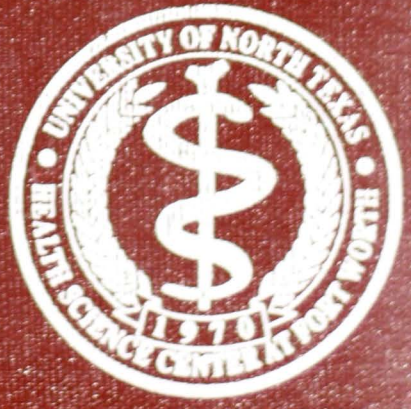
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ABSTRACT

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Primary open-angle glaucoma (POAG) is the second leading cause of irreversible blindness worldwide. Elevated intraocular pressure (IOP) is the major risk factor for POAG and is due to resistance of aqueous humor (AH) outflow through the trabecular meshwork (TM) and Schlemm's canal. Transforming growth factor-beta2 (TGF-β2) is elevated in the aqueous humor of glaucomatous eyes compared to normal eyes. Thus, TGF-β2 may play a role in regulating IOP. Tissue transglutaminase (TGM2) is a member of the transglutaminase family involved in cross-linking ECM proteins. In POAG, there are increased cross-linked extracelluar matrix proteins (ECM) in the TM and, therefore, may result in elevated AH outflow resistance and elevated IOP.

In this study, we examined the differences in both protein expression and enzyme activity of TGM2 between normal and glaucomatous TM cells and tissues. The findings demonstrated the presence of TGM2 in normal and glaucomatous cultured TM cells. We also showed that glaucomatous cultured TM cells and tissues have elevated levels of TGM2. Thus, this data suggest that TGM2 may have a pathogenic role in elevated outflow resistance and

elevated IOP. Second, we observed the induction of TGM2 by TGF-β1, β2, and β3 in cultured TM cells, suggesting TGF- β isoforms regulate TGM2 protein levels. Finally, we observed that R-Smads and P38 regulated TGM2 protein levels, suggesting TGF-β2 acts through both its canonical and non-canonical signaling pathway to regulate TGM2.

TISSUE TRANSGLUTAMINASE ROLE IN GLAUCOMA

DISSERTATION

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LIST OF ABBREVIATIONS

AH - Aqueous Humor

CTGF - Connective Tissue Growth Factor

ECM - Extracellular Matrix

GTM- Glaucomatous Trabecular Meshwork Cells

IOP - Intraocular pressure

ONH - Optic Nerve Head

MMPS - Matrix Metalloproteinases

NTM - Normal Trabecular Meshwork Cells

PAI - Plasminogen Activator Inhibitor

POAG - Primary Open Angle Glaucoma

RGC - Retinal Ganglion Cells

TGFβ – Transforming growth factor beta

TGM2 - Tissue Transglutaminase

TM - Trabecular Meshwork

CHAPTER I

INTRODUCTION TO THE STUDY

Glaucoma

Glaucoma is a group of optic neuropathies defined by damage to the optic nerve head (ONH), loss of retinal ganglion cells (RGC) via apoptosis and subsequent visual field defects. Nearly 70 million people are affected worldwide [1] and at least 3 million people have glaucoma in the United States (Glaucoma Research Foundation). Glaucoma can be categorized into several types including primary open-angle glaucoma, acute closed angle glaucoma, secondary glaucoma, pigmentary glaucoma, and congenital glaucoma [2]. Primary open-angle glaucoma (POAG) accounts for approximately 60% of all cases making it the most common form of glaucoma [3]. Multiple risk factors such as age, ethnicity, family history, myopia, central corneal thickness, long-term glucocorticoid treatment, and intraocular pressure (IOP) are associated with developing POAG [4, 5]. Distinctive features of glaucoma are loss of RGC cell bodies and their axons, notching and thinning of the neuroretinal rim, disc hemorrhages, and deep excavation at the region of the optic nerve head (ONH) due to the progressive bowing and compression of the lamina cribrosa [6,7,2,8]. Proposed mechanisms for optic nerve damage in POAG include (a) blockage of axonal transport resulting in a withdrawal of neurotrophic factors, (b) vascular insufficiency resulting in the loss of RGC due to ONH ischemia, (c) immune abnormality,

(d) glutamate toxicity, and (e) glia activation [4]. Ultimately, glaucomatous progression leads to irreversible peripheral vision loss and eventually blindness.

Human Trabecular Meshwork

The human trabecular meshwork (HTM) is a sieve-like structure and the major site of aqueous humor outflow. Aqueous humor is a translucent nutritive fluid that is responsible for nourishing the tissues found in the anterior chamber of the eye, including the iris, lens, and HTM. The HTM is comprised of four regions into which aqueous humor filters: (a) inner wall of Schlemm's canal, (b) juxtacanalicular tissue, (c) cornealscleral meshwork, and (d) uveal meshwork. Schlemm's canal, a circular channel, is located posterior to the HTM connecting to collector channels (episcleral and conjunctival veins) and exiting via the venous circulation [5]. Schlemm's canal is a vascular endothelial-lined channel containing both gap junctions and elliptical openings[5]. Juxtacanilicular tissue, also known as the cribriform meshwork, is found at the outermost region of the meshwork bordering the inner wall cells of Schlemm's canal [5]. Both the juxtacanilicular tissue and inner wall of Schlemm's canal are the main source of increase resistance to aqueous humor outflow [5]. The juxtacanilicular region of the HTM is composed of connective tissue containing both irregular oriented fibrils and arranged layers of elongated cells [5]. The inner layer of the juxtacanilicular tissue is comprised of the corneoscleral meshwork. Morphologically the corneoscleral meshwork consists of trabecular beams which are perforated by elliptical openings [5]. These elliptical openings diminish in size from 50 μm to 5 μm as it approaches the juxtacanalicular meshwork and Schlemm's canal [5]. Covering the trabecular beams are a single layer of trabecular meshwork cells residing on a basal lamina [5]. The fourth

region of the HTM is the uveal meshwork. It is the region contiguous to the aqueous humor in the anterior chamber. The uveal meshwork is composed of connective tissue and consists of irregular openings ranging in size from 25 μm to 75μm that extend from the iris root and ciliary body to the peripheral cornea [5].

Glaucoma, Exracellular Matrix, and Human Trabecular Meshwork (HTM)

As described previously, the HTM is the major site for aqueous humor outflow. Increased IOP is considered the primary risk factor for the development of glaucoma, and is generated by the outflow pathways of the trabecular meshwork[9]. In susceptible individuals, there is a decrease outflow of aqueous humor through the HTM that is accompanied by increased IOP. Decreased outflow of aqueous humor is due to increased deposition of ECM in the cribriform or juxtacanalicular region of the TM [10-12]. The juxtacanalicular tissue rather than the uveal or cornealscleral meshwork is where "plaque material" or thickened sheaths of elastic fibers accumulate [9]. The modulation of the ECM plays a vital role within the TM tissue. For example, the ECM acts as a supporting framework for cells and is thought to maintain the ECM normal outflow pathway [13]. The ECM components found in the TM include collagen, proteoglycans (e.g. decorin, biglycan, syndecan, versican, perlecan), adhesive glycoproteins (e.g. fibronectin, laminin, vitronectin), and elastin [13]. Normally, there is a delicate balance of deposition and degradation of the ECM in the human TM. However, in POAG, the factors that alter ECM metabolism may compromise the ECM to become more resistant to enzymatic degradation which would result in an alteration of ECM turnover and may tip the balance towards increased accumulation of ECM. Thus, the increased deposition of the ECM would decrease aqueous

humor outflow and cause increase IOP. The increase in pressure would then be displaced towards the back of the eye causing cupping at the optic nerve head (ONH) and damage to the optic nerve resulting in irreversible vision loss [14]. There are approximately 1 million retinal ganglion cell axons in the optic nerve that connect the retina with the visual system of the brain [15, 16]. The optic nerve is accountable for sending visual signals to the brain via RGC, eventually after a period of time, damage to the ONH results in vision loss [14].

Transforming Growth Factors Beta and Smad Dependent Signaling

Transforming growth factor (TGFβ) is a super-family consisting of many factors including TGFβ, bone morphogenetic proteins (BMP), inhibins, and activins which regulate many cellular functions. Originally TGFβ was named sarcoma growth factor, because of its ability to promote normal rat kidney fibroblasts to grow and form colonies [17, 18]. TGFβ isoforms (TGFβ 1-3) are structurally related and consist of a knot motif composed of six cysteine residues and intrachain disulfide bonds that stabilize β sheet bands. A free single cysteine forms one interchain disulfide bond with another monomeric subunit forming two 12.5 kDa TGFβ dimers [19]. These extracellular growth factors are synthesized as dimeric prepropeptide precursors consisting of the mature active TGFβ and the propeptide (TGFβ latent associated protein (LAP)) and this complex is associated by two disulfides bonds to the latent TGFβ binding protein (LTBP)[17]. LAP assists in the proper folding of TGFβ because without LAP, TGFβ leads to accumulation of intracellular disulfide bonded aggregates [17]. LTBP assist in the processing and secretion of TGFβ and without LTBP the TGFβ latent form is retained within the cis-Golgi network and secreted slowly [17]. LTBP and its association with LAP accelerates the

secretion of TGFβ and may target it for deposition into the ECM[17]. After TGFβ is processed and secreted as dimers, TGFβ will bind to and activate their cell surface receptors, type I and type II [20, 21]. In vertebrates there are seven type I receptors, activin-receptor like kinase (ALK 1-7) and five type II receptors [20, 21]. These receptors are commonly called TGFβ-RI and TGFβ-RII, which are serine/threonine kinases and upon ligand binding to TGFβ-RII, a constitutive active receptor, activates TGFβ-RI by its glycine-serine rich domain [20, 21]. This is followed by the activation of downstream mediators called receptor Smad proteins [Mad, mothers against dpp (decapentaplegic)) and C. *elegan Sma*] [20, 17, 21, 22]. The receptor Smad proteins have a conserved amino and carboxyl terminus referred to as the Mad homology (MH1 and MH2), respectively, and a linker region between MH1 and MH2 [23]. The MH2 domain contains a conserved SXS motif responsible for receptor regulated phosphorylation [23].

Smads are classified into three major classes: pathway-specific Smads (Smads 1, 2, 3, 5 and 8), pathway-shared Smads (Smad 4), and inhibitory Smads (Smads 6 and 7). The pathway-specific Smads mediate BMP and TGFβ signaling. For example, Smads 2 and 3 are involved in TGFβ signaling while Smads 1, 5, and 8 are involved in BMP signaling [22, 24]. Pathways that share Smads have a common end point termed co-Smad 4 [24]. Smads 6 and 7 inhibit pathway-specific Smads [24]. Inhibitory Smads may play a role in fine tuning TGFβ signaling through a negative feedback loop [24].

The cellular actions of TGFβ are negatively and positively controlled at every stage of its synthesis, activation, and signaling process [17]. Cellular functions for the TGFβ family include proliferation, differentiation, migration, and ECM synthesis and breakdown [17, 19]. TGFβ utilizes

its signaling mechanism to target genes including ECM genes via activation of Smad canonical or Smad independent pathways [19].

Smad Independent Signaling (Mitogen-Activated Protein Kinase (MAPK) Pathways)

In addition to TGFβ2 activating its canonical signaling pathway, it can also activate Smad independent signaling pathways including the Mitogen-activated protein kinases [25]. Mitogen-activated protein kinases (MAPK) belong to a family of serine/threonine kinases [26]. The MAPK family cascades consist of extracellular-regulating kinase (ERK1/2), the c-jun amino-terminal kinase (JNK 1-3), p38 α/β/δ/γ MAPK, and ERK5 and ERK7 [27, 28, 29]. However, ERK1/2, p38 and JNK 1-3 are the major MAPK pathways studied thus far. ERK1/2 respond to growth factors and hormones, while p38 and JNK 1-3 respond to conditions of cellular stress [30]. Multiple cellular functions are regulated by MAPK including cell proliferation, survival, motility, metabolism, transcription, and translation in order to maintain homeostasis. Thus, unregulated activities of the MAPK pathway are associated with pathological diseases and states ranging from inflammation, unregulated cell proliferation, and tissue remodeling [29, 31]. Signal transduction is initiated at the cell surface and propagates and amplifies the downstream signaling cascade via phosphorylation of the kinases to activate nuclear transcription factors [26]. Activation of the MAPK are dependent upon a five-tier kinase module. For example, the activation of the MAPK is initiated upon an extracellular signal followed by a signaling cascade of phosphorylation by first phosphorylation of MAPK kinase kinase (MKKK), MKKK phosporylates and activates MAPK kinase (MKK), and finally MKK phosporylates the threonine and tyrosine residues of the conserved T-X-Y motif and activates MAPK followed by

phosphorylation of nuclear kinases or transcription factors [25, 32]. Transcription factors acted upon include Elk-1, Ets-2, RSK, MNK, MSK, cPLA2, CHOP, ATF-2, CREB, and MEF2C [33, 25, 31].

Tissue Transglutaminase (TGM2)

The transglutaminase enzyme family consists of 8 members including keratinocyte transglutaminase (TGM1), tissue transglutaminase (TGM2), epidermal transglutaminase (TGM3), prostate transglutaminase (TGM4), TGMX (TGM5), TGM Y (TGM6), TGM Z (TGM7), and factor XIII-A subunit (fibrin-stabiling factor) [34, 35]. Members of the TGM family are calcium-dependent enzymes that catalyze the posttranslational modification of ECM proteins by cross-linking proteins, and making them more resistant to enzymatic degradation [36]. The enzymatic reaction occurs by forming bonds between ε-(γ-glutamyl)lysine or (γ-gluamyl)polyamine [36]. These enzymes also involved in incorporating amines into protein, site-specific deamidation, isopeptidase activity, and promoting cell-matrix interactions [37]. Tissue transglutaminase (TGM2) acts as a classical G protein in its GTP-bound form found in rat liver plasma membranes [38]. Of the TGM family, TGM2 is the most ubiquitous and found in the human peripheral and central nervous system[39]. TGM2 has been reported to be involved in numerous roles including apoptosis [40], cell adhesion [41], and cell matrix interactions resulting in crosslinking of ECM proteins including fibronectin [42]. It has been reported that TGM2 expression is mostly detected in the cytoplasm of cells [43], but also in the plasma membrane [38] and nucleus [44]. There are several reports that indicate increase TGM2 protein levels and activity in several diseases including Alzheimer's, and Huntington's disease, and glaucoma [10, 34, 45, 46].

Transforming Growth Factor Beta/ Tissue Transglutaminase/Trabecular Meshwork

Previous literature suggests that TGF-β2 is involved in the pathogenesis of glaucoma [47]. For example, glaucomatous eyes have increased levels of TGF-β2 in the aqueous humor [48-52]. TGF-β2 can activate several pathways within the TM resulting in increased ECM deposition and irreversible cross-linking by TGM2. This cause and effect relationship would promote increased resistance of aqueous humor outflow. In addition to the initial findings of increased levels of TGF-β2 in the aqueous humor of glaucomatous TM, TGF-β2 increases ECM synthesis in vitro[10] and in vivo within the perfused anterior organ culture model [53]. Welge-Lüssen and coworkers (2000), reported that HTM express and synthesize TGM2 [10]. In addition they also reported that exogenous TGFβ1 and TGFβ2 increased expression of fibronectin and TGM2 [10]. Welge-Lüssen and coworkers (2000) speculate TGF-β1 and TGF-β2 is responsible for the increase of TGM2 resulting in an irreversible increase of cross-linking of ECM proteins, preventing drainage of aqueous humor and eventually leading to increased IOP [10].

Recent studies indicate that TGF-β2 is antagonized by members of the TGF-β family, bone morphogenetic proteins (BMP), in particular BMP4 [54, 55] and BMP7 [56]. However, antagonists proteins such as Gremlins, Smurfs, Bambi, and inhibitory SMADs can contribute to the delicate pathway system by blocking specific sites of either the TGF-β and BMP signaling pathway at the cell membrane or downstream [54]. This is of interest because in mink lung epithelial cell lines (Mv1Lu) and mouse proosteoblastic cells (MC3T3) cell lines, TGF-β, BMP2, and BMP4 regulated the TGM2 promoter through a TGF-β response element [57]. However, this is a cell type specific response because in Mv1Lu cells TGF-β increased the activity of the TGM2 promoter, however, in MC3T3 cells it was the inverse reaction. In Mv1Lu

cells TGF-β increased the activity of the TGM2 promoter in a dose dependent manner, however, when Mv1Lu cells were treated with exogenous BMP2 and BMP4, the TGM2 promoter was inhibited [57]. When both BMP4 and TGF-β were added simultaneously TGFβ could not stimulate TGM2 activity of the promoter [57].

Hypothesis

Glaucoma is the second leading cause of blindness worldwide. A major risk factor in glaucoma is increased intraocular pressure (IOP) due to increased resistance to aqueous humor outflow. The outflow resistance is caused by an increased deposition of extracellular matrix in the trabecular meshwork (TM), preventing drainage of aqueous humor and resulting in increased IOP. At the present time the molecular mechanism responsible for increased IOP is not completely understood. However, increased post-translational modifications of matrix-associated proteins might be responsible in the abnormal function of the TM in glaucoma. TGM2 is a ubiquitous enzyme responsible for the cross-linking of proteins, thus resisting chemical or physical degradation. Preliminary studies in our laboratory, and in others, showing increased expression of TGM2 in glaucomatous human TM suggests a role for TGM2 in the development of ECM changes in glaucoma. Thus, **the goal of this study is to determine the role of TGM2 in the development of glaucoma. The present study will investigate TGM2 differences between normal and glaucomatous cells, the effects of TGF-β2 on TGM2 in the HTM, and identify the molecular pathways utilized for TGM2.**

The canonical pathway for TGF-β signaling is the Smad pathway; however, TGF-β can also directly activate the MAPK signaling pathways involving p38 MAPK, C-JUN, ERK1/2, as

well as via crosstalk mechanisms between TGF-β and MAPK signaling components. To better understand the molecular mechanisms for TGM2 in glaucoma, the following **hypothesis is proposed: TGF-β2 induces increased expression of tissue transglutaminase in trabecular meshwork cells through the Smad and/or the MAPK signaling pathways resulting in extracellular matrix changes in the trabecular meshwork.**

Significance:

The proposed study will elucidate whether TGM2 expression is mediated by Smad phosphorylation or the MAPK pathway in the human glaucomatous trabecular meshwork, an important tissue associated with the pathogenesis of glaucoma. Previous data has shown that TGF-β2 is increased in the aqueous humor of glaucomatous patients. TGF-β2 promotes increased levels of ECM proteins thereby, inhibiting aqueous humor outflow, leading to elevated intraocular pressure in glaucomatous patients. In addition, it has been reported that TGF-β1 and TGF-β2 treatment increases expression of fibronectin and TGM2. Therefore, examining TGF-β and TGM2 will lead to a greater understanding of the signaling regulation. And ultimately TGM2, Smads, or MAPK signaling proteins may be the targets of pharmacological manipulation.

References

1. Quigley HA. Number of people with glaucoma worldwide. *Br.J.Ophthalmol.* 1996;80:389-393.

2. Quigley HA. Neuronal death in glaucoma. *Prog.Retin.Eye Res.* 1999;18:39-57.

3. Liesegang TJ. Glaucoma: Changing concepts and future directions. *Mayo Clin.Proc.* 1996;71:689-694.

4. Pang IH, Clark AF. Rodent models for glaucoma retinopathy and optic neuropathy. *J.Glaucoma* 2007;16:483-505.

5. Wordinger RJ, Clark AF. Effects of glucocorticoids on the trabecular meshwork: Towards a better understanding of glaucoma. *Prog.Retin.Eye Res.* 1999;18:629-667.

6. Quigley HA, Hohman RM, Addicks EM, Massof RW, Green WR. Morphologic changes in the lamina cribrosa correlated with neural loss in open-angle glaucoma. *Am.J.Ophthalmol.* 1983;95:673-691.

7. Varela HJ, Hernandez MR. Astrocyte responses in human optic nerve head with primary open-angle glaucoma. *J.Glaucoma* 1997;6:303-313.

8. Coleman AL. Glaucoma. *Lancet* 1999;354:1803-1810.

9. Tamm ER, Fuchshofer R. What increases outflow resistance in primary open-angle glaucoma? *Surv.Ophthalmol.* 2007;52 Suppl 2:S101-4.

10. Welge-Lussen U, May CA, Lutjen-Drecoll E. Induction of tissue transglutaminase in the trabecular meshwork by TGF-beta1 and TGF-beta2. *Invest.Ophthalmol.Vis.Sci.* 2000;41:2229-2238.

11. Rohen JW, Witmer R. Electrn microscopic studies on the trabecular meshwork in glaucoma simplex. *Albrecht Von Graefes Arch.Klin.Exp.Ophthalmol.* 1972;183:251-266.

12. Lutjen-Drecoll E. Functional morphology of the trabecular meshwork in primate eyes. *Prog.Retin.Eye Res.* 1999;18:91-119.

13. Yue BY. The extracellular matrix and its modulation in the trabecular meshwork. *Surv.Ophthalmol.* 1996;40:379-390.

14. To CH, Kong CW, Chan CY, Shahidullah M, Do CW. The mechanism of aqueous humour formation. *Clin.Exp.Optom.* 2002;85:335-349.

15. Kaushik S, Pandav SS, Ram J. Neuroprotection in glaucoma. *J.Postgrad.Med.* 2003;49:90-95.

16. Osborne NN, Ugarte M, Chao M, et al. Neuroprotection in relation to retinal ischemia and relevance to glaucoma. *Surv.Ophthalmol.* 1999;43 Suppl 1:S102-28.

17. Zhu HJ, Burgess AW. Regulation of transforming growth factor-beta signaling. *Mol.Cell Biol.Res.Commun.* 2001;4:321-330.

18. de Larco JE, Todaro GJ. Growth factors from murine sarcoma virus-transformed cells. *Proc.Natl.Acad.Sci.U.S.A.* 1978;75:4001-4005.

19. Verrecchia F, Mauviel A. Transforming growth factor-beta signaling through the smad pathway: Role in extracellular matrix gene expression and regulation. *J.Invest.Dermatol.* 2002;118:211-215.

20. Massague J. TGF-beta signal transduction. *Annu.Rev.Biochem.* 1998;67:753-791.

21. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr.Rev.* 2002;23:787-823.

22. Schmierer B, Hill CS. TGFbeta-SMAD signal transduction: Molecular specificity and functional flexibility. *Nat.Rev.Mol.Cell Biol.* 2007;8:970-982.

23. Ross S, Hill CS. How the smads regulate transcription. *Int.J.Biochem.Cell Biol.* 2008;40:383-408.

24. Massague J. How cells read TGF-beta signals. *Nat.Rev.Mol.Cell Biol.* 2000;1:169-178.

25. Javelaud D, Mauviel A. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and smad signaling downstream of TGF-beta: Implications for carcinogenesis. *Oncogene* 2005;24:5742-5750.

26. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;298:1911-1912.

27. Zhou G, Bao ZQ, Dixon JE. Components of a new human protein kinase signal transduction pathway. *J.Biol.Chem.* 1995;270:12665-12669.

28. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol.Rev.* 2001;81:807-869.

29. Cuevas BD, Abell AN, Johnson GL. Role of mitogen-activated protein kinase kinase kinases in signal integration. *Oncogene* 2007;26:3159-3171.

30. Owens DM, Keyse SM. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene* 2007;26:3203-3213.

31. Roberts PJ, Der CJ. Targeting the raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* 2007;26:3291-3310.

32. Lowes VL, Ip NY, Wong YH. Integration of signals from receptor tyrosine kinases and g protein-coupled receptors. *Neurosignals* 2002;11:5-19.

33. Hazzalin CA, Mahadevan LC. MAPK-regulated transcription: A continuously variable gene switch? *Nat.Rev.Mol.Cell Biol.* 2002;3:30-40.

34. Lesort M, Tucholski J, Miller ML, Johnson GV. Tissue transglutaminase: A possible role in neurodegenerative diseases. *Prog.Neurobiol.* 2000;61:439-463.

35. Aeschlimann D, Koeller MK, Allen-Hoffmann BL, Mosher DF. Isolation of a cDNA encoding a novel member of the transglutaminase gene family from human keratinocytes. detection and identification of transglutaminase gene products based on reverse transcription-polymerase chain reaction with degenerate primers. *J.Biol.Chem.* 1998;273:3452-3460.

36. Folk JE, Finlayson JS. The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases. *Adv.Protein Chem.* 1977;31:1-133.

37. Fesus L, Piacentini M. Transglutaminase 2: An enigmatic enzyme with diverse functions. *Trends Biochem.Sci.* 2002;27:534-539.

38. Nakaoka H, Perez DM, Baek KJ, et al. Gh: A GTP-binding protein with transglutaminase activity and receptor signaling function. *Science* 1994;264:1593-1596.

39. Tucholski J, Johnson GV. Tissue transglutaminase directly regulates adenylyl cyclase resulting in enhanced cAMP-response element-binding protein (CREB) activation. *J.Biol.Chem.* 2003;278:26838-26843.

40. Fesus L, Davies PJ, Piacentini M. Apoptosis: Molecular mechanisms in programmed cell death. *Eur.J.Cell Biol.* 1991;56:170-177.

41. Cai D, Ben T, De Luca LM. Retinoids induce tissue transglutaminase in NIH-3T3 cells. *Biochem.Biophys.Res.Commun.* 1991;175:1119-1124.

42. Greenberg CS, Birckbichler PJ, Rice RH. Transglutaminases: Multifunctional cross-linking enzymes that stabilize tissues. *FASEB J.* 1991;5:3071-3077.

43. Fesus L, Szondy Z, Uray I. Probing the molecular program of apoptosis by cancer chemopreventive agents. *J.Cell.Biochem.Suppl.* 1995;22:151-161.

44. Piredda L, Farrace MG, Lo Bello M, et al. Identification of 'tissue' transglutaminase binding proteins in neural cells committed to apoptosis. *FASEB J.* 1999;13:355-364.

45. Lesort M, Chun W, Johnson GV, Ferrante RJ. Tissue transglutaminase is increased in huntington's disease brain. *J.Neurochem.* 1999;73:2018-2027.

46. Tovar-Vidales T, Roque R, Clark AF, Wordinger RJ. Tissue transglutaminase expression and activity in normal and glaucomatous human trabecular meshwork cells and tissues. *Invest.Ophthalmol.Vis.Sci.* 2008;49:622-628.

47. Lutjen-Drecoll E. Morphological changes in glaucomatous eyes and the role of TGFbeta2 for the pathogenesis of the disease. *Exp.Eye Res.* 2005;81:1-4.

48. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp.Eye Res.* 1994;59:723-727.

49. Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch.Clin.Exp.Ophthalmol.* 2001;239:109-113.

50. Picht G, Welge-Luessen U, Grehn F, Lutjen-Drecoll E. Transforming growth factor beta 2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. *Graefes Arch.Clin.Exp.Ophthalmol.* 2001;239:199-207.

51. Ochiai Y, Ochiai H. Higher concentration of transforming growth factor-beta in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn.J.Ophthalmol.* 2002;46:249-253.

52. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. *Int.Ophthalmol.* 2004;25:19-22.

53. Gottanka J, Chan D, Eichhorn M, Lutjen-Drecoll E, Ethier CR. Effects of TGF-beta2 in perfused human eyes. *Invest.Ophthalmol.Vis.Sci.* 2004;45:153-158.

54. Wordinger RJ, Agarwal R, Talati M, Fuller J, Lambert W, Clark AF. Expression of bone morphogenetic proteins (BMP), BMP receptors, and BMP associated proteins in human trabecular meshwork and optic nerve head cells and tissues. *Mol.Vis.* 2002;8:241-250.

55. Wordinger RJ, Fleenor DL, Hellberg PE, et al. Effects of TGF-beta2, BMP-4, and gremlin in the trabecular meshwork: Implications for glaucoma. *Invest.Ophthalmol.Vis.Sci.* 2007;48:1191-1200.

56. Fuchshofer R, Yu AH, Welge-Lussen U, Tamm ER. Bone morphogenetic protein-7 is an antagonist of transforming growth factor-beta2 in human trabecular meshwork cells. *Invest.Ophthalmol.Vis.Sci.* 2007;48:715-726.

57. Ritter SJ, Davies PJ. Identification of a transforming growth factor-beta1/bone morphogenetic protein 4 (TGF-beta1/BMP4) response element within the mouse tissue transglutaminase gene promoter. *J.Biol.Chem.* 1998;273:12798-12806.

CHAPTER II

TISSUE TRANSGLUTAMINASE EXPRESSION AND ACTIVITY IN NORMAL AND GLAUCOMATOUS HUMAN TRABECULAR MESHWORK CELLS AND TISSUES

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Purpose. Glaucoma is a leading cause of irreversible visual impairment and blindness in the world. A major risk factor for glaucoma is elevated intraocular pressure due to increased resistance of aqueous humor outflow through the trabecular meshwork (TM). In the glaucomatous TM there is increased accumulation of extracellular matrix (ECM) material due to a disruption of the normal balance between ECM deposition and degradation. Tissue transglutaminase (TGM2) belongs to a family of calcium-dependent enzymes that catalyze the post-translational modification of the ECM by cross-linking proteins, thus making these proteins resistant to enzymatic and physical degradation. It is possible that the increase in ECM proteins seen in the glaucomatous TM is due to increased cross-linking activity of TGM2. The purpose of this study was to determine if there are differences in expression and activity of TGM2 between normal and glaucoma TM cells and tissues.

Methods. Normal (N=3 NTM) and glaucomatous (N=3 GTM) human TM cell lines were grown until confluent. Western immunoblot analysis of cell lysates was used to compare TGM2 protein levels in NTM and GTM cells. TGM2 enzyme activity between NTM and GTM cells was studied using a biotin cadaverine assay. In addition, immunohistochemistry of three normal and three glaucomatous TM tissues was utilized to evaluate the in vivo expression of TGM2, fibronectin (FN) and ε-(γ-glutamyl) lysine (GGEL) proteins.

Results. Western blot analysis and immunohistochemistry demonstrated the presence of TGM2 protein in both NTM and GTM cells. There was an increase in TGM2 protein in GTM cells compared to NTM cells, and GTM cells also had increased in TGM2 enzyme activity compared to NTM cells. Immunohistochemical results demonstrated increased expression of TGM2 and FN in GTM tissues. FN and GGEL proteins were co-localized in GTM tissues, indicating significant cross-linking of FN by TGM2.

Conclusions. This study demonstrated that both NTM and GTM cells express TGM2. In addition, TGM2 protein levels and enzyme activities were elevated in GTM cells. There was also an increase in co-localization of FN and GGEL protein in GTM tissues. These results indicate that TGM2 may play an important role in the pathogenesis of glaucomatous by cross-linking ECM proteins such as FN and thus making the ECM more resistant to degradation.

INTRODUCTION

A major cause of irreversible blindness in the world is primary open-angle glaucoma (POAG). Elevated intraocular pressure (IOP) is an important risk factor in the development of POAG [1]as well as progression of glaucomatous damage [2, 3]. Elevated IOP is due to increased aqueous humor (AH) outflow resistance [4]and appears to be associated with a number of morphological and biochemical changes in the trabecular meshwork (TM). In the normal human TM, a balance exists between extracellular matrix protein (ECM) deposition and degradation. However in POAG, there is increased accumulation of cross-linked ECM proteins in the TM that is believed to result in increased AH outflow resistance and elevated IOP [5, 6].

Several studies have focused on regulators of the ECM in the TM including matrix metalloproteinases (MMPs), plasminogen activators, and growth factors. Reports have indicated that MMPs assist in maintaining AH outflow and this effect can be blocked by synthetic MMP inhibitors or by activating tissue inhibitors of matrix metalloproteinases (TIMPs) [7-9]. Plasminogen activators (PA) are serine proteases involved in degrading fibrin and the ECM, however, the effects of PAs can be blocked by plasmin activator inhibitor-1 (PAI-1) [10, 11].

The transglutaminase (TGM) enzyme family (EC 2.3.2.13) contains several members that are widely distributed in various tissues. Transglutaminase enzymes are encoded by a group of genes that are structurally and functionally related [12]. Family members include keratinocyte transglutaminase (TGM1), tissue transglutaminase (TGM2), epidermal transglutaminase

(TGM3), prostate transglutaminase (TGM4), TGM X (TGM5), TGM Y (TGM 6), TGM Z (TGM 7) and factor XIII-A subunit (fibrin-stabilizing factor) [13, 14]. Although identified at the gene level, TGM5, TGM6 and TGM7 have unknown functions [12]. Transglutaminase enzymes are known to catalyze the post-translational modification of proteins via formation of isopeptide bonds [15] . The enzymatic reaction occurs by forming bonds between ε-(γ-glutamyl) lysine or (γ-glutamyl) polyamine [16]. The resultant cross-linked proteins are highly resistant to both physical and enzymatic degradation [16].

Tissue transglutaminase (TGM2) is a unique transglutaminase family member. Within the TGM family, TGM2 is the most ubiquitous enzyme and is found in numerous tissues including the human peripheral and central nervous system [17]. It has been reported that TGM2 expression is mostly detected in the cytoplasm of cells [18], but it is also found in the plasma membrane [19] and the nucleus [20]. In addition, TGM2 can be secreted into the ECM. The membrane bound form of TGM2 binds GTP and may function as a G protein [19]. This enzyme incorporates amines into proteins, regulates site-specific deamination and isopeptidase activity, and promotes cell-matrix interactions [21]. TGM2 is involved in apoptosis [22], cell adhesion [23], and cell matrix interactions resulting in crosslinking of ECM proteins including fibronectin [24]. TGM2 can be upregulated and/or activated in a variety of diseases associated with enhanced accumulation of cross-linked proteins in the ECM, and therefore may also be involved in the pathophysiology of glaucoma.

Welge-Luβen and colleagues reported increased mRNA expression and protein levels of TGM2 in cultured human TM cells after exogenous treatment with either transforming growth factor beta-1 (TGF-β1) or transforming growth factor beta -2 (TGF-β2). In addition to TGM2 expression, they showed that TGM2 enzymatic activity was also stimulated by TGF-β1 and TGF-β2 in normal cultured TM cells [25] . TGF-β2 treatment of cultured optic nerve head (ONH) astrocytes also upregulated TGM2 expression [26]. Interestingly, Fuchshofer and colleagues also demonstrated TGF-β2 induced TGM2 expression was mediated through the action of connective tissue growth factor.

These above reports are very insightful because a number of studies have previously demonstrated that glaucoma patients have elevated TGF-β2 levels in both the AH [27-29] and the ONH [30, 31]. This has led to the concept that elevated TGF-β2 levels may increase cross-linked proteins in the ECM of the glaucomatous TM and ONH. However, we are not aware of any studies that have compared TGM2 expression or enzyme activity in normal and glaucomatous TM cells and/or tissues. The purpose of this study was to expand our knowledge of TGM2 in the pathophysiology of POAG by examining differences in both protein expression and enzyme activity of TGM2 between normal and glaucoma TM cells and tissues.

METHODS

Trabecular Meshwork Dissection and Cell Culture

Human TM cells were isolated from carefully dissected human TM tissue explants and characterized as previously described [32-34]. Isolated TM cells were grown in Dulbecco's Modified Eagle's Medium (low glucose) supplemented with 10% fetal bovine serum (FBS) (both from HyClone Labs, Logan, UT), L-glutamine (0.292 mg/ml), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and amphotericin B (4 μg/ml). Antibiotics were purchased from Gibco BRL, Grand Island, NY. Medium was exchanged every 2-3 days and near-confluent cultures were trypsinized and seeded in 25 cm^2 cell culture flasks (Corning Inc, Corning, NY) and maintained at 37°C in 5% CO_2-95% air. A total of three normal (aged 2 (HTM 2), 54 (HTM10A) and 72 (NTM115-01) yrs.) and three glaucomatous (aged 60 (GTM75A, 72 (GTM60A) and 87 (GTM152-99) yrs.) human TM cell lines were used in the experiments.

Protein Extraction and Western Blot Analysis of Tissue Transglutaminase (TGM2) Protein Levels in Normal and Glaucomatous Trabecular Meshwork Cell Lines

Total cellular protein was isolated from cultured cells using M-PER extraction buffer (#78501, Pierce Biotech, Rockford, IL.) and Protease Inhibitor Cocktail (#78415, Pierce Biotech) or a lysis buffer containing 10 mM Tris-HCl, 0.5% NP40, 150 mM NaCl, 0.5% sodium

deoxycholate, 0.1% SDS, 0.2 mM PMSF in ethanol, 1 μg/ml aprotinin, 4 μg/ml pepstatin, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate (10 μl/ml) [35]. Protein concentration was determined by using the Bio-Rad D_c Protein Assay System as described by the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA). A standard curve was generated using bovine serum albumin (BSA) and absorbance at 750nm was read within 15 minutes.

A total of 20 ug of protein was loaded per well and separated on a SDS-PAGE denaturing polyacrylamide gel 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 approximately 60 minutes in order to block non-specific binding. Blots were processed using a mouse anti-TGM2 monoclonal primary antibody (#MS-300-P1, Neomarkers, Fremont, CA) and the secondary consisted of goat anti-mouse antibody (#SC 2005, Santa Cruz Biotechnololgy, Santa Cruz, CA). The Super Signal West Dura Extended Duration Substrate (#34075, Pierce Biotech) was used for detection and blots were exposed to a Fluorchem 8900 imager (Alpha Innotech, San Leandro, CA).

Tissue Transglutaminase Enzyme Activity

Cell Enzyme Activity Assay: TGM2 enzyme activity was measured using a microscopic assay as previously described [38, 39]. Briefly, TGM2 activity was assessed by exposing cells to 1mM biotin cadaverine, a pseudo-substrate of TGM2, for 48 hours. Cells were fixed for 30

minutes in 3.7% volume per volume basis (v/v) formaldehyde in PBS and permeabilized with 0.2% (v/v) Triton X-100 in PBS for 30 minutes at room temperature. Cells were further incubated with an Alexa Fluor 488 streptavidin-conjugate (1:1000, v/v) in PBS for 1 hour at room temperature, washed, and mounted. The cells were observed with an Olympus Model BX51 trinocular research microscope (Leeds Instruments, Irving TX). TGM 2 activity was seen as increased fluorescence labeling.

Western Blot Analysis: Details of the TGM2 activity assay have been published previously [36] with slight modifications. N-(5-aminopentyl)biotinamide, trifluoroacetic acid salt (biotin cadaverine, 1mM) (Molecular Probes, Eugene, Oregon), a pseudo-substrate for TGM2, was added to TM cells in serum free medium for 48 hours. Cell lysate and conditioned medium were collected, separated by SDS-PAGE, and transferred to PVDF membranes. TGM2 mediated incorporation of biotin cadaverine into proteins was detected by streptavidin-HRP using Super signal West Dura Extended Substrate. Following the detection of proteins, the blots were then stripped and re-probed with anti-fibronectin.

Immunolocalization of Tissue Transglutaminase (TGM2), Fibronectin (FN) and ε- (γ-glutamyl) lysine in Normal and Glaucomatous Trabecular Meshwork Tissues

Details of the immunocytochemistry of TGM2 in TM tissues have been published previously [37]. In order to document the presence of TGM2, FN, and cross-linked ε-(γ-glutamyl) lysine (GGEL)

isopeptide in the TM, three sets of normal and glaucomatous age-matched human eyes were used (e.g. normal donors of 72, 88 and 94 yrs; glaucomatous donors of 76, 87, and 92 yrs.). Briefly, eyes were obtained from regional eye banks within 6 hours of death and fixed in 10% formalin. Tissues were dehydrated and embedded in paraffin and stored until further use. TM tissues were deparaffinized in xylene, then treated with 0.02M glycine for 15 minutes and dehydrated twice with 100%, 95%, 70%, 50% ethanol for 5 minutes each. After boiling TM sections in 10mM citrate buffer, pH 6.0, the TM tissues were incubated with normal serum and 3% BSA in PBS for 30 minutes. TM sections were washed and processed with primary antibodies for TGM2 (#MS-300-P1, Neomarkers), FN (# F-3648, Sigma Aldrich, St. Louis, MO), and GGEL (#ab424, Abcam, Cambridge MA). The primary antibodies were detected with appropriate secondary antibodies (Alexa Fluor 488, 564, and/or 633; Molecular Probes) for 45 minutes. The co-localization of FN and GGEL protein indicates transamidation of FN by TGM2 in TM tissues. The visualization of cell nuclei was performed by staining tissue sections with DAPI (300 μm) for 10 minutes. Controls consisted of omission of primary antibodies. Images were captured using a Zeiss 410 confocal imaging system (Carl Zeiss, Thornwood, N.Y.)

Statistics

The average values for NTM and GTM are stated as means +/- standard deviation (SD). Statistical analysis was performed using Microsoft Excel software, and the data was analyzed by t-test (two-sample assuming unequal variances). All differences were considered statistically significant at $p<0.005$ between NTM and GTM.

RESULTS

Tissue Transglutaminase (TGM2) Protein Levels in Normal and Glaucomatous Trabecular Meshwork Cells

We examined TGM2 protein expression in the lysates of 3 normal and 3 glaucomatous TM cell lines. TGM2 was expressed in all six cell lines as an 77 kDa protein band on the western blots (Figure 1A). The 3 GTM cell lines appeared to have increased TGM2 protein levels compared to NTM cell lines. All western blots were re-probed for β-actin (Figure 1), which served as a loading control. Relative TGM2 intensity levels were measured via densitometry (Figure 1B). These data indicate that both NTM and GTM cell lines contain TGM2 protein, and that the protein levels of TGM2 were significantly elevated ($p<0.005$) in the GTM cell lines.

Immunohistochemical Localization of Tissue Transglutaminase (TGM2) Protein in Normal and Glaucomatous Human TM Tissue

We next examined the protein levels of TGM2 in three normal and three glaucomatous TM tissues from human donors. TGM2 was present in all six human TM samples. Figure 2 is a representative example of one set of age-matched eyes. In agreement with TM cell lysate western blot data, we found that TGM2 appeared elevated in the TM of glaucomatous donor eyes

(Figure 2B) compared to age-matched controls (Figure 2A), and this was seen in all 3 sets of glaucomatous donor eyes

Tissue Transglutaminase (TGM2) Enzyme Activity in Normal and Glaucomatous Trabecular Meshwork Cells

We next examined TGM2 enzyme activity in both normal and glaucomatous cultured TM cells. To analyze TGM2 activity, a biotin labeled cadaverine / streptavidin immunohistochemical staining assay was performed in NTM and GTM cells. Cells were labeled with biotin cadaverine for 48 hours prior to fixation and staining, and TGM2 enzyme activity was detected by the Alexa Fluor 488 streptavidin-conjugate. GTM cells contained higher TGM2 activity compared to NTM cells (Figure 3). Control experiments included incubation of both cell types in the DMSO carrier in the absence of biotin cadaverine.

TGM2 activity was also assessed by western immunoblot analysis in three independent experiments comparing normal and glaucomatous cell lines by supplementing serum free medium with biotin cadaverine (1mM), a pseudo-substrate for TGM2. Samples were extracted, processed together, separated by SDS-PAGE, and, transferred to a PVDF membrane. TGM2 activity was detected using streptavidin-HRP. TGM2 activity was seen in the cell lysates (A) and in the culture medium (B) of both NTM and GTM cells incubated with biotin cadaverine, resulting in the appearance of 220 kDa bands on western blots (Figure 4). The blots were then

stripped and re-probed with an anti-fibronectin antibody that detected an identical 220kDa band, suggesting the cross-linked product was fibronectin.

Immunohistochemical Staining of Fibronectin and ε(γ-glutamly) Lysine Isopeptide in Normal and Glaucomatous Trabecular Meshwork Tissue

To verify TGM2 activity in TM tissues, we examined the expression of fibronectin along with the cross-linked GGEL isopeptide in eyes from three normal and three glaucomatous human donors. Fibronectin and GGEL protein were co-localized in both NTM and GTM tissues (Figure 5), suggesting the transamidation of FN by TGM2 in these tissues. In two of the three GTM tissues, fibronectin was clearly expressed at higher levels compared to NTM. Interestingly, co-localization of FN and GGEL isopeptide was increased surrounding the canal of Schlemn (Figure 5).

DISCUSSION

Elevated IOP is a primary risk factor for the development and progression of glaucoma [1-3]. Glaucomatous elevated IOP is caused by increased aqueous humor outflow resistance [4] and is closely associated with morphological and biochemical changes in the TM. There is an increased accumulation of ECM proteins in the glaucomatous TM [4, 40]. The increased deposition of ECM proteins in the glaucomatous TM may be due to increased ECM synthesis and/or decreased degradation. ECM metabolism within the human TM is regulated by matrix metalloproteinases [7, 41], plasmin activator inhibitor-1 [11, 42], and growth factors[37, 40, 43]. Human TM cells express numerous growth factor receptors and respond to exogenous growth factors [32]. Growth factors within the aqueous humor also appear to be involved in regulating ECM metabolism in the TM and aqueous outflow pathway.

Numerous studies have reported elevated levels of TGF-β2 in the AH of glaucomatous patients [27-29]. Members of the TGFβ superfamily including TGF-β2 regulate ECM synthesis and deposition. Welge-Luβen and colleagues have reported that TGM2 mRNA expression and protein levels of TGM2 are increased in TGF-β1 or TGF-β2 treated cultured human TM cells. In addition to TGM2 expression, TGM2 enzymatic activity was also increased in TGF-β treated TM cells [25]. TGM2 can also increase the conversion of latent TGF-β to its biologically active form [44-46], providing a potential feedback mechanism in the glaucomatous TM and leading to further TGM2 induction.

Transglutaminase enzymes catalyze the post-translational modification of proteins via formation of isopeptide bonds [15]. The resultant cross-linked proteins are highly resistant to both physical and enzymatic degradation [16]. Of the various members of the transglutaminase family of enzymes, TGM2 has been implicated in numerous fibrotic disorders such as pulmonary fibrosis, renal fibrosis, and atherosclerosis [47-55]. Although TGM2 can be induced by TGF-β1 and TGF-β2 in TM cells [25], little is known about the role for TGM2 in glaucoma pathogenesis. Therefore, the purpose of this study was to determine whether there are any differences in TGM2 protein levels and activity between NTM and GTM cells and tissues.

Western immunoblot and immunohistochemical analyses showed that TGM2 is present in both NTM and GTM cells and tissues. Our results support the previous study by Welge-Luβen and colleagues who first reported the presence of TGM2 in cultured TM cells [25]. More importantly, we demonstrated significantly increased protein levels of TGM2 in cultured TM cells and TM tissues obtained from glaucomatous patients. We believe that this is the first report that TGM2 is upregulated in glaucomatous TM cells and tissues.

However, the presence of TGM2 protein in cells or tissues does not necessarily mean that the enzyme is biologically active. Therefore, the enzymatic activity of TGM2 was measured by the incorporation of biotin-cadaverine, a pseudo-substrate for TGM2, into cells. We looked for the presence of GGEL bonds in the TM, since TGM2 inserts these bonds between proteins that it cross-links, such as FN. This action makes ECM proteins more resistant to proteolytic degradation. Since FN can serve as a substrate in the TGM2 catalyzed reactions, we looked at

co-localization of both FN and the GGEL proteins in normal and glaucomatous cultured TM cells and tissues. Our immunohistochemical results indicated increased co-localization of FN and GGEL proteins associated with Schlemn's canal in glaucomatous human tissues. These results suggest that TGM2 may render the ECM resistant to proteolytic degradation at the site of aqueous humor outflow.

In conclusion, our results confirm the presence of TGM2 in normal cultured human TM cells. In addition we demonstrated that cultured glaucomatous TM cells and glaucomatous TM tissues from human donors have elevated levels of TGM2. We also showed that TGM2 enzymatic activity is elevated in glaucomatous cells and tissues. Lastly, there is co-localization of GGEL proteins with FN in the area of Schlemn's canal. This area of the human TM is known to be involved in AH outflow resistance. Proteins cross-linked by TGM2 exhibit resistance to proteolytic degradation and thus may restrict AH outflow from the TM. The normal function of the TM is dependent on a balance between ECM deposition and degradation. Increased cross-linked proteins in the TM would result in the ECM being more resistant to enzymatic degradation and turnover. Taken together, these findings suggest that TGM2 protein and enzyme activity are elevated in the glaucomatous TM and may play a pathogenic role in increased outflow resistance and glaucomatous ocular hypertension.

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Figure Legends

Figure 1. Chemiluminiscent Detection of Tissue Tranglutaminase Protein in Normal and Glaucomatous Human Trabecular Meshwork Cells.

(A) Total protein was collected from 3 normal (N) and 3 glaucomatous (G) cell lines and electrophoresed in SDS-PAGE gels followed by western immunoblotting for tissue transglutaminase (77 kDa). All cell lines express tissue transglutaminase proteins. Protein levels of TGM2 were higher in GTM compared to NTM cell lines. β-actin was used as an internal loading control. (B) Densitometric readings of TGM2 normalized to β-actin for 3 NTM and 3 GTM cell lines. * Statistical difference at the $p<0.005$ level (+/- SD).

Figure 2. Immunofluorescent staining of Tissue Transglutaminase in Normal and Glaucomatous Trabecular Meshwork tissues.

Six different human eyes, 3 NTM (72, 88, 94 yrs.) and 3 GTM (76, 87, 92 yrs.), were fixed, sectioned and stained with antibodies for TGM2. Negative controls consisted of PBS-BSA without primary antibody, normal IgG, and mouse ascites. TGM2 expression appeared to be higher in GTM tissues compared to NTM tissues: NTM (A) and GTM (B); TGM2 at 400x (A-B), (C) representative of a no primary antibody control, (D) a representative of an ascites control. TGM2 was localized to the cytoplasm, in agreement with previous reports. DAPI staining was used for nuclear staining.

Abbreviations: AC- anterior chamber, TM- trabecular meshwork, SC- Schlemn's Canal

Figure 3. Transglutaminase activity in NTM and GTM cells. The cells were incubated with vehicle (DMSO) control or biotin-labeled cadaverine (1mM). Transamidated and cross-linked cadaverine was detected by Alexa Fluor 488 streptavidin-conjugate (green).

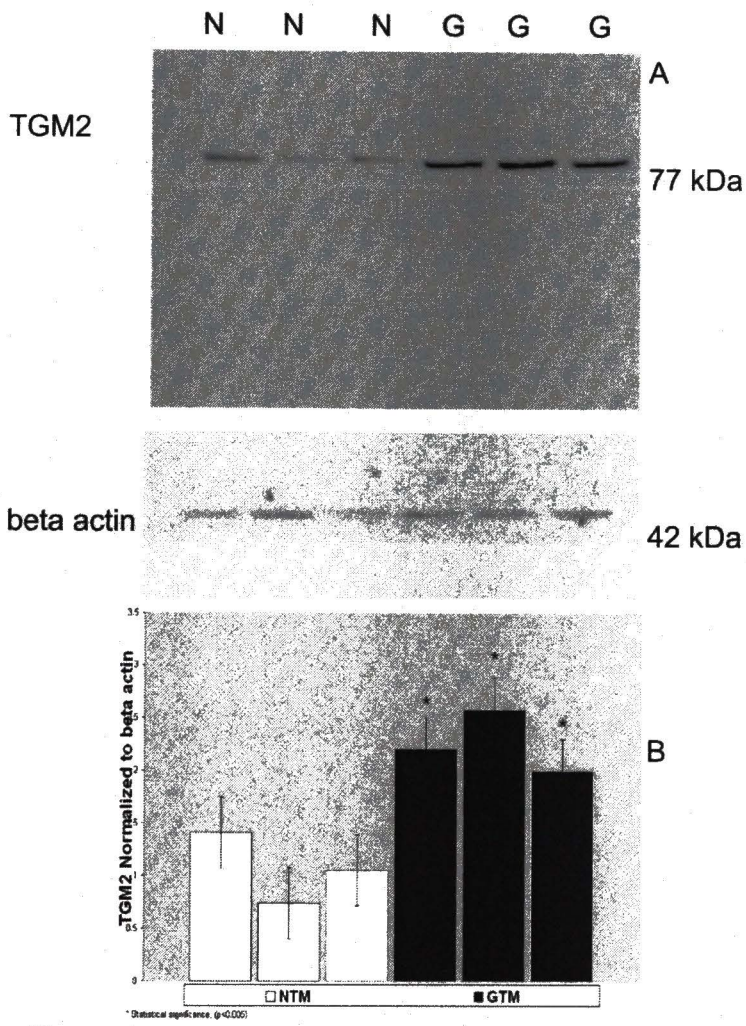
Figure 4. Western immunoblot of TGM2 activity in NTM and GTM cells.

TGM2 enzyme activity was present in cell lysate (A) and culture medium (B) of NTM and GTM cells. The cells were incubated with vehicle (DMSO) control or biotin-labeled cadaverine (1mM). TGM2 mediated incorporation of biotin cadaverine into cells was detected by western immunoblotting using streptavidin-HRP conjugated secondary antibody. The blots were then stripped and re-probed with an anti-fibronectin antibody, corresponding to the 220 kDa band.

Figure 5. Immunoflourescent staining to γ-glutamyl-ε-lysine (GGEL) and fibronectin (FN) in normal and glaucomatous TM tissues.

Six different human eyes, 3 NTM (72, 88, 94 yrs.) and 3 GTM (76, 87, 92 yrs.), were fixed, sectioned and stained with antibodies for GGEL and FN. Negative controls consisted of PBS-BSA without primary antibody, IgG, and mouse ascites (data not shown). GTM tissues had increased expression of GGEL, increased FN expression, and co-localization of GGEL and FN: (A) NTM at 100x, (B) GTM at 100x, (C) NTM at 400x, (D) GTM at 400x. Both GGEL and FN were localized to the cytoplasm in TM tissues.

Abbreviations: AC- anterior chamber, TM- trabecular meshwork, SC- Schlemn's Canal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A
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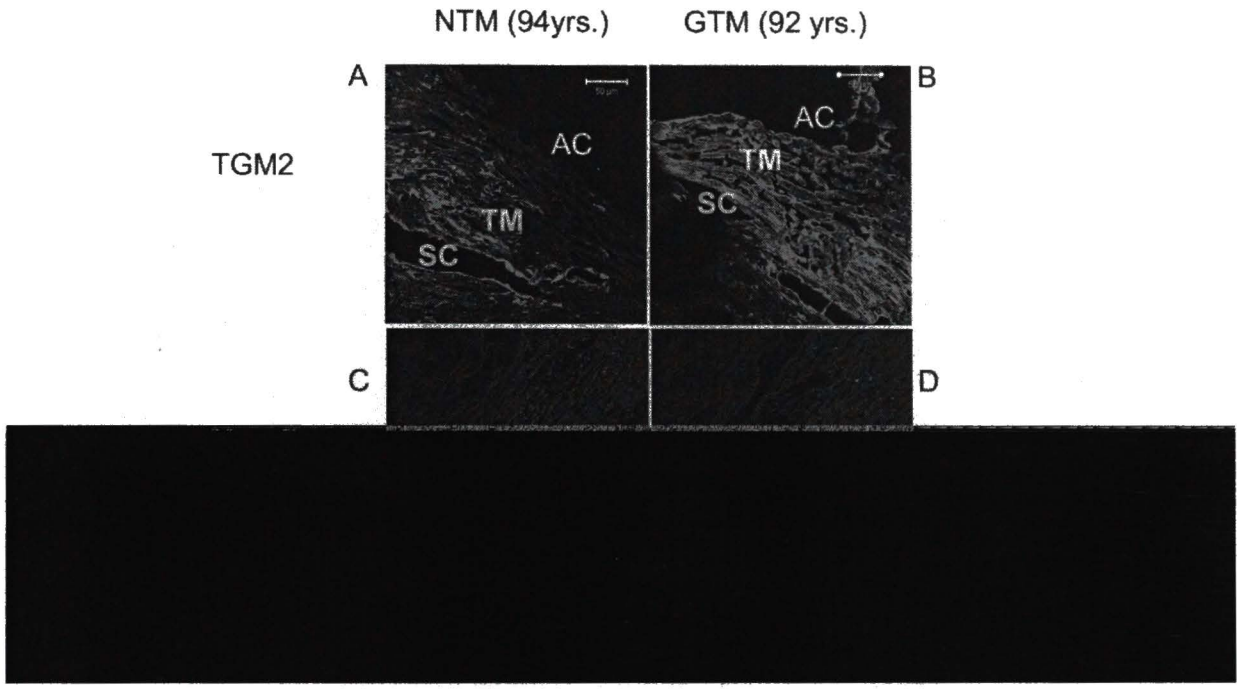
Figure 1.


NTM (94yrs.)
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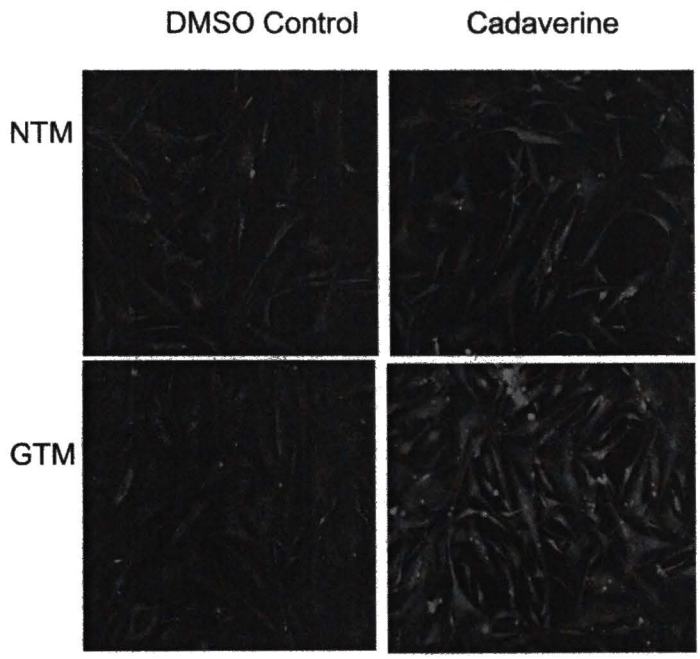
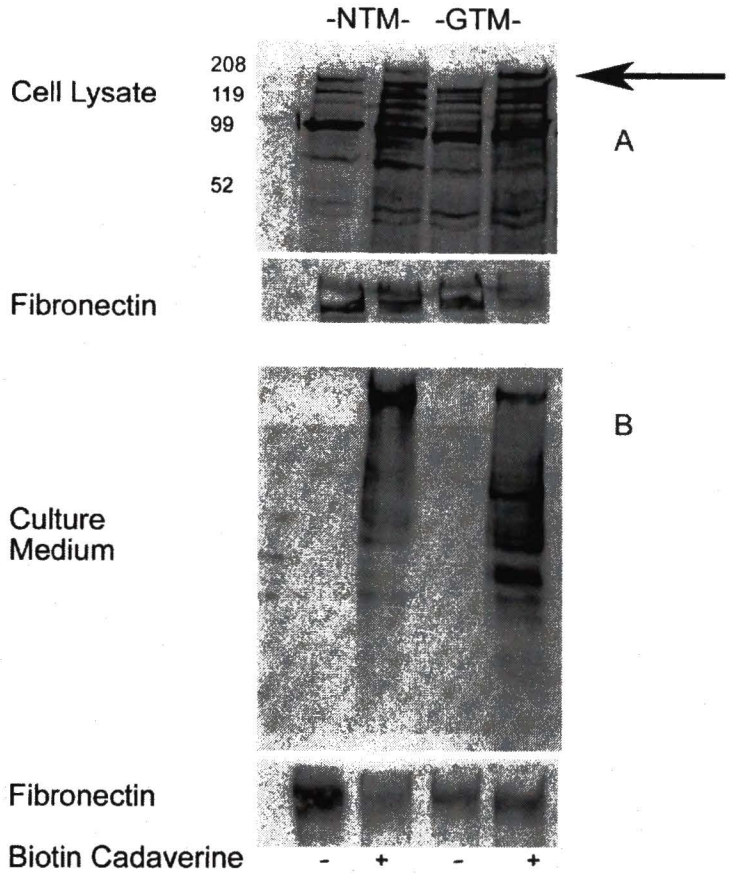


Figure 3.


-NTM- -GTM-
Cell Lysate
208
119
99
52
A
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Fibronectin
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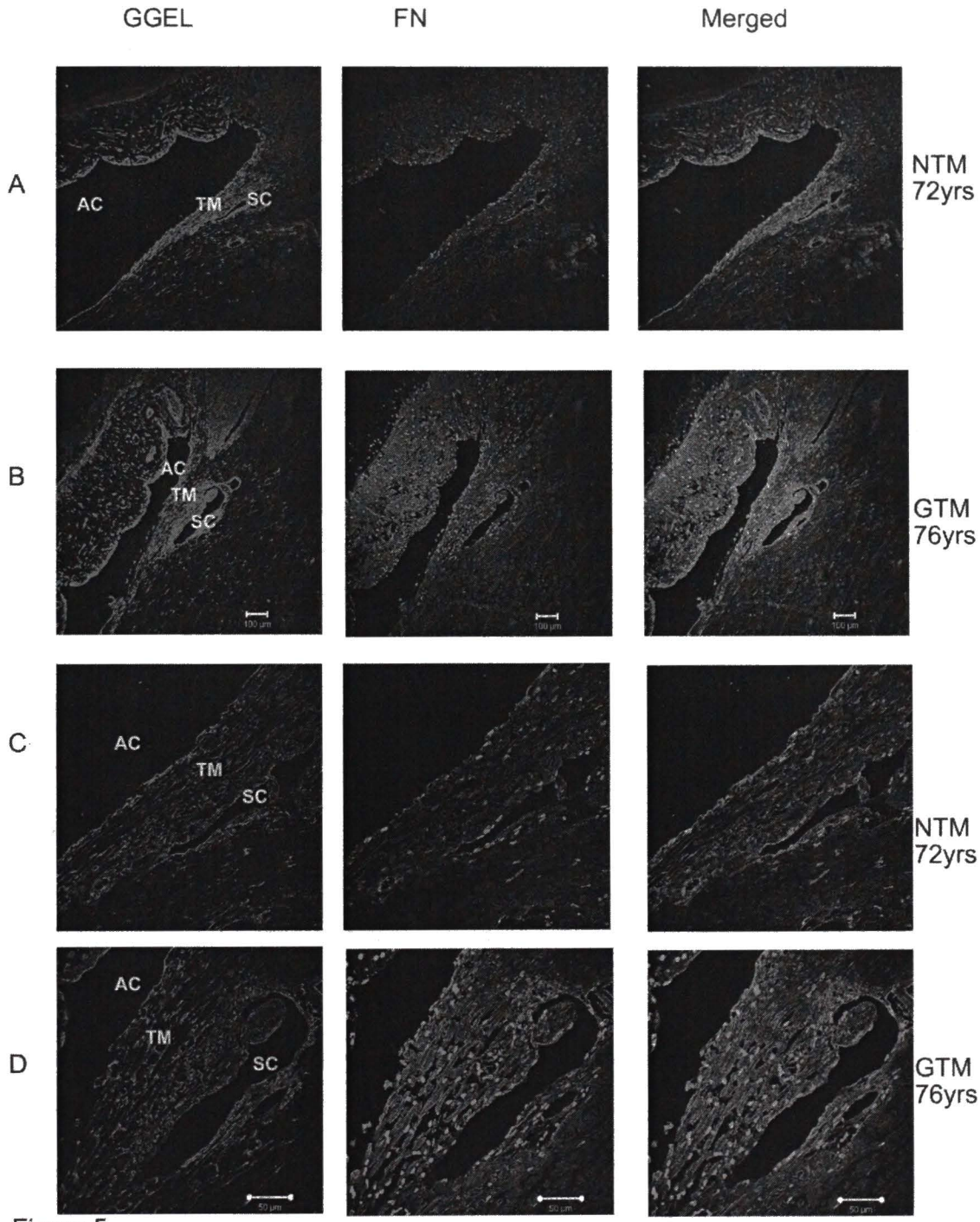
Figure 4.


GGEL
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TM
SC
NTM
72yrs
D
AC
TM
SC
GTM
76yrs

Figure 5.

References

1. Gordon MO, Beiser JA, Brandt JD, et al. The ocular hypertension treatment study: Baseline factors that predict the onset of primary open-angle glaucoma. Arch.Ophthalmol. 2002; 120:714-20; discussion 829-30.

2. Kass MA, Heuer DK, Higginbotham EJ, et al. The ocular hypertension treatment study: A randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma. Arch.Ophthalmol. 2002; 120:701-13; discussion 829-30.

3. AGIS Investigators. The advanced glaucoma intervention study (AGIS): 7. The relationship between control of intraocular pressure and visual field deterioration. The AGIS investigators. Am.J.Ophthalmol. 2000; 130:429-440.

4. Rohen JW. Why is intraocular pressure elevated in chronic simple glaucoma? anatomical considerations. Ophthalmology 1983; 90:758-765.

5. Rohen JW, Witmer R. Electron microscopic studies on the trabecular meshwork in glaucoma simplex. Albrecht Von Graefes Arch.Klin.Exp.Ophthalmol. 1972; 183:251-266.

6. Lutjen-Drecoll E. Functional morphology of the trabecular meshwork in primate eyes. Prog. Retin. Eye Res. 1999; 18:91-119.

7. Bradley JM, Vranka J, Colvis CM, et al. Effect of matrix metalloproteinases activity on outflow in perfused human organ culture. Invest.Ophthalmol.Vis.Sci. 1998; 39:2649-2658.

8. Pang IH, Hellberg PE, Fleenor DL, Jacobson N, Clark AF. Expression of matrix metalloproteinases and their inhibitors in human trabecular meshwork cells. Invest.Ophthalmol.Vis.Sci. 2003; 44:3485-3493.

9. Fleenor DL, Pang IH, Clark AF. Involvement of AP-1 in interleukin-1alpha-stimulated MMP-3 expression in human trabecular meshwork cells. Invest.Ophthalmol.Vis.Sci. 2003; 44:3494-3501.

10. Eitzman DT, Ginsburg D. Of mice and men. the function of plasminogen activator inhibitors (PAIs) in vivo. Adv.Exp.Med.Biol. 1997; 425:131-141.

11. Fuchshofer R, Welge-Lussen U, Lutjen-Drecoll E. The effect of TGF-beta2 on human trabecular meshwork extracellular proteolytic system. Exp.Eye Res. 2003; 77:757-765.

12. Esposito C, Caputo I. Mammalian transglutaminases. identification of substrates as a key to physiological function and physiopathological relevance. FEBS J. 2005; 272:615-631.

13. Lesort M, Tucholski J, Miller ML, Johnson GV. Tissue transglutaminase: A possible role in neurodegenerative diseases. Prog.Neurobiol. 2000; 61:439-463.

14. Aeschlimann D, Koeller MK, Allen-Hoffmann BL, Mosher DF. Isolation of a cDNA encoding a novel member of the transglutaminase gene family from human keratinocytes. detection and identification of transglutaminase gene products based on reverse transcription-polymerase chain reaction with degenerate primers. J.Biol.Chem. 1998; 273:3452-3460.

15. Beninati S, Piacentini M. The transglutaminase family: An overview: Minireview article. Amino Acids 2004; 26:367-372.

16. Folk JE, Finlayson JS. The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases. Adv.Protein Chem. 1977; 31:1-133.

17. Tucholski J, Johnson GV. Tissue transglutaminase directly regulates adenylyl cyclase resulting in enhanced cAMP-response element-binding protein (CREB) activation. J.Biol.Chem. 2003; 278:26838-26843.

18. Fesus L, Szondy Z, Uray I. Probing the molecular program of apoptosis by cancer chemopreventive agents. J.Cell.Biochem.Suppl. 1995; 22:151-161.

19. Nakaoka H, Perez DM, Baek KJ, et al. Gh: A GTP-binding protein with transglutaminase activity and receptor signaling function. Science 1994; 264:1593-1596.

20. Piredda L, Farrace MG, Lo Bello M, et al. Identification of 'tissue' transglutaminase binding proteins in neural cells committed to apoptosis. FASEB J. 1999; 13:355-364.

21. Fesus L, Piacentini M. Transglutaminase 2: An enigmatic enzyme with diverse functions. Trends Biochem.Sci. 2002; 27:534-539.

22. Fesus L, Davies PJ, Piacentini M. Apoptosis: Molecular mechanisms in programmed cell death. Eur.J.Cell Biol. 1991; 56:170-177.

23. Cai D, Ben T, De Luca LM. Retinoids induce tissue transglutaminase in NIH-3T3 cells. Biochem.Biophys.Res.Commun. 1991; 175:1119-1124.

24. Greenberg CS, Birckbichler PJ, Rice RH. Transglutaminases: Multifunctional cross-linking enzymes that stabilize tissues. FASEB J. 1991; 5:3071-3077.

25. Welge-Lussen U, May CA, Lutjen-Drecoll E. Induction of tissue transglutaminase in the trabecular meshwork by TGF-beta1 and TGF-beta2. Invest.Ophthalmol.Vis.Sci. 2000; 41:2229-2238.

26. Fuchshofer R, Birke M, Welge-Lussen U, Kook D, Lutjen-Drecoll E. Transforming growth factor-beta 2 modulated extracellular matrix component expression in cultured human optic nerve head astrocytes. Invest.Ophthalmol.Vis.Sci. 2005;46:568-578.

27. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. Exp.Eye Res. 1994; 59:723-727.

28. Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. Graefes Arch.Clin.Exp.Ophthalmol. 2001; 239:109-113.

29. Picht G, Welge-Luessen U, Grehn F, Lutjen-Drecoll E. Transforming growth factor beta 2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. Graefes Arch.Clin.Exp.Ophthalmol. 2001; 239:199-207.

30. Pena JD, Taylor AW, Ricard CS, Vidal I, Hernandez MR. Transforming growth factor beta isoforms in human optic nerve heads. Br.J.Ophthalmol. 1999; 83:209-218.

31. Hernandez MR. The optic nerve head in glaucoma: Role of astrocytes in tissue remodeling. Prog.Retin.Eye Res. 2000; 19:297-321.

32. Wordinger RJ, Clark AF, Agarwal R, et al. Cultured human trabecular meshwork cells express functional growth factor receptors. Invest.Ophthalmol.Vis.Sci. 1998; 39:1575-1589.

33. Wordinger RJ, Lambert W, Agarwal R, Talati M, Clark AF. Human trabecular meshwork cells secrete neurotrophins and express neurotrophin receptors (trk). Invest.Ophthalmol.Vis.Sci. 2000; 41:3833-3841.

34. Clark AF, Steely HT, Dickerson JE,Jr, et al. Glucocorticoid induction of the glaucoma gene MYOC in human and monkey trabecular meshwork cells and tissues. Invest.Ophthalmol.Vis.Sci. 2001; 42:1769-1780.

35. Lambert W, Agarwal R, Howe W, Clark AF, Wordinger RJ. Neurotrophin and neurotrophin receptor expression by cells of the human lamina cribrosa. Invest.Ophthalmol.Vis.Sci. 2001; 42:2315-2323.

36. Zhang J, Lesort M, Guttmann RP, Johnson GV. Modulation of the in situ activity of tissue transglutaminase by calcium and GTP. J.Biol.Chem. 1998; 273:2288-2295.

37. Kim SW, Lee ZW, Lee C, Im KS, Ha KS. The role of tissue transglutaminase in the germinal vesicle breakdown of mouse oocytes. Biochem.Biophys.Res.Commun. 2001; 286:229-234.

38. Beck KE, De Girolamo LA, Griffin M, Billett EE. The role of tissue transglutaminase in 1-methyl-4-phenylpyridinium (MPP+)-induced toxicity in differentiated human SH-SY5Y neuroblastoma cells. Neurosci.Lett. 2006; 405:46-51.

39. Wordinger RJ, Fleenor DL, Hellberg PE, et al. Effects of TGF-beta2, BMP-4, and gremlin in the trabecular meshwork: Implications for glaucoma. Invest.Ophthalmol.Vis.Sci. 2007; 48:1191-1200.

40. Lutjen-Drecoll E. Morphological changes in glaucomatous eyes and the role of TGFbeta2 for the pathogenesis of the disease. Exp.Eye Res. 2005; 81:1-4.

41. Pang IH, Fleenor DL, Hellberg PE, Stropki K, McCartney MD, Clark AF. Aqueous outflow-enhancing effect of tert-butylhydroquinone: Involvement of AP-1 activation and MMP-3 expression. Invest.Ophthalmol.Vis.Sci. 2003; 44:3502-3510.

42. Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGFbeta2-induced changes in human trabecular meshwork: Implications for intraocular pressure. Invest.Ophthalmol.Vis.Sci. 2006; 47:226-234.

43. Bradley JM, Anderssohn AM, Colvis CM, et al. Mediation of laser trabeculoplasty-induced matrix metalloproteinase expression by IL-1beta and TNFalpha. Invest.Ophthalmol.Vis.Sci. 2000; 41:422-430.

44. Kojima S, Nara K, Rifkin DB. Requirement for transglutaminase in the activation of latent transforming growth factor-beta in bovine endothelial cells. J.Cell Biol. 1993; 121:439-448.

45. Kojima S, Vernooy R, Moscatelli D, Amanuma H, Rifkin DB. Lipopolysaccharide inhibits activation of latent transforming growth factor-beta in bovine endothelial cells. J.Cell.Physiol. 1995; 163:210-219.

46. Nunes I, Gleizes PE, Metz CN, Rifkin DB. Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. J.Cell Biol. 1997; 136:1151-1163.

47. Verderio EA, Johnson T, Griffin M. Tissue transglutaminase in normal and abnormal wound healing: Review article. Amino Acids 2004; 26:387-404.

48. Boisvert WA, Rose DM, Boullier A, et al. Leukocyte transglutaminase 2 expression limits atherosclerotic lesion size. Arterioscler.Thromb.Vasc.Biol. 2006; 26:563-569.

49. Ikee R, Kobayashi S, Hemmi N, et al. Involvement of transglutaminase-2 in pathological changes in renal disease. Nephron Clin.Pract. 2007; 105:c139-46.

50. Johnson TS, Griffin M, Thomas GL, et al. The role of transglutaminase in the rat subtotal nephrectomy model of renal fibrosis. J.Clin.Invest. 1997; 99:2950-2960.

51. Griffin M, Smith LL, Wynne J. Changes in transglutaminase activity in an experimental model of pulmonary fibrosis induced by paraquat. Br.J.Exp.Pathol. 1979; 60:653-661.

52. Wiebe RI, Tarr AH, Bowness JM. Increased transglutaminase in the aortas of cholesterol-fed rabbits: Occurrence of buffer soluble and insoluble forms and an inhibitor. Biochem.Cell Biol. 1991; 69:821-827.

53. Bowness JM, Tarr AH. Lipoprotein binding of crosslinked type III collagen aminopropeptide and fractions of its antigen in blood. Biochem.Biophys.Res.Commun. 1990; 170:519-525.

54. Bowness JM, Tarr AH, Wiebe RI. Transglutaminase-catalysed cross-linking: A potential mechanism for the interaction of fibrinogen,low density lipoprotein and arterial type III procollagen. Thromb.Res. 1989; 54:357-367.

55. Bowness JM, Venditti M, Tarr AH, Taylor JR. Increase in epsilon(gamma-glutamyl)lysine crosslinks in atherosclerotic aortas. Atherosclerosis 1994; 111:247-253.

CHAPTER III

INTRODUCTION TO CHAPTER IV

The previous chapter focused on differences in TGM2 protein levels between normal and glaucomatous trabecular meshwork (TM) cells. This study demonstrated increased TGM2 protein expression and activity in both glaucomatous TM cells and tissues. Tissue transglutaminase is a multifunctional protein which can catalyze several processes including (a) deamidation of protein substrates, (b) post-translational modification of ECM proteins by the formation of isopeptide bonds between the γ-carboxyl group of a glutamine and the ε-amino group of a lysine (c) and the association with integrins on the cell membrane which would ultimately stabilize the ECM and prevent proteolytic degradation [1-3]. The insertion of isopeptide bonds between the γ-carboxyl group of a glutamine and the ε-amino group of a lysine results in either intra or intermolecular cross-linked proteins [1]. These cross-linked proteins are stable, rigid, and insoluble and as a result resistant to proteolysis [1, 2]. This is of importance since in the glaucomatous eye there is increased deposition of ECM proteins termed "plaque material". ECM deposition is thought to reduce aqueous humor outflow and induce ocular hypertension [4]. The increased plaque material may be due to the cross-linking functions of TGM2 that would prevent

degradation of the ECM proteins. Many investigators have focused on the regulation of ECM protein turn-over via matrix metalloproteinases, plasminogen activators, and growth factors. The previous study focused on the quantitative and qualitative changes of TGM2 in normal and glaucomatous TM cells and tissues.

Next, the molecular mechanism utilized by TGF-β2 to regulate TGM2 was studied. To accomplish this study it was necessary to examine both the canonical and non-canonical signaling pathways (e.g. Smads and MAPK signaling pathways, respectively). TGF-β is a multifunctional protein regulating many cellular processes including proliferation, differentiation, migration, and ECM production and breakdown [5]. In 50% of glaucomatous eyes there are elevated amounts of transforming growth factor β2 (TGF-β2) in the aqueous humor [6-10]. TGF-β2 induces ECM proteins in TM cells such as plasminogen activator inhibitor (PAI), αβ crystalline, myocilin, thrombospondin-1, fibronectin and TGM2 [11-15]. TGF-β is known to regulate genes via the downstream Smad signaling pathway. In addition recent reports indicate that TGF-β can regulate genes via the MAPK signaling pathway or cross-talk mechanisms may occur between TGF-β2 and the MAPK pathways [16, 17]. Therefore. the purpose of the next study was to explore the signaling pathway and possible mediators for TGF-β2 induction of TGM2 via both the Smad and MAPK signaling pathways.

References

1. Wilhelmus MM, van Dam AM, Drukarch B. Tissue transglutaminase: A novel pharmacological target in preventing toxic protein aggregation in neurodegenerative diseases. *Eur.J.Pharmacol.* 2008;585:464-472.

2. Folk JE, Finlayson JS. The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases. *Adv.Protein Chem.* 1977;31:1-133.

3. Lorand L, Graham RM. Transglutaminases: Crosslinking enzymes with pleiotropic functions. *Nat.Rev.Mol.Cell Biol.* 2003;4:140-156.

4. Rohen JW, Witmer R. Electrn microscopic studies on the trabecular meshwork in glaucoma simplex. *Albrecht Von Graefes Arch.Klin.Exp.Ophthalmol.* 1972;183:251-266.

5. Zhu HJ, Burgess AW. Regulation of transforming growth factor-beta signaling. *Mol.Cell Biol.Res.Commun.* 2001;4:321-330.

6. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp.Eye Res.* 1994;59:723-727.

7. Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch.Clin.Exp.Ophthalmol.* 2001;239:109-113.

8. Picht G, Welge-Luessen U, Grehn F, Lutjen-Drecoll E. Transforming growth factor beta 2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. *Graefes Arch.Clin.Exp.Ophthalmol.* 2001;239:199-207.

9. Ochiai Y, Ochiai H. Higher concentration of transforming growth factor-beta in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn.J.Ophthalmol.* 2002;46:249-253.

10. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. *Int.Ophthalmol.* 2004;25:19-22.

11. Fuchshofer R, Welge-Lussen U, Lutjen-Drecoll E. The effect of TGF-beta2 on human trabecular meshwork extracellular proteolytic system. *Exp.Eye Res.* 2003;77:757-765.

12. Welge-Lussen U, May CA, Eichhorn M, Bloemendal H, Lutjen-Drecoll E. AlphaB-crystallin in the trabecular meshwork is inducible by transforming growth factor-beta. *Invest.Ophthalmol.Vis.Sci.* 1999;40:2235-2241.

13. Tamm ER, Russell P, Epstein DL, Johnson DH, Piatigorsky J. Modulation of myocilin/TIGR expression in human trabecular meshwork. *Invest.Ophthalmol.Vis.Sci.* 1999;40:2577-2582.

14. Flugel-Koch C, Ohlmann A, Fuchshofer R, Welge-Lussen U, Tamm ER. Thrombospondin-1 in the trabecular meshwork: Localization in normal and glaucomatous eyes, and induction by TGF-beta1 and dexamethasone in vitro. *Exp.Eye Res.* 2004;79:649-663.

15. Welge-Lussen U, May CA, Lutjen-Drecoll E. Induction of tissue transglutaminase in the trabecular meshwork by TGF-beta1 and TGF-beta2. *Invest.Ophthalmol.Vis.Sci.* 2000;41:2229-2238.

16. Javelaud D, Mauviel A. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and smad signaling downstream of TGF-beta: Implications for carcinogenesis. *Oncogene* 2005;24:5742-5750.

17. Javelaud D, Mauviel A. Mammalian transforming growth factor-betas: Smad signaling and physio-pathological roles. *Int.J.Biochem.Cell Biol.* 2004;36:1161-1165.

CHAPTER IV

TRANSFORMING GROWTH FACTOR-BETA2 REGULATION OF TISSUE TRANSGLUTAMINASE EXPRESSION IN HUMAN TRABECULAR MESHWORK CELLS

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Purpose: Transforming growth factor beta 2 (TGF-β2) has been reported to be elevated in the aqueous humor of glaucomatous patients and to be a causative factor for increased intraocular pressure. Exogenous TGF-β1 and TGF-β2 increase tissue transglutaminase (TGM2) protein levels and enzyme activity in trabecular (TM) cells. TGM2 is a calcium-dependent enzyme that mediates increased cross-linking of proteins, thus making them resistant to enzymatic and physical degradation. To date, it is unknown how TGF-β2 induces TGM2 expression and activity. The signaling pathway by which TGF-β2 stimulates TGM2 production in TM cells was investigated and as well as if CTGF was a mediator of TGF-β2 induction of TGM2 in TM cells. **Methods:** Human NTM cells (N=3) and GTM cells (N=3) were grown until approximately 80% confluent and treated 48 hours with TGF-β2 (5ng/ml) in serum-free medium. Untreated cells acted as controls. Western blot analysis was performed to analyze proteins in various signaling pathways including R-Smad3, R-Smad2, p38, and ERK. Short interfering (si)RNAs were used to suppress the p38, R-Smad3, R-Smad2, Mek1/2 , ERK1/2, and CTGF genes. In addition, immunohistochemistry of 3 NTM and 3 GTM tissues were utilized to evaluate the expression and co-localization of TGF-β2 with TGM2. **Results:** Western blot analysis demonstrated the presence of R-Smad3, p38, and ERK1/2 in human TM cells and their activation by TGF-β2. Inhibition of p38, ERK1/2 and CTGF by siRNA in human TM cells did not prevent TGF-β2 induction of gene expression of TGM2. Inhibition of R-Smad3 via siRNA suppressed R-Smad3 protein levels and caused a decreased in TGM2 protein expression. Immunohistochemistry results demonstrated increased expression and increased co-localization of TGF-β2 and TGM2 in GTM tissues. **Conclusions:** These studies demonstrated that human TM cells express proteins for the canonical and non-canonical signaling pathways. Trabecular meshwork cells respond to exogenous TGF-β2 by the phosphorylation of downstream signaling proteins. In addition, TGF-

β2 appears to act through the canonical Smad signaling pathway to increase TGM2 protein levels and enzyme activity. Selective knockdown of R-Smad2 increased R-Smad3 protein levels, thus compensating for the loss of R-Smad, and the reciprocal occurred with selective knockdown of R-Smad3. There was also increased levels and co-localization of TGF-β2 with TGM2 in the glaucomatous TM cells.

INTRODUCTION

Glaucoma is the second leading cause of irreversible blindness. Primary open-angle glaucoma (POAG) belongs to a group of heterogeneous optic neuropathies characterized by optic nerve head damage and visual field defects. In POAG, three ocular tissues are effected including the optic nerve, retina, and the trabecular meshwork (TM) which is the major aqueous humor outflow pathway. Impairment of aqueous humor outflow results in ocular hypertension and eventually cupping, excavation of the optic nerve head and vision loss.

Extracellular matrix (ECM) remodeling plays a role in the TM tissue architecture. For example, the ECM acts as a supporting framework for cells and is thought to maintain the ECM normal outflow system [1]. The ECM proteins found in the TM include collagens, proteoglycans (e.g. decorin, biglycan, syndecan, versican, perlecan), adhesive glycoproteins (e.g. fibronectin, laminin, vitronectin), and elastin [1]. Normally, there is a delicate balance of deposition and degradation of the ECM in the human TM. In POAG, there is excessive amounts of cross-linked ECM proteins and this is associated with the development of increased AH outflow resistance and elevated IOP [2, 3].

Tissue transglutaminase (TGM2) may play a role in modulating the ECM protein turn-over in glaucoma [4, 5]. TGM2 (EC 2.3.2.13) is a member of the transglutaminase enzyme family and is known to catalyze the posttranslational modification of proteins by inserting highly stable bonds between ε-(γ-glutamyl) lysine or (γ-glutamyl) polyamine [6]. This renders the ECM resistant to both physical and enzymatic degradation [6].

The exact molecular mechanism on how increased cross-linked ECM proteins occur in POAG is not known. Tissue transglutaminase has been referred to as having a "split personality" due to both its ability to cross-linking proteins and its ability to hydrolyze guanosine 5'–triphosphate and adenosine triphosphate [7]. In addition, TGM2 has been implicated in eye diseases including cataracts, pseudoexfoliation syndrome, and glaucoma [4, 8, 9]. Several regions of the anterior eye segment express TGM2, including the lens, iris, sclera, ciliary muscle, ciliary processes, and the trabecular meshwork [4].

Several insightful articles have appeared concerning TGM2 and TGF-β2 and their potential association with glaucoma [4, 5]. The first report indicated exogenous TGF-β1 and TGF-β2 increased mRNA expression, protein levels, and enzymatic activity of TGM2 in cultured human TM cells [4]. Differences in TGM2 protein levels and enzyme activity were reported comparing normal and glaucomatous human TM cells and tissues [10]. In addition, Fuchshofer and co-workers (2005) reported exogenous treatment of TGF-β2 increased TGM2 expression in optic nerve head (ONH) astrocytes and this action was mediated through CTGF/CCN2 [5]. TGF-β2 regulates ECM deposition by CTGF[11]. Interestingly, Fuchshofer and co-workers (2005) also demonstrated that TGF-β2 induced CTGF mRNA expression and protein levels in optic nerve head astrocytes and HTM cells [5, 12].

To date, there is no clear understanding how TGF-β2 induces TGM2 expression and enzyme activity. This study was an attempt to elucidate the signaling pathways that regulate TGF-β2 regulation of TGM2. In addition, the involvement of CTGF was investigated to determine if CTGF acts as a mediator for TGF-β2 induction of TGM2 in TM cells.

METHODS

Trabecular Meshwork Dissection and Cell Culture

Primary human TM cells were obtained from Alcon Laboratories (Fort Worth, Texas). Human TM cells were grown in Dulbecco's Modified Eagle's Medium (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. Fresh medium was exchanged every 2-3 days and cells were maintained at 37°C in 5% CO_2-95% air.

Treatent with Exogenous TGF-β

Human TM cells were grown in DMEM plus 10% FBS until they were 70% to 80% confluent. The cells were washed with serum-free DMEM and cultured in serum-free DMEM for 24 hours. The cells were then treated with recombinant TGF-β protein (R&D Systems, Minneapolis, MN).

Protein Extraction and Western Blot Analysis

Total cellular protein was isolated from cultured cells using M-PER extraction buffer (#78501, Pierce Biotech, Rockford, IL.) and Protease Inhibitor Cocktail (#78415, Pierce Biotech) or isolated using Laemmli Sample buffer with 5% β-mercaptoethanol. Protein concentration was determined by using the Bio-Rad D_c Protein Assay System according to manufacturer's

instructions (Bio-Rad Laboratories, Richmond, CA) or EZQ Protein Quantitation Kit according to manufacturer's instructions (#R33200, Molecular Probes).

A total of 20 ug of protein was loaded per well and separated on a SDS-PAGE denaturing polyacrylamide gel 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 approximately 60 minutes in order to block non-specific binding. Blots were processed using appropriate antibodies and appropriate secondary antibodies. The Super Signal West Femto Maximus Sensitivity Substrate (#34095, Pierce Biotech) was used for detection and blots were exposed to a Fluorchem 8900 imager (Alpha Innotech, San Leandro, CA).

Concentrating Conditioned Medium

Cultured TM cells were plated with equal number of cells and equal volume of medium in 12-well plates. Conditioned medium was collected and concentrated by using the StrataClean Resin according to manufacturer's instructions (#400714, Stratagene, La Jolla, CA).

Immunolocalization of Tissue Transglutaminase (TGM2) and Tranforming Growth Factor beta2 (TGFβ2) in Normal and Glaucomatous Trabecular Meshwork Tissues

Details of the immunocytochemistry of TGM2 in TM tissues have been published previously[10]. In order to document the presence of TGM2 and TGF-β2 in the TM, three sets of normal and glaucomatous age-matched human eyes were used (e.g. normal donors of 72, 88 and

94 yrs; glaucomatous donors of 76, 87, and 92 yrs.). Paraffin TM sections were processed with primary antibodies for TGM2 (#MS-300-P1, Neomarkers) and TGF-β2 (#sc-90, Santa Cruz Biotechnology). The primary antibodies were detected by incubation with appropriate secondary antibodies (Alexa Fluor 488, and 633; Molecular Probes) for 45 minutes. The visualization of cell nuclei was performed by staining tissue sections with DAPI (300 μm) for 10 minutes. Controls consisted of omission of primary antibodies, control IgG, and/or mouse ascites. Images were captured using a Zeiss 410 confocal imaging system (Carl Zeiss, Thornwood, N.Y.)

Immunocytochemistry for Trabecular Meshwork Cells

Cells were grown on glass coverslips in 24 well plates. At about 80% confluency, cells were fixed with 3.5% formaldehyde (Fisher Scientific, Pittsburgh, PA) in PBS for 20 minutes. Cells were treated with 0.2% Triton X-100 in 1 X PBS for 20 minutes. Incubation took take place for 1 hour with 5% normal blocking serum and 0.3% in PBS. Cells were then incubated with primary antibodies overnight at 4° C and secondary antibodies at 1:200 and 1%BSA in PBS for 1 hour at room temperature. Negative controls consisted of omission of primary antibody, control IgG, and/or mouse ascites. To visualize nuclei, cells were treated with 300 nM DAPI nuclear stain and mounted using Aqua-Mount (Lerner Laboratories, Pittsburgh, PA). Slides will be stored in the dark at 4°C until visualized.

Small Interfering RNAs (siRNA) for Smad3, Smad2, p38, and CTGF

The siRNA mediated gene suppression experiments were conducted in TM cells using siGenome On-Target plus SMART pool dublex siRNAs from Dharmacon (Thermo Fisher Scientific., Lafayette, CO). Controls included a RISC-free siRNA (impairs uptake and processing of RISC) and a non-targeted siRNA (ThermoFisher Scientific). Briefly, confluent TM cells were trypsinized, counted, and plated at a density of 8000 cells/well in a 12-well plate in DMEM media containing 10% serum. TM cells were allowed to incubate overnight at 37°C in 5% CO_2-95% air. The following day selected siRNAs were transfected to TM cells using DharmaFECT 1 (Thermo Fisher Scientific) at the desired concentrations in OPTI-MEM media (Gibco, Grand Island, NY) for 24 hours. At the end of 24 hours, medium was changed to either TGF-β2 in serum free medium or serum free medium alone for 48 hours.

Small Interfering RNAs (siRNA) for ERK1/2

The siRNA mediated gene suppression experiments for p42 and p44 were conducted in TM cells using the Cell Signaling Kit System as described by the manufacturer's protocol (Cell Signaling Technology, Inc, Danvers, MA 01923), along with a non-targeted siRNA control (Cell Signaling). Briefly, TM cells were trypsinized, counted, and plated at a density of 8000 cells/well to a 12-well plate in DMEM medium containing 10% serum. The following day siRNAs were transfected to TM cells with the desired concentration of siRNA for 24 hours. At the end of the 24 hours, medium was changed and wells contained TGF-β2 in serum-free medium or serum-free medium alone.

Image J Analysis

Immunohistochemical staining analysis was performed on a PC computer using the public domain NIH Image J program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/.

Statistical Analysis

The statistical significance, defined as $p<0.05$, was evaluated using a statistical software PRISM for Windows. * $p<0.05$ was determined by one-way ANOVA for variance analysis among multiple groups or a paired student's t-test (one tailed t-test).

RESULTS

TGF-β Isoforms Regulate TGM2 protein levels in Human Trabecular Meshwork cells

TGM2 protein expression was examined in the lysates of 3 normal and 3 glaucomatous TM cell lines. The 3 NTM and 3 GTM cell lines were treated separately with each TGF-β isoform (TGF-β1, β2, and β3) at 5ng/ml for a 48 hour time period. TGM2 was expressed in all cell lines as a 77 kDa protein band on the Western blots (Figure 1A). The expression of TGM2 was induced by all three TGF-β isoforms compared to untreated controls in both NTM and GTM cell lines. These data suggest that TGM2 is induced by all three isoforms and thus not isoform specific. Interestingly, the GTM cell lines were more responsive to TGF-β treatment than the NTM cell lines. After treatment with either TGF-β1, TGF-β2, and TGF-β3 the expression of TGM2 increased approximately 5 fold for GTM cells compared to NTM cells (Figure 1). Relative TGM2 intensity levels were normalized to β–actin via densitometry (Figure 1B). These data indicate that both NTM and GTM cell lines contain TGM2 and that the GTM cell lines were more responsive, however, this difference was not statistically significant.

TGM2 Enzyme Activity Following Treatment with TGF-β1, β2, and β3

Having shown that TGM2 protein is induced by all three TGF-β isoforms, the next step was to determine whether TGM2 enzyme activity is elevated in 3 NTM and 3 GTM cell lines following TGF-β treatment. TGM2 activity was analyzed utilizing a biotin labeled cadaverine-streptavidin immunohistochemical assay (Figure 2A). Figure 2 is a representative example of

one cell line. The enzyme activity of TGM2 with TGF-β1, β2, or β3 treatment was statistically elevated ($p<0.005$) in all three TGF-β groups compared to untreated control with cadaverine alone (Figure 2B). Previous investigators have published that the TGF-β2 is the isoform elevated in POAG, thus the remainder of this study focuses on TGF-β2 mediated effects on TGM2.

TGFβ–2 Protein Levels in Normal and Glaucomatous Trabecular Meshwork Cells

The protein expression of TGF-β2 in the lysates of three normal and three glaucomatous TM cell lines were examined. Two of 3 GTM cell lines appeared to have increased TGF-β2 protein levels compared with the NTM cell lines. One of the GTM cell lines had less protein loading and which may account for the low expression of TGF-β2. TGF-β2 was expressed at approximately 80 kDa protein band on the Western blot and β-actin served as a loading control (Figure 3). Statistical analysis was performed in 3 NTM and 2 GTM, and these data indicate that the protein levels of TGF-β2 were significantly elevated ($p<0.005$) in the GTM cell lines (Figure 3B).

Immunohistochemical Staining of TGF-β2 and TGM2 in Normal and Glaucomatous TM Tissues

Having shown the endogenous levels of TGF-β2 and TGM2 in NTM and GTM cell lines, co-localization was performed for TGF- β2 and TGM2 in 3 normal and 3 glaucomatous TM tissues from human donors. Immunostaining was performed on paraffin-embedded human TM

sections in glaucomatous donor eyes and compared results to age-matched controls. Immunostaining of TM tissues demonstrated that TGF-β2 and TGM2 were expressed in all six human TM samples. The expression of TGF-β2 was localized uniformly throughout the TM tissues. In two of the three GTM human tissues TGF-β2 appeared to be expressed at a higher level than NTM tissues. TGM2 was present in all six human TM samples. The expression of TGM2 was localized throughout, however, not uniformly in TM tissues. All 3 GTM human TM samples had elevated TGM2 expression compared to age-matched controls. TGF-β2 and TGM2 protein were co-localized in GTM tissues (Figure 4).

Treatment with TGF-β2 Increases TGM2 Levels in NTM and GTM Cells

Western blot was used to analyze for 3 NTM and 3 GTM cell lines using a dose response for TGF-β2 at 0.5 ng/ml to 10.0 ng/ml. Western blot analysis demonstrated that NTM and GTM cell lines responded to TGF-β2 (Figure 5). There was not a dose dependent increase of TGM2 protein levels, thus lower concentrations needed to be evaluated. However, GTM cell lines were more responsive to TGF-β2 compared to NTM (Figure 5 C and D). All Western blots were re-probed for β-actin which served as a loading control (Figure 5).

Phosphorylation of R-Smad3 Following TGF-β Treatment

The canonical downstream signaling pathway for TGF- β uses intracellular R-Smad proteins (R-Smad2 and R-Smad3). To verify whether R-Smad3 was present in TM cells, 3 NTM and 3 GTM cell lines were treated in a time dependent manner with TGF- β2 (5ng/ml). Western

blot analysis detected phosphorylated R-Smad3 protein in cell lysates for NTM cell lines (Figure 6A) and GTM cell lines (Figure 6B). Densitometric analysis demonstrated that phosphorylation of R-Smad3 increased at 15 minutes, with a maximum activation at 30 and 60 minutes followed by a decrease at 2 hours for both NTM cell lines (Figure 6C) and GTM cell lines (Figure 6D).

Specific Inhibitor of Smad3 (SIS3) Reduces TGM2 Protein Levels

Specific inhibitor of R-Smad3 (SIS3) is an inhibitor of TGF- β signaling via inhibition of R-Smad3 phosphorylation. To determine if SIS3 could reduce or inhibit TGM2 protein levels following TGF- β2 treatment was examined in 3 TM cell lines (Figure 7). Treatments included control, TGF-β2 (5ng/ml), and or SIS3 (10uM, 15μM, 25μM, and 50μM) with TGF-β2. Variability was noted between cell lines on SIS3 effects on TGM2. Densitometric analysis revealed a reduction for TGM2 levels, however, statistical analysis demonstrated no statistical difference between groups (Figure 7B).

Suppression of R-Smad3 with siRNA Treatment

R-Smad3 siRNA mediated gene suppression experiments were conducted in 3 NTM (Figure 8A) and 3 GTM (Figure 8B) cell lines. Controls consisted of cells in serum-free medium (SFM), TGFβ2 treatment, an siRNA control that impairs RISC uptake (Co#1) with TGF-β2, and a non-targeting siRNA (Co#2) with TGF-β2. In all NTM and GTM cell lines, R-Smad3 siRNA at 0.1nm, 10.0nm, and 20.0nm significantly reduced R-Smad3 ($p<0.05$, one tailed t-test as

compared to siRNA Co#1) (Figure 8C). All blots were re-probed for R-Smad2 to determine potential effects of R-Smad3 suppression on R-Smad2 expression. There was a significant induction of R-Smad2 with R-Smad3 siRNA treatment ($p<0.05$, one tailed t-test as compared to siRNA Co#1) (Figure 8C). Next, R-Smad3 siRNA decreased TGM2 protein levels at all siRNA concentrations. There was a modest decrease of TGM2 protein levels at 10.0 nm and 20.0 nm siRNA in both the NTM and GTM cell lines. β-actin served as a loading control for all the Western blots. In addition, the conditioned medium was collected for both NTM and GTM cells. The medium was concentrated and proteins analyzed by Western blots. Interestingly, a reduction of R-Smad3 gene expression resulted in reduced TGM2 protein levels in all cell lines tested (Figure 8).

Immunostaining of R-Smad3 and TGM2 with Smad3 siRNA Treatment

A previous study showed that R-Smad3 siRNA treatment resulted in down-regulation of R-Smad3 and reduced levels of TGM2 protein levels. In order to expand on this observation, 3 TM cell lines were used for immunostaining. Controls consisted of cells in serum-free medium (SFM), TGF-β2 treatment, a siRNA control that impairs RISC uptake (Co#1) with TGF-β2, and a non-targeting siRNA (Co#2) with TGF-β2. Receptor Smad3 treatments at 1.0nM, 10.0nM, and 20.0nM were used to down-regulate R-Smad3. In all 3 cell lines, R-Smad3 siRNA at 1.0nM, 10.0nM, and 20.0nM down-regulated R-Smad3 expression and reduced TGM2 expression (Figure 9).

Suppression of R-Smad2 with siRNA Treatment

The Smad2 siRNA mediated gene suppression experiments were conducted in 2 NTM (Figure 10A) and 2 GTM (Figure 10B) cell lines. Controls consisted of cells in serum-free medium (SFM), TGF-β2 treatment, a siRNA control that impairs RISC uptake (Co#1) with TGF-β2, and a non-tarageting siRNA (Co#2) with TGF-β2. In all NTM and GTM cell lines, R-Smad2 siRNA at 0.1nM, 1.0nM, 10.0nM, and 20.0nM significantly reduced total Smad2 ($p<0.05$, one tail t-test) (Figure 10C). All blots were re-probed for R-Smad3 to determine the effects of R-Smad2 siRNA on R-Smad3. There was a significant induction in R-Smad3 protein levels in the R-Smad2 siRNA treatment at siRNA concentrations of 1.0nM, 10.0nM, and 20.0nM (Figure 10A). In GTM cell lines there was an increased in R-Smad3 protein levels in the R-Smad2 siRNA treatments at siRNA concentrations of 10.0nM and 20.0nM (Figure 10B). Next, R-Smad2 siRNA suppression of TGM2 protein levels was determined in TM cells. There appeared to be variability between cell lines on R-Smad2 siRNA effects on TGM2. Some cell lines appeared to have a modest decrease in TGM2 protein levels, while others had no apparent effect of TGM2 protein levels. Densitometric analysis with the combination of both NTM and GTM cell lines revealed a statistical decrease for R-Smad2 siRNA and a statistical increase for R-Smad3 effects compared to siRNA Co#1 ($p<0.05$, one tailed t-test as compared to siRNA Co#1) (Figure 10C). β-actin served as a loading control for all the Western blots (Figure 10).

Suppression of CTGF with siRNA Treatment

Having shown reduced TGM2 protein levels with R-Smad3 and R-Smad2 siRNA treatment, CTGF, a downstream regulator of TGFβ2, was examined to determine its role as a mediator to regulate TGM2 levels in 3 NTM (Figure 11A) and 3 GTM (Figure 11B) cell lines. Controls consisted of cells in serum-free medium (SFM), TGF-β2 treatment, a siRNA control that impairs RISC uptake (Co#1) with TGF-β2, and a non-targeting siRNA (Co#2) with TGF-β2. Human TM cell lines were treated with CTGF siRNA at 0.1nM, 1.0nM, 10.0nM, and 20.0nM. In all cell lines, upon siRNA treatment at 1.0nM, 10.0nM, and 20.0nM CTGF was significantly reduced ($p<0.05$, one tailed t-test as compared to siRNA Co#1) (Figure 11C). All Western blots were re-probed to determine CTGF siRNA effects on TGM2. There was no apparent effect of CTGF suppression on TGM2. β-actin was used as a loading control (Figure 11).

Phosphorylation of p38 MAP Kinase Following TGF-β2 Treatment

The non-canonical signaling pathway for TGF-β2 includes the MAP kinase signaling pathway. Therefore, the determination of TGF- β 2 (5ng/ml) phosphorylation of p38 in 2 NTM and 2 GTM cell lines in a time dependent manner was examined in TM cells. Western blot analysis detected phosphorylated p38 protein in cell lysates for NTM cell lines (Figure 12A) and GTM cell lines (Figure 12B). Phosphorylation of the 42 kDa band of p38 MAP Kinase was detectable at 15 minutes, reached a maximum at 30 minutes, was down-regulated at 60 minutes, but increased at 2 hours in NTM cells (Figure 12A). In GTM cell lines, the 42 kDa band of p38 MAP Kinase was slightly phosphorylated at 15 minutes, reached a maximum at 30 minutes, was

down-regulated at 60 minutes, but slightly increased at 2 hours (Figure 12B). Densitometric analysis demonstrated no statistical significance for TGF-β2 effects on p38 (data not shown).

Suppression of p38 MAP Kinase with siRNA Treatment

The p38 siRNA mediated gene suppression experiments were conducted in one NTM (Figure 13A) and 2 GTM (Figure 13B) cell lines. Controls consisted of cells in serum-free medium (SFM), TGF-β2 treatment, a siRNA control that impairs RISC uptake (Co#1) with TGF-β2, and a non-targeting siRNA (Co#2) with TGF-β2. In all NTM and GTM cell lines, p38 siRNA at 0.1nM, 1.0nM, 10.0nM, and 20.0nM reduced p38. All Western blots were re-probed to determine p38 siRNA effects on TGM2. There was an increase in TGM2 protein levels, with a statistical significance between p38 siRNA at 1.0nM and 20nM compared to siRNA Co#1 treatment ($p < 0.05$, one tailed t-test as compared to siRNA Co#1) (Figure 13C). β-actin was used as a loading control (Figure 13).

Phosphorylation of ERK 1/2 MAP Kinase

TGF- β2 phosphorylation of the ERK1/2 MAP kinase signaling pathway was examined in TM cells. Therefore, 2 NTM and 2 GTM cell lines were treated in a time dependent manner with TGF- β2 (5ng/ml). Western blot analysis detected phosphorylated ERK1/2 protein in cell lysates for NTM cell lines (Figure 14A) and GTM cell lines (Figure 14B). Densitometric analysis demonstrated that phosphorylation of ERK1/2 increased at 15 minutes with a maximum activation at 30 minutes, and decreased at 60 minutes for NTM cell lines (Figure 14C). In GTM

cell lines ERK1/2 increased at 5 minutes with a maximum activation at 15 minutes and remained phosphorylated up to 2 hours (Figure 14D).

ERK 1/2 Inhibition with PD98059 and UO126

Two upstream inhibitors for ERK1/2 are PD98059 and UO126 which inhibit MEK1 and MEK1/2, respectively. Inhibition of PD98059 and UO126 on the ERK1/2 signaling pathway in 2 NTM (Figure 15A) and 1 GTM (Figure 15B) cell lines were examined. All 3 cell lines were under conditions either in serum-free, TGF-β2, PD98059 (10μM, 20μM, and 30μM) and UO126 (10μM, 20μM, and 30μM) with TGF-β2. Densitometric analysis revealed a non-significant reduction of ERK1/2 with PD98059 and UO126 treatment (Figure 15C). Western blots were re-probed for TGM2 to determine if PD98059 and/or UO126 had effects for TGM2 protein levels. Thus, densitometric analysis revealed a modest, but no significant effects in TGM2 protein levels (Figure 15C).

Suppression of MEK 1 and MEK2 with siRNA Treatment

Having shown there was a decrease in TGM2 levels with the PD98059 and UO126 inhibitor, although not statistical significant, experiments in 2 NTM and 1 GTM cell lines for MEK1 and/or MEK2 siRNA (Figure 16) was examined. Controls consisted of cells in serum-free medium (SFM), TGFβ2 treatment, and a non-targeting siRNA control (siCO) with TGF-β2. Figure 16 is a representative cell line for MEK1 siRNA (Figure 16A) and MEK2 siRNA (Figure 16B). For MEK1 siRNA experiments, total protein for MEK1 was suppressed, however, there

was an increase in MEK2 siRNA samples. For MEK2 siRNA experiments, total protein for MEK2 was suppressed and no effects on MEK1 siRNA samples for MEK2 (Figure 16B). Next, MEK1 and/or MEK2 siRNA had no effect on TGM2 protein levels. β-actin was used as a loading control. Densitometric analysis revealed no statistical significance for MEK1, MEK2, and TGM2 protein levels (Figure 16C).

Suppression of ERK 1/2 (p44/p42) MAP Kinase with siRNA Treatment

To determine if ERK1/2 siRNA has any effects on TGM2 protein levels, experiments were conducted in 2 NTM and 2 GTM cell lines. Figure 17 is one representative cell line. Controls consisted of cells in serum-free medium (SFM), TGFβ2 treatment, and a non-targeting siRNA control (siCO) with TGF-β2. ERK1 siRNA treatment suppressed total ERK1 protein levels, however, there were suppressive effects on ERK2 and the reciprocal was true for ERK2 siRNA (Figure 17). There was no apparent effect of ERK1 or ERK2 siRNA on TGM2 protein levels. β-actin was used as a loading control. Densitometric analysis revealed statistical decrease for ERK2 (p42) at 10nm and 20nm ($p < 0.05$, one tailed t-test as compared to siCo) (Figure 17B).

DISSCUSSION

A major risk factor for the development of glaucoma is elevated IOP [13-15]. Elevated IOP is due to the increased AH outflow resistance and as a result is associated with morphologic and biochemical changes in the TM [16]. Within the glaucomatous TM there is an increased accumulation of ECM proteins [16, 17] which may be as a result of abnormal turnover of ECM proteins that is regulated by MMPs [18, 19], PAI-1 [20, 21], and growth factors [17, 22, 23]. Human TM cells express many growth factors and their receptors in vitro [24]. Growth factors present in the TM and AH are believed to maintain homeostasis and altered growth factor expression may result in deleterious consequences to the TM and/or regulation of AH outflow pathways.

TGF-β2 influences many aspects of cellular behavior including proliferation, differentiation, migration, and ECM synthesis and breakdown [25, 26]. With respect to glaucoma, a number of research studies have reported elevated amounts of TGF-β2 in the AH of glaucomatous eyes compared to normal eyes [27-31]. The perfusion of TGF-β2 decreases the outflow facility in ex-vivo anterior segment organ culture models [32]. Fleenor and co-workers, recently reported the perfusion of TGF-β2 in ex-vivo anterior segment organ culture models elevated IOP in a time dependent manner and also increased of fibronectin and PAI-I levels in the elutes of organ culture models [21]. Thus, it is reasonable to suspect TGF-β2 may have a direct role in ECM deposition and accumulation and elevated IOP in glaucoma.

Welge-Lüssen and co-workers first reported TGM2 induction by TGF-β1 and β2 in cultured HTM cells at both the mRNA and protein levels. Of interest, TGM2 can convert the latent TGF-β form to its active form. Thus, it is reasonable to suspect in the glaucomatous TM,

TGF-β2 induces TGM2, and the induction of TGM2 increases the conversion of latent TGF-β to its biological active form, consequently providing a further induction of TGM2. Despite the fact that TGF-β1 and β2 induce TGM2 and are elevated in GTM cells, the exact role or molecular mechanism for TGM2 in the pathogenesis of glaucoma is not understood. Therefore, the purpose of this study was two-fold: (a) to examine the induction of TGM2 by TGF-β isoforms, and (b) to identify the signaling pathway utilized by TGF-β2 to regulated TGM2 in cultured TM cells.

Western blot analysis showed that TGM2 was induced by TGF-β1, -β2, or -β3 in both NTM and GTM cells and that GTM cells were more responsive to treatment with all TGF-β isoforms in comparison to NTM cell lines. These results support the previous study by Welge-Lüssen and co-workers [4], who first reported the presence of TGM2 induction by TGF-β1 and β2 in cultured TM cells. These results also demonstrate that TGF-β3 can also increase TGM2 protein levels in cultured TM cells. Next, the induction of TGM2 enzyme activity by TGF-β1, -β2, or -β3 in cultured TM cells was examined. Enzyme activity was measured by the incorporation of biotin-cadaverine, a pseudosubstrate for TGM2. These results demonstrated significantly increased enzymatic activity levels of TGM2 in cultured TM cells following exogenous treatment by all three TGF-β isoforms. These results demonstrate that the TGM2 enzyme is biologically active and is induced by all TGF-β isoforms.

The endogenous expression of TGF-β2 in NTM and GTM cell lines and tissues significantly increased protein levels of TGF-β2 in GTM cells in comparison to NTM cells. To verify if TGF-β2 is also increased in vivo, 3 NTM and 3 GTM tissues were evaluated for TGF-

β2 and TGM2 with immunohistochemical staining. These immunohistochemical results indicated increased expression and increased co-localization for both TGF-β2 and TGM2 in GTM tissues in comparison to NTM tissues. These results suggest a direct role of TGM2 induction by TGF-β2 in glaucomatous eyes.

Within the glaucomatous eye, TGF-β2 has been reported to be as high as 2.7ng/ml compared to 1.48ng/ml in normal eyes. For our NTM and GTM cell cultures we wanted to perform a dose response for TGF-β2 at concentrations of 0.5ng/ml - 10.0ng/ml. Our data did not show a dose dependent increase with TGF-β2. We observed a slight induction of TGF-β2 at all concentrations used in comparison to untreated controls in NTM cell cultures. However, in the GTM cell cultures TGF-β2 stimulated a greater response as high as one fold in comparison to NTM for TGM2 protein levels.

As a first step to evaluate TGF-β2 regulation of TGM2, it was necessary to determine if TM cells express the proteins for the canonical signaling pathway of TGF-β2. Isolated TM cells expressed and phosphorylated R-Smad3 of the canonical TGF-β2 signaling pathway and was activated in response to TGF-β2 in a time dependent manner. These data demonstrate that both NTM and GTM cells express the phosphorylated R-Smad3 and respond to exogenous TGF-β2 with increased activation of R-Smad3 signaling. SIS3, a inhibitor of the phosphorylation of R-Smad3, down-regulatd TGM2 protein levels. In addition, R-Smad3 was knock-down with R-Smad3 siRNA. The silencing of R-Smad3 gene expression decreased TGM2 protein levels. These finding demonstrated a reduction of TGM2 protein levels by Western blots and immunocytochemistry for R-Smad3 siRNA. For Western blots a modest reduction of TGM2 at the concentration of 10.0nM and 20.0nM of R-Smad3 siRNA was observed. Western blots were

re-probed to determine if R-Smad3 siRNA has an effect on R-Smad2 protein levels. Interestingly, R-Smad3 siRNA resulted in an increase in R-Smad2 protein levels. These results suggest R-Smad2 was compensating for the loss of R-Smad3 and may explain why only small decreases in TGM2 protein levels were noted.

Because of the effect of R-Smad3 siRNA on R-Smad2 expression, the effects of R-Smad2 siRNA on R-Smad3 and TGM2 protein levels was investigated. R-Smad2 knock-down with R-Smad2 siRNA was observed. These findings demonstrated a modest decrease in TGM2 protein levels, however, there was an increase with respect to R-Smad3 protein levels. Again, suggesting R-Smad3 was compensating for the loss of R-Smad2 and explaining small decreases in TGM2 protein levels were detected.

The effects of TGF-β2 on ECM turn over has been reported to be mediated by CTGF [11]. Fuchshofer and co-workers demonstrated that TGF-β2 induces TGM2 mRNA and protein expression and this action was mediated via CTGF in optic nerve head astrocytes [5]. In addition, Fuchshofer and co-workers demonstrated that TGF-β2 induces CTGF in TM cell cultures. These results are in agreement with Fuchshofer and co-workers (2007) in that there was an induction of CTGF with TGF-β2. The upregulation of TGM2 by TGF-β2 mediated by CTGF was examined in both NTM and GTM cell lines. CTGF was knock-down with CTGF siRNA, however, there was no effect on TGM2 levels, which suggests that CTGF does not mediate the induction of TGM2 in TM cells by TGF-β2.

TGF-β2 has been reported to activate the MAPK (p38, ERK1/2, and JNK) pathways in various cell types [33]. Thus, the next approach was to evaluate if TGF-β2 stimulates the p38 signaling pathway and to determine if p38 regulated TGM2 in TM cells. The data demonstrated

that both NTM and GTM cells expresses p38 and that p38 responds to exogenous TGF-β2 with increased activation of p38 signaling. P38 silencing in TM cells with p38 siRNA would determine if the silencing of p38 would lead to a reduction of TGM2 protein levels in TM cells. Unexpectantly, the data demonstrated an increase of TGM2 protein levels with p38 siRNA.

In a review by Ross and Hill (2008), post-translational sites of all R-Smad protein are present. For example, the R-Smad signaling proteins contain phosphorylation sites at their linker region and R-Smad2 contains phosphorylation sites at both its linker region and MH1 domain for MAPK [33]. In addition, the linker region of R-Smad2 and R-Smad3 may be phosphorylated by an upstream activator of p38 and by JNK, respectively [33]. This may explain why p38 silencing increase TGM2 protein levels, p38 may be regulating TGF-β2 by phosphorylation at the MH1 domain or linker regions of R-Smads to regulate TGM2 protein levels.

In addition to the p38 signaling pathway, the presence of another MAPK signaling pathway in TM cells was examined. TGF-β2 has been reported to stimulate ERK1/2 in a cell type specific manner [33]. This study, demonstrated NTM and GTM cells expressed ERK1/2 and ERK1/2 respond to exogenous TGF- β2 with increased activation of ERK1/2 signaling. Two upstream inhibitors, PD98059 and UO126, which inhibit MEK1 and MEK1/2, respectively were examined in TM cells. The results demonstrated no significant decrease of TGM2 protein levels with PD98059 and UO126 inhibitors. Next, MEK1 and MEK2 siRNA effects on MEK1, MEK2, and TGM2 were examined in TM cells. MEK2 silencing effected MEK1 levels by increasing total protein levels, suggesting MEK1 compensated for the loss of MEK2, however, the opposite was not true with MEK1 silencing. With respect to TGM2 expression, no apparent change in protein levels was detected following the use of siRNA. To expand this study and examine

further down-stream ERK1 and 2 was knock-down with ERK 1 and 2 siRNA, however, there was no effect on TGM2 protein levels by in TM cells.

In conclusion, the results of this study demonstrated that cultured glaucomatous TM cells and tissues from human donors have elevated levels of TGF-β2. NTM and GTM cells expressed TGM2 and that TGM2 is stimulated by all three TGF-β isoforms. In addition, the canonical and non-canonical signaling pathways were stimulated by TGF- β2 in NTM and GTM cells. R-Smad2 and R-Smad3 siRNA and SIS3 down-regulated TGM2 protein levels. Unexpectantly, the silencing of p38 up-regulated TGM2 protein levels, possible through the phosphorylation of R-Smads, and ERK1/2 had no apparent role in regulating TGM2 protein levels.

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Figure Legends

Figure 1. Western blot analysis of TGM2 in NTM and GTM cells after treatment with TGF-β1, β2, or β3.

(A) Three NTM and 3 GTM cell lines were treated with either TGF -β1, -β2, or -β3 (5 ng/ml). Cell lysates were collected and electrophoresed in SDS-PAGE gels followed by Western blots for TGM2 (77 kDa). All cell lines expressed TGM2 proteins. Protein levels of TGM2 were higher in GTM compared to NTM cell lines. (B) Densitometric readings of TGM2 normalized to β-actin for 3 NTM and 3 GTM cell lines.

Figure 2. TGM2 Enzyme Activity via Biotinlated Cadaverine Assay Following Treatment with TGF-β1, β2, or β3

(A) A representative TM cell staining incubated with vehicle (DMSO) control, biotin-labeled cadaverine (1mM), and biotin-labeled cadaverine with either TGF -β1, -β2, or -β3 (5ng/ml). Transamidated and cross-linked cadaverine was detected by Alexa Fluor 488 streptavidin-conjugate (green). TGM2 induction was observed with all three TGF- β isotypes. (B) Image J Analysis was performed to quantitate the intensity levels, * statistical difference at the $p<0.05$ level (+/- SEM).

Figure 3. Western blot of TGF- β2 in NTM and GTM cells

(A) Up-regulation of TGF- β2 in glaucomatous cells compared to normal TM cells. Six different trabecular meshwork cell lines, 3 normal (NTM) and 3 glaucomatous (GTM) cell lines were grown in DMEM-containing serum. Cells were rinsed with PBS and collected with cell lysis buffer. Cell lysate were subjected to 10% SDS-PAGE and Western blot analysis using anti-TGFβ2 antibody. The molecular weight for the monomer is 12.5 kDa, the dimer is 25kDa, and the unprocessed precursor form is 48kDa. Western blot analysis shows a molecular weight at approximately 80 kDa. β-actin was used as a loading control. (B) Densitometric reading of TGF-β2 normalized to β-actin for 3NTM and 2 GTM cell lines. * Statistical difference at the $p<0.005$ level (+/- SEM).

Figure 4. Immunocytochemical staining for TGF-β2 and TGM2 in NTM and GTM tissues.

Age-matched tissues NTM (88 yrs.) and GTM (87 yrs.), were fixed, sectioned and stained with antibodies for TGF-β2 and TGM2. Negative controls consisted of PBS-BSA without primary antibody, control IgG, and mouse ascites (data not shown). GTM tisues showed an increased expression of co-localization of TGM2 and TGF- β2. (A) NTM (10X), (B) NTM (40X), (C) GTM (10X), and (D) GTM (40X)

abbreviations: AC- anterior chamber, TM- trabecular meshwork, SC- Schlemn's Canal

Figure 5. Dose Response for TGF-β2 Induction of TGM2

A representative Western blot of one (A) NTM and one (B) GTM cell line treated with TGF-β2 at 0.5ng/ml, 1.25ng/ml, 2.5ng/ml, 5.0ng/ml, and 10.0ng/ml. Densitometric analysis demonstrated (D) GTM cell lines had a greater response for TGF-β2 compared to (C) NTM.

Figure 6. Western Blot analysis of phosphorylated R-Smad3 in TGF-β2 treated NTM and GTM cells.

Western blot analysis for phosphorylated R-Smad3 (p-Smad3) in (A) NTM and (B) GTM cells treated with exogenous TGF-β2 (5ng/ml) for 0 minutes, 5 minutes, 15 minutes, 30 minutes, 60 minutes, and 2 hours. Blots were stripped, washed thoroughly and re-probed for total R-Smad3. Phosphorylated R-Smad3 was measured by Western blot and analyzed by densitometry for (C) NTM (n=3) and (D) GTM (n=3) cell lines.

Figure 7. Western Blot analysis of R-Smad3 Following SIS3 Treatment

(A) Western blot analysis in TM cells for R-Smad3. TM cells were pre-treated with SIS3 (an inhibitor of R-Smad3) at various concentrations (10μM, 15μM, 25μM, 50μM) for 1 hour and then treated with TGF- β2 for 12 hours. Untreated TM cells served as controls. β-actin was used as a loading control. (B) Densitometric Analysis demonstrated decreased levels of TGM2.

Figure 8. Effects of R-Smad3 siRNA Treatment in NTM and GTM Cells

Western blot analysis of R-Smad3, R-Smad2, and TGM2 with treatment of R-Smad3 siRNA followed by TGF-β2 (5ng/ml) for 48 hours. Representative blots are shown from one (A) NTM and one (B) GTM cell line. Blots were stripped, washed thoroughly and re-probed for each of the following antibodies: R-Smad2, TGM2, and β-actin. Conditioned medium (CM) was collected from samples with same volume and cell numbers and run on a Western blot. (C) Densitometric analysis was performed for R-Smad3, R-Smad2, and TGM2 normalized to β-actin. * Statistical difference at the $p<0.05$ level (one tailed t-test, compared to Co#1).

Figure 9. Immunohistochemical Staining of NTM and GTM Cells after R-Smad3 siRNA

A representative immunostaining for TM cells transfected with R-Smad3 siRNA (1.0nM, 10.0nM, and 20.0nM) and then treated with TGF-β2 for 48 hours. Human TM cells were fixed and stained with antibodies for R-Smad3 (red), TGM2 (green), and co-localization (merged) expression.

Figure 10. Effects of R-Smad2 siRNA Treatment in NTM and GTM Cells

Western blot analysis of R-Smad2, R-Smad3, and TGM2 with treatment of R-Smad2 siRNA followed by TGF-β2 for 48 hours. Representative blots are shown from one (A) NTM and one (B) GTM cell line. Blots were stripped, washed thoroughly and re-probed for each of the following antibodies, R-Smad3, TGM2, and β-actin. β-actin served as a loading control. (C)

Densitometric analysis was performed for R-Smad2, R-Smad3, and TGM2 normalized to β-actin. * Statistical difference at the $p<0.05$ level (one tailed t-test, compared to Co#1).

Figure 11. Effects of CTGF siRNA Treatment in NTM and GTM Cells

Western blot analysis of CTGF and TGM2 with treatment of CTGF siRNA followed by TGF-β2 (5ng/mL) for 48 hours. Representative blots are shown from one (A) NTM and one (B) GTM cell line. Blots were stripped, washed thoroughly and re-probed for TGM2 and β-actin. β-actin served as a loading control. (C) Densitometric analysis was done for CTGF and TGM2 normalized to β-actin. * Statistical difference at the $p<0.05$ level (one tailed t-test, compared to Co#1).

Figure 12. Western Blot analysis of phosphorylated p38 in TGF-β2 treated NTM and GTM cells

Western blot analysis of phosphorylated p38 (p-p38) in (A) NTM and (B) GTM cells following treatment with exogenous TGF-β2 (5ng/ml) for various times (0 minutes, 5 minutes, 15 minutes, 30 minutes, 60 minutes, and 2 hours). Blots were stripped, washed thoroughly and re-probed by antibodies to total p38 and β-actin.

Figure 13. Effects of p38 siRNA Treatment in NTM and GTM Cells

Western blot analysis of p38 and TGM2 with treatment of p38 siRNA followed by TGF-β2 (5ng/mL) for 48 hours. Representative blots is shown from one (A) NTM and one (B) GTM cell line. Blots were stripped, washed thoroughly and re-probed for p38, TGM2, and β-actin. β-actin served as a loading control. (C) Densitometric analysis was performed for p38 and TGM2 normalized to β-actin. * Statistical difference at the $p<0.05$ level (one tailed t-test, compared to Co#1).

Figure 14. Western Blot analysis of phosphorylated ERK1/2 in TGF-β2 treated NTM and GTM cells

Western blot analysis of phosphorylated ERK1/2 (pERK1/2) in (A) NTM and (B) GTM cells were treated with exogenous TGF-β2 (5ng/mL) for various times (0 minutes, 5 minutes, 15 minutes, 30 minutes, 60 minutes, and 2 hours). Blots were stripped, washed thoroughly and re-probed with total ERK1/2. Phosphorylated ERK1/2 was measured by Western blot and analyzed by densitometry for (C) NTM and (D) GTM cell lines.

Figure 15. Effects of PD98059 and UO126 on TGM2 Protein Levels

Western blot analysis of ERK 1/2 in (A) NTM and (B) GTM cell lines, pre-treated for 1 hour with PD98059 or UO126 and then treated with TGF-β2 for 1 hour, or TGF-β2 alone. Untreated TM cells served as controls. Cellular lysate was collected, separated by SDS-PAGE, transferred

to PVDF membranes and blotted with ERK1/2. Blots were stripped, washed thoroughly and re-probed with TGM2 antibody. β–actin served as a loading control. Densitometric analysis was done for ERK1/2 and TGM2 normalized to β-actin.

Figure 16. Effects of MEK 1 and 2 siRNA Treatment in NTM and GTM Cells

Western blot analysis of MEK1 and 2, and TGM2 treatment with MEK1 or 2 siRNA followed by TGF-β2 (5ng/mL) for 48 hours. Representative blots are shown from one (A) NTM and one (B) GTM cell line. Blots were stripped, washed thoroughly and re-probed for each of the following antibodies, TGM2 and β-actin. β-actin served as a loading control. (C) Densitometric analysis was done for MEK1 and 2 and TGM2 normalized to β-actin.

Figure 17. Effects of ERK1 and 2 siRNA Treatment in NTM and GTM Cells

Western blot analysis of ERK 1 and 2 and TGM2 with treatment of either ERK 1 and 2 siRNA. (A) A representative blot is shown for one TM cell line for ERK1 and 2 siRNA treatment. Blots were stripped, washed thoroughly and re-probed for TGM2 and β-actin. β-actin served as a loading control. (C) Densitometric analysis was done for ERK 1 and 2 and TGM normalized to β-actin. * Statistical difference at the $p<0.05$ level (one tailed t-test, compared to siCo).

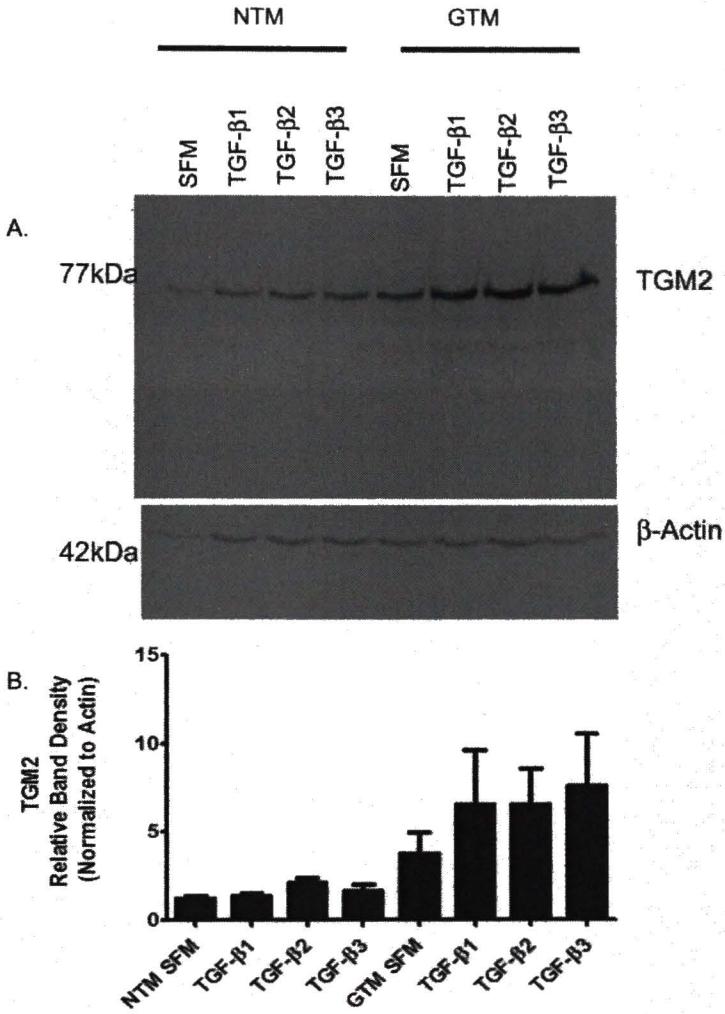
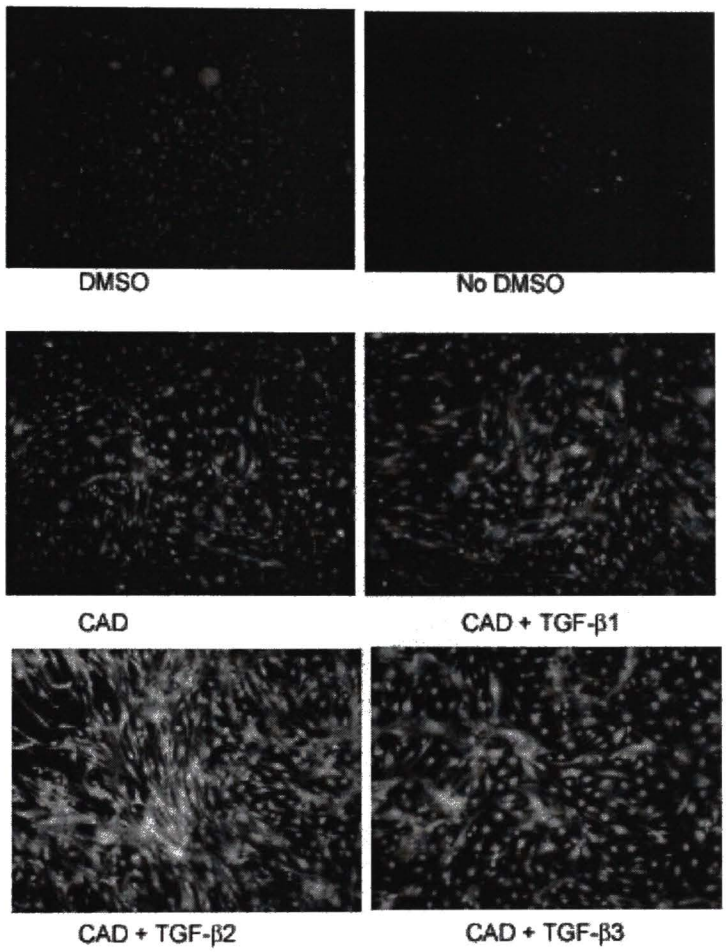

NTM
GTM
SFM
TGF-β1
TGF-β2
TGF-β3
SFM
TGF-β1
TGF-β2
TGF-β3
A.
77kDa
TGM2
42kDa
β-Actin
B.
TGM2
Relative Band Density
(Normalized to Actin)
15
10
5
0
NTM SFM
TGF-β1
TGF-β2
TGF-β3
GTM SFM
TGF-β1
TGF-β2
TGF-β3

Figure 1.

A.


DMSO
No DMSO
CAD
CAD + TGF-β1
CAD + TGF-β2
CAD + TGF-β3

B.

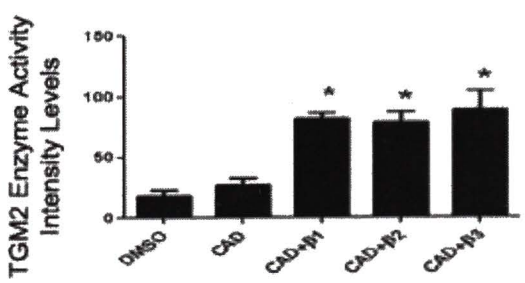

TGM2 Enzyme Activity Intensity Levels
150
100
50
0
*
*
*
DMSO
CAD
CAD+β1
CAD+β2
CAD+β3

Figure 2.

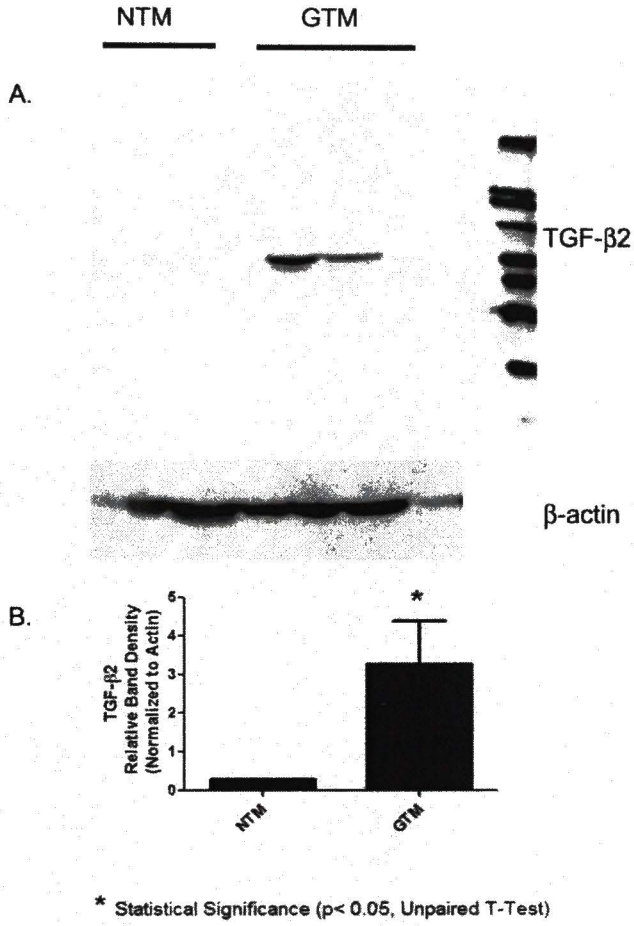

NTM
GTM
A.
TGF-β2
β-actin
B.
TGF-β2
Relative Band Density
(Normalized to Actin)
0
1
2
3
4
5
*
NTM
GTM
* Statistical Significance (p< 0.05, Unpaired T-Test)

Figure 3.

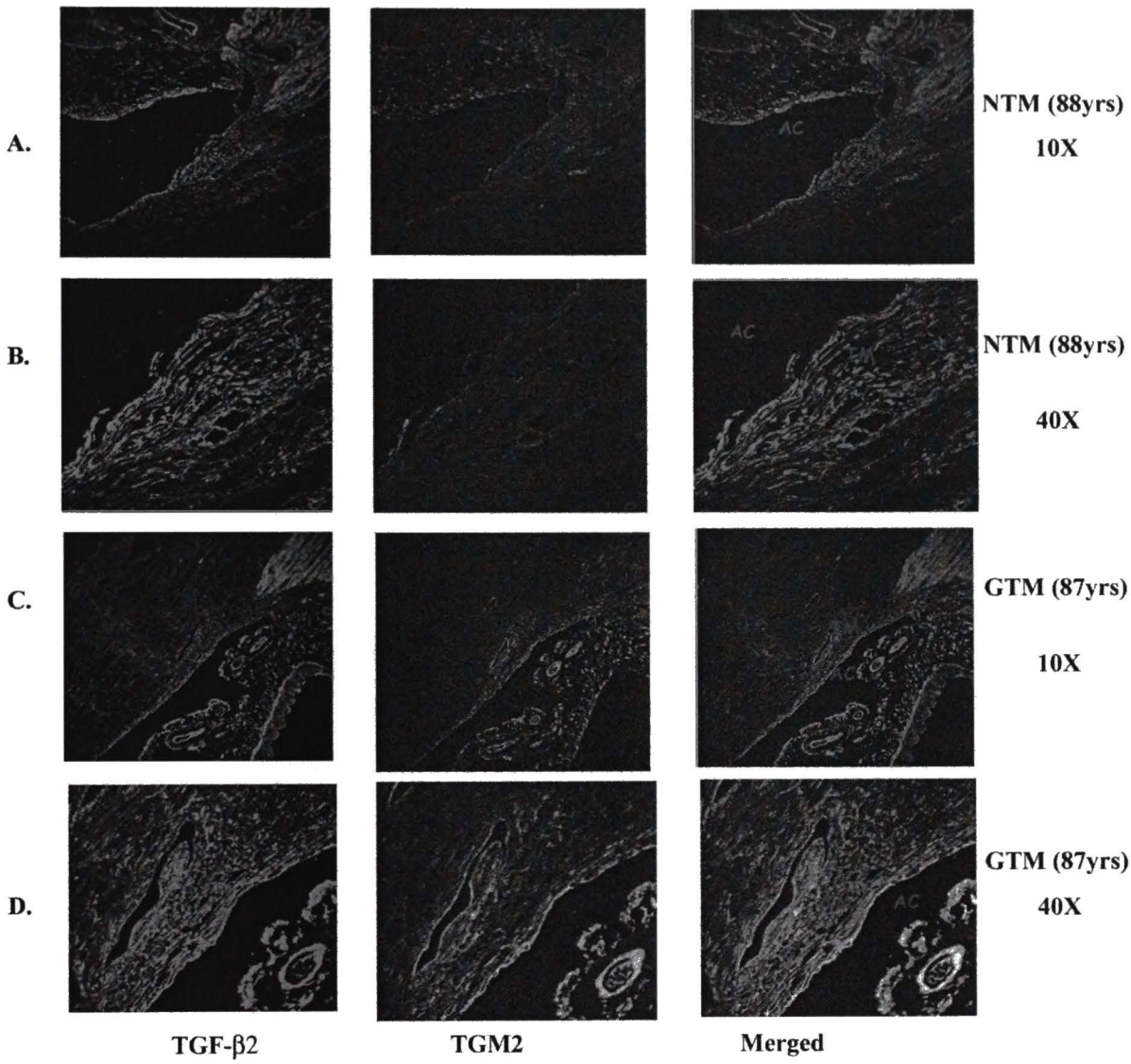

A.
B.
C.
D.
AC
NTM (88yrs)
10X
NTM (88yrs)
40X
GTM (87yrs)
10X
GTM (87yrs)
40X
TGF-β2
TGM2
Merged

Figure 4.

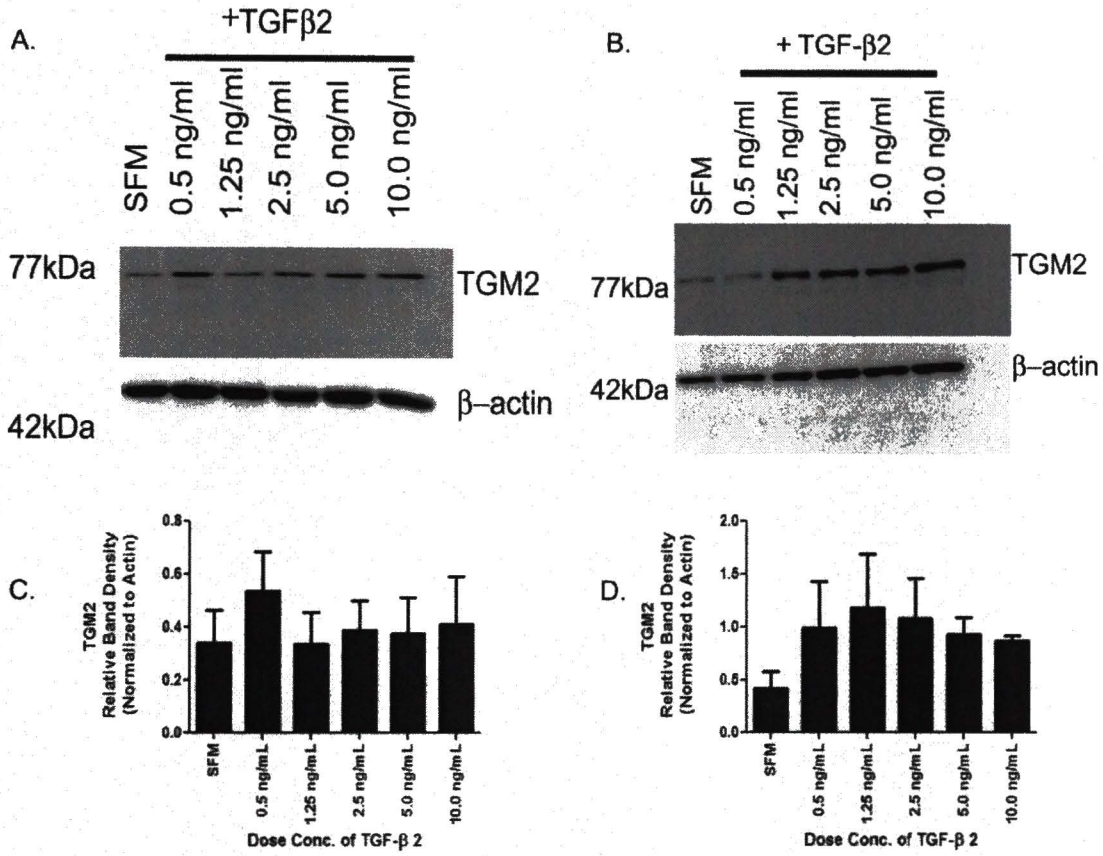

A.
+TGFβ2
SFM
0.5 ng/ml
1.25 ng/ml
2.5 ng/ml
5.0 ng/ml
10.0 ng/ml
77kDa
TGM2
42kDa
β–actin
B.
+ TGF-β2
SFM
0.5 ng/ml
1.25 ng/ml
2.5 ng/ml
5.0 ng/ml
10.0 ng/ml
77kDa
TGM2
42kDa
β–actin
C.
TGM2 Relative Band Density (Normalized to Actin)
0.0
0.2
0.4
0.6
0.8
SFM
0.5 ng/mL
1.25 ng/mL
2.5 ng/mL
5.0 ng/mL
10.0 ng/mL
Dose Conc. of TGF-β 2
D.
TGM2 Relative Band Density (Normalized to Actin)
0.0
0.5
1.0
1.5
2.0
SFM
0.5 ng/mL
1.25 ng/mL
2.5 ng/mL
5.0 ng/mL
10.0 ng/mL
Dose Conc. of TGF-β 2

Figure 5.

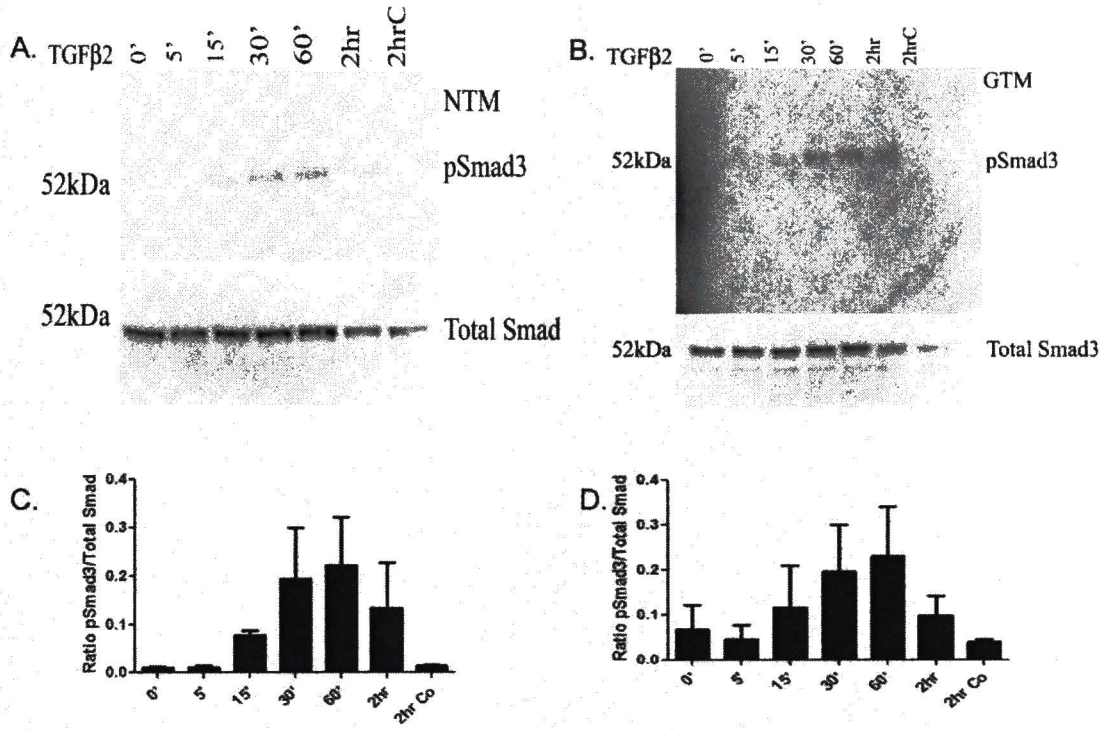

A.
TGFβ2
0'
5'
15'
30'
60'
2hr
2hrC
NTM
52kDa
pSmad3
52kDa
Total Smad
B.
TGFβ2
0'
5'
15'
30'
60'
2hr
2hrC
GTM
52kDa
pSmad3
52kDa
Total Smad3
C.
Ratio pSmad3/Total Smad
0.4
0.3
0.2
0.1
0.0
0'
5'
15'
30'
60'
2hr
2hr Co
D.
Ratio pSmad3/Total Smad
0.4
0.3
0.2
0.1
0.0
0'
5'
15'
30'
60'
2hr
2hr Co

Figure 6.

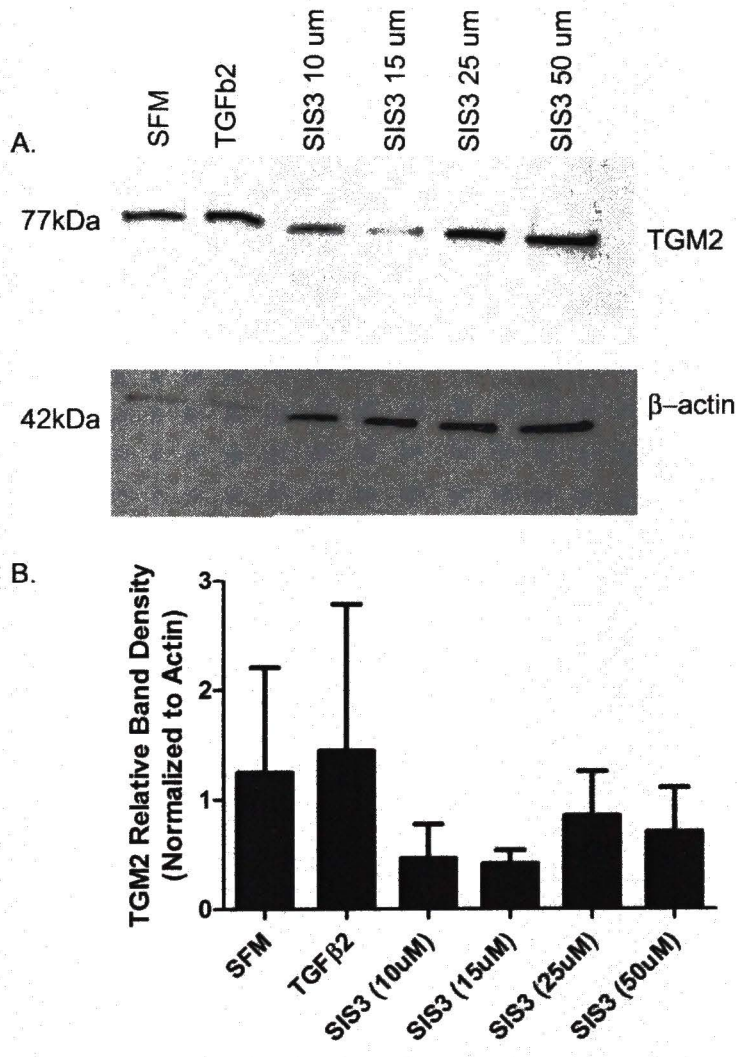

A.
SFM
TGFb2
SIS3 10 um
SIS3 15 um
SIS3 25 um
SIS3 50 um
77kDa
TGM2
42kDa
β–actin
B.
TGM2 Relative Band Density (Normalized to Actin)
0
1
2
3
SFM
TGFβ2
SIS3 (10uM)
SIS3 (15uM)
SIS3 (25uM)
SIS3 (50uM)

Figure 7.

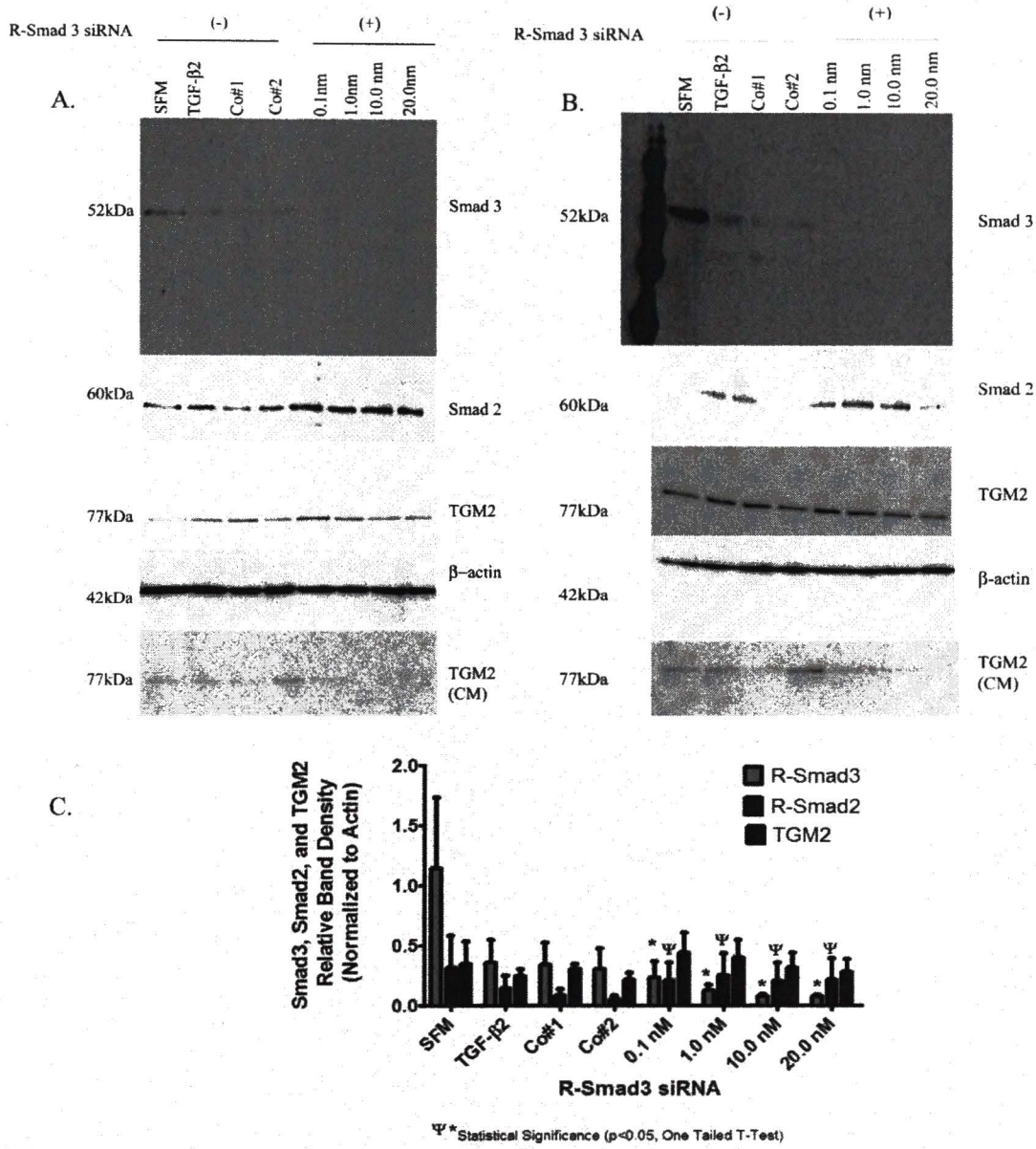

A.
R-Smad 3 siRNA
(-)
(+)
SFM
TGF-β2
Co#1
Co#2
0.1nm
1.0nm
10.0 nm
20.0nm
52kDa
Smad 3
60kDa
Smad 2
77kDa
TGM2
β–actin
42kDa
77kDa
TGM2 (CM)
B.
R-Smad 3 siRNA
(-)
(+)
SFM
TGF-β2
Co#1
Co#2
0.1 nm
1.0 nm
10.0 nm
20.0 nm
52kDa
Smad 3
60kDa
Smad 2
77kDa
TGM2
β-actin
42kDa
77kDa
TGM2 (CM)
C.
Smad3, Smad2, and TGM2 Relative Band Density (Normalized to Actin)
2.0
1.5
1.0
0.5
0.0
R-Smad3
R-Smad2
TGM2
SFM
TGF-β2
Co#1
Co#2
0.1 nM
1.0 nM
10.0 nM
20.0 nM
R-Smad3 siRNA
Ψ*Statistical Significance (p<0.05, One Tailed T-Test)

Figure 8.

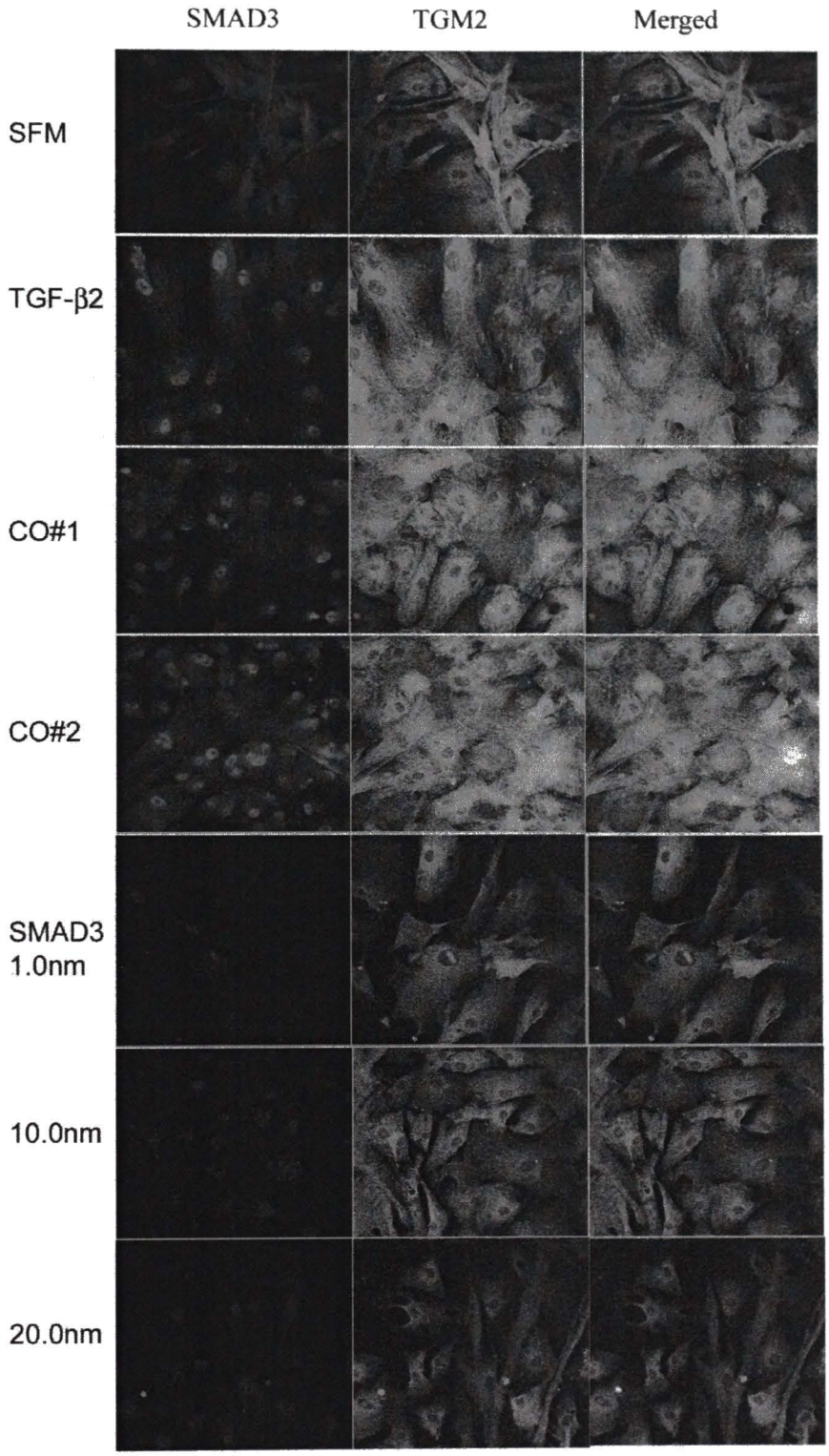


Figure 9.

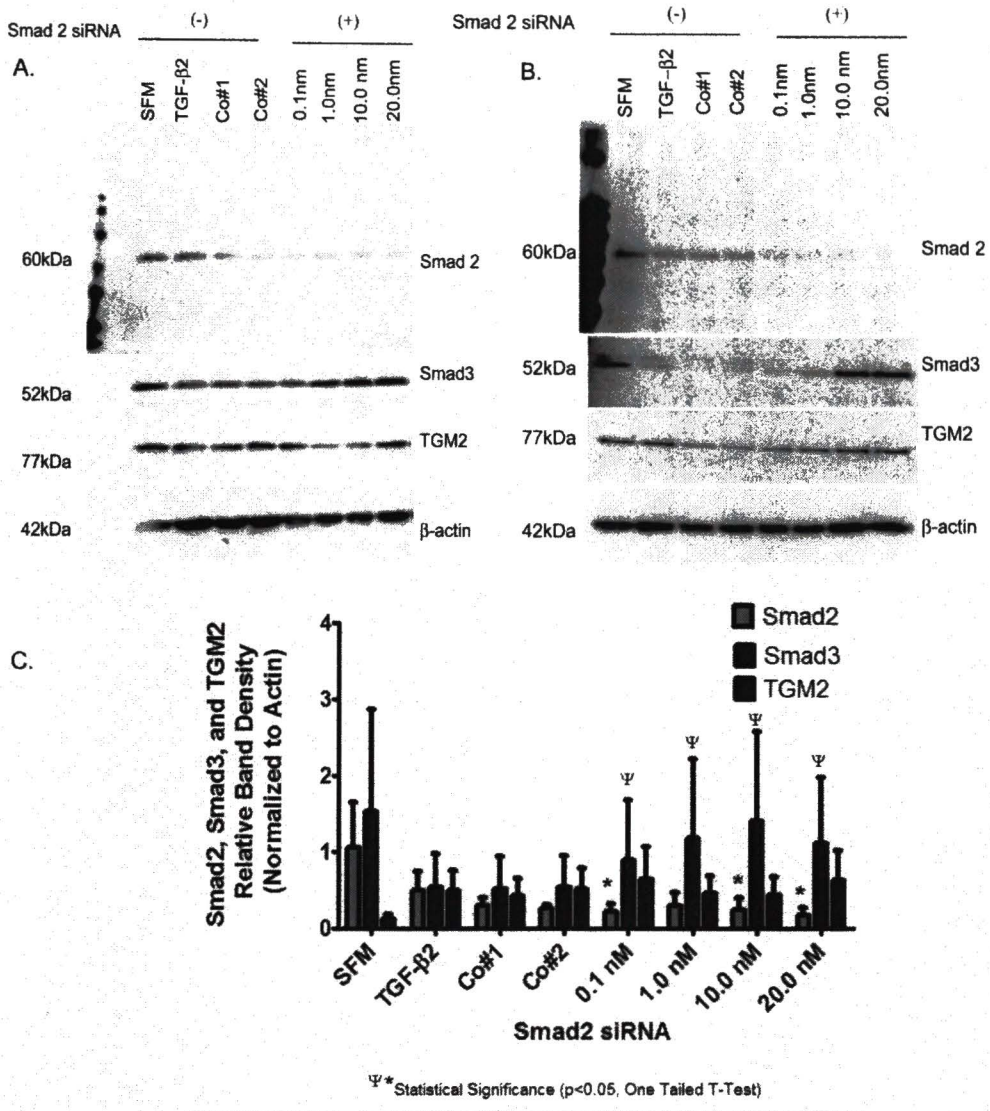

Smad 2 siRNA
(-)
(+)
A.
SFM
TGF-β2
Co#1
Co#2
0.1nm
1.0nm
10.0 nm
20.0nm
60kDa
Smad 2
52kDa
Smad3
77kDa
TGM2
42kDa
β-actin
B.
C.
Smad2
Smad3
TGM2
Smad2, Smad3, and TGM2
Relative Band Density
(Normalized to Actin)
0.1 nM
1.0 nM
10.0 nM
20.0 nM
Smad2 siRNA
Ψ*Statistical Significance (p<0.05, One Tailed T-Test)

Figure 10.

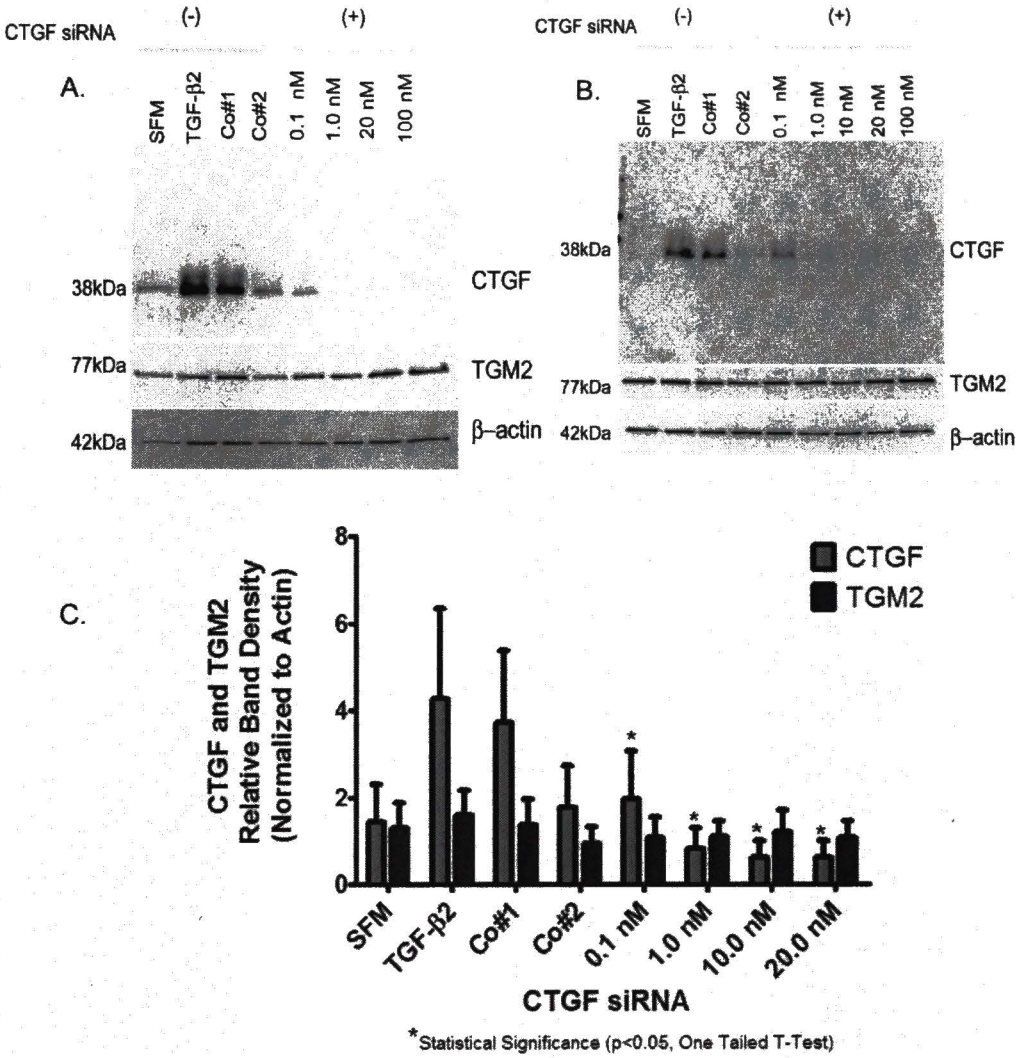

CTGF siRNA
(-)
(+)
A.
SFM
TGF-β2
Co#1
Co#2
0.1 nM
1.0 nM
20 nM
100 nM
38kDa
77kDa
42kDa
CTGF
TGM2
β–actin
B.
CTGF siRNA
(-)
(+)
SFM
TGF-β2
Co#1
Co#2
0.1 nM
1.0 nM
10 nM
20 nM
100 nM
38kDa
77kDa
42kDa
CTGF
TGM2
β–actin
C.
CTGF and TGM2
Relative Band Density
(Normalized to Actin)
CTGF
TGM2
SFM
TGF-β2
Co#1
Co#2
0.1 nM
1.0 nM
10.0 nM
20.0 nM
CTGF siRNA
*Statistical Significance (p<0.05, One Tailed T-Test)

Figure 11.

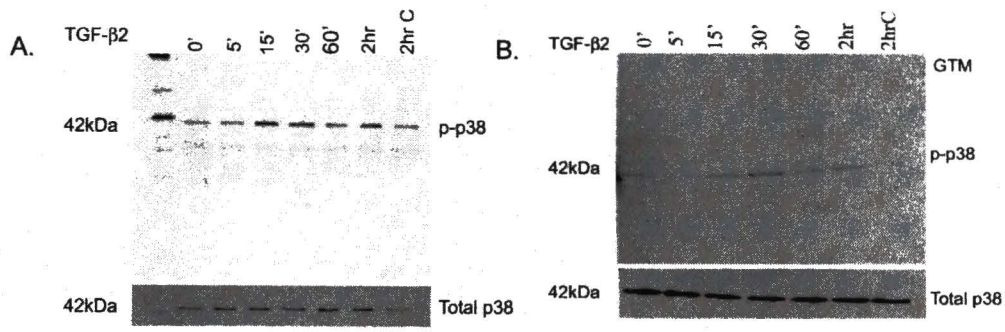

A.
TGF-β2
0'
5'
15'
30'
60'
2hr
2hr C
42kDa
p-p38
42kDa
Total p38
B.
TGF-β2
0'
5'
15'
30'
60'
2hr
2hrC
GTM
42kDa
p-p38
42kDa
Total p38

Figure 12

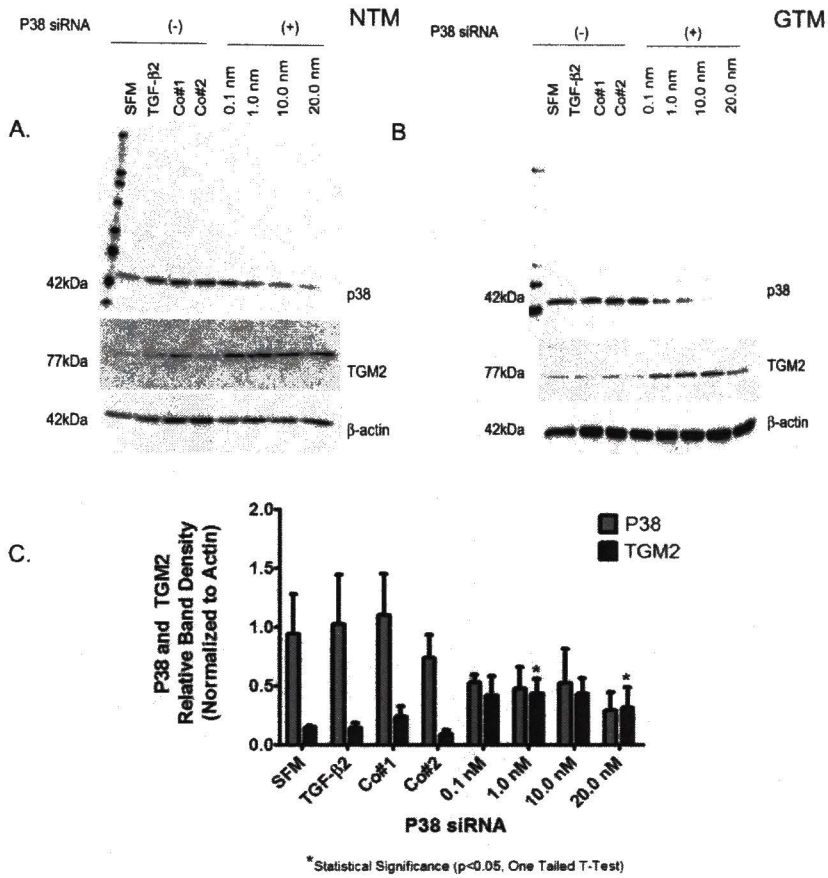

P38 siRNA
(-)
(+)
NTM
SFM
TGF-β2
Co#1
Co#2
0.1 nm
1.0 nm
10.0 nm
20.0 nm
A.
42kDa
p38
77kDa
TGM2
42kDa
β-actin
B
GTM
C.
P38 and TGM2
Relative Band Density
(Normalized to Actin)
2.0
1.5
1.0
0.5
0.0
P38
TGM2
0.1 nM
1.0 nM
10.0 nM
20.0 nM
P38 siRNA
*Statistical Significance (p<0.05, One Tailed T-Test)

Figure 13.

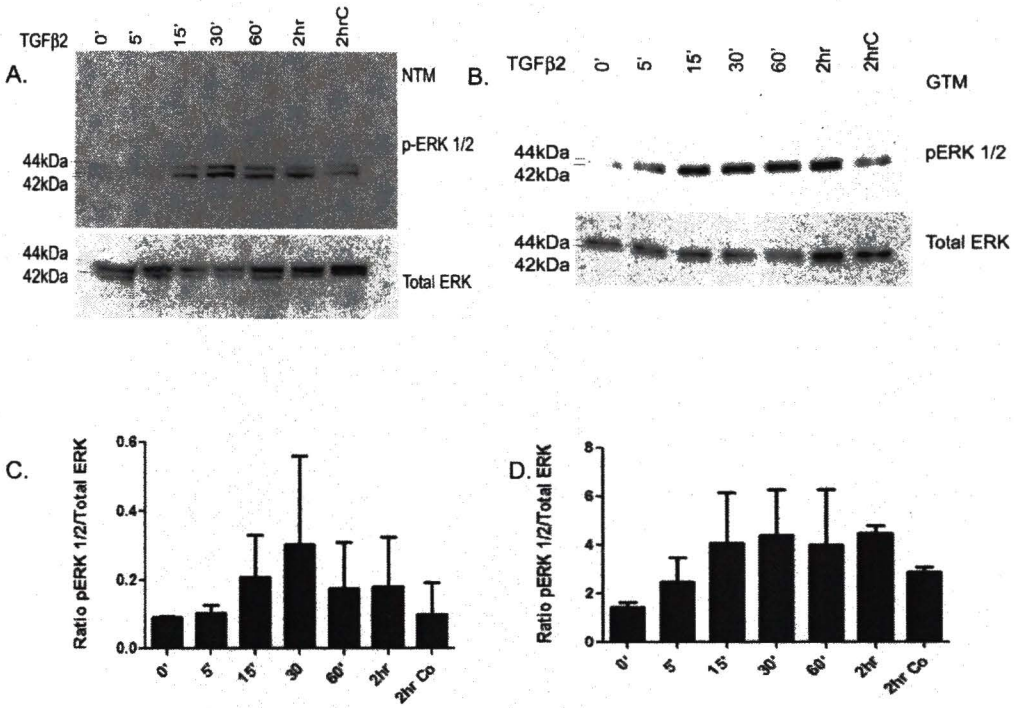

A.
TGFβ2
0'
5'
15'
30'
60'
2hr
2hrC
NTM
p-ERK 1/2
44kDa
42kDa
44kDa
42kDa
Total ERK
B.
TGFβ2
0'
5'
15'
30'
60'
2hr
2hrC
GTM
44kDa
42kDa
pERK 1/2
44kDa
42kDa
Total ERK
C.
Ratio pERK 1/2/Total ERK
0.6
0.4
0.2
0.0
0'
5'
15'
30
60'
2hr
2hr Co
D.
Ratio pERK 1/2/Total ERK
8
6
4
2
0
0'
5
15'
30'
60'
2hr
2hr Co

Figure 14

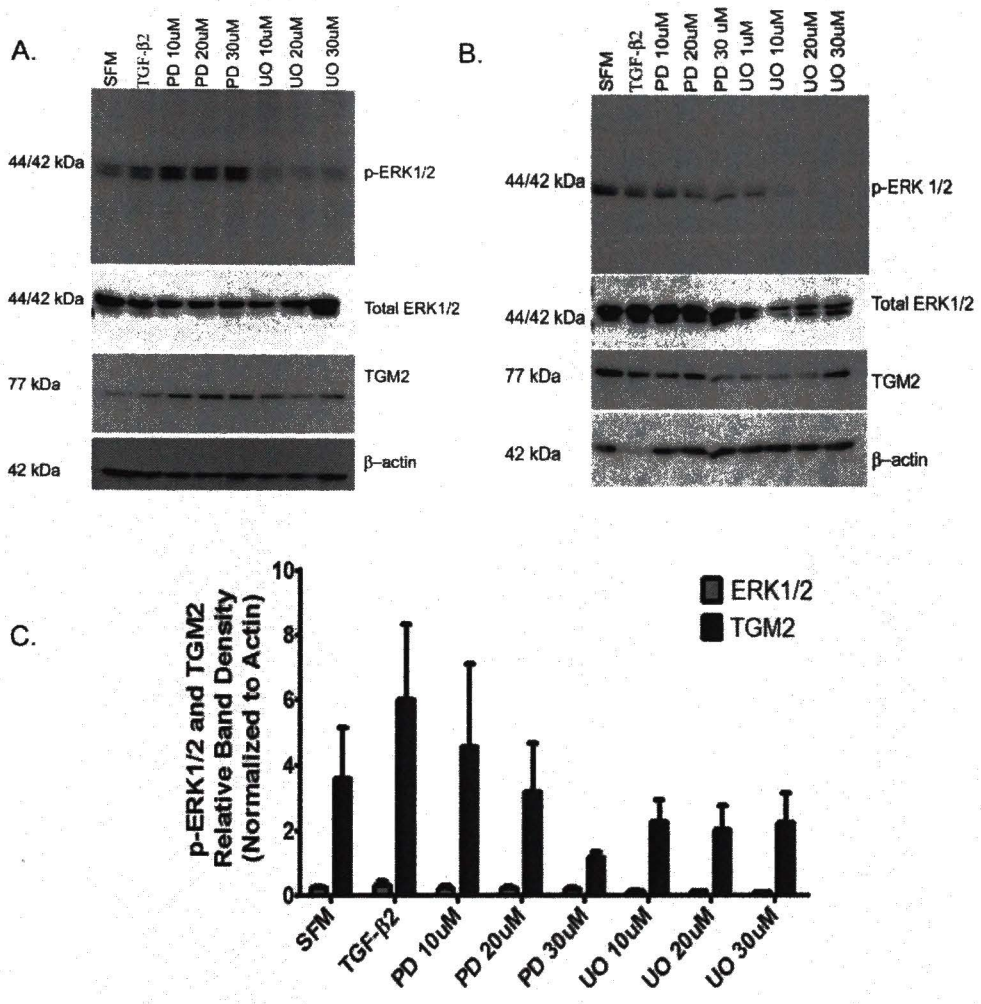

A.
SFM
TGF-β2
PD 10uM
PD 20uM
PD 30uM
UO 10uM
UO 20uM
UO 30uM
44/42 kDa
p-ERK1/2
44/42 kDa
Total ERK1/2
77 kDa
TGM2
42 kDa
β–actin
B.
SFM
TGF-β2
PD 10uM
PD 20uM
PD 30 uM
UO 1uM
UO 10uM
UO 20uM
UO 30uM
44/42 kDa
p-ERK 1/2
44/42 kDa
Total ERK1/2
77 kDa
TGM2
42 kDa
β–actin
C.
p-ERK1/2 and TGM2
Relative Band Density
(Normalized to Actin)
ERK1/2
TGM2
0
2
4
6
8
10
SFM
TGF-β2
PD 10uM
PD 20uM
PD 30uM
UO 10uM
UO 20uM
UO 30uM

Figure 15.

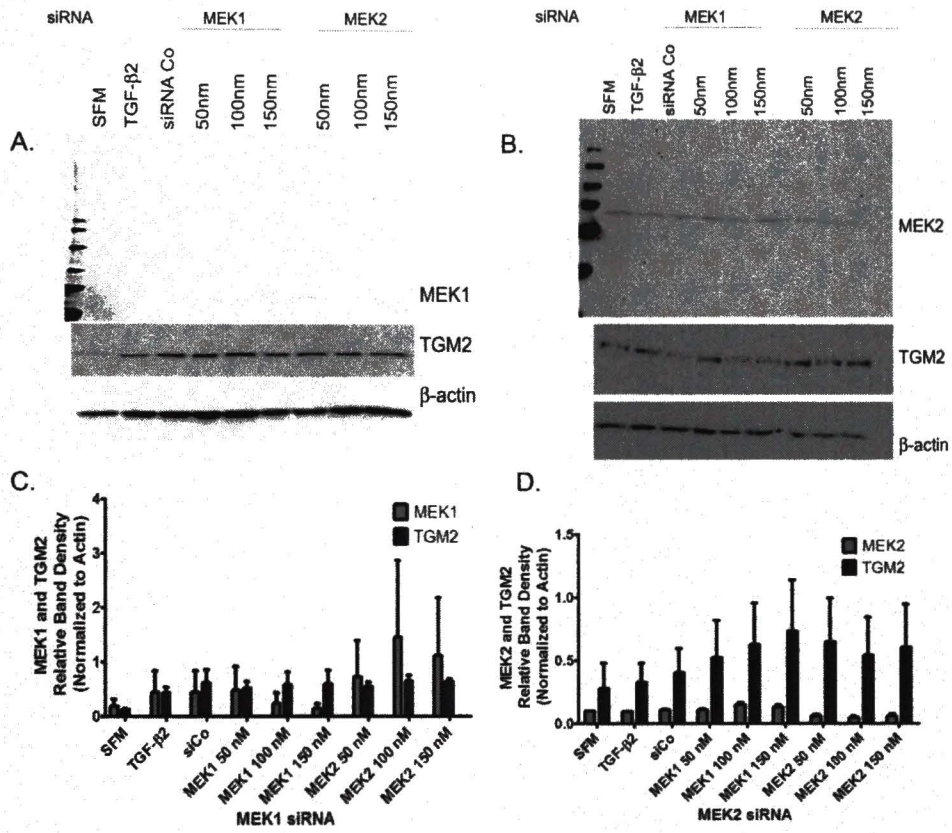

siRNA
MEK1
MEK2
SFM
TGF-β2
siRNA Co
50nm
100nm
150nm
A.
MEK1
TGM2
β-actin
B.
MEK2
TGM2
β-actin
C.
MEK1 and TGM2 Relative Band Density (Normalized to Actin)
MEK1
TGM2
SFM
TGF-β2
siCo
MEK1 50 nM
MEK1 100 nM
MEK1 150 nM
MEK2 50 nM
MEK2 100 nM
MEK2 150 nM
MEK1 siRNA
D.
MEK2 and TGM2 Relative Band Density (Normalized to Actin)
MEK2
TGM2
MEK2 siRNA

Figure 16.

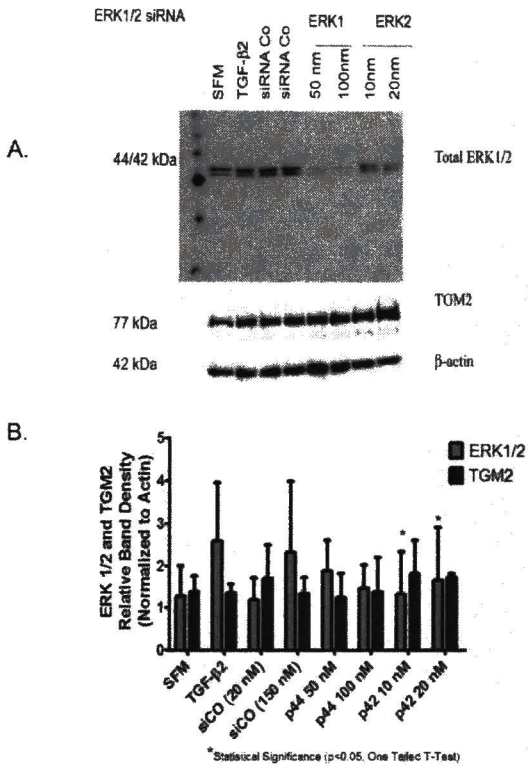

A.
ERK1/2 siRNA
ERK1
ERK2
SFM
TGF-β2
siRNA Co
siRNA Co
50 nm
100nm
10nm
20nm
44/42 kDa
Total ERK1/2
TGM2
77 kDa
42 kDa
β-actin
B.
ERK 1/2 and TGM2 Relative Band Density (Normalized to Actin)
ERK1/2
TGM2
SFM
TGF-β2
siCO (20 nM)
siCO (150 nM)
p44 50 nM
p44 100 nM
p42 10 nM
p42 20 nM
*Statistical Significance (p<0.05, One Tailed T-Test)

Figure 17.

References

1. Yue BY. The extracellular matrix and its modulation in the trabecular meshwork. *Surv.Ophthalmol.* 1996;40:379-390.

2. Rohen JW, Witmer R. Electrn microscopic studies on the trabecular meshwork in glaucoma simplex. *Albrecht Von Graefes Arch.Klin.Exp.Ophthalmol.* 1972;183:251-266.

3. Lutjen-Drecoll E. Functional morphology of the trabecular meshwork in primate eyes. *Prog.Retin.Eye Res.* 1999;18:91-119.

4. Welge-Lussen U, May CA, Lutjen-Drecoll E. Induction of tissue transglutaminase in the trabecular meshwork by TGF-beta1 and TGF-beta2. *Invest.Ophthalmol.Vis.Sci.* 2000;41:2229-2238.

5. Fuchshofer R, Birke M, Welge-Lussen U, Kook D, Lutjen-Drecoll E. Transforming growth factor-beta 2 modulated extracellular matrix component expression in cultured human optic nerve head astrocytes. *Invest.Ophthalmol.Vis.Sci.* 2005;46:568-578.

6. Folk JE, Finlayson JS. The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases. *Adv.Protein Chem.* 1977;31:1-133.

7. Chen JS, Mehta K. Tissue transglutaminase: An enzyme with a split personality. *Int.J.Biochem.Cell Biol.* 1999;31:817-836.

8. Kim SY, Jeitner TM, Steinert PM. Transglutaminases in disease. *Neurochem.Int.* 2002;40:85-103.

9. Zenkel M, Poschl E, von der Mark K, et al. Differential gene expression in pseudoexfoliation syndrome. *Invest.Ophthalmol.Vis.Sci.* 2005;46:3742-3752.

10. Tovar-Vidales T, Roque R, Clark AF, Wordinger RJ. Tissue transglutaminase expression and activity in normal and glaucomatous human trabecular meshwork cells and tissues. *Invest.Ophthalmol.Vis.Sci.* 2008;49:622-628.

11. Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. *FASEB J.* 2004;18:816-827.

12. Fuchshofer R, Yu AH, Welge-Lussen U, Tamm ER. Bone morphogenetic protein-7 is an antagonist of transforming growth factor-beta2 in human trabecular meshwork cells. *Invest.Ophthalmol.Vis.Sci.* 2007;48:715-726.

13. Gordon MO, Beiser JA, Brandt JD, et al. The ocular hypertension treatment study: Baseline factors that predict the onset of primary open-angle glaucoma. *Arch.Ophthalmol.* 2002;120:714-20; discussion 829-30.

14. Kass MA, Heuer DK, Higginbotham EJ, et al. The ocular hypertension treatment study: A randomized trial determines that topical ocular hypotensive medication delays or prevents the onset of primary open-angle glaucoma. *Arch.Ophthalmol.* 2002;120:701-13; discussion 829-30.

15. [Anonymous]. The advanced glaucoma intervention study (AGIS): 7. the relationship between control of intraocular pressure and visual field deterioration.the AGIS investigators. *Am.J.Ophthalmol.* 2000;130:429-440.

16. Rohen JW. Why is intraocular pressure elevated in chronic simple glaucoma? anatomical considerations. *Ophthalmology* 1983;90:758-765.

17. Lutjen-Drecoll E. Morphological changes in glaucomatous eyes and the role of TGFbeta2 for the pathogenesis of the disease. *Exp.Eye Res.* 2005;81:1-4.

18. Bradley JM, Vranka J, Colvis CM, et al. Effect of matrix metalloproteinases activity on outflow in perfused human organ culture. *Invest.Ophthalmol.Vis.Sci.* 1998;39:2649-2658.

19. Pang IH, Fleenor DL, Hellberg PE, Stropki K, McCartney MD, Clark AF. Aqueous outflow-enhancing effect of tert-butylhydroquinone: Involvement of AP-1 activation and MMP-3 expression. *Invest.Ophthalmol.Vis.Sci.* 2003;44:3502-3510.

20. Fuchshofer R, Welge-Lussen U, Lutjen-Drecoll E. The effect of TGF-beta2 on human trabecular meshwork extracellular proteolytic system. *Exp.Eye Res.* 2003;77:757-765.

21. Fleenor DL, Shepard AR, Hellberg PE, Jacobson N, Pang IH, Clark AF. TGFbeta2-induced changes in human trabecular meshwork: Implications for intraocular pressure. *Invest.Ophthalmol.Vis.Sci.* 2006;47:226-234.

22. Wordinger RJ, Fleenor DL, Hellberg PE, et al. Effects of TGF-beta2, BMP-4, and gremlin in the trabecular meshwork: Implications for glaucoma. *Invest.Ophthalmol.Vis.Sci.* 2007;48:1191-1200.

23. Bradley JM, Anderssohn AM, Colvis CM, et al. Mediation of laser trabeculoplasty-induced matrix metalloproteinase expression by IL-1beta and TNFalpha. *Invest.Ophthalmol.Vis.Sci.* 2000;41:422-430.

24. Wordinger RJ, Clark AF, Agarwal R, et al. Cultured human trabecular meshwork cells express functional growth factor receptors. *Invest.Ophthalmol.Vis.Sci.* 1998;39:1575-1589.

25. Zhu HJ, Burgess AW. Regulation of transforming growth factor-beta signaling. *Mol.Cell Biol.Res.Commun.* 2001;4:321-330.

26. Verrecchia F, Mauviel A. Transforming growth factor-beta signaling through the smad pathway: Role in extracellular matrix gene expression and regulation. *J.Invest.Dermatol.* 2002;118:211-215.

27. Tripathi RC, Li J, Chan WF, Tripathi BJ. Aqueous humor in glaucomatous eyes contains an increased level of TGF-beta 2. *Exp.Eye Res.* 1994;59:723-727.

28. Inatani M, Tanihara H, Katsuta H, Honjo M, Kido N, Honda Y. Transforming growth factor-beta 2 levels in aqueous humor of glaucomatous eyes. *Graefes Arch.Clin.Exp.Ophthalmol.* 2001;239:109-113.

29. Picht G, Welge-Luessen U, Grehn F, Lutjen-Drecoll E. Transforming growth factor beta 2 levels in the aqueous humor in different types of glaucoma and the relation to filtering bleb development. *Graefes Arch.Clin.Exp.Ophthalmol.* 2001;239:199-207.

30. Ochiai Y, Ochiai H. Higher concentration of transforming growth factor-beta in aqueous humor of glaucomatous eyes and diabetic eyes. *Jpn.J.Ophthalmol.* 2002;46:249-253.

31. Ozcan AA, Ozdemir N, Canataroglu A. The aqueous levels of TGF-beta2 in patients with glaucoma. *Int.Ophthalmol.* 2004;25:19-22.

32. Gottanka J, Chan D, Eichhorn M, Lutjen-Drecoll E, Ethier CR. Effects of TGF-beta2 in perfused human eyes. *Invest.Ophthalmol.Vis.Sci.* 2004;45:153-158.

33. Ross S, Hill CS. How the smads regulate transcription. *Int.J.Biochem.Cell Biol.* 2008;40:383-408.

CHAPTER V

SUMMARY

The trabecular meshwork (TM) is an active avascular tissue composed of trabecular beams covered with TM cells whose functions include contraction, relaxation, phagocytosis, and synthesis of growth factors and extracellular matrix protein [1-3]. The primary function of the TM is to allow aqueous humor outflow into the canal of Schlemm for regulation of IOP. The trabecular meshwork plays a major role in regulating aqueous humor (AH) outflow and IOP. Trabecular meshwork cells are in constant contact with the AH that contains many growth factors. Transforming growth factor beta2 is elevated in the AH of glaucomatous eyes and TM cells respond to TGF-β2 by increasing the synthesis of ECM components.

According to Welge-Lüssen and co-workers (2000), there is increased expression of tissue transglutaminase (TGM2) after exogenous treatment of TM cells with TGF-β1 and TGF-β2 [4]. This suggests that higher levels of TGF-β2 in the AH of glaucomatous eyes may result in increased expression and activation of endogenous TGM2 in TM cells. The goal of the first study (Chapter II) was to better understand the role of TGM2 in glaucoma. To fulfill this goal, NTM and GTM cells/tissues were evaluated for (a) protein expression and enzyme activity of TGM2, and (b) protein and enzymatic differences of TGM2 between GTM and NTM

cells/tissues. Protein analysis was performed by Western blot and immunohistochemistry, which demonstrated the presence of TGM2 in both NTM and GTM cells/tissues. Enzyme activity was biologically active in both NTM and GTM cells/tissues as measured by the incorporation of biotin-cadaverine, a pseudosubstrate for TGM2. In addition, both protein and enzyme activity levels were induced in GTM compared to NTM cells/tissues. Increased cross-linking of fibronectin by TGM2 was observed utilizing ε–(γ-glutamyl) lysine isopeptide (GGEL) in GTM tissues compared to NTM tissues. Taken together these results suggest that TGM2 is involved in the pathophysiology of glaucoma.

The upregulation of TGM2 expression and activation via TGF-β2 could occur through the canonical TGF-β signaling pathway (Smads) or via another pathway such as the MAPK signaling pathway. The goal of the second study (Chapter IV), was to (a) examine the induction of TGM2 by TGF-β isoforms, and (b) to identify the signaling pathway(s) utilized by TGF-β2 to regulate TGM2 expression. Western blot analysis revealed that all TGF-β isoforms increased TGM2 protein levels. All TGF-β isoforms elevated TGM2 enzymatic levels measured by the incorporation of biotin-cadaverine. However, since the TGF-β2 isoform has been directly linked to glaucoma the remainder of the studies focused on TGF-β2. Since exogenous TGF-β2 regulates TGM2, the endogenous levels for TGF-β2 were examined in NTM and GTM cell lines and tissues. The levels for TGF-β2 were elevated in GTM cells and tissues compared to NTM cells and tissues. This suggests that TGF-β2 may upregulate TGM2 via canonical or non-canonical signaling pathways, resulting in increased expression and activation of TGM2. The canonical downstream signaling pathway for TGF-β2 uses intracellular R-Smad proteins 2/3. Densitometric analysis revealed TGF-β2 activated phosphorylated R-Smad3 at 15 minutes, with

maximum activation at 30 minutes. Next, SIS3 was examined to determine its effect to reduce or inhibit TGM2 protein levels in TM cell lines. Densitometric analysis revealed a reduction of TGM2 protein levels. Then, R-Smad2 and 3 siRNA were examined in both NTM and GTM cell lines. There were significant reductions of R-Smad2 and 3 and a modest decrease of TGM2 protein levels. The effects of TGF-β2 on ECM turnover has been reported to be mediated by connective tissue growth factor (CTGF). Fuchshofer and co-workers (2007), demonstrated that TGF-β2 induces CTGF in TM cell cultures[5]. And Fuchshofer and co-workers (2005), demonstrated that TGF-β2 induces TGM2 mRNA and protein expression and this action was mediated via CTGF in ONH astrocytes[6]. Thus, NTM and GTM cells were treated with CTGF siRNA, however, TM cells did not show the same inhibitory effects on TGM2 as was reported for ONH astrocytes. Next, the non-canonical signaling pathways, such as p38 and ERK1/2 were evaluated to determine if exogenous TGF-β2 would activate their signaling pathways. Both p38 and ERK1/2 responded to TGF-β2, however, p38 and ERK1/2 siRNA did not decrease TGM2 protein levels. Thus, the results suggest, for the first time, that TGM2 is up-regulated in glaucomatous TM cells and tissues and is regulated primarily by R-Smads and the canonical TGF-β signaling pathway.

Based upon the results of this study a schematic model of potential TGF-β2 signaling has been developed (Figure 1). Increased TGF-β2 stimulates its canonical signaling pathway by initiation of ligand binding to TGF-β2 receptors II, transphosphorylation of receptor I, followed by the subsequent phosphorylation R-Smad2/3, which recruit R-Smad4. The entire complex translocates into the nucleus and regulates gene expression. Excessive TGF-β2 signaling has been implicated in the induction of TGM2 and the over abundance of ECM proteins, such as

fibronectin. It is possible that within the TM, TGF-β2 induces TGM2 via activation of its canonical signaling pathway, R-Smad2/3. Thus, TGM is involved in cross-linking ECM proteins resulting in increased outflow resistance followed by elevated IOP and eventually glaucoma (Figure 1).

Future experiments to further elucidate the role of TGM2 in the trabecular meshwork, may include the following: (a) simultaneous siRNA studies for Smad2 and Smad3 to determine if both Smads or one preferentially knocks down TGM2 expression in TM cells; (b) siRNA knockdown of TGM2 to determine if TGM2 siRNA can block TGF-β2 effects on ECM synthesis; (c) over-expression of TGM2 using an adenovirus in the ex-vivo perfused human ocular anterior segments to determine the effects of TGM2 on the IOP; (d) collection of the perfused media of the anterior segments to determine ECM changes in the perfused TM utilizing Western blot and ELISA assays; (e) process the anterior segment TM tissue from the ex-vivo human ocular anterior segments for both light microscopy and electron microscopy to determine ECM build up and/or morphology changes; and (f) determine the role of BMP2 and/or BMP4 and their respective effects on the TGM2 expression and activity.

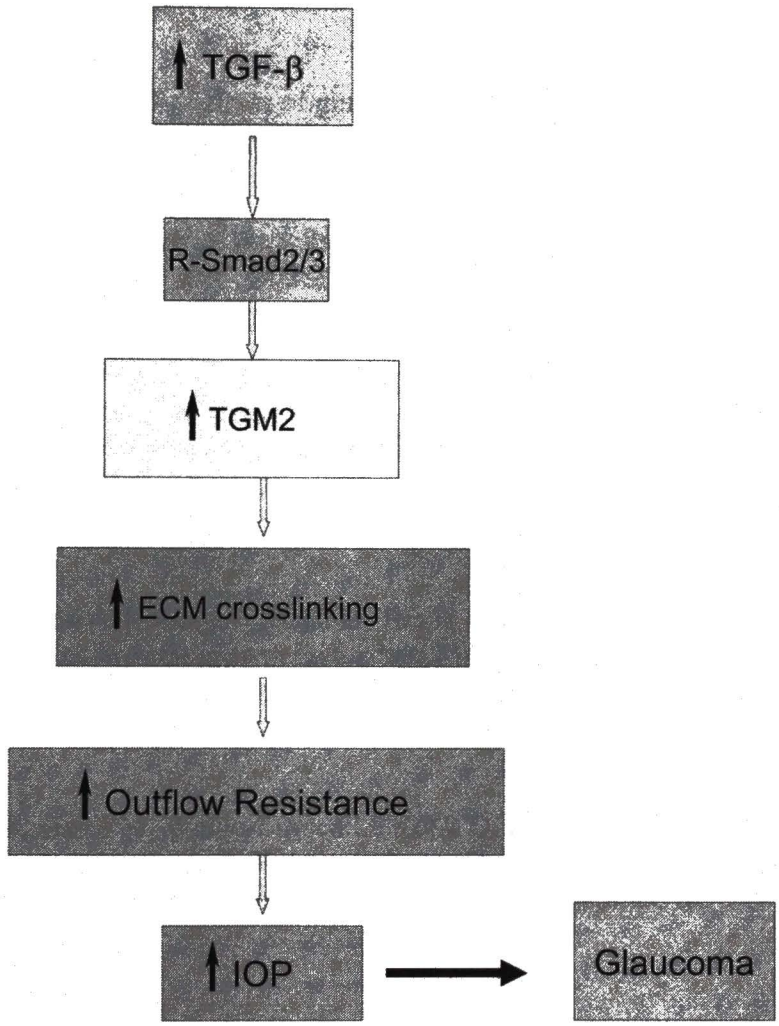

TGF-β
R-Smad2/3
TGM2
ECM crosslinking
Outflow Resistance
IOP
Glaucoma

Figure 1.

References

1. Llobet A, Gasull X, Gual A. Understanding trabecular meshwork physiology: A key to the control of intraocular pressure? *News Physiol.Sci.* 2003;18:205-209.

2. Wordinger RJ, Clark AF. Effects of glucocorticoids on the trabecular meshwork: Towards a better understanding of glaucoma. *Prog.Retin.Eye Res.* 1999;18:629-667.

3. Borras T. Gene expression in the trabecular meshwork and the influence of intraocular pressure. *Prog.Retin.Eye Res.* 2003;22:435-463.

4. Welge-Lussen U, May CA, Lutjen-Drecoll E. Induction of tissue transglutaminase in the trabecular meshwork by TGF-beta1 and TGF-beta2. *Invest.Ophthalmol.Vis.Sci.* 2000;41:2229-2238.

5. Fuchshofer R, Yu AH, Welge-Lussen U, Tamm ER. Bone morphogenetic protein-7 is an antagonist of transforming growth factor-beta2 in human trabecular meshwork cells. *Invest.Ophthalmol.Vis.Sci.* 2007;48:715-726.

6. Fuchshofer R, Birke M, Welge-Lussen U, Kook D, Lutjen-Drecoll E. Transforming growth factor-beta 2 modulated extracellular matrix component expression in cultured human optic nerve head astrocytes. *Invest.Ophthalmol.Vis.Sci.* 2005;46:568-578.