteins to attenuate the TGFβ2-induced ocular hypertension and decreased outflow facility in mice.

Methods: IOP and AH outflow facility changes were studied in female BALB/cJ mice. Ad5.CMV vectors encoding and ID1 and ID3 were injected intravitrealy along with Ad5.CMV.TGFβ2$^{C226S/C288S}$ were injected intravitrealy. Ad5.CMV.TGFβ2$^{C226S/C288S}$ was injected along with Ad5.null vector as a positive control, while Ad5.null injected mice were included as a negative control. IOP was measured using Tonolab impact tonometry, and outflow facility was measure by constant flow infusion method.

Results: Overexpression of ID1 and ID3 in mouse eyes significantly ($p<0.0001$) blocked TGFβ2-induced ocular hypertension and prevented TGFβ2 mediated decreases in AH outflow facility

Conclusions: ID1 and ID3 suppressed the TGFβ2 elevated IOP and decreased outflow facility. This renders ID1 and/or ID3 potentially strong candidates for developing disease-modifying IOP lowering therapies.

INTRODUCTION

Glaucoma is heterogeneous group of optic neuropathies, and a leading cause of irreversible vision loss affecting 64.3-76 million people worldwide.[1-3] The major risk factor associated with primary open angle glaucoma (POAG) is elevated intraocular pressure (IOP).[4, 5] In glaucoma patients, chronic IOP elevation causes progressive retinal ganglion cell death, leading to visual field loss. Lowering IOP reduces the risk of developing glaucoma as well as disease progression.[3] The elevation in IOP is due to disruption in the conventional aqueous humor (AH) outflow pathway and trabecular meshwork (TM) homeostasis, leading to increased in outflow resistance.[6] Various studies have shown the Schlemm's canal (SC) endothelium and the juxtacanalicular region of the TM to be the primary site for AH resistance.[7, 8]

A number of studies have demonstrated increased expression of transforming growth factor beta 2 (TGFβ2) and TGFβ receptors in the AH and TM.[9-16] Elevated TGFβ2 upregulates extracellular matrix protein expression in the TM, including fibronectin, collagen, laminin, and elastin.[17-20] TGFβ2 also induces plasminogen activator inhibitor (PAI-1) expression, which prevents activation of matrix metalloprotease (MMP).[17, 20, 21] Furthermore, TGFβ2 increases ECM crosslinking enzymes such as lysyl oxidases (LOXs), transglutaminase, and bone morphogenetic protein-1 (BMP-1).[22-26] These overall events contribute to increased ECM deposition and decreased ECM turnover in the TM, leading to increased AH resistance and IOP elevation.[17, 27]Further, intravitreal

injection of TGFβ2 increases IOP and AH outflow resistance in rodents, demonstrating the direct relationship between increased TGFβ2 expression and IOP elevation.[27, 28] Intriguingly, BMP (BMP4 and BMP7) antagonize the TGFβ2 effect by decreasing ECM expression in TM. Although BMP7 blocks TGFβ2 via inhibitory smad7 activity, we demonstrate BMP4 inhibits TGFβ2 induced ECM through upregulation of inhibitor of DNA binding proteins ID(ID1 and ID3).[29, 30]

IDs (ID1-4), are transcription regulators, which belong to the superfamily of basic helix loop helix (bHLH) proteins.[31, 32] Each ID is encoded by a different gene on separate chromosomes; however, the HLH domain of ID1-4 is evolutionary conserved across the species.[33, 34] Furthermore, IDs lack the basic domain, which is essential for DNA binding, and therefore negatively regulate E-box transcription factors by forming heterodimeric complexes that suppress transactivation.[35] While ID1- ID3 exhibit some redundancy in their functions, ID4 has a distinct role in neural development and embryogenesis.[36-40] ID1 and ID3 play important roles in cell proliferation and differentiation, angiogenesis, immune cell development and regulation, embryogenesis, neurogenesis, cell division and apoptosis, retina development, as well as circadian rhythm. [41-48] Furthermore, IDs are known to perform important roles in early development of the retina, deciding retinal ganglion cell fate and lens development. Additional reports suggest that ID1-4 are expressed in the cornea and cornea fibroblasts, and the expression is regulated by BMP7 and TGFβ1.[49, 50]. Overall, ID1 and ID3 expression is necessary for healthy eye development. For example the double mutant *Id1*$^{-/-}$ and *Id3*$^{-/-}$ mice exhibit smaller lenses, retinal defects and the development of microphthalmia.[46, 51] Along with the important

roles in development, IDs are also know to negatively regulate fibrosis in pulmonary and corneal fibrotic conditions and suppresses the ECM molecules FN, PAI, thrombospondin-1, and collagen expression during angiogenesis and in human dermal fibroblasts.[50, 52-54]

Previously, we have demonstrated inhibitory effects of ID1 and ID3 in regulating TGFβ2 induced fibronectin and PAI-1 expression in the TM.[29] TGFβ2 and PAI-1 have been shown to elevate IOP in rodents.[27] Additionally, TGFβ2 increases AH outflow resistance by deposition of ECM in the TM in rodents.[27] In this study, we demonstrate intravitreal injection of adenoviral vector expressing active TGFβ2 (Ad5-TGFβ2$^{226/228}$) significantly elevates IOP. Further, we hypothesized that expression of ID1 and ID3 will block the TGFβ2 mediated IOP elevation and prevent the TGFβ2-induced increase in AH outflow resistance.

MATERIALS AND METHODS

Animals

Retired breeder female BALB/cJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) USA. Mice were aged between 40 to 48 weeks old and were maintained on a 12 hr light and dark cycle. All the animal procedures were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic Research and with all the IACUC protocols and regulations at the University of North Texas Health Science

Center. The mice were checked for any ocular abnormalities by ophthalmoscope examination prior to inclusion in studies.

Adenoviral Vectors and Intravitreal Injections

The Ad5.CMV.ID1 (Ad5.ID1) and Ad5.CMV.ID3 (Ad5.ID3) vector stocks prepared in PBS were purchased from Vector Biolabs (Malvern, PA). Mutant TGFβ2 vector Ad5.CMV.TGFβ2$^{226/228}$ (Ad5.TGFβ2) and Ad5.CMV.null vector were purchased from the Viral Vector Core Facility at the University of Iowa. Two intravitreal injections were administered to one eye of each mouse over three days .[55] Intravitreal injections to deliver viral vectors were performed under oxygen (0.8L/min) and 3% isoflurane anesthesia as well as topical ocular 0.5 % proparacaine HCl (Alcaine; Alcon Research, Fort Worth, Tx) for local anesthesia. The animals were divided into 6 groups based on the combinations of injections received (Table 1a). Each group received either Ad5.null, Ad5.ID1 or Ad5.ID3 followed by a second intravitreal injection the second day. The second injections were Ad5.null or Ad5.TGFβ2 vectors. For each injection, 5 x 10^7 pfu of viral vector was injected into left eyes while right eyes were utilized as uninjected control eyes. The intravitreal injection was administered using a Hamilton glass microsyringe fitted with a 33-gauge needle (Hamilton, CO). After injecting 2-3µl, the needle was left in the vitreous cavity for 40sec before swiftly removing the needle. Additionally, buprenex was injected intraperitoneally for managing pain.[27, 56]

Intraocular Pressure And Outflow Facility Measurements

Intraocular pressure was measured in conscious mice as described previously using a rebound tonometer (Tonolab; TiolatOy; Helsinki, Finland).[57] IOPs were measured from -7 days pre viral injection and 21 days post injection three times a week. 21 days post injections, outflow facility measurements were performed as stated previously.[27] In brief, mice were anesthetized using a ketamine/xylazine cocktail (100/10 mg/kg body weight). 20-30 min after administration, a surgical plane of anesthesia was established and maintained during the experiment by administration of an additional one quarter to one half of the original dose as needed. Before intracameral cannulation for outflow facility measurements, 0.5 % proparacaine HCl (Alcaine; Alcon Research, Fort Worth, TX) was applied for local anesthesia.[27, 56] Mice were placed on heating pads, and both eyes of mouse were cannulated with 30 gauge needle attached to tubing connected to an in-line pressure transducer (BLPR; World Precision). The other end was connected to a 50 µl glass microsyringe (Hamilton, CO) filled with filtered PBS that was loaded onto a microdialysis infusion pump (SP101 Syringe Pump; World Precision Instrument) with half stepping capacity. The flow rates were increased by 0.1µl/min every 15 min to a top infusion rate of 0.5µl/min.[58] The stabilized pressure at each flow rate was recorded every 5min. The outflow facilities were calculated as the reciprocal of slopes of respective pressure-flow rate curves.[27] All experiments were conducted in a masked manner.

Statistical analysis

The paired Student's t-test was used to compare two groups (treated v/s. control eyes paried within each animal). Comparison between multiple groups was performed using one way ANOVA. Statistical tests applied for each experiment is stated in their respective figure legends. Graph Pad Prism 6 software (Graph Pad Prism, Inc. San Diego; CA.) was used to perform the statistical analysis. The average value for each group was presented as mean ±S.E.M, and $p<0.05$ was considered to be statistically significant.

RESULTS

Intravitreal injection of Ad5.TGFβ2 elevates IOP in BALB/cJ mice

Several studies have previously demonstrated that intravitreal injection of Ad5.CMV.TGFβ2$^{226/228}$ elevates IOP in various strains of mice, including BALB/cJ.[27, 28, 56] We first tested the efficiency of Ad5.TGFβ2 to elevate IOP in conscious BALB/cJ female mice. Baseline IOPs were measured prior to intravitreal injections of viral vectors. The left eye was either injected with Ad5-TGFβ2$^{226/228}$ or Ad5-null vector, while right eyes served as uninjected controls (Table 1b). We observed significantly ($p<0.0001$) increased IOP from day 5 until 21 days (Figure 1), similar to previously published data.

ID1 and ID3 blocks TGFβ2-induced IOP elevation in BALB/cJ mice

TGFβ2 expression is increased in the TM and AH from POAG eyes. TGFβ2 also increases IOP in rodents and in ex vivo human perfusion cultured anterior segments, which is associated with increased TM ECM deposition.[19, 27, 28, 59-61] We previously showed that ID1 and ID3 block the TGFβ2 induction of FN and PAI-1 in TM cells.[29] In order to determine whether ID1 and ID3 also block TGFβ2-induced IOP elevation, we injected Ad5.ID1 or Ad5.ID3 viral vectors along with Ad5.TGFβ2 while the contralateral eyes remained as uninjected controls. We included viral vector control groups, by injecting Ad5.TGFβ2, Ad5.null and Ad5.ID1 or Ad5.ID3 along with Ad5.null vectors (Table 1b). The Ad5.TGFβ2 injected group of mice demonstrated significantly increased IOP, while mice injected Ad5.null and Ad5.ID1 or Ad5.ID3 along with Ad5.null showed no change from baseline IOP (Figures 2 B and 2 D). In contrast to the Ad5.TGFβ2 group, eyes injected with Ad5.ID1 or Ad5.ID3 viral vectors along with Ad5.TGFβ2 did not develop ocular hypertension and retained baseline IOPs (~13±2 mmHg) (Figures 2A and 2C) similar to the uninjected eyes. These results show that ID1 and ID3 significantly ($p<0.001$) blocked Ad5.TGFβ2- induced IOP. This implies that ID1 and ID3 are important negative regulators of TGFβ2-induced ocular hypertension.

ID1 and ID3 inhibit TGFβ2 mediated increase in aqueous humor outflow resistance

Shepard and colleagues demonstrated that TGFβ2 increased IOP and decreased the AH outflow facility in mouse eyes compared to contralateral uninjected eyes.[27] In addition, perfusion culture of human anterior segments also demonstrated a TGFβ2 mediated reduction in the outflow facility.[59] To study the inhibitory effect of overexpressed ID1 and ID3 on TGFβ2 decreased outflow facility, we used a constant flow perfusion method as described earlier.[58] Twenty-one days post intravitreal injection of Ad5.TGFβ2$^{226/228}$ with or without Ad5.ID1 or Ad5.ID3 viral vectors, outflow facilities were measured. The contralateral uninjected eyes were used as paired controls. Mice receiving Ad5.TGFβ2$^{226/228}$ exhibited a significant decrease in AH outflow facility compared to their contralateral control eyes (Figure 3A). In contrast, mice that received Ad5.ID1 or Ad5.ID3 along with Ad5.CMV.TGFβ2$^{226/228}$ showed no significant change in outflow facility compared to their contralateral eyes (Figure 3B and 3C). Therefore, ID1 and ID3 blocked the TGFβ2 reduction of outflow facility, thereby maintaining normal aqueous humor outflow.

DISCUSSION

Elevated IOP remains a major risk factor associated with the development and progression of glaucoma. Current treatment to alleviate the progression of glaucomatous neurodegeneration is achieved by IOP lowering drug therapy and surgical intervention.[62] However, therapeutic complications, side effects, progressive loss of efficacy and poor patient compliance limit the success of current therapies. In addition, all approved IOP lowering therapies do not address the glaucomatous pathologic damage to the TM, and new disease modifying therapies would directly prevent disease progression. In this study, we explore the potential therapeutic role of transcription regulators ID1 and ID3 in regulating TGFβ2 mediated increased IOP.

A number of investigators have contributed to our current understanding of TGFβ2 effects in TM that lead to IOP elevation and increased AH outflow resistance.[11, 12, 60, 61, 63-65] In the normal eye, TGFβ2 is secreted in small amounts that contributes to normal TM homeostasis and immune privilege by suppressing the immune response in the anterior chamber. However, in POAG TGFβ2 levels are increased in the AH and TM cells and tissues.[10, 12, 13, 15, 66, 67] The elevated TGFβ2 expression increases the ECM proteins fibronectin, collagen I and IV, laminin, tenascin C, versican and elastin.[17-19, 68, 69] Additionally, FN along with specific integrins play important roles in increased deposition of other ECM proteins to form extracellular scaffolds.[70, 71] TGFβ2 also increases PAI-1 expression, which inhibits plasmin activation, thereby inhibiting MMP activation and decreasing ECM turnover.[19, 72-74] Further, increased PAI-1 expression

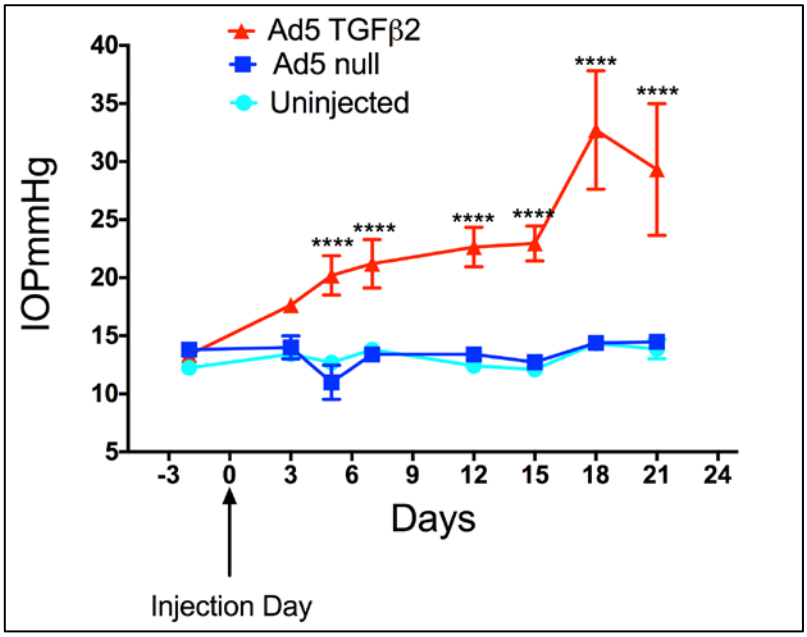
occurs in TGFβ2-induced ocular hypertension in mice.[27] Hence, PAI-1 plays an important role in upregulating IOP by reduction of ECM turnover. Previously, we demonstrated that overexpression ID1 and ID3 inhibits TGFβ2 induced PAI-1 and FN expression.[29] Therefore, TGFβ2-induced ocular hypertension in mice would be an appropriate model to study the effects ID1 and ID3 in regulating TGFβ2 increased IOP.

First, We demonstrate that Ad5.CMV.TGFβ2$^{226/228}$ elevated mouse IOP, which is in agreement with previously published data.[27] Further, we also demonstrated that Ad5.ID1 and Ad5.ID3 blocked the TGFβ2-induced IOP elevation. ID1 is known to regulate uPA expression and MMP2, MMP9 and MMP14 activity.[75-77] In mouse fibroblast cells, ID1 suppresses the fibrotic markers thrombospondin-1, LOX, βV-integrin, inhibin βA and FN.[53] Recent reports suggest that ID3 plays a critical role in regulating corneal fibrosis by blocking collagen I, α-SMA, fibronectin expression.[50] Since ID1 and ID3 regulate fibrotic expression and activation of MMPs, these IDs may regulate ECM turnover, which may result in maintenance of AH outflow homoeostasis.[74] However, Ad5.TGFβ2$^{226/228}$ decreases the outflow facility.[27] Therefore, we examined whether Ad5.ID1 or Ad5.ID3 could inhibit the Ad5.TGFβ2$^{226/228}$ decrease in outflow facility. Ad5.TGFβ2$^{226/228}$ injected eyes demonstrated a significant reduction of outflow facility, Ad5.ID1 or Ad5.ID3 blocked the Ad5-TGFβ2$^{226/228}$ reduced outflow facility showing that ID1 and ID3 play negatively regulate TGFβ2 induced changes in AH outflow resistance.

In summary, we confirm that Ad5.TGFβ2$^{226/228}$ significantly elevates IOP and reduces outflow facility. Ad5.ID1 and Ad5.ID3 blocked Ad5.TGFβ2$^{226/228}$ elevated IOP and

decreased AH outflow facility. These results show that ID1and ID3 are important negative regulators of TGFβ2 elevated IOP and outflow changes and appear to be novel targets for developing disease-modifying therapies in the glaucomatous eye.

Figure 1.



Intravitreal injection of Ad5.TGFβ2$^{226/228}$ elevates IOP in BALB/cJ mice. Mice were injected with Ad5.TGFβ2$^{226/228}$ (n=5) or Ad5.null (n=3) viral vectors in left eyes, while right eyes were uninjected controls. Mean ± SEM, ****P< 0.0001

Figure 2

ly

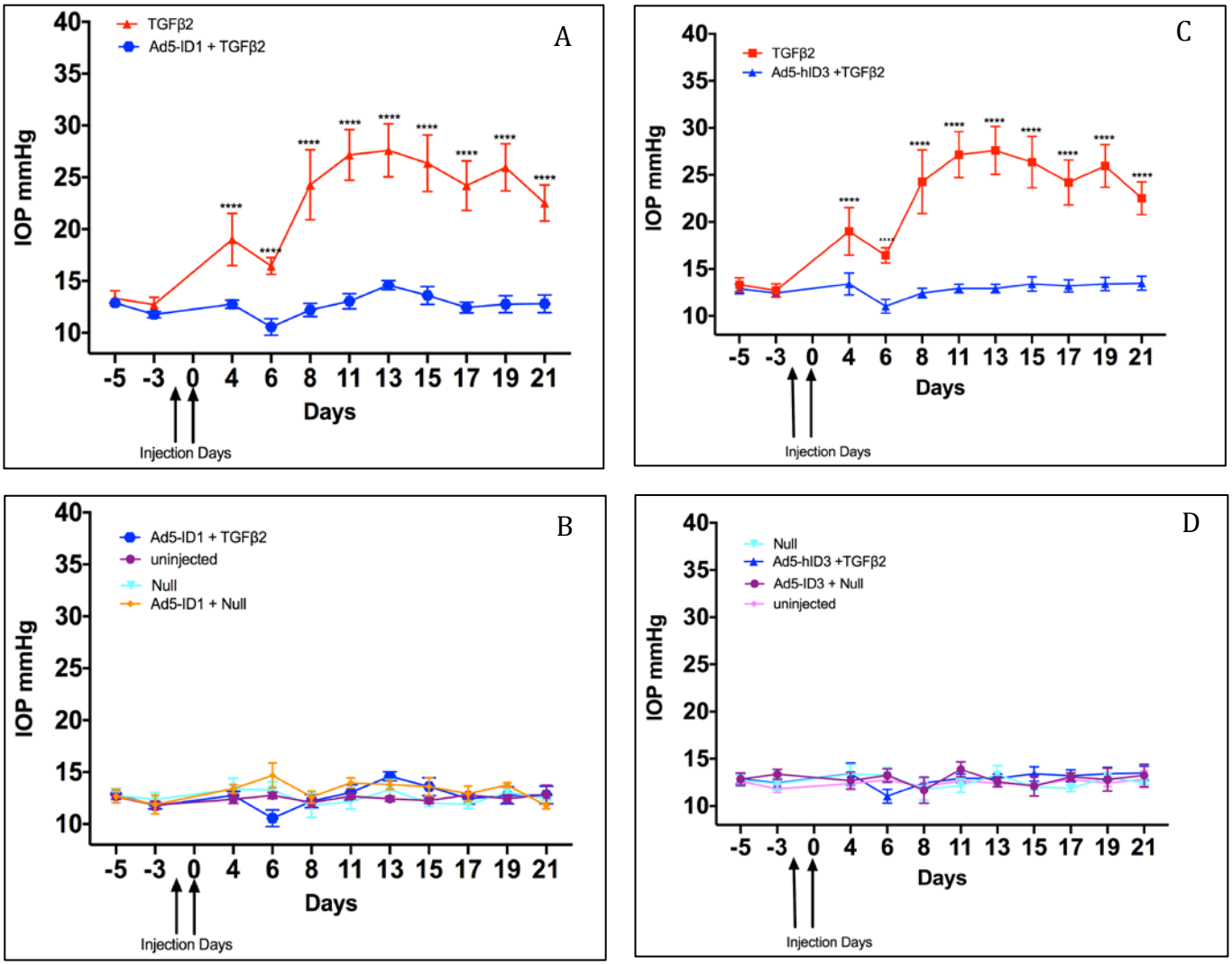


Figure 2. ID1 and ID3 block TGFβ2-induced IOP elevation

(A) Intravitreal injected Ad5.TGFβ2$^{226/228}$ left eyes (n=5) showed significant IOP elevation starting 4 days post injection (n=5; $p<0.0001$). This TGFβ2-induced IOP elevation was completely blocked by concomitant treatment with Ad5.ID1 (n=5). (B) There were no significant differences in IOPs between uninjected eyes, and eyes injected

with Ad5.null, Ad5.null + Ad5.ID1, or Ad5.TGFβ2 + Ad5.ID1 vectors (n=5 each group). (C) Intravitreally injected Ad5.TGFβ2$^{226/228}$ left eyes (n=5) showed significant IOP elevation starting 4 days post injection (n=5; $p<0.0001$). This TGFβ2-induced IOP elevation was completely blocked by concomitant treatment with Ad5.ID3 (n=5). (D) There were no significant differences in IOPs between uninjected eyes, and eyes injected with Ad5.null, Ad5.null + Ad5.ID3, or Ad5.TGFβ2 + Ad5.ID3 vectors (n=5 each group). The data shown are means ± SEM, **** $P< 0.0001$.

Figure 3.

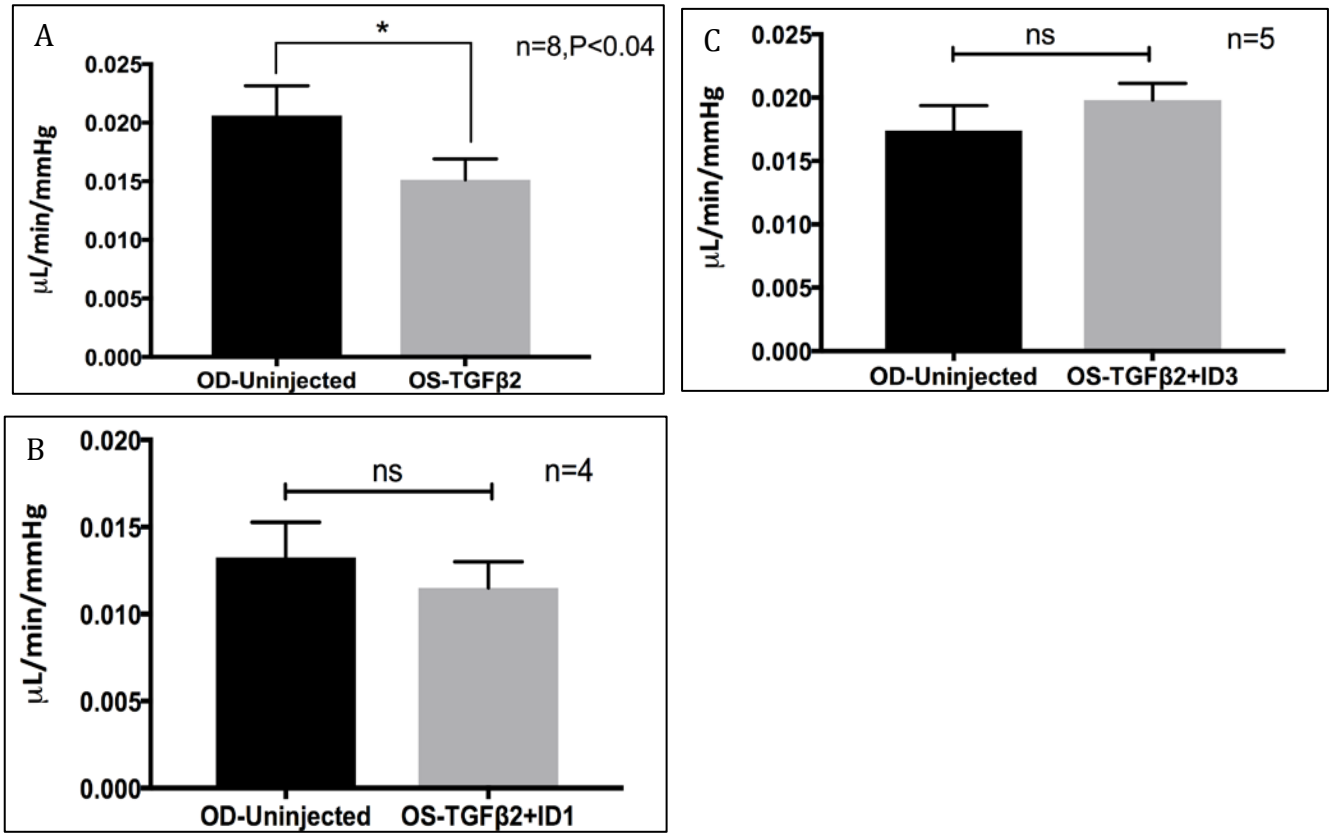
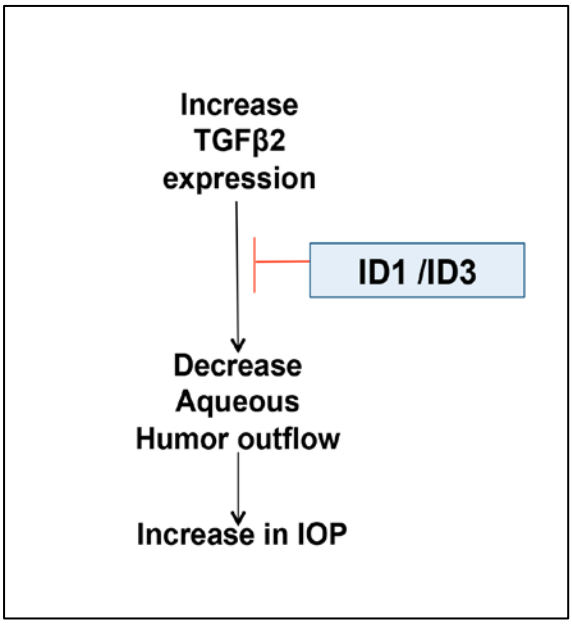


Figure 3. ID1 and ID3 prevent TGFβ2-induced reduction in aqueous outflow facility

(A) 21 days post intravitreal injection, mouse eyes from groups 2, 4, and 6 (Table 1b) where cannulated to measure outflow facilities. Mice injected with Ad5.TGFβ2$^{226/228}$ displayed significantly decreased outflow facility when compared to their contralateral uninjected eyes (n=8; p<0.04). Mice injected Ad5.ID1 along with Ad5.TGFβ2$^{226/228}$ (B) (n=4) or Ad5.ID3 along with Ad5.TGFβ2$^{226/228}$ (C) (n=5) exhibited no significant change in outflow facilities. The bars represent mean ± SEM, * P< 0.04 (paired Student' t-test).

Figure 4



Overexpression of ID1 or ID3 block TGFβ2-induced IOP elevation and decreased AH outflow facility.

TABLE

Table 1a. Experimental design for intravitreal injection to test Ad5.TGFβ2$^{226/228}$ vector

Injection Day	Day 0	Mice (n)
Group of animals	Injection received	
1	Ad-null-vector (2 µl, titer 5X10^7)	3
2	Ad-TGFβ2$^{226/228}$ (2 µl, titer 5X10^7)	5

Table 1b. Experimental design for intravitreal injections to determine effects of ID1 and ID3 on TGFβ2-induced ocular hypertension

Injection Day	Day -2	Day 0	Mice (n)
Group of animals	First injection received	Second injection received	
1	Ad5-null-vector (2 µl, titer 5X10^7)	Ad5-null-vector (2 µl, titer 5X10^7)	5
2	Ad5-null-vector (2 µl, titer 5X10^7)	Ad5-TGFβ2$^{226/228}$ (2 µl, titer 5X10^7)	5
3	Ad5-hID1 (2 µl, titer 5X10^7)	Ad5-null-vector (2 µl, titer 5X10^7)	5
4	Ad5-hID1 (2 µl, titer 5X10^7)	Ad5-TGFβ2$^{226/228}$ (2 µl, titer 5X10^7)	5
5	Ad5-hID3 (3 µl, titer 5X10^7)	Ad5-null-vector (2 µl, titer 5X10^7)	5
6	Ad5-hID3 (3 µl, titer 5X10^7)	Ad5-TGFβ2$^{226/228}$ (2 µl, titer 5X10^7)	5

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CHAPTER IV

ID1 and ID3 induce NFκB, RBP-Jκ and cAMP/PKA promoter activity in trabecular meshwork cells

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Abstract

Elevated IOP, due to altered ECM and decreased aqueous humor (AH) outflow flacility through the trabecular meshwork (TM), is the major risk factor associated with primary open angle glaucoma (POAG). TGFβ2 expression in the TM and AH is elevated in the eyes of POAG patients. TGFβ2 contributes to the pathogenesis of POAG by modifying the ECM and reorganizing the cytoskeleton in the TM, which causes decreased AH outflow facility and elevated intraocular pressure (IOP). In our previous findings, we demonstrated that ID1 and ID3, the downstream targets of BMP4, can inhibit TGFβ2 increased FN and PAI-1 expression in TM cells. In addition, we also demonstrated that ID1 and ID3 block TGFβ2-induced IOP elevation and decreased outflow facility. However, the signaling mechanism(s) by which ID1 and ID3 negatively regulates TGFβ2 effects in the TM remains undetermined.

ID1 and ID3 are transcription regulators that negatively regulate bHLH transcription by inhibiting the transcription complex binding to specific promoters on DNA. In order to determine signaling pathways regulated by ID1 and ID3, we performed luciferase promoter activity assays. Treatment of cultured human TM cells with TGFβ2 alone for 24 hours did not change the promoter activities of NFκB, NOTCH, and cAMP/PKA. In contrast, we observed significant ($p<0.01$) increases in NFκB, Notch, and cAMP/PKA activity by over expression of ID1 or ID3 along with TGFβ2.

These data suggest that ID1 and ID3 may activate MMP pathways by upregulating NFκB. Upregulation of Notch/RBP-Jκ may induce TM proliferation and form a positive feedback loop by increasing BMP and repressing BMP inhibitor expression . In addition, increasing cAMP/PKA activity regulates RhoA kinase and actomyosin activity leading to actin filament and myosin light chain rearrangement, which decrease the contractility of the TM cell and increase in outflow facility.

Introduction

Elevated IOP is major risk factor associated with the development and progression of primary open angle glaucoma (POAG).[1-3] The primary cause of increased IOP is due to increased aqueous humor (AH) outflow resistance and decreased outflow facility through the trabecular meshwork (TM).[2, 4] TGFβ2 is increased in the AH of POAG patients, and in TM cells, this profibrotic cytokine disrupts extracellular matrix (ECM) homeostasis, inhibiting ECM turnover and increasing ECM deposition.[5-9] In addition, TGFβ2 increases expression of ECM crosslinking enzymes (TGM2 and LOXs) reorganizes the actin cytoskeleton in TM cells.[10, 11] These TGFβ2-induced changes in the TM leads to increased AH outflow resistance and elevated IOP. Understanding and regulating these molecular events would lead us to develop new disease modifying therapeutic targets that may prevent and reverse glaucoma pathogenesis.

Several ECM components appear to play prominent roles in the development and progression of POAG. Increased PAI-1 expression inhibits MMP activation, thereby inhibiting ECM turnover.[12-14] TGFβ2 also increases the synthesis of ECM molecules fibronectin (FN), collagens IV and VI, and elastin that increase the ECM deposition. In addition to TGFβ2, PAI-1 also increases expression FN in cultured TM cells.[8, 10] PAI-1 alone increases IOP in rodents, and treatment of TM cells with a PAI-1 neutralizing antibody in presence of TGFβ2 increased MMP2 and MMP9 activities. This suggests the important role of PAI-1 in TGFβ2 inhibition of MMPs, leading to ECM deposition and increased AH outflow resistance.[10, 15, 16] In addition, the stretching of TM cells due to IOP elevation as compared with normal increases MMP activation, which suggest increase

stiffness in glaucomatous TM will reduces MMP activity and inhibition of IOP homeostasis.[17, 18]

Furthermore, TGFβ2 reorganizes the actin cytoskeleton in TM cells, which also may be involved in glaucoma pathogenesis. TGFβ2 induces the rearrangement of F-actin to form cross-linked actin networks, which changes TM cell shape and functions.[19] In addition, actomyosin phosphorylation by Rho Kinase decreases outflow facility.[19, 20] TGFβ2 inhibits TM cell proliferation and reduces TM cell number in culture[21], which may be the mechanism for the reduction of TM cellularity in POAG eyes.[22, 23]

In our previous work, we showed that BMP4 blocked TGFβ2 induction of FN and PAI-1 in cultured TM cells.[24] We further demonstrated that BMP-induced ID1 and ID3 can downregulate TGFβ2 profibrotic effects such as increased FN and PAI-1 expression, increased IOP and decreased AH outflow facility.[25] ID1 and ID3 are transcription regulators that negatively regulate various other bHLH transcription factors. In this study, we examined the effect of ID1 or ID3 along with TGFβ2 on promoter activities of a number of prominent transcription factors.

Methods

Cell culture and luciferase reporter arrays

Transformed GTM3 cells developed from glaucomatous TM cells were used because they are easily transfected.[26] The Cignal Finder Reporter Array (Qiagen, Hilden Germany) was utilized, which consists of negative (non inducible firefly plasmid construct) and positive (GFP-firefly) controls. Plate wells contained similar reporter-

firefly constructs per row, for 10 different signaling pathway promoters were tested (Table1). Transfection of GTM3 cells with reporter plasmid was carried out as described by Qiagen with $1x10^5$ cells/well. Subsequently, after 24 hr of transfection with reporter plasmid, ID1 and ID3 were overexpressed by transduction with Ad5.ID1 and Ad5.ID3 viral vectors in serum free medium. Ad5.null was used as a negative control. After 24 hr of viral transduction in serum free media, cells were treated with or without TGFβ2 (5 ng/ml) for additional 24hr. Each treatment was carried out in duplicate, and the each experiment was performed 2-3 times. The plate was developed using the Dual Glow Luciferases Assay System (Promega, Madison WI).

Statistics

Comparison between multiple groups was performed using one way ANOVA. Graph Pad Prism 6 software (Graph Pad Prism, Inc. San Diego; CA.) was used to perform statistical analysis. The average value for each group was presented as mean ±S.E.M and $p<0.05$ was considered statistically significant.

Results

The effects of overexpression of ID1 and ID3 on transcription factor promoter activities with or without TGFβ2 treatment was studied using Cignal Finder Reporter Arrays. Renilla was used as the internal control, while firefly luciferase was the reporter for transcription factor promoter activities. A positive transfection control was added to the assay to assure equal transfection among wells. The study was conducted in GTM3 cells because these transformed cells had better transfection efficiencies. Treatment of GTM3

cells with TGFβ2 alone had no effect on any of the tested signaling pathways including the NFkB, Notch/RBP-Jk, or cAMP/PKA reporter activities. In contrast, overexpression of ID1 ($p<0.01$;n=3) (Figures 1A-C) or ID3 (n=2) (Figures 2A-C) increased NFκB, Notch/RBP-Jκ and cAMP/PKA reporter activity 24 hrs post treatment with or without concomitant TGFβ2 treatment.

Discussion

NFκB, Notch/RBP-Jκ and cAMP/PKA are some of the most versatile transcription factors that regulate a diverse array of cellular functions. Their function depends on the specific phosphorylation site that is activated, the association with and regulation by other transcription factors, the specific cell type, and the cytokine signaling background.

NFκB consists of five transcription factor members, of which p65 and p50 are the most prominent and ubiquitously present. The subunits are bound to IκB in the cytoplasm. Activation of this pathway leads to degradation of IκB, and the activate NFκB complex translocates to the nucleus to either transactivate or transrepress gene expression. The NFκB p65 domain is known to bind to ID1 and regulates TNFα and IL1α pathways to upregulate the activities of MMPs.[27] TNFα and IL1α in TM cells activates MMP3 and MMP12 but not MMP9.[28, 29] In addition to upregulation of MMPs in TM cells, there is crosstalk between JNK1/2, P38α/β and Erk-1/2 signaling pathways.[29] Therefore, our results suggest that ID1 and ID3 increase NFκB activity in TM cells, which may upregulate MMP activities leading to the degradation of FN and other ECM molecules.

Secondly, ID1 and ID3 also upregulates RBP-Jκ promoter activity, which mediates canonical Notch signaling.[30] Although very little is known about Notch signaling in the TM, this pathway plays important roles in development, stem cell biology, cancer biology among others. Notch through RBP-Jκ regulates Smad3 activity by sequestering p300 transcription factor in endothelial cells.[31] Further, Notch also increases cell proliferation and cell survival by regulating apoptosis.[32] In addition, Notch signaling is important in ciliary body morphogenesis and upregulates BMP expression, suggesting a positive feedback loop and by increasing ID expression as well suppressing BMP inhibitors.[33]

ID1 and ID3 also increase cAMP/PKA promoter activity, which may regulate actomyosin contractility by inhibiting Rho-Rho kinase pathway. Increase cAMP reduces RhoA activity by phosphorylating Ser188 and inhibiting endothelin-1, which further leads to increase phoshoryalation of pMLC and inhibition of stress fibers leading to relaxation of the TM cells.[34, 35] Further activation of cAMP/PKA pathway changes the cell-matrix adhesion.[34] These overall changes by cAMP/PKA would increase AH outflow facility and lower IOP.[20]

In summary, ID1 and ID3 regulate three interesting transcription factors: NFκB, Notch/RBP-Jκ, and cAMP/PKA. These data lead us to hypothesize that ID1 and ID3 increase MMP activation, decrease PAI expression, and regulate ECM homeostasis. Secondly, activation of Notch may rescue TM cells from apoptosis, increase TM

proliferation and offer stem cell characteristic, which may lead to increase TM cellularity. Lastly, by upregulating cAMP/PKA activities, IDs may be involved in resisting cytoskeleton changes and inhibit increased ECM-cell adhesion, there by lower IOP by relaxing TM cells and increasing AH outflow facility.

Figure 1

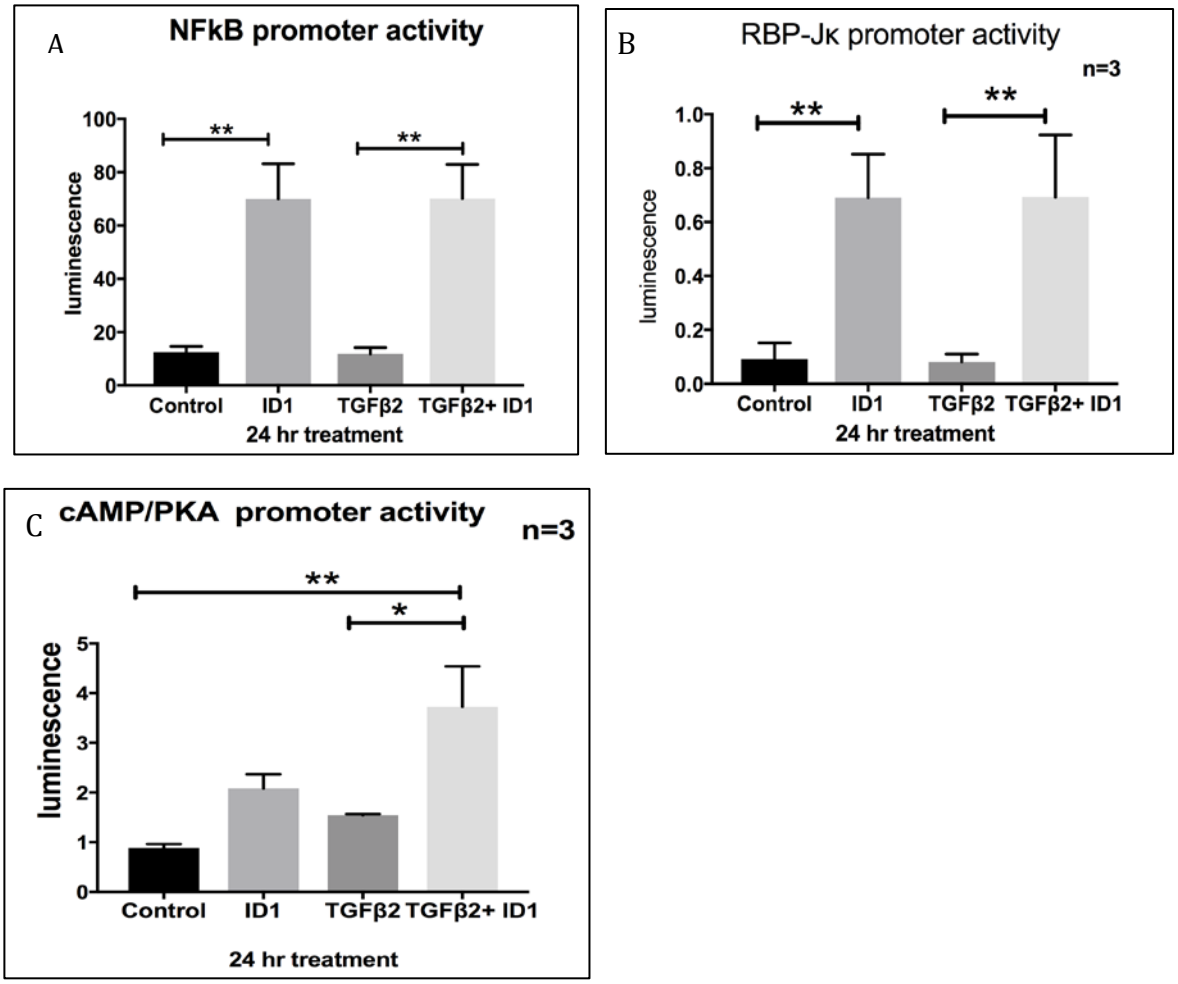

Figure 1. Effect of overexpressing ID1 along with or without TGFβ2 treatment on promoter activity in GTM3 cells. (A) NFκB promoter activity, (B) Notch/RBP-Jκ promoter activity, and (C) cAMP/PKA promoter activity. TGFβ2 does not affect promoter activity, but ID1 with or without TGFβ2 significantly increases promoter activity for these 3 signaling pathways. The bar represents mean ± SEM; (n=3); *p< 0.05, **p<0.01

Figure 2

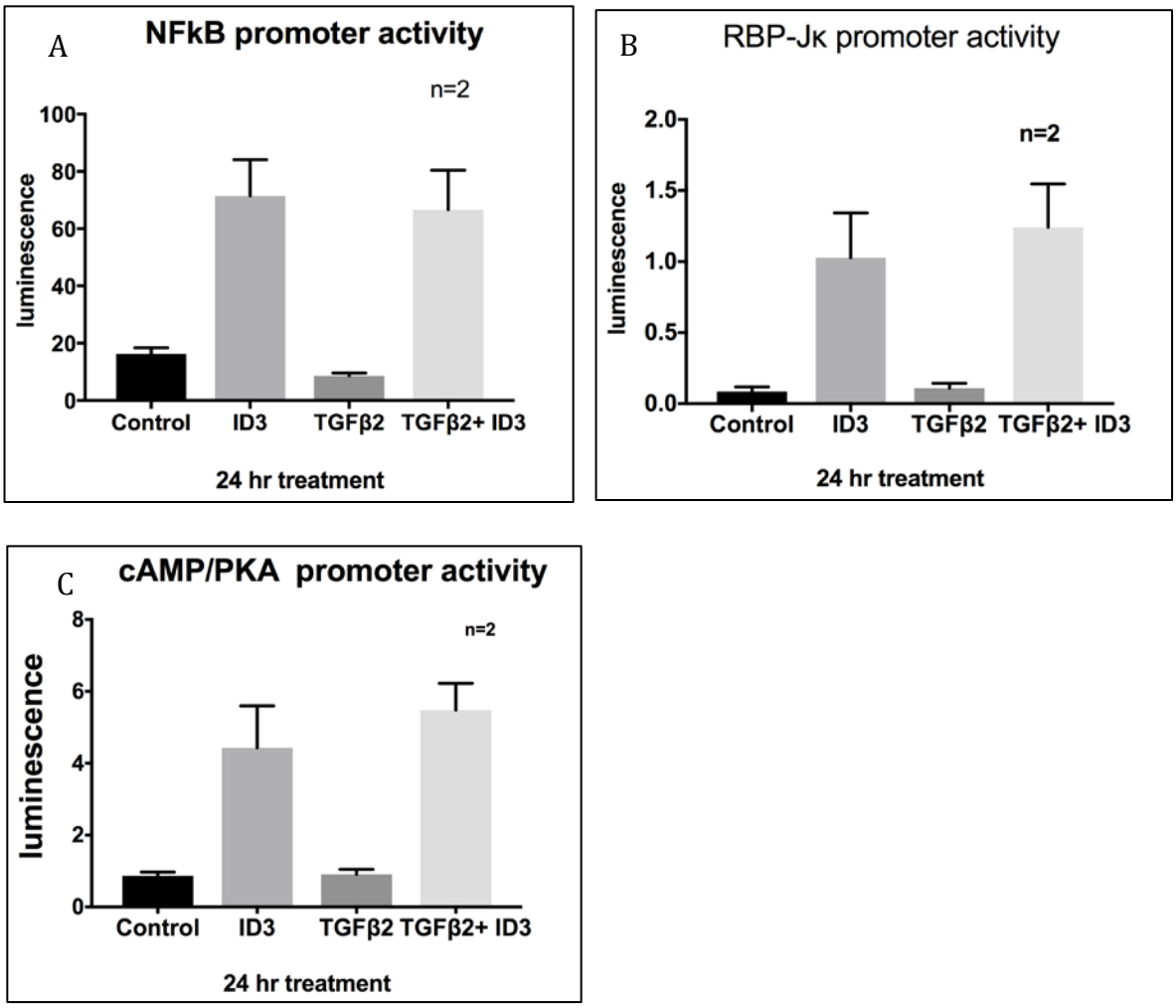

Figure 2. Effect of overexpressing ID3 with or without TGFβ2 treatment on promoter activity in GTM3 cells. (A) NFκB promoter activity, (B) Notch/RBP-Jκ promoter activity, and (C) cAMP/PKA promoter activity. TGFβ2 does not affect promoter activity, but ID3 with or without TGFβ2 significantly increases promoter activity for these 3 signaling pathways. (n=2)

Table 1. **Cignal reporter array assay**

Column number	Pathway	Cignal reporter assay
1	Notch	RBP-Jκ
2	Wnt	TCF/LEF
3	Myc	Myc/Max
4	NFκB	NFκB
5	TGFβ2	SMAD
6	Cell-cycle/pRB-E2F	E2F
7	C/EBP	C/EBP
8	cAMP/PKA	CRE
9	MAPK/ERK	SRE
10	MAPK/JNK	AP-1
11	Negative control Cignal Reporter assay	
12	Positive control Cignal Reporter assay	

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CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Primary open-angle glaucoma (POAG) is one of the leading causes of blindness worldwide. A major risk factor of POAG is increased intraocular pressure (IOP). Elevated IOP results from increased resistance to aqueous humor (AH) outflow through the trabecular meshwork (TM), which is associated with an excessive accumulation of extracellular matrix (ECM) in the TM. The profibrotic growth factor TGFβ2 is elevated in glaucomatous AH and TM that increases synthesis and secretion of the ECM related proteins fibronectin (FN) and plasminogen activator inhibitor-1 (PAI-1) in TM cells.[1-3] Overexpression of TGFβ2 in the rodent eye and in human eye anterior chamber perfusion culture models increases ECM molecules and elevates IOP.[4-7] Additionally, BMP4 and BMP7 counteract TGFβ2 induced fibrosis in the TM by decreasing expression of FN, PAI-1, thrombospondin-1, collagens IV and VI, and MMP2 activity.[8, 9] Although BMP7 inhibits TGFβ2 through induction of inhibitory Smad7 in the TM, the mechanism by which BMP4 inhibits TGFβ2-induced fibrosis remained undetermined.[8, 9] Interestingly, inhibitor of DNA binding proteins (ID1 and ID3), dominant negative transcription regulators and downstream targets of the BMP4 pathway, are known to inhibit TGFβ increased ECM expression in other fibrotic diseases.[10, 11] Hence, we proposed that BMP4 via upregulation of ID1 and ID3 may inhibit TGFβ2 effects such as increased expression of FN and PAI-1 in the TM, as well as elevated IOP and decreased AH outflow facility.

To test our hypothesis, we first determined the basal expression of ID1-ID4 mRNA in the TM and discovered expression of ID1-ID3 in 3 different primary TM cell strains. Although we were unable to detect ID4 mRNA expression in primary TM cells, our lab have previously shown expression of ID4 mRNA in TM tissue.[12] We further studied the BMP4 effects on ID1 and ID3 mRNA and protein expression and their nuclear localization in 3-4 different primary TM cell strains. BMP4 significantly increased early expression of ID1 and ID3 mRNA ($p<0.05$) and protein in primary TM cells. Primary TM cells treated with BMP4 showed increased nuclear and cytoplasmic protein expression of ID1 and increased ID3 expression in the nucleus. An inhibitor of a BMP receptor, LDN193189, abolished BMP4 induction of ID1 and ID3 protein expression ($p<0.05$) in primary TM cells, demonstrating that expression of ID1 and ID3 in TM cells is BMP dependent.

Additionally, we tested whether overexpression of ID1 and ID3 affects TGFβ2 on in TM cells and observed a significant ($p<0.01$) decrease in TGFβ2 elevated FN and PAI-1 expression. This finding suggests that BMP4 downstream targets ID1 and ID3 are important negative regulators of TGFβ2 induced FN and PAI-1 expression.

Overexpression of active TGFβ2 and elevated PAI-1 and FN expression has been associated with elevated IOP in mice and in ex vivo human anterior segment perfusion cultures.[5, 13] In our studies, overexpression of ID1 and ID3 significantly ($p<0.0001$) blocked TGFβ2-induced ocular hypertension in mice. The AH outflow facility was significantly ($p<0.04$) decreased in TGFβ2 ocular hypertensive mice, but no significant change was observed in the outflow facilities of eyes injected with ID1 and ID3 along

with TGFβ2. Furthermore, overexpression of ID1 ($p<0.01$) and ID3 increased NFκB, Notch, and cAMP/PKA promoter activity in presence or absence of TGFβ2.

Our current results demonstrate that ID1 and ID3 can inhibit TGFβ2-induced FN and PAI-1 protein expression in TM cells and increased IOP and decreased AH outflow in mice. This may occur by regulating NFκB, Notch, and cAMP/PKA activities, suggesting ID1 and ID3 as crucial targets in the BMP4 pathway that negatively regulate TGFβ2 profibrotic effects on the TM and offering promising new disease modifying therapeutic targets for lowering IOP in POAG patients.

FUTURE DIRECTIONS

In our studies, we demonstrated that ID1 and ID3 regulate TGFβ2 effects on the TM, IOP and AH outflow facility. However, there are many interesting questions that remain unexplored, such as role of ID1 and ID3 in regulation of other ECM components like ECM crosslinking enzymes and cytoskeletal changes induced by TGFβ2.[14, 15] IDs also regulate Notch activity, suggesting IDs may be involved in a positive feedback loop by increasing BMP expression.[16-18] ID1 and ID3 may increase cAMP activity, and cAMP is known to increase AH outflow facility by regulating actomyosin and phosphorylation of RhoA in bovine TM cells.[19] The potential role of ID1 and ID3 on regulation of actomyosin and cytoskeletal changes should be studied.

Secondly, the precise mechanism(s) by which ID1 and ID3 regulate TGFβ2 activity in the TM remains unmapped. Some of the possible mechanisms by which IDs may negatively regulate TGFβ2 effects in TM are by: regulating their association with CAV-1/2 and internalizing TGFβ and BMP receptors, MMP pathway activation, regulating apoptosis and cell cycle checkpoints, global gene expression by regulating HDACs and modifying cytoskeleton, as well as integrin and ECM interactions.[20-23]

GWAS studies have shown that SNP alleles near *CAV-1* and *CAV-2* are associated with POAG.[24, 25] In addition, Stamer and colleagues demonstrated that knockdown of *Cav-1* increases IOP in mice. ID1 association with CAV-1 may contribute to lowering IOP by modification of E-cadherin, F-actin, ECM and integrin interactions, and TGFβRI, which need to be determined.[20, 26] Furthermore, ID1 and ID3 downregulate PAI-1 expression while upregulating NFκB promoter activity suggesting activation of MMP pathways and regulation of ECM turnover. However, we lack direct evidence of IDs upregulating MMPs in the TM, which further need to be studied.[23, 27] IDs are also known to negatively regulate apoptosis and stimulate cell cycle progression by inhibiting all the cyclin dependent kinase inhibitors (p27, p21, p16) via suppressing E-box activity.[28-31] IDs are also known to increase Myc/Max promoter activity thereby downregulating thrombospondin expression and cell cycle progression from G1 to S phase.[32, 33] These data suggest IDs may also be involved in increasing TM cell number, which are reduced in glaucoma.[34]

In addition to the TM, IDs are expressed in various ocular tissues including the cornea, lens and retina and regulate their functions.[35-37] IDs regulate corneal fibrosis and are essential for early retina and lens development. In our studies, we examined the functions ID1 and ID3 in the TM. The effects and functions of ID2 and ID4 in the TM and expression and function of ID1-ID4 in other ocular tissues such as the lamina cribrosa and non-pigmented ciliary epithelium remains untested.

In summary, IDs may globally regulate promoter activities of various prominent transcription factors and the functions of other TM proteins, which may negatively regulate TGFβ2 effects in the TM. Furthermore, our data suggest that IDs may be promising and important disease modifying targets in TM by normalizing TM function thereby lowering IOP.

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