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ABSTRACT

Purpose: Glaucoma is a leading cause of blindness worldwide. The leading risk factor is elevated intraocular pressure (IOP) due to fibrotic changes in the trabecular meshwork (TM) tissue. The profibrotic cytokine TGFβ2 is elevated in the aqueous humor and TM of glaucomatous eyes. TGFβ2-mediated extracellular matrix (ECM) deposition in the TM appears to be responsible for increased IOP in *ex vivo* and *in vivo* models. Bone morphogenetic proteins (BMPs) inhibit TGFβ regulation of ECM, and elevated levels of the BMP antagonist gremlin in the glaucomatous TM restores the fibrotic response of TGFβ2. Lysyl oxidase (LOX) is a collagen and elastin polymer crosslinking enzyme, and recent genome wide association studies showed that SNPs in LOXL1, a LOX family member, significantly increased the risk of developing exfoliation glaucoma. The overall aim of my work is to delineate the signaling pathways involved in TGFβ2 and gremlin alteration of the TM ECM and to evaluate the potential role of the LOX family of cross-linking enzymes in this TM ECM remodeling.

Methods: Human TM cells were cultured in the presence or absence of recombinant human TGFβ (0.1-10 ng/ml) or mouse gremlin (100-5000 ng/ml) for 1-72 hours, and total RNA or protein lysates and conditioned medium were harvested from the cells. Effects of gremlin treatment on ECM gene and protein expression were assayed by qRT-PCR and western immunoblotting, respectively. TGFβ2- and gremlin-treated TM cells were also examined for Smad2/3, p38, and JNK activation using western immunoblotting. Cells were treated with

Smad3 inhibitor SIS3 (5 uM), TGFβ receptor inhibitors LY364947 and SB431542 (5 uM), JNK inhibitor SP600125 (10 uM), and AP-1 inhibitor SR11342 (5 uM) with or without TGFβ2/gremlin, to examine the involvement of the TGFβ/Smad signaling pathway.

Results: TGFβ2 and gremlin induced expression of each other in TM cells. TGFβ2 activated both Smad and non-Smad pathways and strongly induced mRNA and protein expression of all 5 LOX genes. Using a novel LOX activity assay, we observed greater ECM crosslinking in TGFβ-treated cells. Gremlin elevated ECM gene and protein expression via the Smad signaling pathway.

Conclusions: The TGFβ induction of LOXs and gremlin-induction of ECM proteins highlight the complex interplay of Smad and the non-Smad signaling in regulating the TGFβ response. TGFβ2 and gremlin are profibrotic in a feed forward loop. The TGFβ response in TM cells involves evasion of BMP inhibition by gremlin induction and also ECM crosslinking through the LOX enzymes.

TGFβ2 AND GREMLIN SIGNALING PATHWAYS REGULATE EXTRACELLULAR MATRIX CHANGES IN TM CELLS: IMPLICATIONS FOR GLAUCOMA

DISSERTATION

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By

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TGFβ2 AND GREMLIN SIGNALING PATHWAYS REGULATE EXTRACELLULAR MATRIX CHANGES IN TM CELLS: IMPLICATIONS FOR GLAUCOMA

Anirudh Sethi

Chapter I

INTRODUCTION

Primary Open Angle Glaucoma (POAG)

Glaucoma is a leading cause of blindness in the world and affects about 60-70 million people, accounting for 1% of the world population [1-3]. Glaucoma represents a group of optic neuropathies that result in death of retinal ganglion cells leading to slow and progressive vision loss [4]. Primary open angle glaucoma (POAG) is the most prevalent form of glaucoma in the United States [3]. The loss of vision in POAG is slow, painless and asymptomatic. Elevation of intraocular pressure (IOP) is regarded as the most significant risk factor for POAG [5]. IOP is a measurement of the fluid pressure inside the eye generated by the flow of aqueous humor (AH) in the anterior segment of the eye. The AH is secreted from the ciliary body, flows into the anterior chamber and exits through the trabecular meshwork (TM) into Schlemm's canal and episcleral veins. The flow of AH is regulated by the TM tissue [6-7].

Trabecular Meshwork (TM)

The TM cells lie on the extracellular matrix (ECM) beams, and TM cells regulate their ECM environment. Histologically, the TM can be divided into three distinct layers. The outer uveal and corneoscleral portions of the TM are highly fenestrated and are composed of several irregular layers of ECM covered by the TM cells. The trabecular beams become more flattened and sheet-like in the deeper portions of this region [8]. The center or stroma of the beams exhibits typical collagen fibrils, elastic fibers and microfibril sheath-derived material [9]. Between the

beams and sheets, the irregular intertrabecular spaces form channels leading to the juxtacanalicular (JCT) region, which lies adjacent to the endothelial lining of Schlemm's canal (SC) [10-11]. The cells on the outer layers of the TM are actively phagocytic and are thought to act primarily as pre-filters, removing cellular debris from the aqueous humor prior to its passage through the less porous inner JCT and SC regions. The JCT region is composed of an amorphous ECM with a discontinuous scattering of several layers of TM cells embedded within the ECM. Basement membrane proteins, type IV collagen, fibronectin and laminin, and basement membrane proteoglycans have been identified in the JCT region [8, 12-17].

Extracellular Matrix (ECM) in the TM

A variety of ECM components have been identified within the TM. Most of these are similar to those found in other tissues, although a few unique isoforms have been identified as well. Studies with electron microscopy and with general microscopic stains identify glycosaminoglycans (GAGs), collagens, elastic fibrils, basement membrane and several non-specific ECM components [8]. Based on several staining techniques trabecular GAGs have been identified including hyaluronic acid, chondroitin and dermatan sulfates, keratin and heparin sulfates [18]. Proteoglycans in the TM include decorin, biglycan, versican, perlecan, and syndecan amongst others [19]. Several intrinsic basement membrane proteins, particularly laminin, type IV collagen and perlecan, as well as integrin receptors are expressed in the outflow pathway and have been localized to basement membranes of TM beam cells, SC inner wall cells and JCT cells [8].

One of the major TM ECM proteins is fibronectin (FN). It is found abundantly in TM beams, the JCT region, the basement membranes of TM beam cells and the SC inner wall cells [20]. TM beams are also comprise of collagen I and III (COL1 and COL3) which provide tensile strength

to the beams [21]. Collagen IV (COL4) is present in the basement membrane and provides support to JCT cells. Collagen V and VI are also present in both TM beams and JCT region within the collagen and elastin (ELN) rich regions [22]. Non-fibrillar collagens are also associated with the TM beams and the JCT are extremely rich in elastin (ELN) fibrils which are responsible for elastic characteristics of this tissue. ELN is also present in the core of the TM beams [16, 23].

The TM actively regulates its ECM and the rate of the ECM turnover. Various proteinases and their inhibitors are expressed in TM tissues and in cultured TM cells. These include matrix metalloproteinases (MMPs), membrane type (MT) MMPs, ADAMs (A Disintegrin-like And Metalloproteinase), ADAMTSs (ADAM with ThromboSpondin type 1 motifs), plasminogen activators (PA), tissue inhibitors of metalloproteinases (TIMPs), PA inhibitors (PAIs), and serine proteinase inhibitors (serpins) [24-29].

Extracellular Matrix Turnover in POAG: Role of Transforming Growth Factor Beta 2

There are multiple reports that illustrate remodeling of ECM in the glaucomatous TM. It is believed that the high ECM remodeling in the TM results in increased resistance to the AH outflow, which increases IOP and may lead to POAG [8, 30-32]. ECM deposition and remodeling are the highlight of fibrotic diseases of the ECM-rich connective tissues of the lung, heart and kidney. ECM turnover is a highly regulated process that involves deposition, cross-linking, and degradation of the various ECM components [33]. Cellular growth factors including transforming growth factor beta (TGFβ) have been reported to regulate the ECM turnover [34-37]. There are three known TGFβ isoforms: TGFβ1, TGFβ2 and TGFβ3. TGFβ2 is the most abundant TGFβ isoform in the eye, and elevated levels of TGFβ2 have been reported in the AH of glaucomatous patients [38-41]. We and others have previously reported that TGFβ2 is profibrotic in the TM and regulates

several stages of ECM turnover. TGFβ2 treatment increases synthesis and deposition of ECM proteins like fibronectin-1 (FN1), collagens (COL), and elastin (ELN) [31, 42-43]. TGFβ2 down regulates ECM degradation by inducing plasminogen activator inhibitor-1 (PAI1), a key player in inhibiting MMP activation [42-43]. More recently, TGFβ2 has been reported to induce expression of the ECM cross-linking enzyme tissue transglutaminase-2 (TGM2) [44-45]. There is evidence for both Smad and non-Smad signal transduction pathways in TGFβ regulation of ECM remodeling in several breast, liver, colon, and lung cancerous and normal cell lines [46-49].

Importantly, TGFβ2 elevates IOP in isolated perfusion cultured human anterior eye segments [50]. Interestingly, the eyes with elevated IOP were positively correlated to increased ECM proteins like FN1 and PAI1 [51]. Adenoviral overexpression of TGFβ2 has also been reported to elevate IOP in mice and rats. The role of TGFβ2 is summarized in Figure 1.

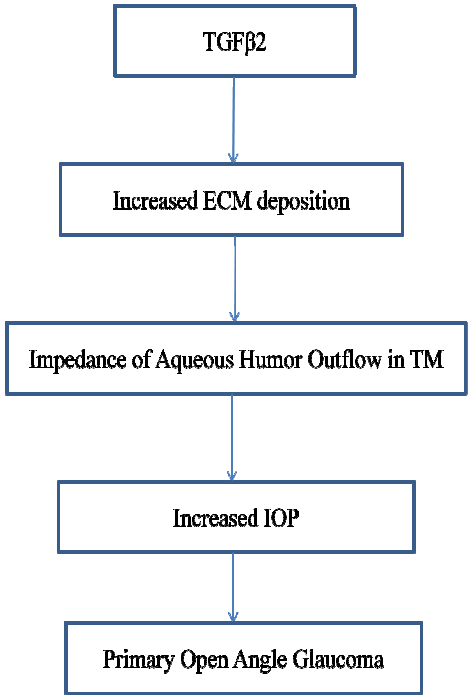

Figure 1: Elevated TGFβ2 induces ECM protein deposition in TM resulting in elevated IOP.

TGFβ Signaling Pathways

In most cell types, TGFβ isoforms utilize the canonical Smad signaling pathway. However, under specific pathological conditions in some cells, these cytokines also utilize non-canonical signaling pathways including c-Jun N-terminal Kinase 1/2 (JNK1/2), extracellular signal-regulated kinases (ERK) and P38 mitogen-activated protein kinase (MAPK) (Fig. 2).

TGFβ ligands bind directly to TGFβ type II receptors (TGFBR2) on target cells, which leads to the recruitment of TGFβ type I receptors (TGFBR1). TGFBR2 then trans-phosphorylates TGFBR1, enabling the TGFBR1 kinase domain to act on cytoplasmic proteins and thereby propel downstream signaling actions. Receptor regulated (R-) Smads (Smad2 and Smad3) are direct targets of the ligand-receptor complex and substrates of the TGFBR1. Once activated, TGFBR1 phosphorylates R-Smads which form trimeric complexes involving two R-Smads and one common (Co-) Smad, Smad4. Once formed in the cytoplasm, these trimeric Smad complexes relocate to the nucleus in order to regulate transcription from a wide variety of promoters [52-53].

TGFβ induction of gene expression often requires the transcriptional cofactor p300; the TGFβ-mediated phosphorylation of Smad3 elevates the association between Smad3 and p300. Smad2 is not believed to bind DNA directly, but rather requires a nuclear DNA binding protein of the Fast family (Fast-1) to bind DNA, in association with Smad4, and activate transcription in response to TGFβ. In fibroblasts obtained from adult *Smad3*$^{-/-}$ and *Smad3*$^{+/+}$ mice, TGFβ1 was unable to induce transcription in *Smad3*$^{-/-}$ fibroblasts. Mice homozygous for a deletion in Smad2 die during embryogenesis, whereas mice homozygous for a deletion of Smad3 are viable and fertile [54].

In non-Smad signaling pathways TGFβ is known to activate P38 MAPK, ERK and/or JNK-c-JUN pathways. There are three different mechanisms by which these non-Smad pathways transmit their signals. First, the activated receptor directly phosphorylates these non-Smad kinases and initiates parallel signaling pathways. Additionally, non-Smad signaling proteins directly phosphorylate Smad proteins and modify the Smad activity. Also, activated Smads directly interact with one or more of the MAPKs modulating their activity, thus transducing their signals to non-Smad pathways [55].

Emerging evidence also indicates that to regulate the expression of target genes, Smads cooperate with other transcription factors implicated in TGFβ signaling. One of those transcription factors is AP-1, a heterodimer of c-Fos and c-Jun family members that binds specific sequences in target promoters. Stimulation of AP-1-dependent transcription can be achieved by phosphorylation of the c-Jun transactivation domain JNK, a member of the MAPK family. SMAD- and AP-1-binding elements are juxtaposed in the TGFβ-responsive regions of PAI-1 promoter and both have been positively implicated in PAI-1 induction by TGF-β [56]. Furthermore, a direct, TGFβ-inducible interaction between Smad3 and c-Jun has also been described [57].

Interestingly, TGFβ2 has been shown to phosphorylate and activate canonical Smad2/3 and the non-Smad pathways JNK1/2, ERK and P38 MAPK in the TM [53, 58-59].

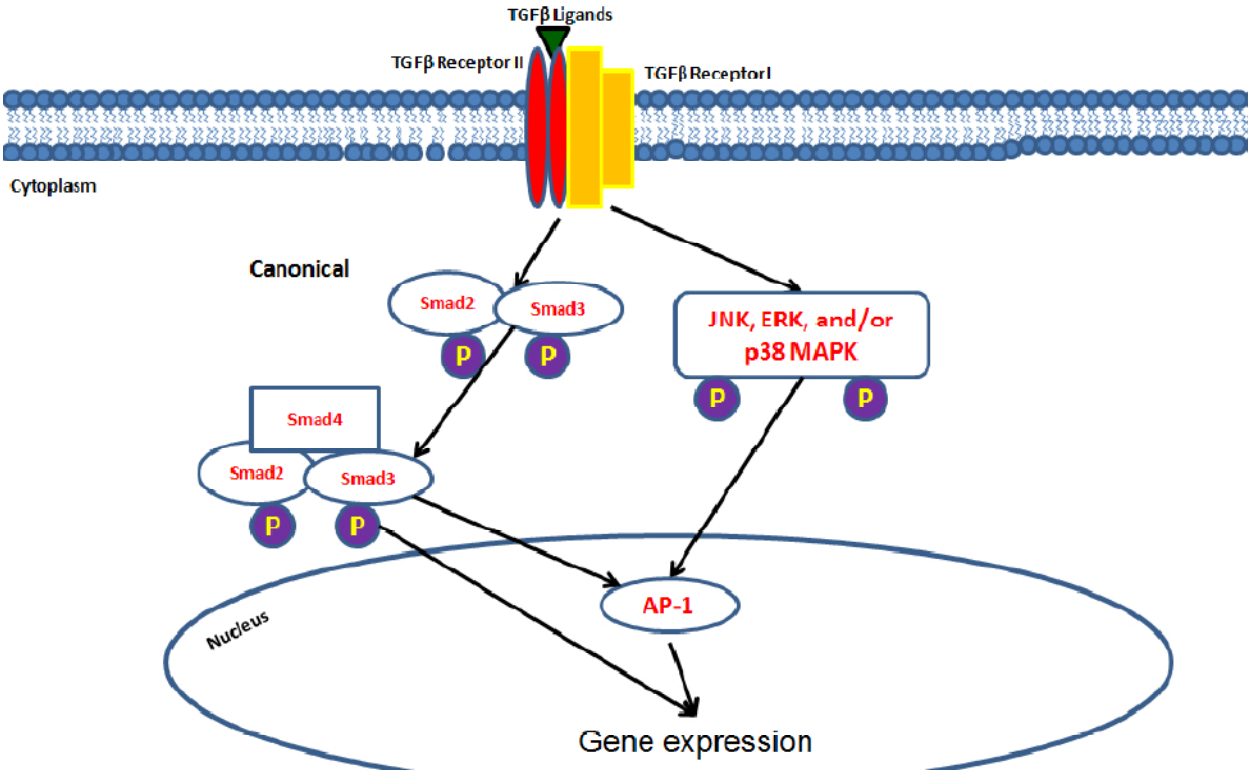

Figure 2: TGFβ signaling mechanism in TM and other cell types

Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs) were originally identified as osteoinductive cytokines that promote bone and cartilage formation but are now known to control multiple functions in a variety of cells [60]. Knockout studies in mice indicate that BMP4 is essential in early morphogenesis of the eye [61]. A heterozygous deficiency of BMP4 results in anterior segment dysgenesis of the eye and elevated IOP [61]. Previous work from our laboratory demonstrated that TM cells and tissues express BMPs, BMP receptors and BMP antagonists [62]. The exact role of BMPs in human TM cells in unknown. However, several reports indicate that BMPs function as anti-fibrotic agents [63-64]. Some of the anti-fibrotic effects BMPs are mediated primarily via inhibiting the pro-fibrotic actions of TGFβ2 [65]. BMPs have been shown to antagonize TGFβ

signaling in fibrotic diseases in the kidney, lungs and liver [66-68]. BMP4 and BMP7 inhibit TGFβ2-stimulated ECM synthesis and deposition in the cultured human TM cells [42-43]. These data suggest a functional role of BMPs in cultured human TM cells. However, whether BMPs modulate TGFβ2 elevation of IOP is thus far not known.

Gremlin

Several secreted BMP antagonists have been identified including noggin, chordin, follistatin, Dan, cerebus, caronte and gremlin. Gremlin is a highly conserved 184 amino acid protein (20.7 kDa). The human gremlin gene (GREM1) has been mapped to chromosome 15q13–q15. Gremlin exists in both secreted and cell-associated (e.g. membrane associated) forms. Gremlin inhibits BMP signaling via binding to and forming heterodimers with BMP-2, BMP-4, and BMP-7. The binding of gremlin to BMPs prevents BMP ligand–receptor interaction and subsequent downstream signaling. In tissues a delicate balance exists between BMP activity and its inhibition through spatial and temporal expression of specific BMPs and the BMP inhibitors. BMP antagonists such as gremlin, play an important role in regulating multiple cell functions both during early development and in adult tissues. Gremlin inhibition of BMPs is important for the development of limb and retina. Gremlin knockout mice are neonatally lethal as they lack kidneys and have lung defects. In adults, gremlin is responsible for regulating cell proliferation and stem cell differentiation [69].

In addition to the ability of gremlin to directly bind and inhibit BMP action, gremlin may exert direct effects on cell function via BMP-independent mechanisms. Exogenous gremlin may bind to and act directly on endothelial cells to modulate angiogenesis including endothelial cell migration. Recently, it was also reported that gremlin can physically interact with VEGF

receptor 2 to activate VEGF signaling [70]. Thus a receptor-mediated mechanism of action may exist for gremlin. Gremlin interacts with Slit proteins and acts as a direct negative regulator of monocyte chemotaxis [71].

The involvement of gremlin in various diseases has primarily centered on fibrotic changes in the kidney, lung, liver, and osteoarthritis. The most widely studied fibrotic disease is kidney fibrosis where neutralization of BMP7 via gremlin increased the expression of FN and COL3. In addition, both gremlin and connective tissue growth factor (CTGF) are upregulated by TGFβ1 in kidneys of diabetic animals. With respect to the pathophysiology of ocular diseases there are reports that elevated glucose, mechanical strain, and TGFβ2 stimulate gremlin expression in retinal pericytes [72]. Thus the involvement of gremlin in ocular diseases such as diabetic retinopathy (e.g. high glucose levels) and glaucoma (e.g. elevated TGFβ2 in aqueous humor, TM and the optic nerve head and mechanical strain) is of great interest [69].

We have reported that BMP4 selectively counteracts the action of TGFβ2 on ECM-related proteins in cultured human TM cells [42]. Thus, it appears that BMP4 may play a role in maintaining the normal function of the TM by modifying the fibrotic actions of TGFβ2. Gremlin inhibits BMP4 activity in cultured TM cells and increases outflow resistance in a perfusion cultured human eye anterior segment model. Significantly, we noted that both gremlin mRNA and protein are increased in glaucomatous human TM cell lines [42]. We have suggested that, in POAG, elevated gremlin expression by TM cells inhibits BMP4 antagonism of TGFβ2 leading to increased ECM deposition and elevated IOP. However, whether gremlin alone can induce pro-fibrotic changes in the TM cells is not very well established. Also, the molecular mechanism of gremlin upregulation of ECM proteins is also not clear. The following chapters address these questions.

Lysyl Oxidase (LOX) and LOX Like (LOXL) Genes

The ECM environment of the TM and other connective tissues is very dynamic and involves constant ECM turnover. The ECM turnover and remodeling is highly regulated and involves active deposition, crosslinking and degradation of the ECM components. ECM crosslinking involves enzyme-mediated inter-chain or intra-chain covalent bond formation between ECM molecules (collagen, elastin, fibronectin), thereby making them more resistant to degradation [73-74]. Lysyl oxidase (LOX) and lysyl oxidase like 1-4 (LOXL1-4) are a family of elastin and collagen crosslinking enzymes. As the name suggests these enzymes covalently link the lysine residues in elastin and collagen fibers [75]. The crosslinking activity of these genes resides within their C-termini which are 100% conserved amongst the five LOX genes. The N-terminus regions between the various LOX genes are not fully conserved, which may confer specific activities that are yet to be determined.

The role of LOX in the fibrosis of heart and liver has been known for a long time. These fibrotic diseases are associated with extensive remodeling of elastin and collagen fibers. It is likely that these enzymes also play a crucial role in other fibrotic diseases such as glaucoma. Elastin, fibrillins, and collagen are substrates for the LOX enzymes, and increased levels of these molecules in glaucoma TM tissues may be due to decreased turnover of these cross-linked molecules. A recent report used atomic force microscopy was used to show that TM tissues from glaucoma eyes are significantly stiffer than the TM from non-glaucoma eyes [76]. Greater cross-linking of the TM ECM could lead to greater tissue stiffness. Overexpression of both TGFβ1and TGFβ2 has been shown to increase ECM crosslinking. Other ECM crosslinking enzymes like transglutaminase 2 (TGM2) have been previously reported as downstream targets of TGFβ ligands in both connective tissues and the TM [77-79]. TGFβ induction of the 2 major classes of

ECM cross-linking enzymes, LOXs and TGM2, may be involved in enhanced ECM deposition and greater TM stiffness, which ultimately may be responsible for the increased outflow resistance and elevated IOP in POAG.

There is a confirmed genetic association of LOXL1 with exfoliation glaucoma [80-81]. Allele frequency differences in LOXL1 SNPs significantly increase the risk for developing exfoliation glaucoma. Interestingly, TGFβ1 has been reported to be elevated in the aqueous humor of patients with exfoliation syndrome and exfoliation glaucoma [82], and we have shown that TGFβ1 enhances LOXL1 expression in the TM. Exfoliation syndrome and exfoliation glaucoma are associated with increased levels of exfoliation material in the anterior segment and elsewhere in the body [83]. This exfoliation material comprises a variety of ECM proteins, including elastic fibrillins, among others. It is possible that the LOXL1 protein of exfoliation glaucoma patients has altered cross-linking activity, which may cause the formation and deposition of exfoliation material in the anterior segment leading to compromised aqueous outflow and elevated IOP.

LOX and LOXL1 enzymes are over-expressed in several types of tissue fibrosis [75, 84-86]. Therefore, understanding the roles of LOX and LOXL enzymes in diseases like glaucoma may have broader implications for other serious fibrotic diseases.

SPECIFIC AIMS:

Glaucoma is the leading cause of irreversible vision loss and blindness affecting about 60 million people worldwide [1-3]. Among the multiple risk factors that have been identified, elevated IOP is considered as the primary causative risk factor for development and progression of the disease. Increased IOP is considered to be the result of decreased outflow of aqueous humor (AH) through the TM [5, 87-89]. Elevated IOP damages the retinal ganglion cell axons resulting in eventual vision loss. Eight independent studies have reported elevated levels TGFβ2 in the AH of primary open angle glaucoma patients. We have previously reported elevated levels of BMP antagonist gremlin in glaucomatous TM cells and tissues. Both TGFβ2 and gremlin increase IOP in isolated perfusion cultured human anterior eye segments [42, 50]. Increased TGFβ and Gremlin are classically associated with fibrotic diseases of the lung, liver and kidney and are associated with heavy ECM remodeling [90-93]. Previous studies have identified ECM remodeling in the glaucomatous TM as well as in TGFβ2-treated human eye cultures [8, 30-31]. Our laboratory has previously demonstrated that gremlin blocks BMP4 inhibition of TGFβ2-mediated ECM remodeling in cultured TM cells [42-43].

Lysyl oxidase (LOX) and LOX-like (LOXL) 1-4 is a family of collagen and elastin-crosslinking enzymes associated with fibrosis of the heart, lung and kidney [91, 94-98]. Interestingly, TGFβ1 has been reported to induce LOX overexpression in some fibrotic pathogenesis [97, 99-100]. The expression of LOXs and the role of TGFβ2 regulation of LOXs have not been reported previously in TM.

Both TGFβ2 and gremlin have profibrotic effects on the TM. However, the role and signaling mechanisms employed by gremlin to upregulate the ECM genes and proteins in the TM cells are

not very clear. Also, to the best of our knowledge TGFβ2 induction of LOXs and the signaling pathways employed by TGFβ2 to regulate LOXs have not been studied in great detail.

We hypothesize that TGFβ2 and Gremlin employ both Smad dependent and independent signaling pathways to induce several ECM changes in the TM. The ECM changes include induction of ECM-related proteins like FN1, COL1, PAI1 and ELN as well as the LOX family of cross-linking enzymes (Fig. 3-4).

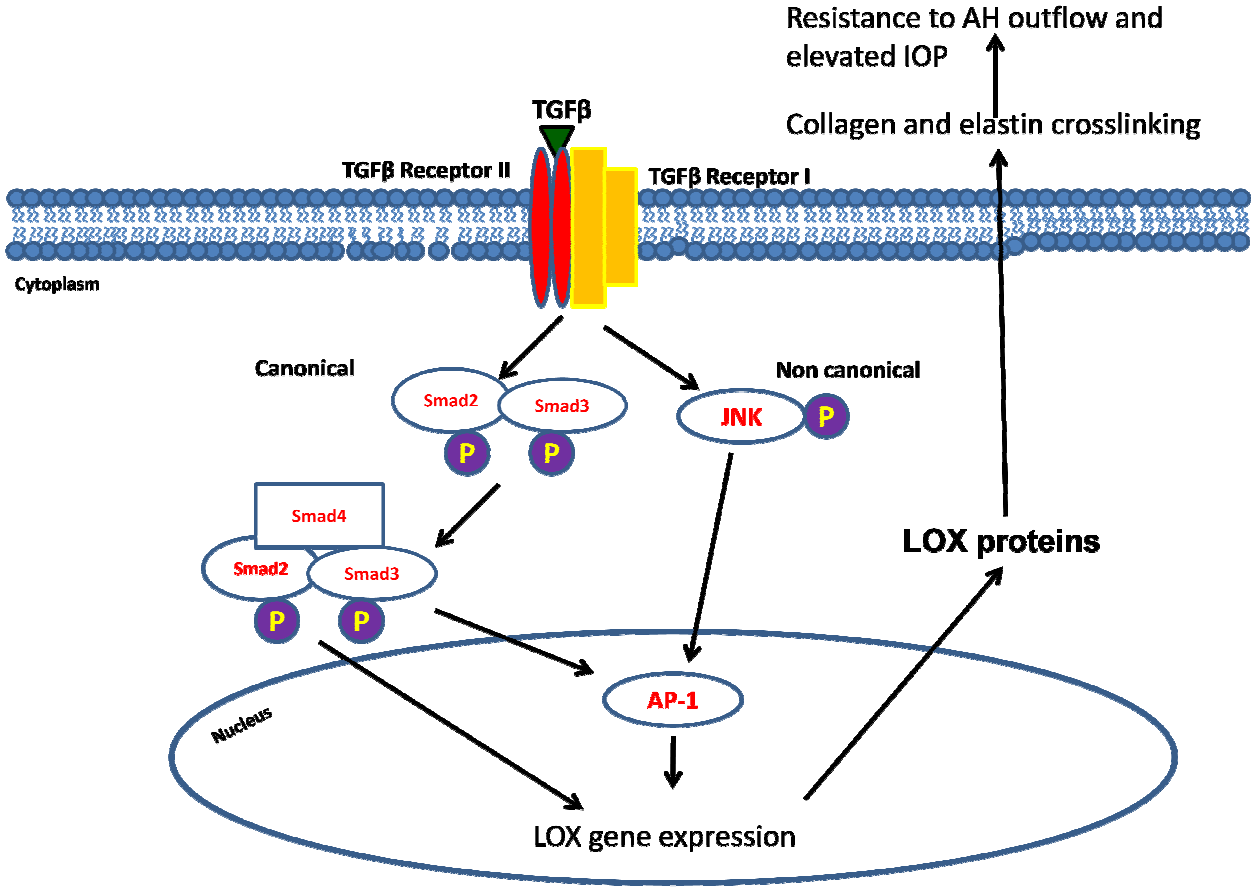

Figure 3: Hypothesis

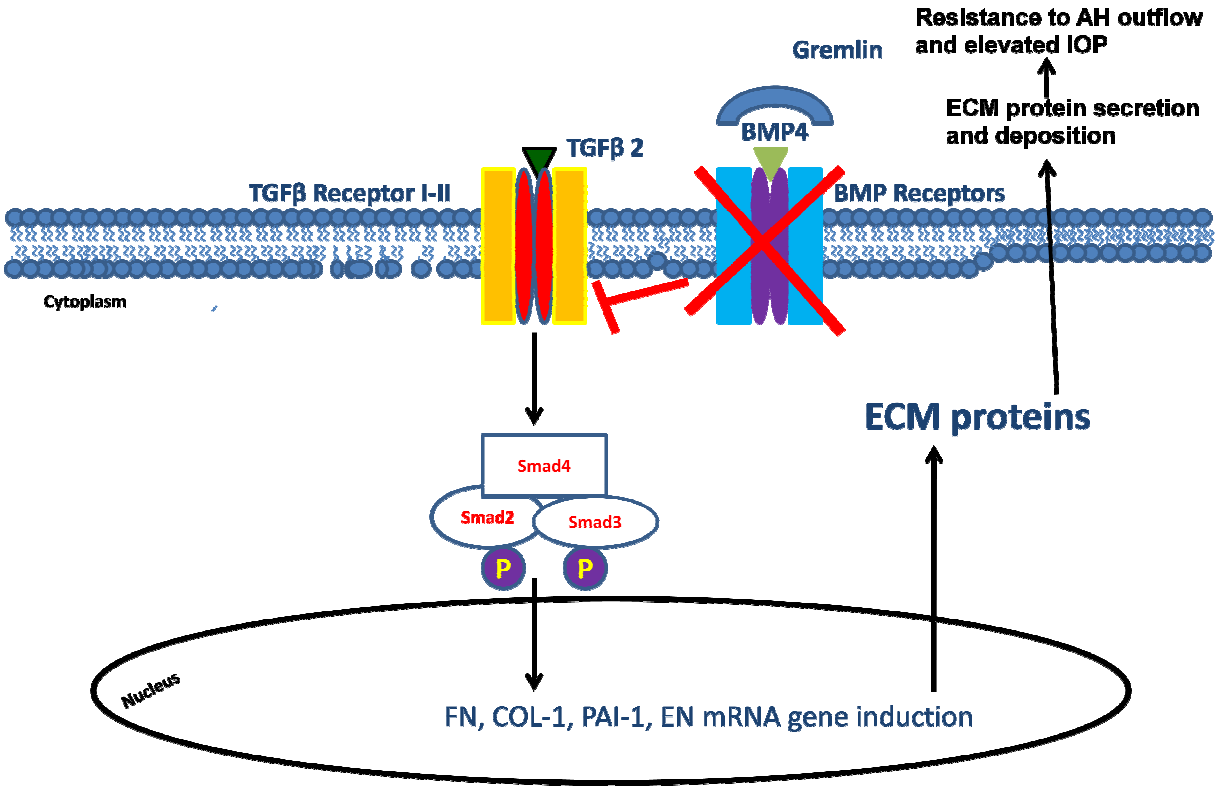

Figure 4: Hypothesis

We propose to determine ECM crosslinking effects of LOXs in cultured human TM cells. We propose to determine the role of TGFβ2 signaling in LOXs induction. We also aim to study the TGFβ2 signaling mechanisms employed by gremlin to upregulate ECM proteins in the TM cells. To address these goals the following specific aims have been designed:

1. ***To determine the signaling mechanism(s) of TGFβ-mediated induction of LOXs***

Confluent TM cells will cultured and treated with 5 ng/ml of TGFβ1-3 for 12 and 48 hours for isolating total RNA and cellular proteins. The RNA will be subjected to reverse transcription (RT) followed by quantitative real-time PCR (qRT-PCR) and proteins will be subjected to western immunoblotting for analyzing LOX genes and proteins. We will also analyze TGFβ1-3 concentration- and time-dependent induction of LOXs mRNA and proteins. We will further

inhibit Smad2-3, JNK and AP-1 signaling by small molecule inhibitors and siRNA to determine their role in TGFβ induction of LOXs. Lastly, we will also analyze the elastin cross-linking activity of LOX proteins in TM cells.

2. ***To determine the role of Gremlin in inducing ECM proteins***

Confluent TM cells will cultured and treated with 1 μg/ml of recombinant gremlin for 24 hours for isolating total RNA and cellular proteins. The RNA will be subjected to RT followed by qRT-PCR and cellular proteins will be subjected to western immunoblotting for analyzing ECM proteins like FN, COL1, ELN and PAI1. We will also determine gremlin concentration- and time-dependent induction of ECM genes mRNA and proteins. We will further inhibit TGFBR1, TGFβ2, Smad2-3-4, and CTGF signaling by small molecule inhibitors and/or siRNA to determine their role in gremlin induction of LOXs.

Significance: This will be the first study focusing on the significance of TGFβ2/Gremlin signaling pathways in regulating several aspects of ECM environment in TM cells. This will also be the first study aimed at understanding the role and regulation of LOXs in the TM. This study will be useful in understanding the specific TGFβ2/Gremlin mechanisms involved in glaucoma development that can be targeted therapeutically.

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Chapter II

TRANSFORMING GROWTH FACTOR BETA INDUCES EXTRACELLULAR MATRIX PROTEIN CROSSLINKING LYSYL OXIDASE (LOX) GENES IN HUMAN TRABECULAR MESHWORK CELLS*

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Abstract

Purpose: The profibrotic cytokine TGFβ is associated with glaucoma and plays an important role in the regulation of extracellular matrix metabolism in the trabecular meshwork (TM). The purpose of this study is to determine whether expression of the ECM cross-linking LOX genes is regulated by TGFβ in the TM cells.

Methods: Expression of the 5 LOX genes (LOX, LOXL1, LOXL2, LOXL3, and LOXL4) was examined in cultured human TM cells using RT-PCR, quantitative RT-PCR, and western immunoblotting. TM cells were treated with recombinant TGFβ1-3 to determine the effects on LOX and LOXL1-4 expression. TM cells were pretreated with TGFBR inhibitors (LY364947, SB431542), inhibitors of the canonical Smad signaling pathway (SIS3 or Smad2/3/4 siRNAs), or inhibitors of the nonSmad signaling pathways (SP600125, SR11302) to identify the signaling pathway(s) involved in TGFβ induction of LOX and LOXL gene and protein expression. A novel LOX activity assay was used to determine the effects of LOX inhibitor BAPN on tropoelastin crosslinking.

Results: All five LOX genes (LOX, LOXL1-4) are expressed in cultured human TM cells and are induced by all three isoforms of TGFβ. This TGFβ induction of LOX and LOXL expression was blocked by TGFR inhibitors as well as by inhibitors of the canonical Smad2/3/4 signaling and nonSmad JNK/AP-1 signaling pathways ($p<0.05$).

Conclusions: Both Smad and nonSmad signaling pathways are involved in TGFβ LOX induction, suggesting complex regulation of these important extracellular matrix cross-linking enzymes. Increased LOX activity may be at least partially responsible for TGFβ-mediated IOP elevation and increased aqueous humor outflow resistance.

Introduction

Glaucoma is a leading cause of irreversible visual impairment and blindness in the world, with primary open-angle glaucoma (POAG) the major form of glaucoma [1-2]. Elevated intraocular pressure (IOP) is a major risk factor for the development and progression of glaucoma [3-4], and this ocular hypertension is due to increased aqueous humor outflow resistance in the trabecular meshwork (TM) and is associated with increased deposition of extracellular matrix (ECM) material within the TM. The profibrotic cytokine TGFβ2 has been implicated in the pathogenesis of POAG [5]. Aqueous humor levels of TGFβ2 are elevated in the aqueous humor [6-8] and TM (Tovar-Vidalez T et al. submitted for publication) of POAG patients. TM cells express TGFβ receptors, and TGFβ2 has direct effects on the TM [9]. TGFβ2 has been shown to increase aqueous outflow resistance and elevate IOP in perfusion cultured human and bovine eyes [10-12] as well as in rodent eyes [13]. TGFβ 1 is elevated in the aqueous humor of exfoliation glaucoma patients [14], suggesting that this TGFβ isoform may be associated with the accumulation of exfoliation material, including ECM proteins, in the anterior segments of patients with this syndrome.

TGFβ2 regulates ECM metabolism in TM cells and tissues. This cytokine increased expression of a variety of ECM proteins, including fibronectin, collagen, elastin, and proteoglycans as well as increased levels of PAI-1 and TIMP-1, inhibitors that suppress proteolytic degradation of the ECM [15]. In addition, TGFβ2 increased expression of the ECM cross-linking enzyme transglutaminase-2 (TGM2) in TM cells [16]. The combination of increased ECM synthesis, increased crosslinking, and decreased degradation would cause increased ECM deposition in the TM, which may be responsible for the TGFβ2-mediated increased resistance to aqueous humor outflow. Similar changes occur in the TM of POAG patients, with increased levels of fibronectin [17], collagen [18], PAI-1 [19], and TGM2 [20].

In addition to TGM2, there is a second important class of ECM cross-linking enzymes. The lysyl oxidase (LOX) family contains 5 genes (LOX and LOXL1-4) encoding enzymes that covalently cross-link elastin and collagens via generation of aldehydes on lysine residues [21-22]. This cross-linking reaction provides additional mechanical strength to the ECM and also makes the ECM more resistant to degradation. LOX enzymes play a role in a variety of fibrotic diseases [21-25]. Single nucleotide polymorphisms (SNPs) in LOXL1 are associated with significantly increased risk for developing exfoliation glaucoma [26-27] further suggesting potential roles for LOXs in glaucoma pathogenesis. The purpose of the present study was to determine: (1) whether the LOX and LOXL genes and proteins are expressed in human TM cells, (2) whether TGFβ induces LOX gene expression and activity in the TM, and (3) which TGFβ signaling pathway(s) regulate LOX expression in the TM.

Methods

TM Cell Culture

Human TM cells were isolated from carefully dissected human TM tissue explants derived from patients with glaucoma or from normal donors and characterized as previously described [9]. All donor tissues were obtained from regional eye banks and managed according to the guidelines in the Declaration of Helsinki for research involving human tissue. Isolated TM cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) containing L-glutamine (0.292 mg/mL; Gibco BRL Life Technologies), penicillin (100units/ml)/streptomycin (0.1 mg/mL; Gibco BRL Life Technologies), and 10% fetal bovine serum (Gibco BRL Life Technologies).

TM Cell Treatments

TM cells were grown to 100% confluency and then substituted to serum free medium for 24 hours prior to treatments to avoid effects of serum proteins on treatments. TM cells were incubated with fresh medium containing specific signaling inhibitors for 1-12 hrs. prior to the addition of varying concentrations of recombinant human TGFβ-1, 2 or 3 proteins (R&D System, Minneapolis, MN). Small molecule inhibitors LY364947 (5μM, Cat. No. 2718, Tocris biosciences, Ellisville, MO) and SB431542 (5μM, Prod. No. S4317, Sigma-Aldrich, St. Louis, MO) were used to examine the effects of inhibition of TGFβ Receptor-1/2. Smad-3 phosphorylation inhibitor SIS3 (10μM, Prod. No. S0447, Sigma-Aldrich, St. Louis, MO), JNK inhibitor SP600125 (10μM, Prod. No. S5567, Sigma-Aldrich, St. Louis, MO), and AP-1 inhibitor SR11302 (5μM, Cat. No. 2476, Tocris biosciences, Ellisville, MO) were used to examine effects of inhibition on canonical Smad, JNK, and AP-1 signaling. Varying concentrations of the LOX

inhibitor β-aminopropionitrile (BAPN) (Prod. No. A3134, Sigma-Aldrich, St. Louis, MO) were used for the LOX activity assay.

Small Interfering RNA and Transfection

SMARTpool siRNAs for Smad2, Smad3, Smad4, TGFBR1 and non-targeting control siRNAs were purchased from Dharmacon (Lafayette, CO). Transfection of siRNA was performed as described previously [28-29]. Three TM cell strains were grown in 12-well plates containing DMEM with 10% FBS. In one tube, 4 μL of DharmaFECT 1 Transfection Reagent (T-2001-01; Dharmacon, CO) was mixed gently with 200 μL of Opti-MEM medium (Invitrogen) and incubated for 5 min at room temperature. In separate tubes, various concentrations of siRNA were mixed gently with 200 μL of Opti-MEM medium. These two tubes were combined, gently mixed, and incubated for 20 min at room temperature. After incubation, DMEM without FBS and antibiotics was added to obtain a final volume of 2 mL for each well (10 nM of test siRNA and 10 nM of control siRNA). Cells were washed with sterile PBS and incubated with siRNA transfection solution for 24 h at 37°C. Cells were washed with sterile PBS and incubated with 10% FBS containing DMEM for 24 h at 37°C. Cells were then washed with serum-free DMEM medium for 24 hours and treated with TGFβ2 in serum-free DMEM medium for 48 h. Cell lysates were analyzed for various proteins by the western immunoblotting (Table 1).

RNA isolation, RT-PCR and agarose gel electrophoresis

Total cellular RNA was prepared from cultured TM cells using TRI Reagent RT extraction (Cat. # RL-311, MRC Inc., Cincinnati, OH), and the SuperScript VILO cDNA Synthesis kit (Cat. # 11754, Invitrogen) was used for first strand cDNA synthesis with 1 μg of total RNA. Primers for the various LOX proteins were designed using Primer3 software

(http://frodo.wi.mit.edu/primer3/). The primer pairs, expected product sizes, and annealing temperatures are listed in Table 2. The PCR products were loaded and electrophoresis performed on a 1.5% agarose gel.

Quantitative Real Time PCR

Real-time PCR was performed as described previously [30]. Briefly, 2.5 µL (approx. 200 ng) of cDNA was used in a reaction consisting of 1.5 units per reaction of antibody-bound Taq enzyme (Jump Start; Sigma-Aldrich, St. Louis, MO), 10x PCR buffer, 1.5 mM $MgCl_2$, 200 nM dNTP mix, 100 nM respective primers, 2.5 µL green nucleic acid dye (EvaGreen; Biotium, Hayward, CA), as well as 30 nM passive reference dye (Rox; USB, Cleveland, OH) per 50-µL reaction. PCR was performed on a real-time thermal cycler (model Mx3000p; Stratagene, La Jolla, CA), with cycling parameters of initial denaturation at 95°C; 40 cycles of 95°C 30 seconds, 60°C 30 seconds, and 72°C 60 seconds, and a denaturation cycle for creation of a dissociation curves. Reactions for each sample and gene of interest were run in duplicate, cycle thresholds (Ct) were normalized to beta-actin expression as a housekeeping gene, and comparative quantitation was performed using MxPro ver. 4.0 software (Stratagene). Only individual PCR samples with single-peak dissociation curves were selected for data analysis.

Protein Extraction and Western Immunoblot Analysis

Total cellular protein was extracted from the TM cells using mammalian protein extraction buffer (MPER, Cat # 78501; Pierce Biotech, Rockford, IL) containing protease inhibitor (Cat. # 78415, Pierce Biotech) and phosphatase inhibitor (Cat. # 78420, Pierce Biotech) cocktails. Protein concentration was determined using the Bio-Rad Dc protein assay system (Cat. # 500-0111, Bio-Rad Laboratories, Richmond, CA). The cellular proteins were separated on denaturing

polyacrylamide gels and then transferred to PVDF membranes by electrophoresis. Blots were blocked with 5% Fat-free Dry Milk in tris-buffered saline tween buffer (TBST) for 1 h and then incubated overnight with primary antibodies (Table 1). The membranes were washed with TBST and processed with corresponding horseradish peroxidase-conjugated secondary antibodies (Table 2). The proteins were then visualized in a Fluor ChemTM 8900 imager (Alpha Innotech, San Leandro, CA) using ECL detection reagent SuperSignal West Femto Maximum Sensitivity Substrate (Cat. # 34096, Pierce Biotechnology). To ensure equal protein loading, the same blot was subsequently developed for β-actin and GAPDH expression.

Statistical Analysis

For comparing results between two groups Student's t test was performed. For comparison of results between more than two groups One-Way ANOVA was employed. Data reported are mean ± standard deviation.

Results

Expression of LOX family members in TM cells

The expression of all five members of the LOX gene family in the TM has not been previously studied. Therefore, we determined whether LOX and LOXL mRNA and proteins are expressed in the cultured human TM cells. Utilizing RT-PCR, we profiled the cDNA samples obtained from eleven TM cell strains (Fig. 1A), and the protein expression was studied in six TM cell strains (Fig. 1B). LOX and LOXL1-4 mRNA (Fig. 1A) and LOX, LOXL1,2,4 proteins were expressed in multiple TM cell strains, although there appeared to be differences in their basal LOX protein expression among the TM cell strains. We could not study LOXL3 protein expression due to the lack of a commercially available antibody.

TGFβ1-3 induce LOXs in TM cells

TM cells were treated with TGFβ1-3 (5 ng/ml) for 12 hours followed by RNA extraction and qRT-PCR. Each TGFβ isoform significantly induced LOX and LOXL1-4 mRNA (n=3, $p<0.05$)(Fig. 2A). We also treated six TM cell strains with TGFβ1-3 (5 ng/ml of) for 48 hours, and western immunoblotting of TM cell lysates was used to study the effects on LOX and LOXL protein expression. TGFβ1-3 induced LOX, LOXL1, LOXL2 and LOXL4 protein expression in all the TM cell strains tested. The blot represents data generated in two of the six cell strains employed for the experiment (Fig. 2B).

TGFβ1-3 induce LOXs in a concentration- and time-dependent fashion

TM cell strains (n=3) were treated with increasing concentrations of TGFβ1-3 (0-10 ng/ml) for 48 hours. The mRNA and protein expression of LOX and LOXLs were determined using qRT-PCR and western immunoblotting respectively. TGFβ1 (Figs. 3A-B), TGFβ2 (Figs 3C-D), and TGFβ3 (Figs. 3E-F) each induced LOX and LOXL1-4 mRNA and LOX, LOXL1, 2, and 4 protein expression in a concentration-dependent manner. The maximum induction for mRNA appeared to be around 10 ng/ml while the maximum induction of proteins was seen at around 1 ng/ml. TM cells were treated with TGFβ1-3 for 6, 12 and 48 hours to examine whether TGFβ induces LOXs mRNA in a time-dependent manner. The greatest mRNA induction of LOXs was observed at 12 hours ($p<0.05$), and by 48 hours there was little or no statistical difference between untreated and TGFβ1-3 treated groups (Figs. 4A, C, E). Similarly, TM cell strains (n=2) were treated with TGFβ1-3 (5 ng/ml) for 6, 12, 24, 48 and 72 hours to evaluate effects on LOX and LOXL protein expression. TGFβ ligands induced LOX AND LOXL proteins as early as 24 hours and maintained the induction up to 72 hours (Fig. 4B, D, F). Therefore, TGFβ induction of LOX and LOXL gene and protein expression was both time and dose dependent.

TGFβ signaling in LOX and LOXL induction

We employed various small molecule inhibitors to determine the TGFβ signaling pathway(s) involved in LOX and LOXL induction. SB431542 is a widely used selective TGFBR1 and TGFBR2 receptor inhibitor [31]. LY364947 is a relatively selective inhibitor for the TGFBR2 receptor [32]. We treated TM cell strains (n=3) with recombinant TGFβ1-3 (5 ng/ml) for 12 hours with or without one hour pre-treatment with 5 μM SB431542 or LY364947. Total RNA was isolated for qRT-PCR analysis. Each TGFβ isoform elevated LOX and LOXL1-4 expression

compared to untreated or inhibitor only-treated samples ($p<0.01$). Pretreatment with either of the two inhibitors, LY364947 (Fig. 5A) or SB431542 (Fig. 5C) blocked the TGFβ-mediated induction in all the cell strains ($p<0.05$).

We also treated three TM cell strains (n=4) with or without TGFβ1-3 (5 ng/ml) for 48 hours, with or without a one hour pre-treatment with 5 μM SB431542 or LY364947. The LOX and LOXL proteins were analyzed using western immunoblotting. TGFβ1-3 each elevated LOX and LOXL1, 3, 4 compared to untreated or vehicle-treated samples. Each of the two inhibitors, LY364947 (Fig. 5B) or SB431542 (Fig. 5D), inhibited the TGFβ-mediated induction. Treatment with the inhibitors alone did not have any effect on the LOX and LOXL expression (data not shown).

In addition to these TGFBR1/2 inhibitors, we also used siRNA-mediated TGFB receptor 1 (TGFBR1) knockdown to confirm the role of TGFβ receptor signaling in LOX and LOXL induction. TGFβ2-treated TM cells were untransfected or transfected with a non-targeting siRNA control, or TGFBR1 siRNA. As previously shown, TGFβ2 induced LOX and LOXL protein expression. Control siRNAs did not affect endogenous TGFBR1 levels and did not affect TGFβ2-induction of LOX and LOXL expression. Consistent with the data with small molecule TGFBR1 or TGFBR2 inhibition, TGFBR1 knockdown inhibited TGFβ2-induction of LOX and LOXL proteins (Fig. 5E). These results strongly support TGFβ receptor-dependent regulation of LOX and LOXL protein expression.

TGFβ induces LOX and LOXLs utilizing both Smad and JNK signaling pathways

The profibrotic cytokine TGFβ has been shown to activate both canonical Smad and non-canonical signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway in various cells and tissues [33-35]. TGFβ signaling is complex because these different signal transduction pathways can interact with each other [36-37]. We wanted to determine which of these TGF□ signaling mechanism(s) are involved in LOX and LOXL induction in TM cells.

TGFβ2 activates canonical Smad and MAPK signaling in the TM cells [38]. Three primary TM cell strains were treated with TGFβ1-3 (5 ng/ml) for 15, 30, 60, 120 and 240 minutes, and total and phosphorylated Smad2, Smad3, and JNK1/2 proteins were evaluated by western immunoblotting. All three TGFβ ligands phosphorylated both Smad2 (Fig. 6A), Smad3 (Fig. 6B), and JNK1/2 (Fig. 6C) proteins during this time course. There were no changes in total Smad2, Smad3, or JNK1/2 levels.

Phosphorylated Smads 2 and 3 form a complex with co-Smad4 to regulate transcription of their target genes. To determine if the Smad2/3 complex transcriptionally regulates the LOXs, we employed a selective small molecule inhibitor of Smad3, SIS3. Three TM cell strains were treated with SIS3 (10 μM) six hours prior to treating with recombinant human TGFβ1-3 for 12 or 48 hours to study mRNA and protein expression of LOXs, respectively. Untreated cells, DMSO-treated cells, and SIS3 alone treated cells were the negative controls. TGFβ-induction of LOX and LOXL mRNA and protein expression were inhibited by SIS3 pretreatment ($p<0.01$) (Figs. 7A & 7B). Therefore, TGFβ mediated Smad2/3 signaling in TM cells induced all members of the LOX gene family.

To confirm the role of Smad signaling in regulation of LOX expression, we employed siRNA-mediated knockdown of Smad2, Smad3, and Smad4. Non-targeting siRNA served as the

negative control. Cells transfected with Smad2 (Fig. 7C), Smad3 (Fig. 7D), or Smad4 (Fig. 7E) siRNAs were subsequently treated with or without TGFβ2. Untransfected and untreated cells served as negative controls, while untransfected cells treated with TGFβ2 served as the positive control. TGFβ2 induced expression of LOX and LOXL proteins. Control siRNAs neither affected TGFβ2-induction of LOXs nor did they affect the endogenous Smad2/3/4 levels. As expected, knockdown of Smads 2, 3, or 4 inhibited TGFβ2-induction of the LOX and LOXL proteins.

In addition to activating Smad signaling, TGFβ also activated the non-Smad JNK1/2 signaling pathway (Fig. 6C). We used the selective JNK inhibitor SP600124 to determine whether TGFβ-activated JNK signaling regulates LOX and LOXL induction. Three TM cell strains were pretreated for 6 hours with SP600125 (10 μM) prior to incubation with or without recombinant human TGFβ1-3 (5 ng/ml) for 12 and 48 hours, to study LOX and LOXL mRNA and protein expression, respectively. Untreated cells, DMSO-treated cells, and SP600125-only treated samples served as negative controls. SP600125 pretreatment inhibited TGFβ-induction of LOX and LOXL mRNA ($p<0.05$) (Fig. 8A) and protein (Fig. 8B) expression in TM cells. SP600125 alone did not alter endogenous LOX or LOXL mRNA (Fig. 8A) or protein (data not shown) expression. Taken together, these data strongly suggest that both Smad and JNK signaling regulate LOXs in TM cells.

AP-1 regulates TGFβ-induction of LOXs

The transcription factor AP-1 is comprised of two units, c-Fos and c-Jun. The Jun subunit is phosphorylated by active JNK1/2, which subsequently activates transcription of AP-1 target genes [39]. However, AP-1 also acts as a co-transcription factor of other gene regulators like the Smad2/3/4 complex [40]. Our data strongly indicate that both Smad (Fig. 7) and JNK1/2 signaling (Fig. 8) regulate LOX and LOXL expression, so we wanted to determine the role of AP-1 as the transcriptional regulator of the LOXs. We treated TM cell strains (n=3) with SR11302 (5μM), a small molecule AP-1 inhibitor [41], for twelve hours prior to treatment for 12 or 48 hours with 5ng/ml of TGFβ1-3. The 12 hour TGFβ treatment group was analyzed for effects on mRNA and the 48 hour treatment group for effects on LOX and LOXL protein expression. Pretreatment with the AP-1 inhibitor significantly reduced the TGFβ1-3-induction of LOXs and LOXL mRNA ($p<0.01$) (Fig. 9A) and protein (Fig. 9B) expression. These data indicate that regulation of LOX genes by TGFβ involves both canonical and non-canonical signaling pathways, which may interact by sharing common transcription factors like AP-1.

TGFβ regulates LOX enzymatic activity in the cultured TM cells

Finally, we wanted to determine whether TGFβ regulates the enzymatic activity of LOXs in TM cells. Since LOXs catalyze elastin and collagen cross-linking, we wanted to determine the potential role of LOXs in TGFβ cross-linking of elastin in the TM cells. We utilized the irreversible LOX inhibitor, β-aminopropionitrile (BAPN), to block elastin cross-linking [42-43]. TM cells were treated for 48 hours with increasing concentrations of BAPN, and cell lysates were analyzed by western immunoblotting for tropoelasin, the soluble non-crosslinked form of elastin. BAPN should decrease the crosslinking of elastin leading to higher levels of the substrate,

tropoelastin. Indeed, we observed that increasing concentrations of BAPN elevated tropoelastin levels in the TM cells (Fig. 10A) (n=3).

We next examined the role of TGFβ-induced LOXs in regulating elastin crosslinking. TM cells were treated with increasing concentrations of TGFβ2 for 48 hours, and levels of tropoelastin were determined by western immunoblotting. Increasing TGFβ2 concentrations increased tropoelastin in the TM cells (Fig. 10B). We followed these studies by treating TM cells with increasing concentrations of TGFβ2 along with BAPN (1 mM) for 48 hours. Cells treated with TGFβ2 and BAPN showed greater tropoelastin levels, even at lower TGFβ2 concentrations (Fig. 10C). We observed similar results with TGFβ1 and TGFβ3 treatment (data not shown). In addition to inducing LOX and LOXL expression, TGFβ2 also increased tropoelastin expression. Since BAPN is an irreversible inhibitor of LOX, it blocks the LOX enzymatic activity, and appears to result in higher levels of the LOX substrate tropoelastin, both in the absence and presence of TGFβ2.

We also used the reverse strategy, treating TM cells with a single concentration of TGFβ2 (5 ng/ml) along with increasing concentrations of BAPN. TGFβ2 elevated tropoelastin levels. However, cells co-treated with varying BAPN concentrations further increased tropoelastin levels (Fig. 10D). The effect of BAPN appeared to be concentration-dependent at 1-10 mM BAPN concentration but not at 30 mM, perhaps due to BAPN toxicity at this concentration. TGFβ2 induced both tropoelastin and LOXs, but inhibiting LOXs decreased tropoelastin crosslinking thereby increasing levels of uncrosslinked tropoelastin. Similar results were observed with TGFβ1 and TGFβ3 (data not shown), indicating that the three TGFβ ligands

increase LOX activity in TM cells. Taken together, these data show that LOXs are enzymatically active in TM cells and that TGFβ regulates LOX activity.

Discussion

We have shown that all 5 LOX genes are expressed in multiple human TM cell strains and that all 3 TGFβ isoforms induce mRNA and protein expression of these LOX and LOXL genes. We developed a novel LOX activity assay and have shown basal LOX enzyme activity in TM cells, which can be further induced by TGFβ. Finally, we demonstrated that, canonical Smad as well as non-Smad JNK1/2 and AP-1 signaling pathways are involved in the TGFβ induction of the LOX and LOXL genes. Figure 11 schematically summarizes these data. It is somewhat surprising to find that all 5 LOX genes are expressed in the TM and induced by TGFβ. Why would the TM require this redundancy in this class of enzymes? The LOX and LOXL enzymes may have subtle differences in enzyme activity, secondary regulation, and/or cellular or tissue localization, which warrants further investigation.

These LOX enzymes may play a role in the pathogenesis of glaucoma. TGFβ2 levels are higher in the aqueous humor [5-8] and TM (Tovar-Vidales et al. submitted for publication) of POAG patients, and TGFβ2 also regulates ECM metabolism in the TM [11, 15-16]. The TM of POAG patients has increased elastic sheath derived material [44-45] and increased type VI "curly" collagen [18]. Elastin, fibrillins, and collagen are substrates for the LOX enzymes, and increased levels of these molecules in glaucoma TM tissues may be due to decreased turnover of these cross-linked molecules (Fig. 11). A recent report used atomic force microscopy to show that TM tissues from glaucoma eyes are significantly stiffer than the TM from non-glaucoma eyes [46]. Greater cross-linking of the TM ECM could lead to greater tissue stiffness. TGFβ induction of the 2 major classes of ECM cross-linking enzymes, LOXs and TGM2, may be involved in enhanced ECM deposition and greater TM stiffness, which ultimately may be responsible for the increased outflow resistance and elevated IOP in POAG.

There is a confirmed genetic association of LOXL1 with exfoliation glaucoma [26-27]. Allele frequency differences in LOXL1 SNPs significantly increase the risk for developing exfoliation glaucoma. Interestingly, TGFβ1 has been reported to be elevated in the aqueous humor of patients with exfoliation syndrome and exfoliation glaucoma [14], and we have shown that TGFβ1 enhances LOXL1 expression in the TM. Exfoliation syndrome and exfoliation glaucoma are associated with increased levels of exfoliation material in the anterior segment and elsewhere in the body [47]. This exfoliation material comprises a variety of ECM proteins, including elastic fibrillins, among others. It is possible that the LOXL1 gene of exfoliation glaucoma patients has altered cross-linking activity, which may cause the formation of exfoliation material in the anterior segment leading to compromised aqueous outflow and elevated IOP. The morphologic changes in exfoliation glaucoma may be due to TGFβ1 induced LOX crosslinking of exfoliation material in the outflow pathway. In contrast, elevated TGFβ2 levels in POAG may activate LOXs to crosslink endogenous ECM molecules in the TM. Therefore, it is not surprising that the two forms of glaucoma have differing morphologies.

TGFβ1 and TGFβ2 are profibrotic cytokines that play a pathogenic role in other fibrotic diseases, such as sclerosis, fibrosclerosis, as well as kidney, lung, and liver fibrosis. Similar to POAG, the fibrotic changes at the cellular and tissue level arise from disordered and exaggerated deposition of the ECM, including collagens, elastin, and fibronectin. Fibrotic ECM remodeling also involves cross-linking of ECM molecules. LOX and LOXL1 enzymes are over-expressed in several types of tissue fibrosis [21-22, 48-49]. Therefore, understanding the roles of LOX and LOXL enzymes in glaucoma may have broader implications for other serious fibrotic diseases.

The potential relationship between the LOX and LOXL genes in regulating aqueous outflow in TGFβ2-induced ocular hypertension, POAG, and exfoliation glaucoma warrants further studies.

Are all 5 LOX genes functionally redundant or does each serve a specific role in TM ECM metabolism? Do any of these LOX genes play a direct role in TGFβ2-induced ocular hypertension? Which LOX genes are more important for normal TM homeostasis and are any of these genes directly involved in glaucoma pathogenesis? Will increased expression of any of the LOX or LOXL genes cause glaucoma-like morphological changes in the TM and directly cause IOP elevation? Our current results provide a foundation to address these issues.

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Tables

Table 1

Antibody	Ab. Dilution	Source
Rabbit Anti-LOX	1:10,000	Novus Biologicals (Cat. # NB100-2530)
Rabbit Anti-LOXL1	1:500	Abnova (Cat. # H00004016-D01P)
Mouse Anti-LOXL2	1:2,000	R&D Systems (Cat. # MAB2639)
Mouse Anti-LOXL4	1:250	Abcam (Cat. # ab25904)
Mouse Anti-ACTB	1:1,000	Millipore (Cat. # MAB1501)
Rabbit Anti-GAPDH	1:1,000	Cell Signaling (Cat. # 2118)
Rabbit Anti-TGFBR1	1:250	Abcam (Cat. # ab67492-100)
Rabbit Anti-SMAD2	1:1,000	Cell Signaling (Cat. # 3107)
Rabbit Anti-Phos-SMAD2	1:1,000	Cell Signaling (Cat. # 3122)
Rabbit Anti-SMAD3	1:1,000	Cell Signaling (Cat. #9532S)
Rabbit Anti-Phos-SMAD3	1:1,000	Cell Signaling (Cat. # 9520S)
Rabbit Anti-SMAD4	1:1,000	Cell Signaling (Cat. # 9515)
Rabbit Anti-JNK1/2	1:1,000	Cell Signaling (Cat. # 9258)
Rabbit Anti-Phos-JNK1/2	1:1,000	Cell Signaling (Cat. # 9251S)
Mouse Anti-ELN	1:500	Millipore (Cat. # MAB2503)
Donkey anti-mouse IgG	1:10,000	Santa Cruz Biotechnology (Cat. # sc-2314)
Goat anti-rabbit IgG	1:10,000	Santa Cruz Biotechnology (Cat. # sc-2004)

Table 1: List of various antibodies used for western immunoblotting studies

Table 2

Gene		Primer (5’ → 3’)
LOX	Left	CGACCCTTACAACCCCTACA
	Right	AAGTAGCCAGTGCCGTATCC
LOXL1	Left	AGAGCCTCTCTGTCCACCAG
	Right	GTACACCTGCCCGTTGTTGTTCT
LOXL2	Left	CCTGGGGAGAGGACATACAA
	Right	CTCGCAGGTGACATTCTTCA
LOXL3	Left	CAACGCGGCCTTCTACAG
	Right	GGTGTCATTGGCACGATAGA
LOXL4	Left	CGACAGCCACTACTACAGGAAA
	Right	CTGGTGGATCCAGAAGGAGTT
ACTB	Left	GTCCACCTTCCAGCAGATGT
	Right	AAAGCCATGCCAATCTCATC

Table 2: List of the primers used for PCR studies

Figure Legends

Figure 1: **Expression of LOX and other LOX Like genes in TM**. (A) RT-PCR on RNA samples from 11 TM cell strains for the five LOX genes and β-actin as the housekeeping gene. The agarose gel image represents data generated in three independent experiments. (B) Western immunoblots of protein samples from 6 TM cell strains. Antibodies specific to LOX, LOXL1, LOXL2 and LOXL4 were used with β-actin (ACTB) as the loading control. The blot represents data from three independent experiments.

Figure 2: **TGFβ1-3 induce LOXs in TM cells**. (A) Induction of LOX/LOXL mRNA in three TM cell strains treated with TGFβ1-3 (5 ng/ml) for 12 hours. The graph values represent the fold induction of LOXs normalized to ACTB. Three replicates of each sample were employed. All 3 TGFβ isoforms induced LOXs in all the three cell lines. Student t test was used for statistical analyses. * $0.01<p<0.05$; ** $0.0001<p<0.01$ and *** $p<0.0001$ (B) Western immunoblots of LOX/LOXL in two TM cell strains treated with TGFβ1-3 (5 ng/ml) for 48 hours. The TGFβ isoforms induced LOX proteins as compared to ACTB. Similar results were observed in four additional TM cell strains. The image is representative for three independent experiments.

Figure 3: **Concentration-dependent TGFβ-induction of LOXs:** Dose dependent induction of LOX and LOXL mRNA (A, C, E) and protein (B, D, F) by 0-10 ng/mL TGFβ1 (A,B), TGFβ2 (C,D), and TGFβ3 (E,F) in cultured TM cell strains (n=3). qRT-PCR values (A, C, E) represent TGFβ fold induction compared to controls and normalized to ACTB as housekeeping gene. Three replicates of each sample were employed. One-Way ANOVA was used for statistical analyses. * $0.01<p<0.05$, ** $0.0001<p<0.01$ and *** represent $p<0.0001$. Western immunoblots

(B, D, F) are representative of data obtained in the three TM cell strains. 5 ng/ml of TGFβ1-3 showed maximum LOX/LOXL induction.

Figure 4: **Time-dependent TGFβ-induction of LOXs:** Time course induction (0-72 hours) of LOX and LOXL mRNA (A, C, E) and protein (B, D, F) 5 ng/mL TGFβ1 (A,B), TGFβ2 (C,D), and TGFβ3 (E,F) in cultured TM cell strains (n=2). qRT-PCR values (A, C, E) represent TGFβ fold induction compared to controls and normalized to ACTB as housekeeping gene. Three replicates of each sample were employed. One-Way ANOVA was used for statistical analyses. * $0.01<p<0.05$, ** $0.0001<p<0.01$ and *** represent $p<0.0001$. Western immunoblots (B, D, F) are representative of data obtained in the three TM cell strains. 5 ng/ml of TGFβ1-3 showed maximum LOX/LOXL induction.

Figure 5: **TGFβ receptor inhibition blocks TGFβ-induction of LOXs. :** Effect of TGFBR inhibitors LY364947 (A,B) and SB431542 (C,D) on TGFβ1-3 induction of LOX/LOXL mRNA (A,C) and protein (B,D) expression. qRT-PCR values (A,C) represent fold gene induction normalized to ACTB as the housekeeping gene in treated samples compared to controls (triplicates of 3 TM strains). One-Way ANOVA was used for statistical analyses. *and # $0.01<p<0.05$, ** and ## $0.0001<p<0.01$, *** and ### represent $p<0.0001$. "*" = differences between TGFβ samples vs. TGFβ + inhibitor samples, while "#" = differences between TGFβ treated and the untreated cells. (B,D) Western immunoblots of TM cells treated with 5 ng/ml of TGFβ1-3 for 48 hours along with 5 μM of LY364947 (B) or SB431542 (D). Untreated and DMSO-treated cells served as negative controls. GAPDH was used as loading control. Blots shown are representative of data from 4 different TM cell strains. (E) Western immunoblots of LOX and LOXL proteins after siRNA mediated TGFBR1 knockdown followed by TGFβ2 treatment. TM cells were treated with TGFBR1 or control siRNA, followed by treatment with 5

ng/ml of TGFβ2 for 48 hours. GAPDH was used as loading control. Blots are representative data from 2 different TM cell strains.

Figure 6: TGFβ activates both canonical and non-canonical signaling pathways in TM cells. Western immunoblots of Smad2/pSmad2 (A), Smad3/pSmad3 (B), and JNK1/2/pJNK1/2 (C) in 4 TM cell strains treated for 0-240 minutes with TGFβ1-3. TGFβ1-3 treatment caused a time-dependent increase in pSmad2, pSmad3, and pJNK1/2 expression.

Figure 7: **Smad2/3/4 inhibition blocks TGFβ-induction of LOXs.** Treatment of TM cells with Smad3 inhibitor SIS3 blocks TGFβ1-3 induction of LOX/LOXL mRNA (A) and protein (B) expression. (A) qRT-PCR analysis of the TGFβ1-3-induction of LOXs in presence of specific inhibitor of Smad3 (SIS3). qRT-PCR values represent fold induction of LOX/LOXL genes normalized to ACTB as housekeeping gene in TGFβ treated samples as compared to controls (triplicates of 3 TM cell strains). One-Way ANOVA was used for statistical analyses. *and # $0.01<p<0.05$, ** and ## $0.0001<p<0.01$, *** and ### $p<0.0001$. "*" = differences between TGFβ1-3 samples vs. TGFβ + inhibitor samples; "#" = differences between TGFβ1-3 treated vs. the untreated cells. (B) Western immunoblots of LOX/LOXL proteins after pretreatment with SIS3 followed by TGFβ treatment. Immunoblots are representative of three different TM cell strains treated with 5 ng/ml of TGFβ1-3 for 48 hours along with 10 μM of SIS3. GAPDH was used as loading control. Untreated and DMSO-treated cells served as negative controls. (C,D,E) Western immunoblots of LOX/LOXL proteins in TM cells pretreated with Smad2 (C), Smad3 (D), or Smad4 (E) siRNAs followed by TGF□2 treatment. Control cells were transfected with non-targeting siRNA. Immunoblots are representative of results from 2 TM cell lines. Each Smad siRNA not only knocked down its target protein, but also suppressed TGFβ2 induction of LOX/LOX proteins.

Figure 8: **JNK1/2 inhibition blocks TGFβ-induction of LOXs.** Effect of JNK1/2 inhibitor SP600125 on TGFβ1-3 induction of LOX/LOXL mRNA (A) and protein (B) expression in cultured TM cells. (A) qRT-PCR analysis values represent fold induction of LOX/LOXL genes normalized to ACTB in treated samples as compared to controls. Cumulative data for experiments performed in triplicate in two TM strains. One-Way ANOVA was used for statistical analyses. *and # $0.01<p<0.05$, ** and ## $0.0001<p<0.01$, *** and ### $p<0.0001$. "*" = differences between TGFβ samples vs. TGFβ + inhibitor samples; "#" = differences in TGFβ-treated vs. the untreated cells. (B) Western immunoblots of LOX/LOXL proteins in TM cells pretreated with SP600125 followed by TGFβ1-3 treatment. Immunoblots are representative of three different TM cell strains studied. GAPDH was used as loading control. Untreated and DMSO-treated cells served as negative controls. SP600125 suppressed TGFβ induction of LOX/LOXL mRNAs and proteins suggesting involvement of the JNK1/2 pathway.

Figure 9: **AP-1 inhibition blocks TGFβ-induction of LOXs.** Effect of AP1 inhibitor SR11302 on TGFβ1-3 induction of LOX/LOXL mRNA (A) and protein (B) expression in cultured TM cells. (A) qRT-PCR analysis values represent fold induction of LOX/LOXL genes normalized to ACTB in treated samples as compared to controls. Cumulative data for experiments performed in triplicate in two TM strains. One-Way ANOVA was used for statistical analyses. *and # $0.01<p<0.05$, ** and ## $0.0001<p<0.01$, *** and ### represent $p<0.0001$. "*" = differences between TGFβ-samples vs. TGFβ + inhibitor samples; "#" = differences for TGFβ-treated vs. the untreated cells. (B) Western immunoblots of LOX/LOXL proteins in TM cells pretreated with SR11342 followed by TGFβ1-3 treatment. Immunoblots are representative of three different TM cell strains studied. GAPDH was used as loading control. Untreated and DMSO-treated cells

served as negative controls. SP600125 suppressed TGFβ induction of LOX/LOXL mRNAs and proteins supporting involvement of the JNK1/2 and AP-1 signaling pathways.

Figure 10: LOX activity assay. (A) Representative western immunoblot for tropoelastin (normalized to ACTB) in TM cells treated with increasing concentrations (1, 3, 10 and 30 mM) of LOX inhibitor β-aminopropionitrile (BAPN). There was a concentration-dependent increase in tropoelastin levels. (B) Representative western immunoblot for tropoelastin (normalized to ACTB) in TM cells treated with increasing concentrations of TGFβ2 (0.1, 0.25, 0.5, 1, 2.5, 5 and 10 ng/ml). There was a concentration dependent increase in tropoelastin levels. (C) Representative western immunoblot for tropoelastin (normalized to ACTB) in TM cells treated with 1mM of BAPN along with increasing concentrations of TGFβ2 (0.1, 0.25, 0.5, 1, 2.5, 5 and 10 ng/ml). (D) TM cells were treated with 5 ng/ml of TGFβ2 along with increasing concentrations of BAPN (1, 3, 10 and 30 mM) and probed for tropoelastin levels. Each of these western blots are representative for independent experiments performed in three different TM cell strains.

Figure 11: **Proposed mechanism of TGFβ-regulation of LOXs in TM and implications in glaucoma**. TGFβ ligands bind to the TGFBR1-2 receptor heterotetramer complex on the cell surface and activate the Smad2/Smad3 complex, which utilizes Smad4 to translocate to nucleus. This complex may bind by itself or with AP-1 to the upstream promoter regions of LOX genes to regulate their transcription. TGFβ receptor activation also appears to phosphorylate and activate JNK1/2, which phosphorylates the Jun component of the transcription factor AP-1. AP-1 binds to upstream promoter region of LOXs to regulate their gene transcription. LOX mRNA transcribes to proteins and cross-links collagen and elastin fibrils in the ECM of TM cells.

Figures

Fig. 1A

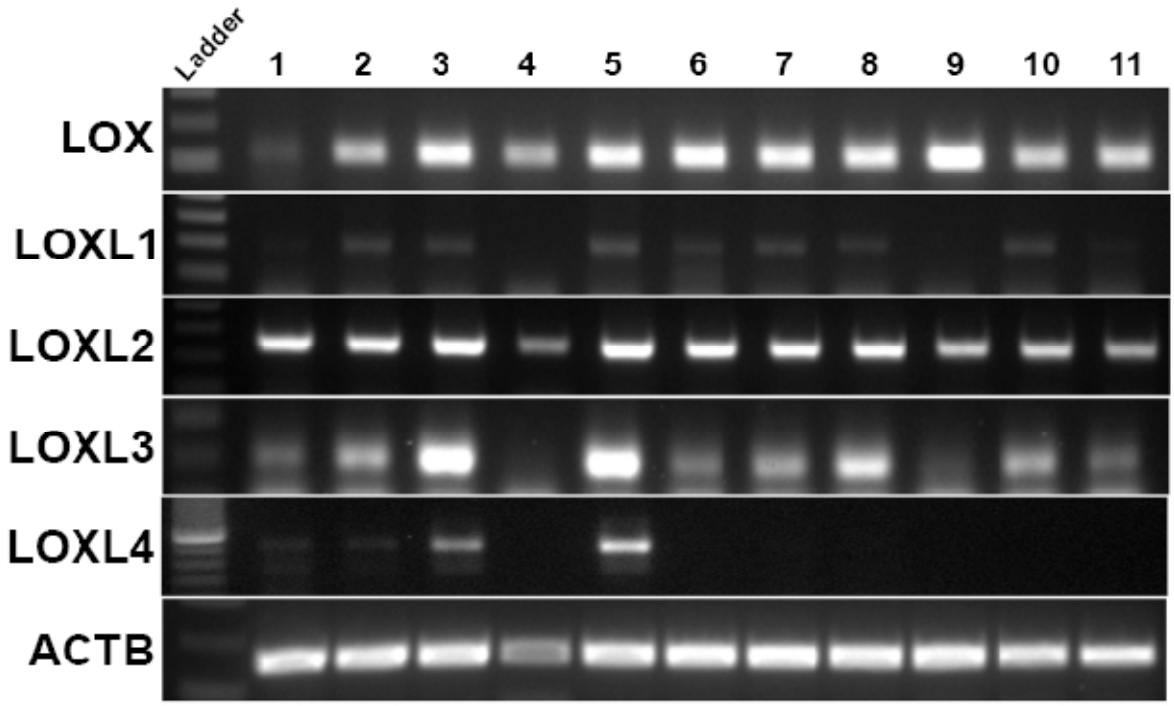

Fig. 1B

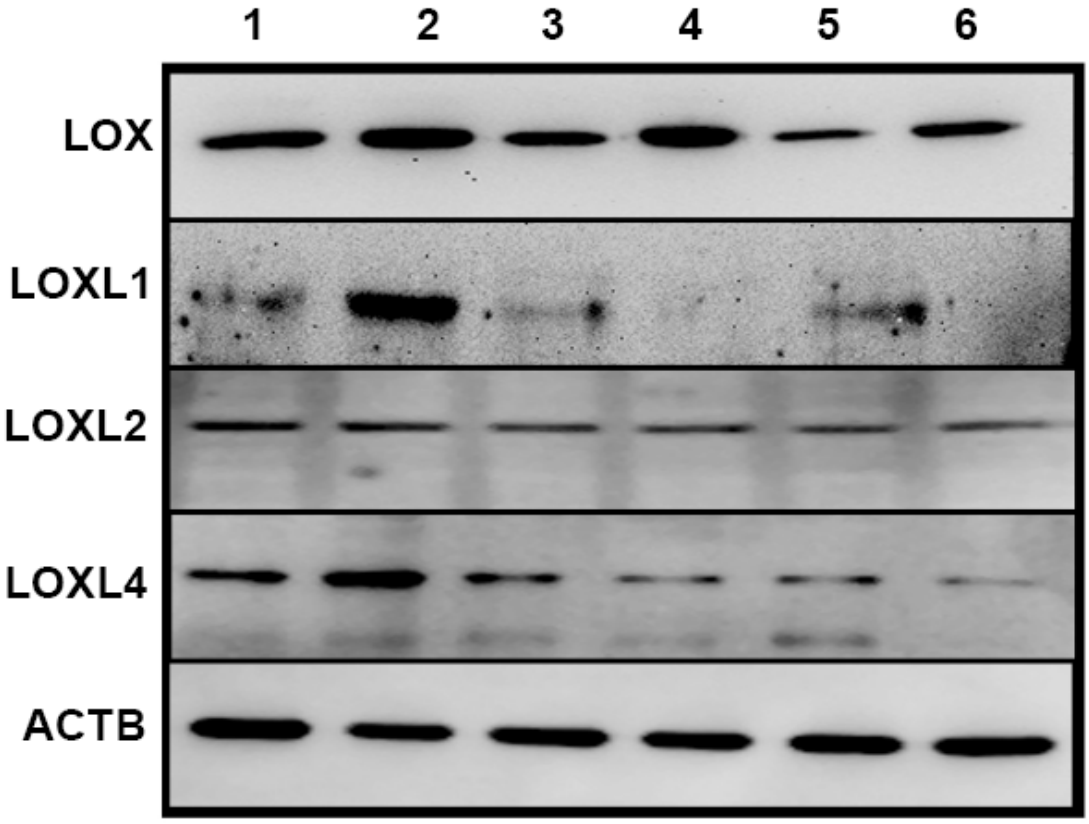

Fig. 2A

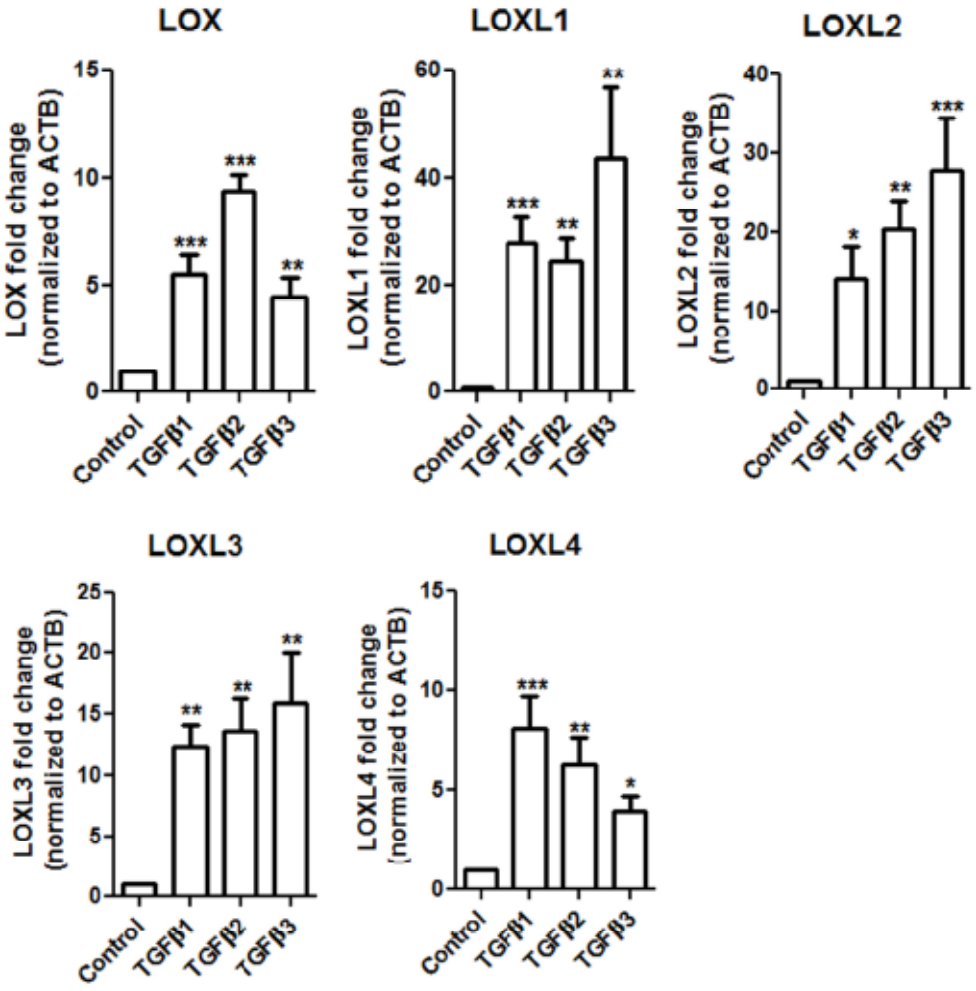
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Fig. 2B

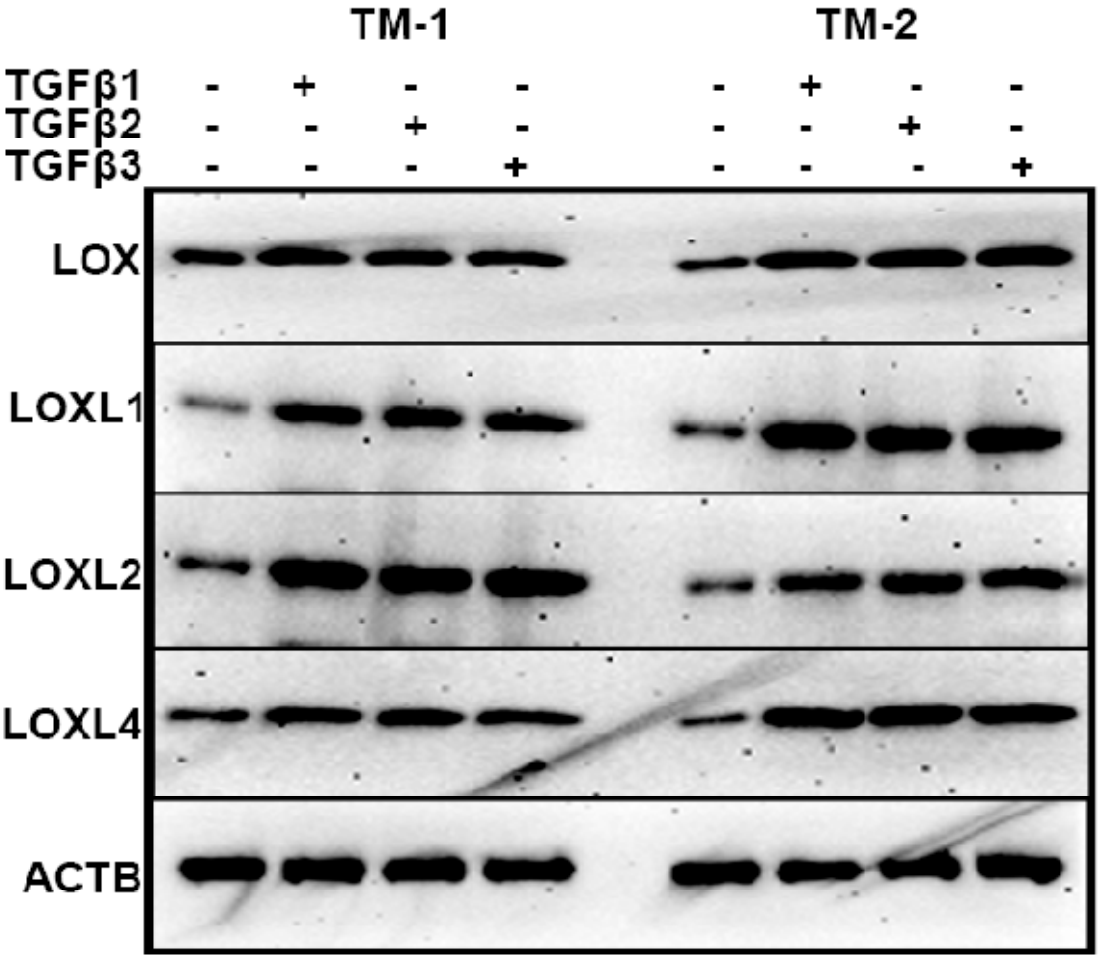
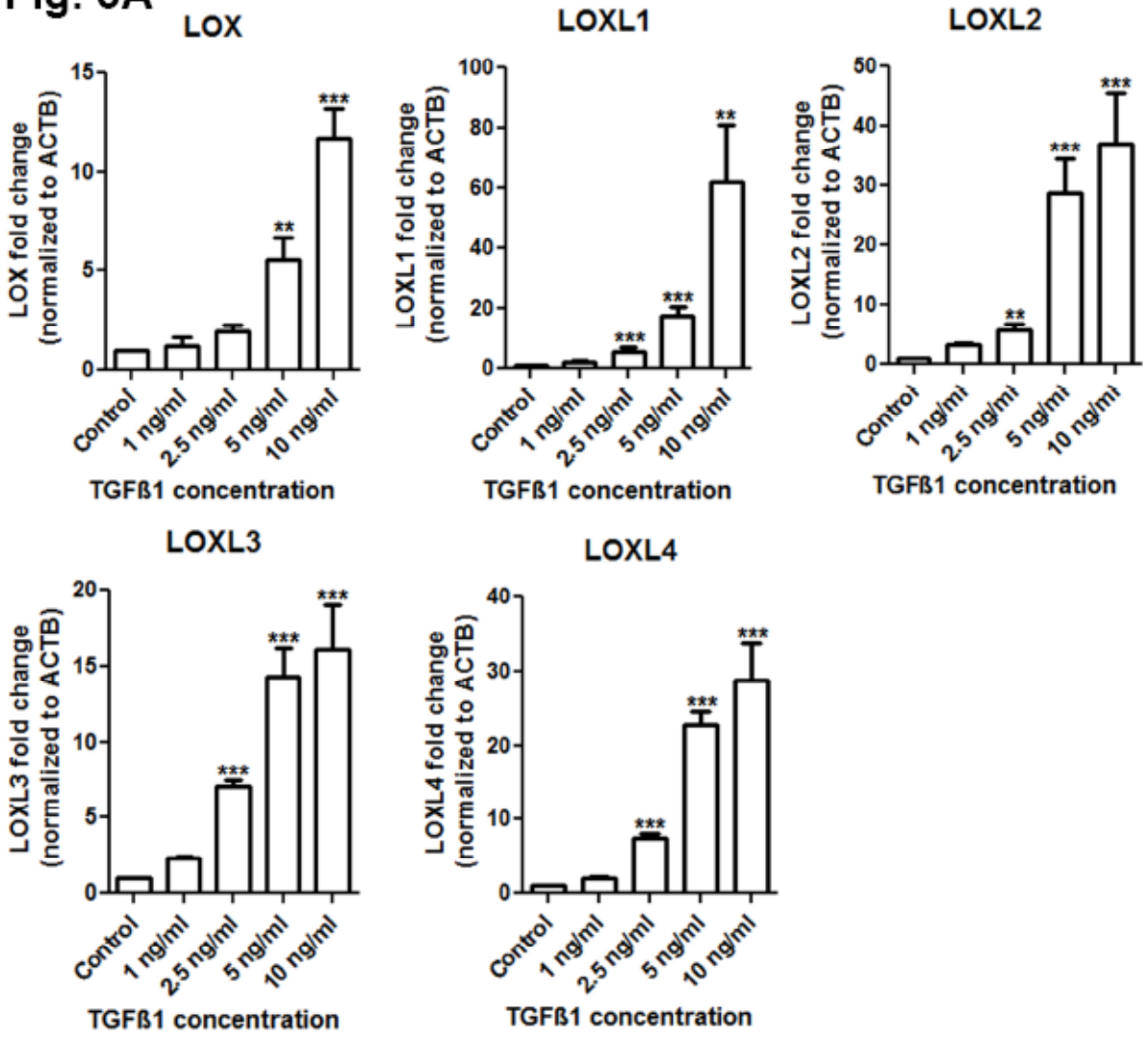


Fig. 3A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TGFß1 concentration
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LOXL3 fold change (normalized to ACTB)
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5 ng/ml
10 ng/ml
TGFß1 concentration

Fig. 3B

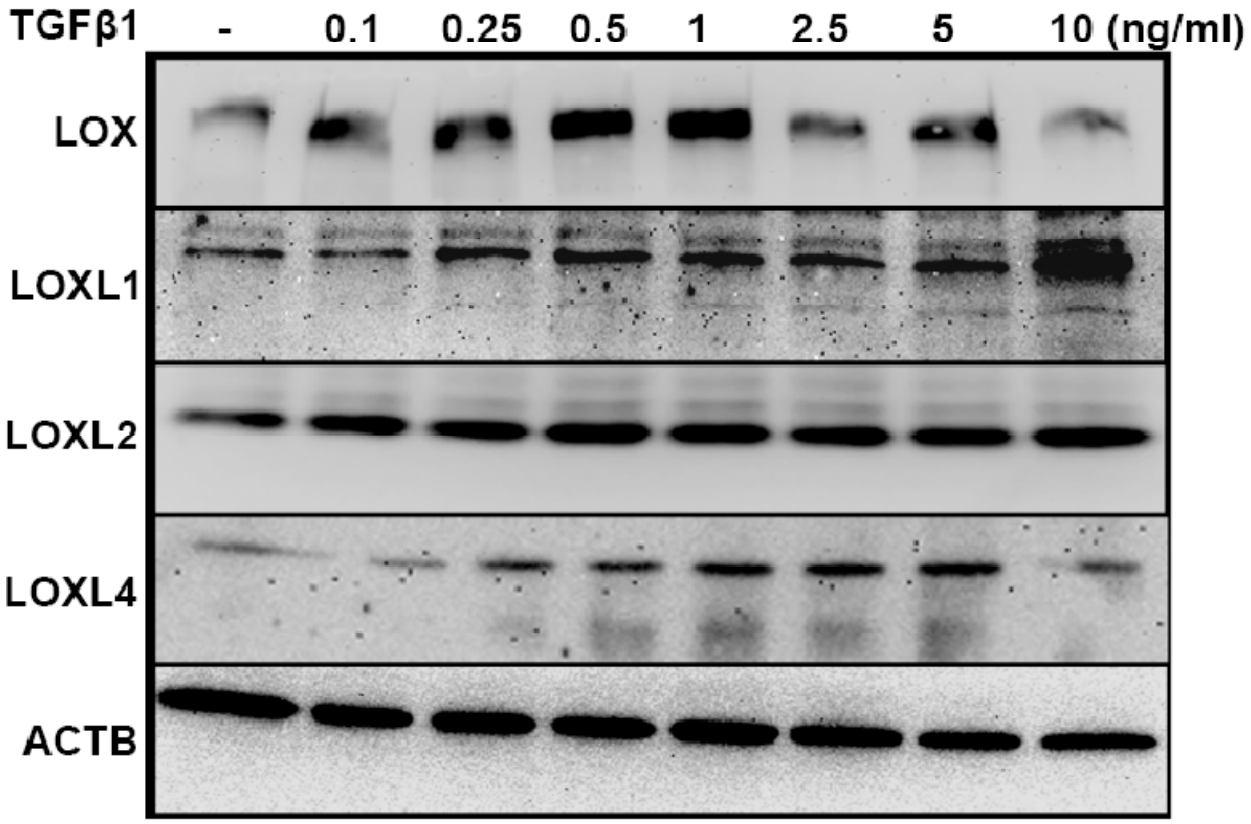


Fig. 3C

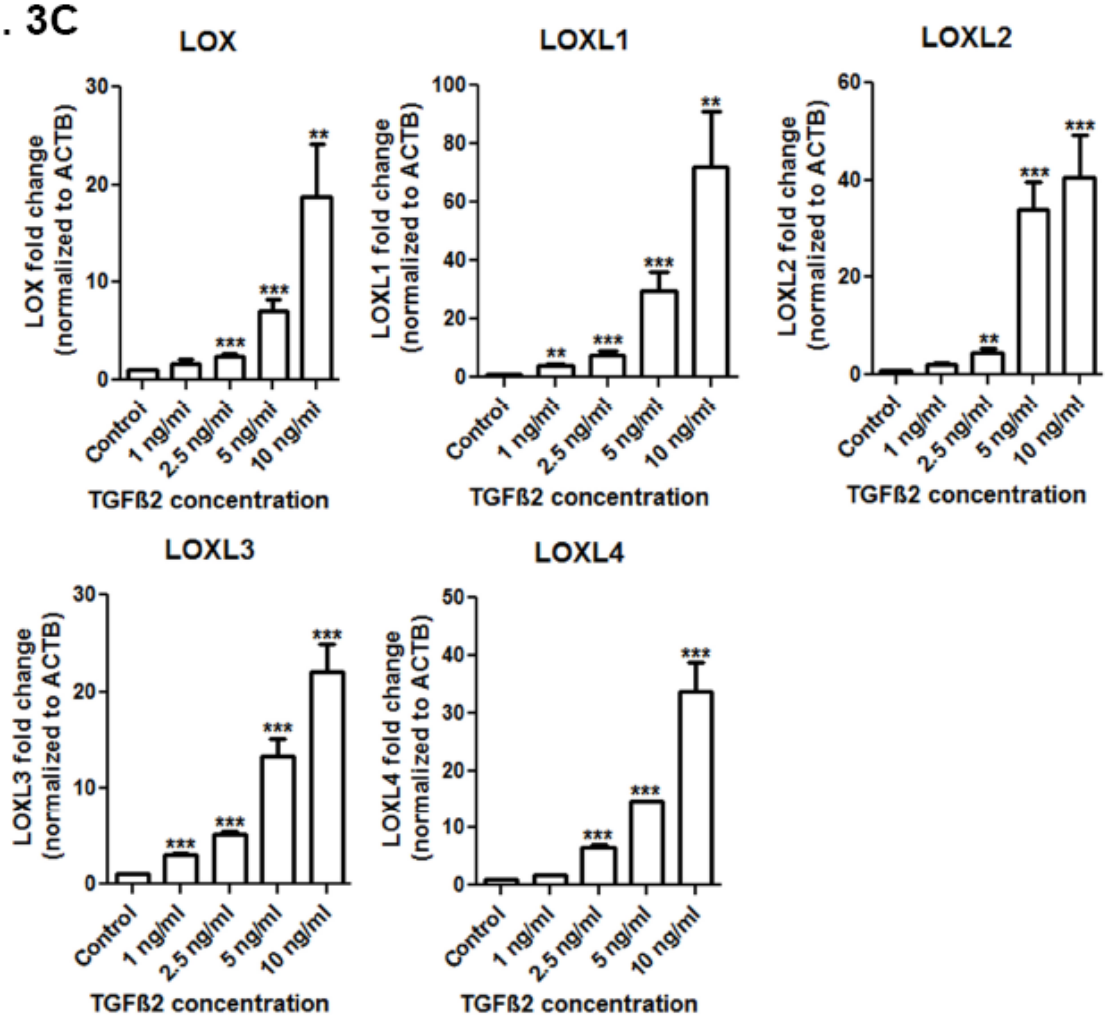

LOX
LOX fold change (normalized to ACTB)
30
20
10
0
**
***
***
Control
1 ng/ml
2.5 ng/ml
5 ng/ml
10 ng/ml
TGFß2 concentration
LOXL1
LOXL1 fold change (normalized to ACTB)
100
80
60
40
20
0
**
**
***
***
Control
1 ng/ml
2.5 ng/ml
5 ng/ml
10 ng/ml
TGFß2 concentration
LOXL2
LOXL2 fold change (normalized to ACTB)
60
40
20
0
**
***
***
Control
1 ng/ml
2.5 ng/ml
5 ng/ml
10 ng/ml
TGFß2 concentration
LOXL3
LOXL3 fold change (normalized to ACTB)
30
20
10
0
***
***
***
***
Control
1 ng/ml
2.5 ng/ml
5 ng/ml
10 ng/ml
TGFß2 concentration
LOXL4
LOXL4 fold change (normalized to ACTB)
50
40
30
20
10
0
***
***
***
Control
1 ng/ml
2.5 ng/ml
5 ng/ml
10 ng/ml
TGFß2 concentration

Fig. 3D

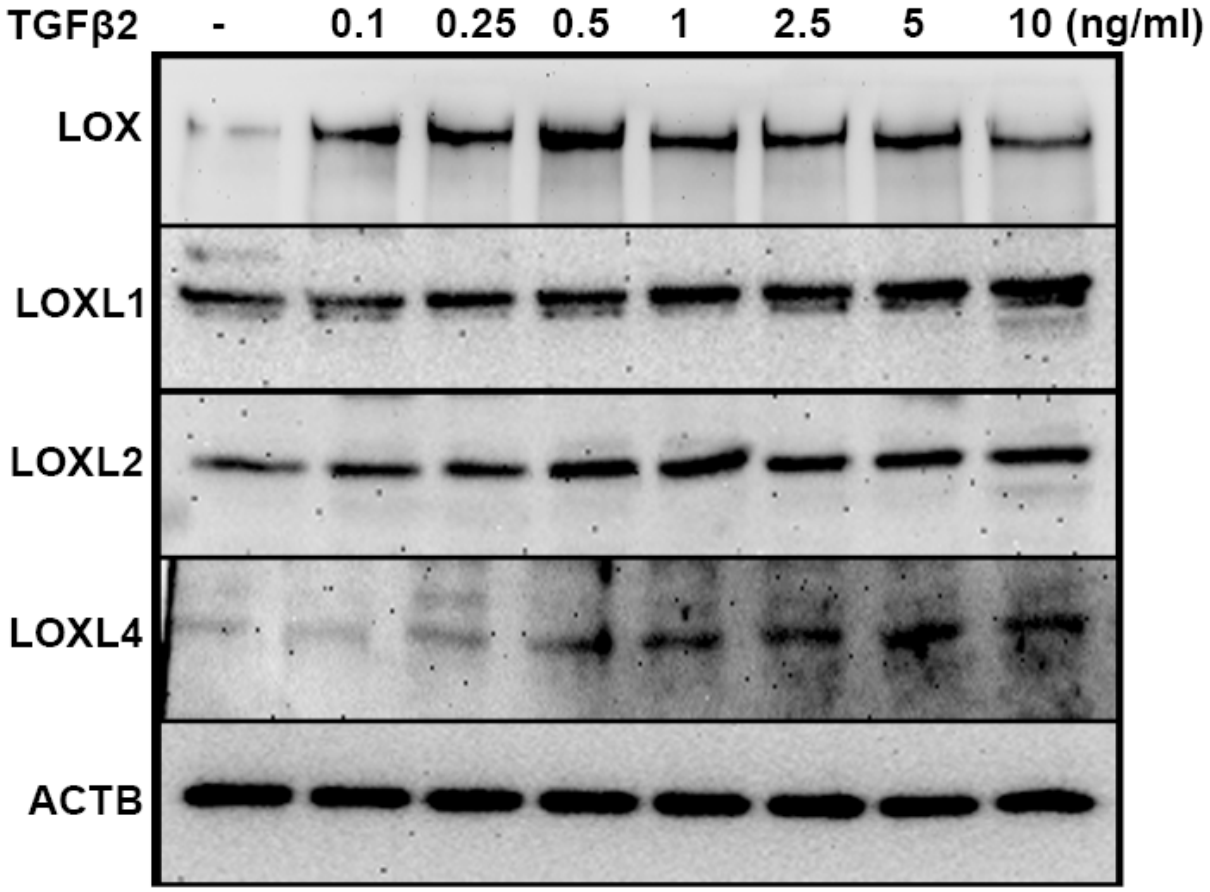


Fig. 3E

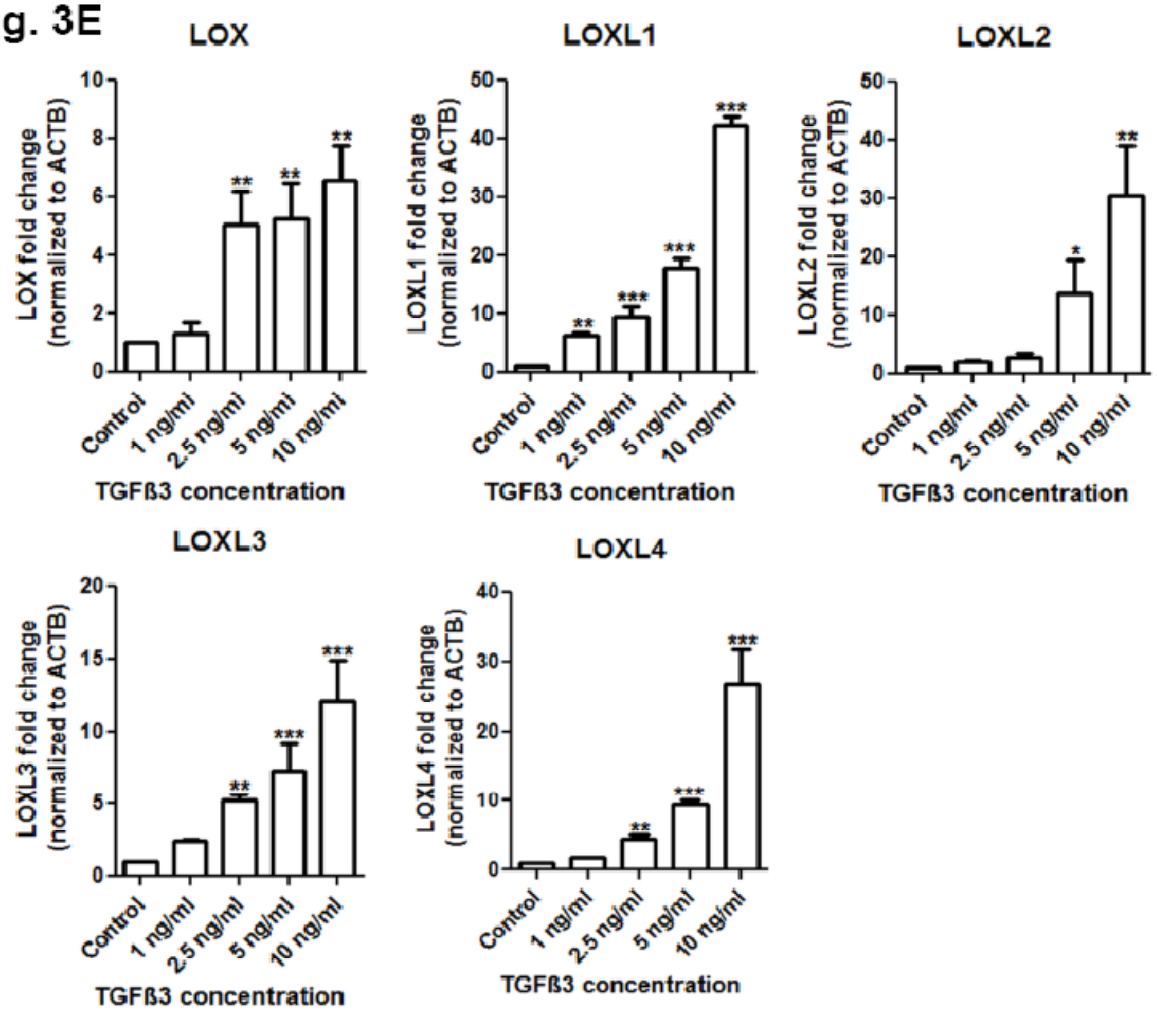

LOX
LOX fold change (normalized to ACTB)
LOXL1
LOXL1 fold change (normalized to ACTB)
LOXL2
LOXL2 fold change (normalized to ACTB)
LOXL3
LOXL3 fold change (normalized to ACTB)
LOXL4
LOXL4 fold change (normalized to ACTB)
Control
1 ng/ml
2.5 ng/ml
5 ng/ml
10 ng/ml
TGFß3 concentration

Fig. 3F

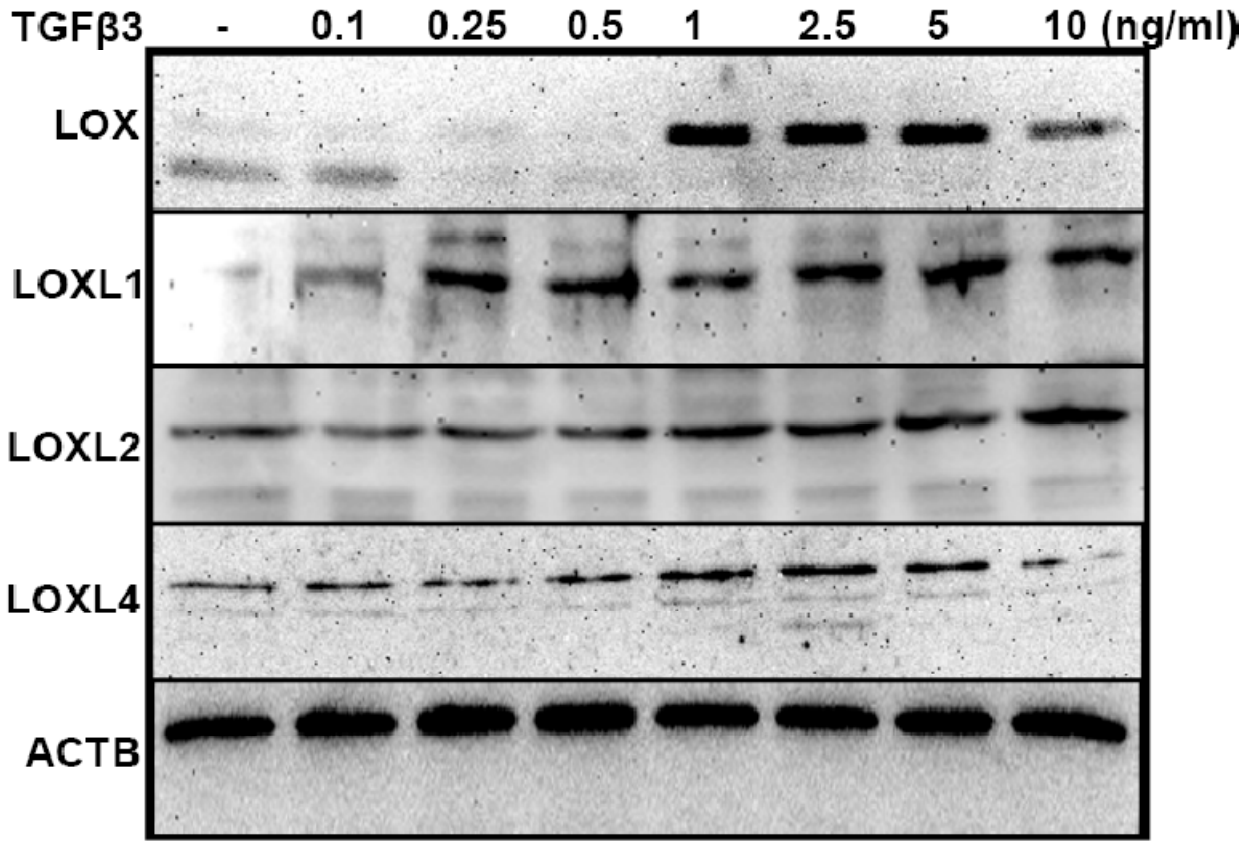


Fig. 4A

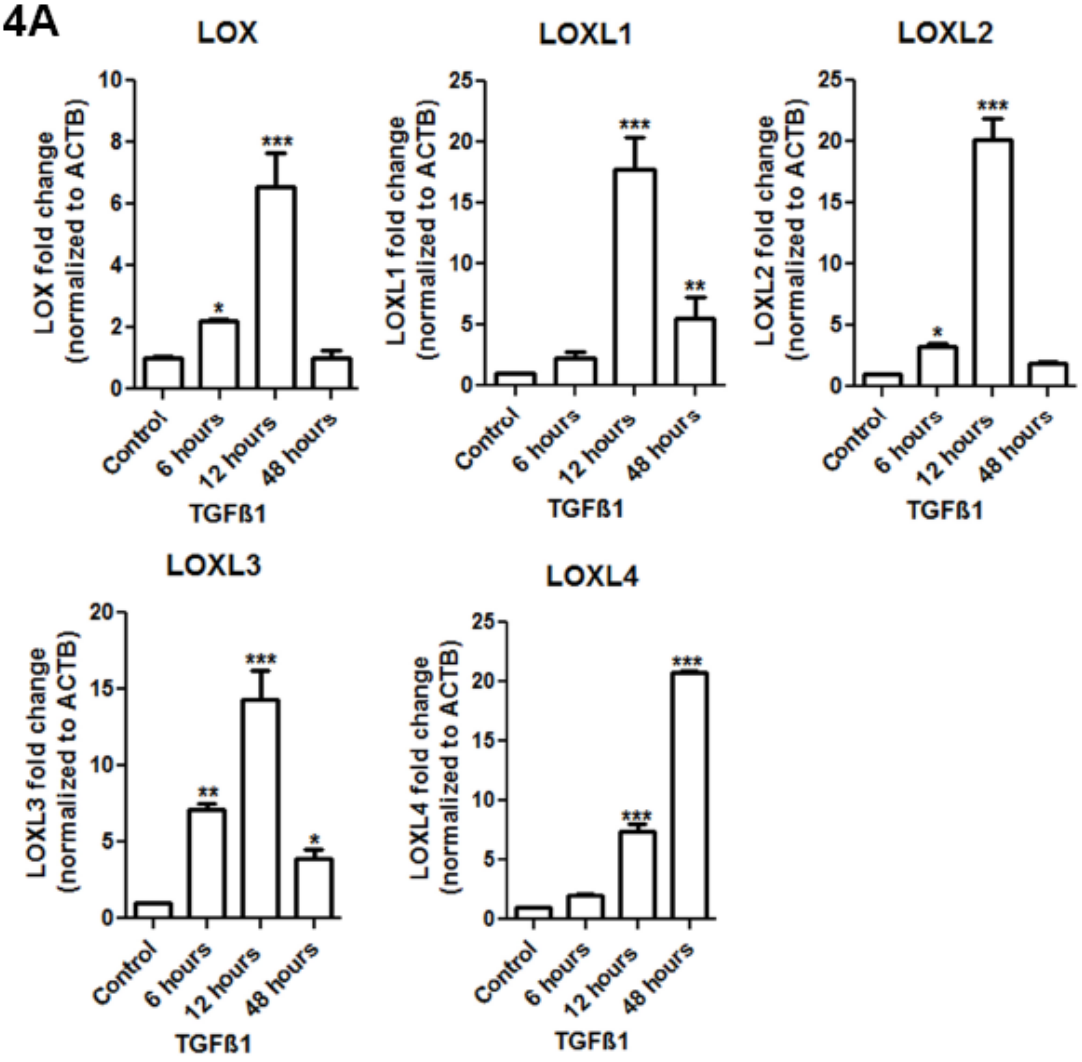

LOX
LOX fold change (normalized to ACTB)
10
8
6
4
2
0
*
***
Control
6 hours
12 hours
48 hours
TGFß1
LOXL1
LOXL1 fold change (normalized to ACTB)
25
20
15
10
5
0
***
**
Control
6 hours
12 hours
48 hours
TGFß1
LOXL2
LOXL2 fold change (normalized to ACTB)
25
20
15
10
5
0
*
***
Control
6 hours
12 hours
48 hours
TGFß1
LOXL3
LOXL3 fold change (normalized to ACTB)
20
15
10
5
0
**
***
*
Control
6 hours
12 hours
48 hours
TGFß1
LOXL4
LOXL4 fold change (normalized to ACTB)
25
20
15
10
5
0
***
***
Control
6 hours
12 hours
48 hours
TGFß1

Fig. 4B

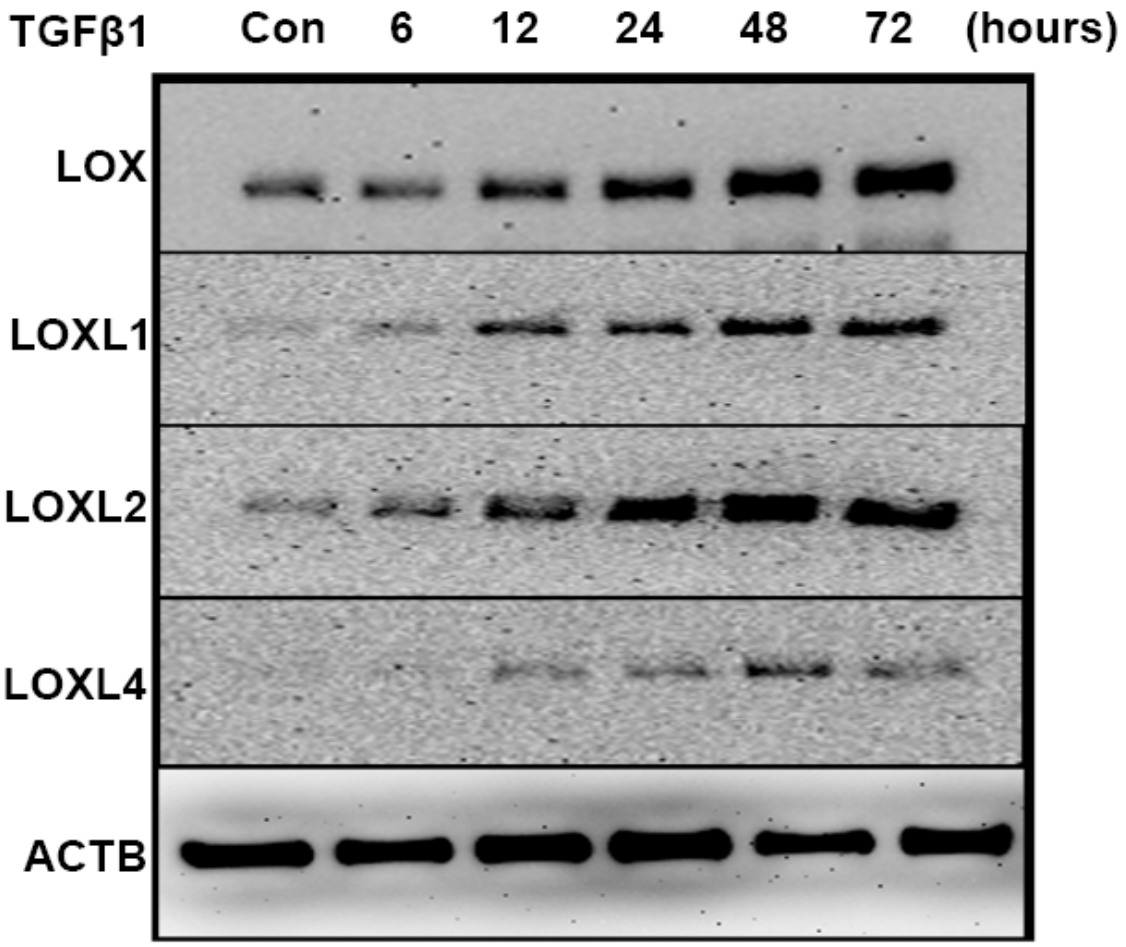


Fig. 4C

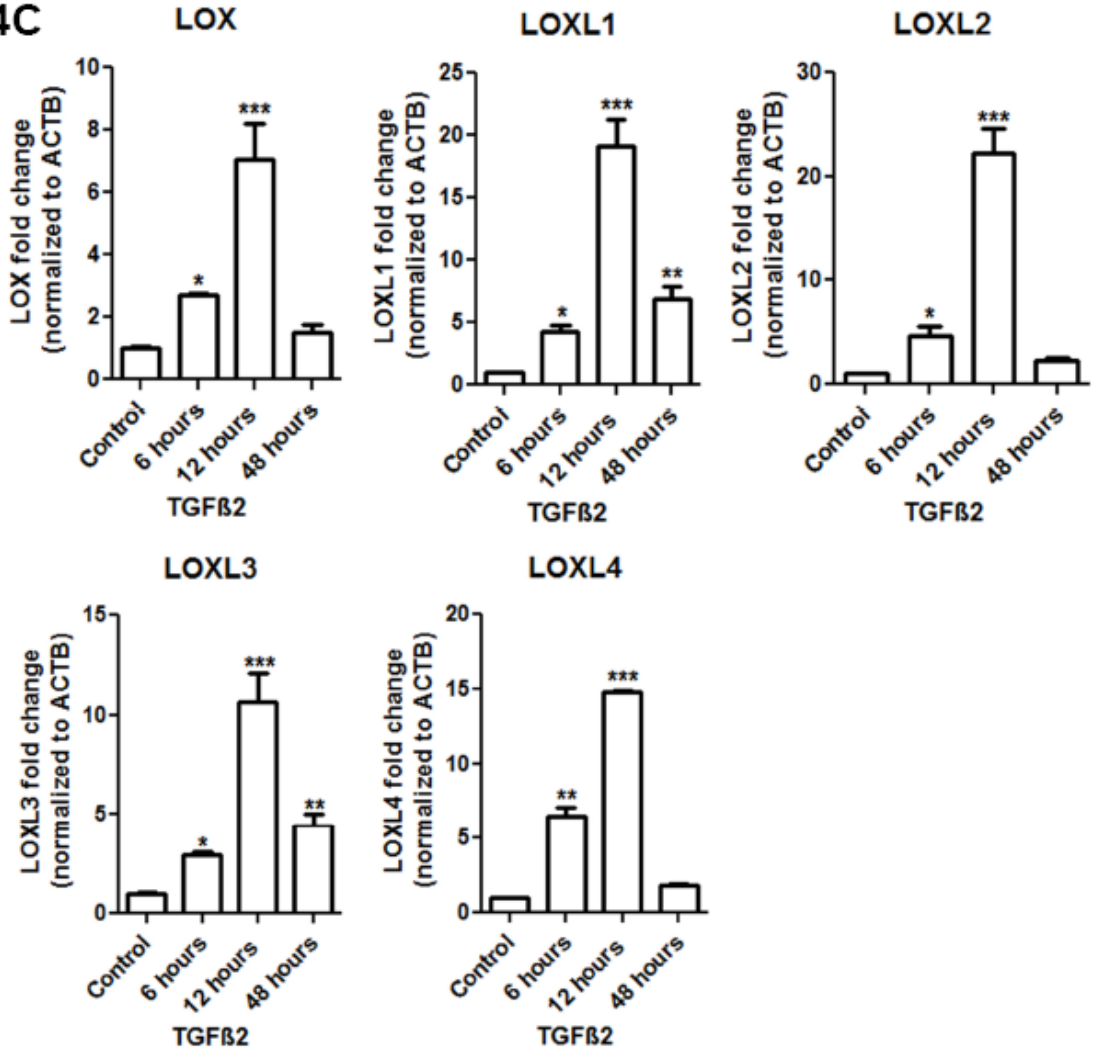

LOX
LOX fold change (normalized to ACTB)
10
8
6
4
2
0
*
***
Control
6 hours
12 hours
48 hours
TGFß2
LOXL1
LOXL1 fold change (normalized to ACTB)
25
20
15
10
5
0
*
***
**
Control
6 hours
12 hours
48 hours
TGFß2
LOXL2
LOXL2 fold change (normalized to ACTB)
30
20
10
0
*
***
Control
6 hours
12 hours
48 hours
TGFß2
LOXL3
LOXL3 fold change (normalized to ACTB)
15
10
5
0
*
***
**
Control
6 hours
12 hours
48 hours
TGFß2
LOXL4
LOXL4 fold change (normalized to ACTB)
20
15
10
5
0
**
***
Control
6 hours
12 hours
48 hours
TGFß2

Fig. 4D

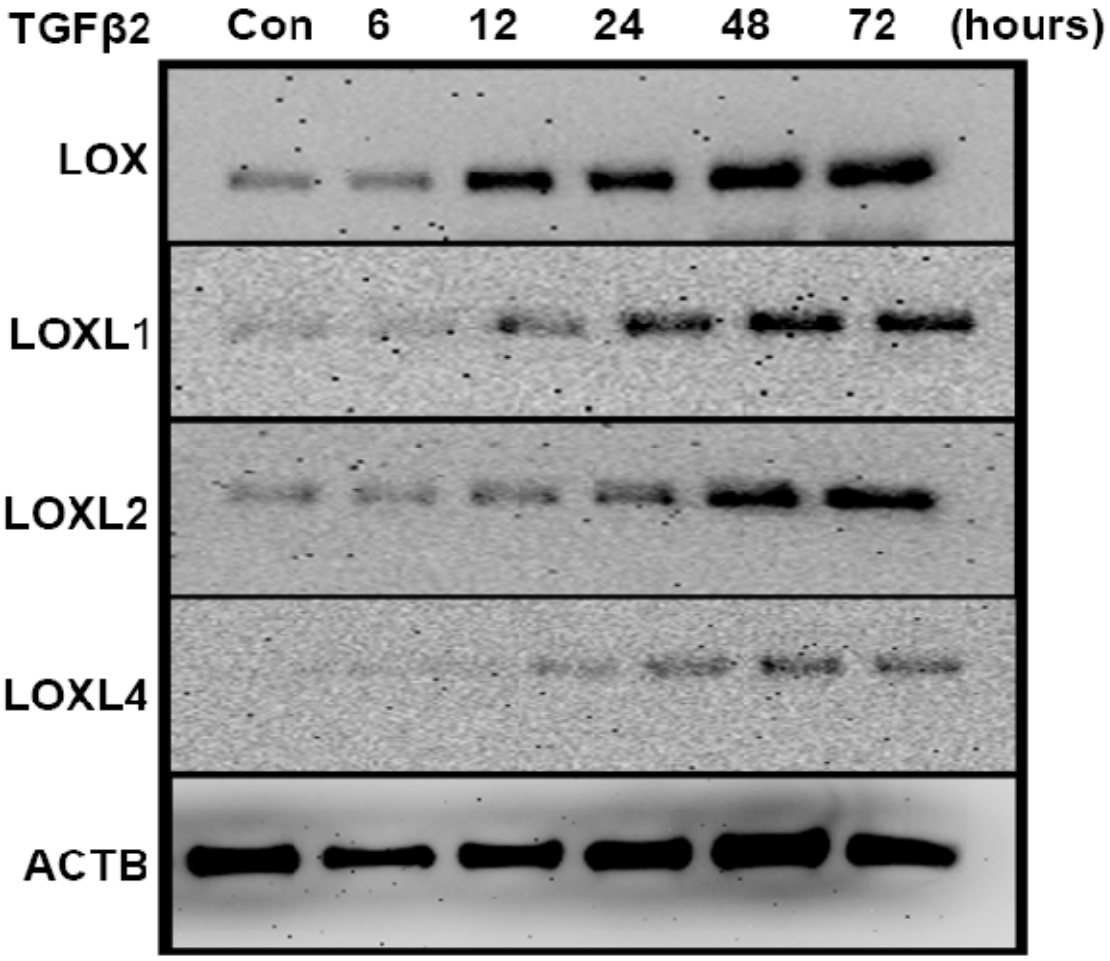


Fig. 4E

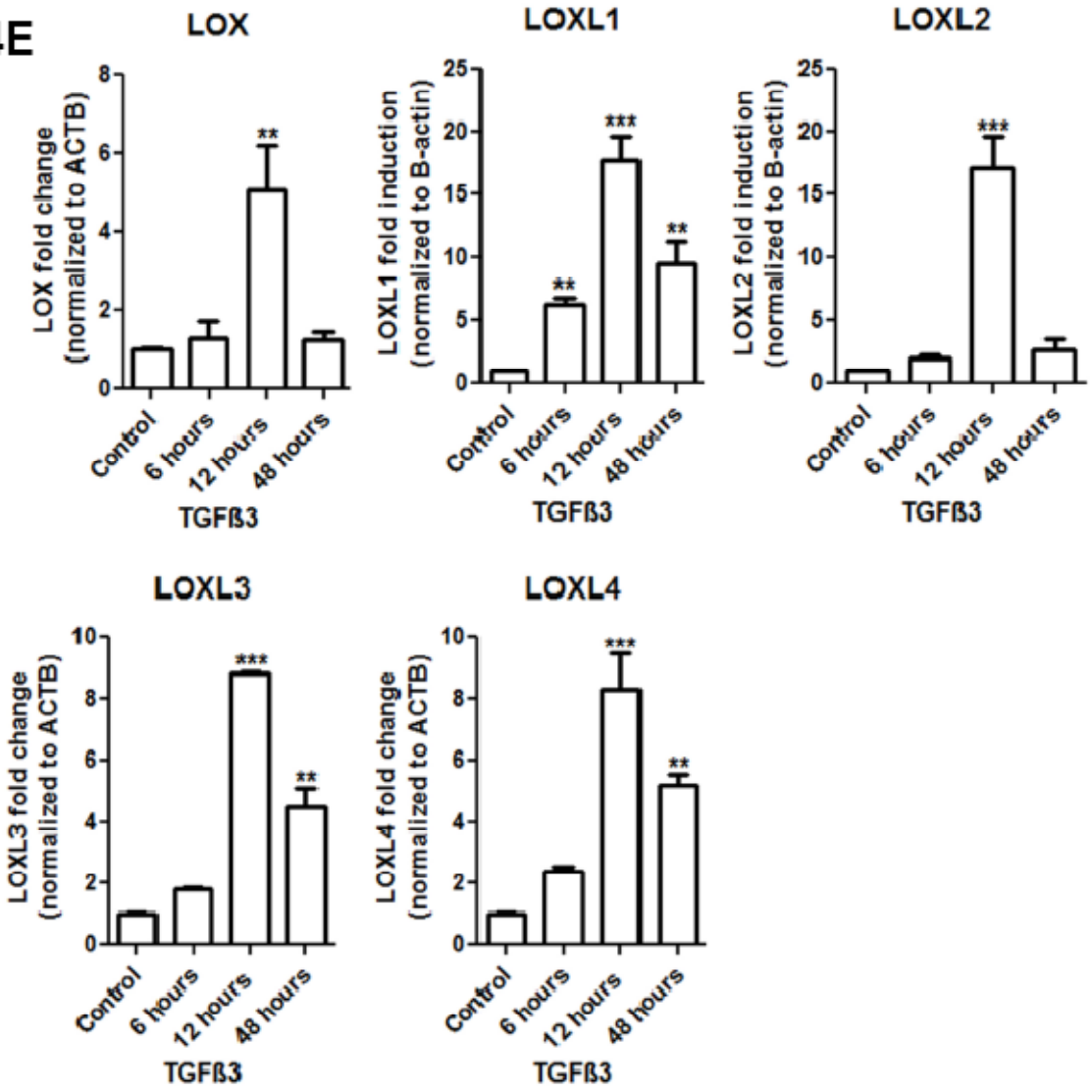

LOX
LOX fold change (normalized to ACTB)
LOXL1
LOXL1 fold induction (normalized to B-actin)
LOXL2
LOXL2 fold induction (normalized to B-actin)
LOXL3
LOXL3 fold change (normalized to ACTB)
LOXL4
LOXL4 fold change (normalized to ACTB)
Control
6 hours
12 hours
48 hours
TGFß3

Fig. 4F

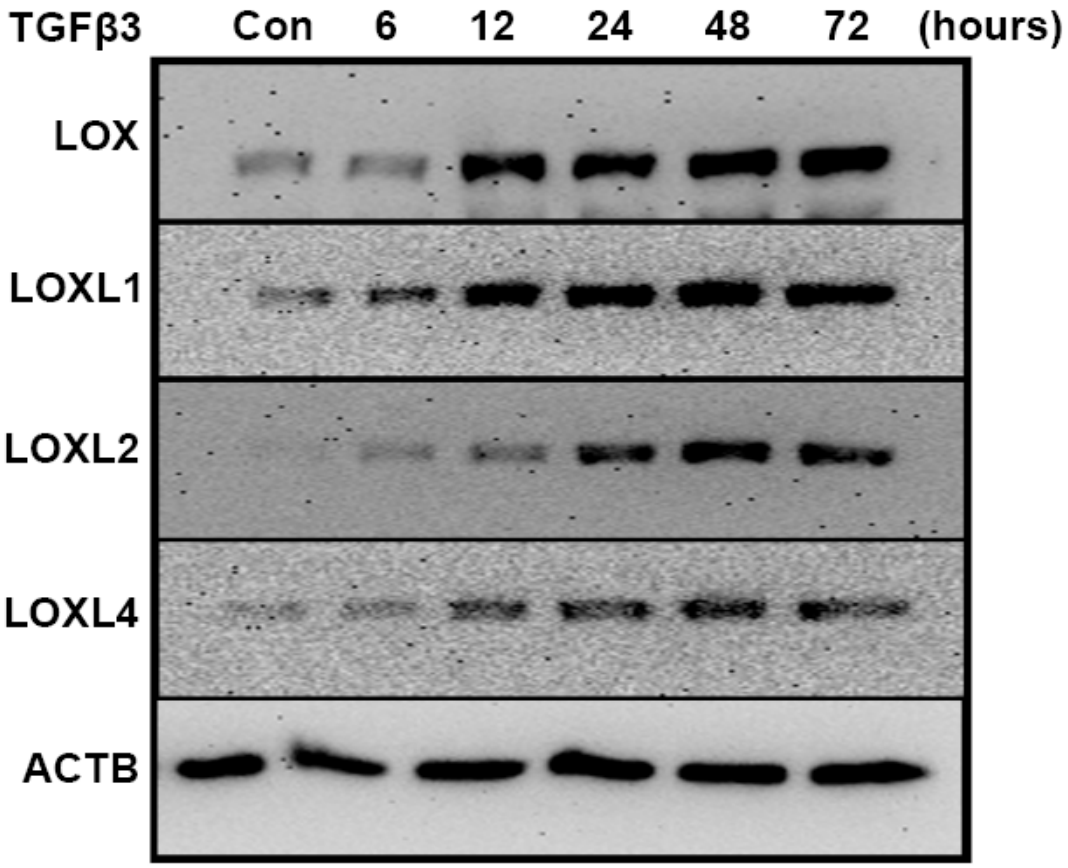


Fig. 5A

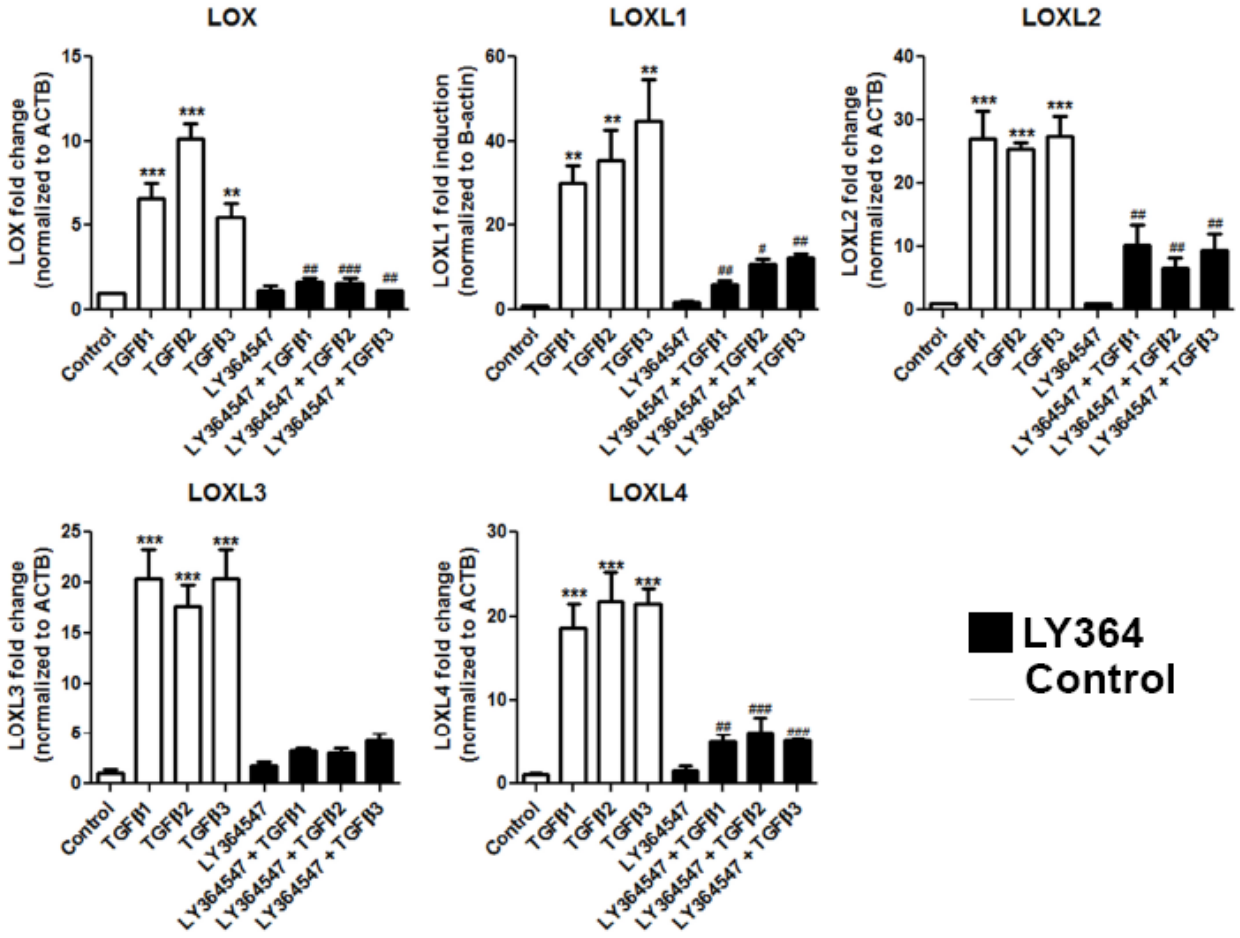

LOX
LOX fold change (normalized to ACTB)
LOXL1
LOXL1 fold induction (normalized to B-actin)
LOXL2
LOXL2 fold change (normalized to ACTB)
LOXL3
LOXL3 fold change (normalized to ACTB)
LOXL4
LOXL4 fold change (normalized to ACTB)
Control
TGFβ1
TGFβ2
TGFβ3
LY364547
LY364547 + TGFβ1
LY364547 + TGFβ2
LY364547 + TGFβ3
LY364
Control

Fig. 5B

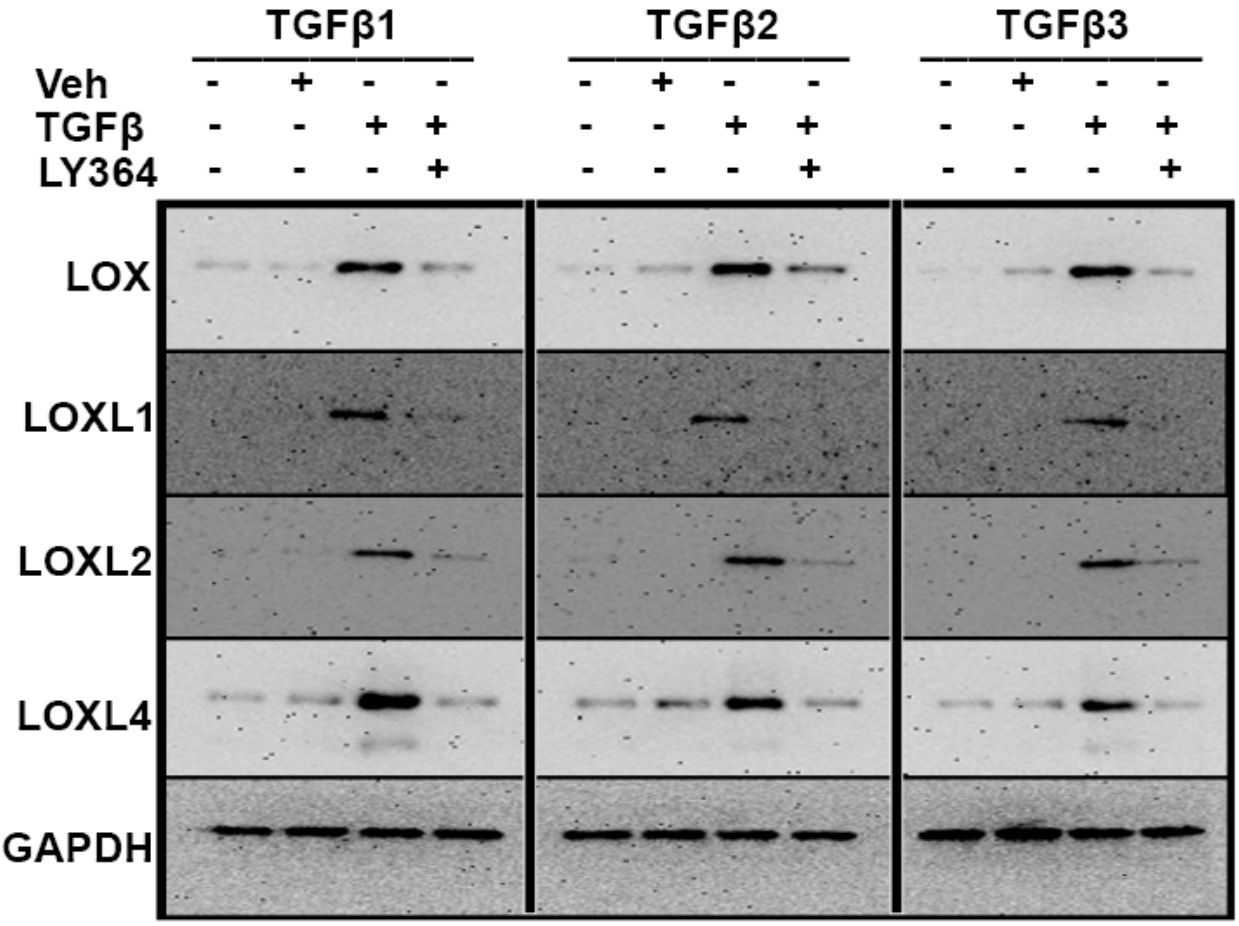

TGFβ1
TGFβ2
TGFβ3
Veh - + - - - + - - - + - -
TGFβ - - + + - - + + - - + +
LY364 - - - + - - - + - - - +
LOX
LOXL1
LOXL2
LOXL4
GAPDH

Fig. 5C

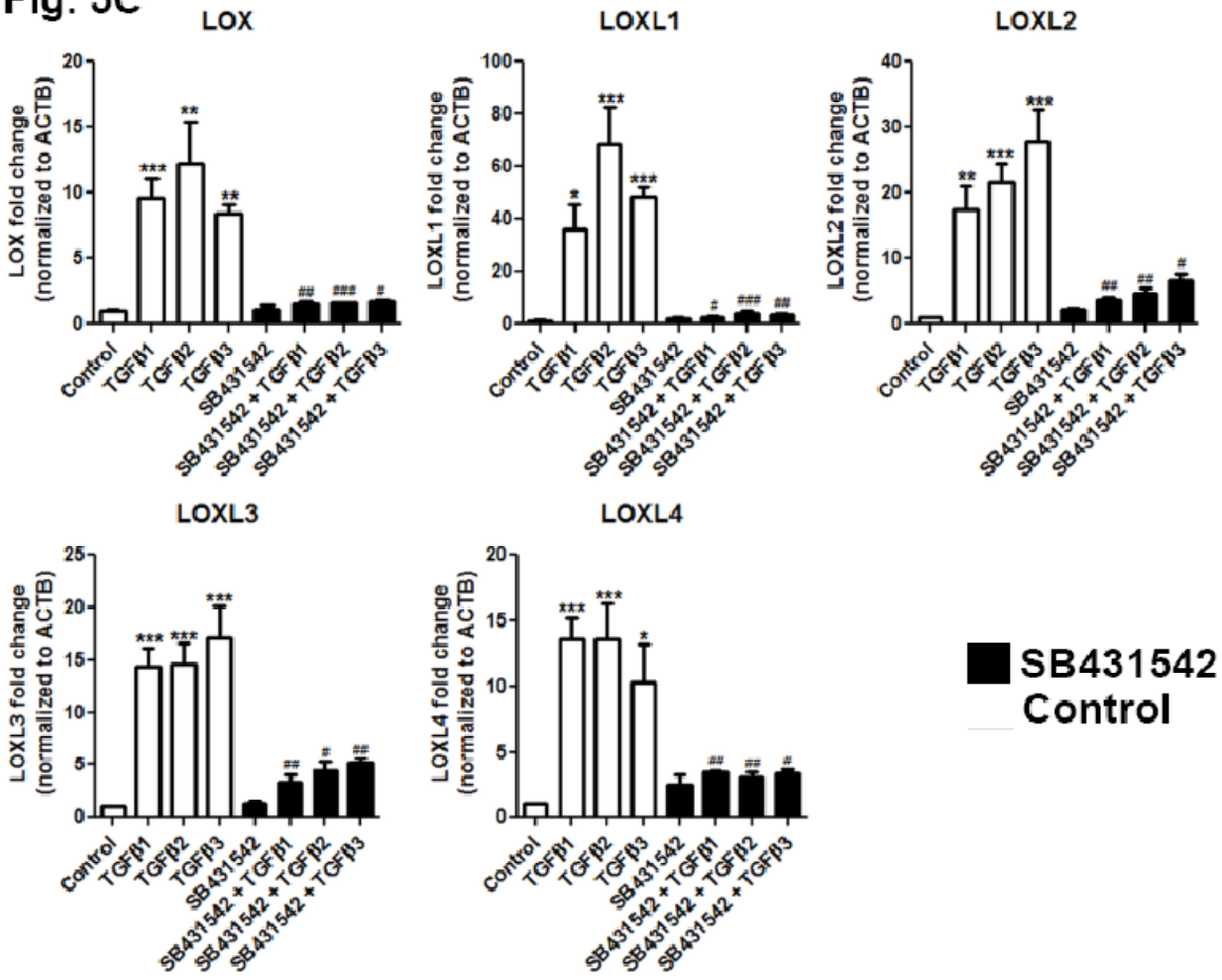

LOX
LOXL1
LOXL2
LOXL3
LOXL4
LOX fold change (normalized to ACTB)
LOXL1 fold change (normalized to ACTB)
LOXL2 fold change (normalized to ACTB)
LOXL3 fold change (normalized to ACTB)
LOXL4 fold change (normalized to ACTB)
Control
TGFβ1
TGFβ2
TGFβ3
SB431542
SB431542 + TGFβ1
SB431542 + TGFβ2
SB431542 + TGFβ3
SB431542
Control

Fig. 5D

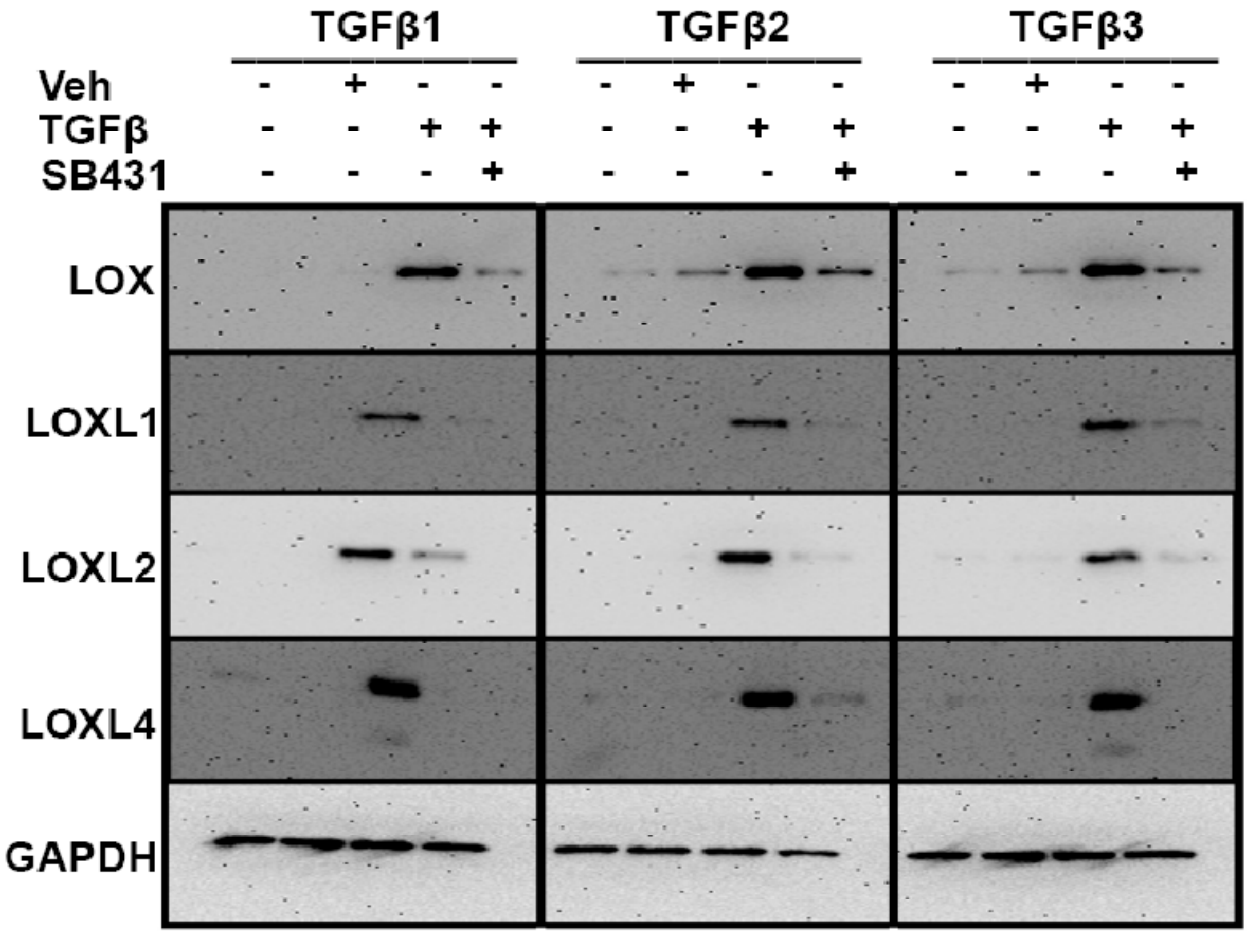

TGFβ1
TGFβ2
TGFβ3
Veh - + - - - + - - - + - -
TGFβ - - + + - - + + - - + +
SB431 - - - + - - - + - - - +
LOX
LOXL1
LOXL2
LOXL4
GAPDH

Fig. 5E

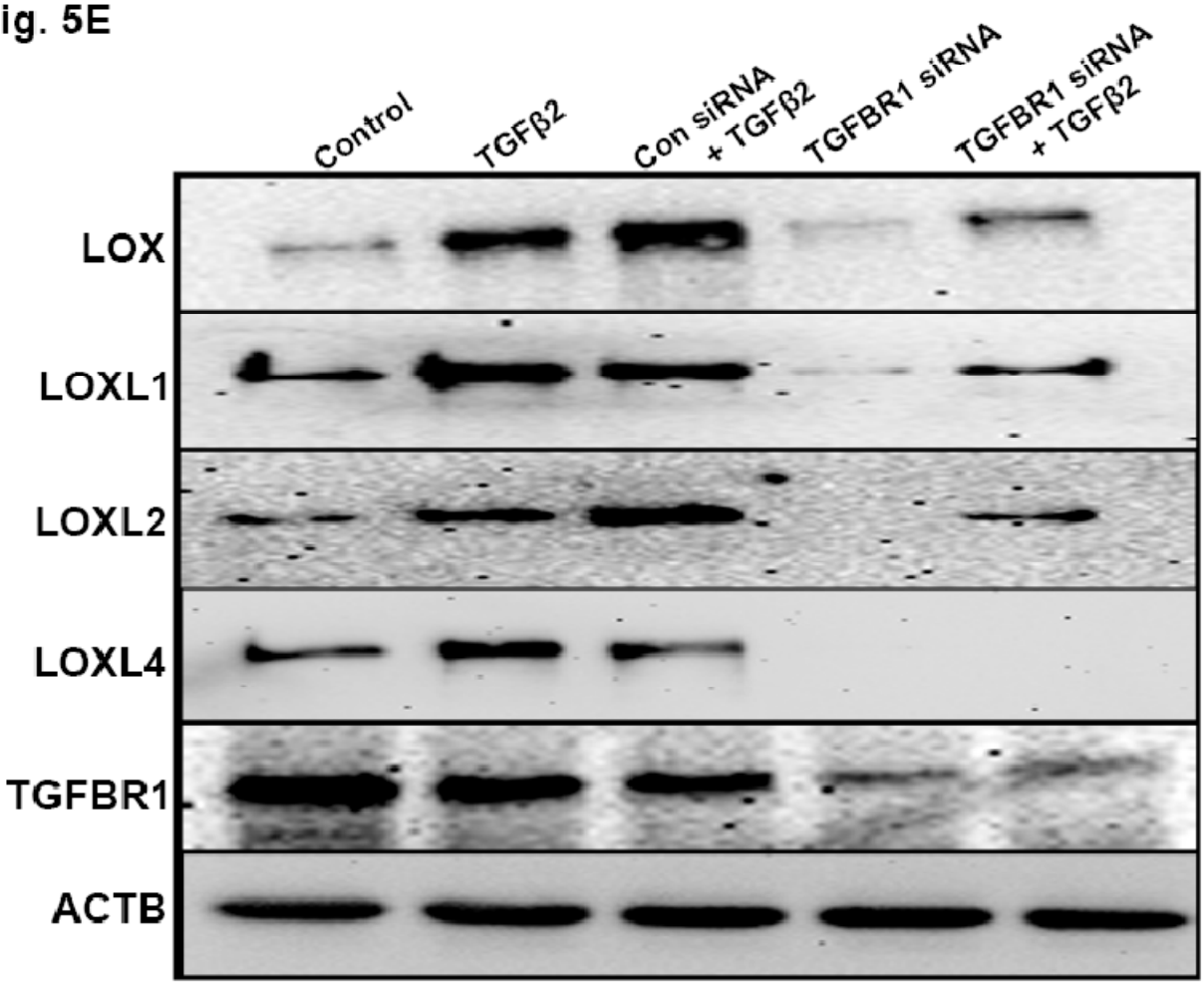

Control
TGFβ2
Con siRNA + TGFβ2
TGFBR1 siRNA
TGFBR1 siRNA + TGFβ2
LOX
LOXL1
LOXL2
LOXL4
TGFBR1
ACTB

Fig. 6A

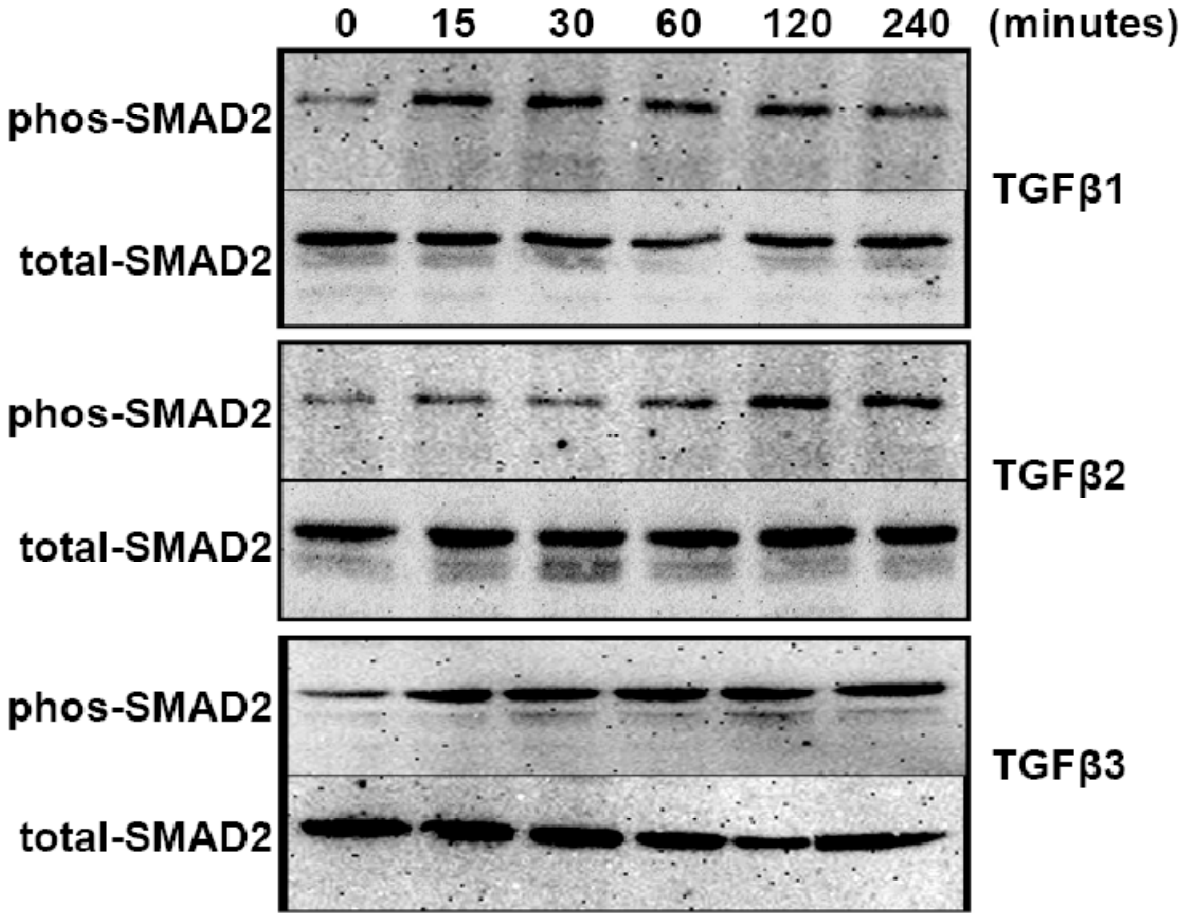

0
15
30
60
120
240
(minutes)
phos-SMAD2
total-SMAD2
TGFβ1
phos-SMAD2
total-SMAD2
TGFβ2
phos-SMAD2
total-SMAD2
TGFβ3

Fig. 6B

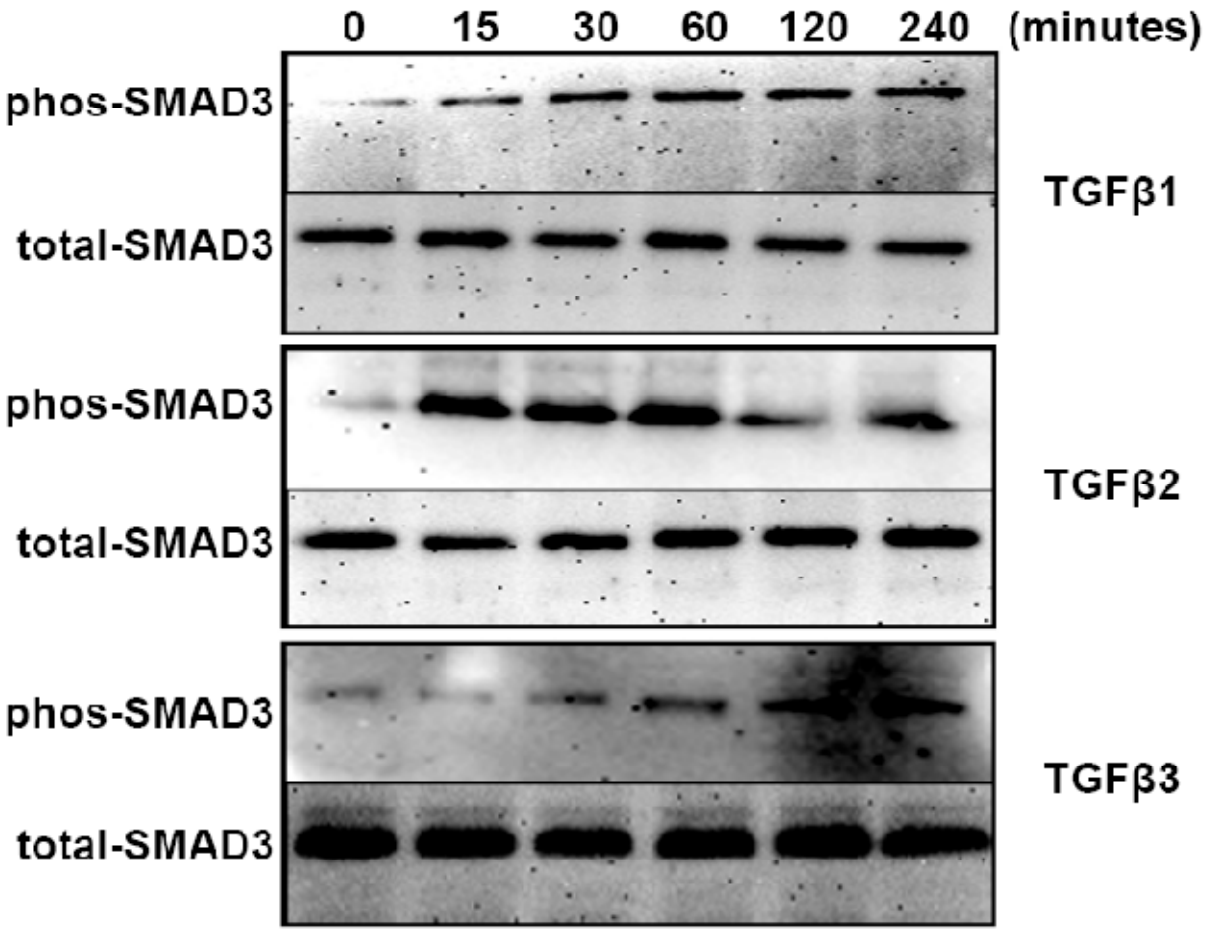

0
15
30
60
120
240
(minutes)
phos-SMAD3
total-SMAD3
TGFβ1
phos-SMAD3
total-SMAD3
TGFβ2
phos-SMAD3
total-SMAD3
TGFβ3

Fig. 6C

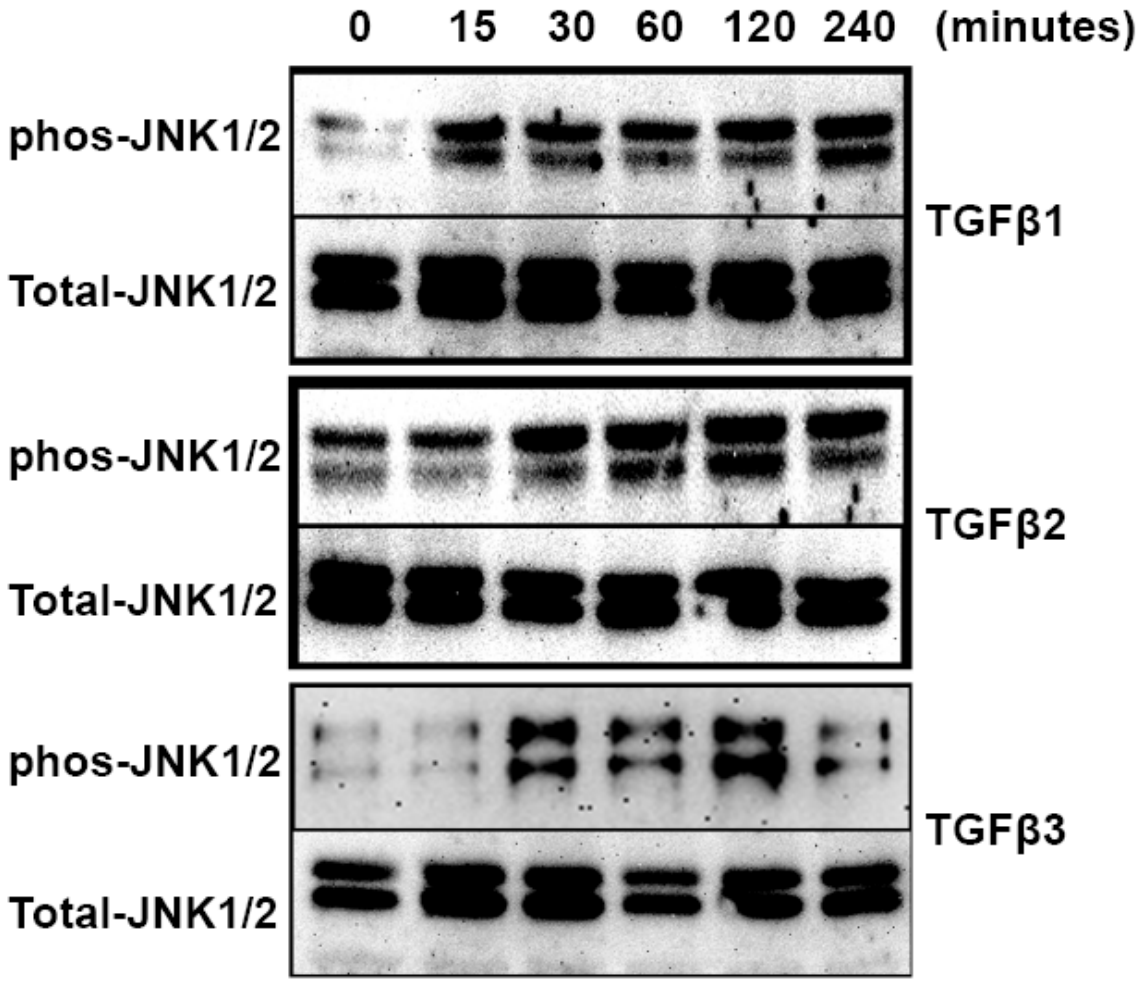

0
15
30
60
120
240
(minutes)
phos-JNK1/2
Total-JNK1/2
TGFβ1
phos-JNK1/2
Total-JNK1/2
TGFβ2
phos-JNK1/2
Total-JNK1/2
TGFβ3

Fig. 7A

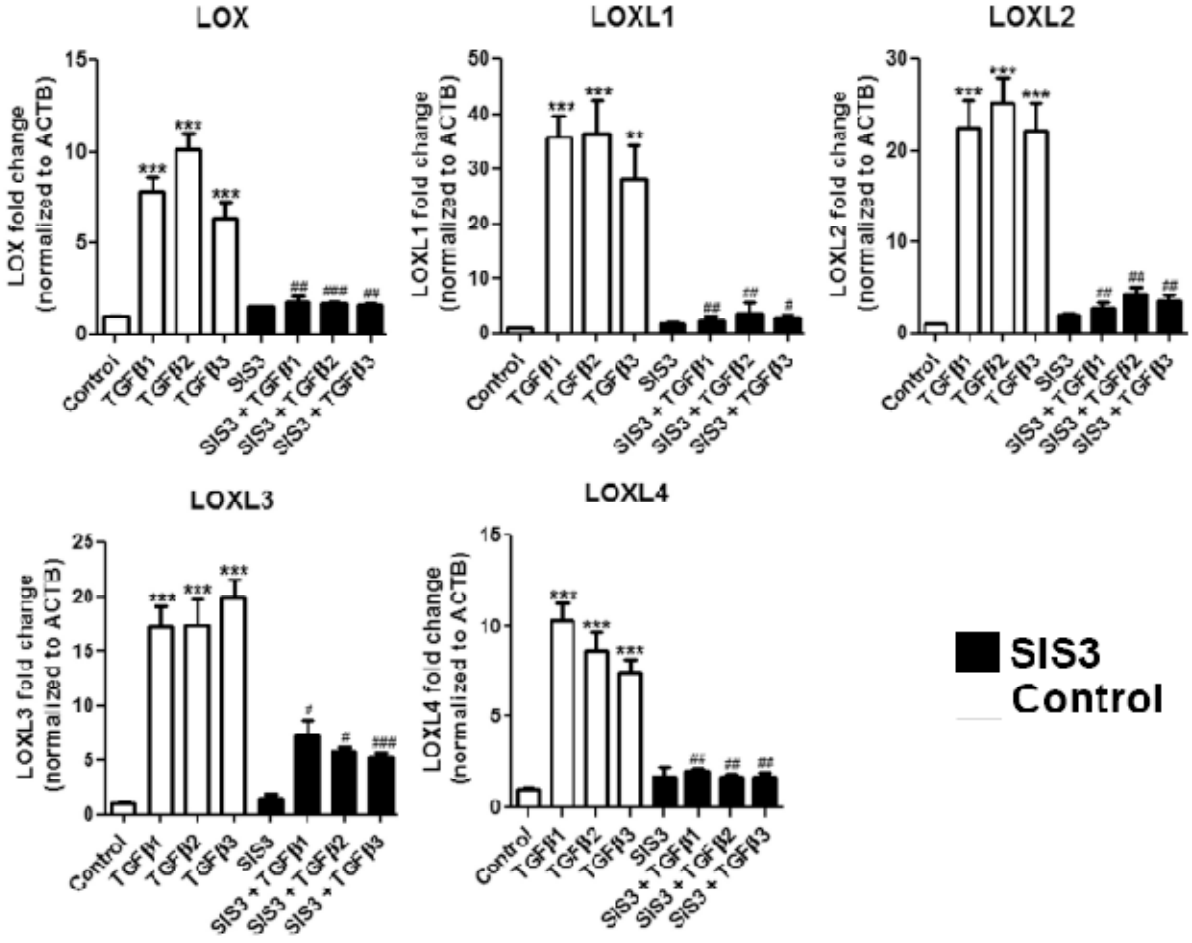

LOX
LOXL1
LOXL2
LOXL3
LOXL4
LOX fold change (normalized to ACTB)
LOXL1 fold change (normalized to ACTB)
LOXL2 fold change (normalized to ACTB)
LOXL3 fold change (normalized to ACTB)
LOXL4 fold change (normalized to ACTB)
Control
TGFβ1
TGFβ2
TGFβ3
SIS3
SIS3 + TGFβ1
SIS3 + TGFβ2
SIS3 + TGFβ3
SIS3
Control

Fig. 7B

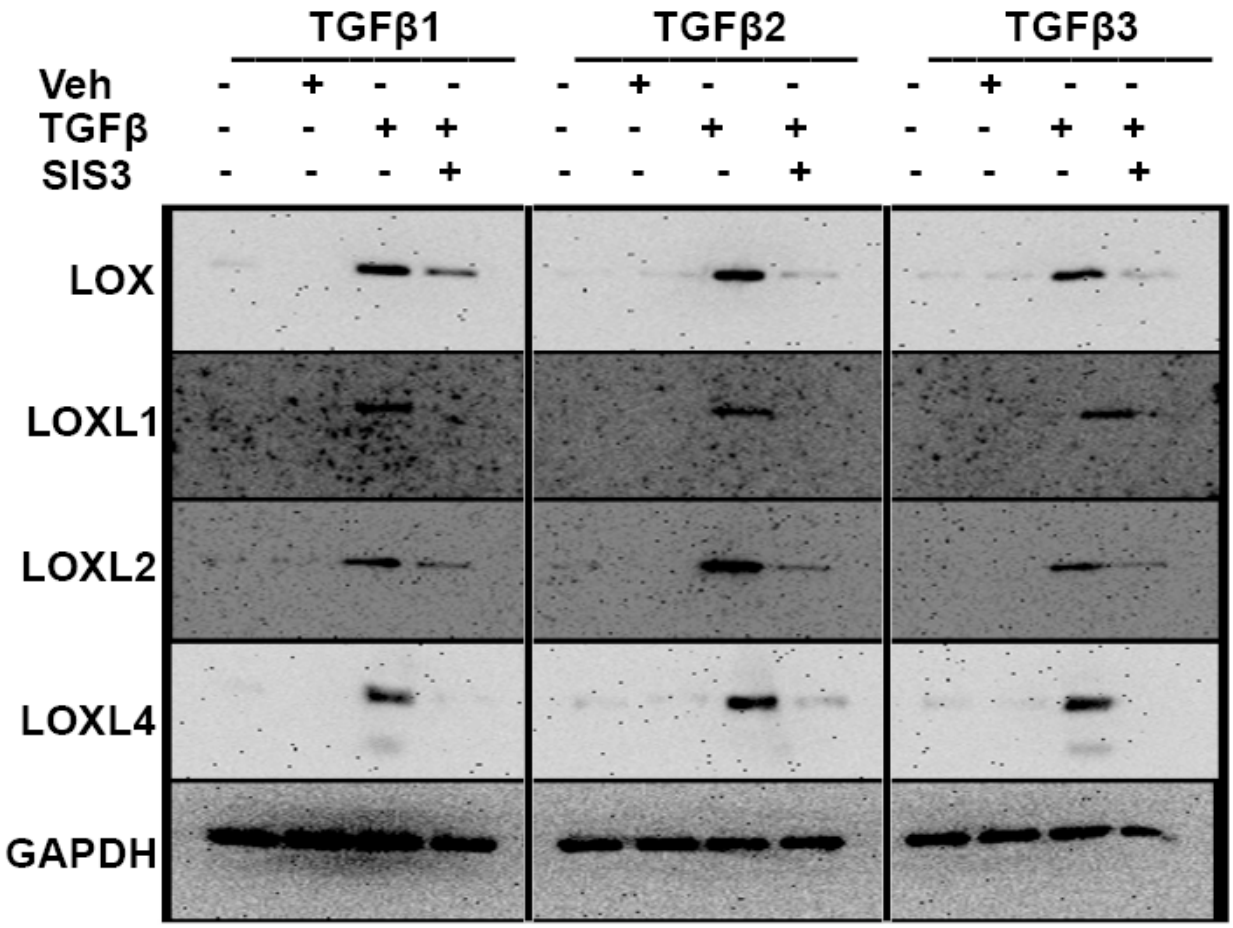


Fig. 7C

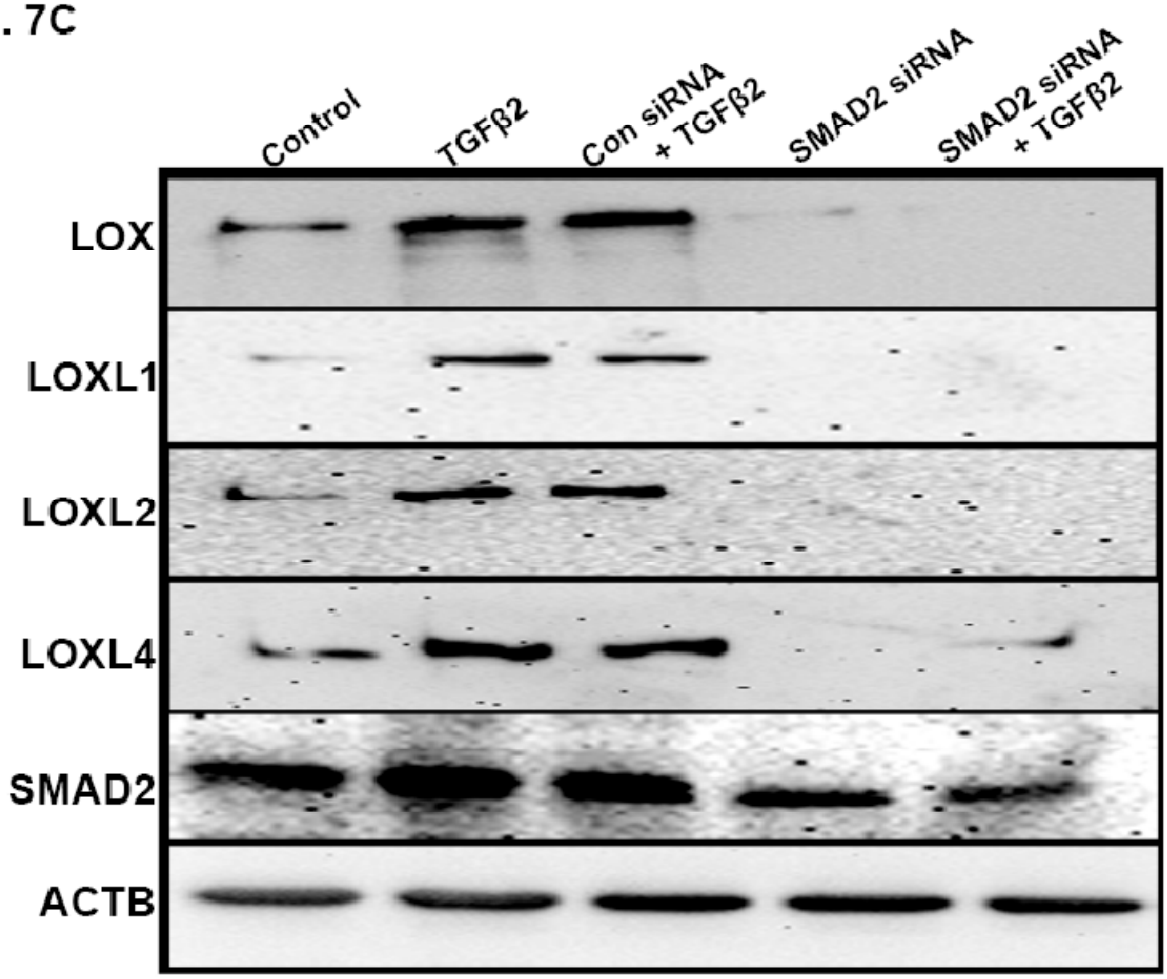

Control
TGFβ2
Con siRNA + TGFβ2
SMAD2 siRNA
SMAD2 siRNA + TGFβ2
LOX
LOXL1
LOXL2
LOXL4
SMAD2
ACTB

Fig. 7D

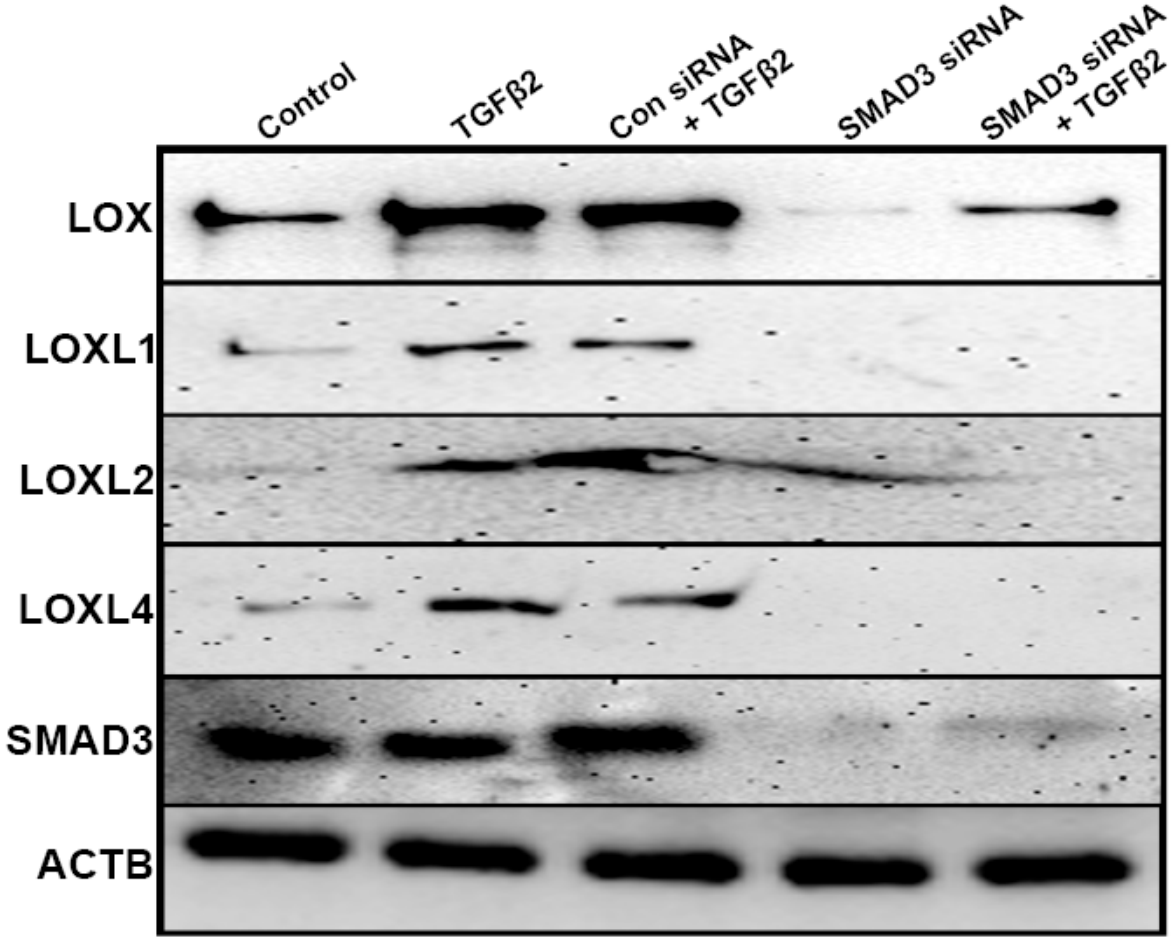

Control
TGFβ2
Con siRNA + TGFβ2
SMAD3 siRNA
SMAD3 siRNA + TGFβ2
LOX
LOXL1
LOXL2
LOXL4
SMAD3
ACTB

Fig. 7E

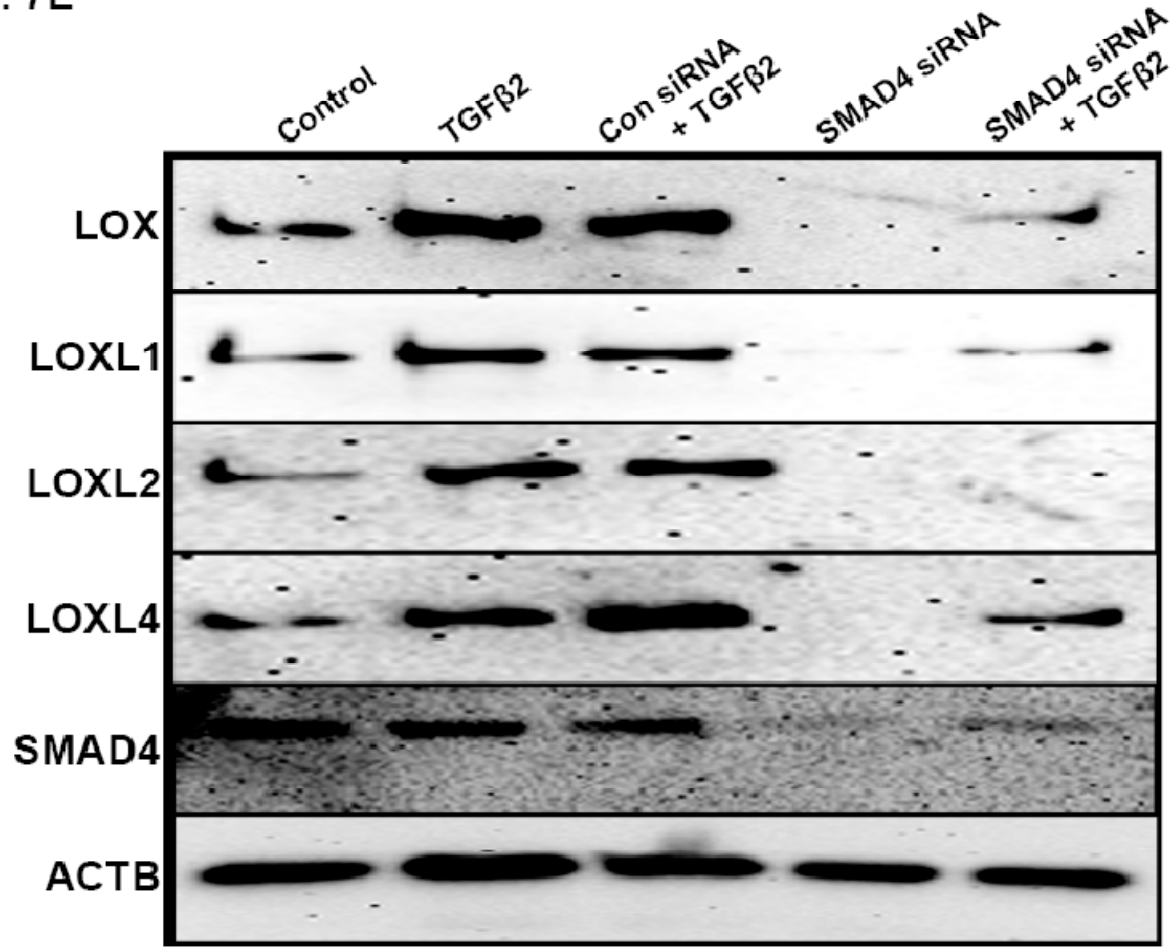

Control
TGFβ2
Con siRNA + TGFβ2
SMAD4 siRNA
SMAD4 siRNA + TGFβ2
LOX
LOXL1
LOXL2
LOXL4
SMAD4
ACTB

Fig. 8A

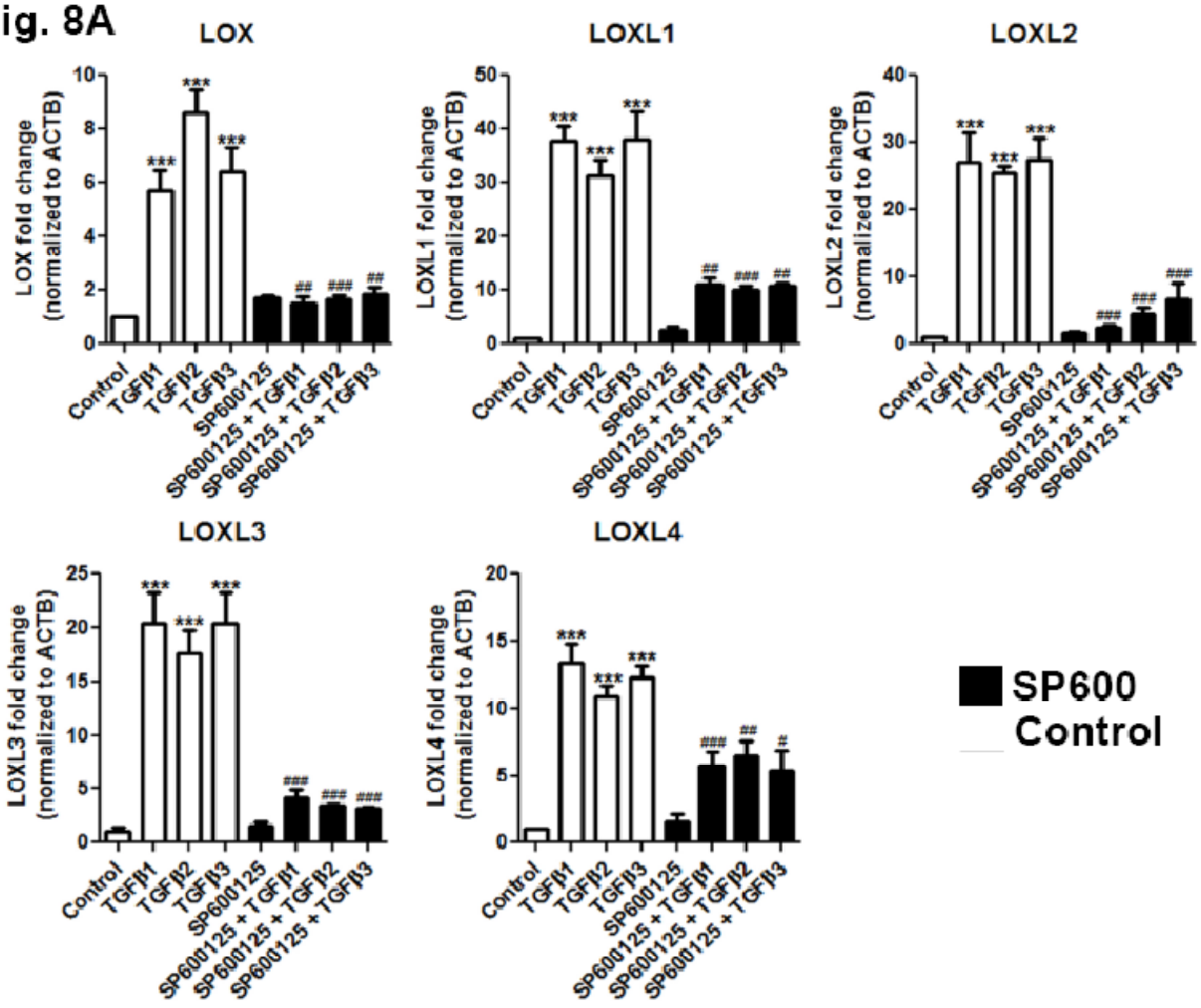

LOX
LOXL1
LOXL2
LOXL3
LOXL4
LOX fold change (normalized to ACTB)
LOXL1 fold change (normalized to ACTB)
LOXL2 fold change (normalized to ACTB)
LOXL3 fold change (normalized to ACTB)
LOXL4 fold change (normalized to ACTB)
Control
TGFβ1
TGFβ2
TGFβ3
SP600125
SP600125 + TGFβ1
SP600125 + TGFβ2
SP600125 + TGFβ3
SP600
Control

Fig. 8B

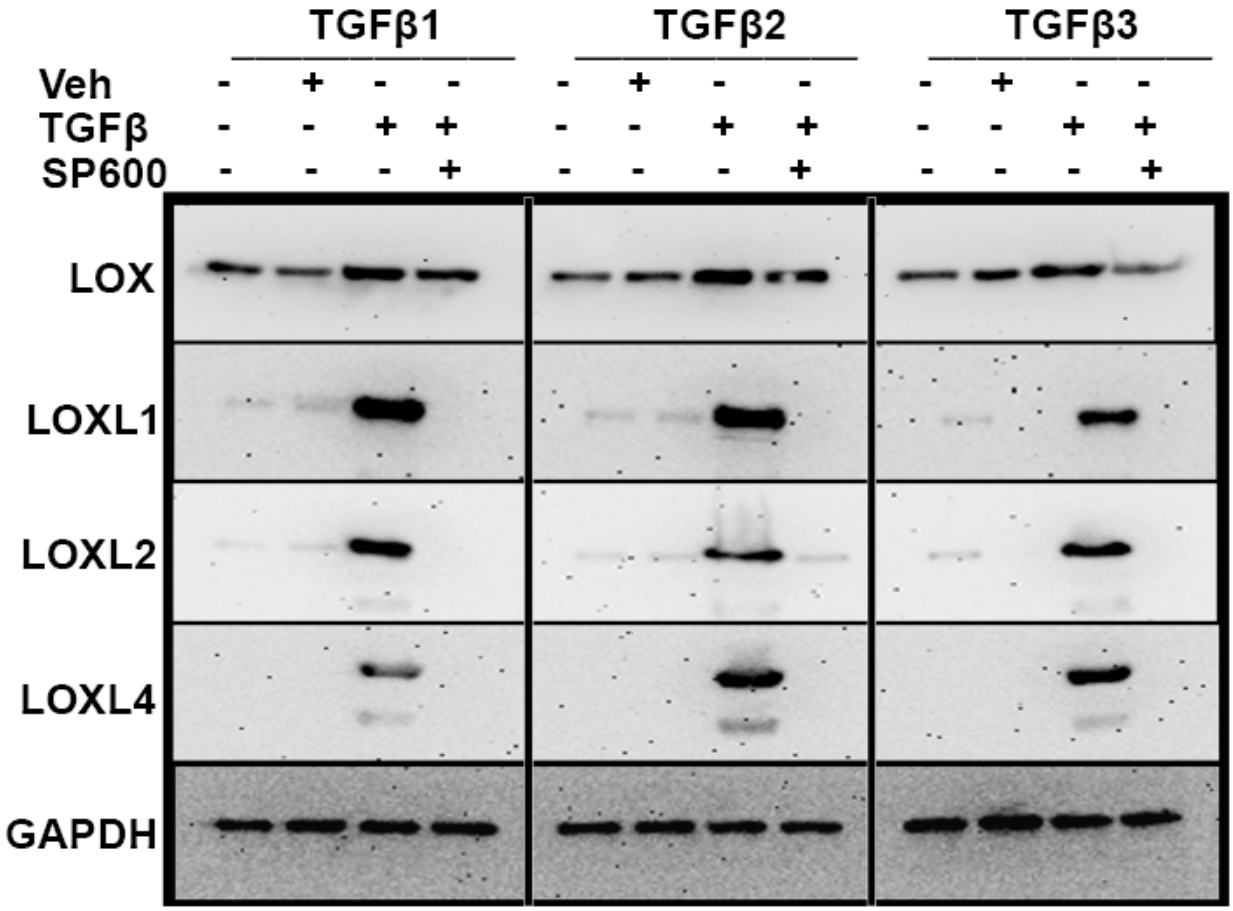

TGFβ1
TGFβ2
TGFβ3
Veh - + - - - + - - - + - -
TGFβ - - + + - - + + - - + +
SP600 - - - + - - - + - - - +
LOX
LOXL1
LOXL2
LOXL4
GAPDH

Fig. 9A

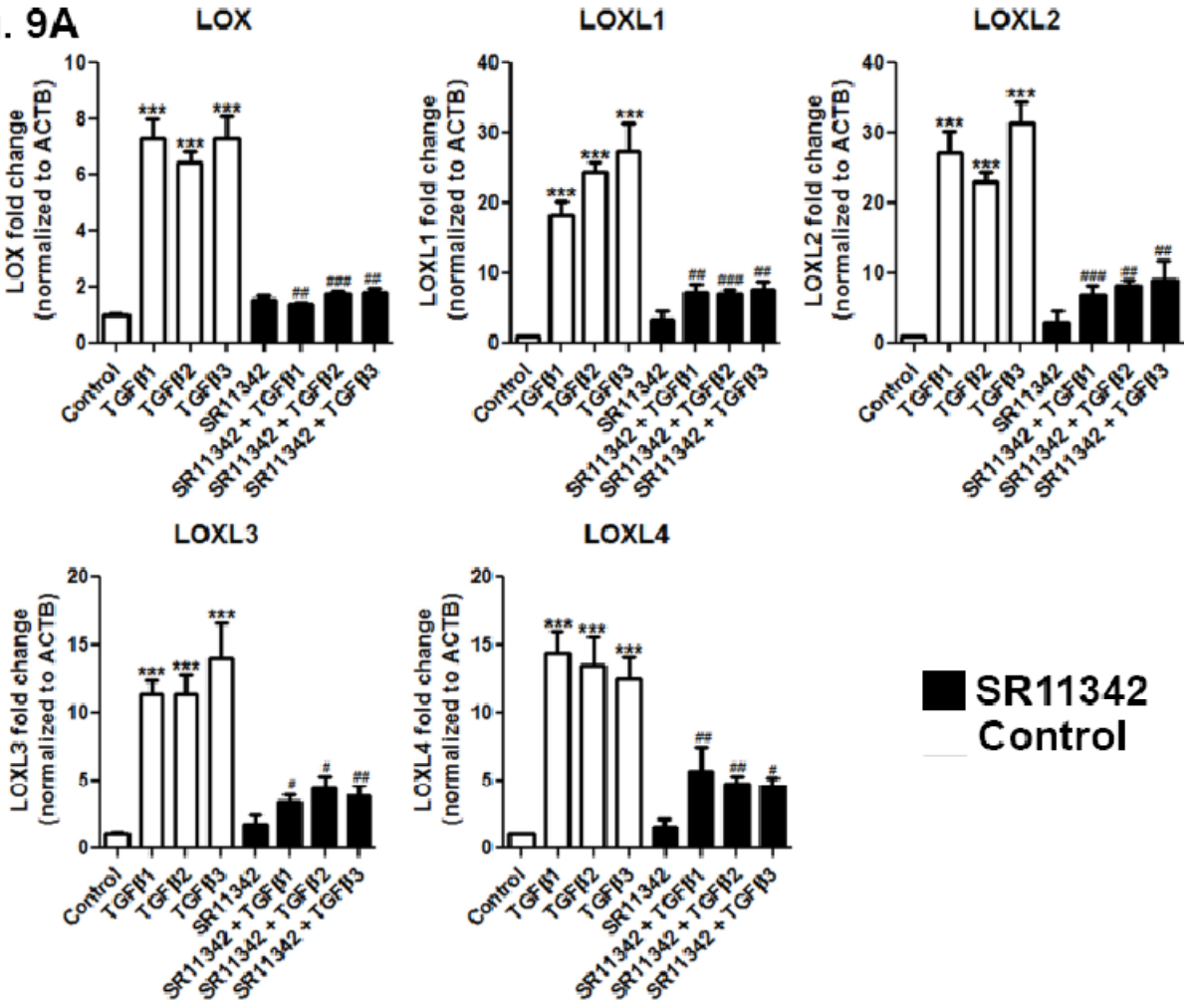

LOX
LOXL1
LOXL2
LOXL3
LOXL4
LOX fold change (normalized to ACTB)
LOXL1 fold change (normalized to ACTB)
LOXL2 fold change (normalized to ACTB)
LOXL3 fold change (normalized to ACTB)
LOXL4 fold change (normalized to ACTB)
Control
TGFβ1
TGFβ2
TGFβ3
SR11342
SR11342 + TGFβ1
SR11342 + TGFβ2
SR11342 + TGFβ3
SR11342
Control

Fig. 9B

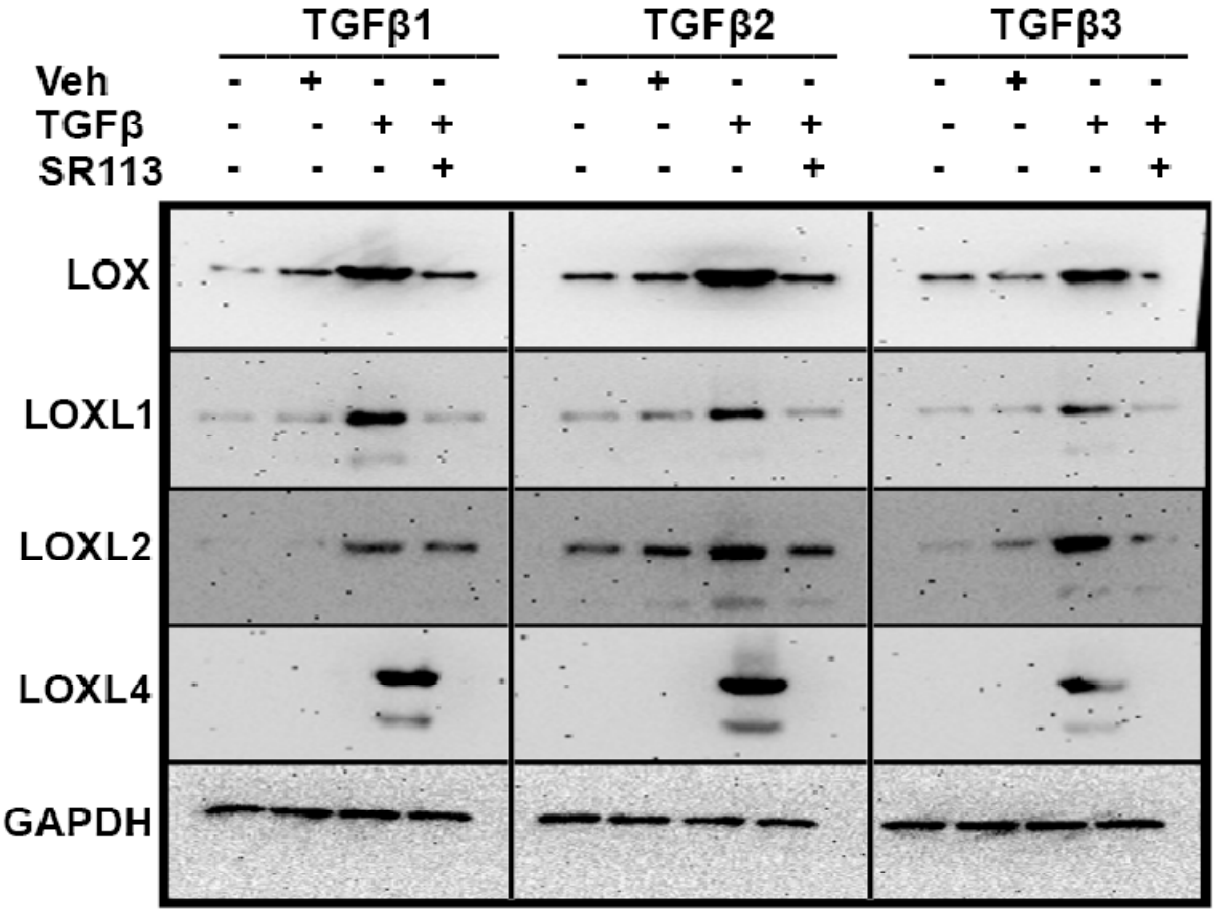

TGFβ1
TGFβ2
TGFβ3
Veh - + - - - + - - - + - -
TGFβ - - + + - - + + - - + +
SR113 - - - + - - - + - - - +
LOX
LOXL1
LOXL2
LOXL4
GAPDH

Fig. 10A

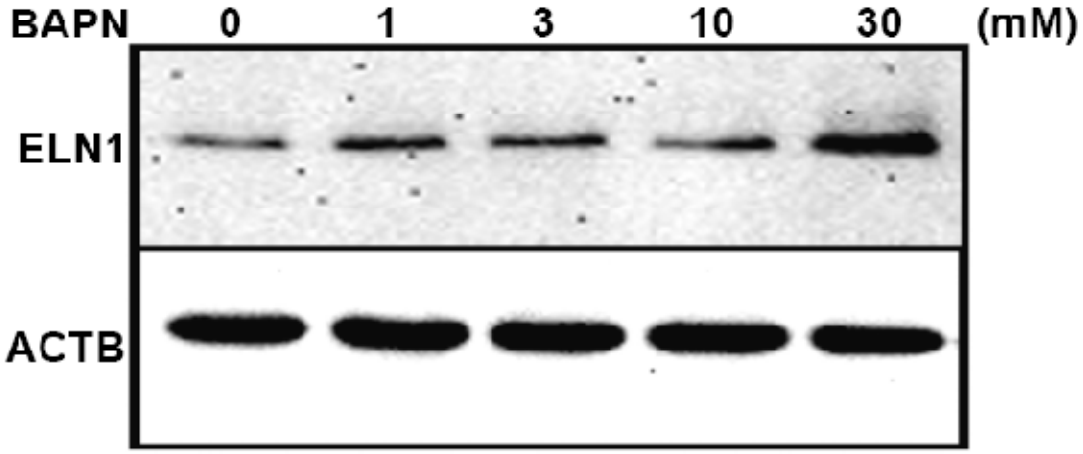


Fig. 10B

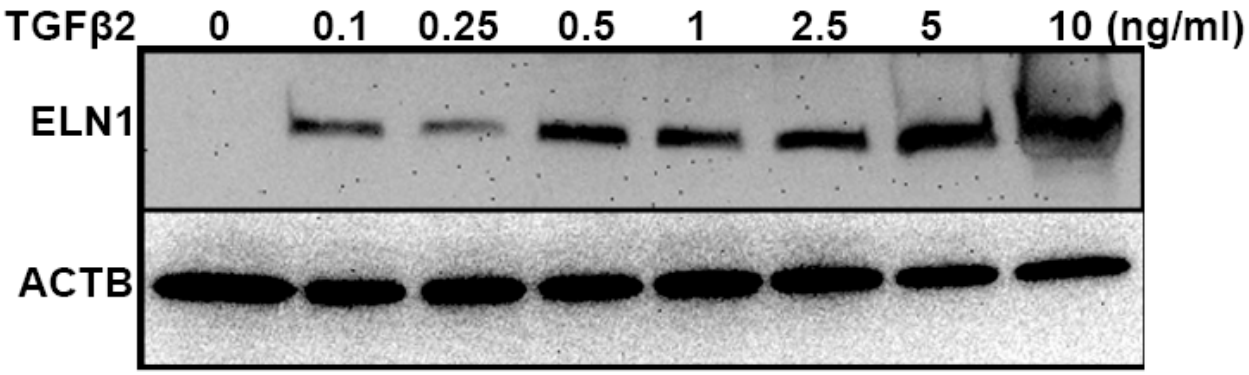


Fig. 10C

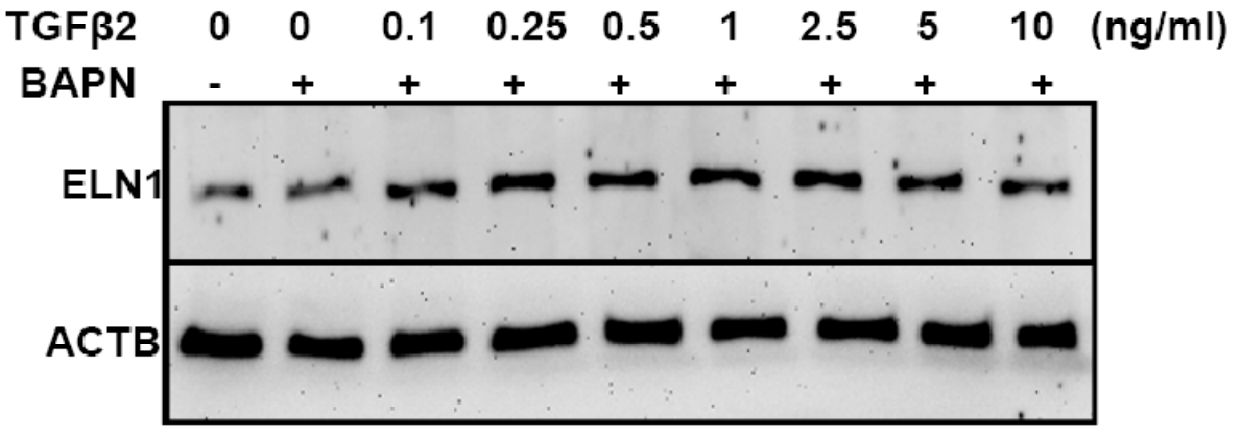


Fig. 10D

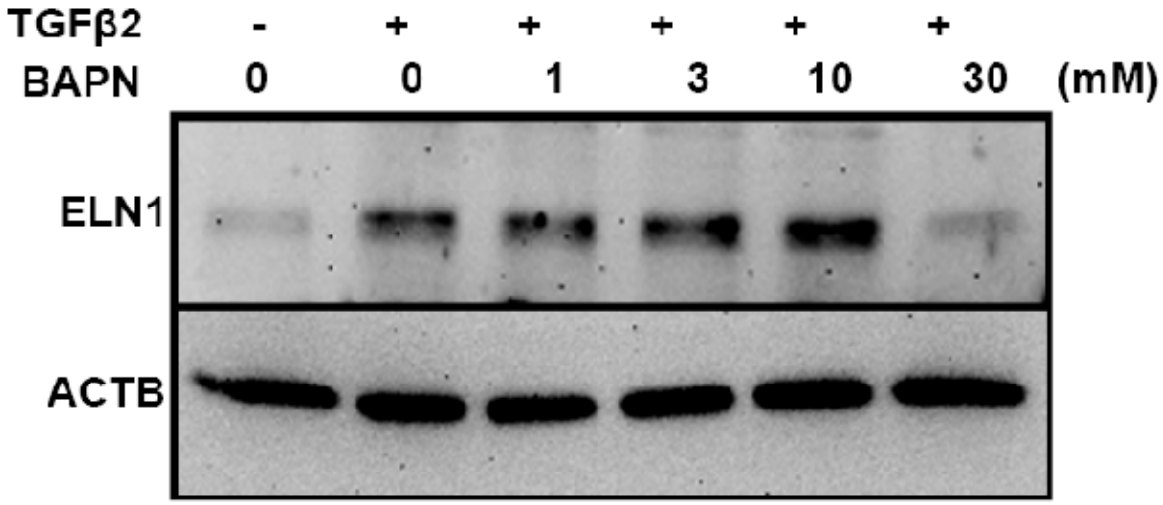
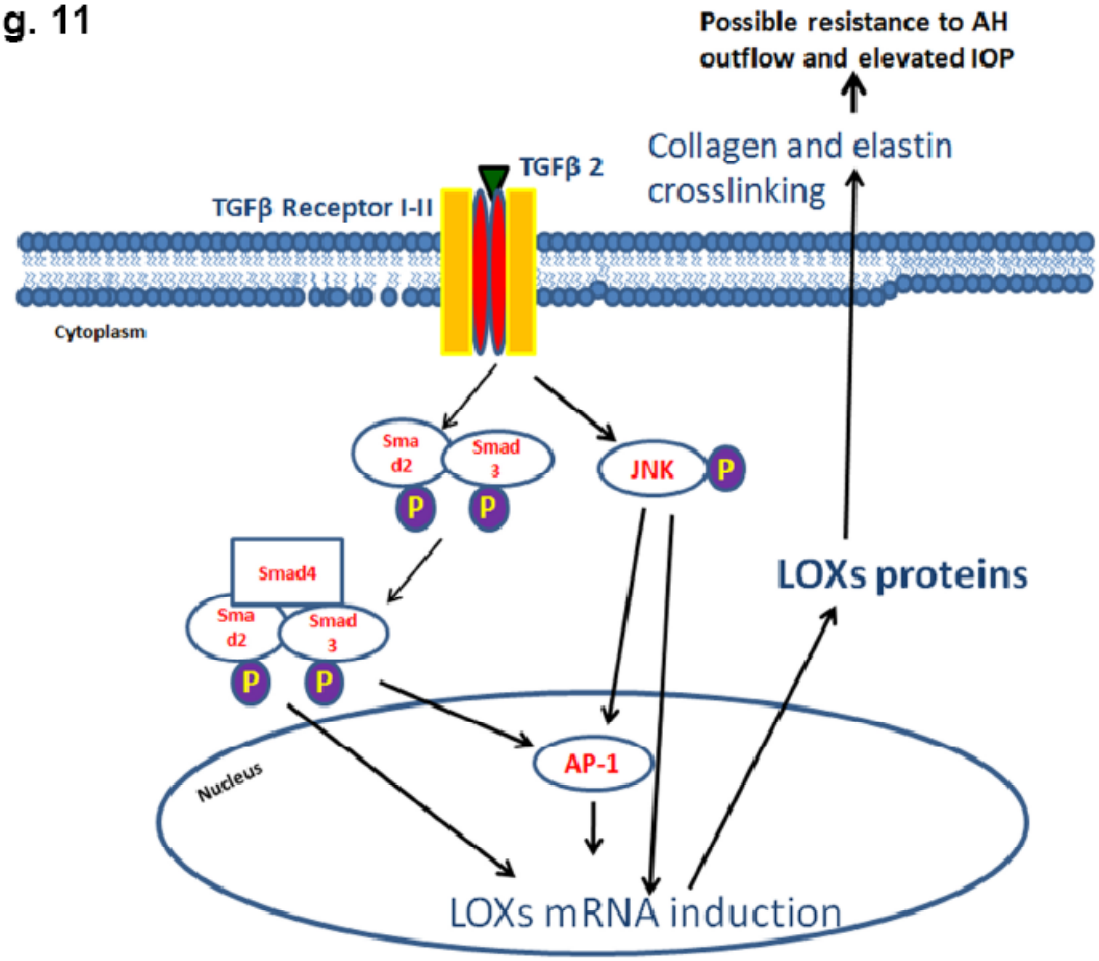


Fig. 11


Possible resistance to AH outflow and elevated IOP
Collagen and elastin crosslinking
TGFβ 2
TGFβ Receptor I-II
Cytoplasm
Sma d2
Smad 3
P
P
JNK
P
Smad4
Sma d2
Smad 3
P
P
LOXs proteins
AP-1
Nucleus
LOXs mRNA induction

References

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Chapter III

ROLE OF TGFβ/SMAD SIGNALING IN GREMLIN INDUCTION OF HUMAN TRABECULAR MESHWORK EXTRACELLULAR MATRIX PROTEINS*

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Abstract

Purpose: The BMP antagonist Gremlin is elevated in glaucomatous TM cells and tissues and elevates intraocular pressure (IOP). Gremlin also blocks BMP4 inhibition of TGFβ2 induction of TM ECM proteins. The purpose of this study is to determine whether Gremlin regulates ECM proteins in the cultured human TM cells.

Methods: Human TM cells were treated with recombinant gremlin to determine the effects on ECM gene and protein expression. Expression of the ECM genes FN, COL1, PAI1, and ELN was examined in cultured human TM cells by quantitative RT-PCR, and western immunoblotting. TM cells were pretreated with TGFBR inhibitors (LY364947, SB431542 or TGFBR1/TGFB2 siRNAs), inhibitors of the Smad signaling pathway (SIS3 or Smad2/3/4 siRNAs) or with CTGF siRNA to identify the signaling pathway(s) involved in gremlin induction of ECM gene and protein expression.

Results: All ECM genes analyzed (FN, COL1, PAI1 and ELN) were induced by Gremlin. This gremlin induction of ECM genes and proteins expression was blocked by TGFBR inhibitors as well as by inhibitors of the canonical Smad2/3/4 and CTGF signaling pathways.

Conclusions: Gremlin employs canonical TGFβ2/Smad signaling to induce ECM genes and proteins in cultured human TM cells. Gremlin also induces both TGFβ2 and CTGF, which can act downstream of gremlin to mediate some of these ECM changes in TM cells.

Introduction

Glaucoma is a leading cause of irreversible visual impairment and blindness in the world, with primary open-angle glaucoma (POAG) being the major form of glaucoma [1-2]. Elevated intraocular pressure (IOP) is a major risk factor for the development and progression of glaucoma [3-4], and this ocular hypertension is due to increased aqueous humor outflow resistance in the trabecular meshwork (TM) and is associated with increased deposition of extracellular matrix (ECM) material within the TM. TGFβ2 levels are elevated in the aqueous humor [5-7] and TM (Tovar-Vidales T et al. submitted for publication) of POAG patients. Trabecular meshwork cells express TGFβ2 receptors, and TGFβ2 has several effects on the TM [8]. TGFβ2 has been shown to increase aqueous outflow resistance and elevate IOP in perfusion cultured human and bovine eyes [9-11] as well as in rodent eyes [12].

TGFβ2 modulates ECM metabolism in TM cells and tissues. This cytokine increased expression of a variety of ECM proteins, including fibronectin (FN), collagen (COL), elastin (ELN), and proteoglycans as well as increased levels of plasminogen activator inhibitor-1 (PAI1) and tissue inhibitor of metalloproteinase-1 (TIMP1), inhibitors that suppress proteolytic degradation of the ECM [13]. In addition, TGFβ2 increased expression of the ECM cross-linking enzymes transglutaminase-2 (TGM2) [14] and lysyl oxidases (LOXs) (Sethi *et. al.* submitted for publication).

We have previously reported that TM cells express several members of the bone morphogenetic (BMP) family, including BMP ligands (BMP2, BMP4, BMP5 and BMP7), BMP receptors (BMPR1a, BMPR1b, BMPR2), and the BMP antagonists gremlin, follistatin and norrin [15-16]. BMPs are members of the TGFβ superfamily of proteins that control multiple functions in a

variety of cell types [17-18]. BMP4 and BMP7 block the TGFβ2-induction of a variety of ECM proteins, including fibronectin-1, collagen IV & VI, TSP-1, and PAI1 [18-19].

Several structurally distinct BMP antagonists tightly regulate BMP cellular activity. BMP antagonists like Gremlin directly bind BMP ligands and block BMP binding to their receptors [20-21]. We have reported that there are greater levels of gremlin in glaucomatous TM cells and tissues [18]. Gremlin antagonized BMP4 inhibition of TGFβ2-induced ECM proteins like FN and PAI1 in TM cells and elevated IOP in perfusion cultured human anterior segments [18]. Gremlin is a highly conserved 20.7 kDa glycoprotein that heterodimerizes with BMP-2, -4 and -7 and plays a key role in regulating multiple cellular functions both during early development as well as adult tissue homeostasis [21-22].

Gremlin may potentiate the profibrotic effects of TGFβ2 by blocking the BMP4 regulation of TGFβ2 activity. However, whether Gremlin alone can induce fibrosis-like activities in cultured TM cells is currently unknown, and the potential signaling mechanisms involved have not been previously characterized. The purpose of the present study was to determine: (1) whether gremlin induces ECM genes and proteins in cultured TM cells and (2) the signaling mechanisms involved in gremlin-induction of ECM genes and proteins.

Methods

TM Cell Culture

Human TM cells were isolated from carefully dissected human TM tissue explants derived from patients with glaucoma or from normal donors and characterized as previously described [10, 15, 18, 23-24]. All donor tissues were obtained from regional eye banks and managed according to the guidelines in the Declaration of Helsinki for research involving human tissue. Isolated TM cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) containing L-glutamine (0.292 mg/mL; Gibco BRL Life Technologies), penicillin (100units/ml)/streptomycin (0.1 mg/mL); Gibco BRL Life Technologies), and 10% fetal bovine serum (Gibco BRL Life Technologies).

TM Cell Treatments

TM cells were grown to 100% confluency and then kept in serum-free medium for 24 hours prior to treatments to avoid the effect of serum proteins. TM cells were incubated with fresh medium containing specific signaling inhibitors for 1-6 hrs., prior to the addition of varying concentrations of recombinant gremlin protein (R&D System, Minneapolis, MN). Small molecule inhibitors LY364947 (5μM, Cat. No. 2718, Tocris biosciences, Ellisville, MO) and SB431542 (5μM, Prod. No. S4317, Sigma-Aldrich, St. Louis, MO) were used to examine the effects of inhibition of TGFβ Receptor-1/2. Smad-3 phosphorylation inhibitor SIS3 (10μM, Prod. No. S0447, Sigma-Aldrich, St. Louis, MO), JNK inhibitor SP600125 (10μM, Prod. No. S5567, Sigma-Aldrich, St. Louis, MO), and P-38 inhibitor SB203580 (5μM, Cat. No. 1202, Tocris Biosciences, Ellisville, MO) were used to examine effects of inhibition on canonical Smad, JNK, and P-38 signaling pathways.

Small Interfering RNA and Transfection

siRNAs for Smad2, Smad3, Smad4, TGFBR1, TGFB2, and CTGF as well as non-targeting control siRNAs (SMARTpool) were purchased from Dharmacon (Lafayette, CO). siRNA transfection was performed as described previously [25-26]. Three different TM cell strains were grown in 12-well plates containing DMEM with 10% FBS. In one tube, 4 μL of DharmaFECT 1 Transfection Reagent (T-2001-01; Dharmacon, CO) was mixed gently with 200 μL of Opti-MEM medium (Invitrogen, Carlsbad, CA) and incubated for 5 min at room temperature. In separate tubes, siRNAs were mixed gently with 200 μL of Opti-MEM medium. These two tubes were combined, gently mixed, and incubated for 20 min at room temperature. After incubation, DMEM without FBS and antibiotics was added to obtain a final volume of 2 mL for each well (10 nM of test and control siRNAs). Cells were washed with sterile PBS and incubated with siRNA transfection solution for 24 h at 37°C. Cells were washed with sterile PBS and incubated with 10% FBS containing DMEM for 24 h at 37°C. Cells were then washed with serum-free DMEM medium for 24 hours and treated with TGFβ2 in serum-free DMEM medium for an additional 48 hrs. Cell lysates and conditioned medium were analyzed for various proteins by the western blotting (see Table 1 for list of antibodies used).

RNA isolation and RT-PCR

Total cellular RNA was extracted from cultured TM cells using TRI Reagent RT extraction (Cat. # RL-311, MRC Inc., Cincinnati, OH), and the SuperScript VILO cDNA Synthesis kit (Cat. # 11754, Invitrogen) was used for first strand cDNA synthesis. Primers for the various LOX proteins were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/). The primer pairs, expected product sizes, and annealing temperatures are listed in Table 2.

Quantitative Real Time PCR

Real-time PCR was performed as described previously [18]. Briefly, 2.5 µL of cDNA was used in a reaction consisting of 1.5 units per reaction of antibody-bound Taq enzyme (Jump Start; Sigma-Aldrich, St. Louis, MO), 10x PCR buffer, 1.5 mM $MgCl_2$, 200 nM dNTP mix, 100 nM PCR primers (Table 2), 2.5 µL green nucleic acid dye (EvaGreen; Biotium, Hayward, CA), as well as 30 nM passive reference dye (Rox; USB, Cleveland, OH) per 50-µL reaction. PCR was performed on a real-time thermal cycler (model Mx3000p; Stratagene, La Jolla, CA), with cycling parameters of initial denaturation at 95°C; 40 cycles of 95°C 30 seconds, 60°C 30 seconds, and 72°C 60 seconds, and a denaturation cycle for creation of a dissociation curves. Reactions for each sample were run in duplicate, cycle thresholds (Ct) were normalized to either beta-actin or GAPDH expression as housekeeping genes, and comparative quantitation was performed using MxPro ver. 4.0 software (Stratagene). Only individual PCR samples with single-peak dissociation curves were selected for data analysis.

Protein Extraction and Western Blot Analysis

Secreted proteins: ECM proteins secreted by TM cells were determined by Western immunoblot analysis. Conditioned medium was collected from human TM cells after 24-hour treatment with gremlin in serum-free medium containing 0.5 mg/mL BSA. Proteins were separated on a 10% denaturing polyacrylamide gel and transferred by electrophoresis to a PVDF membrane. Blots were blocked with 5% Fat-free Dry Milk in tris-buffered saline tween buffer (TBST) for 1 h and then incubated overnight with primary antibodies (Table 1). The membranes were washed with TBST and processed with corresponding horseradish peroxidase-conjugated secondary antibodies (Table1). The proteins were then visualized in a Fluor ChemTM 8900

imager (Alpha Innotech, San Leandro, CA) using ECL detection reagent SuperSignal West Femto Maximum Sensitivity Substrate (Cat. # 34096, Pierce Biotechnology Rockford, IL)

Cell-associated proteins: Total cellular protein was extracted from the TM cells using mammalian protein extraction buffer (MPER, Cat # 78501; Pierce Biotechnology), containing protease inhibitor (Cat. # 78415, Pierce Biotechnology) and phosphatase inhibitor (Cat. # 78420, Pierce Biotechnology) cocktails. Protein concentration was determined using the Bio-Rad Dc protein assay system (Cat. # 500-0111, Bio-Rad Laboratories, Richmond, CA). The cellular proteins were separated on denaturing polyacrylamide gels and then transferred to PVDF membranes by electrophoresis. Blots were blocked with 5% Fat-free Dry Milk in TBST for 1 h and then incubated overnight with primary antibodies (Table 1). The membranes were washed with TBST and processed with corresponding horseradish peroxidase-conjugated secondary antibodies (Table 1). The proteins were then visualized in a Fluor ChemTM 8900 imager (Alpha Innotech) using ECL detection reagent SuperSignal West Femto Maximum Sensitivity Substrate (Cat. # 34096, Pierce Biotechnology). To ensure equal protein loading, the same blot was subsequently developed for β-Actin expression.

Fibronectin ELISA

Conditioned medium of human TM cells was collected and evaluated for gremlin effects on fibronectin production using a commercially available Quantimatrix Human Fibronectin ELISA kit (Cat. # ECM300; Chemicon International, Billerica, MA). We previously demonstrated that treatment of cultured human TM cells with TGF2 significantly increases fibronectin levels in the culture medium [10, 18].

Statistical Analysis

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

Results

Gremlin induces ECM proteins in TM cells

Gremlin has been previously reported to antagonize BMP4 inhibition of TGFβ2-induction of ECM proteins like FN and PAI1 in human TM cells [18]. However, it is not known whether gremlin alone can induce these ECM proteins in TM cells. Therefore, we determined the effect of gremlin on FN, COL1a, PAI1 and ELN expression in cultured human TM cells. Treatment with gremlin (1 μg/ml) for 24 hours significantly induced FN, COL1a, PAI1 and ELN mRNA expression ($n=3$, $p<0.05$) (Fig. 1A) as well as ECM protein expression in cell lysates and conditioned medium (Fig. 1B). We also performed quantitative FN ELISA on the conditioned medium samples of the four TM strains utilized for the western immunoblotting assay (Fig. 1B), and gremlin significantly elevated the amount of secreted FN in the treated TM cells compared to controls ($n<0.001$) (Fig. 1C).

Gremlin induces ECM genes and proteins in a concentration- and time-dependent fashion

Trabecular meshwork cell strains ($n=3$) were treated with increasing concentrations of gremlin (0-5 μg/ml) for 24 hours. The mRNA and protein expression of FN, COL1, PAI1 and ELN were determined using qRT-PCR and western immunoblotting respectively. Gremlin induced expression of ECM mRNA (Fig. 2A), cell-associated (Fig. 2B) and secreted ECM proteins (Fig. 2C) in a concentration-dependent manner. Gremlin also significantly elevated the amount of secreted FN assessed by ELISA in a concentration-dependent manner (Fig. 2D). TM cells were treated with gremlin for 6, 12 and 24 hours to determine the time dependence of ECM mRNA induction. Gremlin significantly ($p<0.01$) induced FN, PAI-1, COL1, and ELN mRNA expression, although the time course of induction varied slightly for each gene (Fig. 3A).

Similarly, TM cell strains (n=3) were treated with gremlin (1 μg/ml) for 3, 12, 24, 48 and 72 hours to evaluate effects on ECM protein expression. Gremlin induced both cell-associated and secreted ECM proteins as early as 12 hours and maintained this induction for up to 72 hours (Fig. 3B and C). Gremlin also significantly elevated the amount of secreted FN in a time-dependent manner (Fig. 3D). Therefore, gremlin induction of ECM mRNA and proteins was both time and dose dependent.

TGFβ signaling in Gremlin induction of ECM proteins

Gremlin was previously reported to antagonize the BMP4 inhibition of TGFβ2-induced ECM proteins in human TM cells [18], but the signaling mechanism(s) involved was not determined. We used various small molecule inhibitors to explore the involvement of TGFβ signaling pathway(s) in gremlin-mediated ECM induction. SB431542 is a selective TGFBR1 and TGFBR2 receptor inhibitor [27], while LY364947 is a relatively selective inhibitor for the TGFBR2 receptor [28]. We pretreated TM cell strains (n=3) for 1 hr with or without 5 μM SB431542 or LY364947 followed by treatment with recombinant Gremlin (1 μg/ml) for 24 hours. Gremlin elevated FN, COL1, PAI1 and ELN mRNA expression compared to untreated or inhibitor only-treated samples ($p<0.001$). Pretreatment with either of the two inhibitors, LY364947 or SB431542 completely blocked gremlin-mediated mRNA induction in all the cell strains ($p<0.001$) (Fig. 4A).

We used the same strategy to evaluate the effects of these TGFBR inhibitors on gremlin induction of ECM proteins. Gremlin elevated cell associated as well as secreted FN, COL1, PAI1 and ELN protein levels compared to untreated or vehicle-treated samples. Each of the two inhibitors, LY364947 (Fig. 4B) or SB431542 (Fig. 4C), completely inhibited the Gremlin-mediated ECM protein induction. Treatment with the inhibitors alone did not have any effect on

the ECM proteins expression. We also analyzed the conditioned medium samples using FN ELISA. Gremlin treatment significantly elevated the amount of secreted FN, which was blocked by pretreatment with the two TGFBR inhibitors (Fig. 4D). The FN ELISA data agreed with our western immunoblotting data (Figure 4B and C).

In addition to TGFBR1/2 inhibitors, we also used siRNA-mediated TGFBR1 knockdown to confirm the role of TGFβ receptor signaling in gremlin induction of ECM proteins. Gremlin-treated TM cells were untransfected or transfected with a non-targeting siRNA control or TGFBR1 siRNA. As previously shown, Gremlin induced ECM protein expression. Control siRNA did not affect endogenous TGFBR1 levels and did not affect gremlin-induction of ECM proteins expression. Consistent with the data with small molecule TGFBR1 or TGFBR2 inhibition, TGFBR1 siRNA knockdown inhibited Gremlin-induction of ECM proteins (Fig. 4E).

TM cells endogenously express TGFβ2 [6, 29] and gremlin [15, 18] proteins, and both TGFβ2 [10, 30] and gremlin (Fig. 1-3) induce ECM proteins in TM cells. We wanted to determine whether Gremlin treatment alters endogenous levels of TGFβ2 and vice-versa, which in turn may regulate gremlin's ECM induction profile. TM cell strains (n=3) were treated with increasing concentrations of gremlin (0-5 μg/ml) or TGFβ2 (0-10 ng/ml) for 24 hours. The mRNA and protein expression of TGFβ2 and gremlin were determined using qRT-PCR and western immunoblotting, respectively. Gremlin induced TGF□2 and TGFβ2 induced Gremlin mRNA (Fig. 5A) and protein (Fig. 5B) expression in a concentration-dependent manner.

We employed siRNA-mediated TGFB2 knockdown to confirm the role of TGFβ2 in gremlin induction of ECM proteins. Gremlin-treated TM cells were either untransfected or transfected with a non-targeting siRNA control or TGFB2 siRNA. Gremlin induced both ECM proteins and

TGFβ2. Control siRNA did not affect endogenous TGFB2 levels and did not alter gremlin-induction of ECM proteins expression. However, TGFB2 knockdown blocked Gremlin-induction of ECM proteins (Fig. 5C). Taken together, our results (Figs. 4 and 5) strongly support the roles of TGFβ2 and TGFBR in gremlin induction of ECM protein expression.

Role of Connective tissue growth factor (CTGF) in Gremlin induction of ECM proteins

CTGF regulates several ECM proteins in cultured human TM cells and mediates TGFβ2 induction of FN, collagens I, II, IV and integrins [31]. We wanted to determine whether CTGF is involved in gremlin induction of ECM proteins in TM cells. Gremlin-treated TM cells were either untransfected or transfected with a non-targeting siRNA control or CTGF siRNA. Gremlin induced both ECM proteins and CTGF. Control siRNA did not affect endogenous CTGF levels and did not affect gremlin-induction of ECM proteins expression. However, CTGF knockdown blocked Gremlin-induction of cell-associated and secreted ECM proteins FN and COL1 but not ELN or PAI1 (Fig. 6).

Gremlin induces ECM proteins utilizing Smad signaling pathway

The profibrotic cytokine TGFβ2 can activate both canonical Smad and non-canonical signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway in various cells and tissues [32-34]. TGFβ signaling is complex because these different signal transduction pathways can interact with each other [35-36]. We wanted to determine whether gremlin treatment activates any of the TGFβ signaling pathways and whether any of the TGFβ signaling mechanism(s) are involved in gremlin-induction of ECM proteins in TM cells.

A previous study showed that TGFβ2 activates both canonical Smad and MAPK signaling in TM cells [37]. We treated 4 primary TM cell strains with TGFβ2 (5 ng/ml) or gremlin (1 μg/ml) for 15, 30, 60, 120 and 240 minutes, and total and phosphorylated Smad2 and Smad3,as well as P38 and JNK1/2 MAPK proteins were evaluated by western immunoblotting. TGFβ2 phosphorylated both canonical (Smad2 and Smad3) as well as non-canonical (P38 and JNK1/2) pathways (Fig. 7A-B). In contrast, Gremlin activated only the Smad pathway (Fig. 7A) but not the P38 or JNK1/2 pathways (Fig. 7B). There were no changes in total Smad2, Smad3, P38, and JNK1/2 levels.

Phosphorylated Smads 2 and 3 together or individually form a complex with co-Smad4 to regulate transcription of their target genes [38-39]. To determine if Smad3 transcriptionally regulates the ECM proteins, we employed a selective small molecule inhibitor of Smad3, SIS3. Three TM cell strains were treated with SIS3 (10 μM) six hours prior to treating with recombinant gremlin for an additional 24 hours to study mRNA and protein expression of FN, COL1, PAI1 and ELN. Untreated cells and SIS3 alone treated cells served as negative controls. Gremlin-induction of ECM mRNA and cell-associated protein expression was inhibited by SIS3 pretreatment ($p<0.01$) (Figs. 8A & 8B). SIS3 treatment also blocked gremlin induction of cell associated and secreted ECM proteins as well as soluble FN in conditioned medium analyzed by western immunoblotting (Fig. 8B) and ELISA (Fig. 8C), respectively. These results concurred with our mRNA results (Fig. 8A). Therefore, gremlin induction of ECM mRNA and proteins is mediated by Smad3 signaling.

To confirm the role of Smad signaling in gremlin regulation of ECM protein expression, we employed siRNA-mediated knockdown of Smad2, Smad3, and Smad4. Non-targeting siRNA

served as the negative control. Cells transfected with Smad3 (Fig. 8D), Smad2 (Fig. 8E), or Smad4 (Fig. 8F) siRNAs were followed by treatment with or without gremlin. Untransfected and untreated cells served as negative controls, while untransfected cells treated with gremlin served as the positive control. Gremlin increased the expression of ECM proteins. Control siRNAs neither affected gremlin-induction of ECM proteins nor did they affect the endogenous Smad2/3/4 levels. As expected, knockdown of Smad3 completely inhibited gremlin-induction of the cell-associated and secreted ECM proteins (Fig. 8D). Smad2 and Smad4 knockdown also completely inhibited gremlin-induction of the cell-associated and secreted FN and PAI1 proteins. However, Smad2 or Smad4 knockdown did not consistently inhibit gremlin-induction of COL1 and ELN even though levels of each Smad were sufficiently reduced (Fig. 8E and F). This variable effect was seen within the same cell strain as well as between different cell strains. Taken together, these results (Fig. 8) strongly support Smad3-dependent regulation of gremlin induction of all ECM protein expression.

Discussion

Interactive TGFβ/BMP signaling plays an important role in ECM homeostasis, and perturbation in the balance of this signaling is associated with fibrotic diseases, including glaucoma. TGFβ2 plays an important role in glaucoma pathogenesis [5]. Aqueous humor levels of TGFβ2 are significantly elevated in POAG patients [5-7], and TGFβ2 is also elevated in glaucomatous TM cells and tissues (Tovar-Vidales, submitted for publication). TGFβ2 increases the expression of a number of ECM proteins in the TM, and also elevates IOP in perfusion cultured anterior segments (B) and rodent eyes [12]. Trabecular meshwork cells and tissues express BMPs, BMP receptors and BMP antagonists, and BMP4 and BMP7 inhibit TGFβ2 induction of ECM proteins [18-19]. Inhibition of BMP signaling exacerbates the TGFβ2 effect on the TM ECM. Gremlin protein levels are higher in GTM cells, and gremlin blocks BMP suppression of TGFβ2 mediated effects on the TM ECM [18]. In addition, Gremlin treatment alone elevates IOP in perfusion cultured anterior segments [18], suggesting that perturbation of normal TGFβ2/BMP homeostasis can play a role in ocular hypertension.

To directly test this latter hypothesis, we examined the effect of gremlin on TM ECM expression. We found that gremlin increased ECM mRNA and protein expression. However, in contrast to TGFβ2 that activates both the Smad and nonSmad MAPK signaling pathways, gremlin only activated the canonical Smad 2/3 pathway. Inhibition of Smad signaling, but not nonSmad signaling, blocked the gremlin effect on TM ECM expression. Connective tissue growth factor is induced by TGFβ2 and acts as a downstream mediator of TGFβ signaling, regulating the induction of multiple ECM proteins including FN, collagen types I, II, IV, and VI [31]. Interestingly, our results show that gremlin induces CTGF, and that the gremlin induction of FN and COL1 is dependent on CTGF. In contrast, gremlin induction of PAI1 and ELN was not

dependent on CTGF. Others have also reported that CTGF does not induce PAI1 expression in human TM cells [31]. We did not examine if CTGF can also induce gremlin in a feed-forward loop and if gremlin can act also as a mediator of CTGF signaling. These experiments are currently under investigation.

Most studies focusing on gremlin have been focused on its role in development or in several fibrotic diseases. It is not uncommon to find developmental genes re-expressed in several diseased conditions including several kinds of cancer. However, there need to be more studies that need to be designed to address such hypothesis.

It also appears that Gremlin and TGFβ2 are involved in a "feed forward" pathogenic pathway. We have shown that gremlin increases TGFβ2 expression, and TGFβ2 increases gremlin expression in TM cells. This process would further exacerbate ECM deposition within the TM, potentially leading to increased aqueous humor outflow resistance and IOP elevation. Levels of both TGFβ2 and Gremlin are elevated in the anterior segment in glaucoma, but the primary cause of increased expression of these signaling molecules in glaucomatous eyes is currently unknown. Mechanical stress (i.e. cyclic stretch) and substrate elasticity have been shown to increase TGFβ expression in the TM **[40-41]**. The effects of these perturbations on gremlin expression have not been evaluated. It is plausible to hypothesize that elevated levels of gremlin in glaucoma patients may lead to higher TGFβ2 levels in the TM.

TGFβ and gremlin also play a role in other fibrotic diseases. Gremlin has been associated with several fibrotic diseases of lungs [42], kidneys [43-44] and in osteoarthritis [45]. Gremlin was shown to induce expression of FN [26] and several types of collagens [46]. Gremlin has also been reported as a

downstream mediator of TGFβ's fibrotic effects in the kidney [44]. Several growth factors like CTGF have been reported to induce ECM proteins like TGFβ2 in several kinds of cells [47].

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

Acknowledgements

The authors would like to acknowledge grant support from the National Institute of Health-National Eye Institute (EY-017374 awarded to RJW). The authors will also like to thank Jessica Chiou for her help with this project.

Figure Legends

Figure 1: **Gremlin induces ECM mRNA and proteins in TM cells**. (A) Induction of FN, COL1a, PAI1 and ELN mRNA in three TM cell strains treated with Gremlin (1 μg/ml) for 24 hours. The graph values represent the fold induction of ECM genes normalized to GAPDH. Three replicates of each sample were employed. Gremlin significantly induced ECM genes in all the three cell lines. ** $0.0001<p<0.01$ and *** $p<0.0001$ (B) Western immunoblots of cell-associated and secreted ECM proteins in two TM cell strains treated with Gremlin (1 μg/ml) for 24 hours. Gremlin induced cell-associated ECM proteins were normalized as compared to ACTB, while equal volumes of conditioned medium. Similar results were observed in two additional TM cell strains. These western blot images are representative of three independent experiments. (C) Induction of secreted FN in four TM cell strains treated with Gremlin (1 μg/ml) for 24 hours. FN ELISA values represent the ng/ml of FN. Three replicates of each sample were employed. *** $p<0.0001$.

Figure 2: **Concentration-dependent Gremlin-induction of ECM mRNA and proteins.** Concentration dependent induction of ECM genes mRNA (A), cell-associated protein (B), and secreted proteins (C) by 0-5 μg/mL Gremlin in cultured TM cell strains (n=3). qRT-PCR values (A) represent fold Gremlin induction compared to controls and normalized to ACTB as housekeeping gene. Three replicates of each sample were employed. Concentration dependent induction of cell associated (B) and secreted FN proteins (C, D) by 0-5 μg/mL Gremlin in cultured TM cell strains (n=3). Western immunoblots (B, and C) are representative of data obtained in the three TM cell strains. Three replicates of each sample were employed for the FN

ELISA (D). One-Way ANOVA was used for statistical analyses. * $0.01<p<0.05$, ** $0.0001<p<0.01$ and *** $p<0.0001$.

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

Figure 4: TGFβ receptor inhibition blocks Gremlin-induction of ECM mRNA and proteins. Effect of TGFBR inhibitors LY364947 and SB431542 on Gremlin induction of ECM mRNA (A) and protein expression (B-D). qRT-PCR values (A) represent fold induction normalized to ACTB in treated samples compared to controls (triplicates of 3 TM strains). Cell associated and secreted proteins were analyzed with western immunoblotting (B, C) and with FN ELISA (D). (B, C) Western immunoblots of TM cells treated with 1 μg/ml of Gremlin for 24 hours along with 5 μM of LY364947 (B) or SB431542 (C). Untreated and inhibitor-treated cells served as negative controls. ACTB was used as loading control. Blots shown are representative of data from 3 different TM cell strains. (D) FN ELISA of conditioned medium. (E) Western immunoblots of ECM proteins after siRNA mediated TGFBR1 knockdown followed by Gremlin treatment. TM cells were treated with TGFBR1 or control siRNA, followed by treatment with 1 μg/ml of Gremlin for 24 hours. ACTB was used as loading control. Blots are representative data from 3 different TM cell strains. One-Way ANOVA was used for statistical analyses (A,D). *and

0.01<p<0.05, ** and ## 0.0001<p<0.01, *** and ### represent p<0.0001. “#” = differences between Gremlin samples vs. Gremlin + inhibitor samples, while “*” = differences between Gremlin treated and the untreated cells.

Figure 5: TGFβ2 knockdown inhibits Gremlin induction of ECM proteins. TGFb2 and Gremlin reciprocally induce the expression of each other’s mRNA (A) and protein (B). TGFβ2 treatment induced expression of Gremlin mRNA (A) and protein (B) expression, while Gremlin treatment induced TGFβ2 mRNA (A) and protein (B) expression. Three replicates of each sample were employed. One-way ANOVA was used for statistical analyses. ** 0.0001<p<0.01; *** p<0.0001. (C) Western immunoblots of ECM proteins after siRNA mediated TGFβ2 knockdown followed by Gremlin treatment. TM cells were treated with TGFβ2 or control siRNA, followed by treatment with 1 μg/ml of Gremlin for 24 hours. ACTB was used as loading control. Blots are representative data from 3 different TM cell strains.

Figure 6: CTGF knockdown inhibits Gremlin induction of ECM proteins. Western immunoblots of ECM proteins after siRNA mediated CTGF knockdown followed by Gremlin treatment. TM cells were treated with CTGF or control siRNA, followed by treatment with 1 μg/ml of Gremlin for 24 hours. ACTB was used as loading control. Blots are representative data from 3 different TM cell strains. CTGF knockdown blocked Gremlin induction of FN and COL1, but had no effect on Gremlin induction of PAI1 and ELN.

Figure 7: TGFβ2 but not Gremlin activates both canonical and non-canonical signaling pathways in TM cells. Representative western immunoblots of canonical Smad2/pSmad2 and Smad3/pSmad3 signaling (A), as well as noncanonical JNK1/2/pJNK1/2 and P38/pP38 signaling (B) in 4 TM cell strains treated for 0-240 minutes with TGFβ2 and Gremlin. TGFβ2 and Gremlin

treatment caused a time-dependent increase in pSmad2 and pSmad3 expression. TGFβ2 treatment caused a time-dependent increase in pJNK1/2 and pP38 expression while Gremlin did not affect pJNK1/2 or pP38 levels.

Figure 8: **Smad2/3/4 inhibition blocks Gremlin-induction of ECM proteins.** Treatment of TM cells with Smad3 inhibitor SIS3 blocks Gremlin induction of ECM mRNA (A), cell-associated protein (B), and secreted protein expression (B, C). (A) qRT-PCR analysis of the Gremlin-induction of ECM genes mRNA in presence of specific inhibitor of Smad3 (SIS3). qRT-PCR values represent fold induction of ECM mRNA normalized to ACTB in Gremlin treated samples compared to controls (triplicates of 3 TM cell strains). One-Way ANOVA was used for statistical analyses of qRT-PCR (A) and FN ELISA (C) results. "#" = differences between Gremlin samples vs. Gremlin + inhibitor samples; "*" = differences between Gremlin treated vs. the untreated cells; *and # $0.01<p<0.05$, ** and ## $0.0001<p<0.01$, *** and ### $p<0.0001$. (B) Western immunoblots of cell-associated and secreted ECM proteins after pretreatment with SIS3 (10 □M) followed by Gremlin (1 μg/ml) treatment for 24 hrs. Immunoblots are representative of three different TM cell strains. ACTB was used as a loading control. Untreated and SIS3 only-treated cells served as negative controls. (C) FN ELISA of secreted FN from Gremlin +/- SIS3 treated TM cells (n=3 strains). (D, E, F) Western immunoblots of ECM proteins in TM cells pretreated with Smad3 (D), Smad2 (E), or Smad4 (F) siRNAs followed by Gremlin treatment. Control cells were transfected with non-targeting siRNA. Immunoblots are representative of results from 3 TM cell lines. Each Smad siRNA knocked down its target protein. SIS3 siRNA suppressed the Gremlin induction of all 4 ECM proteins. Smad2 and Smad4 siRNAs consistently suppressed Gremlin induction of FN and PAI1, but variably suppressed Gremlin induction of ELC and COL1 proteins.

Tables

Table 1

Antibody	Ab. Dilution	Source
Rabbit Anti-FN	1:1000	Chemicon (Cat. # NB100-2530)
Rabbit Anti-PAI1	1:500	Novus Biologicals (Cat. # NBP1-19773)
Rabbit Anti-COL1	1:500	Abcam (Cat. # ab6308)
Mouse Anti-ELN	1:250	Abcam (Cat. # ab21605)
Mouse Anti-ACTB	1:1000	Millipore (Cat. # MAB1501)
Rabbit Anti-TGFβ2	1:500	Santa Cruz Biotechnology (Cat. # sc-90)
Rabbit Anti-Gremlin	1:250	Abcam (Cat. # ab22138)
Rabbit Anti-TGFBR1	1:250	Abcam (Cat. # ab67492-100)
Rabbit Anti-CTGF	1:500	Novus Biologicals (Cat. # NB100-724)
Rabbit Anti-SMAD2	1:1000	Cell Signaling (Cat. # 3107)
Rabbit Anti-Phos-SMAD2	1:1000	Cell Signaling (Cat. # 3122)
Rabbit Anti-SMAD3	1:1000	Cell Signaling (Cat. #9532S)
Rabbit Anti-Phos-SMAD3	1:1000	Cell Signaling (Cat. # 9520S)
Rabbit Anti-JNK1/2	1:1000	Cell Signaling (Cat. # 9258)
Rabbit Anti-Phos-JNK1/2	1:1000	Cell Signaling (Cat. # 9251S)
Rabbit Anti-SMAD4	1:1000	Cell Signaling (Cat. # 9515)
Rabbit Anti-P38 MAPK	1:1000	Cell Signaling (Cat. # 9212)
Rabbit Anti-Phos-P38	1:1000	Cell Signaling (Cat. # 9211)
Donkey anti-mouse IgG	1:10,000	Santa Cruz Biotechnology (Cat. # sc-2314)
Goat anti-rabbit IgG	1:10,000	Santa Cruz Biotechnology (Cat. # sc-2004)

Table 1: List of various antibodies used for western immunoblotting studies

Table 2

Gene		Primer (5’ → 3’)
FN	Left	AGCGGACCTACCTAGGCAAT
	Right	GGTTTGCGATGGTACAGCTT
COL1a	Left	GGAATGAAGGGACACAGAGG
	Right	TAGCACCATCATTTCCACGA
PAI1	Left	CCACTTCTTCAGGCTGTTCC
	Right	CCGTTGAAGTAGAGGGCATT
ELN	Left	AGCCAAGTATGGAGCTGCTG
	Right	GCTGCTTCTGGTGACACAAC
TGFB2	Left	CCGGAGGTGATTTCCATCTA
	Right	CTCCATTGCTGAGACGTCAA
GREM1	Left	AAGCGAGACTGGTGCAAAAC
	Right	CTTGCAGAAGGAGCAGGACT
ACTB	Left	GTCCACCTTCCAGCAGATGT
	Right	AAAGCCATGCCAATCTCATC

Table 2: List of the primers used for PCR studies

Figures

Fig. 1A

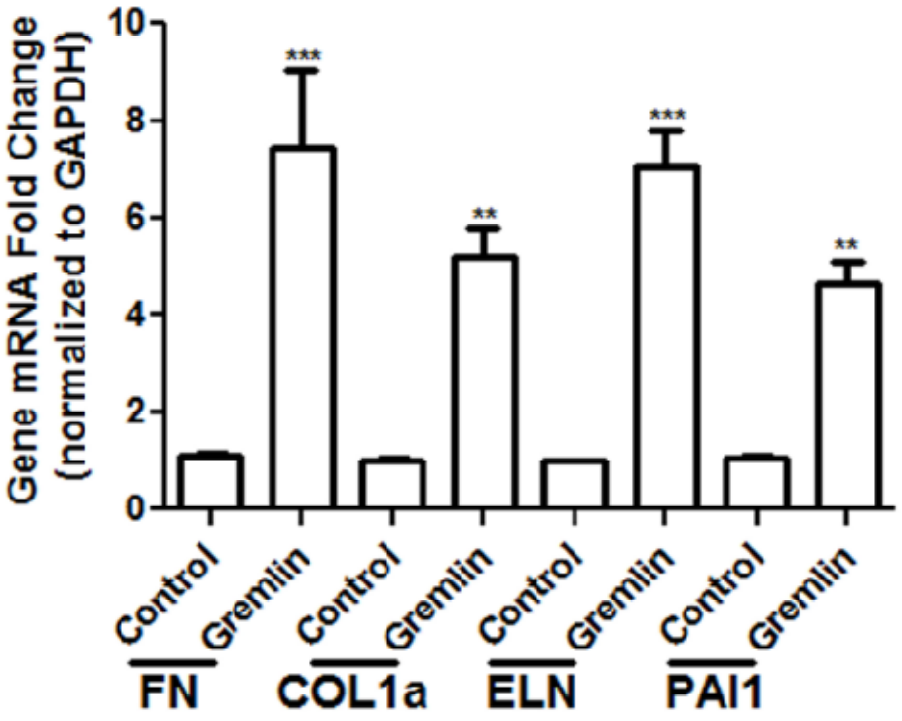


Fig. 1B

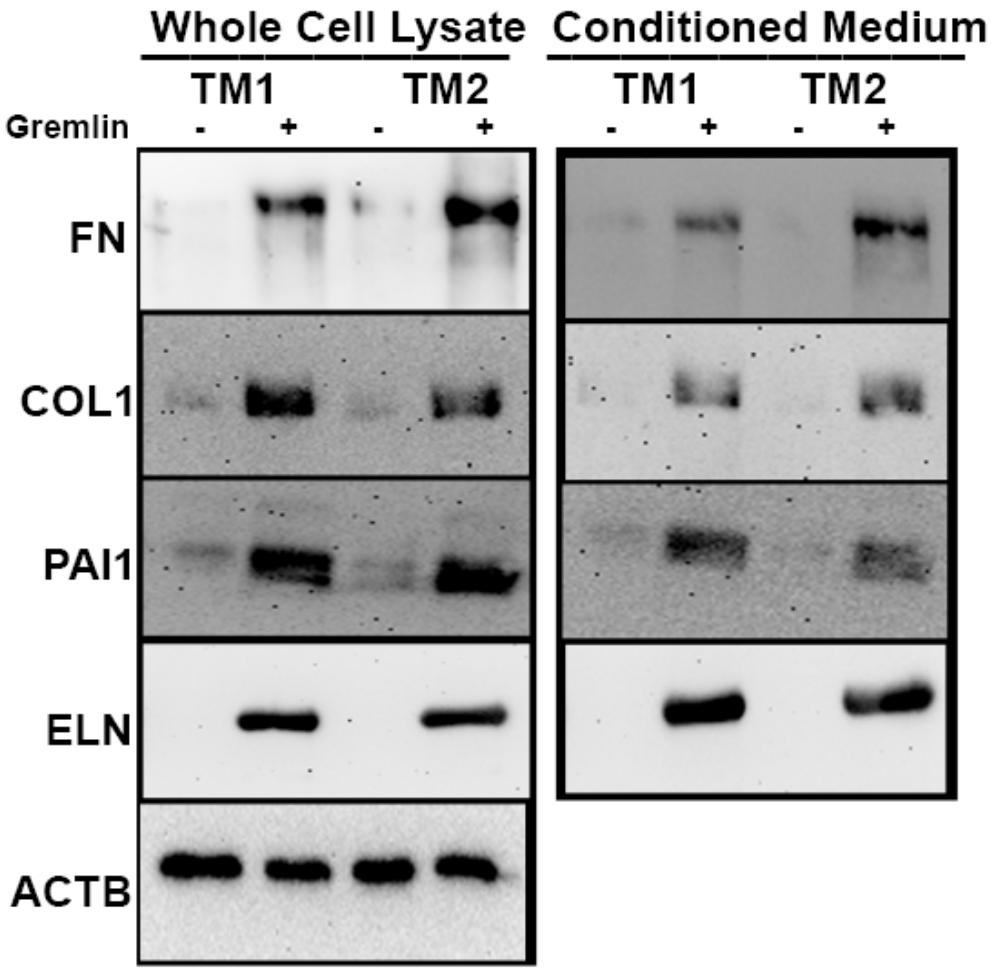

Whole Cell Lysate
Conditioned Medium
TM1
TM2
TM1
TM2
Gremlin
- + - +
- + - +
FN
COL1
PAI1
ELN
ACTB

Fig. 1C

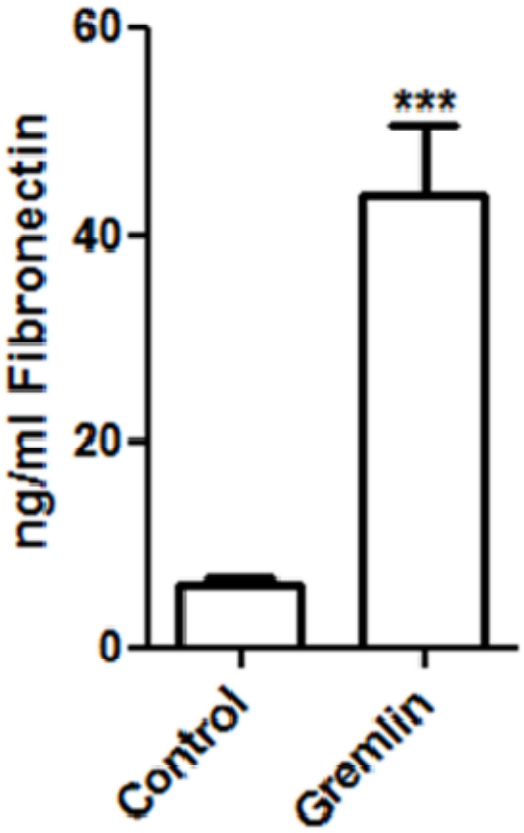


Fig. 2A

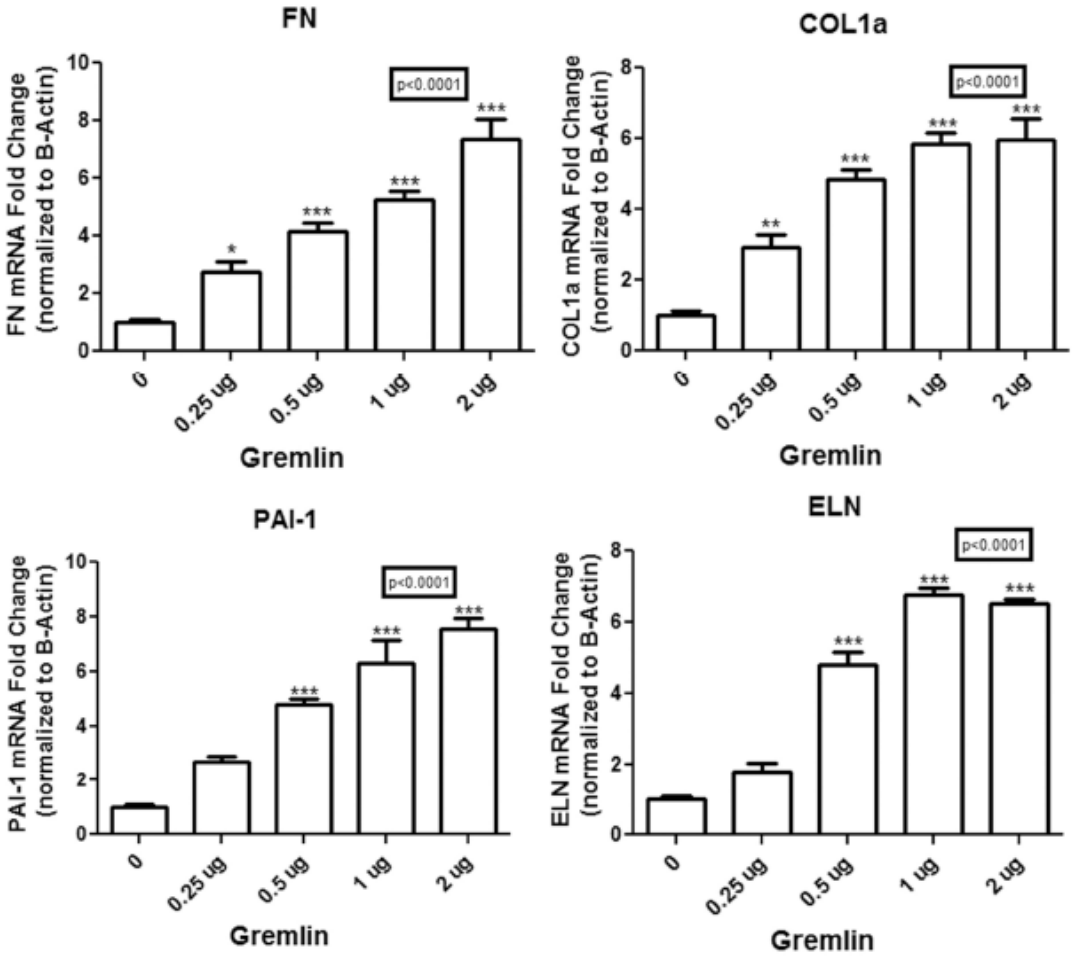

FN
FN mRNA Fold Change (normalized to B-Actin)
p<0.0001
0
0.25 ug
0.5 ug
1 ug
2 ug
Gremlin
COL1a
COL1a mRNA Fold Change (normalized to B-Actin)
p<0.0001
Gremlin
PAI-1
PAI-1 mRNA Fold Change (normalized to B-Actin)
p<0.0001
Gremlin
ELN
ELN mRNA Fold Change (normalized to B-Actin)
p<0.0001
Gremlin

Fig. 2B

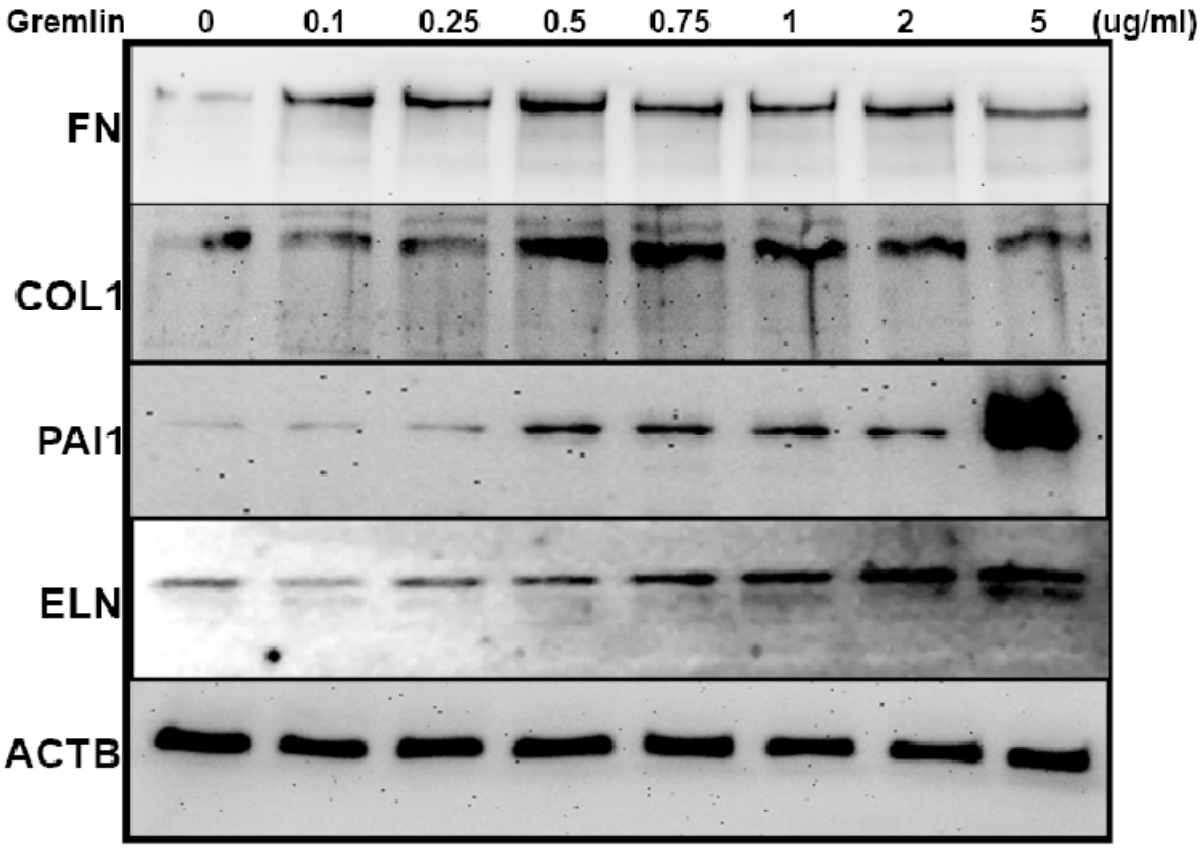


Fig. 2C

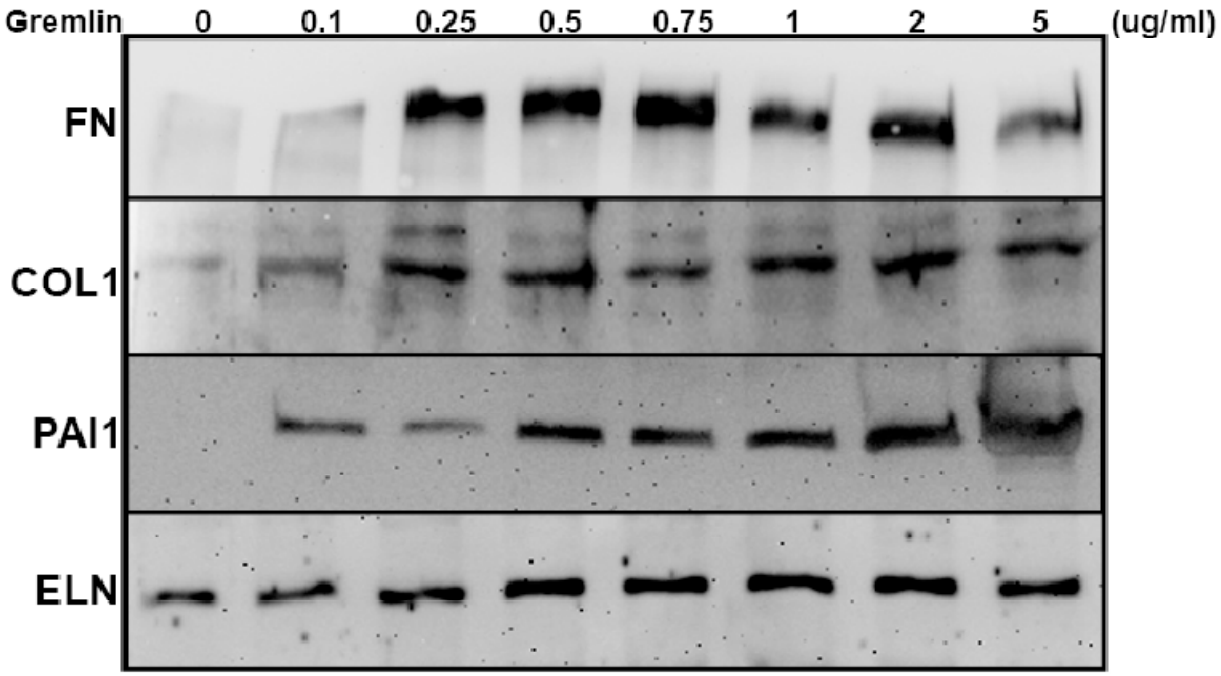


Fig. 2D

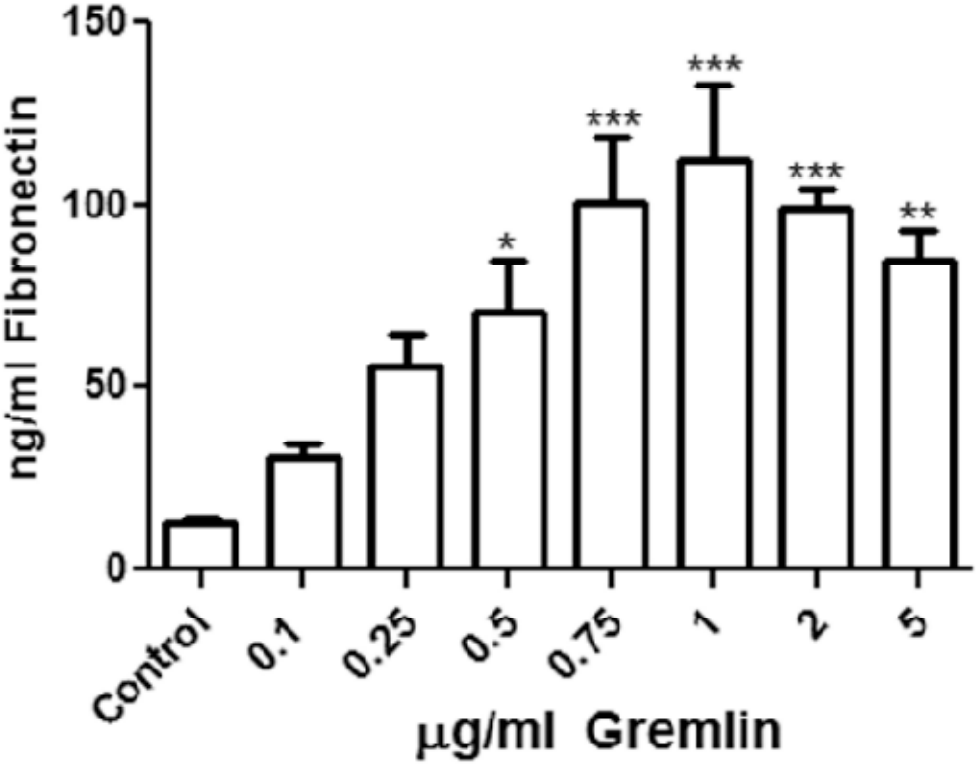

150
100
50
0
ng/ml Fibronectin
*
***
***
***
**
Control
0.1
0.25
0.5
0.75
1
2
5
μg/ml Gremlin

Fig. 3A

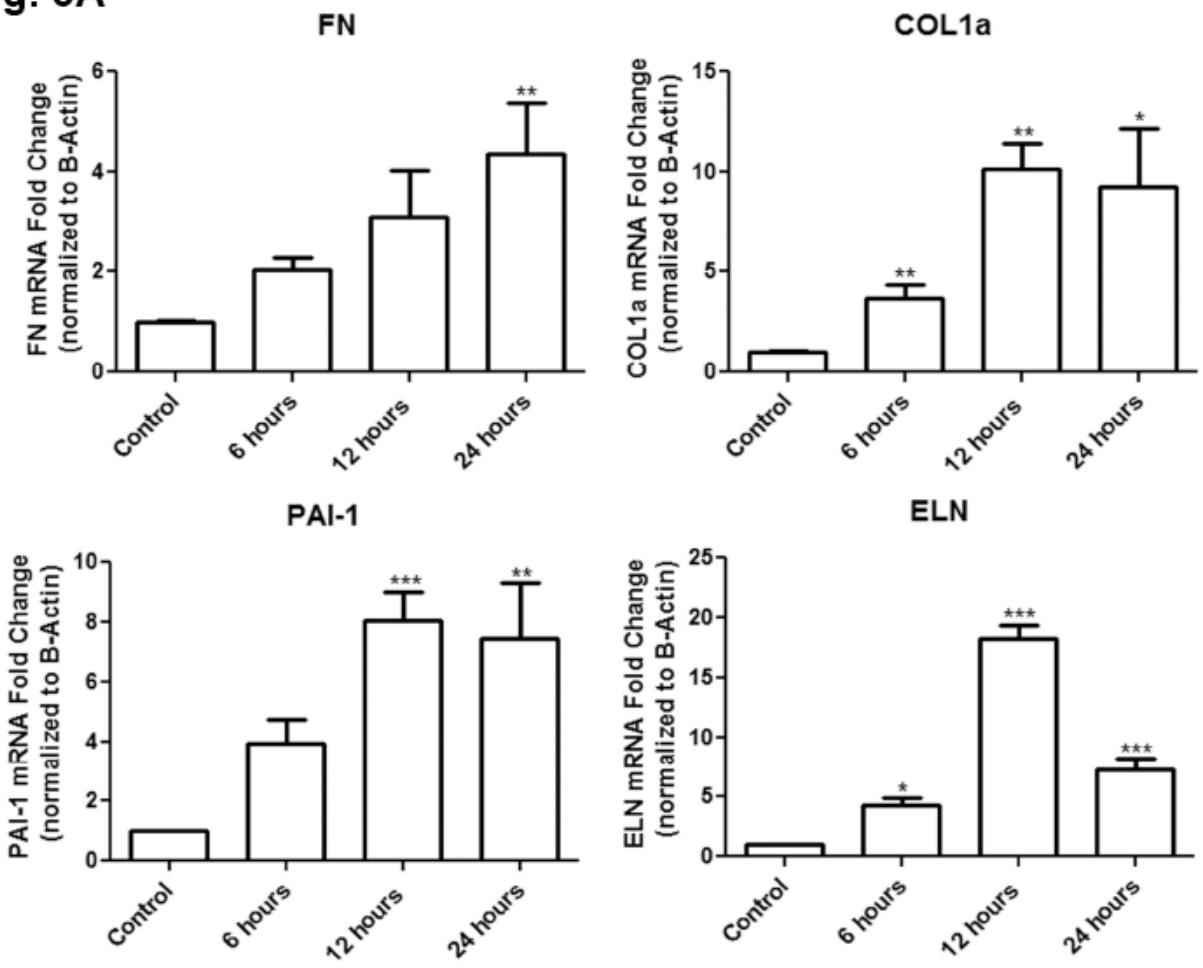

FN
FN mRNA Fold Change (normalized to B-Actin)
0
2
4
6
**
Control
6 hours
12 hours
24 hours
COL1a
COL1a mRNA Fold Change (normalized to B-Actin)
0
5
10
15
**
**
*
Control
6 hours
12 hours
24 hours
PAI-1
PAI-1 mRNA Fold Change (normalized to B-Actin)
0
2
4
6
8
10
***
**
Control
6 hours
12 hours
24 hours
ELN
ELN mRNA Fold Change (normalized to B-Actin)
0
5
10
15
20
25
*
***
***
Control
6 hours
12 hours
24 hours

Fig. 3B

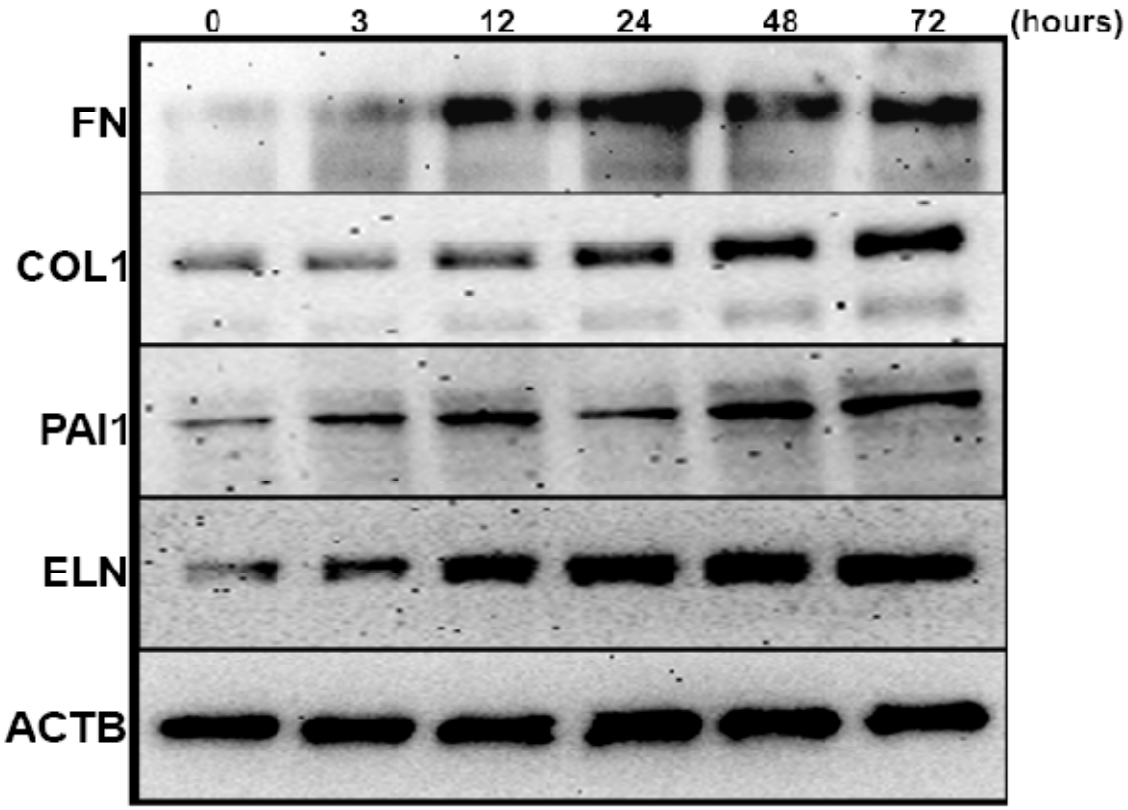


Fig. 3C

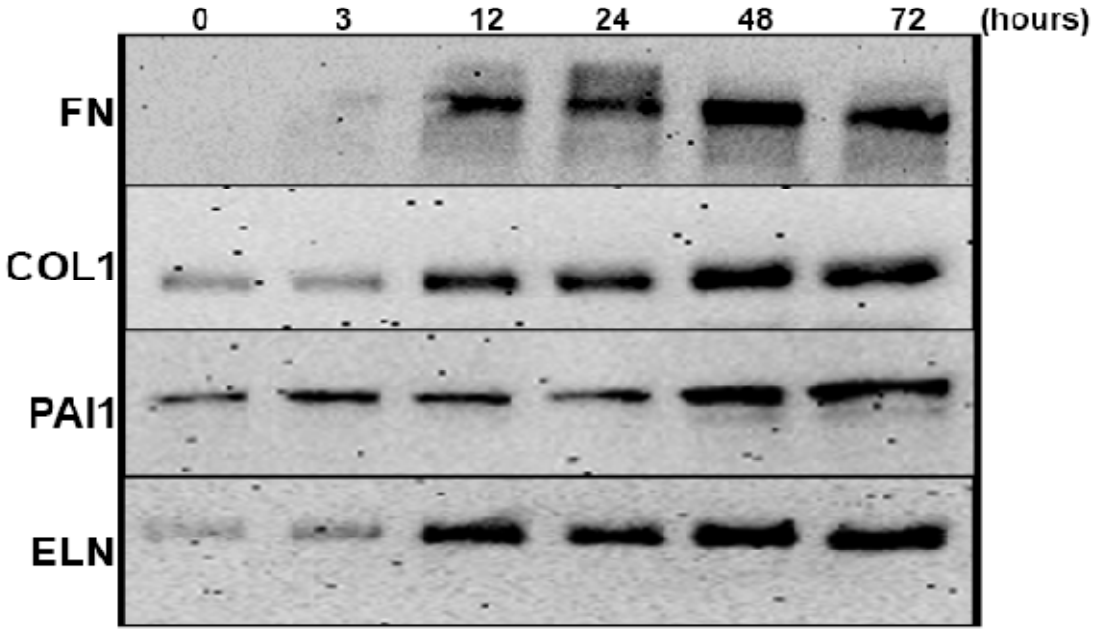


Fig. 3D

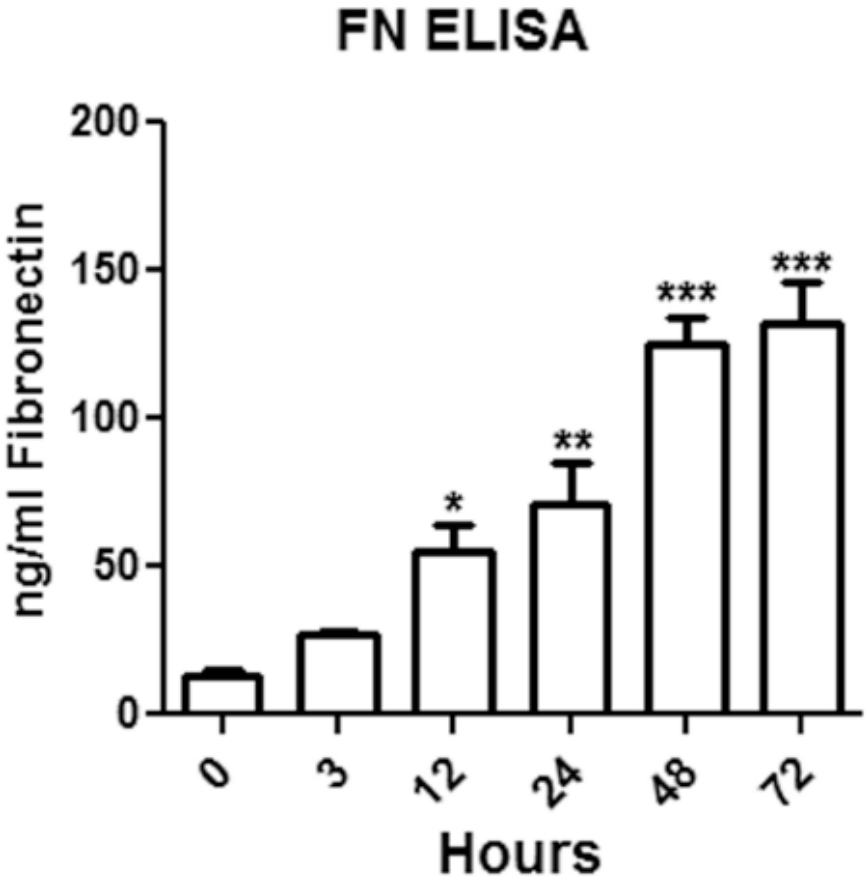


Fig 4A

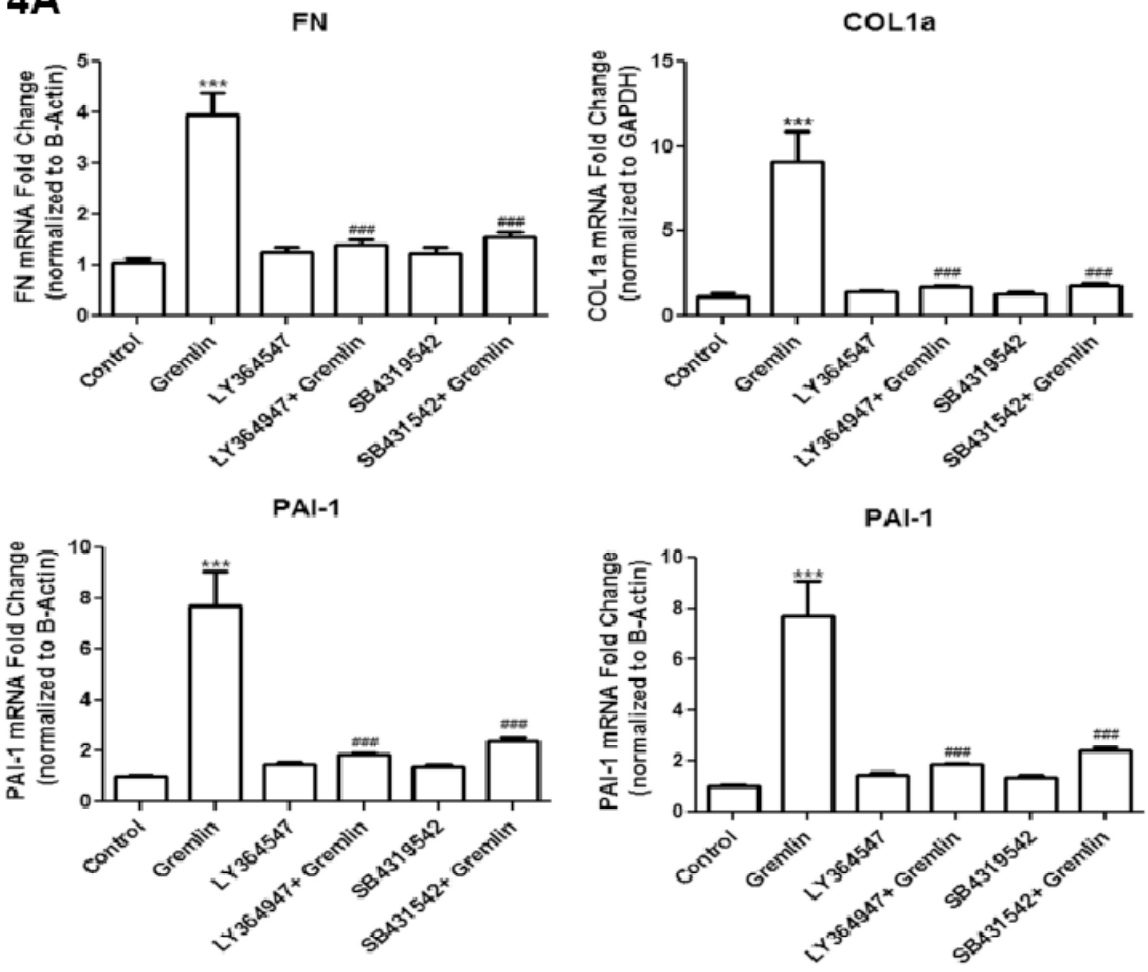

FN
FN mRNA Fold Change (normalized to B-Actin)
***
###
###
Control
Gremlin
LY364547
LY364947+ Gremlin
SB4319542
SB431542+ Gremlin
COL1a
COL1a mRNA Fold Change (normalized to GAPDH)
***
###
###
Control
Gremlin
LY364547
LY364947+ Gremlin
SB4319542
SB431542+ Gremlin
PAI-1
PAI-1 mRNA Fold Change (normalized to B-Actin)
***
###
###
Control
Gremlin
LY364547
LY364947+ Gremlin
SB4319542
SB431542+ Gremlin
PAI-1
PAI-1 mRNA Fold Change (normalized to B-Actin)
***
###
###
Control
Gremlin
LY364547
LY364947+ Gremlin
SB4319542
SB431542+ Gremlin

Fig 4B

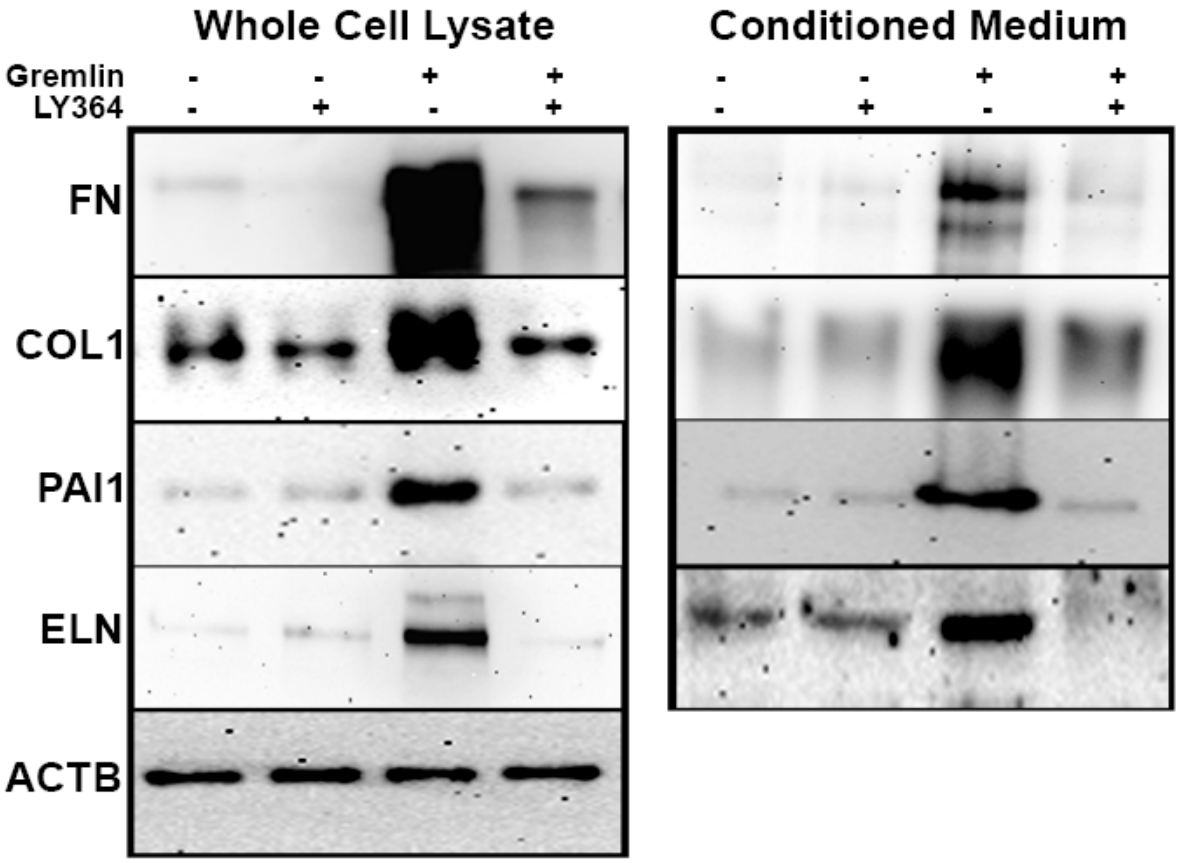

Whole Cell Lysate
Conditioned Medium
Gremlin - - + +
LY364 - + - +
- - + +
- + - +
FN
COL1
PAI1
ELN
ACTB

Fig 4C

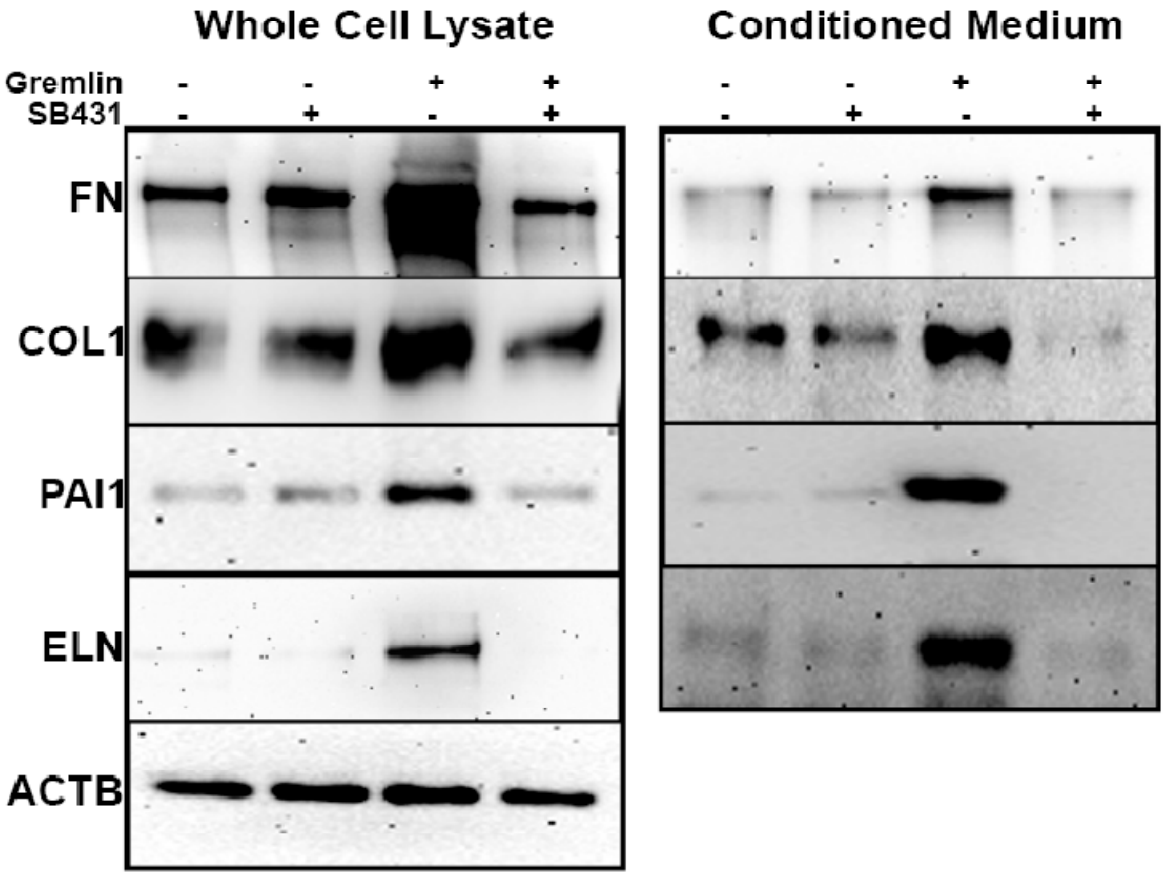

Whole Cell Lysate
Conditioned Medium
Gremlin - - + +
SB431 - + - +
FN
COL1
PAI1
ELN
ACTB

Fig 4D

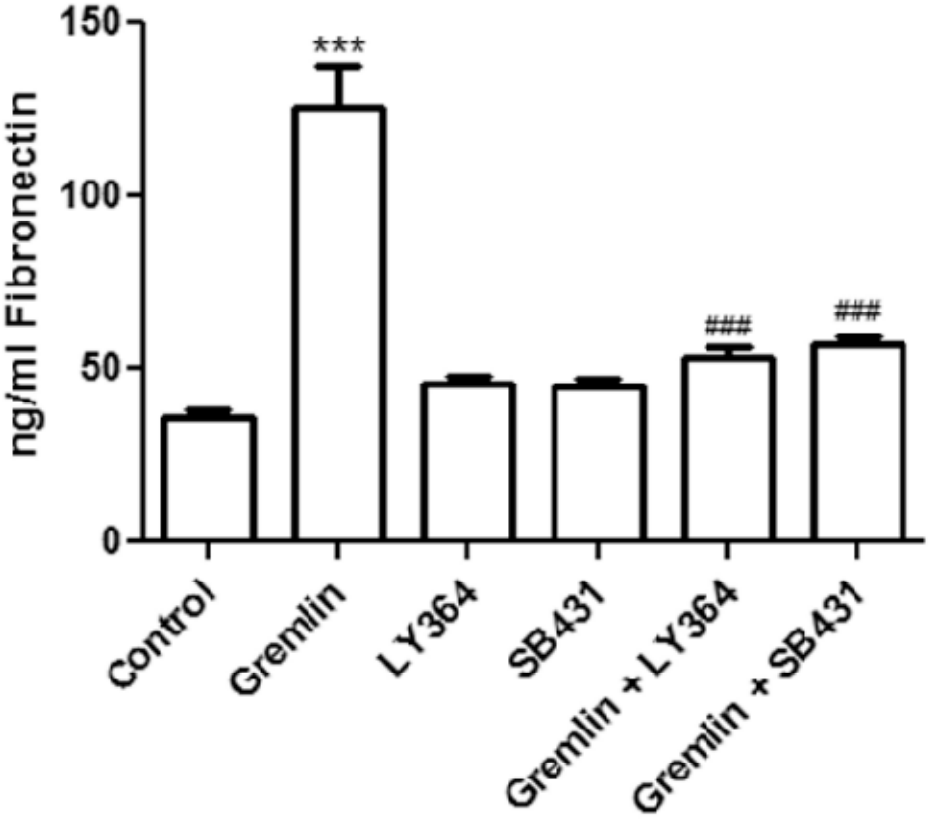

150
100
50
0
ng/ml Fibronectin
***
###
###
Control
Gremlin
LY364
SB431
Gremlin + LY364
Gremlin + SB431

Fig 4E

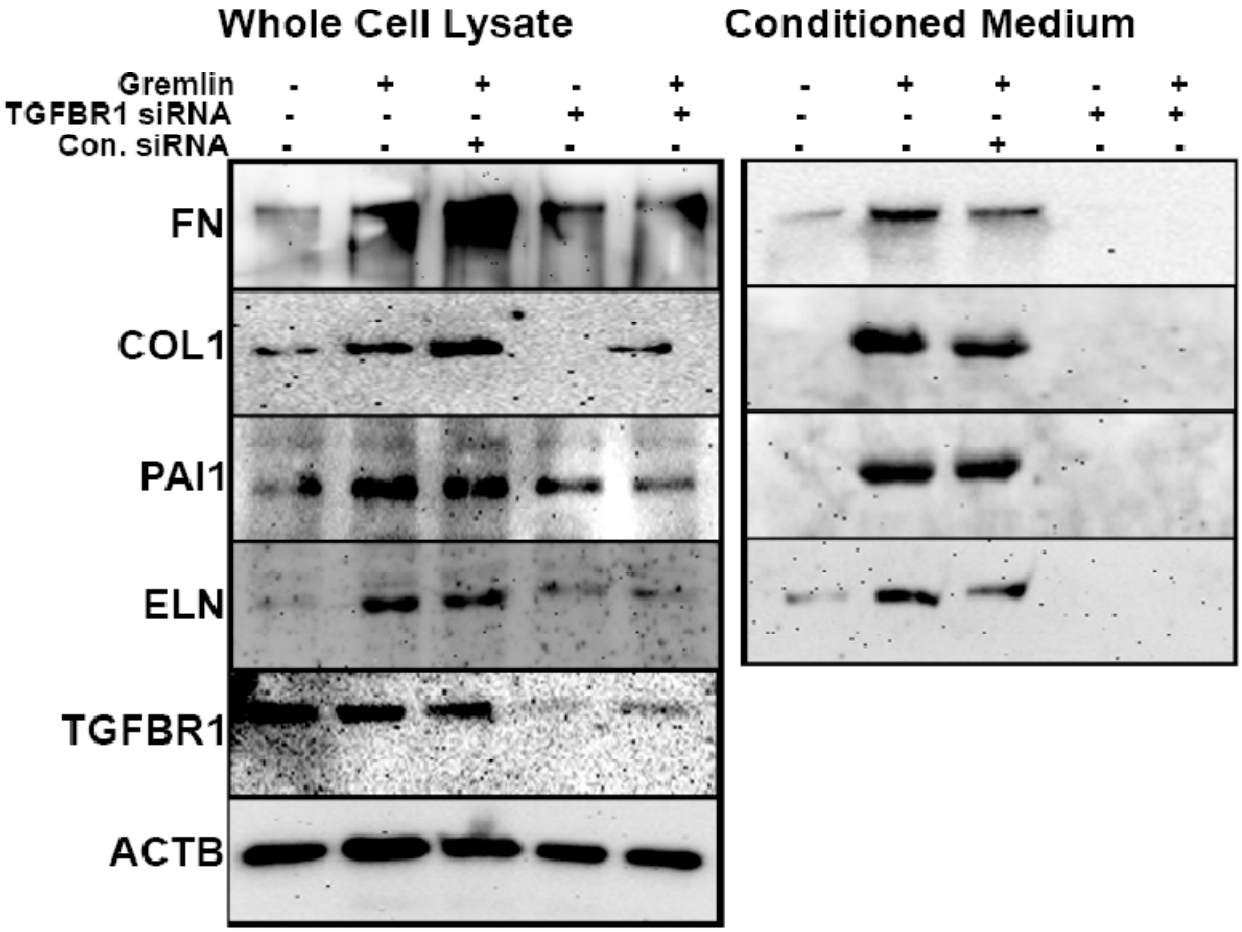

Whole Cell Lysate
Conditioned Medium
Gremlin - + + - + - + + - +
TGFBR1 siRNA - - - + + - - - + +
Con. siRNA - - + - - - - + - -
FN
COL1
PAI1
ELN
TGFBR1
ACTB

Fig. 5A

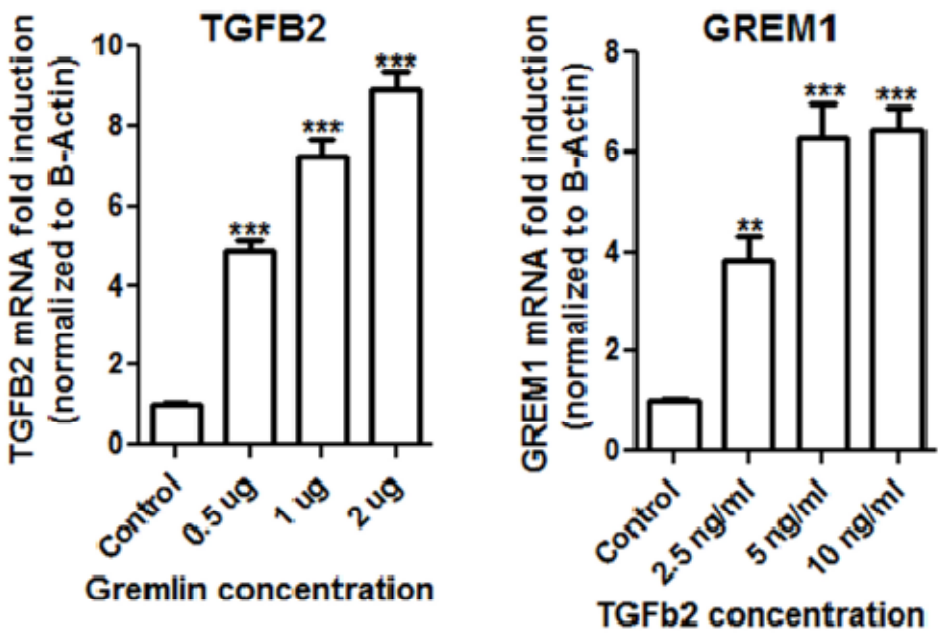

TGFB2
TGFB2 mRNA fold induction (normalized to B-Actin)
0
2
4
6
8
10
***
***
***
Control
0.5 ug
1 ug
2 ug
Gremlin concentration
GREM1
GREM1 mRNA fold induction (normalized to B-Actin)
0
2
4
6
8
**
***
***
Control
2.5 ng/ml
5 ng/ml
10 ng/ml
TGFb2 concentration

Fig 5B

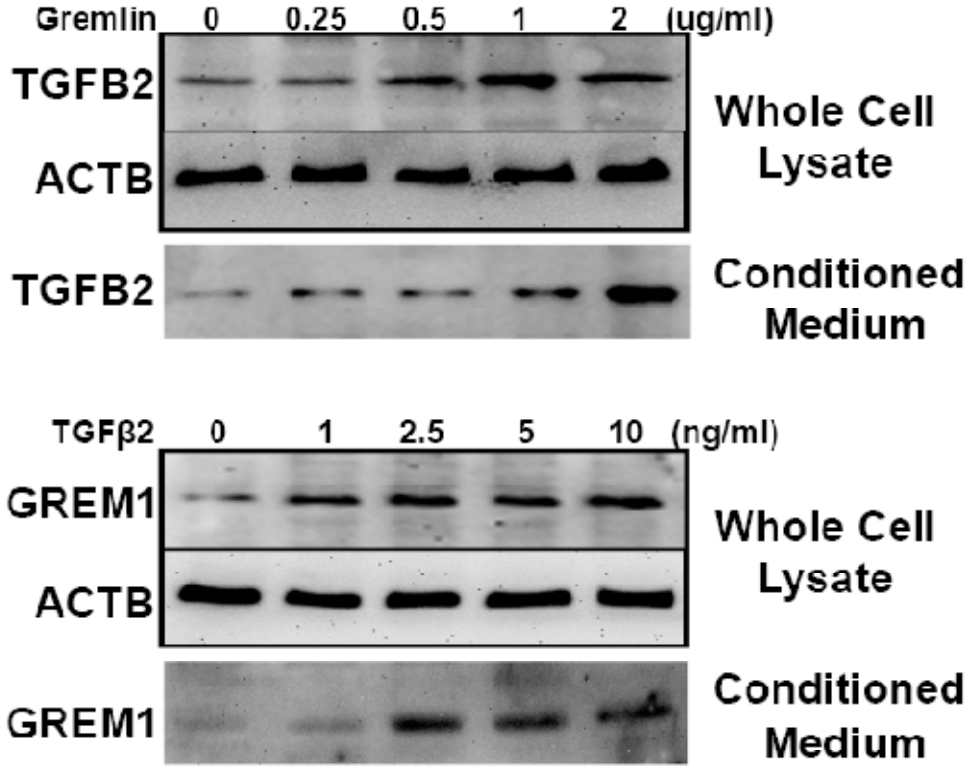

Gremlin 0 0.25 0.5 1 2 (ug/ml)
TGFB2
ACTB
Whole Cell Lysate
TGFB2
Conditioned Medium
TGFβ2 0 1 2.5 5 10 (ng/ml)
GREM1
ACTB
Whole Cell Lysate
GREM1
Conditioned Medium

Fig 5C

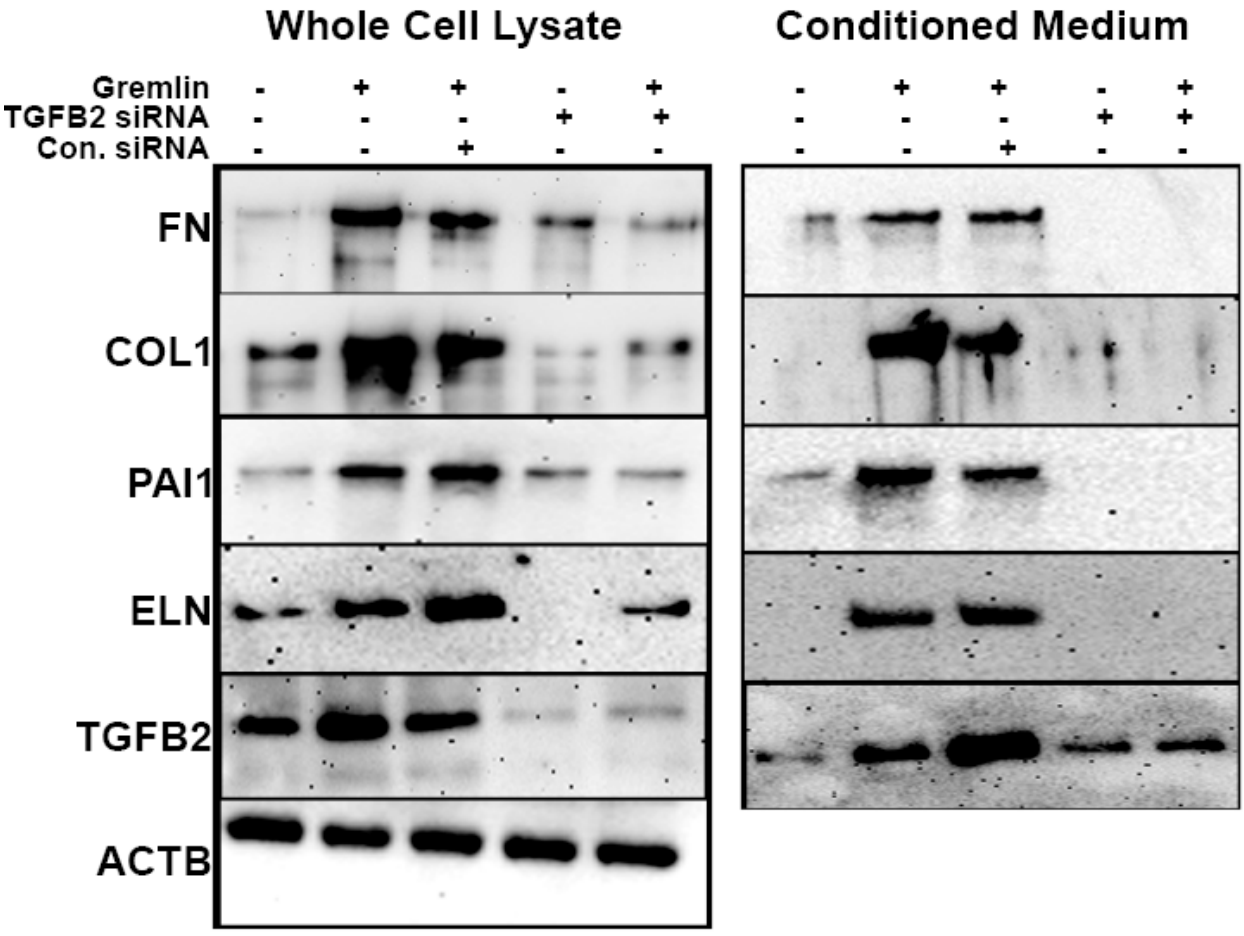

Whole Cell Lysate
Conditioned Medium
Gremlin - + + - + - + + - +
TGFB2 siRNA - - - + + - - - + +
Con. siRNA - - + - - - - + - -
FN
COL1
PAI1
ELN
TGFB2
ACTB

Fig 6

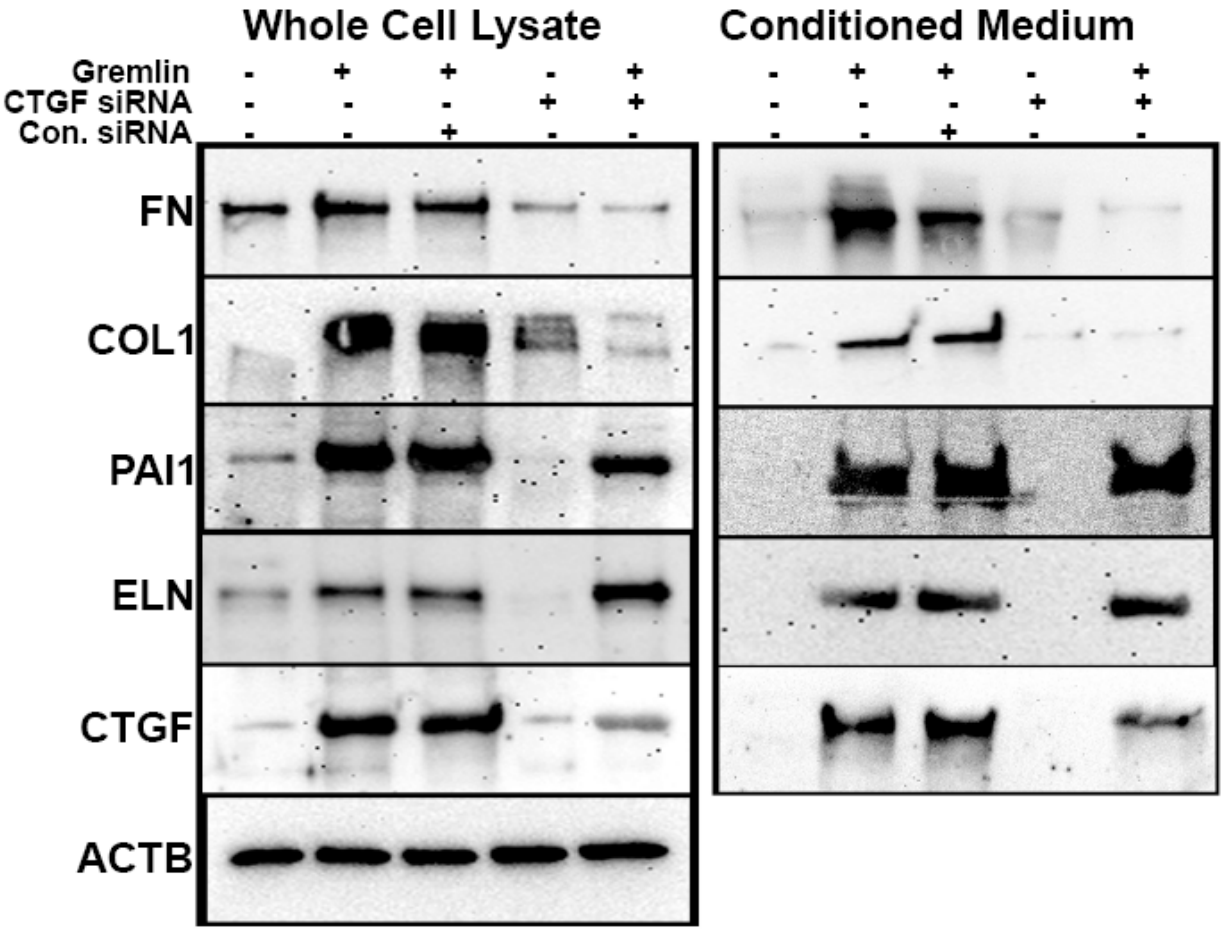
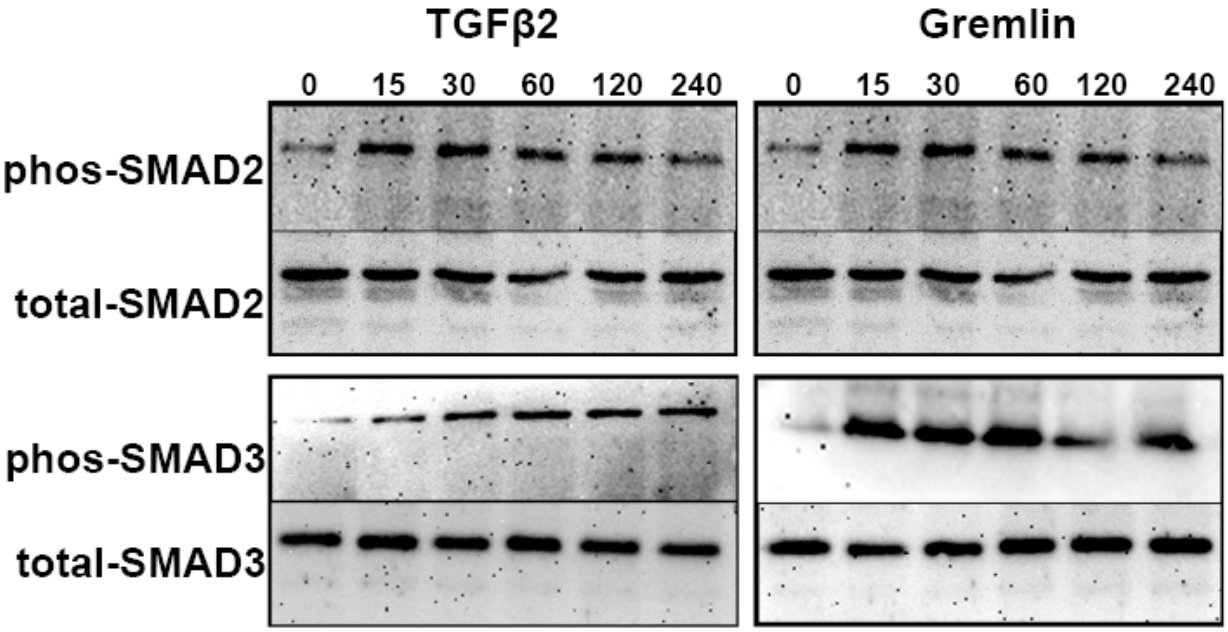

Whole Cell Lysate
Conditioned Medium
Gremlin - + + - + - + + - +
CTGF siRNA - - - + + - - - + +
Con. siRNA - - + - - - - + - -
FN
COL1
PAI1
ELN
CTGF
ACTB

Fig. 7A


TGFβ2
Gremlin
0 15 30 60 120 240
0 15 30 60 120 240
phos-SMAD2
total-SMAD2
phos-SMAD3
total-SMAD3

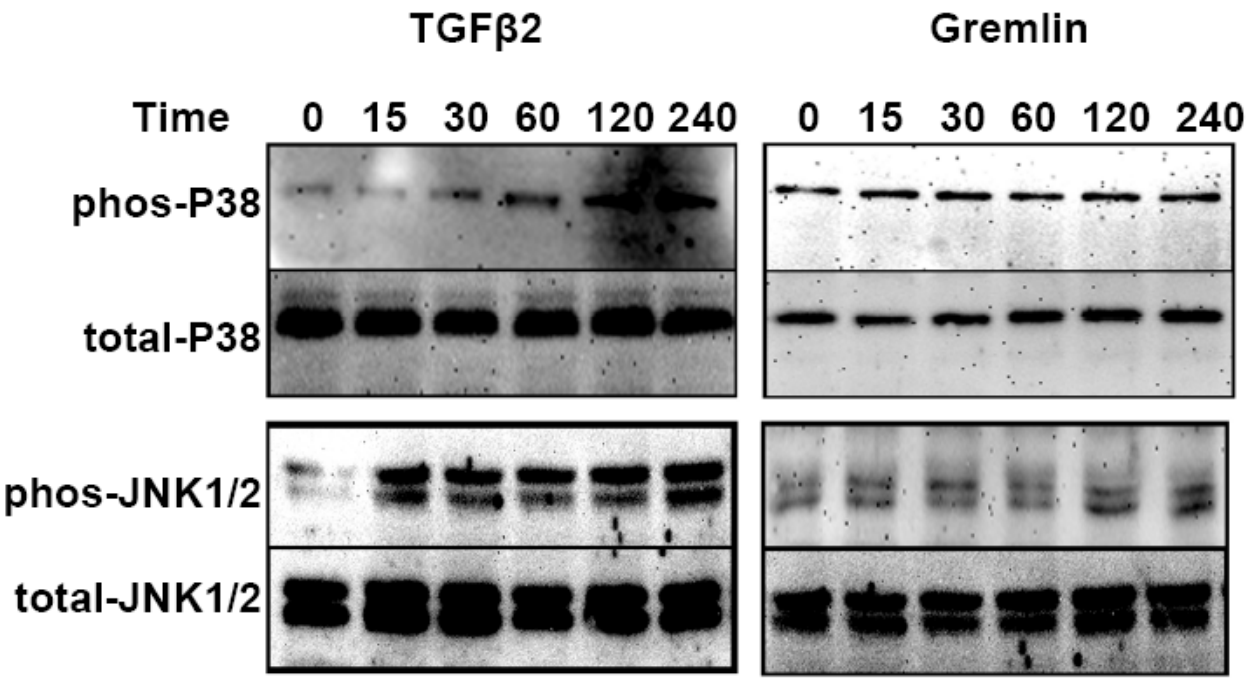

TGFβ2
Gremlin
Time
0 15 30 60 120 240
0 15 30 60 120 240
phos-P38
total-P38
phos-JNK1/2
total-JNK1/2

Fig 8A

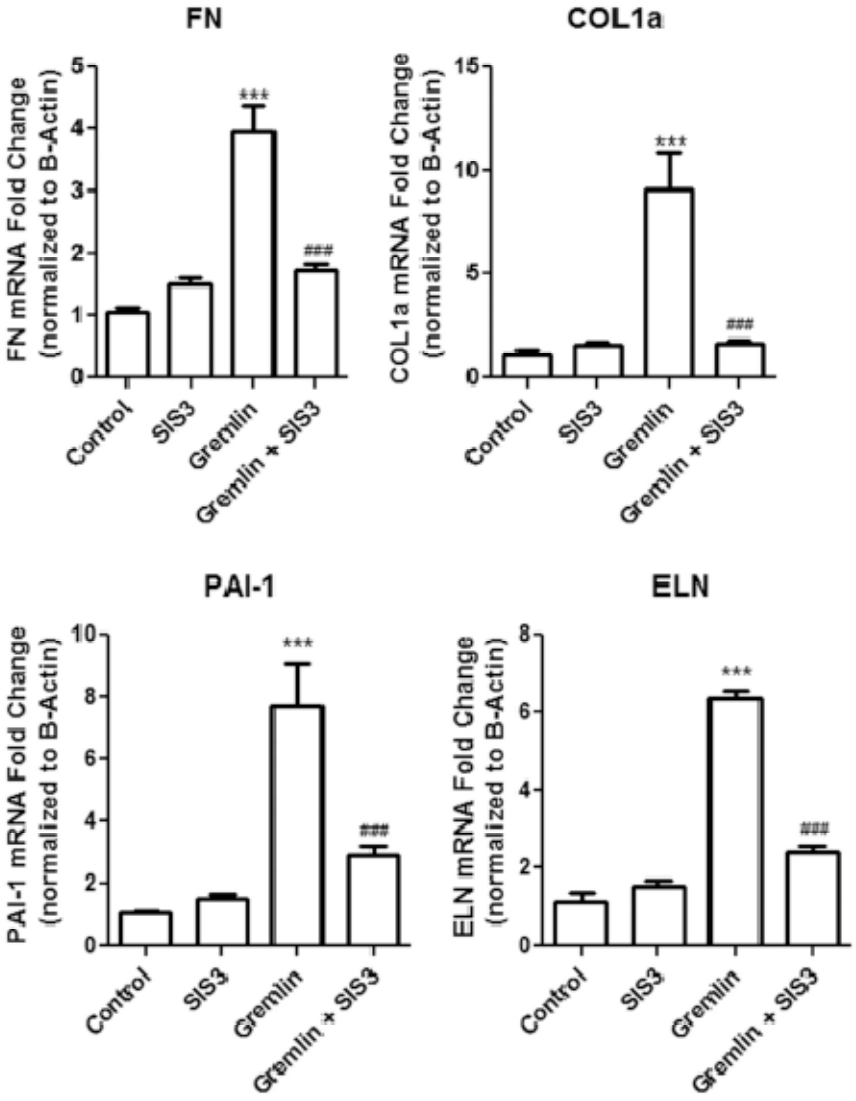

FN
FN mRNA Fold Change (normalized to B-Actin)
0
1
2
3
4
5
***
###
Control
SIS3
Gremlin
Gremlin + SIS3
COL1a
COL1a mRNA Fold Change (normalized to B-Actin)
0
5
10
15
***
###
Control
SIS3
Gremlin
Gremlin + SIS3
PAI-1
PAI-1 mRNA Fold Change (normalized to B-Actin)
0
2
4
6
8
10
***
###
Control
SIS3
Gremlin
Gremlin + SIS3
ELN
ELN mRNA Fold Change (normalized to B-Actin)
0
2
4
6
8
***
###
Control
SIS3
Gremlin
Gremlin + SIS3

Fig 8B

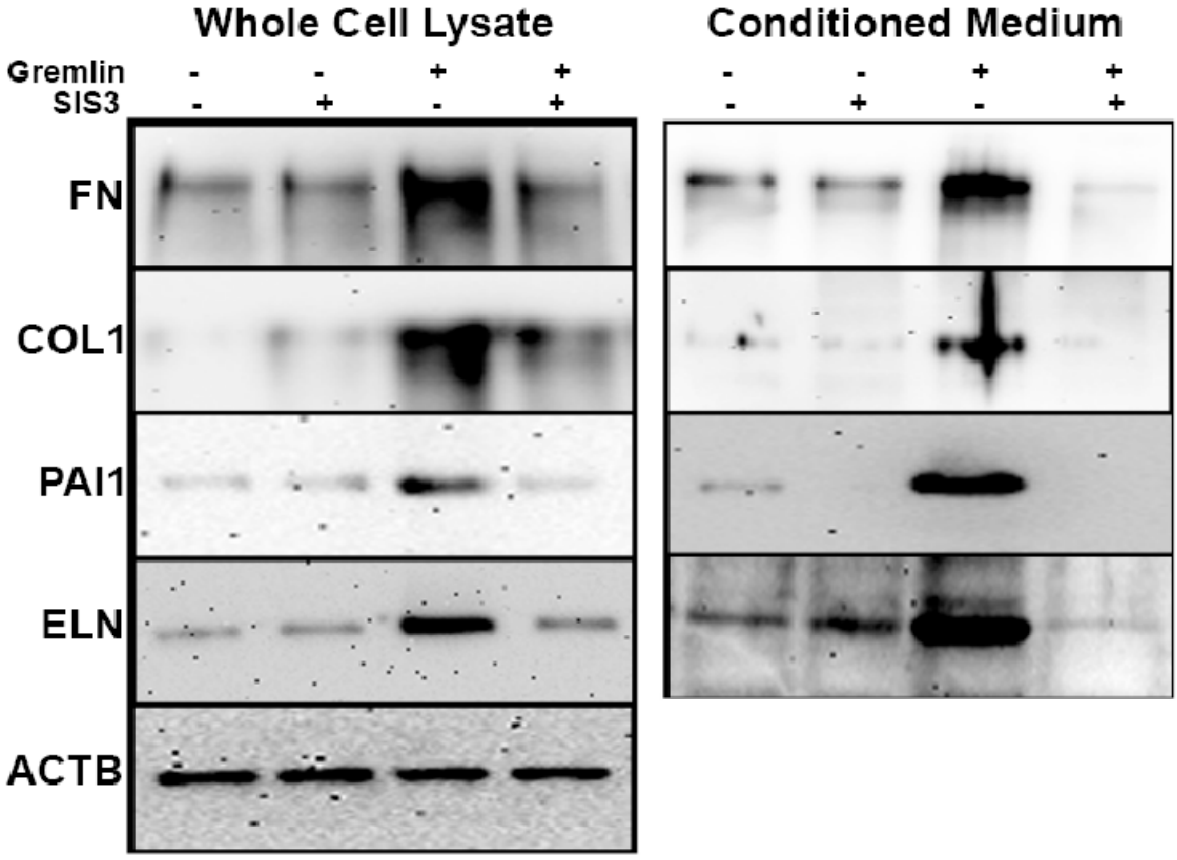

Whole Cell Lysate
Conditioned Medium
Gremlin - - + +
SIS3 - + - +
- - + +
- + - +
FN
COL1
PAI1
ELN
ACTB

Fig 8C

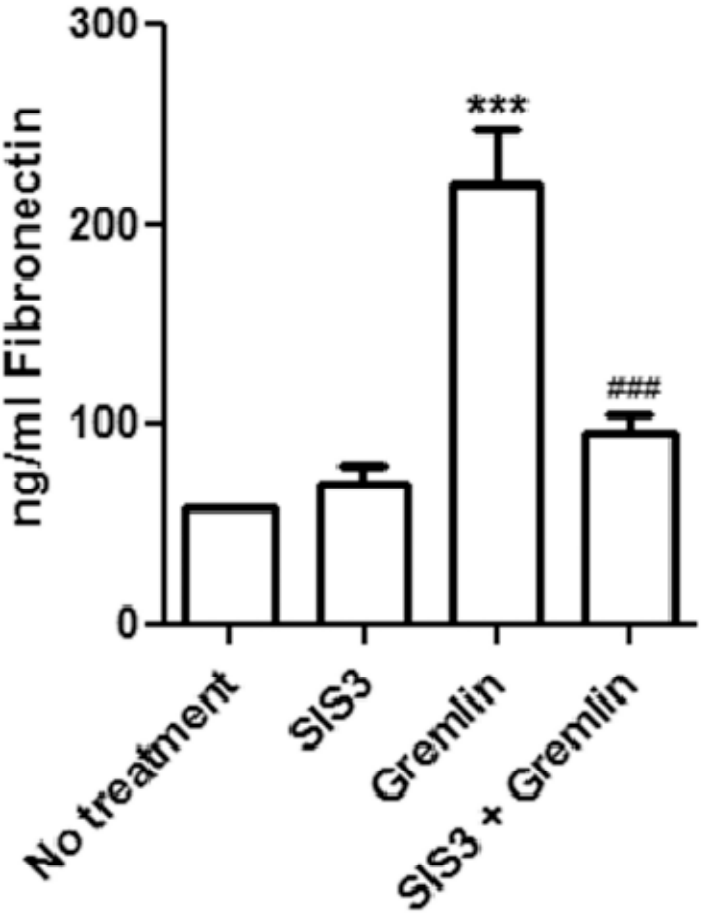

300
200
100
0
ng/ml Fibronectin
***
###
No treatment
SIS3
Gremlin
SIS3 + Gremlin

Fig 8D

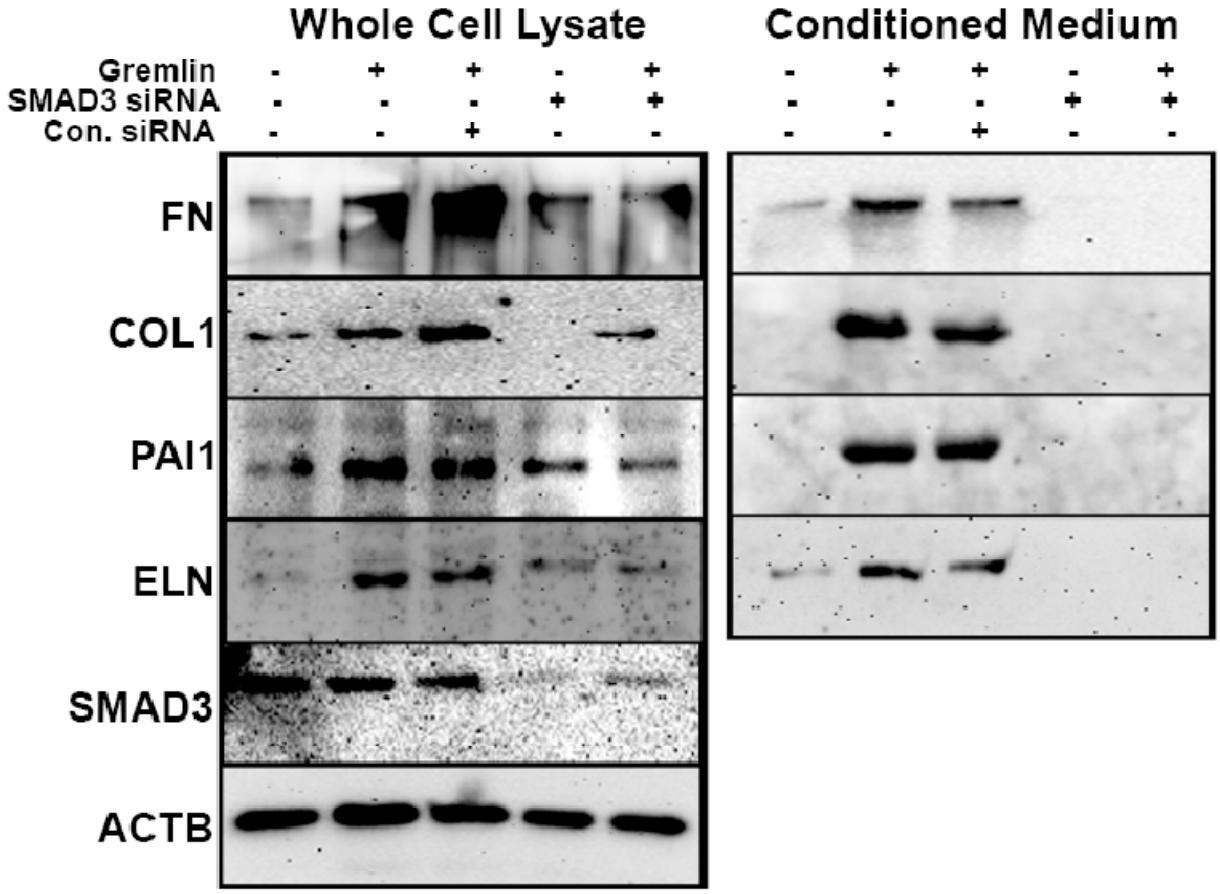

Whole Cell Lysate
Conditioned Medium
Gremlin - + + - + - + + - +
SMAD3 siRNA - - - + + - - - + +
Con. siRNA - - + - - - - + - -
FN
COL1
PAI1
ELN
SMAD3
ACTB

Fig 8E

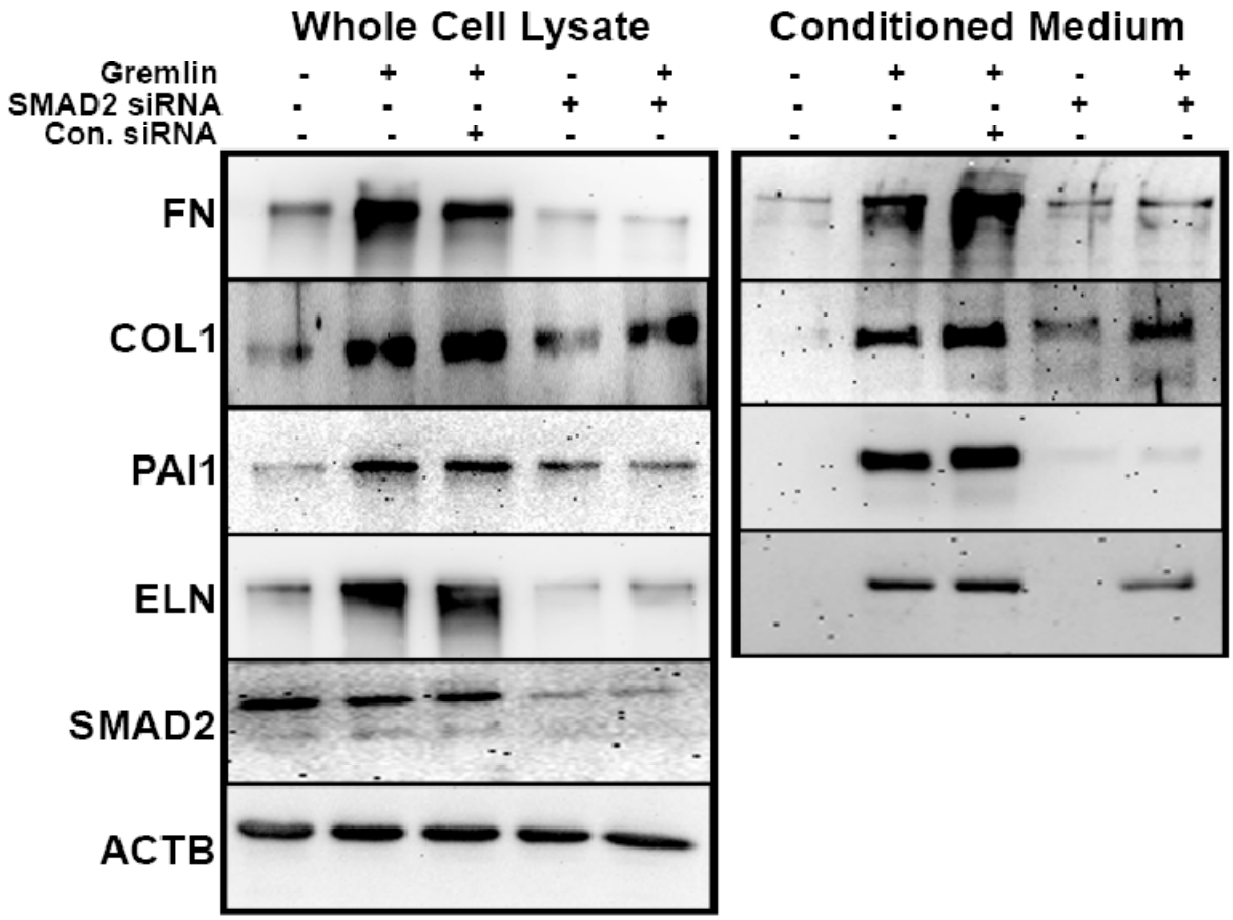
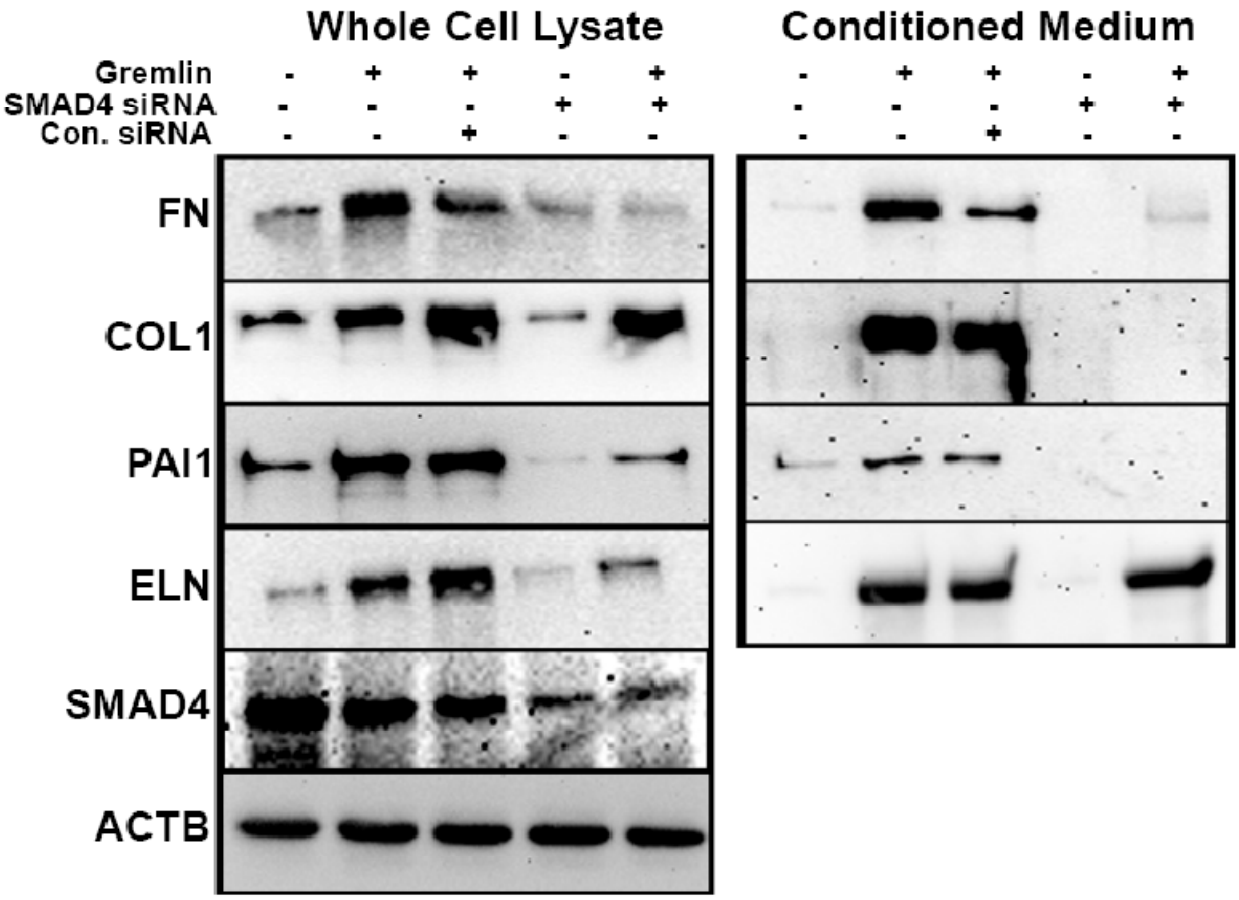

Whole Cell Lysate
Conditioned Medium
Gremlin - + + - + - + + - +
SMAD2 siRNA - - - + + - - - + +
Con. siRNA - - + - - - - + - -
FN
COL1
PAI1
ELN
SMAD2
ACTB

Fig 8F


Whole Cell Lysate
Conditioned Medium
Gremlin - + + - + - + + - +
SMAD4 siRNA - - - + + - - - + +
Con. siRNA - - + - - - - + - -
FN
COL1
PAI1
ELN
SMAD4
ACTB

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Chapter IV

CONCLUSIONS

The results from the present studies support our hypothesis that TGFβ1-3 induced the expression of LOX and LOXL genes and proteins in cultured human TM cells. We also observed that TGFβ utilizes both canonical Smad and the non canonical JNK signaling to induce LOXs. We also report that the BMP antagonist gremlin strongly induces the ECM genes and proteins FN, ELN, COL1 and PAI1. However, unlike TGFβ2 that activates both Smad and MAPK signaling, gremlin only activates Smad signaling in TM cells. Gremlin relies mainly on Smad signaling to regulate induction of ECM genes and proteins.

In the first set of studies (Chapter II) we report that all 5 LOX genes are expressed across multiple human TM cell strains. We also report that all 3 TGFβ isoforms induce mRNA and protein expression of these LOX and LOXL genes. TGFβ induces LOXs in both a concentration- and time-dependent fashion. We developed a novel LOX activity assay and have shown basal LOX enzyme activity in TM cells, which can be further increased by TGFβ. We showed that TGFβ1-3 phosphorylate and activate Smad2/3 and JNK1/2 signaling in human TM cells. Finally, we demonstrated that inhibitng canonical Smad signaling either by small molecule Smad inhibitor SIS3 or via siRNA mediated knock down of Smad2,3,4 blocks TGFβ induction of LOXs. Similarly, JNK1/2 inhibitor, SP600125 and the AP-1 inhibitor SR11302 attenuated TGFβ1-3 induction of LOX genes and proteins in TM cells. Taken together, these results highlight the importance of both Smad and nonSmad pathways in TGFβ induction of LOXs in human TM cells.

In the second set of experiments (Chapter III) we show that gremlin significantly upregulates the ECM proteins FN, COL1, PAI1 and ELN in cultured human TM cells. We observed a concentration- and time-dependent gremlin induction of these ECM proteins. Interestingly, blocking TGFβ receptors by either small molecule inhibitors (SB431542 or LY364947) or by siRNA-mediated knock down of TGFBR1 blocked the gremlin induction of ECM proteins. siRNA knockdown of TGFβ2 also blocked gremlin induction of ECM proteins. However, CTGF knockdown inhibited gremlin induction of FN and COL1 but not ELN and PAI1 indicating that different downstream signaling molecules regulate specific proteins in TM cells. While TGFβ2 activated both Smad and nonSmad signaling pathways, gremlin only phosphorylates and activates Smad2/3 proteins but failed to phosphoryate JNK1/2 and P38 MAPK proteins in TM cells. Interestingly, Smad3 knockdown blocked gremlin induction of all ECM proteins tested. In contrast, Smad2 or Smad4 knockdown failed to consistently inhibit gremlin induction of all ECM proteins. These data point to the importance of specific signaling cues that direct important functions of TM cells such as ECM turnover.

Significantly, these observations confirm that both TGFβ2 and gremlin play important roles in glaucoma pathogenesis in the TM. Elevated TGFβ2 and gremlin levels in glaucoma induce ECM remodeling that may increase AH outflow resistance and elevate IOP. TGFβ2 signaling is also complex involving both canonical Smad and the non-Smad signaling in TM cells. Interestingly, gremlin selectively activates Smad signaling to bring about TM cell fibrosis. Our experiments with gremlin suggest CTGF as a potential mediator of TM fibrosis. We also reveal that LOX enzymes are present in TM cells and are regulated by TGFβ. More importantly, we report that LOXs are enzymatically active in TM cells and TGFβ2 modulates their activity. Thus,

modulation of LOXs might provide a novel therapeutic target to prevent ECM remodeling and IOP elevation in glaucoma.

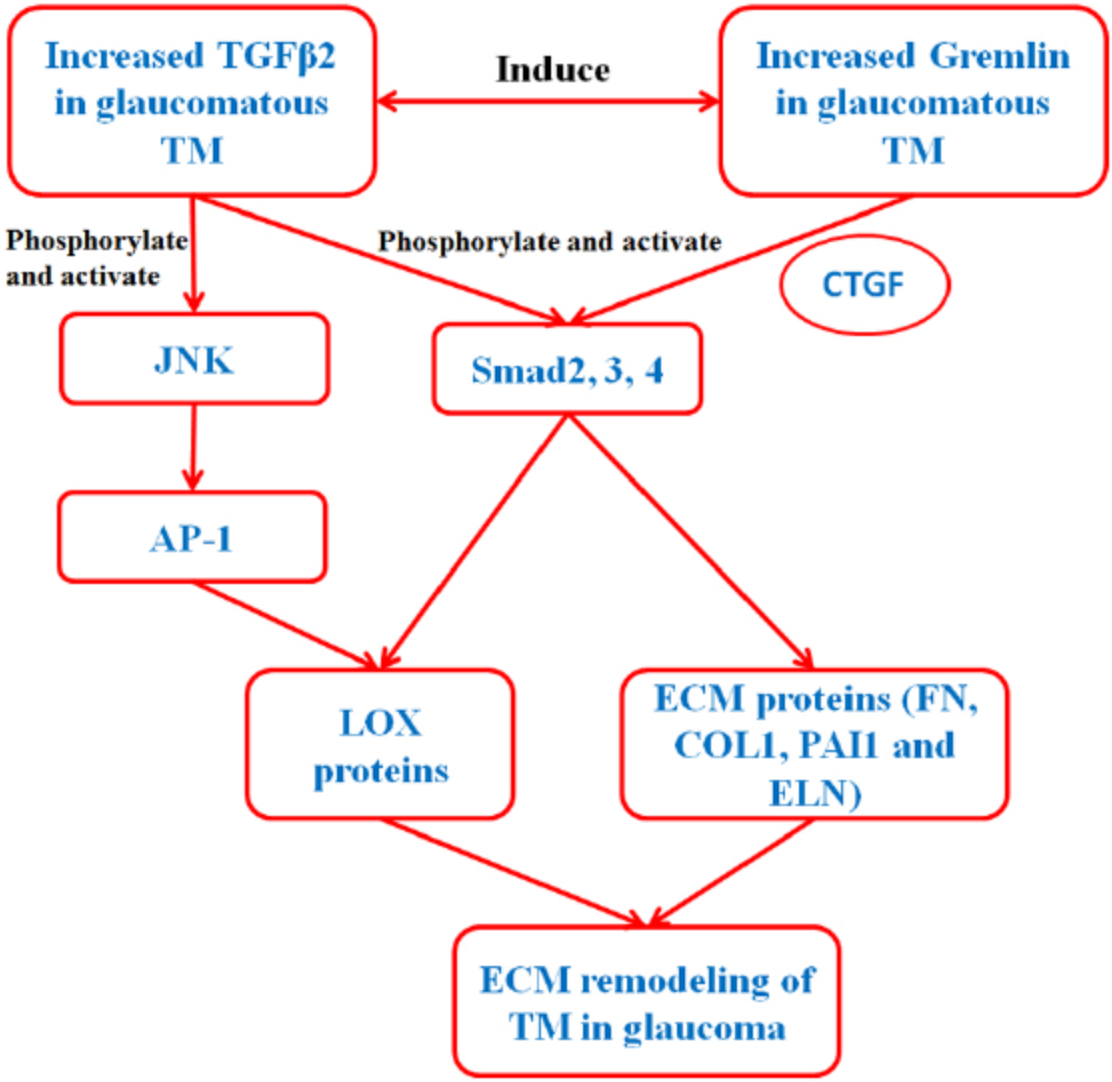

Figure 1: TGFβ2 and Gremlin induce expression of different ECM proteins in glaucomatous TM

Chapter V

UNRESOLVED QUESTIONS/ISSUES AND FUTURE DIRECTIONS

Our studies elucidate the importance of TGFβ induction of LOXs and gremlin's profibrotic effects in TM cells. These data highlight some important gaps in the data and raise very pertinent questions for future experiments. The role of LOXs in TGFβ2 induction of IOP arises as the most important question. Also, the role of JNK vs Smad signaling in regulating IOP remains unanswered. We also did not determine if gremlin also regulates LOXs. The role of Smad signaling in regulating gremlin induction of IOP is also a very important question that warrants further investigation. The following are some of the experiments designed to address some of the concerns and questions that we raised.

1. *Ex-vivo* examination of the role of LOXs in regulating IOP.
    - Effect of TGFβ2 induction of IOP will be examined using perfusion cultured anterior bovine eye segments. The anterior eye segments will be perfused with 5 ng/ml recombinant human TGFβ2 and pressure will be monitored for 7 days. To study TGFβ2 induction of LOX genes and proteins in the bovine TM qRT-PCR and western immunoblotting will be employed.
    - Next, effect of LOX and LOXL1 on regulating IOP will be examined by adenoviral LOX and LOXL1 transduction of the TM. IOP will be monitored constantly and elastin crosslinking in TM will be studied with the LOX activity assay.
    - Finally, the role of LOX and LOXL1 in TGFβ2 induction of IOP will be studied. Eyes will be transduced with adenoviral LOX and LOXL1 shRNA a couple of

days before continuous perfusion with 5 ng/ml recombinant human TGFβ2. The pressure will be monitored for 9 days. Knockdown of LOXs will be examined with western immunoblotting.

2. *In-vivo* examination of the role of LOXs in regulating IOP in mice
    - The effects of LOX and LOXL1 on regulating IOP will be examined by intravitreal injection of adenoviral LOX and LOXL1. IOP will be monitored constantly and LOX overexpression in TM will be examined with western immunoblotting. Elastin crosslinking in TM will be studied with the LOX activity assay
    - Next the role of LOX and LOXL1 in TGFβ2 induction of IOP will be studied. Adenoviral LOX and LOXL1 shRNA will be injected in the eyes intravitrealy for a couple of days before adenoviral injection of TGFβ2. The pressure will be monitored. Knockdown of LOXs will be examined with western immunoblotting.
3. Role of BMP4 and gremlin in regulating TGFβ2 induction of LOXs
    - Since BMP4 has been reported to inhibit TGFβ2 induction of ECM proteins the effects of BMP4 on TGFβ2 induction of LOXs will be studied by treating cultured human TM cells simultaneously with BMP4 and TGFβ2. We will also evaluate the effect of gremlin in blocking BMP4 inhibition of TGFβ2 induction of LOXs.
    - We will also evaluate whether gremlin alone can increase expression of LOXs in cultured human TM cells. We will also determine the gremlin signaling mechanisms involved in LOXs induction.

4. *Ex-vivo* examination of the role of Smad3 in regulating IOP.
    - The role of Smad signaling on regulating IOP will be examined by infusing 10 μM of a small molecule inhibitor of Smad3, SIS3. After two days of SIS3 infusion, we will perfuse the anterior bovine segments with recombinant gremlin. IOP will be monitored constantly and TM will be studied for any SIS3 toxicity.
    - Finally, the role of Smad signaling in gremlin induction of IOP will be studied. Adenoviral Smad3 shRNA will be injected in the eyes a couple of days before continuous perfusion with recombinant gremlin. The pressure will be monitored for 9 days. Knockdown of Smad3 will be examined with western immunoblotting.