Fitzgerald, Ashley Michelle, **Characterization and Function of Follistatin in Human Trabecular Meshwork Cells and Tissues.** Doctor of Philosophy (Cell Biology and Anatomy), March 2013

Primary Open Angle Glaucoma (POAG) is a leading cause of blindness affecting over 70 million people worldwide. The most important risk factor for developing POAG is elevated intraocular pressure (IOP), which results from increased resistance of aqueous humor (AH) through the trabecular meshwork (TM) outflow pathway. Transforming growth factor- beta II (TGF-β2) is elevated in the AH and TM of glaucoma patients. Recent evidence indicate an extracellular BMP antagonist, gremlin, regulates BMP signaling and TGF-β2 activity. Follistatin (FST), another secreted BMP antagonist is recognized for its ability to bind BMPs and their type I receptor, sequestering BMP signaling. The purpose is to evaluate the presence and relevant activity of follistatin in TM tissues and cells. We hypothesize expression of follistatin in human trabecular meshwork cells alters the expression of extracellular matrix (ECM) deposition seen in the pathogenesis of glaucoma.

First, we examined differential FST expression in human trabecular meshwork cells and tissues. We observed a significant increase in expression of FST in glaucomatous as compared to normal protein and mRNA expression.

Next, we determined if FST could be induced upon treatment of exogenous TGF-ß2 protein in human TM cells. Studies showed TGF-ß2 up-regulated FST mRNA transcript

in a time dependent manner. FST protein secretion was increased in a time and does dependent manner.

Third, we assessed FST effects on induction or inhibition of ECM proteins in human TM cells. ECM protein and mRNA expression was time dependent; nevertheless the response of ECM protein to FST treatment is different depending on isoform presence. Additional studies will be done to further elucidate these findings.

Lastly, we evaluated FST-288 and FST-315 inhibition of BMP4 attenuation of TGF-ß2 induced ECM expression. Data suggest FST-315 to suppress BMP-4 effects on TGF-ß2 induced ECM and FST-288 enhanced BMP-4 effects on TGF-ß2 induced ECM.

The goal is to evaluate additional factors that contribute to the pathogenesis of POAG and assess how these factors can provide possible therapeutic mechanisms for the treatment of glaucoma.

CHARACTERIZATION AND FUNCTION OF FOLLISTATIN IN HUMAN TRABECULAR MESHWORK CELLS AND TISSUES

Ashley M. Fitzgerald, B.S.

APPROVED:

Major Professor: _____
Robert Wordinger,PhD

Committee Member: _____
Abbot F. Clark, PhD

Committee Member: _____
Harlan Jones, PhD

Committee Member: _____
Rustin Reeves, PhD

University Member: _____
Peter Raven, PhD

Graduate Advisor: _____
Robert Wordinger, PhD

Department Chair: _____
Anuja Ghorpade, PhD

ACCEPTED:

Graduate Dean: _____
Jamboor Vishwanatha, PhD

Characterization and Function of Follistatin in Human Trabecular Meshwork Cells and Tissues

DISSERTATION

Present to the Graduate Council of the Graduate School of Biomedical Science University of North Texas Health Science Center at Fort Worth In Partial Fulfillent of the Requirements For the Degree of Doctor of Philosophy

By

Fitzgerald Ashley Michelle

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CHAPTER I

INTRODUCTION

Glaucoma

Glaucoma is a group of progressive optic neuropathies affecting approximately 1% of the population worldwide.[1-3] Primary open-angle glaucoma (POAG), the most prevalent form of glaucoma, can result in irreversible blindness and is estimated to affect more that 70 million people. Important risk factors for POAG include age, race, genetics, and elevated intraocular pressure (IOP). Elevated IOP results from increased resistance to aqueous humor (AH) outflow through the trabecular meshwork (TM). Aqueous humor is produced by the ciliary body in the posterior chamber of the eye and contains growth factors and other nutrients that support and bathe avascular tissues of the eye (e.g. cornea, and lens). Aqueous humor exits the anterior segment through the trabecular meshwork (TM) and into the canal of Schlemm and subsequently into the intrascleral and episcleral venous plexuses. Studies suggest that excess accumulation of extracellular matrix (ECM) proteins in the TM may contribute to increased resistance to AH outflow, increased IOP and death of retinal ganglion cells (RGC). [4-6]

Trabecular Meshwork (TM)

The TM is composed of the following three regions: (a) uveal meshwork, (b) corneoscleral meshwork, and (c) juxtacanalicular (JCT) meshwork. The uveal meshwork and corneoscleral meshwork form trabecular beams, or lamellae. Transmission electron microscopy has demonstrated that TM cells reside on a basal lamina (BL) and cover the trabecular beams. The core of the trabecular beams is composed of extracellular matrix (ECM) proteins including type I

collagen, fibronectin and elastin. The BL is known to be composed, in part, of laminin and type IV collagen. The uveal and corneoscleral regions of the TM do not provide significant resistance to AH outflow due to their high porosity. [7] Support for this concept comes from experimental studies, which demonstrated that cutting through the inner parts of the TM did not affect outflow facility. [7, 8]

However, the JCT meshwork does not form trabecular beams, since it is a typical loose connective tissue with resident cells that are embedded in a loosely arranged fibrillar ECM. The JCT meshwork is localized directly adjacent to the inner wall of Schlemm's canal. Cells within the JCT form long cellular processes that attach to (a) adjacent cells, (b) extracellular matrix fibrils, and (c) endothelial cells of Schlemm's canal. Together with the endothelium of Schlemm's canal and its BL, the JCT forms the inner wall region. [9] There is general agreement that the inner wall region of the TM is the site of AH outflow resistance in normal eyes; however, there is still active debate concerning the molecular mechanisms contributing to outflow resistance in the glaucomatous eye. While the ECM environment is critical for normal function of the TM, a consensus is lacking as to an understanding of what factors modulate ECM synthesis and degradation in the normal and glaucomatous TM.

Transforming Growth Factor-beta2 (TGF-β2) Signaling

The TGF-β superfamily of secreted growth factors is divided into 4 subgroups: Mullerian inhibitory substance family, inhibin/activin family, bone morphogenetic protein (BMP) family and the TGF-β family. Within the TGF-β family, 3 distinct isoforms have been identified in

mammals (e.g. TGF-β1, TGF-β2 and TGF-β3). All 3 isoforms can initiate several intracellular signaling cascades and modulate transcription of numerous target genes.

The TGF-β receptor is a heteromeric transmembrane complex that consists of both TGF-β receptor I (TGF-βRI) and TGF-β receptor II (TGF-βRII). The TGF-β receptor complexes possess serine/threonine kinase activity. Secreted, active TGF-β2 first binds TGF-βRII and subsequently initiates phosphorylation and activation of TGF-βRI within the cytoplasmic domain. In the TGF-β canonical signaling pathway, signaling leads to phosphorylation and activation of receptor mediators R-Smad2 and R-Smad3. Both R-Smad2 and R-Smad3 can form oligomeric complexes with common Smad4 prior to translocation into the nucleus and regulation of gene transcription (Figure 1)

The Relationship of Transforming Growth Factor-beta2 (TGF-β2) to Fibrosis and Glaucoma

TGF-β2 is the most abundant TGF-β isoform in the eye [10, 11]. A number of studies have reported elevated levels of TGF-β2 (2-5ng/ml) in the AH of patients with POAG [10, 12-14]. Endogenous TGF-β2 levels are elevated in both cultured glaucomatous TM cell stains and glaucomatous TM tissues [15]. In other tissues, TGF-β2 signaling has been shown to mediate fibrotic changes, including increased ECM protein deposition [16-18]. Our laboratory and others have suggested a similar role for TGF-β2 in the TM, reporting increased synthesis and secretion of ECM proteins and a potential role for ECM deposition in POAG [19-22]. In addition, TGF-β2 treatment of cultured human TM cells induces cross-linking of fibronectin via induction of tissue transglutaminase [23, 24]. We have also recently reported that TGF-β2 simulates the synthesis and secretion of lysyl oxidases; enzymes that cross-link ECM collagen and elastin fibers [25]. In the human anterior segment organ culture model, perfusion with TGF-β2 promotes a focal accumulation of fine

fibrillar extracellular material in the TM, increased fibronectin levels, and elevated IOP [26, 27, 28]. In addition, intraocular injection of a viral vector encoding bioactive TGF-β2 induced ocular hypertension in rats and mice, and significantly decreased AH outflow facility in the mouse [28].

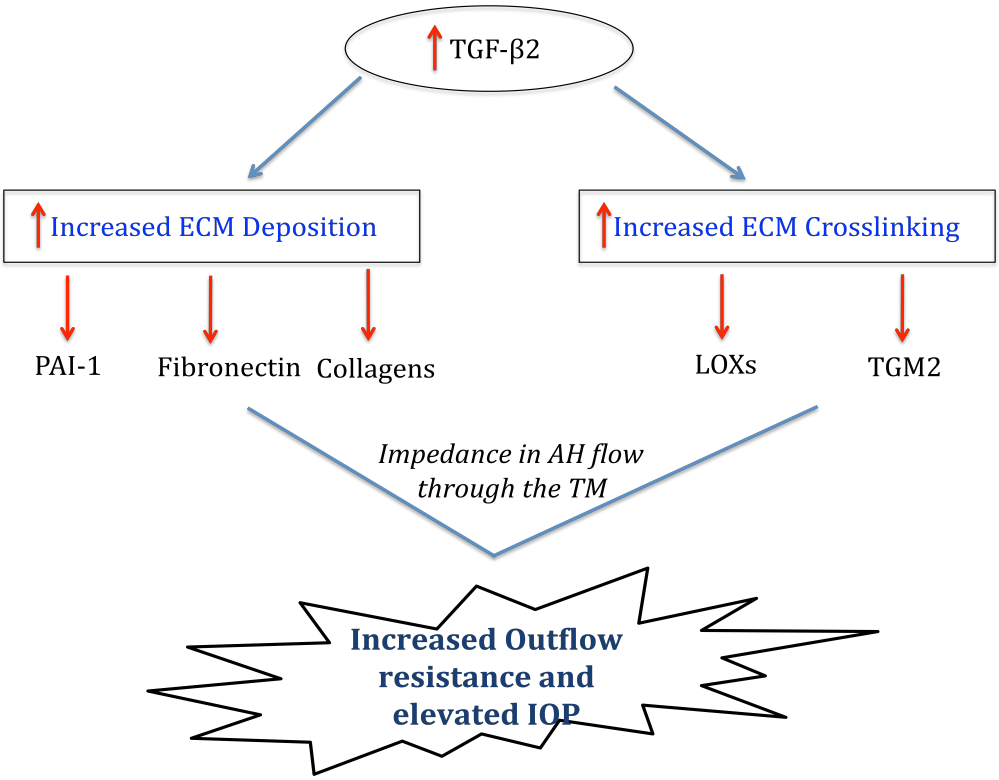

Figure 1: Elevated TGF-ß2 in the AH in glaucoma leads to (a) increased ECM deposition and crosslinking, (b) increased AH outflow resistance in the TM and (c) elevated IOP.

Bone Morphogenetic Proteins (BMP)

Bone morphogenetic proteins (BMP) are members of the TGF-β superfamily of growth factors. BMPs were originally identified as osteoinductive growth factors that promoted bone and cartilage formation. However, they are now known to control multiple cellular functions (e.g. development, morphogenesis, cell proliferation, and apoptosis) in a wide variety of cells.

BMP initiate signaling by binding to cell surface type I and type II serine/threonine kinase receptors (Figure 2). Initially, BMPs bind the type II BMP receptor and subsequently transphosphorylates the type I BMP receptor. Downstream BMP signaling involves Smad proteins. Receptor-regulated Smads (R-Smad1, R-Smad5 and R-Smad8) transiently associate with the type I BMP receptor and undergo direct phosphorylation (Figure 2). Subsequently, phosphorylated R-Smad proteins associate with common Smad 4 (Co-Smad4) and the heteromeric complex translocates to the nucleus to regulate target genes (Figure 2). Although the signaling mechanisms utilized by different BMP appear to be similar, specific cellular responses vary widely.

We have previously reported BMP4 inhibitory effects on TGF-β2 mediated ECM remodeling in cultured TM cells. [22] Similar to BMP4, Tamm et al reported that BMP7 can also inhibit TGF-β2 mediated ECM deposition in TM cells. [29, 30] These data indicated the ability of BMPs to regulate the effect of TGF-β2 in the TM; however, the regulation of these signaling pathways is still not very well understood. Tamm et al recently showed that I-Smad 7 is responsible for BMP7 attenuation of TGF-β2 induced ECM synthesis and secretion. The exact mechanisms of BMP4

inhibition of TGFβ2 response in the TM and other ocular tissue remain to be elucidated. Taken together aberrant TGFβ signaling in POAG could be a consequence of a loss of BMP regulation.

BMP Antagonists and Follistatin (FST)

The activity of BMP in vivo is subject to tight control mechanisms that may be modified in patients with POAG. BMP associated proteins that function as BMP binding proteins or BMP antagonists may alter biological activity of BMPs and their regulation of TGF-β2 in glaucoma pathogenesis. Gremlin, a secreted BMP antagonist, has been reported by our laboratory to attenuate TGF-β2 induced ECM secretion of FN.[22] Secreted BMP antagonists regulate signaling of members of the BMP family, including that of BMP-4, by binding to BMP ligands and/or preventing their interaction with the cellular receptor complex.[22, 31, 32] (Fig.2) Some BMP-associated molecules (e.g. follistatin, gremlin, chordin, and noggin) are expressed in TM cells.[32, 33] Wordinger et al. list BMP antagonists and the specific BMP protein binding affinity.[34] TGF-β2 up-regulates the expression of the BMP antagonist gremlin in TM cells.[35] Certainly, more information on the expression of BMPs and their antagonists in the TM of patients with POAG would be of interest, and needs to be further studied in greater depth.

Follistatin (FST) was first identified as a follicle-stimulating hormone inhibiting protein present in ovarian follicular fluids. It has since been shown to be a multifactorial regulatory protein exerting a majority of its effects via neutralization of activin molecules or inhibiting BMPs and BMP receptor I. Follistatin (FST) is a monomeric binding protein characterized by its cysteine-rich sequence, and belongs to a family of proteins which contain the well conserved FST domain. Follistatin plays a role in several physiological processes including regulating pituitary FSH production, liver homeostasis[36], wound repair, and response to inflammatory stimuli.

Recent studies have shown FST inhibits BMP 2, 4, and 7. Follistatin (FST) binds to BMPs and neutralizes their activities, forming a trimeric complex of FST, BMP, and the BMP receptor. [37-39] For Example FST has been shown to inhibit the role of BMP7 and Activin A in the differentiation of cornea fibroblasts. [40]

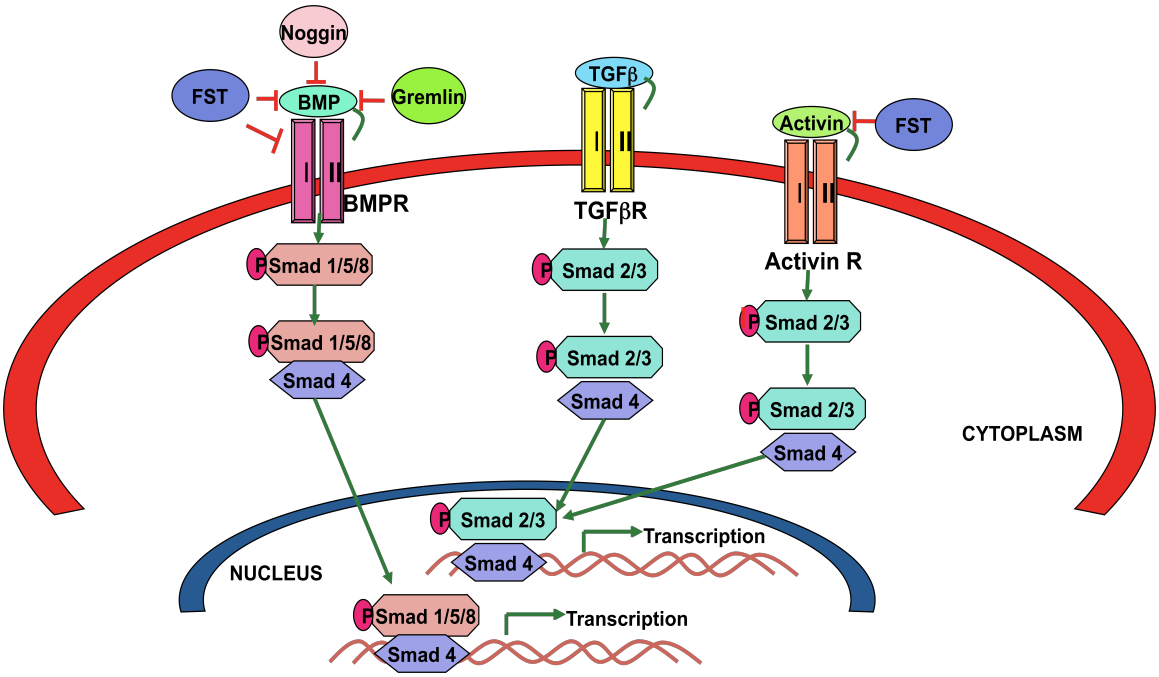

Figure 2: TGF-ß and BMP signaling regulation

Hypothesis and Specific Aims

The primary FST transcript undergoes alternative splicing to produce mRNAs that encode two FST proteins, FST-288 and FST-315. [41, 42] The FST315 isoform contains all six exons, whereas the FST-288 isoform is missing exon 6, which codes for the acidic C-terminal tail. A third isoform, FST-303, appears to arise from proteolytic cleavage of the FST-315 C-terminal tail between residues 300 and 303.[43] All three isoforms contain a region of basic residues known as the heparin-binding sequence (HBS), which is essential for binding to cell-surface heparin-sulfated proteoglycans.[44-46] It has been proposed that the acidic tail in FST-315 interacts with the basic residues within the HBS, thereby suppressing the cell-surface binding activity of FST-315. [43] These biochemical distinctions suggest that each isoform may be responsible for different subsets of biological activities depending on their degree of cell-surface localization and subsequent compartmentalization within the body. This concept is supported by the finding that FST-315 is the predominant circulating FST isoform in human serum [47], whereas ovarian follicular fluid contains primarily FST-303. [46] Nevertheless, differential biological activity, as well as the underlying mechanisms among the FST isoforms remain to be fully elucidated in the human TM.

We hypothesize that FST is expressed in TM cells and tissues, and plays a role in ECM remodeling in the TM.

Specific Aim #1: Determine the differential expression of FST in normal and glaucomatous human TM cells and tissues.

In these experiments we will determine the presence of the FST mRNA in human cultured TM cells, and FST proteins in human TM cells and tissues. The presence of FST mRNA and the differences in FST expression in normal vs. glaucomatous human cultured TM cells will be

determined by RT-PCR, and quantitative real time PCR (qPCR) using specific FST primers. To assess the presence and differential expression of proteins in normal TM (NTM) vs. glaucomatous TM (GTM), samples will subjected to western blotting analysis. The differences in FST protein NTM vs. GTM cells will be confirmed by immunohistochemical staining of NTM and GTM tissues. Follistatin isoform FST-288 and FST-315 protein will be evaluated by immunohistochemical analysis of NTM and GTM tissues.

Specific Aim 2: Determine if FST is induced in human TM cells by exogenous human recombinant TGF-β2 protein.

We will determine the induction of the FST mRNA and protein following TGF-β2 treatment in primary TM cells. A dose response will be conducted in primary human TM cells with or without recombinant human TGF-β2 at concentrations of 0,1.0, 2.5, 5.0, 7.5 and 10 ng/ml of for 48 hours in serum-free conditions. Primary human TM cells will also be treated at a fixed concentration of 2.5ng/ml of TGF-β2 in a time course for 6, 12, 24, 48, and 72 hours. The cells will be processed for QRT-PCR and western blotting analysis. Conditioned media will be collected to determine the effect of TGF-ß2 on FST secretion in primary TM cells.

Specific Aim # 3: Determine the Effect of Exogenous FST isoforms on the expression of ECM protein synthesis and secretion in human TM cells.

The induction of the ECM proteins FN, PAI-1, Col1A, and BMP4 mRNA will be evaluated following treatment with human recombinant protein FST-288, and FST-315 in primary TM cells. Primary TM cells will be treated with a fixed concentration of 50ng/ml of human recombinant FST-288 or FST-315 for 3, 6, 12, 24 and 48 hours in serum-less conditions. The

cells will be processed for QRT-PCR and western blotting analysis. Conditioned media will be collected to determine exogenous FST-288 and FST-315 effects on ECM protein secretion in primary TM cells.

Specific Aim # 4: Determine if FST isoforms block BMP4 inhibition of TGF-β2 induced ECM deposition in human TM cells

Primary human TM cells will be treated in combination and alone with human recombinant BMP4 (10ng/ml) protein, and human recombinant FST-288 (50ng/ml), or FST-315 (50ng/ml) proteins for 24 and 48 hours in serum-less conditions. Cells will also be treated alone with TGF-β2 (5ng/ml). Fibronectin, and PAI-1 expression will be studied by western blotting analysis.

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Chapter II

THE EFFECTS OF TRANSFORMING GROWTH FACTOR- BETA2 (TGF-β2) ON THE EXPRESSION OF FOLLISTATIN AND ACTIVIN A IN NORMAL AND GLAUCOMATOUS HUMAN TRABECULAR MESHWORK CELLS AND TISSUES

Ashley M. Fitzgerald, Cecila Benz, Abbot F. Clark, and Robert J. Wordinger

Abstract

This study compared follistatin (FST) and Activin (Act) expression in normal (NTM) and glaucomatous (GTM) trabecular meshwork cells and tissues and determine if exogenous TGF-β2 regulates the expression of FST and Acts in TM cell. Total RNA was isolated from TM cell strains, and mRNA expression for FST317/344 isoforms, and Acts was determined via RT-PCR and Q-PCR. Western immunoblotting and immunocytochemistry determined FST and Act A protein levels in NTM and GTM cells. Cells were treated with recombinant human TGF-β2 protein at 0-10 ng/ml for 0-72 hrs. Q-PCR, western immunoblotting, immunocytochemistry, and ELISA immunoassay were utilized to determine changes in FST and Act A mRNA and protein levels. In addition, NTM and GTM tissue samples were examined by immunohistochemistry for expression of FST, FST-315, FST-288 and Act A. Both FST mRNA and protein levels were significantly elevated in GTM cells. FST mRNA transcripts FST317/344 were also significantly elevated in GTM cells. Immunohistochemistry showed FST levels were significantly elevated in GTM tissues. Exogenous TGF-β2 significantly induced FST mRNA and protein expression. Immunohistochemistry demonstrated Act A protein levels were significantly higher in NTM tissues compared to GTM tissues. FST is elevated in GTM cells and tissues. FST is known to be an inhibitor of BMPs, which coupled with the ability of TGF-β2 to upregulate FST levels, may indicate a possible role of FST in the pathogenesis of glaucoma. These results suggest additional endogenous molecules in the human TM may regulate TGF-β2 signaling via inhibition of BMP family members.

Keywords: trabecular meshwork, follistatin, Activin A, glaucoma, TGF-β2, BMP

Introduction

Glaucoma is a group of progressive optic neuropathies affecting about 1% of the population worldwide[2-4], Primary open angle glaucoma (POAG), the most prevalent form of glaucoma, results in irreversible blindness and is estimated to affect more than 60 million people[3]. Important risk factors for POAG include age, race, and elevated intraocular pressure (IOP). Elevated IOP results from increased resistance of aqueous humor (AH) outflow through the trabecular meshwork (TM) due to excess accumulation of extracellular matrix (ECM) proteins [5-7].

TGF-β2 is the most abundant TGF-β isoform in the eye [14, 60]. A number of studies have reported elevated levels of TGF-β2 (2-5ng/ml) in the AH of patients with POAG [1, 13-15]. Endogenous TGF-b2 levels are elevated in both cultured GTM cell stains and glaucomatous TM tissues [61]. In other tissues, TGF-β signaling has been shown to mediate fibrotic changes, including increased ECM protein deposition [16-18]. Our laboratory and others have suggested a similar role for TGF-β2 in the TM, reporting increased synthesis and secretion of ECM proteins and a potential role for ECM deposition in POAG [19-22]. In addition, TGF-β2 treatment of cultured human TM cells induces cross-linking of fibronectin via induction of tissue transglutaminase [23, 24]. We have also recently reported that TGF-β2 simulates the synthesis and secretion of lysyl oxidases, enzymes that also cross-link ECM collagen and elastin fibers [25]. In the human anterior segment organ culture model, perfusion with TGF-β2 promotes a focal accumulation of fine fibrillar extracellular material in the TM, increased fibronectin levels, and elevated IOP [27 28, 29]. In addition, intraocular injection of a viral vector encoding bioactive TGF-β2 induced ocular hypertension in rats and mice, and significantly decreased AH outflow facility in the mouse [29].

Our laboratory has previously reported that TM cells express several members of the BMP family, including BMP ligands (BMP2, BMP4, BMP5, and BMP7), receptors (BMPR1a, BMPR1b, and BMPR2), and BMP antagonists gremlin, noggin, and follistatin [36, 62]. BMPs elicit multiple functions in a variety of ocular tissues [62] and other cell types [63, 64]. For example, BMP4 and BMP7 blocked TGFβ2-induction of a variety of ECM proteins, including fibronectin-1, collagens IV and VI, TSP-1, and PAI-1 [20, 35]. BMP antagonists tightly regulate BMP cellular activity by either binding directly to BMP ligands or to the type I BMP receptor[65, 66]. We reported greater levels of the BMP antagonist gremlin in GTM cells and tissues. [20] In addition, gremlin antagonizes BMP4 inhibition of TGFβ2-induced cellular ECM proteins FN and PAI-1 and elevates IOP in perfusion-cultured human anterior segment[20]. We have proposed that gremlin potentiates the profibrotic effects of TGF-β2 in the TM by blocking BMP4 regulation of TGF-β2 activity[20]. However, whether gremlin is the only mediator that blocks BMP activity in the TM is currently unknown, and the role(s) of other potential BMP antagonists in the TM has not been reported.

Follistatin (FST) is also a secreted BMP antagonist whose mRNA expression has been previously reported in TM cells [36]. FST was first identified as a follicle-stimulating hormone (FSH) inhibiting molecule present in ovarian follicle fluid. It has since been shown to be a multifactorial regulatory protein exerting a majority of its effects by neutralization of activin molecules (Act) or inhibiting BMPs [49-51, 67]. FST and Act are usually co-expressed, and FST is known to bind and inhibit Acts with high affinity.

The primary FST transcript undergoes alternative splicing to produce mRNAs (FST317/344) that encode two FST proteins, FST-288 and FST-315 (Figure 1) [53, 54]. The FST-315 isoform is

encoded by all six exons, whereas the FST-288 isoform lacks expression of exon 6, which encodes the acidic C-terminal tail. Both isoforms contain the heparin-binding sequence (HBS) of basic residues, which is essential for binding to cell-surface heparin-sulfated proteoglycans [56-58]. It has been proposed that the acidic tail in FST-315 interacts with the basic residues within the HBS, thereby suppressing the cell-surface binding activity of FST-315 [55]. These biochemical distinctions suggest that each isoform may be responsible for different subsets of biological activities depending on their degree of cell-surface localization and subsequent compartmentalization within tissues. The finding that FST-315 is the predominant circulating FST isoform in human serum supports this concept [59].

The biological activities as well as the underlying mechanisms for FST and Act involvement in the TM have not been explored. The purpose of this study was to (a) assess FST and Act expression in NTM and GTM cells and tissues and (b) determine whether exogenous TGF-β2 regulates the expression of FST in cultured NTM and GTM cells. A better understanding of the role of BMP antagonists in the human TM may identify potential therapeutic targets for the treatment of glaucoma.

Methods and Materials

Trabecular Meshwork Cell Culture

Well-characterized, primary human TM cell strains were obtained from Alcon Research, Ltd. (Fort Worth, Texas) as previously reported [23, 25, 28, 29, 36, 61, 68]. Human TM cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (low glucose) supplemented with 10% fetal

bovine serum (FBS) (HyClone Labs, Logan, UT), L-glutamine (0.292 mg/ml), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and amphotericin B (4 mg/ml). Antibiotics were purchased from Gibco BRL (Grand Island, NY). Cells were maintained at 37ºC in 5% CO_2-95% air, and fresh medium was exchanged every 2-3 days. No evidence of cellular senescence was observed.

When the cells were 80% to 90% confluent, the cells were washed with serum-free DMEM and cultured in serum-free DMEM for 24 hrs. They were then treated with or without recombinant TGF-β2 protein (#302-B2, R&D Systems, Minneapolis, MN) at selected concentrations (0, 1, 2.5, 5.0, 7.5 and 10 ng/ml) for 6, 12, 24, 48 and 72 hrs.

RNA Extraction and Polymerase Chain Reaction (PCR)

RNA was isolated using TRI reagent RT (MRC, Inc., Cincinnati, OH), and cDNA was synthesized using a Superscript c-DNA kit (Invitrogen). PCR primers were designed using Primer 3 software (http://frodo.wi.mit.edu/) (Table 1) and were obtained from Sigma Aldrich. RT-PCR reactions were run in a PTC-100 thermal cycler (MJ Research, Inc., Ramsey, MN) for 28-35 cycles. PCR amplified products were run on 1% agarose gels and analyzed with the Fluorchem 8900 UVP system (Alpha Innotech, Logan, UT).

In addition, quantitative PCR (Q-PCR) was performed as previously described[26] using PCR primers (Table 1). Briefly, 2.5 µL of cDNA was used in a reaction consisting of 1.5 units 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 1), 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 version 4.0 software (Stratagene La Jolla, CA). PCR samples with single-peak dissociation curves were selected for data analysis. TaqMan gene expression probes were used to analyze the presence of FST-317 and FST-344 in TM cells. TaqMan® gene expression assays (Applied BioSystems, Carlsbad, CA) were used according to the manufacture's instructions.

Enzyme-Linked Immunosorbent Assay (ELISA)

Briefly, cells were placed in serum-free DMEM for 24 hrs. followed by treatment with or without TGF-β2 at 2.5ng/ml for 48 hrs. Conditioned medium was collected from primary TM cells and centrifuged at 2,000 rpm for 5 mins to remove cellular debris. FST secretion was quantified using a commercially available ELISA kit (#DFN00 R&D systems, Minneapolis, MN) as directed by manufacturer's instructions. Quantification of FST was measured using a SpectraMax 340PC (Molecular Devices, Sunnyvale, CA). Data were plotted and analyzed using Graph-Pad Prism 5.

Protein Extraction and Western Blot Analysis

Total cellular protein was isolated from cultured TM cells using either (a) M-PER extraction buffer (#78501, Pierce Biotech, Rockford, IL) and Protease Inhibitor Cocktail (#78415, Pierce Biotech, Rockford, IL) or (b) Laemmli sample buffer (#161-0737, Bio-Rad, Hercules, CA) containing 5% beta-mercaptoethanol. Protein concentrations were determined using the Bio-Rad D_c protein assay system according to manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA) or the EZQ protein quantitation kit according to manufacturer's instructions (#R33200, Molecular Probes, Grand Island, NY).

A total of 60 mg of protein was loaded per well and separated by denaturing SDS-PAGE and then transferred by electrophoresis to PVDF membranes. The PVDF Membranes were incubated in 5% milk in Tris Buffered Saline Tween (TBST - 20mM Tris, 0.5M NaCl, and 0.05% Tween 20, pH 7.4) for 60 minutes in order to block non-specific binding. Blots were processed using primary antibodies and appropriate secondary antibodies (Table 2). The Super Signal West Femto Maximus sensitivity substrate (#34095, Pierce Biotech, Rockford, IL) was used for detection of proteins, and blots were exposed in a Fluorchem 8900 Imager (Alpha Innotech, San Leandro, CA).

Immunohistochemistry of TM Tissues

Three pairs of normal human eyes (ages 79, 80, and 82 yrs.) and 3 pairs of glaucoma age-matched eyes (ages 79, 80, and 82 yrs.) were used to demonstrate the presence of full length FST, FST-288, FST-315, and Act A proteins in TM tissues. Paraffin sections were

deparaffinized, rehydrated, and placed in 0.1% Trition-X100 or citrate buffer (pH 6) for antigen retrieval, followed by 20mM glycine for 15 mins. Sections were blocked in 10% normal serum. Subsequently, primary antibodies for FST-288, FST-315, full length FST, and Activin A were incubated at 4°C overnight. Secondary staining was performed for one hour at room temperature with either goat anti-mouse IgG FITC antibody (AbD Serotec, Raleigh, NC), or goat anti-rabbit Alexa 568 conjugated secondary antibody (Molecular Probes, Grand Island, NY). Antibodies used and respective dilutions are provided in Table 2. Visualization of cell nuclei was performed by staining tissue sections with DAPI (300nM) for 10 minutes. Images were captured using a Zeiss 510 confocal microscope (Carl Zeiss, Thornwood, N.Y.) or a Nikon Eclipse Ti-U microscope (Melville, NY) containing the Nuance FX imaging system (CRI, Burlington, MA). Analysis of staining intensity was performed using Image J (NIH). Two images were quantified per sample and no primary controls were used for background subtraction. The background was subtracted based on the primary controls corresponding to each sample. The area extending from the Schlemm's canal to the uveal scleral layer of the TM was used for quantification.

Immunocytochemistry of TM Cells

Primary human TM cells were grown on glass coverslips in 24 well plates. At 80% confluency, cells were fixed with 3.5% formaldehyde (Fisher Scientific, Pittsburgh, PA) in 1 X PBS for 20 minutes. Cells were treated with 0.2% Triton X-100 in PBS for 20 minutes. Cells were then blocked for 1 hour with 5% normal blocking serum in 1 X PBS. Cells were then incubated with primary antibodies overnight at 4º C. The next day, cells were washed three times with 1 X PBS solution and then incubated with secondary antibodies in 1% BSA\PBS at a 1:200 dilution for 1

hour at room temperature. To visualize nuclei, cells were treated with DAPI (300nM) nuclear stain for 10 mins and coverslips were mounted using Aqua-Mount (Lerner Laboratories, Pittsburgh, PA). Slides were stored in the dark at 4º C until visualized with a Nikon Eclipse Ti-U microscope (Melville, NY) containing the Nuance FX imaging system (CRI, Burlington, MA).

Statistical Analysis

For comparison of statistical difference between two groups, the Student's t-test was performed. One-way ANOVA was used for comparison of results between more than two groups. Statistical significance was evaluated with *p*-values less than or equal to 0.05.

Results

FST mRNA Expression in Normal and Glaucomatous TM Cells

Four NTM and three GTM cell strains were examined for the presence of FST mRNA by RT-PCR (Fig.2A). FST was expressed in all seven TM cell strains, and densitometry showed total FST mRNA expression was significantly higher in GTM cells compared to NTM cells (Fig. 2B; $p < 0.05$).

Specific FST317 and FST344 PCR by Q-PCR were used to further analyze FST isoform expression in TM cells. mRNA expression of each cell strain was normalized to its β-actin control. Both FST317 (Fig. 2C) and FST344 (Fig. 2D) mRNAs were expressed in both NTM and GTM cells. The expression of each isoform was greater in GTM in comparison to NTM cells ($p < 0.05$).

FST Protein Expression in NTM and GTM Cells

Western immunoblotting analyses confirmed FST protein expression with a band of 35 kDa in primary cultured NTM (n=3) and GTM (n=3) cells (Fig. 3A). A non-specific 75kDa band (*), as reported by the manufacturer, was apparent. Densitometry analyses of FST protein levels demonstrated significantly more expression of FST protein in GTM cells as compared to NTM

cells (Fig. 3B; $p<0.05$). Positive controls were used to confirm specific binding of pan FST antibody to FST isoforms (data not shown).

FST Expression in NTM and GTM Tissues

Representative images of FST expression in human TM tissues were taken from three normal and three glaucoma age-matched donor eyes. All sections were stained with DAPI to visualize nuclei. FST was expressed in normal (Fig. 4C and G) and glaucomatous (Fig. 4K and O) TM tissues. FST expression was dispersed throughout the TM with more intense staining in the uveal region of the TM. Image J was used to quantify staining intensity in order to evaluate the difference in expression of FST in NTM versus GTM tissues. FST expression levels were significantly higher ($p<0.05$) in GTM tissues as compared to NTM tissues (Fig. 4Q). No primary antibody and goat IgG (Fig. 4A, E, I and M) were used as negative controls, and rat testes were used as a positive control for FST staining (data not shown).

We next wanted to assess the expression of specific FST isoforms in TM tissues. Two normal and two glaucomatous age-matched samples were subjected to immunohistochemical staining using antibodies specific for FST 288 and FST 315. FST 315 expression appeared to be greater in NTM tissues (Fig. 5C) as compared to GTM tissue (Fig. 5G), although this when quantified by imager difference was not statistically significant (Fig. 5Q). FST 288 was also present in NTM tissue (Fig. 5K), but appeared to have greater expression in GTM tissue (Fig. 5O). In contrast to FST 315 results, FST 288 protein levels appeared higher in GTM tissue compared to NTM tissue (Fig. 5R), but this difference was not statistically significant.

TGF-β2 Induction of FST in TM cells

We previously have shown cross-talk between TGF-β and BMP signaling pathways in TM cells [20] and that TGF-β2 increased the expression of the BMP antagonist gremlin [48]. To evaluate the effects of TGF-β2 on FST expression, we treated NTM and GTM cells with or without TGF-β2 (2.5ng/ml) for 48 hrs. and mRNA levels of FST-317 and FST-344 were subsequently quantified by Q-PCR. There was a significant increase of FST-317 ($p < 0.01$) (Fig. 6A) and FST-344 ($p<0.05$) (Fig. 6B) expression following exogenous TGF-β2 treatment.

To further evaluate whether TGF-β2 could modulate FST protein expression in TM cells, we treated TM cells with varying concentrations of TGF-β2 (0-10 ng/ml) at different time points (0-72 hrs.). Representative immunoblots of conditioned medium showed a dose dependent increase of FST secretion (Fig. 7A) and a time dependent increase of FST secretion (Fig. 7B). Q-PCR analysis also showed a TGF-β2 mediated increase in FST expression, peaking at 6 hrs. (Fig. 7C). FST ELISA results confirmed increased FST secretion by TM cells after TGF-β2 treatment ($p<0.05$) (Fig. 7D). These results demonstrated that exogenous TGF-β2, increased FST mRNA expression and FST protein secretion in both a time and dose dependent manner in TM cells.

We also examined the immunocytochemical localization of FST following TGF-β2 treatment using an antibody that recognizes all isoforms of FST (Fig. 8). There was low expression of FST in untreated control TM cells (Fig. 8B). Treatment with TGF-β2 (5ng/ml) for 48 hrs. markedly up-regulated FST protein levels (Fig. 8E). This increased FST protein expression was localized

perinuclear (Fig. 8F), suggesting increased synthesis of FST within the secretory pathway as a result of TGF-β2 treatment.

Activin mRNA Expression in Human TM cells

We evaluated the mRNA expression of Acts using seven TM cells strains (four NTM and three GTM). Act A and Act B were expressed in most of the NTM and GTM cell strains (Fig. 9). Act A expression appeared to be greater than Act B in both cell types (Fig 9A). mRNA expression of each cell strain was normalized to its β-actin control densitometry of RT-PCR amplified products showed no statistically significant differences in the expression of Act A (Fig. 9B) or Act B (Fig. 9C) in NTM vs. GTM cells. Act C and Act E mRNA was not expressed in NTM or GTM cell strains. Commercially available human normal liver tissue (L) was used as a positive control (data not shown).

Activin A Protein Expression in NTM and GTM Tissues

Since FST and Act A have been reported to be co-expressed in many tissues, we wanted to determine whether Act A protein was also expressed in human TM tissues (Fig. 10). The absence of primary antibody and/or rabbit IgG were used as negative controls, while human liver was used as a positive control for Act A staining (data not shown). Act A was expressed in NTM tissues (Fig. 10B and F). The expression appeared to be concentrated in the juxtacanalicular (JCT) region of the TM, and inner and outer wall endothelium of Schlemm's canal (Fig. 10D and H). In contrast, FST has a more uniform distribution throughout all regions of the TM. Act A

was also expressed in GTM tissues (Fig. 10J and N). Act A protein levels were significantly lower in GTM as compared to NTM tissues ($p < 0.05$) (Fig. 10Q).

Discussion

Our current results demonstrate that the BMP antagonist FST is present in both TM cells and tissues. The primary FST transcript undergoes alternative splicing to produce mRNAs (FST317/344) that encode for proteins that are proteolytically cleaved, yielding FST-288 and FST-315 [53, 54]. Both FST mRNA and protein are expressed in human NTM and GTM cells, with significantly higher expression in GTM cells as compared to NTM cells. In addition, FST isoforms, FST-288 and FST-315 are present in human NTM and GTM tissues. Our results are the first to demonstrate FST-288 and FST-315 protein expression in TM tissues.

Immunohistochemical staining also suggested a difference in FST isoform protein levels in human NTM vs. GTM tissues. FST-288 is bound by heparin on the cell surface and FST-315 is present in the extracellular space [56, 69, 70], which may be responsible for the expression patterns of the FST isoforms in TM tissues. FST-315 appeared to be less in GTM vs. NTM tissues. This may be due to lower cellularity in the glaucomatous TM. Also, the profibrotic growth factor TGF-β2 induced FST mRNA and protein expression in a dose and time dependent manner in cultured TM cells. TGF-β2 induction of FST is similar to our previous report of gremlin induction by TGF-β2 in TM cells[19]. Thus it is possible that, similar to gremlin, the up-regulation of FST may also block BMP-4 inhibition of TGF-β2 induction of ECM proteins in the TM. In addition, the potential role(s) of FST-288 and FST-315 in the pathophysiology of glaucoma is currently not known and will form the basis of future studies.

Although FST is a BMP antagonist, it also inhibits Act signaling [49, 71]. We demonstrated mRNA expression for Act A and Act B in both NTM and GTM cells. mRNA expression of other activin genes was not detected. To our knowledge, this is the first report of the presence of Act A and B mRNA and Act A protein in human TM tissues. These findings are not totally unexpected, since previous studies reported that FST and Act A are usually co-expressed [71, 72]. Due to the low expression of Act B in TM cells, we focused our attention on Act A, whose mRNA expression was robust in TM cells. Act A protein was significantly lower in GTM compared to NTM tissues. Since TGF-β2 increased FST expression and TGF-β2 protein levels are elevated in glaucomatous AH, this may allow elevated levels of FST to function primarily as a BMP antagonist in the glaucomatous TM. Furthermore, the FST/Act complex can potentially bind BMPs and the BMP type I receptor, thus also inhibiting BMP activity in the TM [73].

Taken together, our results highlight the complex relationship of TGF-β2, BMPs and BMP antagonists in the human TM. Additional studies will further assess the relationship of Act A and TGF-β2, the function of FST in TM cells, and their potential role in the pathophysiology of glaucoma.

Acknowledgements

We would like to thank Anne-Marie Brun for providing valuable assistance with immunohistochemical staining and I-fen Chang for assistance with confocal imaging.

Table 1. PCR primers

Gene Name	Primer Pair	Product Size (base pairs)
Follistatin	ctctgccagttcatggagga	
	tccttgctcagttcggtctt	107
Activin A	tctcctgggcaagaagaaga	
	atgttgaccttgccatcaca	195
Activin B	tgaaactcctgccctacgtc	
	tgcacgtctaggttgagtcg	208
Activin C	ggagctgcttcttgatctgg	
	tcctgttccctgttgtcctc	165
Activin E	caatgtggtcaagacggatg	
	tcccataggggtcaagtgag	202
Beta- Actin	cctgtacggtccactgctta	
	tggacttgcatccaggttca	350

Note: Primers for FST-317 and FST-344 mRNA analysis are tagged with TaqMan probes provided in the TaqMan® gene expression assays. Specific primer sequences are not applicable.

Table 2. Antibodies

Antibody(cat. #)	IHC	Western Blot	Source
Follistatin (AF669)	1:7	1:500	R&D system (Minneapolis, MN)
Follistatin (ab64490)	--	1:500	Abcam (Cambridge, MA)
Follistatin288 (mca4736ga)	1:100	--	AbD Serotec (Raleigh, NC)
Follistatin315 (mca4735ga)	1:100	--	AbD Serotec (Raleigh, NC)
Activin A (NB110-61019)	1:50	--	Novus Biological (St. Charles, MO)
Beta-Actin (MAB1501)	--	1:1000	Millipore (Billerica, MA)
Donkey anti-goat Alexa- 568 (A11011)	1:200	--	Molecular Probes (Grand Island, NY)
Donkey anti-rabbit Alexa-488 (A21206)	1:200	--	Molecular Probes (Grand Island, NY)
Goat anti-mouse FITC-488 (STAR70)	1:100	--	AbD Serotec (Raleigh, NC)
Mouse IgG (I-2000)	1:100	--	Vector Labs (Burlingame, MA)
Rabbit IgG (I-1000)	1:50	--	Vector Labs (Burlingame, MA)
Goat IgG (I-5000)	1:7	--	Vector Labs (Burlingame, MA)
Donkey anti-goat (sc-2020)	--	1:10,000	Santa Cruz (Santa Cruz, CA)
Goat anti-mouse (sc-2005)	--	1:10,000	Santa Cruz (Santa Cruz, CA)
Goat anti-rabbit (32460)	--	1:10,000	Thermo Scientific (Pittsburgh, PA)

Figure Legends

Figure 1. ***Schematic representation of the FST gene.*** Schematic representation of nuclear and protein processing of the follistatin (FST) gene, including alternative splicing and post-translational modification. The human FST gene is composed of six exons alternatively spliced to produce FST-317 and FST-344 mRNA transcripts. These mRNA transcripts are then translated into pre-proteins FST-317 (translated from FST-317 mRNA) and FST-344 (translated from FST-344 mRNA). Pre-protein FST-317 and FST-344 signal peptides are cleaved, yielding FST active forms FST-288 and FST-315, respectively. FST-288 and FST-315 can undergo further proteolytic cleavage and glycosylation. Both active proteins contain an N-domain, FST I, II, and III domains, and an additional C-domain (acidic tail) in FST-315. Figure modified from Lin et al [53].

Figure 2. ***Follistatin mRNA is present in NTM and GTM cell strains.*** (**A**) Ethidium bromide-stained agarose gel of RT-PCR amplified products for FST and actin from cultured NTM and GTM cells. (**B**) Densitometric analysis of FST mRNA expression. Expression was significantly higher in GTM compared to NTM cell (* = $p<0.05$;n=7) (C-D) FST-317 and FST-344 isoforms are expressed in NTM and GTM cells. (C) TaqMan Q-PCR products of FST-317 normalized to actin showed expression in NTM and GTM cells. FST-317 expression in GTM cells was significantly higher than NTM cells (* = $p< 0.05$; n=10) (D) TaqMan Q-PCR products of FST-344 normalized to actin showed expression in NTM and GTM cells. FST-344 expression in GTM cells was significantly higher than NTM cells (* = $p< 0.05$; n=10).

Figure 3. ***Follistatin protein is present in NTM and GTM cells.*** (**A**) Western immunoblot of cellular FST protein in TM cells with bands at 35 kDa. A non-specific 75-kDa band (Ψ) was apparent. (**B**) FST protein expression normalized to actin was measured by densitometry. FST expression was significantly higher in GTM cells. (* = $p< 0.05$; n=4)

Figure 4. ***Immunohistochemical staining of FST expression in NTM and GTM tissues.*** Representative images (200X) of FST expression in the TM from six age-matched normal and glaucomatous ocular tissues. Negative goat IgG control (**A**, **E**, **I**, **M**). DAPI stained nuclei (**B**, **F**, **J**, **N**). FST expression in NTM (**C** and **G**) and GTM (**K** and **O**) tissues. FST staining merged with DAPI in normal TM (**D** and **H**) and glaucomatous (**L** and **P**) TM tissues. (**Q**) Relative intensity measurements of FST in six age-matched NTM and GTM tissues. FST expression was significantly greater in GTM tissues (* = $p<0.05$; n=6) Scale bar =100µm

Figure 5. ***Immunohistochemical staining for FST-315 and FST-288 proteins in NTM and GTM tissues.*** Representative images (200X) of immunohistochemical staining for FST-315 and FST-288 proteins in four age-matched NTM and GTM tissues were observed. No primary control (**A**, **E**, **I**, **M**). DAPI stained nuclei (**B**, **F**, **J**, **N**). FST-315 expression in NTM (**C**) and GTM (**G**) tissues. FST-288 expression in NTM (**K**) and GTM (**O**) tissues. FST-315 and FST-288 staining merged with DAPI in NTM (**D** and **L**) and GTM (**L** and **P**) TM tissues. Relative intensity measurements of FST-315 in four age-matched NTM and GTM tissues (**Q**). Relative intensity measurements of FST-288 in four age-matched NTM and GTM tissues (**R**). The relative differences in staining intensities between NTM and GTM were not statistically significant. Scale bar =100µm

Figure 6. ***TGF-β2 stimulates FST 317 and FST 344 mRNA expression in cultured primary TM cells.*** (**A**) FST 317 mRNA is significantly increased in primary cultured TM cells upon TGF-β2 (2.5ng/ml) treatment (** = p< 0.01; n=5). (**B**) FST-344 mRNA is significantly increased FST-344 expression in primary cultured TM cells upon TGF-β2 (2.5ng/ml) treatment (* = p < 0.05 ; n=5).

Figure 7. ***TGF-β2 dose and time dependently increased FST mRNA and protein expression in cultured TM cells.*** (**A**) Representative western immunoblot images of TM cell conditioned medium. TM cells treated with varying concentrations (0.5, 1.0, 2.5, 5.0, or 10ng/ml) of human recombinant TGF-β2 for 24 hrs. (n=3). (**B**) TM cells treated with or without 2.5 ng/ml of TGF-β2 for 12, 24, 48 and 72 hrs. (n=5). (**C**) Q-PCR amplified products of TM cells treated with and without 2.5 ng/ml of TGF-β2 for 6, 12, 24, 48, and 72 hrs. FST mRNA expression was normalized to actin and compared to the zero time control (n=3). (**D**) Effects of TGF-β2 (0 and 2.5 ng/ml for 48 hrs.) on FST secretion measured by ELISA in six different human TM cell lines (** = p< 0.01; n=6).

Figure 8. ***TGF-β2 induces FST protein expression in primary cultured primary TM cells assessed by immunostaining.*** DAPI staining of nuclei **(A, D)**. FST staining in HTM cells treated without (**B)** or with (**E**) TGF-β2 (5ng/ml) for 48 hrs. Merge of FST staining and DAPI in control (**C**) and TGF-β2 treated cells (F) FST staining was increased in the perinuclear region (F; arrow heads) in the TM cells. Scale bar = 100μm

Figure 9. ***Act A and Act B mRNA expression in cultured NTM and GTM cells.*** (**A**) Ethidium bromide-stained gel of RT-PCR amplified products for Act A and B, and actin from cultured NTM (n=4) and GTM (n=3) cells. (**B**) Act A mRNA expression in NTM vs. GTM was not significantly different. (**C**) Act B mRNA expression in NTM vs. GTM was not significantly different.

Figure 10. ***Act A expression in NTM and GTM tissues.*** Representative (200X) images of Act A in four age-matched (79 and 82 yrs.) NTM and GTM tissues. DAPI stained nuclei **(A, E, I, M)**. Act A expression in NTM **(B, F)** and GTM **(J, N)** tissues. Act A staining merged with DAPI in NTM **(C, G)** and GTM **(K, O)** tissues. Scale bar = 100mm Higher magnification images (400x) of Act A staining merged with DAPI in NTM **(D, H)** and GTM **(L, P)** tissues. **(Q)** Relative staining intensity measurements of Act A expression in age-matched NTM (n=2) and GTM (n=2) tissues demonstrates significantly decreased expression of Act A in GTM tissues (* = $p < 0.05$; n=4). Scale bar = 20μm

Figures

Figure 1

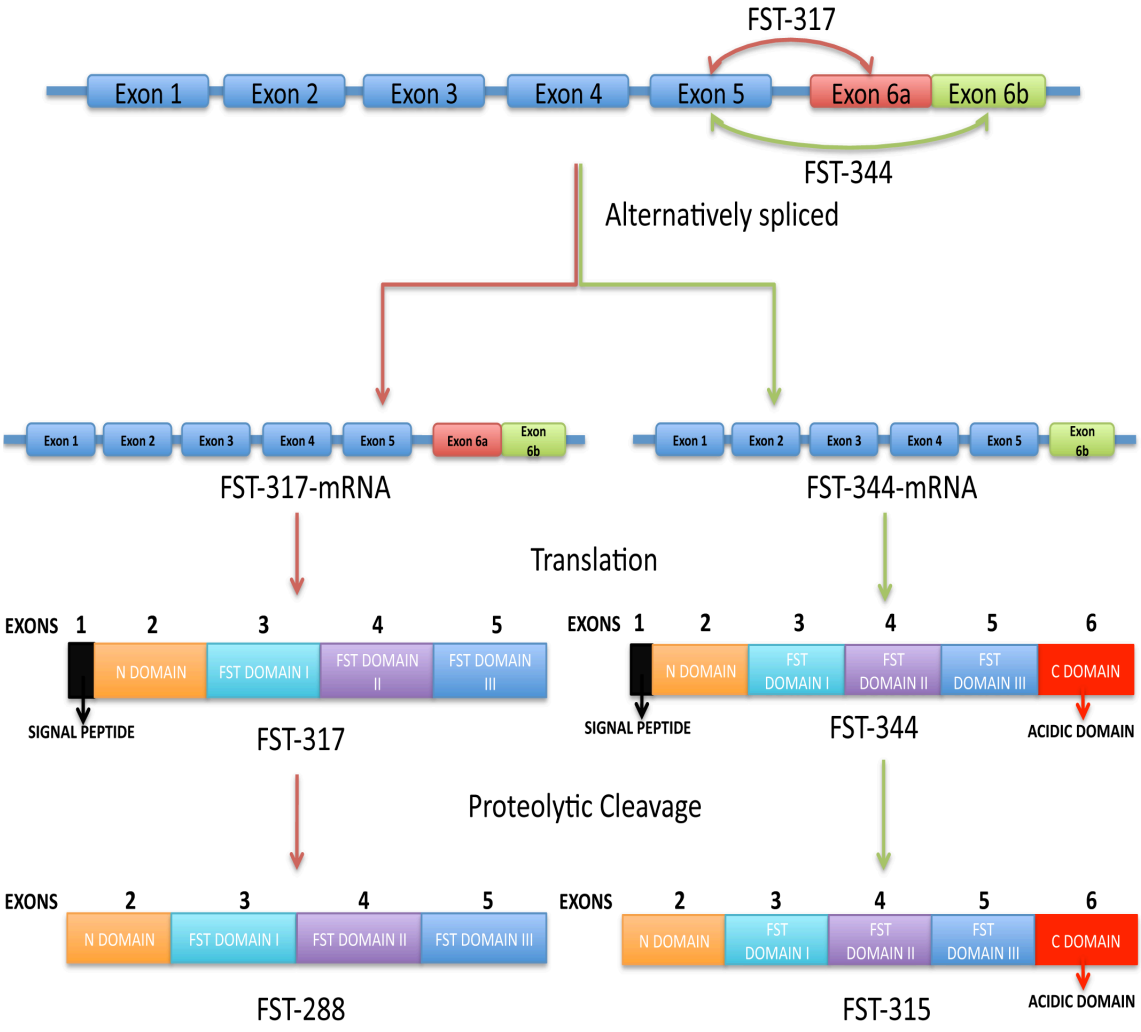
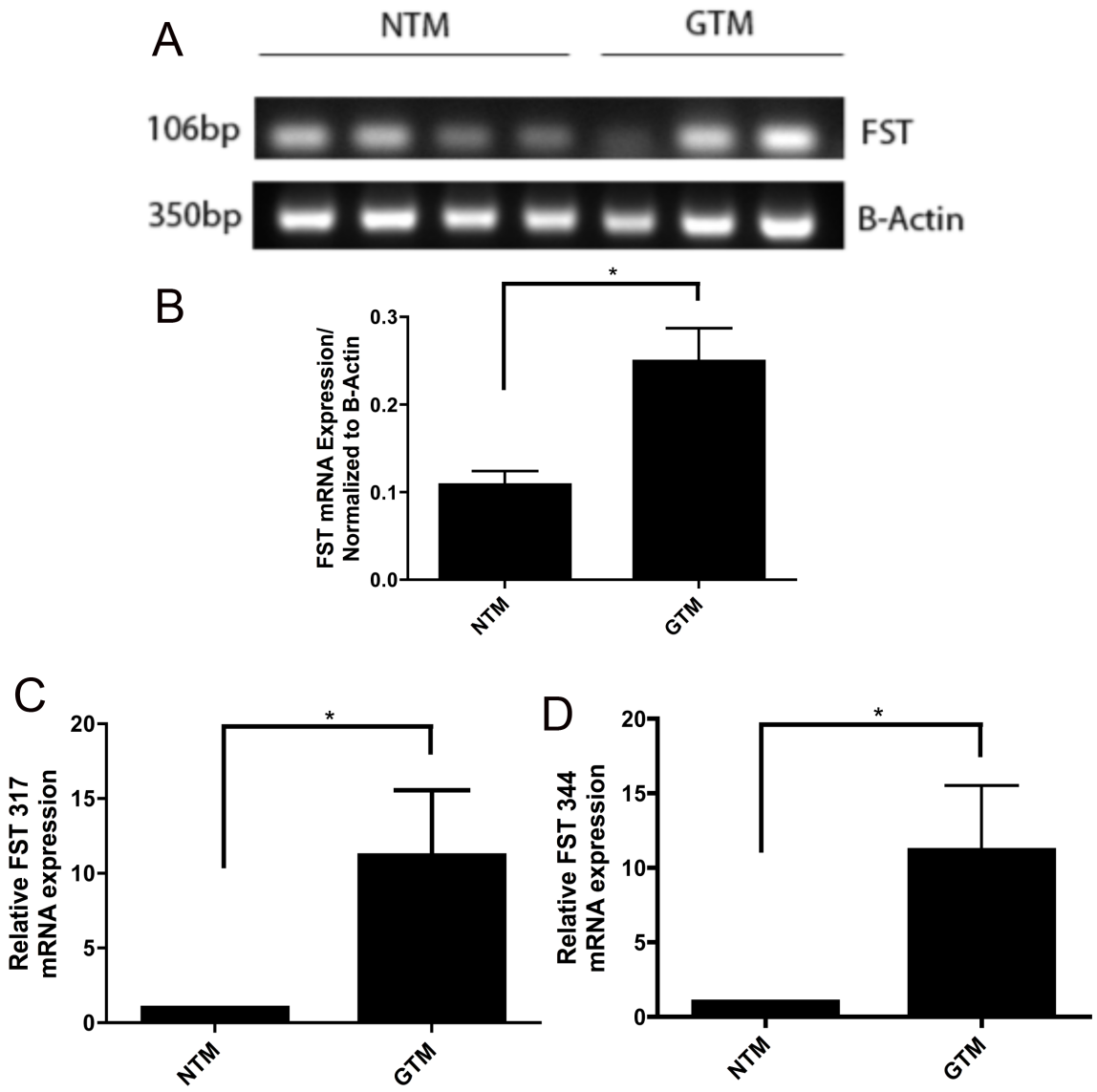


Figure 2


A
NTM
GTM
106bp
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B
FST mRNA Expression/ Normalized to B-Actin
0.3
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Relative FST 317 mRNA expression
20
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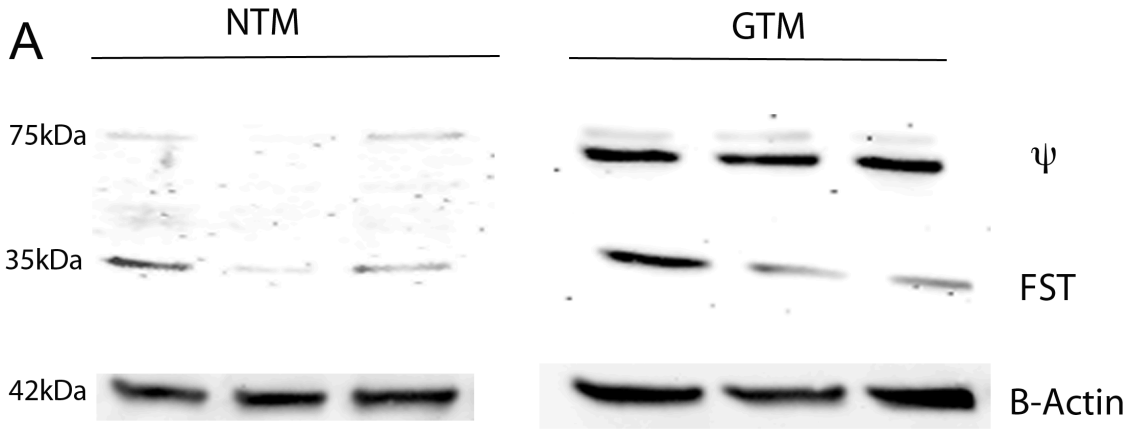
NTM
GTM
D
Relative FST 344 mRNA expression
20
15
10
5
0
*
NTM
GTM

Figure 3


A
NTM
GTM
75kDa
35kDa
42kDa
ψ
FST
B-Actin

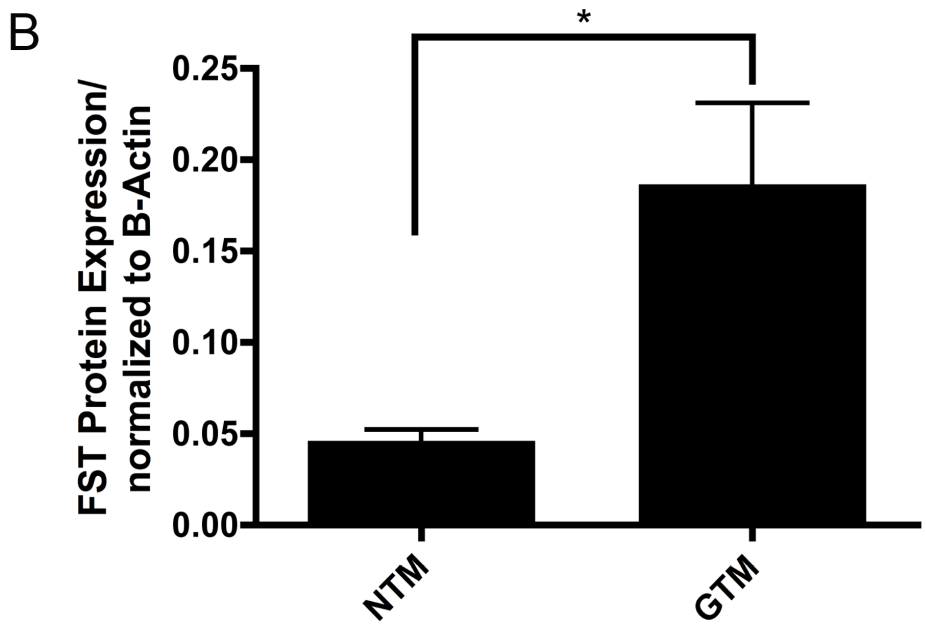
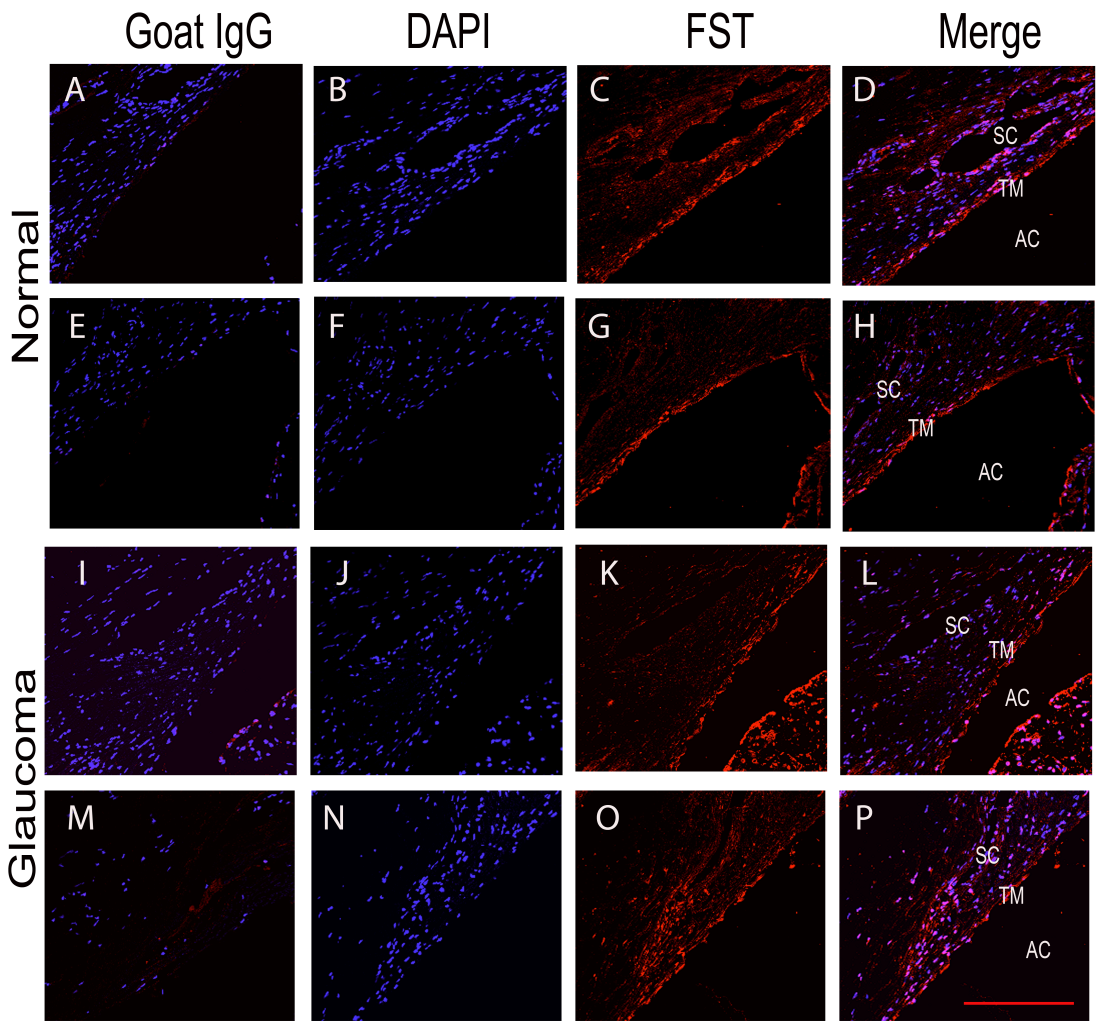

B
*
FST Protein Expression/
normalized to B-Actin
0.25
0.20
0.15
0.10
0.05
0.00
NTM
GTM

Figure 4


Goat IgG
DAPI
FST
Merge
Normal
Glaucoma
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
SC
TM
AC

Q.

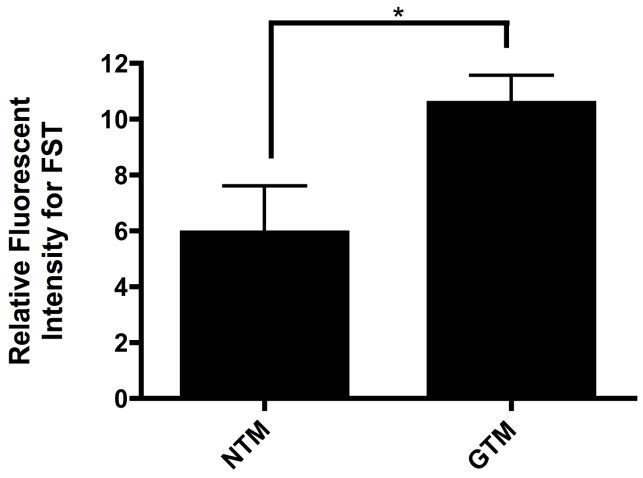


Figure 5

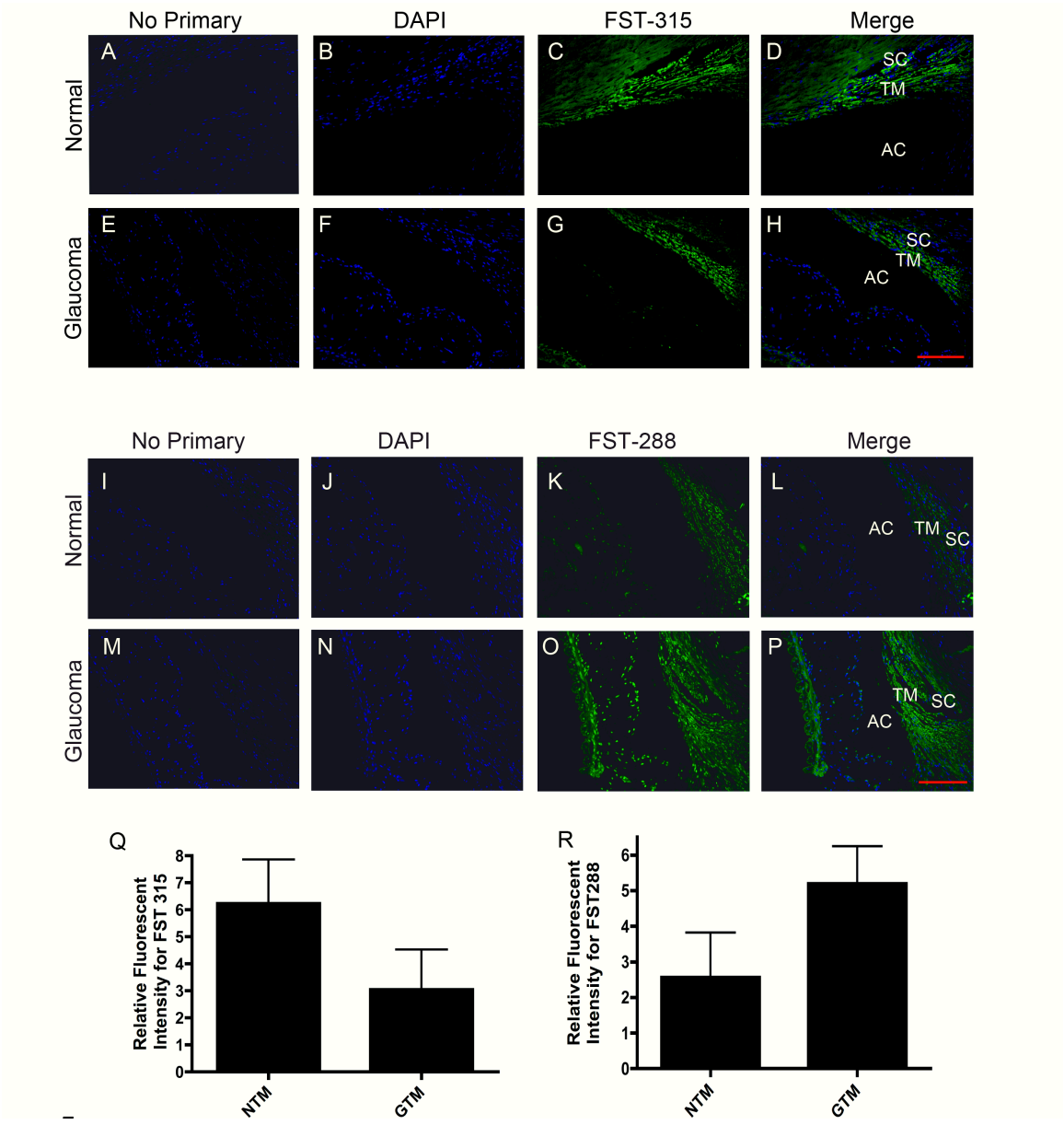

No Primary
DAPI
FST-315
Merge
Normal
Glaucoma
A
B
C
D
E
F
G
H
SC
TM
AC
No Primary
DAPI
FST-288
Merge
I
J
K
L
M
N
O
P
Q
Relative Fluorescent Intensity for FST 315
NTM
GTM
R
Relative Fluorescent Intensity for FST288
NTM
GTM

Figure 6

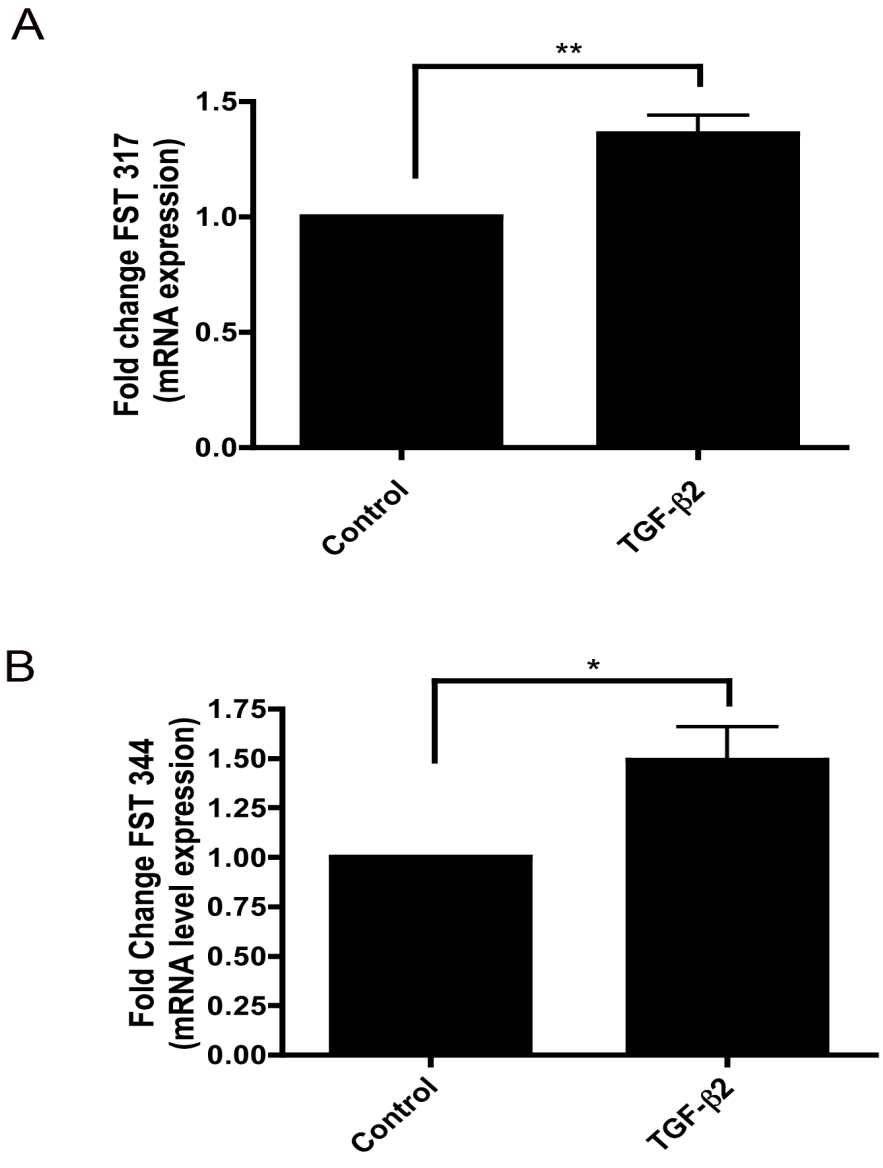

A
**
1.5
1.0
0.5
0.0
Fold change FST 317
(mRNA expression)
Control
TGF-β2
B
*
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Fold Change FST 344
(mRNA level expression)
Control
TGF-β2

Figure 7

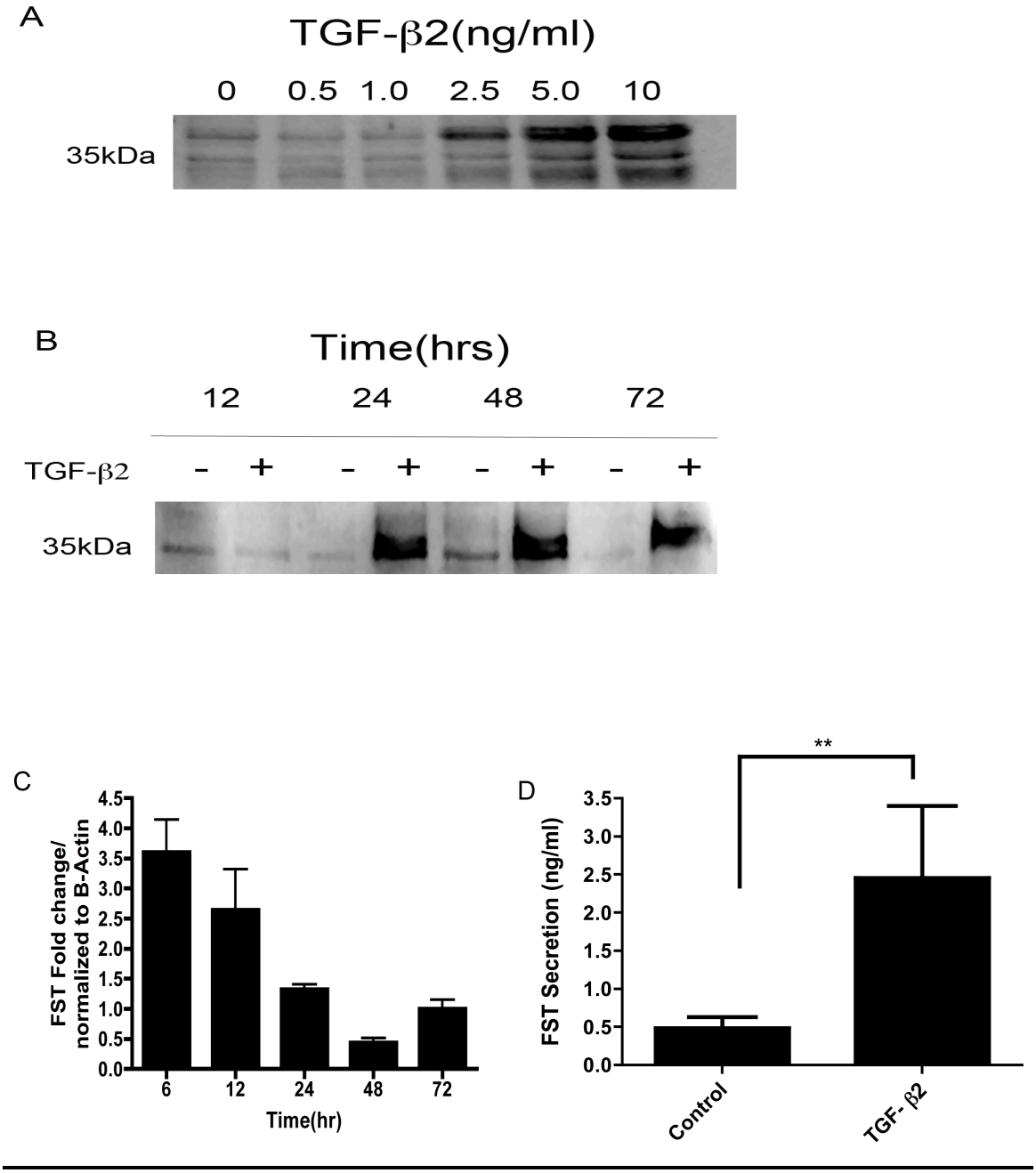

A
TGF-β2(ng/ml)
0 0.5 1.0 2.5 5.0 10
35kDa
B
Time(hrs)
12 24 48 72
TGF-β2 - + - + - + - +
35kDa
C
FST Fold change/ normalized to B-Actin
4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
6 12 24 48 72
Time(hr)
D
**
FST Secretion (ng/ml)
3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
Control
TGF- β2

Figure 8

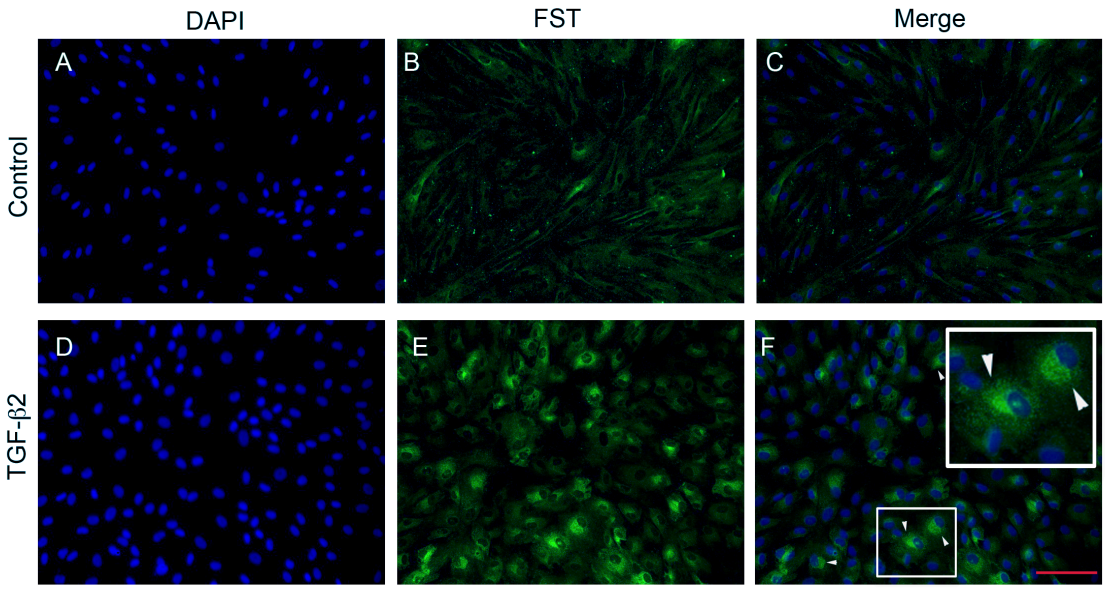


Figure 9

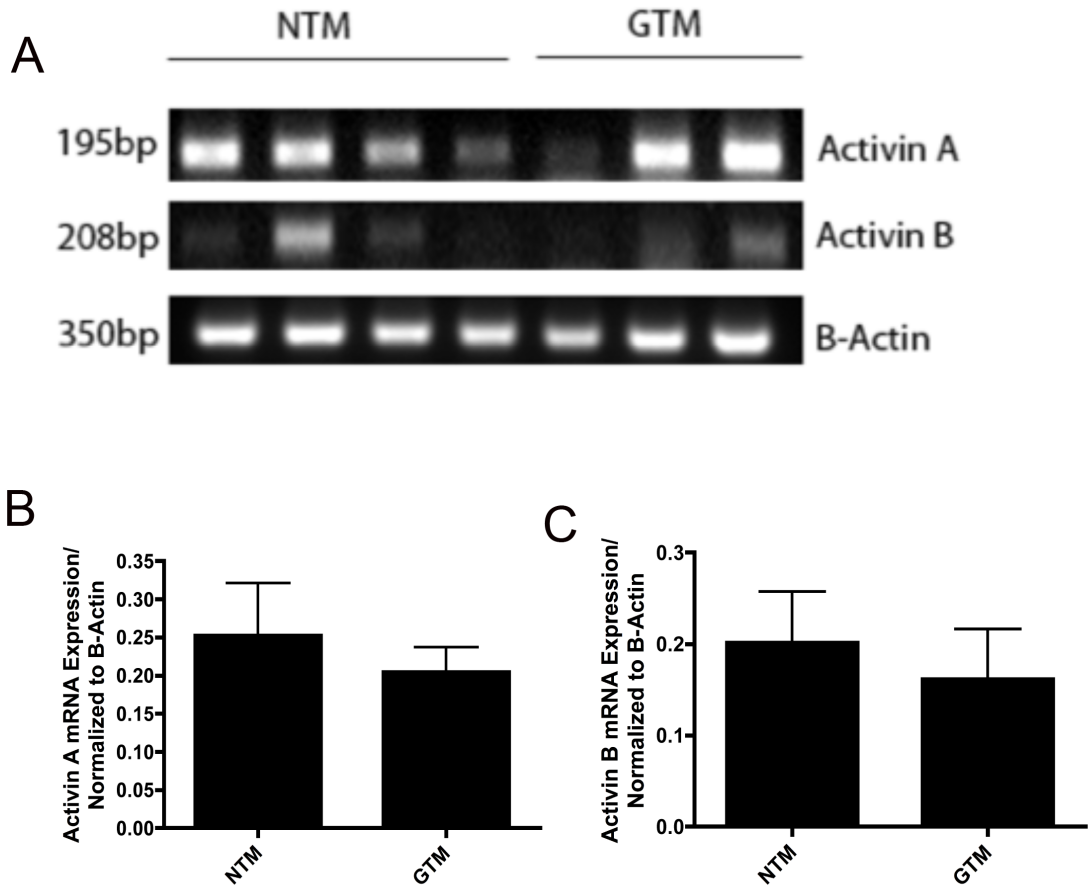
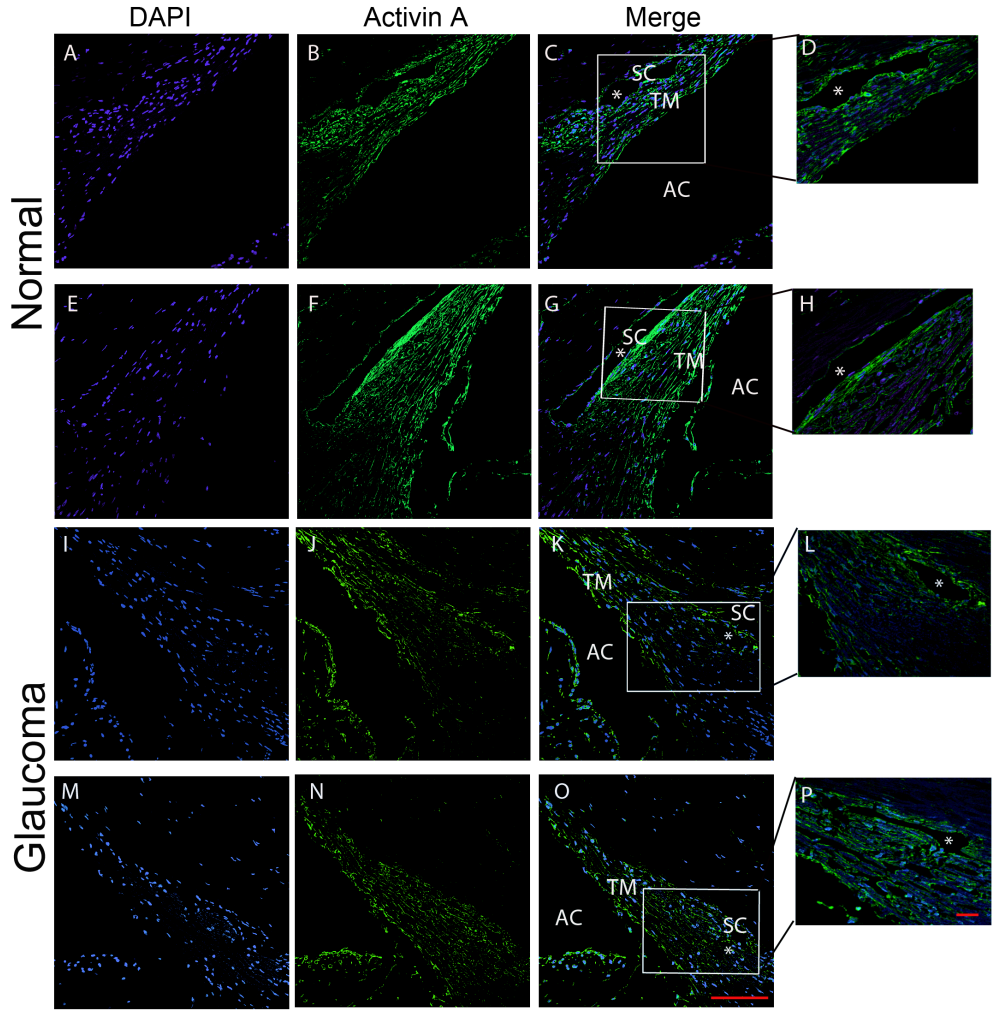
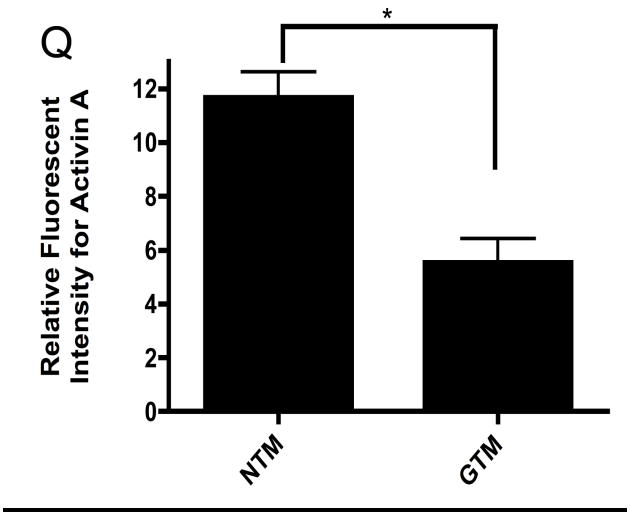

A
NTM
GTM
195bp
Activin A
208bp
Activin B
350bp
B-Actin
B
Activin A mRNA Expression/
Normalized to B-Actin
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
NTM
GTM
C
Activin B mRNA Expression/
Normalized to B-Actin
0.3
0.2
0.1
0.0
NTM
GTM

Figure 10


DAPI
Activin A
Merge
Normal
Glaucoma
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
SC
TM
AC
*


Q
Relative Fluorescent Intensity for Activin A
12
10
8
6
4
2
0
*
NTM
GTM

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Chapter III

WHY FOLLISTATIN

Chapter III

In order to maintain a homeostatic environment growth factors and associated molecules regulate cell signaling and cellular processes. In the trabecular meshwork (TM), bone morphogenetic proteins (BMP) have been shown to regulate transforming growth factor- beta (TGF-ß) signaling. [1, 2] It has also been shown that the BMP antagonist, gremlin and inhibitory Smad7 suppresses BMP signaling and activity.[2,3] TGF-ß2 is significantly increased in the aqueous humor and TM of glaucomatous patients as compared to normal patients.[4-7] Gremlin, a BMP antagonist, inhibits BMP4 attenuation of TGF-ß2 induced ECM production in the TM.[8] We previously reported that both gremlin mRNA expression and protein levels are increased in human glaucoma cell strains as compared to normal cell strains.[8] A recent study by Sethi *et al.* showed that gremlin induced ECM proteins fibronectin (FN), collagen 1 (Col1), plasminogen activating inhibitor-1 (PAI-1), elastin (ELN), and TGF-ß2 in TM cells.[9] It is plausible to suggest that gremlin may contribute to development and progression of glaucoma pathogenesis by increasing ECM proteins resulting in a disruption in the TM structural components, and inhibiting BMP4 regulation of TGF-ß2 activity in the TM.

Interestingly, several other proteins (e.g. noggin, chordin, and follistatin) also inhibit BMPs.[10-12] Chordin and FST mRNA expression were reported by Wordinger *et al.* to be expressed in ocular cells and tissues including the TM.[13] It is intriguing that FST also regulates other members of the TGF-ß superfamily, including activins, growth differentiation factors (GDFs), and myostatin.[14] We have shown that FST is expressed in both human TM cells and tissues, and is increased by exogenous TGF-ß2 treatment of primary TM cell strains.[15]

There is a great deal of promiscuity between FST and TGF-ß superfamily members. FST is widely known for its inhibitory effects on activins. There are several reports of FST isoforms and how they function in regulating activins. It is known that FST-288 has a higher affinity to bind actvins at the cell surface resulting in the neutralization of activin, while FST-315 binds activins with a lower affinity. FST-288 also promotes activin degradation, while FST-315 is thought to act as a reservoir for activins. How FST isoform specificity in the inhibition of these molecules in the TM still remains to be elucidated. In particular, FST inhibition of BMPs is of interest to us. Follistatin has been reported to neutralize BMP-2 and BMP-4 action in embryogenesis[16] and may influence cartilage formation, maturation, and bone development through interactions with BMP-7. [17] Reports have also shown the BMP-6 and BMP-7 potentially interact with FST to control pituitary follicular stimulating hormone secretion.[18]

As previously stated, FST isoforms (FST-288 and FST-315) are present in TM cells and tissues [15] however further examination of how these isoforms might function in TM cells remains to be investigated. FST-288 is known to localize at the cell surface via heparin sulfate binding[19, 20]. FST-315 is reported as the predominant circulating form, due to its 27 acidic residues at the C-terminus preventing its association with the cell surface.[21, 22] Although they elicit similar functions in their capacity to antagonize activins and BMPs, due to differences in localization, they may function differently in TM cells.[16,23, 24] The function of FST in the TM have never been study, thus we wanted to investigate the novel roles of FST-288 and FST-315 in human TM cells.

The next study seeks to determine additional functions of FST-288 and FST-315 in TM cells and whether FST may be involved in ECM and BMP4 induction or inhibition. We will also examine if FST-288 and FST-315 might contribute to BMP4 regulation in TM cells, by attenuating BMP4 ability to suppress TGF-ß2 induced ECM deposition of FN. **We hypothesize that FST-288 and FST-315 may induce ECM protein synthesis and deposition and inhibit BMP-4 action in TM cells.**

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24. Sidis Y, Mukherjee A, Keutmann H, Delbaere A, Sadatsuki M, Schneyer A. Biological activity of follistatin isoforms and follistatin-like-3 is dependent on differential cell surface binding and specificity for activin, myostatin, and bone morphogenetic proteins. *Endocrinology* 2006;147:3586-3597.

Chapter IV

THE EFFECTS OF FOLLISTATIN ON EXTRACELLULAR MATRIX REMODELING AND BONE MORPHOGENETIC PROTEIN-4 ACTIVITY IN HUMAN TRABECULAR MESHWORK CELLS AND TISSUES

Ashley M. Fitzgerald, Abbot F. Clark, and Robert J. Wordinger

Abstract

Primary open angle glaucoma (POAG) is a leading cause of blindness affecting 70 million people worldwide**.** The major risk factor for developing POAG is elevated intraocular pressure (IOP) resulting from increased resistance of aqueous humor outflow through the trabecular meshwork (TM). We have previously reported that BMP-4 attenuates TGF-β2 induced extracellular matrix (ECM) deposition in TM cells. BMP-4 inhibitory effects are blocked by a BMP antagonist, gremlin. Follistatin (FST) has also been reported to inhibit BMPs as well as activins (Act). Two isoforms of FST (288 and 315) have been described. FST function in normal (NTM) and glaucomatous (GTM) TM cells and tissues are unknown. The purpose of this study was to determine if FST: (a) induces ECM mRNA expression and protein levels in TM cells, (b) blocks BMP4 inhibition of TGFβ-2 activity, and (c) regulates BMP-4 and TGF-β2 protein expression in TM cells. QRT-PCR was used to determine mRNA expression of ECM proteins collagen 1A, PAI-1, and fibronectin (FN) 3, 6, and 12 hrs. after FST treatment. Western immunoblot (WB) analysis was used to evaluate FST effects on ECM proteins FN, PAI-1, collagen1A, and growth factors (TGF-β2, and BMP4). WB was also used to evaluate FST effects at 48hrs. on BMP4 inhibition of TGFβ-2 activity by assessing FN and PAI-1 expression. FST-315 and FST-288 appeared to have a bi-phasic effect on mRNA expression of FN and Collagen-1A that was time dependent in TM cells. FST-288 and FST-315 both induced FN and PAI-1 secretion in TM cells. FST-315 blocked BMP4 inhibition of TGFβ-2 activity, while FST-288 appeared to enhance BMP4 activity. FST-288 increased BMP4 secretion in a time dependent manner. FST-288 slightly increased TGF-ß2 protein secretion at 48hrs, while increasing BMP-4 secretion at 24 and 48hrs. FST-315 had no effect on TGF-ß2 secretion and slightly increased BMP-4 expression at 48hrs. This is the first documentation of FST function in TM cells.

Exogenous FST elicited a bi-phasic effect on ECM protein and mRNA expression in TM cells. FST 315 inhibited BMP4 activity, while FST 288 enhanced BMP4 activity. These results further our knowledge of the potential role of BMP antagonists in the human TM and their potential roles in the pathogenesis of glaucoma.

Keywords: trabecular meshwork, follistatin, glaucoma, BMP

Introduction

Primary open-angle glaucoma (POAG) is a major form of glaucoma leading to irreversible visual impairment and blindness worldwide.[1, 2] The major causative risk factor for developing POAG is elevated intraocular pressure (IOP).[3, 4] Ocular hypertension is due to an imbalance between aqueous humor (AH) production and outflow facility through the trabecular meshwork (TM).[5, 6] Elevated IOP is also associated with increased extracellular matrix (ECM) deposition within the TM. The exact mechanisms for pathological changes in the TM are not known.

The expression of TGF-ß2 is increased in the AH and TM of glaucoma patients.[7-10] Extracellular matrix proteins fibronectin (FN)[11], plasminogen inhibitor-1 (PAI-1), collagens I and IV, and crosslinking enzymes tissue transglutaminase (TGM2) [12, 13] and lysyl oxidases (LOXs)[14] are induced by TGF-ß2 in TM cells.[15] TGF-ß2 induced ECM deposition in TM cells can be attenuated by bone morphogenetic protein-4 (BMP-4) and BMP-7.[16, 17] BMPs are members of the TGF-ß superfamily of proteins. While it is evident that BMPs can regulate TGF-ß2 activity in the TM, TGF-ß2 also regulates BMPs by inducing proteins that inhibit BMP signaling.[16, 17]

Previously, we reported that TGF-ß2 increases the BMP antagonist protein gremlin in primary TM cell strains. We also demonstrated that gremlin is increased in glaucomatous TM cells and tissues.[16] Gremlin attenuates BMP4 inhibition of TGF-β2 induction of ECM components (e.g. FN, and PAI-1) in TM cells and increased IOP in the perfused human organ culture model.[16] While we have identified a role of gremlin in the TM[16, 18] other BMP antagonizes like gremlin may also regulate BMP activity in the TM.

A study conducted by Wordinger et. al identified several other BMP antagonists expressed by TM cells and tissues.[19] To date there has been not further examination of these molecules. Follistatin (FST) mRNA was expressed similarly to that of gremlin in TM cells. Follistatin (FST) is alternatively spliced to yield two mRNA transcripts (FST-317 and FST-344). These mRNA transcripts encode for pre-proteins FST-317 and FST-344. Both pre-proteins are post-translationally modified into their active forms (e.g. FST-288 and FST-315). FST-288 is known to be associated with cell surfaces, while FST-315 is present in the circulation or extracellular matrix.[20]

FST-288 and FST-315 inhibit Activin A and BMP.[21, 22] Follistatin antagonistic affects on Activin A and BMPs play a major role in development and reproduction.[23, 24] We have previously shown FST and FST isoform expression in human TM cells and tissues.[25] We also recently reported FST induction by TGF-β2 treatment in human primary cultured TM cells.[25] In addition, there are greater levels of FST in glaucoma TM cells and tissues.[25] Thus it is plausible that FST may directly affect ECM and also block BMP4 inhibition of TGFβ-2 in TM cells. The potential for FST involvement in TM dysfunction has never been studied. We wanted to further characterize how FST isoforms, FST-288 and FST-315 function in cultured primary human TM cells.

The purpose of this study was to (a) assess the affects of exogenous FST-288 and FST-315 on ECM protein expression and secretion in primary TM cell strains, (b) determine FST-288 or FST-315 effects the expression of BMP4 and TGF-ß2 in primary human TM cell strains, and (c)

determine if FST has the ability to inhibit BMP4 attenuation of TGF-β2 induced FN and PAI-1 expression.

Methods and Materials

Trabecular Meshwork Cell Culture

Well-characterized, primary human TM cell strains were obtained from Alcon Research, Ltd. (Fort Worth, Texas) as previously reported [13, 14, 19, 26-29]. Human TM cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (low glucose) supplemented with 10% fetal bovine serum (FBS) (HyClone Labs, Logan, UT), L-glutamine (0.292 mg/ml), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and amphotericin B (4 mg/ml). Antibiotics were purchased from Gibco BRL (Grand Island, NY). Cells were maintained at 37ºC in 5% CO_2-95% air, and fresh medium was exchanged every 2-3 days. No evidence of cellular senescence was observed.

When the cells were 100% confluent, the cells were washed with serum-free DMEM and cultured in serum-free DMEM for 24 hrs. They were then treated with or without recombinant FST-288 (#5836-FS, R&D Systems, Minneapolis, MN) or FST-315 (#4889-FN, R&D Systems, Minneapolis, MN) at 50ng/ml for 3,6, 12, 24, 48 and 72 hrs.

RNA Extraction and Polymerase Chain Reaction (PCR)

RNA was isolated using TRI reagent RT (MRC, Inc., Cincinnati, OH), and cDNA was synthesized using a Superscript c-DNA kit (Invitrogen). PCR primers were designed using Primer 3 software (http://frodo.wi.mit.edu/) (Table 1) and were obtained from Sigma Aldrich.

In addition, quantitative PCR (QPCR) was performed as previously described[26] using PCR primers (Table 1). Briefly, 2.5 µL of cDNA was used in a reaction consisting of 1.5 units 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 1), 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 version 4.0 software (Stratagene La Jolla, CA). PCR samples with single-peak dissociation curves were selected for data analysis.

Protein Extraction and Western Blot Analysis

Total cellular protein was isolated from cultured TM cells using laemmli sample buffer (Bio-Rad, Hercules, CA) containing 5% beta-mercaptoethanol. Protein concentrations were

determined using the EZQ protein quantitation kit according to manufacturer's instructions (#R33200, Molecular Probes, Grand Island, NY).

A total of 30µg or 15µg of protein was loaded per well and separated by denaturing SDS-PAGE and then transferred by electrophoresis to PVDF membranes. The PVDF membranes were incubated in 5% milk in Tris Buffered Saline Tween (TBST - 20mM Tris, 0.5M NaCl, and 0.05% Tween 20, pH 7.4) for 60 minutes in order to block non-specific binding. Blots were processed using primary antibodies and appropriate secondary antibodies (Table 2). The Super signal west femto maximus sensitivity substrate (#34095, Pierce Biotech, Rockford, IL) was used for detection of proteins, and blots were exposed in a fluorchem 8900 imager (Alpha Innotech, San Leandro, CA). Conditioned medium was concentrated using a resin (Stratagene, Santa Clara, CA), as per manufacturer's instructions. To assess presence and differential expression of secreted proteins, samples were subjected to western blotting for further analysis. Equal volumes of conditioning medium were be used as a loading control.

Results

Effect of Exogenous FST Isoforms on ECM Gene Expression and Protein Levels in Human TM Cells

FST isoforms (FST 288 and FST 315) have been previously reported to be expressed in TM cells and tissues.[24] However, the function of FST isoforms in human TM cells still remains to be elucidated. We first sought to determine if exogenous FST isoforms induced ECM protein synthesis and secretion (e.g. FN, PAI-1 and Col1A) in cultured human TM cells.

Transformed Human TM cells

FST 288 and FST 315 induction of ECM proteins as dose dependent in transformed human TM cells. GTM3 cells were treated at doses of 0,5,15,50,100, and 300 ng/ml for 48 hrs. with FST-288 or FST-315. FST-288 increased FN expression at low doses, while appearing to decrease FN expression at doses of 100 and 300 ng/ml. FST-315 increased FN expression in a dose dependent manner. (data not shown).

Primary Human TM cells

Treatment with FST 288 (50ng/ml) for 3, 6, and 12 hours had a significant bi-phasic effect on FN gene expression in TM cells (Fig.1A; $P< 0.01$,N=3). FST-288 decreased FN expression from 3 to 6hrs. then increased FN expression at 12hrs. Exogenous FST-288 (50ng/ml) treatment, although not statistically significant, appeared to decrease PAI-1 gene expression at 6 and 12hrs. in TM cells (Fig. 1B), while significantly decreasing Col1A gene expression from 3 to 6hrs. (Fig. 1C; $P< 0.05$). Treatment with FST 315 (50ng/ml) for 3, 6, and 12 hours also had a significant bi-phasic effect on FN gene expression in TM cells (Fig.1A; $P< 0.001$, N=3). FST-315 increased FN expression from 3 to 6hrs. then decreased FN expression 6 to 12hrs Exogenous FST-315

(50ng/ml) treatment did not alter PAI-1 gene expression in TM cells (Fig. 1B). Col1A mRNA was increased by FST-315 treatment in TM cells in a time-dependent manner (Fig.1C).

We next determined if exogenous FST 288 and/or FST 315 induced ECM proteins in primary human TM cells. Western blotting analysis on whole cell lysate (WCL) and conditioned medium (CM) samples of human TM cells (N=3) treated with FST 288 or FST 315 (50ng/ml for 24 and 48 hours) are shown in Figures 2 and 3 respectively. FST 288 appeared to have a bi-phasic effect on cellular FN expression, increasing FN expression at 24 hours while decreasing FN expression at 48 hours. (Fig. 2A) Densitometric analysis of FN induction by FST-288 at 24 hrs were statistically significant (Fig. 2B, $P< 0.05$). PAI-1 expression was induced by FST-288 at 48hours (Fig. 2A). Densitometric analysis of these changes was also not statistically significant (Fig. 2C). FST-288 increased FN expression at 24 and 48 hours, and PAI-1 expression at 48 hours. These data suggest that FST-288 can alter mRNA and protein expression of PAI-1 and FN in human TM cells.

Exogenous FST-315 significantly decreased both cellular FN and PAI-1 expression at 24 and 48 hours in human TM cells (Fig. 3A, N=3). Densitometric analysis of FN expression in TM cells showed this decrease in cellular FN expression to be significant (Fig. 3B; $P<0.05$). Densitometric analysis indicated an overall decrease in PAI-1 protein expression (Fig. 3C). We also wanted to assess the effects of FST-315 on ECM protein secretion. Treatment of FST-315 up-regulated FN secretion at 48 hours, and PAI-1 secretion at 24 and 48 hours in human TM cells (Fig. 3D).

Effect of Exogenous FST Isoforms on BMP-4 Attenuation of TGF-β2 Induced FN Expression in Human TM Cells

Human TM cells strains (N=3) were treated with or without TGF-β2 (5ng/ml), BMP4 (10ng/ml), and/or FST-288 (50ng/ml) for 48h hours (Fig. 4A). Cellular FN and PAI-1 protein levels were determined by western blotting analysis. TGF-β2 induced both FN and PAI-1 proteins, while BMP4 attenuated this induction, as we previously reported.[16] BMP-4 and FST-288 decreased FN expression. FN expression in TM cells co-treated with TGF-β2, BMP-4, and FST-288 was equal to that of control. Thus, these data indicate that FST-288 does not directly antagonize BMP-4 activity in human TM cells. However, interestingly, FN expression was even lower than that of TGF-ß2/BMP-4 treated TM cells suggesting an enhancement of BMP-4 inhibition when human TM cells are treated in combination with BMP-4 and FST-288.

Human TM cells were treated with or without TGF-β2 (5ng/ml), BMP-4 (10ng/ml), and/or FST-315 (50ng/ml) for 48hrs. (Fig.4B; n=3). TGF-β2 increased expression of FN and PAI-1, which was partially block by co-treatment with BMP-4. FST-315 alone had no effect FN and PAI-1 expression. FN expression in the co-treatment group with TGF-β2, BMP-4, and FST-315 was similar to that of the TGF-β2 treated TM cells. In contrast, FST-315 did not appear to block the BMP-4 reduction of TGF-β2-induced PAI-1 expression. These data indicate that FST-315 has the potential to block BMP-4 activity in human TM cells, but this regulation may be specific to certain ECM proteins.

Effect of Exogenous FST Isoforms on BMP-4 and TGFβ-2 Protein Levels in Human TM Cells

We also wanted to further assess the effects of FST-288 on TGF-β2 and BMP-4 protein expression in human TM cells. Primary TM cells were treated with FST-288 (50ng/ml) for 24 and 48 hours. (N=3). Western blotting analysis (Fig. 5) of cells treated with FST-288 showed FST-288 to have no effect on TGF-ß2 expression (Fig. 5B), conversely it appeared to slightly increase TGF-ß2 secretion (Fig. 5D). FST-288 also had no effect on BMP4 expression (Fig. 5A). Interestingly FST-288 increased BMP4 secretion at 24 and 48 hours in human TM cells (Fig. 5D).

FST-315 had a different effect on BMP4 inhibition in TM cells than that of FST-288. TM cells (N=3) were treated with FST-315 (50ng/ml) for 24 and 48 hours. Western blotting analysis (Fig. 6) indicated that exogenous FST-315 did not induce TGF-β2 expression (Fig. 6B) or secretion (Fig. 6D) TGF-β2. FST-315 also had no effect on BMP4 expression (Fig. 6C) and slightly increased BMP4 secretion at 48 hours (Fig. 6D).

Discussion

Primary open-angle glaucoma is characterized as a progressive optic neuropathy resulting from elevated IOP in the TM. Increases in ECM deposition are implicated to play a key role in the biochemical and structural changes exhibited in the glaucomatous TM.[30-32] The molecular mechanisms responsible for these changes in the TM leading POAG are still not very well understood. There is evidence to show that TGF-β2 is an important component for changes seen in the TM.[14, 29, 33, 34] TGF-β2 is up-regulated in the AH and TM of glaucomatous patients compared to normal patients.[7-10, 35] Our laboratory has previously shown that TGF-β2 increases ECM proteins and crosslinking enzymes (e.g. FN, PAI-1, Collagen I and IV[16], TGM2[13, 28], and LOXs[14]) in primary cultured human TM cells. Additional studies have also showed that TGF-β2 increased IOP in perfusion culture anterior segment and rodent eyes.[16, 29]

TGF-β2 induces both gremlin and FST mRNA and protein expression in TM cells.[18, 25] Gremlin and FST are BMP antagonists and have been implicated in glaucoma pathology.[16] Gremlin also induces TGF-β2 and ECM proteins FN, PAI-1, elastin, and collagen 1A.[18] Gremlin also inhibits BMP-4 activity in primary cultured TM cells. BMP-4 is known for counteracting TGF-β2 effects on ECM and IOP in the TM. Interestingly, there are other factors such as FST that can alter BMP activity. This will be important for developing therapeutic strategies for regulating ECM deposition in the TM.

Follistatin antagonistic effects on BMPs play a major role in reproduction.[23, 36, 37] You et al showed FST inhibits Smad1 in the BMP signaling pathway.[38] FST is also implicated in liver physiology and cancer.[39] FST is a key mediator of cell development and differentiation in a

number of tissue and organ systems.[40] The primary FST transcript undergoes alternative splicing to produce mRNAs that encode two FST proteins, FST-288 and FST-315. An important feature of FST is the FST domain region and its ability to bind to cell-surface heparin sulfate proteoglycans via the consensus heparin-binding sequence within FST domain 1.[41] This property appears essential to FST-288 function as a local cellular regulator. Cell surface bound FST has been postulated to form a barrier.[42-45] High local FST concentrations may be sufficient to regulate the local actions of other TGFβ-related factors such as the BMPs, despite their lower affinity for FST.[46-48] FST-288 and FST-315 may play a role in regulating BMPs in the TM. Several, ECM proteins including testican, agrin, SPARC, and FN bind FST via the FST domains. Argin bear proteoglycan side-chains that recognize binding proteins on cell surfaces.[49-52] It is possible that FST is involved in ECM deposition in the TM seen in glaucoma. SPARC has been shown to interact with both collagen and vitronectin.[53] Agrin also has recognition sequences for laminin and heparin.[54, 55]

In this study, we wanted to demonstrate FST role(s) in the TM. We reported for the first time the presence of FST isoforms FST-288 and FST-315 mRNA and protein in TM cells and tissues. We also showed that FST was expressed significantly more in glaucomatous TM cells and tissues as compared to normal TM cells and tissues.[25] FST expression and secretion was induced by exogenous TGF-β2 treatment. These studies provided a foundation for our current study.

We evaluated the function of FST in the TM by treating cells with recombinant FST-288 or FST-315 proteins. We wanted to know what role each isoform elicits in TM cells. This is critical because these isoforms are localized to different compartments of the cell. FST-288 is localized to the cell surface by its heparin sulfate binding domain and is known to be the more active form,

while FST-315 is known as the circulating FST, due to its acidic domain at the C-terminus. FST-288 and FST-315 had a bi-phasic effect on FN mRNA expression in TM cells. FST-288 also elicited bi-phasic effects on Col 1A. FST-288 significantly decreased and increased FN and Col 1A expression, respectively over time, while FST-315 increased and decreased FN mRNA expression respectively overtime. FST-288 decreased PAI-1 mRNA expression in a timely manner. FST-315 slightly decreased PAI-1 expression at 12 hours and increased Col 1A expression at 12 hrs. FST-288 and FST-315 both increased PAI-1 and FN secretion in TM cells. The ability of these isoforms to alter ECM protein expression did not coincide with one another. FST-288 increased FN secretion at 24 and 48 hrs, which FST-315 induced FN secretion at 48 hrs. This may be due to the fact that FST-288 is localized to the cell surface and is the more potent form. FST-315 acts as a reservoir binding molecules and affecting the expression of molecules.

We also evaluated the effects of FST isoforms on BMP-4 activity in TM cells and their ability to modulate BMP-4 and TGF-β2 expression in TM cells. Data showed that while FST-315 attenuated BMP-4 effects on TGF-β2 induced FN protein expression, FST-288 appeared to enhance BMP-4 inhibition of TGF-β2 induced FN and PAI-1 protein expression in TM cells. This latter finding is controversial because FST is known to antagonize BMP activity. [56] Interestingly, this is the first known report of differences in FST isoform antagonistic effects on BMP-4 in a cell culture system and in the TM. These data highlight the possible differences in FST-288 and FST-315 function in TM cells. Also, FST-288 increased BMP-4 secretion at 24 and 48 hrs, with a slight increase in TGF-β2 secretion at 48 hrs. FST changes to ECM, its effects on BMP-4 inhibition, and BMP-4 and TGF-β2 appear to be independent of one another. Further

studies are needed to assess how FST affects ECM proteins in TM cells. It is also of great importance to know whether gremlin and FST work in concert or independently of one another when inhibiting BMP activity in TM cells.

We have proposed a mechanism (Fig. 7) for the role of FST isoforms in TM cells. We have previously shown that FST isoforms are expressed and induced by TGF-β2 in TM cells. There were some differences in profile expression, FST-288 being expressed more in GTM tissues as compared to NTM tissues, and FST-315 with greater expression in the NTM as compared to GTM cells.[25] This may implicate some difference in function of these isoforms. We speculate that FST function in TM cells is diverse. FST-288 and FST-315 both increased ECM protein secretion in TM cells. Due to the differences in localization of these isoforms, increases in ECM deposition could have varying outcomes. FST-288 binds to the cell surface via binding ECM components; this may indeed be a precursor to increased ECM and resistance in the TM. FST-315 does not bind to the cell surface, but can binds molecules such as BMPs possibly regulating expression of these proteins. It is possible that there could be a "good" and "bad" FST. One that helps to regulate ECM protein expression, while the other increases expression of ECM proteins. Also, FST-315 inhibited BMP-4 activity in TM cells, while FST-288 appeared to enhance BMP-4 activity in TM cells. Further studies are needed and our current studies will serve as a basis for future studies in identifying long-term effects of FST-288 or FST-315 in the TM. It is our thought that one FST may act to maintain homeostasis in the TM, conversely the other aids in increased ECM deposition leading to increased resistance and increased IOP in the TM. These studies expand the current knowledge of the complex regulation of the ECM, and BMP signaling in the TM.

Table 1. PCR Primers

Gene Name	Primer Pair	Product Size (base pairs)
Fibronectin	agcggacctacctaggcaat ggtttgcgatggtacagctt	222
Pai-1	ccacttcttcaggctgttcc ccgttgaagtagagggcatt	186
Collagen 1A	ggaatgaagggacacagagg tagcaccatcatttccacga	196
Beta- Actin	cctgtacggtccactgctta tggacttgcatccaggttca	350

Table 2. Antibodies

Antibody(cat. #)	Western Blot	Source
Fibronectin (AB1945)	1:1000	Millipore (Billerica, MA)
PAI-1 (sc-5297)	1:200	Santa Cruz (Santa Cruz, CA)
BMP-4 (4680)	1:500	Cell Signaling (Beverly, MA)
TGF-ß2 (NBP1-59437)	1:500	Novus (Littleton, CO)
Beta-Actin (MAB1501)	1:1000	Millipore (Billerica, MA)
Goat anti-mouse (sc-2005)	1:10,000	Santa Cruz (Santa Cruz, CA)
Goat anti-rabbit (32460)	1:10,000	Cell Signaling (Pittsburgh, PA)

Figure Legends

Figure 1. ***FST-288 and FST-315 effects on ECM genes.*** Q-PCR amplified products of TM cells treated with and without 50 ng/ml of FST-288 or FST-315 for 3,6,and 12 hrs. (A) Q-PCR analysis of FN induction by FST-288 (**p<0.01) or FST-315 (*p<0.05, ***p< 0.001). (B) Q-PCR products for PAI-1 expression in TM cells treated with FST-288 or FST-315. (C) Q-PCR analysis of Col 1A fold change in FST-288 (*p<0.05) or FST-315 treated TM cells. mRNA level expression was normalized to beta-actin and compared to its respective control time point n=3.

Figure 2. ***FST-288 effects on ECM protein expression and secretion.*** Primary TM cells were treated for 24 and 48 hrs. with or with out 50ng/ml of human recombinant FST-288 protein. (A) Western blot analyses of FST-288 effects on cellular FN and PAI-1 proteins in TM cells. (B) FN protein changes normalized to beta-actin were measured by densitometry. Control vs. treated; n=3 (C) PAI-1 protein changes were normalized to beta-actin was measured by densitometry. Control vs. treated; n=3 (D) western blot analyses of conditioned medium for FN and PAI-1 from primary TM cells treated with or with out FST-288.

Figure 3. ***FST-315 effects on ECM protein expression and secretion.*** Primary TM cells were treated for 24 and 48 hrs. with or with out 50ng/ml of human recombinant FST-315 protein.(n=3) (A) Western blot analyses of FST-315 effects on cellular FN and PAI-1 proteins in TM cells. (B) FN protein changes normalized to beta-actin were measured by densitometry. Control vs. treated; n=3 (C) PAI-1 protein changes normalized to beta-actin were measured by densitometry. Control

vs. treated; n=3 (D) western blot analyses of conditioned medium for FN and PAI-1 from primary TM cells treated with or with out FST-315.

Figure 4. ***FST-288 and FST-315 effects on BMP4 inhibition in primary cultured TM cells.*** (A) TM cells were treated with or without TGF-ß2 (5ng/ml), BMP-4 (10ng/ml), and/or FST-288 (50ng/ml) for 48 hrs. Cells were also treated in combination with TGF-ß2 (5ng/ml)/BMP4 (10ng/ml)/FST-288 (50ng/ml) for 48 hrs. Changes in cellular FN and PAI-1 expression were assessed by immunoblotting (n=3). (B) TM cells were treated with or without TGF-ß2 (5ng/ml), BMP-4 (10ng/ml), and/or FST-315 (50ng/ml) for 48 hrs. Changes in cellular FN and PAI-1 expression were assessed by immunoblotting (n=3).

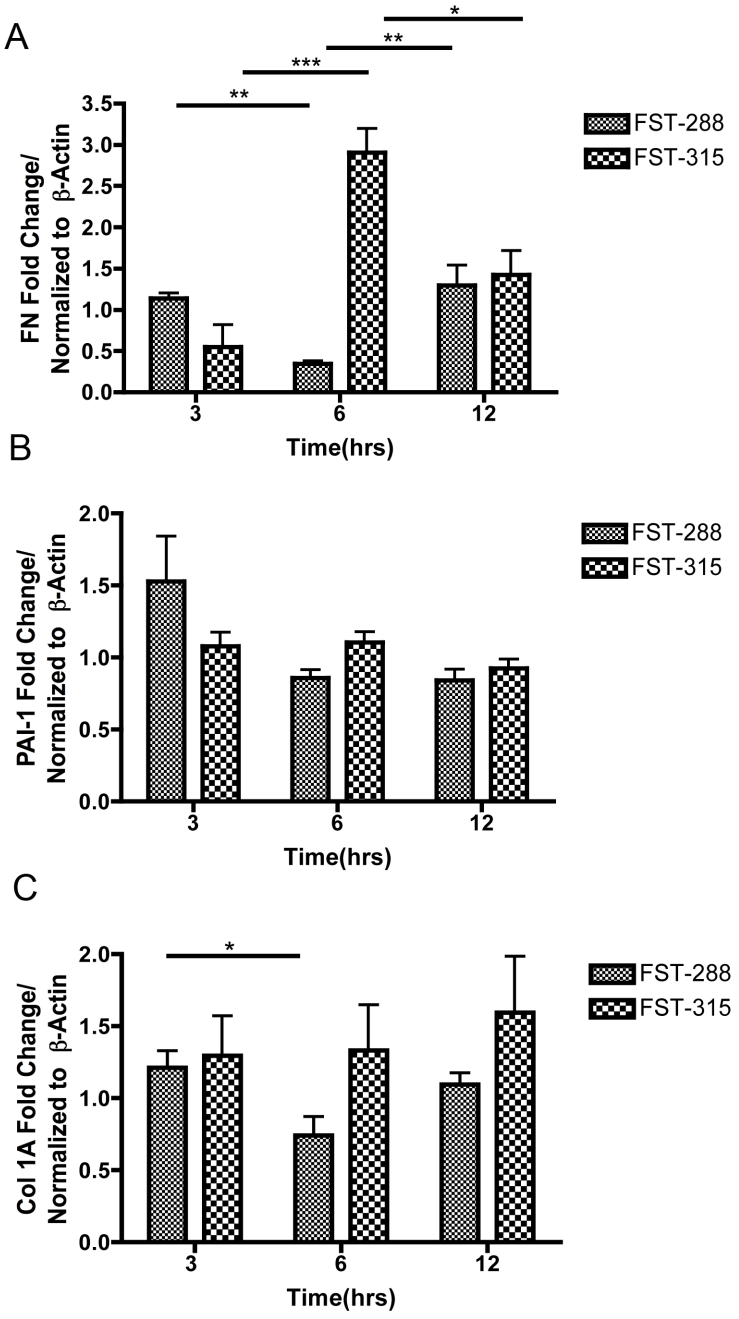
Figure 5. ***FST-288 effects on TGF-ß2 and BMP4 expression in TM cells.*** (A) Primary TM cells were treated for 24 and 48 hrs. with or without 50ng/ml of human recombinant FST-288 protein. (A) Western blot analyses of FST-288 effects on cellular TGF-ß2 and BMP4 protein in TM cells. (B) TGF-ß2 protein changes normalized to beta-actin were measured by densitometry. Control vs. treated; n=3 (C) BMP4 protein changes normalized to beta-actin were measured by densitometry. Control vs. treated; n=3 (D) western blot analyses of conditioned medium for TGF-ß2 and BMP4 from primary TM cells treated with or with out FST-288.

Figure 6. ***FST-315 effects on TGF-ß2 and BMP4 expression in TM cells.*** Primary TM cells were treated for 24 and 48 hrs. with or without 50ng/ml of human recombinant FST-315 protein.(n=3) (A) Western blot analyses of FST-315 effects on cellular TGF-ß2 and BMP4 protein in TM cells. (B) TGF-ß2 protein changes normalized to beta-actin were measured by

densitometry. Control vs. treated; n=3 (C) TGF-ß2 protein changes normalized to beta-actin were measured by densitometry. Control vs. treated; n=3 (D) western blot analyses of conditioned medium for TGF-ß2 and BMP4 from primary TM cells treated with or with out FST-315.

Figure 7. ***Schematic Representation of FST-288 and FST-315 effects on ECM proteins and BMP-4 activity in TM cells.*** FST-288 decrases ECM production, while increasing ECM secretion. FST-315 also decreases ECM production while increasing ECM secretion. FST-315 creates a reservoir for ECM proteins increasing ECM deposition in TM cells, which may result in increased IOP leading to POAG.

Figure 1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FST-288
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B
PAI-1 Fold Change/ Normalized to β-Actin
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Time(hrs)
FST-288
FST-315
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3
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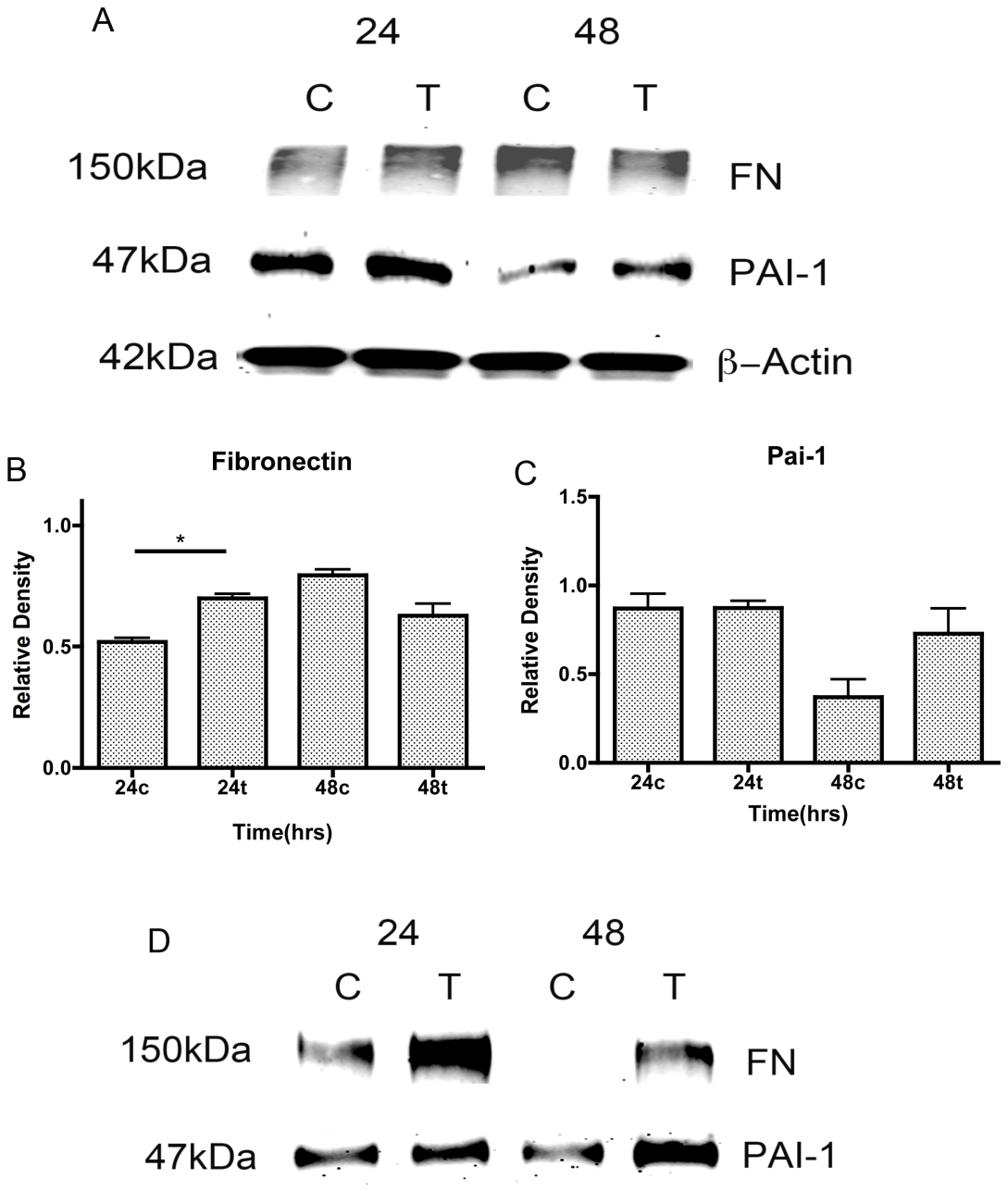
Time(hrs)
FST-288
FST-315

Figure 2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PAI-1

Figure 3

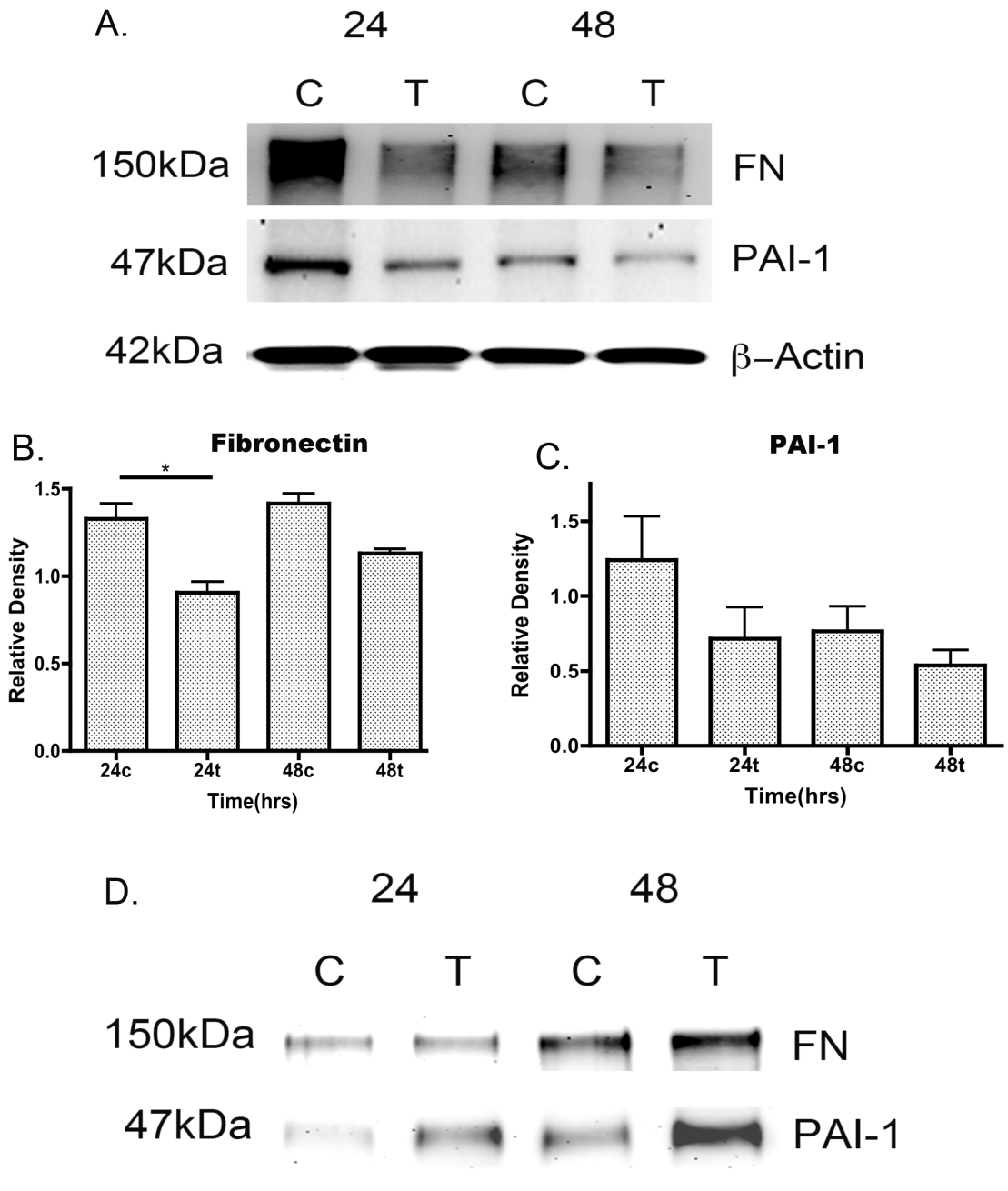

A.
24
48
C
T
C
T
150kDa
FN
47kDa
PAI-1
42kDa
β–Actin
B.
Fibronectin
*
Relative Density
1.5
1.0
0.5
0.0
24c
24t
48c
48t
Time(hrs)
C.
PAI-1
Relative Density
1.5
1.0
0.5
0.0
24c
24t
48c
48t
Time(hrs)
D.
24
48
C
T
C
T
150kDa
FN
47kDa
PAI-1

Figure 4

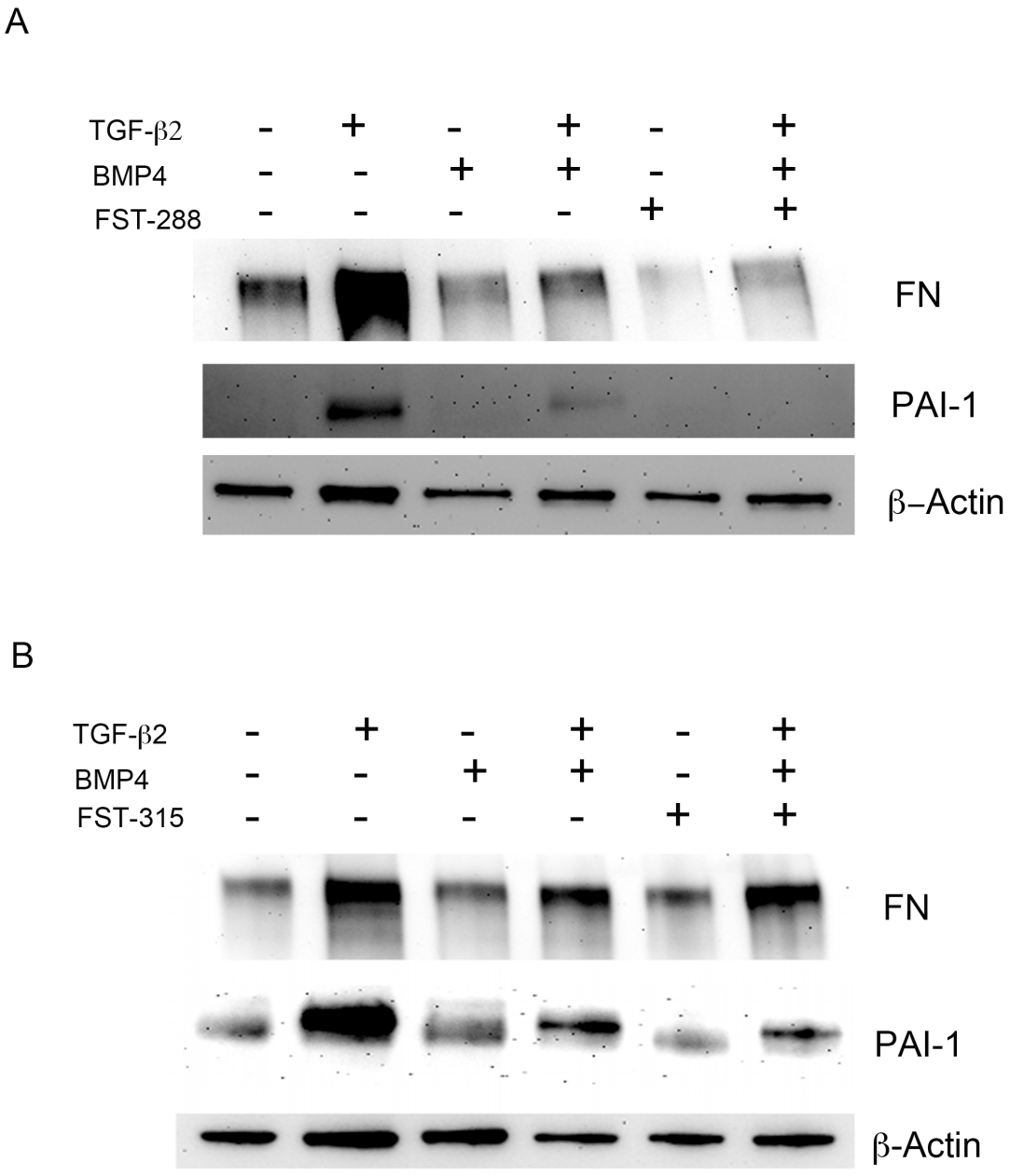

A
TGF-β2 - + - + - +
BMP4 - - + + - +
FST-288 - - - - + +
FN
PAI-1
β–Actin
B
TGF-β2 - + - + - +
BMP4 - - + + - +
FST-315 - - - - + +
FN
PAI-1
β-Actin

Figure 5

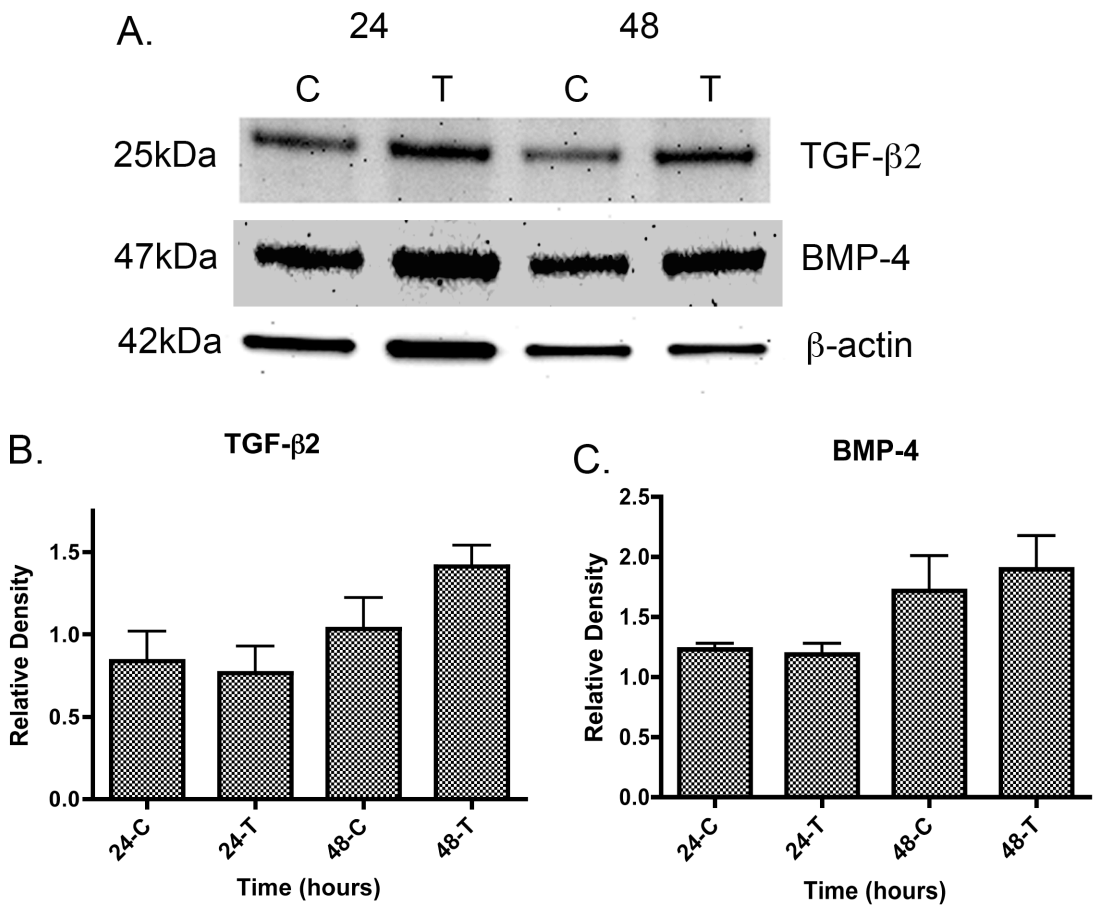

A.
24
48
C
T
C
T
25kDa
TGF-β2
47kDa
BMP-4
42kDa
β-actin
B.
TGF-β2
Relative Density
1.5
1.0
0.5
0.0
24-C
24-T
48-C
48-T
Time (hours)
C.
BMP-4
Relative Density
2.5
2.0
1.5
1.0
0.5
0.0
24-C
24-T
48-C
48-T
Time (hours)
D.
24
48
C
T
C
T
25kDa
TGF-β2
47kDa
BMP4

Figure 6

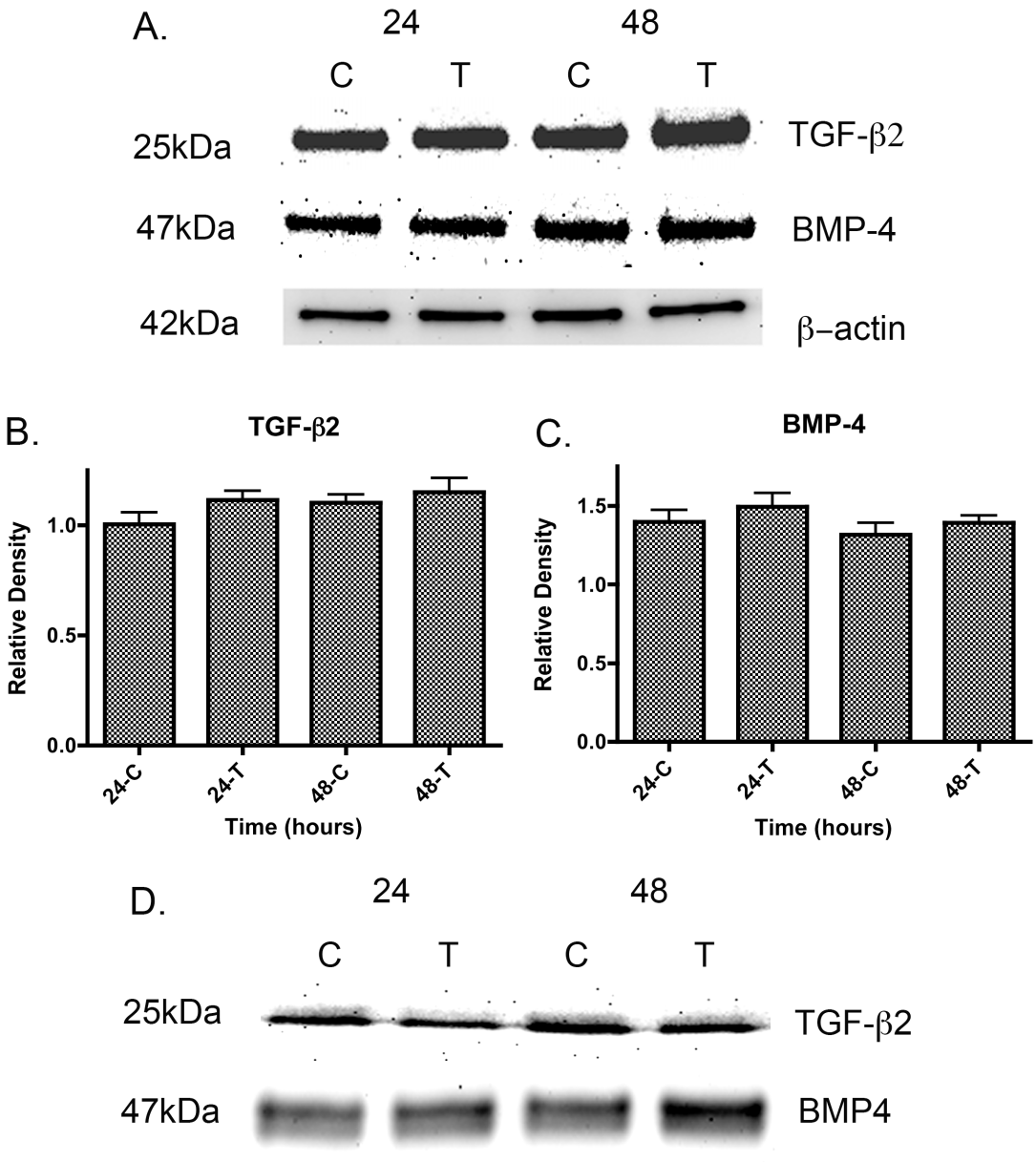
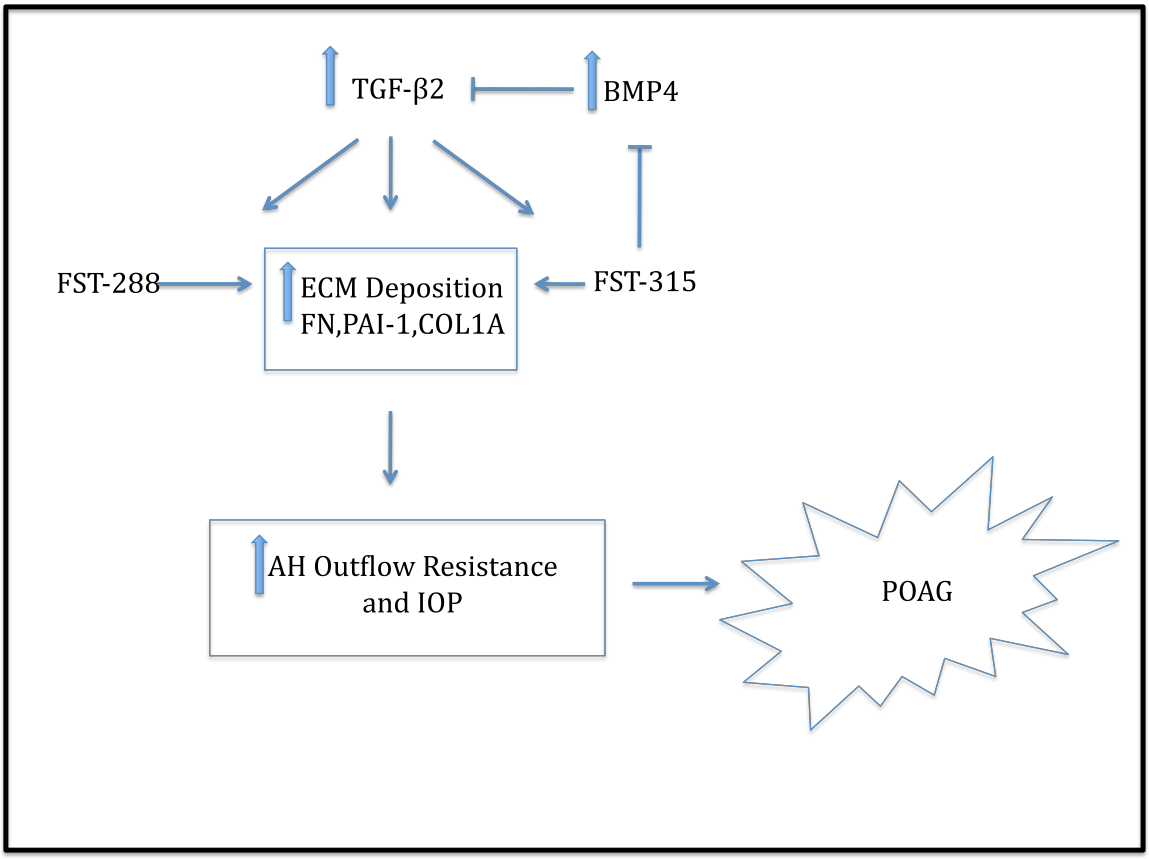

A.
24
48
C
T
C
T
25kDa
TGF-β2
47kDa
BMP-4
42kDa
β–actin
B.
TGF-β2
Relative Density
1.0
0.5
0.0
24-C
24-T
48-C
48-T
Time (hours)
C.
BMP-4
Relative Density
1.5
1.0
0.5
0.0
24-C
24-T
48-C
48-T
Time (hours)
D.
24
48
C
T
C
T
25kDa
TGF-β2
47kDa
BMP4

Figure 7


TGF-β2
BMP4
FST-288
ECM Deposition
FN,PAI-1,COL1A
FST-315
AH Outflow Resistance
and IOP
POAG

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Chapter V

Conclusions and Future Directions

Conclusions

The results of these studies highlight the importance in understanding the complex TGF-ß/BMP signaling pathways in the TM. For example, TGF-ß2 is capable of inducing factors such as FST that are important for regulation of the activity of TGF-ß2. BMP-4 inhibits TGF-ß2 induced ECM deposition in TM cells. Elevated TGF-ß2 induces gremlin expression, which results in BMP-4 attenuation of TGF-ß2 signaling in TM cells.

The first investigation sought to identify other proteins involved in a positive feed-forward loop in TGF-ß2 activation. Results demonstrated that FST mRNA was present in TM cells and expressed significantly more in GTM cells as compared to NTM cells. Follistatin protein was also expressed in NTM cells and tissues and significantly elevated in GTM cells and tissues. FST mRNA transcripts FST317/344 were also significantly elevated in GTM cells. Immunohistochemistry showed FST levels were significantly elevated in GTM tissues. Data implicated differences in FST isoform expression (FST-288 and FST-315) in NTM as compared to GTM tissues. FST-288 was expressed more in GTM tissues; conversely FST-315 was expressed more in NTM tissues. Exogenous TGF-ß2 significantly induced FST mRNA and protein expression in TM cells. Thus, FST a BMP antagonist was identified as possible regulator of TGF-ß/BMP activation.

The second study demonstrated that FST-288 and FST-315 affects ECM protein expression and secretion as well as BMP-4 inhibition of TGFβ-2 induced ECM in TM cells. Results from this

investigation support the hypothesis that FST plays a role in ECM deposition and BMP-4 inhibition that may contribute to glaucoma pathology. Exogenous FST elicited a bi-phasic effect on ECM protein and mRNA expression in TM cells. However the ability of FST isoforms to alter ECM protein expression did not coincide with one another. For example, FST-288 increase FN secretion at 24 and 48 hrs conversely, FST-315 induced FN secretion at 48 hrs. This may be due to the fact that FST-288 is localized to the cell surface and is the more active isoform. FST-315 acts as a reservoir, over time affecting the expression of molecules.

FST 315 inhibited BMP4 activity, while FST 288 enhanced BMP4 activity. These data highlight the possible differences in FST-288 and FST-315 function in TM cells. Also, FST-288 increased BMP-4 secretion at 24 and 48 hrs, with a slight increase in TGF-ß2 secretion at 48 hrs. FST changes to ECM, its effects on BMP-4 inhibition, and BMP-4 and TGF-ß2 appear to be independent of one another. These results further our knowledge of the potential role of BMP antagonists in the human TM and the possibility that multiple regulators of BMPs exist in the human TM.

Taken together, our results highlight the complex relationship of TGF-ß2 and BMPs in the human TM. Other factors may play an important role in regulating these pathways in both normal TM homeostatis, but more importantly in glaucoma pathology. The TM expresses several factors that are critical in maintaining normal homeostasis of ECM protein expression in the TM. These data suggest that the expression of FST in the TM may be important in this maintenance. Ultimately, identifying key factors that regulate ECM remodeling and BMP-4 inhibition will

provide a better understanding in the regulation of the TGF-ß/BMP signaling, and a novel therapeutic target in the prevention or treatment of POAG.

Future Directions

1. Further assess how FST regulates ECM deposition in TM cells.
    - Effects of FST on other ECM components such as elastin, LOXs, and TGM-2 in TM cells will be assessed by treating human primary TM cells with varying concentrations of FST-288 or FST-315. Any changes will be examined by QPCR or western blot analysis.

    - Next, we will assess canonical and non-canonical signaling pathways for TGF-ß2 and BMPs. We will determine if FST-288 and FST-315 regulate downstream TGF-ß2 signaling molecules Smad 2 and 3, and BMP signaling molecules Smad 1/5/8 in TM cells.

    - Since FST isoforms may not alter TGF-ß/BMP canonical signaling pathways, we will also examine non-canonical pathways such as ERK and p38MAPK in TM cells.

2. Determine if FST and gremlin can elicit a synergistic effect or compete for the binding and inhibition of BMP-4 in TM cells.

- FST and gremlin synergism will be tested by co-treating TM cells with fixed concentration of FST and gremlin for several time points with or without exogenous BMP-4 treatment. We will further assess the expression of FN via western blot analysis.

- FST and gremlin binding studies of BMP-4 will be performed with the use of ligand-binding assays. This study will assess the binding affinity of FST and gremlin to BMP-4.

3. *Ex-vivo* examination of FST effects on IOP elevation in the TM.
    - Using the bovine perfusion organ culture model we will assess FST-288 and FST-315 effects on IOP and ECM deposition in the TM. This will be determined by perfusion the bovine TM with human recombinant FST-288 or FST-315 and monitoring IOP. ECM changes will be assessed with perfused medium after the completion of experiment.