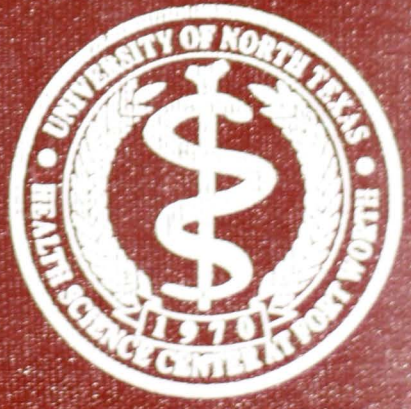
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Glaucoma, of which primary open-angle glaucoma (POAG) is the most common form, is the second leading cause of irreversible blindness in the world. Ocular hypertension is an important risk factor in the development of POAG. The human trabecular meshwork (HTM) is the major regulation site for aqueous humor outflow thus controlling intraocular pressure. In POAG, there are specific morphological and pathological changes in the HTM, including an increase in extracellular matrix components and a decrease in the number of HTM cells.

Myocilin (also known as GLC1A or TIGR) is associated with hypertensive POAG by both genetic linkage analysis and glucocorticoid induction studies. Brain-derived neurotrophic factor (BDNF) and transforming growth factor-beta isoforms (TGFβ1–3) have been shown to be present both in normal cultured HTM cells and aqueous humor. In addition, biologically active TGFβ2 levels are increased in the aqueous humor of POAG patients. Mechanical stretch, an important factor in HTM during intraocular hypertension, may up-regulate the expression of BDNF in the HTM cells. Therefore, BDNF and TGFβ2 may be modulators of extracellular proteins in response to the hypertensive glaucomatous injury. However, the regulation of myocilin expression by these growth

factors in the HTM has not been studied. Moreover, HTM cells may signal each other via paracrine and autocrine pathways involving BDNF and TGFβ2.

In this study, HTM cells were isolated and cultured in vitro. Myocilin gene expression and protein secretion by normal and glaucomatous HTM cells were compared. The regulatory effects of BDNF and/or TGFβ2 on myocilin gene expression and protein secretion by normal and glaucomatous HTM cells were also examined, as well as the reciprocal induction between BDNF and TGFβ2 gene expression and protein secretion. The interdenpendence between BDNF and TGFβ2 in regulating myocilin expression was determined. The results of the study established the regulatory effects of BDNF and TGFβ2 on myocilin expression as well as on each other. It is possible that both BDNF and TGFβ2 interact with each other in response to an increase of intraocular pressure through paracrine/autocrine mechanisms, resulting in differential gene expression of myocilin.

MYOCILIN REGULATION BY BRAIN-DERIVED NEUROTROPHIC FACTOR AND TRANSFORMING GROWTH FACTOR-BETA2 IN NORMAL AND GLAUCOMATOUS HUMAN TRABECULAR MESHWORK CELLS

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CHAPTER I

INTRODUCTION

Glaucoma is the second leading cause of irreversible blindness in the world, affecting approximately 70 million people (Liesegang, 1996). It is a progressive optic neuropathy characterized by optic nerve head cupping and visual field loss as a result of retinal ganglion cell apoptosis and nerve fiber layer damage (Shields *et al.*, 1996). The risk factors for glaucoma are intraocular hypertension, old age, African descent, myopia and a family history of the disease (Flanagan, 1998). Detection of glaucoma includes both functional and structural measurements. Standard perimetry is the most common assessment for visual field loss. Other methods such as short-wavelength automated perimetry and frequency-doubling perimetry are also used. Optic disc morphological changes, such as increased cupping, neuroretinal rim thinning are essential for structurally identifying glaucoma. The management of glaucoma is based on lowering intraocular pressure to prevent further optic nerve head damage. Drug therapy as well as laser trabeculoplasty and incisional glaucoma surgery are currently used to achieve the goal of maintaining adequate vision for patients during their lifetime (Medeiros and Weinreb, 2002).

A. Primary Open Angle Glaucoma and Intraocular Pressure

Primary open-angle glaucoma (POAG) is the most common form of glaucoma in the U.S. and Europe, accounting for over 60% of all cases (Liesegang, 1996). POAG is characterized by recognizable glaucomatous structural and functional damage with a normal appearing anterior chamber angle (Liesegang, 1996). POAG can be subclassified into juvenile (<40 years old) and adult onset (>40 years old) forms (Shimizu *et al.*, 2000). It can also be divided using intraocular pressure into two subtypes, hypertensive (>21 mmHg) and normal tensive (<21 mmHg) (Medeiros and Weinreb, 2002). Although the exact etiology of POAG is still a mystery, elevated intraocular pressure is by far the most important risk factor. Among patients with asymmetrical IOP and visual field loss in one eye, the eye with the field loss almost invariably has the higher IOP (Mao *et al.*, 1991). Several observational studies of patients treated for glaucoma indicate that better controlled IOP is associated with a better prognosis (Odberg, 1987; Heijl *et al.* 2002; AGIS, 2000; Lee *et al.*, 2003).

B. Aqueous Humor and Intraocular Pressure

Aqueous humor (a) conserves the structural integrity and normal optical functioning of the eye, (b) maintains the metabolic balances for avascular tissues including the cornea, lens, and trabecular meshwork, (c) facilitates cellular and humoral immune responses in inflammation and infection, and (d) provides a reducing environment via through control of ascorbate levels (Krupin and Civan, 1996). Aqueous humor is derived from blood circulating in the capillaries of the ciliary processes of the ciliary body. When the normal blood-aqueous barrier is intact, aqueous humor production is dependent upon

energy-consuming, active-transport secretory processes of the ciliary epithelium, and secondary ultrafiltration in the ciliary process vasculature and the adjacent stromal layer (Lutjen-Drecoll and Rohen, 1996). Aqueous humor exits the anterior segment of the eye through three routes: pressure-sensitive flow (trabecular meshwork flow, the major portion), pressure-insensitive flow (uveoscleral flow), and across the iris. Intraocular pressure reflects a balance between the rate of aqueous humor formation and the rate of fluid outflow through the trabecular meshwork and the uveoscleral pathways (Kaufman, 1996).

C. Human Trabecular Meshwork (HTM)

The human trabecular meshwork is the primary regulator of the rate and conductance of aqueous humor outflow, and hence controls ocular rigidity and intraocular pressure (Kaufman, 1996). The trabecular meshwork (TM) is located at the corneoscleral junction in the anterior segment of the human eye (Junqueira *et al.*, 1998). It functions as a one-way self-cleaning filter that allows cellular debris and particles to be flushed out, and prevents backflow from Schlemm's canal. The human TM consists of four parts: the endothelial layer of the inner wall of Schlemm's canal (outermost layer), the juxtacanalicular tissue (also known as the cribriform layer), the corneoscleral meshwork and the uveal meshwork (innermost layer) (Lutjen-Drecoll and Rohen, 1996).

D. Human Trabecular Meshwork Cells

The trabecular meshwork normally contains three basic cells: trabecular cells, cribriform cells, and the endothelial cell lining of Schlemm's canal (Lutjen-Drecoll and Rohen, 1996). The trabecular cells, are derived from neural crest cells and reside on a

basal lamina within the corneoscleral and uveal meshwork. Trabecular meshwork cells are involved in the self-cleaning processes of the trabecular meshwork via phagocytosis, and tissue repair via fibrillogenesis. The corneoscleral region was initially considered to be the "reticuloendothelial system (RES) of the eye", and was thought to be capable of cleaning the intertrabecular spaces of debris and particles by active cellular processes such as phagocytosis (Rohen, 1978). The cribriform cells are scattered randomly within the extracellular matrix of the cribriform layer underneath the endothelial lining of the Schlemm's canal. They contain mushroom-like cytoplasmic processes for contacting the canal endothelium. They may be responsible for extracellular matrix production. The endothelial lining of Schlemm's canal, derived from mesodermal tissue, produces the underlying basement membrane material and is capable of developing pores, vacuoles, and transcellular microchannels, through which aqueous fluid and particles can pass. The biological activities of the trabecular cells may be as important for the regulation of aqueous outflow as the number and size of pores in the inner wall endothelium, which are responsible for the aqueous humor outflow resistance (Ritch *et al.*, 1996).

E. Extracellular Matrix (ECM) within the Human Trabecular Meshwork

The ECM of the human trabecular meshwork is the structural support that allows the human TM cells to function as a tissue to contribute significantly to aqueous humor resistance. The extracellular matrices of human TM contains many basement membrane-associated and ground substance components including collagen subtype I, III-VI, laminin, fibronectin, heparan sulfate proteoglycan, elastic fibers, and dermatan sulfate, chondroitin sulfate proteoglycans. Heparan sulfate proteoglycan expression decreases

with age, while fibronectin expression increases with glaucoma and aging. Trabecular meshwork ECM can be regulated via changing the rate of either matrix synthesis or turnover, which in turn alters the outflow facility. Ascorbate mediates the synthesis of glycosaminoglycan, fibronectin, laminin, and collagen. The family of matrix metalloproteinase (MMP) is also involved in the regulation of the aqueous humor outflow pathway. Stromelysin (MMP-3) cleaves laminin, fibronectin, types IV and V collagen, proteoglycans, which increases the outflow facility (Hernandez and Gong, 1996).

F. Human Trabecular Meshwork and Primary Open Angle Glaucoma

In POAG, there is increased deposition of extracellular plaque material in the cribriform layer and beneath the inner wall of the canal of Schlemm (Rohen *et al.*, 1993; Alvarado *et al.*, 1986; Lutjen-Drecoll *et al.*, 1986). It was suggested that a depletion of hyaluronic acid and the accumulation of chondroitin sulfates increase aqueous outflow resistance in POAG (Knepper *et al.*, 1996). In eyes with advanced POAG, axon counts in the optic nerve head showed a significant negative correlation with the amount of sheath-derived (SD) plaque material in the cribriform region (i.e. eyes with lower axon counts had larger amounts of SD plaques). Thus, increasing severity of optic nerve damage in POAG is accompanied by an increase in the amount of SD plaque material in the trabecular meshwork (Gottanka *et al.*, 1997). There is also hyalinization of the trabecular beams, thickening of the basement membrane lining the beams, loss of trabecular cells covering the beams, and fusion of denuded beams (Rohen *et al.*, 1993). The pathophysiology of the human TM in POAG has been characterized by an increase in extracellular matrix components (Rohen *et al.*, 1993; Yue, 1996). However, the

phagocytic ability of the trabecular meshwork appears similar between eyes with POAG and normal eyes in perfusion organ culture (Matsumoto and Johnson, 1997). Human TM cells usually appear as small inactive cells lining the trabecular beams, but they are capable of being transformed into metabolically active cells containing many specific active organelles (Ritch *et al.*, 1996). In fact, human TM cells appear to enlarge, spread, and show signs of activation with enlarged nuclei and increased free ribosomes in POAG eyes (Rohen *et al.*, 1993). These signs of activation suggest increased protein synthesis in human TM cells.

G. Glucocorticoids and Primary Open Angle Glaucoma

As dexamethasone (DEX) and other potent anti-inflammatory steroids were developed and used as topical eye drops, a clinically significant rise in IOP was observed in many patients receiving long-term therapy. Patients who developed corticosteroid-related elevations in IOP were referred to as "steroid responders", and showed a marked decrease in outflow facility with a normal-appearing angle, which simulates some of the characteristics of POAG (Armaly, 1963a). In DEX-induced ocular hypertension in perfusion-cultured human eyes, TM cells were also activated, along with the appearance of thickened trabecular beams and juxtacanalicular tissue, decreased intertrabecular spaces, and increased amounts of amorphogranular extracellular material (Clark *et al.*, 1995). These changes are similar to changes that occur in POAG. This may be the result of DEX regulation of the extracellular matrix in human TM. Dexamethasone treatment can alter the glycosaminoglycan (GAG) profile by increasing chondroitin sulfate and decreasing hyaluronate deposition, increase the deposition of fibronectin and laminin,

inhibit matrix metalloproteinases stromelysin and gelatinase as well as tissue plasminogen activator activities (Wordinger and Clark, 1999). Besides the morphological changes in the human TM following DEX administration, the magnitude of the DEX-induced ocular hypertension in glaucomatous eyes is much greater than that of normal eyes. This phenomenon is also observed in low-tension glaucoma patients whose ocular pressures usually do not differ significantly from normal (Armaly, 1963b). In contrast to the general population, patients with POAG (Becker and Mills, 1963; Armaly, 1963b), and descendants or siblings of POAG patients (Paterson, 1966; Bartlett *et al.*, 1993), are at greater risk for being steroid responders. These observations suggest that steroid responsiveness is closely associated with POAG. Therefore glucocorticoid administration has been suggested to be a model for POAG.

H. Myocilin

Myocilin was originally discovered as a cytoskeletal associated protein involved in the morphogenesis of the basal body, a major microtubule organization center in cilia (Kubota, 1997). The MYOC gene contains 3 exons. The N-terminal part of the myocilin protein is a hydrophobic region with a cleavable signal peptide. There are also leucine and arginine repeats that may be involved in protein-protein interaction (Ortego *et al.*, 1997), such as myocilin-myocilin oligomerization. The C-terminal part has significant homology to olfactomedin, which has been determined to be a major component of the extracellular matrix of the olfactory neuroepithelium (Bal and Anholt, 1993). Myocilin is expressed intracellularly in punctate deposits surrounding the nucleus (Clark *et al.*, 1998) and is associated with intracellular vesicles (Stamer *et al.*, 1998). Recent studies showed

that myocilin is also present in the aqueous humor (Jacobson *et al.*, 2001; Russell *et al.*, 2001), and binds to fibronectin in the extracellular matrix of human TM (Filla *et al.*, 2002). In addition, myocilin is present in the aqueous humor in the form of oligomers ranging from 120 kDa to 250 kDa (Russell *et al.*, 2002; Fautsch *et al.*, 2000). There are motifs in the promoter region upstream of the myocilin gene that suggest a basis by which putative genetic / environment challenges can regulate myocilin production (Polansky *et al.*, 1997). Among these motifs are a stretch-response element, NF-κB, an AP-1 and AP-2 sites (Kirstein *et al.*, 2000). The presence of glucocorticoid response element (GRE) half sites suggests a possible regulatory mechanism of myocilin expression by glucocorticoids (Nguyen *et al.*, 1998; Fingert *et al.*, 1998). Recent work suggests that these GRE sites do not function (Shepard *et al.*, 2001). There appears to be a major induction of a 55kDa cellular form of myocilin in cultured human TM cells treated with glucocorticoids for 2-3 weeks as demonstrated by SDS-and 2-dimensional polyacrylamide gel electrophoresis (Polansky *et al.*, 1997; Nguyen *et al.*, 1998; Clark *et al.*, 2001). In addition, a larger 66kDa glycosylated isoform of this protein, which is immunoreactive with anti-myocilin antibodies, is secreted into the medium of DEX-treated human TM cells (Nguyen *et al.*, 1998). Consequently, myocilin was once named trabecular meshwork-induced glucocorticoid response protein (TIGR) (Polansky *et al.*, 1997; Nguyen *et al.*, 1998). The delayed induction of myocilin by glucocorticoid closely matches the propensity of topical ocular glucocorticoid administration to raise intraocular pressure. Accordingly, this protein has been suggested to be associated with steroid-induced ocular hypertension. However, glucocorticoid effects on myocilin do not seem to

be direct (Shepard *et al.*, 2001) and disease causing MYOC mutations are evenly distributed among steroid responders and non-responders (Fingert *et al.*, 2001). Therefore, the relationship between myocilin expression and the ocular hypertension is still unknown.

I. Myocilin and Primary Open Angle Glaucoma

GLC1A was the first glaucoma locus identified by Sheffield et al. (1993) via genetic linkage analysis. Stone et al. (1997) mapped the gene (myocilin, MYOC) causing GLC1A glaucoma to the GLC1A locus by positional cloning methods. Upon isolation and characterization from a human retinal cDNA library (Kubota *et al.*, 1997), the MYOC gene was found to have 100% homology to the first glaucoma gene at the GLC1A locus (Ortego *et al.*, 1997; Nguyen *et al.*, 1998; Fingert *et al.*, 1998). The MYOC gene has been identified to be responsible for inheritable forms of juvenile as well as some adult onset POAG (Stone *et al.*, 1997; Alward *et al.*, 1998). Despite intensive studies on myocilin, the basic function of this protein is still a puzzle. Mutations in MYOC that are responsible for POAG are concentrated in the olfactomedin-homologous region (Adam *et al.*, 1997; Fingert *et al.*, 1999). Although disease-causing MYOC mutations in heterozygotes can lead to POAG (Morissette, 1998), several research groups have reported that absence or decreased expression of myocilin does not lead to elevated IOP (Kim *et al.*, 2001) or POAG (Wiggs and Vollrath, 2001). In addition, only native myocilin can be secreted (Jacobson *et al.*, 2001), while mutant myocilin not only undergoes proteolysis in cells but also negatively affects native myocilin secretion (Caballero *et al.*, 2000; Zimmerman *et al.*, 1999). Therefore, disease-causing mutations in

humans are more than likely of the gain of function type. The above studies have led to the theory that mutant myocilin accumulation within the intracellular compartments of TM cells disrupts normal cellular scavenging function thus obstructing aqueous humor outflow (Fingert *et al.*, 2002). Between normal and glaucomatous individuals, the differences in myocilin immunolocalization in TM tissue are conflicting. Lutjen-Drecoll *et al.* (2000) demonstrated that myocilin staining was increased in glaucomatous human TM tissue. However, Tawara *et al.* (2000) detected no notable differences of myocilin staining between normal and glaucomatous human TM tissue. Therefore, the role of myocilin in glaucoma is still unknown.

J. Endogenous Growth Factors and the Human TM

Growth factors acting through high-affinity cell surface receptors expressed by target cells, activate a cascade of intracellular pathways, inducing transcriptional regulation (Brugge, 1993). They are responsible for controlling cellular functions, including proliferation, motility, differentiation, phagocytosis, extracellular matrix synthesis and degradation. In POAG, there are specific morphological and pathological changes in the human TM, including an increase in extracellular matrix components and a decrease in the number of TM cells (Rohen *et al.*, 1993). Therefore, growth factors may play key roles in the pathophysiology of glaucoma. It has been reported that aqueous humor contains various growth factors, including TGFβ isoforms (Tripathi *et al.*, 1991; Tripathi *et al.*, 1994). Previous work in our lab (Wordinger *et al.*, 1996; Wordinger *et al.*, 1998; Wordinger *et al.*, 1999) showed that specific growth factors including BDNF, TGFβ isoforms (TGFβ1-3) and their functional receptors are expressed in normal cultured

human TM cells. Since human TM cells are continuously bathed in the aqueous humor, growth factors may function via autocrine or paracrine mechanisms to regulate cell functions, which in turn may modulate the outflow pathway. Consequently, any condition or agent that alters the normal expression of growth factors or growth factor receptors by TM cells may lead to an abnormal microenvironment within the tissue and result in pathological changes of the human TM. For example, TGFβ inhibits human TM cell proliferation thus aggravating POAG (Wordinger *et al.*, 1998). Growth factor receptor activation can also affect calcium mobilization and transportation (Steinhausen *et al.*, 2000; Collison and Duncan, 2001), which may influence the exchange of components between human TM intracellular fluid and the aqueous humor via secretion modification.

1). Transforming growth factor beta-2 (TGFβ2) - Among various growth factors, biologically active TGFβ2 levels are increased in the aqueous humor of glaucoma patients (Tripathi *et al.*, 1994; Inatani *et al.*, 2001; Picht *et al.*, 2001). TGFβ isoforms control the development and homeostasis of most tissues through regulating the cell cycle and extracellular matrix production (Schuster and Kriedglstein, 2002). TGFβ isoforms can initiate cell cycle arrest by inducing cyclin-dependent kinase inhibitors through sp-1 like sites, and stimulate expression of interstitial collagens as well as other extracellular matrix proteins, such as collagen α1(I), collagen α2(I), type 1 plasminogen activator inhibitor (PAI-1), elastin, and perlecan through AP-1 sites (Massague, 1998). It has also been shown that TGFβ isoforms can down-regulate secretory protease stromelysin (or transin, MMP-3) via a Fos-containing repressor (Kerr *et al.*, 1990). TGFβ isoforms signal via sequential binding to two serine/threonine kinase receptors on the cell surface, TβRI

and TβRII. When complexed with the ligand and TβRII, TβRI can regulate gene expression through phosphorylation of downstream signaling elements, such as SMAD proteins (Massague, 2000), TAK1 (TGF-β-activated kinase 1) (Shibuya *et al.*, 1996). Phosphorylated SMADs translocate into the nucleus and generate transcription complexes that recognize specific DNA-binding site (Massague, 2000). Alternatively, TGFβ isoforms not only elevate the expression of TAK1 but also phosphorylate it, which subsequently activates the MAP kinase cascade (i.e. phosphorylation of ERK1) to stimulate transcription factors containing AP-1 sites (Shibuya *et al.*, 1996; Kerr *et al.*, 1990; Wakefield and Robert, 2002). Interestingly, phosphorylation of MAP-kinase sites in the linker region of SMADs inhibits its nuclear accumulation (Kretzschmar *et al.*, 1997 and 1999). This suggests an auto-regulatory mechanism between the two pathways of TGFβ isoforms.

Another inhibitory mechanism in the SMAD system for TGFβ isoforms signaling is the presence of SMAD7 (Nakao *et al.*, 1997; Hayashi *et al.*, 1997), which can be induced by shear stress in vascular endothelial cells (Topper *et al.*, 1997). This suggests an autocrine TGFβ response to environmental stress including intraocular hypertension. TGFβ1 and 2 enhance the synthesis and secretion of fibronectin by modifying its pre-mRNA alternative splicing pattern in porcine trabecular meshwork cells (Li *et al.*, 2000). They also increase tissue transglutaminase (tTgase), an enzyme that cross-links extracellular matrix (ECM) proteins, especially fibronectin in cultured human TM cells (Welge-Lussen *et al.*, 2000). Additionally, it has been demonstrated that TGFβ1 treatment induced a 3.8- and 4-fold increase in myocilin mRNA levels after 3 to 12 days

in *in vitro* cultured human TM cells (Tamm *et al.*, 1999). DNAse I footprinting experiments showed AP-1-like sequence protection in the MYOC promoter region (Kirstein *et al.*, 2000), which is also a transcriptional activation site of TGFβ signal cascade downstream to TAK1. Therefore, TGFβ2 may be a direct regulator of myocilin expression in response to glaucomatous injury.

2). Brain derived neurotrophic factor (BDNF) – BDNF belongs to the neurotrophin family that also includes, nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (Barbacid, 1994). BDNF not only regulates the development, survival and differentiation of neurons through TrkB and p75 receptors, but also regulates synaptic transmission and plasticity through TrkB and ion channels (Barde, 2002). Although BDNF and TrkB have been demonstrated predominantly in the nervous system, they are expressed in several nonneuronal tissues, including muscle, heart and the vasculature, at levels comparable to those of the brain (Donovan *et al.*, 1995; Hiltunen *et al.*, 1996). In heart, BDNF plays an essential role in maintaining vessel stability through direct *in vivo* angiogenic actions on endothelial cells (Donovan *et al.*, 2000). In the circulatory system, laminar fluid shear stress can induce a rapid release of BDNF sequestered in platelets (Fujimura *et al.*, 2002) while down-regulating BDNF synthesis by endothelial cells (Nakahashi *et al.*, 2000). Mechanical stretch of an organotypic brain slice culture for twenty-four hours significantly increases the expression of BDNF (Morrison *et al.*, 2000). Mechanical stretch is an important factor in the trabecular meshwork during POAG, especially with elevated IOP. Therefore, BDNF may play an important role in modulating both cell survival and extracellular environment of human

TM in response to elevated intraocular pressure. So far there are three pathways identified downstream to TrkB (Yuen *et al.*, 1999):

(1) TrkB can phosphorylate the tyrosine residues on phospholipase C-γ1 (PLC-γ1), releasing diacylglycerol (DAG) for protein kinase C activation and inositol 1,4,5-triphosphate (IP_3) for calcium mobilization from intracellular stores. This pathway accounts mostly for BDNF's effects on synaptic plasticity.

(2) Activated TrkB can also bind to and phosphorylate SHC, which subsequently stimulates ras-mediated activation of the MAP kinase cascades.

(3) Phosphatidylinositol-3 kinase (PI-3K) is recognized as an anti-apoptotic regulator downstream to TrkB.

Previous results from our lab demonstrated that the human TM cells express BDNF and one form of truncated TrkB receptor but not p75 (Wordinger *et al.*, 2000). Truncated TrkB has been reported mainly in non-neuronal tissues and lacks the intracellular tyrosine kinase domain (Luikart *et al.*, 2003). Although little is known regarding its function in nonneuronal tissue, truncated TrkB has been demonstrated to be capable of signaling independently of its full-length isoform and can regulate a distinct mode of dendritic growth (Baxter *et al.*, 1997).

C). BDNF and TGFβ2

Growth factors act in concert in various aspects of normal cell function and also under pathological conditions. BDNF and TGFβ2 can have both common and opposing effects on cell survival, proliferation, differentiation and extracellular matrix regulation depending on tissue type. TGFβ2 enhances the synthesis and secretion of fibronectin in

porcine trabecular meshwork cells (Li *et al.*, 2000), and BDNF increases fibronectin and laminin synthesis in neurons (Gruenbaum and Carew, 1999). BDNF supports the survival of pluripotent neural crest cells in the presence of basic fibroblast growth factor (FGF-2), while TGFβ inhibits the survival under the same condition (Sieber-Blum, 1998). BDNF at low levels phosphorylates MAP kinase (ERK1) (Han and Holtzman, 2000), which inhibits SMAD protein accumulation in the nucleus thus abolishing TGFβ effects. It has also been reported that neurotrophins act synergistically with members of the TGFβ superfamily to promote the survival of spiral ganglia neurons *in vitro* (Marzella *et al.*, 1999). In neurons, TGFβ1 can up regulate BDNF mRNA and protein secretion (Sometani *et al.*, 2001). In return, BDNF mediates BMP-2 (bone morphogenetic protein-2, a member of the TGFβ superfamily) effects on cultured neurons (Gratacos *et al.*, 2001). Therefore, BDNF and TGFβ2 may interact with each other in response to elevated intraocular pressure through paracrine/autocrine mechanisms. This may result in differential gene expression (e.g. myocilin), regulating the structure and function of human TM in POAG.

The overall **goal** of this research is to (a) examine the role of BDNF and TGFβ2 in the regulation of myocilin expression, (b) investigate the interaction that may exist between TGFβ2 and BDNF in trabecular meshwork cells, and to (c) determine if there is paracrine signaling involving BDNF and TGFβ2 in human TM cells. The **hypothesis** to be tested is that MYOC gene expression and myocilin protein levels are regulated by BDNF and TGFβ2 in normal and glaucomatous trabecular meshwork cells. The following **specific aims** have been designed to test this hypothesis: (1) Compare and contrast MYOC gene (mRNA) expression and myocilin protein levels and secretion in cultured normal and glaucomatous human TM cell lines; (2) Investigate the synergistic effects of BDNF and TGFβ2 in regulation of MYOC gene (mRNA) expression and myocilin protein levels and secretion in cultured normal and glaucomatous human TM cells; (3) Demonstrate the reciprocal induction between BDNF and TGFβ2 gene (mRNA) and protein secretion in cultured normal and glaucomatous human TM cells; (4) Demonstrate that BDNF and TGFβ2 regulate myocilin levels through distinct signaling pathways.

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CHAPTER II

MATERIAL AND METHODS

Human Trabecular Meshwork Cell Culture

Multiple non-transformed, early passaged human normal (NTM) and glaucomatous (GTM) trabecular meshwork cell lines were utilized in studying the expression of myocilin, BDNF, TGFβ2, TβRI, TβRII, and TrkB. NTMs are from donors of 2, 54, 71, 77, 81, and 87 years, and GTMs are from donors of 72, 75, 78, and 85 years old. The trabecular meshwork cells were grown until confluent or near confluent in Ham's F-10 Medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc. Logan, UT), 2 mM L-glutamine (0.292 mg/ml), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) (Life Technologies, Grand Island, NY). All cell lines were maintained at 37^0C in 5% CO_2-95% room air; and medium was changed every 2 to 3 days. Human trabecular meshwork cells have very slow growth rate. It takes at least two weeks to grow a confluent or near confluent monolayer of a 75 cm^2 flask. The human TM cell lines used for this project are listed in table 1.

Growth Factor Treatment of Human TM Cells

Confluent or near confluent monolayers of human TM cells were washed 4 times with serum free medium before the growth factors were added. The human TM cells were then incubated with individual or combinations of growth factors in serum free medium at

37^0C in 5% CO_2-95% room air for 48 hours except for the time course study. BDNF concentrations ranged from 0.2-50 ng/ml and TGFβ2 concentrations ranged from 0.2-2 ng/ml were utilized to treat the human TM cells. Human TM cells grown in serum-free culture media for 48 hours were used as controls for the treatment. There are variations among cell lines from different donors. Therefore, dose response studies were performed for each growth factor applied for each cell line.

Total Cellular RNA Extraction and cDNA Synthesis

Total cellular RNA was prepared using the guanidinium thiocyanate/ phenol/ chloroform method[31]. Confluent or near confluent human TM cells (5-10 x 10^7 cells) were scraped off the culture flasks, pelleted by centrifugation and lysed in 1 ml TRIzol reagent (Life Technologies) by repetitive pipetting. RNA was precipitated from the resulting aqueous phase by adding isopropyl alcohol followed by centrifugation, then resuspended in 70 µl of water and stored at -80°C. Total cellular RNA was used for cDNA synthesis. To reduce secondary structure, 20 µg RNA was incubated at 85°C for 3 minutes and mixed with 0.75 µg random primers (Promega, Madison, WI). The following were then added to the reaction tube: 80 units RNasin (Promega), 40 units avian myeloblastosis virus (AMV) reverse transcriptase (Promega), 0.625 mM each deoxyribonucleotide, 50 mM Tris-HCl, 75 mM potassium chloride, 10 mM dithiothreitol, and 3 mM magnesium chloride. The reaction tube was incubated at 42°C for 30 minutes, followed by incubating at 94°C for 2 minutes. Synthesis of cDNA was verified by PCR amplification of β-actin. The cDNA was stored at -80°C.

Primer Design and Polymerase Chain Reaction

MYOC, BDNF and TGFβ2 mRNA sequences (accession numbers: NM_000261, AF400438, NM_003238, respectively) were obtained from Entrez (NCBI, Bethesda, MD). Oligo 4.0 (National Biosciences, Plymouth, MN) was used to design PCR primers that have optimal annealing temperatures and magnesium concentrations. Designed primer pairs were submitted through BLAST (National Center for Biotechnology Information, Bethesda, MD) to ensure that they would not hybridize to any other known nucleic acid sequences under the conditions used. The sequences, annealing temperature of the primer pairs, and expected size of products are shown as in table 2. The PCR products were sequenced to ensure specificity and accuracy.

Real-time quantitative PCR

Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) according to the manufacturer's instructions. The amplification reactions were performed in 25 μl total volume with 200 nM primers, and cDNA from 2.5 ng of total RNA in 1X SYBR Green PCR master mix. The results can be displayed as an amplification plot (Figure 1), which reflects the change in fluorescence during cycling. Fluorescence measurements were also taken at every temperature increment from 55°C to 95°C to generate a dissociation curve (Figure 1). Fluorescence was plotted versus temperature. Primer-dimer or nonspecific amplification generally melt at a lower temperature (defined as T_m) than the desired products. The dissociation curve plot shown in figure 1 demonstrated two fluorescence peaks: one centered around 78°C corresponding to TGFβ2; and the other centered around 80°C corresponding to 18S, which is the internal control. There is no primer-dimer peak that centers on 75°C. The

target gene and 18S rRNA amplification reactions were loaded into different wells but on the same plate. Each gene quantification data were normalized to expression of the 18S rRNA amplification reactions performed in the same condition as the target amplification. The PCR reactions were carried out at 95°C for 10 minutes and followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantification of gene expression was performed using the standard curve method according to User Bulletin 2 of the ABI Prism 7700 Sequence Detection System. Briefly, two-fold dilutions of a total cDNA preparation from a control cell line were used to construct a standard curve. For quantitation normalized to an endogenous control (18S rRNA in our case), standard curves were prepared for both the target and 18S rRNA. For each experiment sample, the amount of target and 18S rRNA was determined from the appropriate standard curve. The target amount was divided by the 18S rRNA amount to obtain a normalized target value.

Western Blotting

Confluent or near confluent human TM cells (2 X 10^7 cells) were collected in 0.2 ml protein lysis buffer. The protein lysis buffer consisted of 10 mM Tris-HCl, 0.5% NP40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF in ethanol, 1 μg/ml aprotinin, 4 μg/ml pepstatin, 10 μg/ml leupeptin, and 100 mM sodium orthovanadate (10 μl/ml). Serum-free culture medium was collected and concentrated 20-fold using Centriplus YM-3 centrifugal filter devices (Millipore Corporation, Bedford, MA). Protein concentration was determined by the Lowry method. Cell lysate (40-60 μg) or TM cell culture medium was mixed with a one-third volume of 4X Laemmeli electrophoresis

buffer and boiled for 60 seconds. The Laemmeli electrophoresis buffer consisted of 10% glycerol, 5% β-mercaptoethanol, 2% SDS, 6.25% 1.0 M Tris-HCl pH 6.7, 0.05% bromophenol blue. Proteins were then separated on a 10% denaturing SDS polyacrylamide gel and transferred by electrophoresis to a nitrocellulose membrane. Sypro® Ruby (Molecular Probes, Eugene, Oregon) staining of the membrane before primary Ab incubation was used for quantitation purpose for the Western blots. Non-specific binding was blocked by soaking membranes in 1X TBS, 5% dry milk, and 0.2% Tween-20 for at least 1 hour at room temperature. Membranes were subsequently incubated with primary antibody specific for each target protein at the appropriate concentration overnight at 4°C and then washed four times with TBS plus 0.2% Tween-20. Subsequently, a 45-minute incubation with horseradish peroxidase-conjugated secondary antibody (Promega Corporation, Madison, WI) (1:50,000) was followed by six washes with TBS plus 0.2% Tween-20, and one wash in TBS alone. Chemiluminescence detection was carried out using ECL Western Blotting Kit detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and blots were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) for from 1 second to 30 minutes depending on the amount of target protein present. Chemiluminescence detection also was done using a Chemiluminescence kit (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce) and a cooled, computerized CCD camera-based imaging system (Alpha Innotech, San Leadro, CA) to visualize and record the stained proteins. The digitized images from the gels stained with Sypro® Ruby for total protein, and from the immunostained blots, were quantitatively analyzed using the Chemiimager software (Alpha Innotech). To

quantitate the total protein in each sample, a rectangular box was drawn around the whole lane and a densitometry reading was generated and recorded. To quantitate the specific protein band, a rectangular box was drawn around the band. The area for quantitation remains consistent for each set of membrane. A comparison report of quantitative differences of specific proteins normalized to total protein on each gel was then generated.

Immunofluorescent Staining

To demonstrate cellular myocilin presence in the human TM cells, non-transformed human TM cells were grown on glass coverslips and fixed with 3.5% formaldehyde in PBS for 20 minutes, and then treated with 0.2% Triton X-100 in PBS for 5 minutes. Non-specific binding was blocked by a 20-minute incubation with 10% normal goat serum in PBS. Coverslips were then incubated with primary antibody specific for myocilin (1.0 μg/ml in PBS-BSA) for 60 minutes at room temperature. The cells were then washed and incubated in Alexa Fluor 488 goat anti-rabbit secondary antibodies (Molecular Probes, Eugene, Oregon) 20 μg/ml in 1 X PBS-BSA for 45 minutes. Coverslips were washed, mounted, and viewed using a Nikon Microphot-FXA microscope with an FITC filter. Images were recorded using a Sensys scientific-grade CCD digital camera (Photometrics Ltd., Tucson, Arizona), and processed using IPLab Scientific Imaging Software (Scanalytics, Inc. Fairfax, VA). Omission of primary antibody served as the negative control.

Immunoassays (ELISA) for TGFβ2 and BDNF

The serum-free medium was collected at the end of 48 hours exposure to BDNF or TGFβ2 treatment. A flask of cells grown in serum-free medium without growth factors served as a control. The serum-free medium was then concentrated 20-fold using Centriplus YM-3 centrifugal filter devices (Millipore Corporation, Bedford, MA). Protein concentrations were determined by the Lowry method. Emax™ ImmunoAssay Systems (Promega, Madison WI) specific for BDNF or TGFβ2 were used according to manufacturer's instructions. Nunc ELISA/EIA 96 well plates were coated with anti-BDNF or anti-TGFβ2 monoclonal antibodies (Promega Corporation, Madison, WI) overnight at 4°C. Equal amounts of total protein were added the next day to detect secreted BDNF or TGFβ2 that has been captured by the coated antibody. The secreted growth factors were then detected by treating the plates with the respective polyclonal antibodies (Promega Corporation, Madison, WI. Anti-Human BDNF, 1:500; Anti-Human TGFβ2, 1:2000) followed by a horseradish peroxidase (HRP) conjugated species-specific anti-IgY secondary antibody (1:200). Unbound conjugate was removed by washing. The chromogenic enzyme substrate TMB (3,3', 5, 5'-tetramethylbenzidine was subsequently added to generate a deep blue color during the enzymatic degradation of H_2O_2 by HRP. The reaction was stopped by addition of 1N hydrochloric acid to the wells in the same order in which substrate was added. A clear yellow color was generated whose absorbance was read at 450 nm. Serial two fold dilutions of the recombinant growth factor to be assayed were included in each assay to generate a standard curve. Each sample was assayed in triplicate.

Statistical Analysis

The statistical significance, defined as $p<0.05$, was evaluated using a statistical software SPSS-10 for Macintosh. The mode of data distribution was determined before analysis of variance. For normally distributed data, student t-test was used for variance analysis between two groups; and one-way ANOVA was used for variance analysis among multiple groups. For the data sets that had skewed distribution, nonparametric analysis was used for variance comparison.

Table 1. List of Human TM Cell Lines Used.

Normal Human TM Cells	Glaucomatous Human TM Cells
NTM 2 years old	GTM 72 years old
NTM 54 years old	GTM 75 years old
NTM 71 years old	GTM 79 years old
NTM 77 years old	GTM 85 years old
NTM 81 years old	GTM 78 years old
NTM 87 years old	GTM 84 years old
NTM 5 days old	

HTM = human trabecular meshwork; NTM = normal human trabecular meshwork;

GTM = glaucomatous human trabecular meshwork.

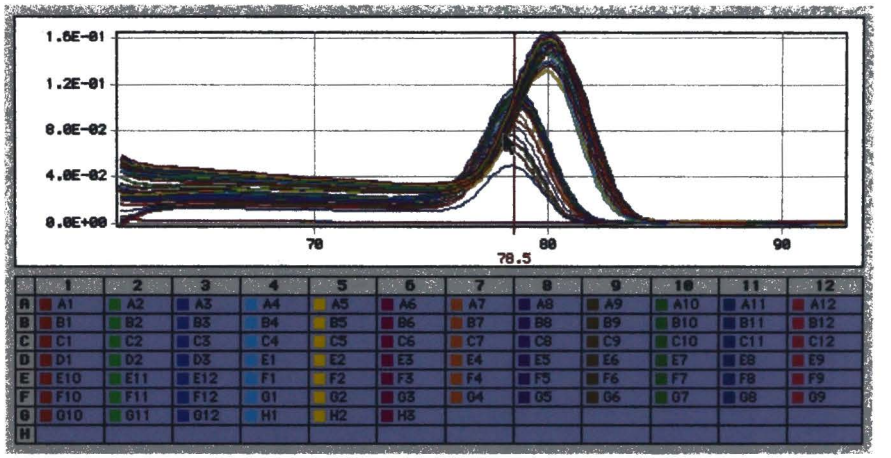
Table 2. Primer Pairs Used for Real-time PCR

Gene	Sequence	Anneal. Temp.	Size (bp)
18S rRNA	5'-GCC GCT AGA GGT GAA ATT CTT G-3' 5'-CAT TCT TGG CAA ATG CTT TCG-3'	60°C	64
MYOC	5'-GCC CAT CTG GCT ATC TCA GG-3' 5'-CTC AGC GTG AGA GGC TCT CC-3'	60°C	81
TGFβ2	5'- GGC GGG ATG GCA TTT TC-3' 5'- CTA CGC CAA GGA GGT TTA CAA AAT A -3'	60°C	67
BDNF	5'- TCG CCA GCC AAT TCT CTT TT –3' 5'- GTG CCG AAC TAC CCA GTC GTA-3'	60°C	66

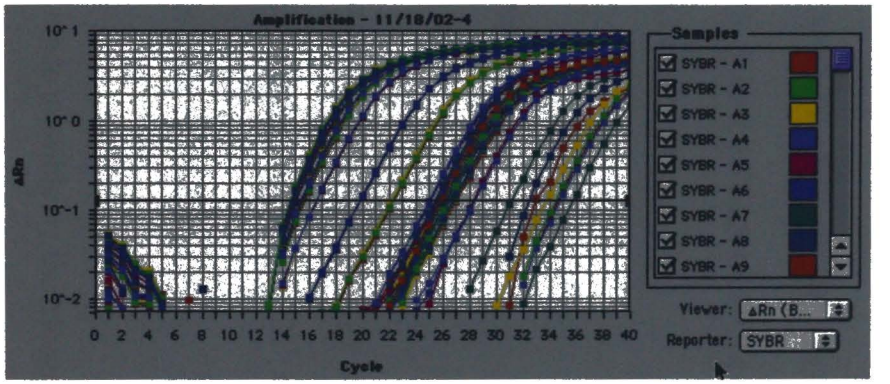
The sequences, annealing temperature of the primer pairs used for real-time PCR, and expected size of products are shown as in table 2.

Figure 1. Amplification and dissociation curve of a reaction with TGFβ2 and 18S rRNA as template. (A) When the amplified products are subjected to dissociation curve analysis, the fluorescence peak corresponding to the 18S rRNA amplicon (center around 80°C) is distinguishable from the peak corresponding to TGFβ2 amplicon (center around 78.5°C). There is no peak due to primer-dimer (center around 75°C). (B) The amplification plot demonstrates the correlation between the cycle number and the fluorescence intensity. The less cycle numbers it takes to pass the fluorescence threshold, the more copy numbers of the target gene in the sample.

A. Dissociation Curve



B. Amplification Curve



CHAPTER III

BRAIN-DERIVED NEUROTROPHIC FACTOR AND TRANSFORMING GROWTH FACTOR-BETA2 REGULATION OF MYOCILIN LEVELS IN HUMAN TRABECULAR MESHWORK CELLS

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Abstract

Purpose: To determine if brain-derived neurotrophic factor (BDNF) and transforming growth factor-beta2 (TGFβ2) regulate MYOC gene transcription and myocilin protein secretion by cultured human trabecular meshwork (TM) cells.

Methods: Multiple non-transformed normal and glaucomatous human TM cell lines were treated with BDNF (0.2-50 ng/ml), or TGFβ2 (0.2-2 ng/ml) in serum free Ham's nutrient mixture F-10 medium for 48 hours. Real Time PCR was used to quantitatively compare growth factor effects on myocilin gene transcription. Western blot analysis was used to determine growth factor effects on intracellular and secreted myocilin protein levels. Immunocytochemistry was used to visualize intracellular myocilin localization in cultured normal human TM cells.

Results: Real time PCR, as well as Western blot analysis, demonstrated that BDNF increased myocilin message, protein levels and secretion in 3 non-transformed normal TM cell lines. In contrast, TGFβ2 down-regulated myocilin message and protein secretion in normal human TM cells compared to non-treated controls. Variable results were seen with BDNF and/or TGFβ2 treatment in glaucomatous human TM cells.

Conclusions: These results demonstrate that BDNF can up-regulate myocilin message and protein levels in normal human TM cells. In addition, exogenous TGFβ2 down-regulates myocilin expression in normal human TM cells. The human TM cells express both BDNF and TGFβ2 and high affinity receptors, so this raises the distinct possibility that myocilin may be controlled through BDNF and TGFβ2 paracrine/autocrine signaling in the human trabecular meshwork.

Introduction

Glaucoma is the second leading cause of blindness in the world, affecting approximately 70 million people. Primary open-angle glaucoma (POAG) is the most common form of glaucoma in the U.S. and Europe, accounting for over 60% of all cases[1]. Although the exact etiology of POAG is still a mystery, ocular hypertension has long been considered a major risk factor[2-5]. The human TM is the major regulatory site of aqueous humor outflow and resistance to outflow[6], consequently affecting intraocular pressure (IOP). The myocilin gene (MYOC, also known as TIGR or GLC1A) has recently attracted the attention of glaucoma researchers for its close association with ocular hypertension[7]. Although MYOC was identified as the first glaucoma gene by genetic linkage analysis, absence or decreased expression of MYOC does not lead to elevated IOP[8] or POAG[9,10]. Patients heterozygous for disease causing mutations develop POAG suggesting that a single copy of this mutation could lead to glaucoma[11]. In addition, there is evidence that native, but not mutant myocilin is secreted by human TM cells[12, 13]. These reports suggested that specific MYOC mutations might cause glaucoma through gain of function (e.g. abnormal intracellular accumulation of mutant myocilin within the TM cells might interfere with normal TM cell function and lead to outflow obstruction).

The present study investigates the regulation of myocilin levels in human TM cells by several growth factors that have been reported to be present in human aqueous humor and expressed by human TM cells. We previously reported that the human TM, which originates from neural crest[14], expresses all four neurotrophins and neurotrophin receptors[15]. Neurotrophins are a family of trophic factors that includes brain derived

neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). They signal through membrane bound tyrosine kinase receptors TrkA, TrkB, TrkC. Several forms of truncated Trk receptors are also present, although the exact function is not known[16]. Known primarily as a neuronal survival factor, BDNF is also expressed in several nonneuronal tissues, including muscle, heart and the vasculature[17-19]. In heart, BDNF plays an essential role in maintaining vessel stability through direct *in vivo* angiogenic actions on endothelial cells[20]. In the circulatory system, lamina fluid shear stress can induce BDNF release by platelets[21], and down-regulating BDNF expression by endothelial cells[22]. Mechanical stretch of an organotypic brain slice culture for twenty-four hours significantly increases the levels of BDNF[23]. It is possible that during intraocular hypertension, mechanical stretch may increase BDNF controlled signaling in the human TM. By activating its receptor, BDNF may exert regulatory effects on the intraocular pressure by modulating the expression of MYOC.

It has also been shown that transforming growth factor beta 2 (TGFβ2) levels in the aqueous humor are higher in glaucomatous patients than those in normal human subjects[24-26]. We have demonstrated that human TM cells synthesize TGFβ2 and its functional receptors[27-29]. Tamm et al reported that TGFβ1 up-regulates myocilin mRNA levels in a TM monolayer cell culture[30]. Although TGFβ1 and TGFβ2 are interchangeable in many *in vitro* systems, they are not functionally redundant in that they appear to possess isoform-specific activities. In the present study, we demonstrated that BDNF up-regulated myocilin mRNA, protein levels, and secretion by cultured normal

human TM cells. Moreover, we reported that in normal human TM cells, TGFβ2 down-regulated myocilin mRNA levels and protein secretion.

METHODS

Human Trabecular Meshwork Cell Culture

Multiple non-transformed, early passaged normal (NTM) and glaucomatous (GTM) human trabecular meshwork cell lines were utilized in studying the expression of myocilin. NTM cell lines were from donors of 2, 54, 71, 77, 81, and 87 years; and GTM cell lines were from donors of 72, 75, 78, and 85 years old. The TM cells were grown until confluent or near confluent in Ham's F-10 Medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc. Logan, UT), 2 mM L-glutamine (0.292 mg/ml), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) (Life Technologies, Grand Island, NY). All cell lines were maintained at 37^0C in 5% CO_2-95% room air; and medium was changed every 2 to 3 days.

Growth Factor Treatment of Human TM Cells

Confluent or near confluent monolayer human TM cells were washed 4 times with serum free medium before the growth factors were added. The human TM cells were then incubated with individual or combinations of growth factors in serum free medium at 37^0C in 5% CO_2-95% room air for 48 hours except for the time course study. BDNF concentrations from 0.2-50 ng/ml and TGFβ2 concentrations from 0.2-2 ng/ml were utilized to treat the human TM cells. Human TM cells grown in serum-free culture media for 48 hours were used as the control treatment.

Total Cellular RNA Extraction and cDNA Synthesis

Total cellular RNA was prepared using the guanidinium thiocyanate/ phenol/ chloroform method[31]. Confluent or near confluent human TM cells (5-10 x 10^7 cells) were scraped off the culture flasks, pelleted by centrifugation and lysed in 1 ml TRIzol reagent (Life Technologies) by repetitive pipetting. RNA was precipitated from the resulting aqueous phase by adding isopropyl alcohol followed by centrifugation, then resuspended in 70 μl of water and stored at -80°C. Total cellular RNA was used for cDNA synthesis. To reduce secondary structure, 20 μg RNA was incubated at 85°C for 3 minutes and mixed with 0.75 μg random primers (Promega, Madison, WI). The following were then added to the reaction tube: 80 units RNasin (Promega), 40 units avian myeloblastosis virus (AMV) reverse transcriptase (Promega), 0.625 mM each deoxyribonucleotide, 50 mM Tris-HCl, 75 mM potassium chloride, 10 mM dithiothreitol, and 3 mM magnesium chloride. The reaction tube was incubated at 42°C for 30 minutes, followed by incubating at 94°C for 2 minutes. Synthesis of cDNA was verified by PCR amplification of β-actin. The cDNA was stored at -80°C.

Real-time quantitative PCR

Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) according to the manufacturer's instructions. For amplification of MYOC, reactions were performed in 25 μl total volume with 200 nM primers[33], (5'-GCC CAT CTG GCT ATC TCA GG-3', forward and, 5'-CTC AGC GTG AGA GGC TCT CC-3', reverse), and cDNA from 2.5 ng of total RNA in 1X SYBR Green PCR master mix. MYOC quantification data were normalized to expression of the 18S rRNA.

The 18S amplification reactions were performed with primers, (5'-GCC GCT AGA GGT GAA ATT CTT G-3', forward and, 5'-CAT TCT TGG CAA ATG CTT TCG-3', reverse), in the same condition as MYOC amplification. The PCR reactions were carried out at 95°C for 10 minutes and followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantification of gene expression was performed using the standard curve method according to User Bulletin 2 of the ABI Prism 7700 Sequence Detection System.

Western Blotting

Confluent or near confluent human TM cells (2 X 10^7 cells) were collected in 0.2 ml protein lysis buffer. The protein lysis buffer consisted of 10 mM Tris-HCl, 0.5% NP40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF in ethanol, 1 μg/ml aprotinin, 4 μg/ml pepstatin, 10 μg/ml leupeptin, and 100 mM sodium orthovanadate (10 μl/ml). Serum-free culture medium was collected and concentrated using Centriplus YM-3 centrifugal filter devices (Millipore Corporation, Bedford, MA). Protein concentration was determined by the Lowry method. Cell lysate (40-60 μg) or TM cell culture medium was mixed with a one-third volume of 4x Laemmeli electrophoresis buffer and boiled for 60 seconds. The Laemmeli electrophoresis buffer consisted of 10% glycerol, 5% β-mercaptoethanol, 2% SDS, 6.25% 1.0 M Tris-HCl pH 6.7, 0.05% bromophenol blue[31]. Proteins were then separated on a 10% denaturing SDS polyacrylamide gel and transferred by electrophoresis to a nitrocellulose membrane. Sypro® Ruby (Molecular Probes, Eugene, Oregon) staining of the membrane before primary Ab incubation was used for quantitation purpose for the Western blots. Non-specific binding was blocked by

soaking membranes in 1X TBS, 5% dry milk, and 0.2% Tween-20 for at least 1 hour at room temperature. Membranes were subsequently incubated with a rabbit polyclonal antibody (1:500) specific for myocilin[34] overnight at 4°C and then washed four times with TBS plus 0.2% Tween-20. Subsequently, a 45-minute incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Promega Corporation, Madison, WI) (1:50,000) was followed by six washes with TBS plus 0.2% Tween-20, and one wash in TBS alone. Chemiluminescence detection was carried out using ECL Western Blotting Kit detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and blots were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) for from 1 second to 30 minutes depending on the amount of target protein present. Chemiluminescence detection also was done using a Chemiluminescence kit (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce) and a cooled, computerized CCD camera-based imaging system (Alpha Innotech, San Leadro, CA) to visualize and record the stained proteins. The digitized images from the gels stained with Sypro® Ruby for total protein, and from the immunostained blots, were quantitatively analyzed using the Chemiimager software (Alpha Innotech). To quantitate the total protein in each sample, a rectangular box was drawn around the whole lane and a densitometry reading was generated and recorded. To quantitate the specific protein band, a rectangular box was drawn around the band. The area for quantitation remains consistent for each set of membrane. A comparison report of quantitative differences of specific proteins normalized to total protein on each gel was then generated.

Immunofluorescent Staining

To demonstrate cellular myocilin presence in the human TM cells, non-transformed human TM cells were grown on glass coverslips and fixed with 3.5% formaldehyde in PBS for 20 minutes, and then treated with 0.2% Triton X-100 in PBS for 5 minutes. Non-specific binding was blocked by a 20-minute incubation with 10% normal goat serum in PBS. Coverslips were then incubated with rabbit polyclonal antibody[34] specific for myocilin (1.0 μg/ml in 1X PBS-BSA) for 60 minutes at room temperature. The cells were then washed and incubated in Alexa Fluor 488 goat anti-rabbit secondary antibodies (Molecular Probes, Eugene, Oregon) 20 μg/ml in 1 X PBS-BSA for 45 minutes. Coverslips were washed, mounted, and viewed using a Nikon Microphot-FXA microscope with an FITC filter. Images were recorded using a Sensys scientific-grade CCD digital camera (Photometrics Ltd., Tucson, Arizona), and processed using IPLab Scientific Imaging Software (Scanalytics, Inc. Fairfax, VA). Omission of primary antibody served as the negative control.

Statistical Analysis

The statistical significance, defined as $p<0.05$, was evaluated using a statistical software SPSS-10 for Macintosh. The mode of data distribution was determined before analysis of variance. For normally distributed data, student t-test was used for variance analysis between two groups; and one-way ANOVA was used for variance analysis among multiple groups. For the data sets that had skewed distribution, nonparametric analysis was used for variance comparison.

Results

Immunocytochemical Localization of Myocilin in Cultured Normal TM cells

Previous studies reported that *in vitro* culture of human TM cells might decrease the expression of MYOC, thus rendering the message undetectable using Northern blot analysis[30]. Cultured normal human TM cells from a donor of 54 years old were used for the immunocytochemistry to localize myocilin protein intracellularly (Figure 1A). Human TM cells were positive for myocilin with a cytoplasmic localization pattern. Myocilin staining was punctate and distributed throughout the cytoplasm, and was more intense in the perinuclear region of the cells, suggesting an endoplasmic reticulum and Golgi-like association. There was no apparent nuclear localization of myocilin protein.

Myocilin mRNA and Protein levels in Cultured Normal and Glaucomatous Human TM Cells

Three normal and three glaucomatous human TM cell lines from donors ranging in age of between 2 years and 87 years were used to compare and contrast the levels of myocilin mRNA and protein (Figure 2. A & B). Not all the adult TM cell lines expressed detectable myocilin mRNA or secreted protein. Myocilin protein appeared on western blot as a characteristic double band at 55-57 kDa[12]. Neither real-time PCR nor western blot analysis demonstrated any significant differences between normal and glaucomatous human TM cell lines with respect to myocilin mRNA levels or protein secretion. However, TM cell lines from younger donors seemed to express considerably lower levels of myocilin when compared with adult normal TM cell lines from 54, 87, and 81 year-old donors (Figure 2B). Another interesting finding is that human TM cells from

different donors demonstrated variable secretory protein profile, which may contribute to the variations we observed across cell lines.

Serum-free Treatment Up-regulates Myocilin mRNA Levels and Protein Secretion by Cultured Normal Human TM Cells

In order to rule out action by regulatory factors in the serum, we first treated human TM cells in serum free medium. Consequently, we studied the effect of serum deprivation on myocilin expression by normal human TM cells. Changes in myocilin mRNA levels and protein secreted with time are shown in Figure 3A and 3B. Upon serum starvation, myocilin mRNA levels reached a peak between 12 and 24 hours, and returned to base level by 48 hours (Figure 3A). In serum free medium, myocilin protein secretion peaked at 30 hours and decreased thereafter (Figure 3B). By 48 hours, protein secretion levels returned to levels seen at 8 hours. Therefore, the length of all the following treatments was set at 48 hours.

BDNF Up-regulates MYOC Expression in Serum-free medium

A normal human TM cell line from a 54 year-old donor was utilized to examine the myocilin gene activity upon BDNF treatment. BDNF can promote neuronal cell proliferation at 50 ng/ml. At the same level, BDNF significantly up-regulated myocilin mRNA levels in human TM cells using real-time PCR (Figure 4A). Intracellular myocilin protein was also elevated by BDNF treatment as demonstrated by western blot (Figure 4B). The blot was reprobed for β-actin to serve as loading control. Furthermore, BDNF also increased myocilin protein levels slightly in the serum-free medium as shown in Figure 4C. The serum-free medium was concentrated and equal amount of total protein

was loaded onto the gel. After transfer, the blot was stained with Sypro® Ruby and the image was recorded for quantitation purpose.

MYOC Expression Up-Regulation by Dexamethasone and BDNF

Three non-transformed normal human TM cell lines from donors of 54, 71 and 77 years old were treated with dexamethasone (10^{-7}M)[34] or BDNF (50 ng/ml) for 48 hours in serum-free medium. At the end of the treatment, the medium was collected and the levels of secreted myocilin were measured using Western blot analysis. The myocilin protein level in the serum-free medium was dramatically up-regulated in all three cell lines upon dexamethasone treatment as shown in Figure 5A, 5B, 5C. Also, BDNF increased the secreted myocilin protein levels in all three cell lines (Figure 5D, 5E, 5F).

BDNF Dose Dependent Regulation of Myocilin Expression in the Serum-free medium

Variation in minimum doses needed to cause an elevation of myocilin expression was demonstrated in a dose-response study with the same three cell lines. BDNF (0.2 ng/ml) is able to up-regulate myocilin protein levels in the serum-free medium of NTM cells from a 71 year-old donor (Figure 6A). A similar response was induced at 50 ng/ml of BDNF in the NTM cells from a 77 year-old donor. In NTM cells from a 54 year-old donor, BDNF treatment at 10 ng/ml elicited similar response as in the other two cell lines (Figure 6B). For NTM cells from the 71 year-old donor, the intensity of individual band was quantitatively analyzed from three sets of experiments. Starting from 2 ng/ml level, BDNF significantly up-regulated myocilin secretion by this cell line ($p<0.05$). A bar

graph was generated based on the readings using one-way ANOVA analysis by a statistical software SPSS10 for Macintosh (Figure 6C).

BDNF treatment results in high levels of myocilin secretion into serum-free medium at 48 hours

Previously, we demonstrated that cell growth in serum-free medium resulted in increased levels of myocilin mRNA through 24 hours and increased protein secretion through 30 hours (Figure 3B). By the end of 48 hours, myocilin mRNA and protein levels returned to basal levels. When BDNF at a concentration of 50 ng/ml was included in the time course study, the pattern of myocilin induction appeared to be similar to serum free treatment for the first 30 hours. However, instead of decreasing, myocilin levels in the medium containing BDNF remained elevated at the end of 48 hours (Figure 7).

TGFβ2 Down-regulates Myocilin mRNA Levels and Protein Secretion in Normal Trabecular Meshwork Cells

The effect of TGFβ2 on myocilin mRNA levels were shown via real time PCR in Figure 8A. The myocilin protein levels in the serum-free medium were examined using western blot analysis and are shown in Figure 8B. Sypro® Ruby staining of the membrane after transfer was recorded for quantitation purpose. TGFβ2 was able to inhibit not only mRNA levels but also protein secretion of myocilin by normal human TM cells.

Figure Legends

Figure 1. Immunocytochemistry staining of cultured normal human trabecular meshwork cells from a 54 year old donor. (A) Myocilin localization in human TM cells. Note the concentrated distribution of myocilin around the nuclei, but the lack of staining within the nuclei. Bars = 20 μm. (B) Higher magnification of myocilin localization in human TM cells. Bars = 10 μm. Note the perinuclear localization in figures A & B. (C) is a control preparation in which saline was substituted for the primary antibody when performing the immunocytochemistry staining. Bars = 20 μm.

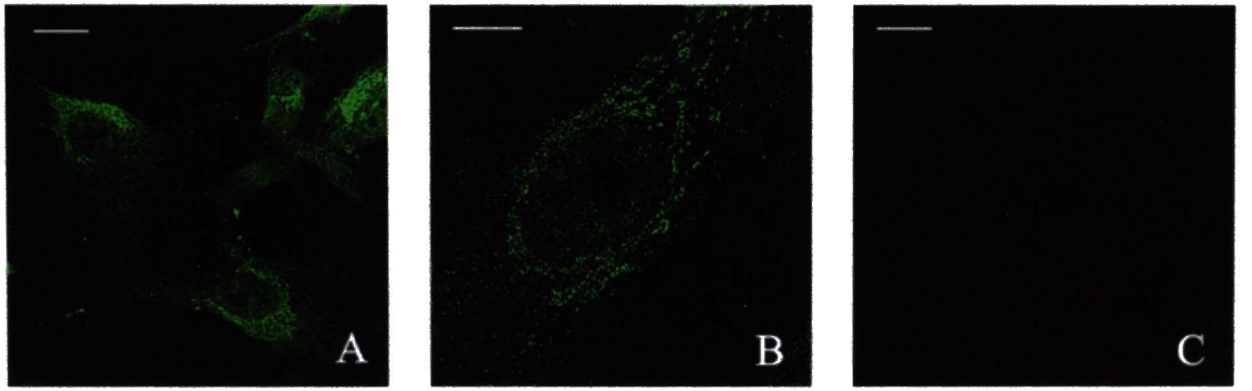
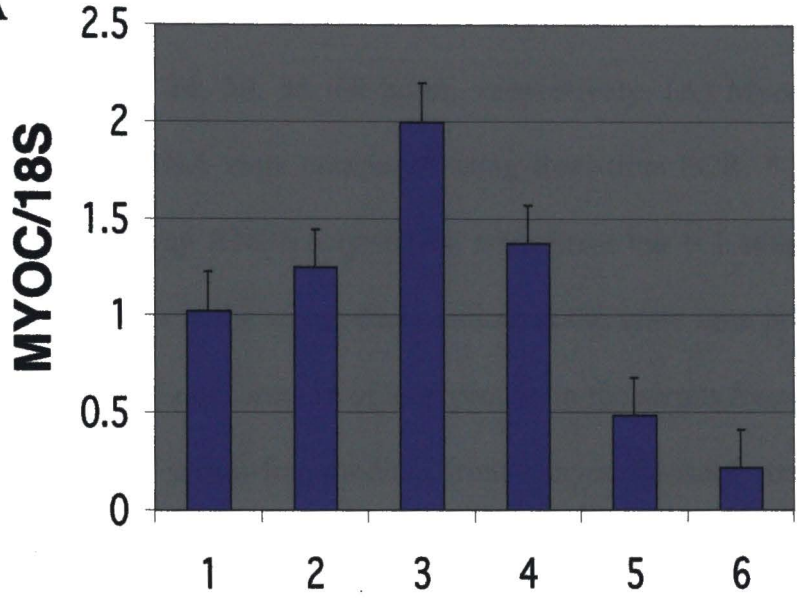

A
B
C

Figure 2. Myocilin mRNA and secreted protein levels from various normal and glaucomatous TM cell lines. (A) Myocilin mRNA levels normalized to 18S rRNA levels were compared using Real-time PCR. Lane 1-3 are samples from normal donors; lane 1: NTM cells from a 54 year old donor; lane 2: NTM cells from an 87 year old donor; lane 3: NTM cells from an 81 year old donor; Lane 4-6 are samples from glaucomatous donors: lane 4: GTM cells from a 75 year old donor; lane 5: GTM cells from a 78 year old donor; lane 6: GTM cells from an 85 year old donor. Error bars = 1 standard deviation. The experiment was repeated four times. (B) Myocilin protein levels in the serum-free medium were shown by western blot analysis, and a Sypro®Ruby staining picture of the blot was also included for quantitation purposes. Lanes 1-4 are samples from normal donors: lane 1: NTM cells from a 2 year old donor; lane 2: NTM cells from a 54 year old donor; lane 3: NTM cells from an 87 year old donor; lane 4: NTM cells from a 71 year old donor. Lanes 5-8 are samples from glaucomatous donors: lane 5: GTM cells from a 78 year old donor; lane 6: GTM cells from a 75 year old donor; lane 7: GTM cells from a 72 year old donor; lane 8: GTM cells from an 85 year old donor. A sample of serum-free medium from a myocilin stably transfected CHO cell line was included to serve as a positive control (PC) for myocilin protein. The experiment was repeated three times.

A


MYOC/18S
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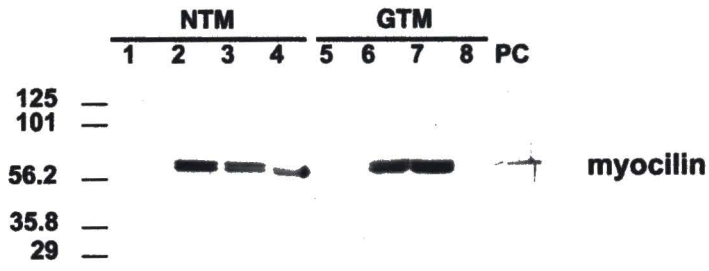
2
1.5
1
0.5
0
1
2
3
4
5
6

B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GTM
1 2 3 4 5 6 7 8 PC
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101
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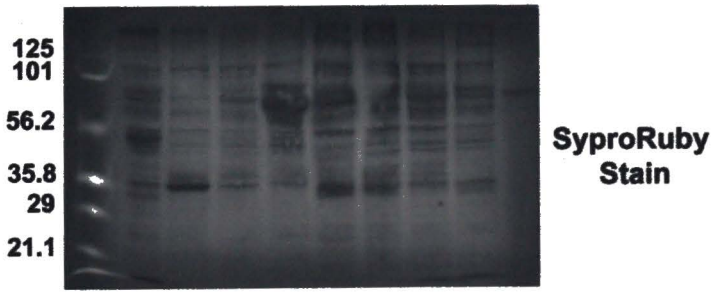
35.8
29
myocilin


125
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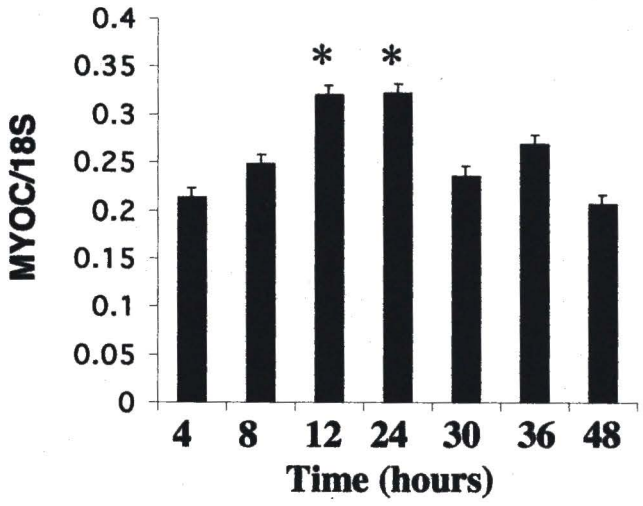
56.2
35.8
29
21.1
SyproRuby
Stain

Figure 3. The effects of serum deprivation on myocilin mRNA levels and protein secretion with time. A cultured normal TM cell line from a 54 years old donor was treated with serum free medium, and mRNA and serum-free medium were collected at time points 4, 8, 12, 24, 30, 36, 48 hours, respectively. (A) Myocilin mRNA levels normalized to 18S rRNA were compared using Real-time PCR. * indicates statistical significance by one-way ANOVA ($p<0.05$), $n=4$. Error bar = 1 standard deviation. (B) Myocilin protein levels in the serum-free medium at the same time points were shown by western blot analysis. Equal amount of total protein in the serum-free medium was loaded per lane. A sample of serum-free medium from a myocilin stably transfected CHO cell line was included to serve as a positive control for the myocilin protein. The experiment was repeated three times.

A


0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
MYOC/18S
*
*
4
8
12
24
30
36
48
Time (hours)

B

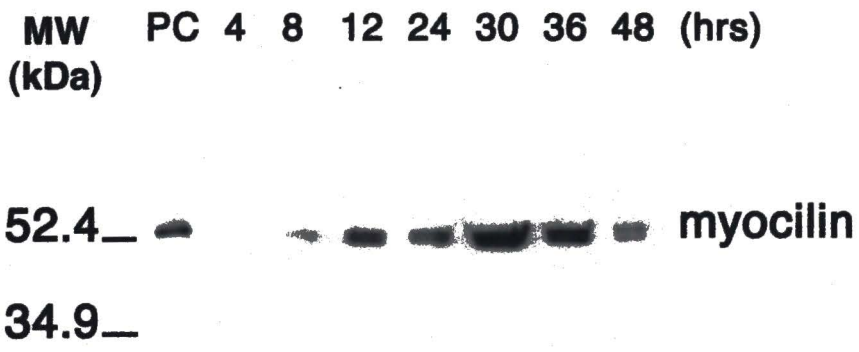
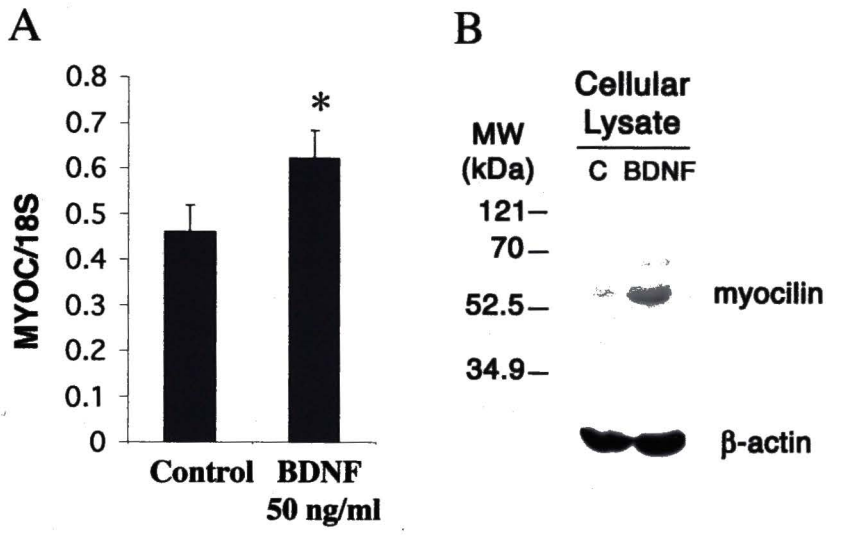

MW (kDa)
PC
4
8
12
24
30
36
48
(hrs)
52.4
34.9
myocilin

Figure 4. The effects of BDNF (50 ng/ml) on myocilin mRNA and protein levels in the serum-free medium of human NTM cells from a 54 years old donor. (A) Real-time PCR demonstrated that BDNF (50 ng/ml) up-regulated myocilin mRNA levels normalized to 18S rRNA at 48 hours compared to non-treatment control. * indicates statistical significance by student T test ($p<0.05$), n=4. Error bar = 1 standard deviation. (B) Western blots of myocilin protein levels in the cellular lysate. The same blot was reprobed with β-actin to serve for quantitation purpose. (C) Western blot of secreted myocilin protein levels with or without BDNF (50 ng/ml) treatment was demonstrated. The serum-free medium was collected at the end of 48 hours and concentrated. Equal amounts of protein were loaded per lane. A sample of serum-free medium from a myocilin stably transfected CHO cell line was included to serve as a positive control for the myocilin protein. Sypro®Ruby staining of the same blot was also included for quantitation purpose. The experiment was repeated three times.


A
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
MYOC/18S
*
Control
BDNF
50 ng/ml
B
MW
(kDa)
Cellular
Lysate
C BDNF
121–
70–
52.5–
34.9–
myocilin
β-actin

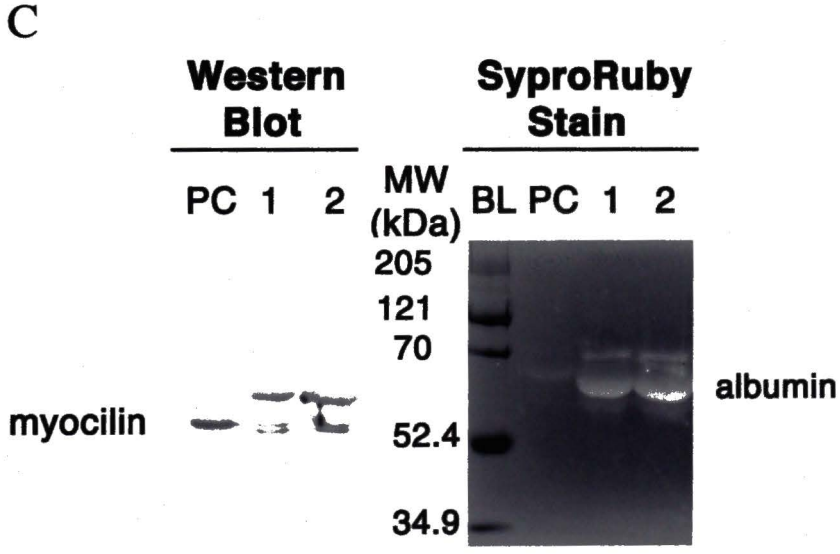

C
Western
Blot
PC 1 2
myocilin
MW
(kDa)
205
121
70
52.4
34.9
SyproRuby
Stain
BL PC 1 2
albumin

Figure 5. The effects of dexamethasone (10^{-7} M) and BDNF (50 ng/ml) on myocilin levels in serum-free medium used to grow three human NTM cell lines by 48 hours. Non-transformed NTM cells from three human donors of 54, 71, and 77 years old, respectively were used. Both treated and non-treated control human TM cells were grown in serum-free media for 48 hours. The serum-free medium was collected and concentrated. An equal amount of protein was loaded per lane. A sample of serum-free medium from a stably transfected CHO cell line was also included to serve as a positive control for myocilin. Panels A, B, C demonstrated that dexamethasone up-regulated myocilin protein levels in the serum-free medium. Panels D, E, F showed the effects of BDNF on myocilin secretion.

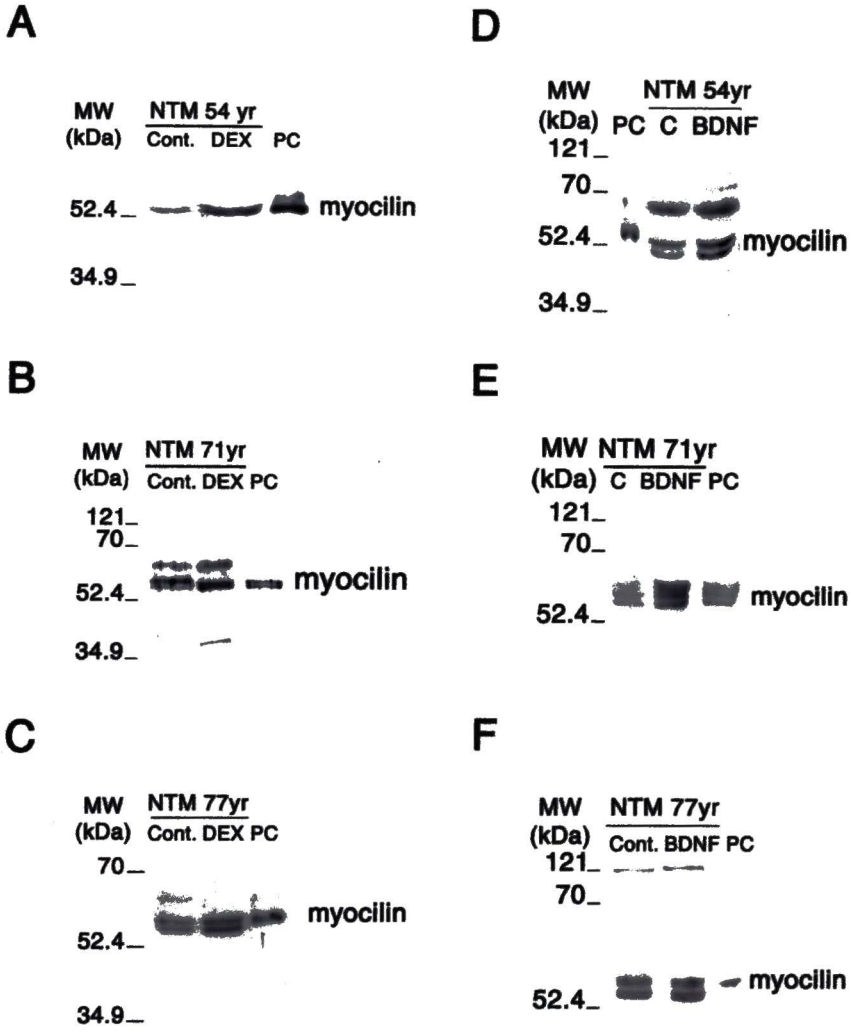
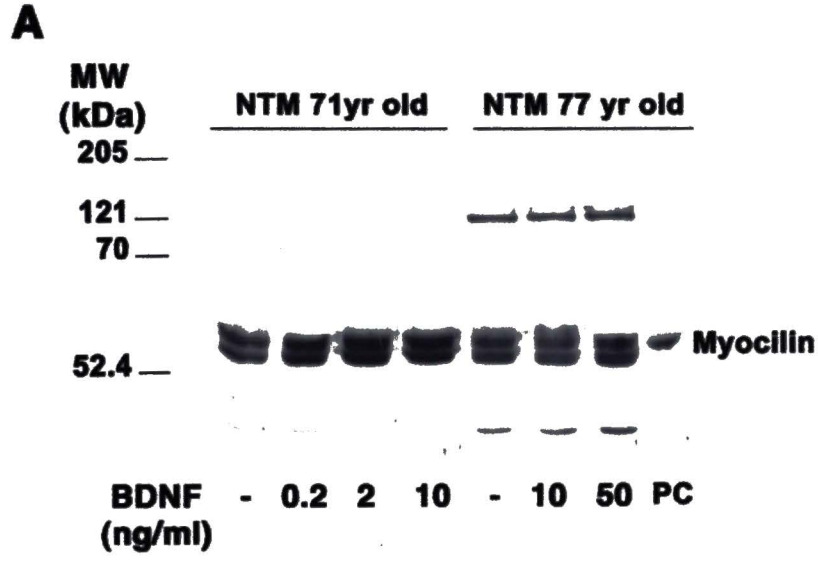
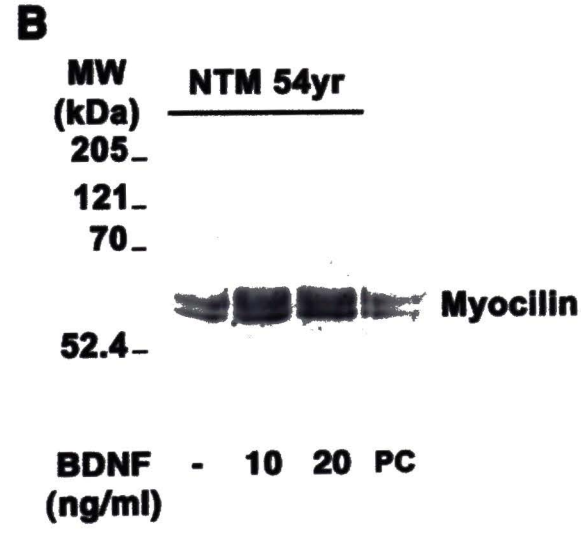
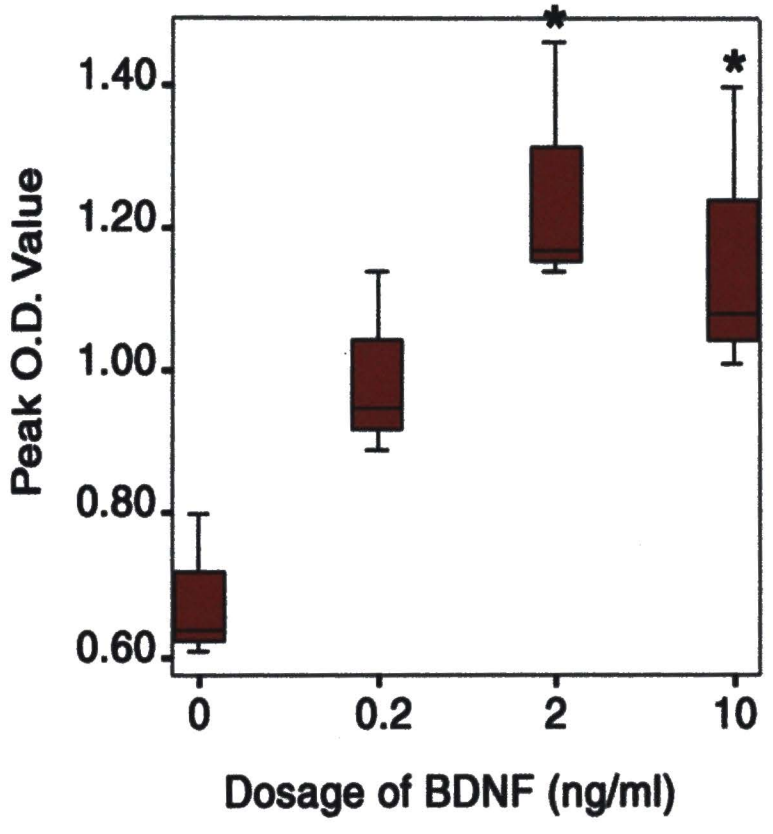

A
MW (kDa)
NTM 54 yr
Cont. DEX PC
52.4_
34.9_
myocilin
B
MW (kDa)
NTM 71yr
Cont. DEX PC
121_
70_
52.4_
34.9_
myocilin
C
MW (kDa)
NTM 77yr
Cont. DEX PC
70_
52.4_
34.9_
myocilin
D
MW (kDa)
NTM 54yr
PC C BDNF
121_
70_
52.4_
34.9_
myocilin
E
MW (kDa)
NTM 71yr
C BDNF PC
121_
70_
52.4_
myocilin
F
MW (kDa)
NTM 77yr
Cont. BDNF PC
121_
70_
52.4_
myocilin

Figure 6. Dose dependent effects of BDNF on myocilin expression in the serum-free medium in human NTM cell cultures. Non-transformed NTM cells from three human donors of 54, 71, and 77 years old were treated with BDNF at dosages ranging from 0.2 to 50 ng/ml in serum free medium. The non-treated human TM cells were also grown in serum-free medium for the same length of time as the controls. The serum-free medium was collected at the end of 48 hours and concentrated. An equal amount of protein was loaded per lane. A sample of serum-free medium from a myocilin stably transfected CHO cell line was also included as a positive control for the myocilin protein. (A) BDNF 0.2 ng/ml, 2 ng/ml, 10 ng/ml were applied to NTM cells from a 71 year-old donor. BDNF 10 ng/ml and 50 ng/ml were applied to NTM cells from a 77 year-old donor. (B) BDNF 10 ng/ml and 20 ng/ml were used to treat NTM cells from a 54 year-old donor. (C) Addition of BDNF of both 2 ng/ml and 10 ng/ml respectively resulted in a statistically significant (one-way ANOVA, $p < 0.05$) increase of myocilin protein levels in the serum-free medium by NTM cells from a 71 year-old donor. * indicates statistical significance, n=4. Error bar = 1 standard deviation.


A
MW
(kDa)
NTM 71yr old
NTM 77 yr old
205
121
70
52.4
Myocilin
BDNF - 0.2 2 10 - 10 50 PC
(ng/ml)


B
MW
(kDa)
NTM 54yr
205
121
70
52.4
Myocilin
BDNF - 10 20 PC
(ng/ml)

C



Statistical significance were determined as $p < 0.05$.

Figure 7. The effects of BDNF treatment on myocilin protein secretion with time. A cultured normal TM cell line from a 54 years old donor was treated with BDNF (50 ng/ml) in serum free medium. The serum-free medium was collected at time point 4, 8, 12, 24, 30, 48 hours, respectively. Myocilin protein levels in the serum-free medium at the above time points were shown by western blot analysis. Equal amount of total protein in the serum-free medium was loaded per lane. A sample of serum-free medium from a myocilin stably transfected CHO cell line was also included to serve as a positive control for myocilin protein.

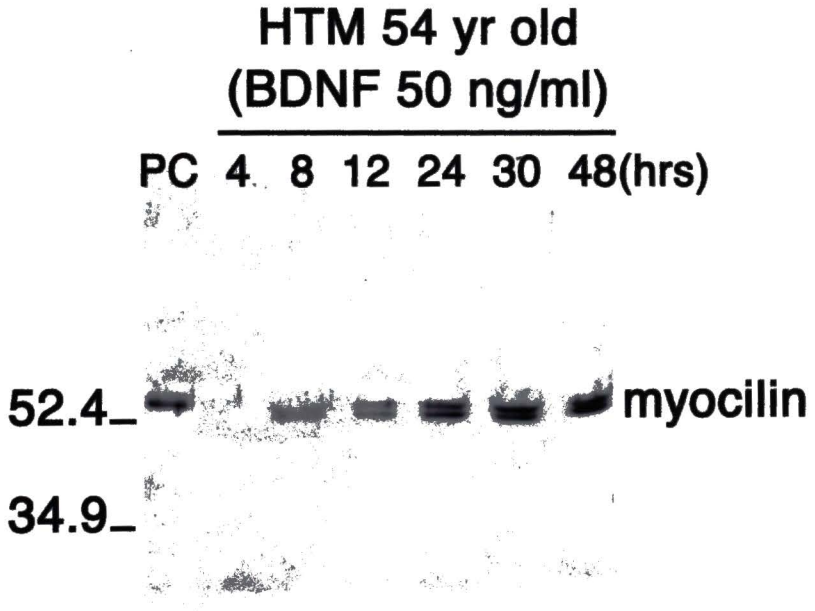
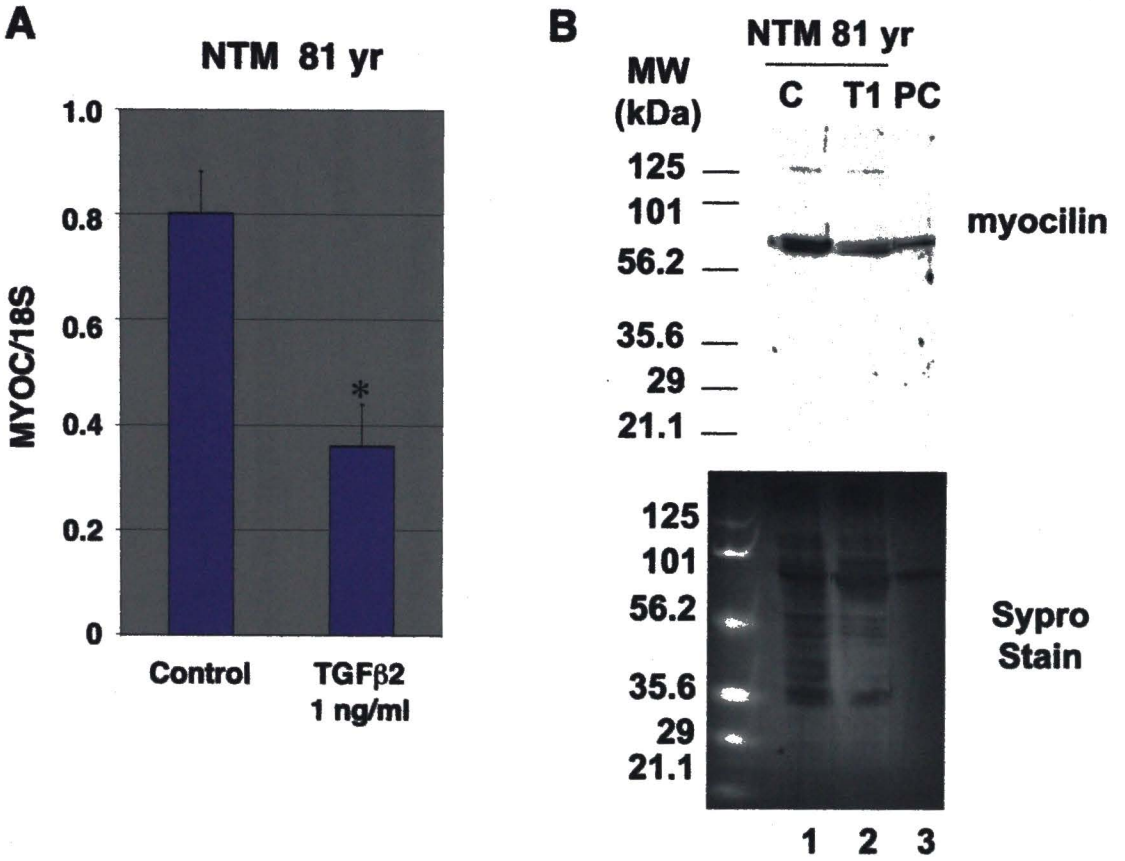

HTM 54 yr old
(BDNF 50 ng/ml)
PC 4 8 12 24 30 48(hrs)
52.4_
34.9_
myocilin

Figure 8. The effects of TGFβ2 (1 ng/ml) on myocilin mRNA levels and protein secretion in human NTM cells. (A) Four non-transformed four human NTM cell lines were treated with TGFβ2 ranging from 0.4 to 1.0 ng/ml. TGFβ2 decreased myocilin mRNA levels when normalized to 18S rRNA expression as determined by Real-time PCR. The result from a representative experiment using a cell line of an 81 year old is shown in the figure. * indicates statistical significance by one-way ANOVA ($p < 0.05$), n=4. Error bar = 1 standard deviation. (B) Secreted myocilin levels were compared by Western blot analysis. Serum-free medium was collected at the end of 48 hours and concentrated. An equal amount of total protein was loaded per lane. A representative blot is shown in (B). C= control; T1= TGFβ2 1 ng/ml treatment. A sample of serum-free medium from a myocilin stably transfected CHO cell line was also included to serve as a positive control for myocilin protein. Sypro® Ruby staining of the same blot was also included for quantitation purposes. 1=control; 2= TGFβ2 1 ng/ml treatment; PC= positive control.


A
NTM 81 yr
MYOC/18S
1.0
0.8
0.6
0.4
0.2
0
*
Control
TGFβ2
1 ng/ml
B
NTM 81 yr
MW
(kDa)
C
T1
PC
125
101
56.2
35.6
29
21.1
myocilin
125
101
56.2
35.6
29
21.1
Sypro
Stain
1
2
3

Discussion

Certain myocilin mutations can lead to a form of glaucoma with an early onset and high intraocular pressure (IOP)[7]. Therefore, myocilin may play a role in regulating IOP during POAG. Mutation knock-in studies of myocilin suggested that mutated myocilin proteins inhibit native myocilin secretion, thereby interfering with normal TM cell functions and resulting in aqueous outflow obstruction[12]. On the other hand, perfusion of the anterior segment of the eye with recombinant myocilin increased intraocular pressure[35]. We are interested in the regulation of MYOC expression and myocilin secretion by endogenous growth factors in the aqueous humor. The immunocytochemistry staining data showed that myocilin protein in human NTM cells is concentrated around the nucleus, suggesting endoplasmic reticulum and Golgi association. In the cytoplasm, myocilin staining appeared punctate, indicating possible association with vesicles. These data agreed with other studies that suggested myocilin is located vesicularly in cells[36].

There have been conflicting results regarding myocilin protein levels in the normal human TM relative to glaucomatous human TM[47, 48]. We initially compared myocilin mRNA and protein levels between normal and glaucomatous human TM cell lines. Of all cultured TM cell lines we studied, secreted myocilin protein levels are not readily detected using Western blot analysis until the medium is concentrated at least 20 fold. Even after concentration, there is minimal detectable secreted myocilin by one TM cell line from a 2 year-old donor. Using Northern blot analysis, Tamm (1999) also reported that TM cell lines from young donors express reduced levels of myocilin mRNA[30].

However, our study demonstrated that both normal and glaucomatous adult TM cell lines secreted myocilin protein. Although different cell lines showed variable amounts of myocilin secretion, there was no significant difference between normal and glaucomatous TM cells. Real time PCR results were consistent with the western blot analysis results. We do not know whether the glaucomatous cell lines we used contain disease-associated mutations of myocilin. Since we were able to detect the secreted form of myocilin, they at least contain one copy of native myocilin.

Interestingly, serum free treatment of cultured human TM cells induced both mRNA levels and protein secretion of myocilin. The elevation of mRNA levels preceded the protein secretion, which indicated that the increased myocilin secretion is most likely due to de novo protein synthesis. It has been reported that MYOC expression can be induced by stretch to the trabecular meshwork and intraocular hypertension[30, 37]. Thus another form of stress, serum deprivation, induces myocilin secretion.

Besides the stretch response element, myocilin also contains an AP-1 site in its promoter region[38]. It has been previously demonstrated that human NTM cells and ex vivo tissue express all four neurotrophins (BDNF, NGF, NT-3 and NT-4) and their functional tyrosine kinase receptors (TrkA, TrkB, TrkC). In addition, human NTM cells are also capable of secreting neurotrophins, which leads to the possibility of a paracrine/autocrine signaling mechanism in the NTM[15]. The results of the present study have shown, for the first time, that BDNF treatment of TM cells results in increased myocilin mRNA levels and enhanced protein secretion. BDNF can signal through its tyrosine kinase receptor (TrkB) in order to phosphorylate Erk[39]. Activated Erk may then

stimulate AP-1 associated transcription factor, thereby up-regulating MYOC expression in trabecular meshwork cells. Although BDNF is well known as a survival factor for both neuronal and nonneuronal cells, it has been shown to enhance fibronectin and laminin synthesis[42]. Fibronectin is an extracellular protein that normally binds to myocilin in the NTM cells[43]. It is also up-regulated by glucocorticoids and glaucomatous condition[44]. It is possible that increased levels of fibronectin may bind to myocilin resulting in decreased aqueous outflow facility. Moreover, the expression of BDNF can be up-regulated by mechanical stretch in organotypic brain slice culture[41]. TM cells in situ are normally under the influence of mechanical factors due to constant fluctuation of the intraocular pressure. Consequently, BDNF may be an important protective modulator of the normal human TM in response to mechanical stretch during elevated changes in intraocular pressure.

It is also notable that all three TM cell lines that responded to BDNF are also glucocorticoid responsive. Glucocorticoids are well known for their delayed induction of MYOC expression and secreted protein in the trabecular meshwork[33], suggesting an indirect effect. Glucocorticoids can also increase intraocular pressure in steroid-responders[40] and mimic glaucomatous pathology. When applied acutely, glucocorticoids can down-regulate the mRNA levels of BDNF but not the full-length TrkB receptor[45], thereby blocking the protective effects of BDNF during the course of stress or injury. BDNF can modulate extracellular matrix via signaling through ERK1. The possible association between glucocorticoid responsiveness and BDNF regulation of myocilin

expression indicates that BDNF may play a direct role in extracellular matrix remodeling in the human TM during intraocular hypertension.

TGFβ2 can also activate AP-1 site through the Erk pathway[46]. Several independent studies showed that in glaucomatous aqueous humor, there are increased levels of both the total and active forms of TGFβ2. However, the exact role TGFβ2 plays during POAG is not clear. A previous report showed that TGFβ1 can up-regulate myocilin mRNA levels in cultured human TM cells[30]. However, our results showed the opposite for TGFβ2. TGFβ2 down-regulated mRNA levels and protein secretion of myocilin in normal human TM cell lines. This result indicated that TGFβ2 possesses an isoform-specific activity[50, 51]. Previous studies from our lab also demonstrated that there is an alternatively spliced form of TβRII present in the human TM. This raises the possibility that TGFβ2 may act differently from TGFβ1 using the TβRII isoform.

We have also tested multiple glaucomatous human TM cell lines for BDNF or TGFβ2 effects on MYOC expression. The results are variable (data not shown). Clark et al. (1995) reported that glaucomatous human TM cells form a geodesic actin filament structure that is not present in normal human TM cells. Hernandez et al. (2002) using cDNA microarray analysis demonstrated that normal and glaucomatous optic nerve head astrocytes display differential gene expression profiles[49]. It is possible that *in vitro* cultured glaucomatous human TM cells conserve some of the *in vivo* phenotypic characteristics of POAG.

In conclusion, myocilin might be a stress response factor, whose levels can be increased by various insults upon the trabecular meshwork. BDNF might be a stress

response-enhancing factor that facilitates the adjustment of the tissue towards the injurious insult. TGFβ2 may be the stabilizing factor trying to minimize the stress response by down-regulating factors such as myocilin in the trabecular meshwork. It is possible that the human trabecular meshwork maintains homeostasis through the concerted efforts of growth factors signaling among the human TM cells via paracrine/autocrine mechanisms.

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PREFACE TO CHAPTER IV

In the previous chapter, both normal and glaucomatous human TM cells expressed varying levels of myocilin mRNA and protein. BDNF up-regulated myocilin mRNA levels and protein secretion, while TGFβ2 down-regulated myocilin expression in normal human TM cells. However, glaucomatous human TM cells responded to the same growth factors treatment differently (data not shown), which suggested that *in vitro* cultured glaucomatous human TM cells might conserve distinct phenotypic characteristics relevant to their roles in the pathogenesis of glaucoma.

To determine if BDNF and TGFβ2 regulate the expression of each other in human trabecular meshwork cells, both normal and glaucomatous human TM cells will be treated with either BDNF and/or TGFβ2. The cellular levels of BDNF or TGFβ2 after cell growth in the presence of each other will be examined. Additionally, changes in the protein levels of membrane bound receptors for both factors (i.e. TβRI, TβRII and TrkB) will be assessed.

CHAPTER IV

EFFECTS OF TRANSFORMING GROWTH FACTOR BETA2 AND BRAIN-DERIVED NEUROTROPHIC FACTOR ON NORMAL AND GLAUCOMATOUS HUMAN TRABECULAR MESHWORK CELLS

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Abstract

Purpose: The human trabecular meshwork (TM) is the main site for aqueous humor drainage and the major regulator of intraocular pressure. Previous studies have shown biologically active TGFβ2 is increased in the aqueous humor (AH) of glaucomatous patients. In addition, we have demonstrated detectable levels of BDNF in human AH. However, the exact role of TGFβ2 and BDNF in the human TM is unknown. The purpose of this study was to compare possible synergistic effects of TGFβ2 and BDNF in glaucomatous and normal human TM cells.

Methods: Well-characterized normal and glaucomatous human TM cells were treated with either BDNF, TGFβ2, or BDNF/TGFβ2 in serum free Ham's nutrient mixture F-10 medium for 48 hours. Real time PCR and ELISA assays were used to examine the regulatory effects of BDNF and TGFβ2 on each other (mRNA levels, and protein secretion). Western blot analysis was used to measure growth factor receptor protein levels.

Results: In normal human TM cells, BDNF up-regulated TGFβ2 mRNA levels and protein secretion but down-regulated TβRI. In return, TGFβ2 promoted not only the transcription of the BDNF gene and BDNF protein secretion, but also increased protein levels of TβRI and truncated TrkB receptor. The combination of BDNF and TGFβ2 enhanced truncated TrkB protein levels without affecting TβRI. In glaucomatous human TM cells, the reciprocal induction between BDNF and TGFβ2 observed in normal human TM cells was not present. BDNF synergistically increased the expression of TβRI in combination with TGFβ2. BDNF abolished the induction of TrkB by TGFβ2.

Conclusions: This study demonstrates, for the first time, the differential response to TGFβ2 and BDNF by normal and glaucomatous human TM cells. In addition, this study demonstrates the reciprocal up-regulation between TGFβ2 and BDNF in cultured normal human TM cells. This raises the distinct possibility that paracrine/autocrine signaling via the interaction between BDNF and TGFβ2 may occur within normal human TM cells and may be altered in glaucomatous human TM cells.

Introduction

The human trabecular meshwork (TM) is the major site for regulating aqueous outflow resistance, and intraocular pressure[1]. Ocular hypertension has long been considered as a major risk factor for primary open angle glaucoma (POAG), the most common form of glaucoma in the U.S. and Europe, accounting for over 60% of all cases[2]. In POAG, an increase in extracellular matrix components and a decrease in the number of TM cells have characterized the pathophysiology of the human TM. Moreover, human TM cells from POAG donors appear to show signs of "activation" with enlarged nuclei and increased free ribosomes indicating protein synthesis[4]. The molecular basis of the change in glaucomatous human TM cells may reflect differential gene expression via various growth factors found in the aqueous humor.

Transforming growth factor beta 2 (TGFβ2) levels are increased in the aqueous humor of glaucomatous patients when compared to normal human samples[5-7]. However, it is still unknown whether this increase of TGFβ2 levels is a cause or an effect. TGFβ2 can be secreted from multiple sites along the aqueous flow pathway[4]. Previous reports from our laboratory demonstrated that human TM cells express TGFβ2 and its high affinity functional receptors[8-10]. TGFβ isoforms regulate the cell cycle and the production of the extracellular matrix proteins[11-13]. For instance, TGFβ2 enhances the synthesis and secretion of fibronectin and laminin in porcine trabecular meshwork cells[14]. Under normal culture conditions, TGFβ2 inhibits human trabecular meshwork cell proliferation induced by epidermal growth factor[9]. Therefore, increased TGFβ2 levels in human

trabecular meshwork may lead to decreased human TM cell number and increased extracellular matrix deposition resembling POAG.

Normal human trabecular meshwork also contains various protective mechanisms against injurious assault in order to retain normal functions during stressful events, such as intraocular hypertension. During ocular hypertension, mechanical stretch plays an important role in the trabecular meshwork pathology. In neuronal tissue, mechanical stretch can induce the expression of brain-derived neurotrophic factor (BDNF)[15]. BDNF belongs to the neurotrophin family that also includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Human trabecular meshwork was originated from neural crest. It has also been reported that human TM[19] expresses all four neurotrophins and their tyrosine kinase (Trk) receptors (e.g. TrkA, TrkB, TrkC). Although BDNF is a survival factor for neuronal cells, it also plays an essential role in maintaining vessel stability through direct *in vivo* angiogenic actions on endothelial cells[16-18].

It is possible that ocular hypertension may induce BDNF expression, which in turn may activate the Erk[20] pathway through TrkB phosphorylation, thereby modulating the TGFβ2 regulatory effects on extracellular environment. When two growth factors share a common signaling pathway and regulate similar processes, it is possible that there may be crosstalk between them. It has been reported[21] that neurotrophins act synergistically with members of the TGFβ superfamily to promote the survival of spiral ganglia neurons *in vitro*. In neurons, TGFβ1 can up-regulate BDNF mRNA and protein levels in the cultured medium[22]. In return, BDNF mediates BMP-2 (bone morphogenetic protein-2, a member

of the TGFβ superfamily) effects on cultured neurons[23]. We therefore hypothesized that BDNF and TGFβ2 may have regulatory effects on each other or on their high affinity specific receptors. In the present study, we demonstrated the reciprocal up-regulation of both mRNA and protein levels between TGFβ2 and BDNF in normal human TM cells. Interestingly, we further report that this reciprocal effect is lacking in glaucomatous human TM cells.

METHODS

Human Trabecular Meshwork Cell Culture

Five non-transformed normal human TM cells from donors of different age (e.g. 2 years, 54 years, 71 years, 77 years, and 87 years) and three non-transformed glaucomatous human TM cells from donors of different age (e.g. 72 years, 75 years, and 78 years) were utilized in studying mRNA levels and secretion of BDNF and TGFβ2. The trabecular meshwork cells were grown until confluent or near confluent in Ham's F-10 Medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Inc. Logan, UT), 2 mM L-glutamine (0.292 mg/ml), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) (Life Technologies, Grand Island, NY). All cell lines were maintained at 37°C in 5% CO_2-95% room air; medium was changed every 2 to 3 days.

Growth Factor Treatment of Human TM Cells

Confluent or near confluent monolayer human TM cells were washed 4 times with serum-free medium before the growth factors were added. The human TM cells were

then incubated with individual or combinations of growth factors in serum free medium at 37°C in 5% CO_2-95% room air for 48 hours. BDNF ranging in concentration from 10 to 50 ng/ml and TGFβ2 ranging in concentration from 0.1 to 2 ng/ml were used in the treatments. Human TM cells growing in serum-free media without growth factors was used as controls for the treatment.

Total Cellular RNA Extraction and cDNA Synthesis

Total cellular RNA was prepared using the guanidinium thiocyanate/ phenol/ chloroform method[24]. Confluent or near confluent human TM cells (5-10 x 10^7 cells) were scraped off the culture flasks, pelleted by centrifugation and lysed in 1 ml TRIzol reagent (Life Technologies) by repetitive pipetting. RNA was precipitated from the resulting aqueous phase by adding isopropyl alcohol followed by centrifugation. RNA was resuspended in 70 µl of water and stored at -80°C. Total cellular RNA was used for cDNA synthesis. To reduce secondary structure, 20 µg RNA was mixed with 0.75 µg random primers (Promega, Madison, WI) and incubated at 85°C for 3 minutes. The following were then added to the reaction tube: 80 units RNasin (Promega), 40 units avian myeloblastosis virus (AMV) reverse transcriptase (Promega), 0.625 mM each deoxyribonucleotide, 50 mM Tris-HCl, 75 mM potassium chloride, 10 mM dithiothreitol, and 3 mM magnesium chloride. The reaction tube was incubated at 42°C for 30 minutes, followed by incubating at 94°C for 2 minutes. Synthesis of cDNA was verified by PCR amplification of β-actin. The cDNA was stored at -80°C.

Primer Design and Polymerase Chain Reaction

BDNF and TGFβ2 mRNA sequences (accession numbers: AF400438, NM_003238, respectively) were obtained from Entrez (NCBI, Bethesda, MD). Oligo 4.0 (National Biosciences, Plymouth, MN) was used to design PCR primers that have optimal annealing temperatures and magnesium concentrations. Designed primer pairs were submitted through BLAST (National Center for Biotechnology Information, Bethesda, MD) to ensure that they would not hybridize to any other known nucleic acid sequences under the conditions used. For TGFβ2, the specific 3' downstream primer (5'- GGC GGG ATG GCA TTT TC-3') and the 5' upstream primer (5'- CTA CGC CAA GGA GGT TTA CAA AAT A -3') were used. For BDNF, the specific 3' downstream primer (5'- TCG CCA GCC AAT TCT CTT TT –3') and the 5' upstream primer (5'- GTG CCG AAC TAC CCA GTC GTA-3') were used. The annealing temperature of the primer pairs used in this experiment was 60°C. These primer designs yielded an expected product of 66 base pairs for TGFβ2 or 67 base pairs for BDNF, respectively.

Real-time quantitative PCR

Real-time PCR was performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) according to the manufacturer's instructions. For amplification of BDNF and TGFβ2, reactions were performed in 25 μl total volume with 200 nM of the above primers, and cDNA from 2.5 ng of total RNA in 1X SYBR Green PCR master mix. BDNF and TGFβ2 quantification data were normalized to expression of the 18S. The 18S amplification reactions were performed with primers, (5'-GCC GCT AGA GGT GAA ATT CTT G-3', forward and, 5'-CAT TCT TGG CAA ATG CTT TCG-3',

reverse), in the same condition as BDNF and TGFβ2 amplification. The PCR reactions were carried out at 95°C for 10 minutes and followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantification of gene expression was performed using the standard curve method according to User Bulletin 2 of the ABI Prism 7700 Sequence Detection System. The PCR products were sequenced to confirm the accuracy.

Western Blotting

Confluent or near confluent human TM cells were collected in 0.2 ml protein lysis buffer. The protein lysis buffer consisted of 10 mM Tris-HCl, 0.5% NP40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM PMSF in ethanol, 1 μg/ml aprotinin, 4 μg/ml pepstatin, 10 μg/ml leupeptin, and 100 mM sodium orthovanadate (10 μl/ml). The conditioned culture medium was collected and concentrated using Centriplus YM-3 centrifugal filter devices (Millipore Corporation, Bedford, MA). Protein concentration was determined by the Lowry method. Lysate (40-60 μg) or the TM culture medium were mixed with a one-third volume of Laemmeli electrophoresis buffer and boiled for 60 seconds. Laemmeli electrophoresis buffer consisted of 1.0 ml glycerol, 0.5 ml β-mercaptoethanol, 3.0 ml of 10% SDS, 1.25 ml 1.0 M Tris-HCl pH 6.7, 1-2 mg bromophenol blue. Proteins were then separated on a denaturing SDS polyacrylamide gel and transferred by electrophoresis to a nitrocellulose membrane. Sypro® Ruby (Molecular Probes, Eugene, Oregon) staining of the membrane was used to record the total protein profile. Non-specific binding were subsequently blocked by soaking membranes in 1X TBST (1X TBS and 0.2% Tween-20), 5% dry milk for at least 1 hour at room temperature. Membranes were then incubated with primary antibody (1:666.7)

(Santa Cruz Biotechnology, Inc. Santa Cruz, CA) for overnight at 4°C, and followed by four times washing with 1X TBST. A 45-minute incubation with horseradish peroxidase-conjugated secondary antibody (Promega Corporation, Madison, WI) (1:10,000) was applied. Six washes with 1X TBST, and one final wash in 1X TBS alone was followed. Chemiluminescence detection was done using a Chemiluminescence kit (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce) and a cooled, computerized CCD camera-based imaging system (Alpha Innotech, San Leadro, CA) to visualize and record the stained proteins. The digitized images from the gels stained with Sypro® Ruby for total protein, and from the immunostained blots, were quantitatively analyzed using the Chemiimager software (Alpha Innotech). To quantitate the specific protein band, a rectangular box was drawn around the band. The area for quantitation remains consistent for each set of membrane. A comparison report of quantitative differences of specific proteins normalized to total protein on each gel was then generated.

Immunoassays (ELISA) for TGFβ2 and BDNF

The serum-free medium was collected at the end of 48 hours exposure to BDNF or TGFβ2 treatment. The serum-free medium was then concentrated 20-fold using a Centriplus YM-3 centrifugal filter device (Millipore Corporation, Bedford, MA). Protein concentrations were determined by the Lowry method. Emax™ ImmunoAssay Systems (Promega, Madison WI) specific for BDNF or TGFβ2 were used according to manufacturer's instructions. Nunc ELISA/EIA 96 well plates were coated with anti-BDNF or anti-TGFβ2 monoclonal antibodies (Promega Corporation, Madison, WI) overnight at 4°C. Equal amount of total protein was added the next day for the secreted

BDNF or TGFβ2 to be captured by the coated antibody. The secreted growth factors were then detected by treating the plates with the respective polyclonal antibodies (Anti-Human BDNF, 1:500; Anti-Human TGFβ2, 1:2000) (Promega Corporation, Madison, WI) followed by a Anti-IgY horseradish peroxidase conjugated secondary antibody (1:200). Enzyme substrate was subsequently added to generate a color product whose absorbance was read at 450 nm. Serial two-fold dilutions of the recombinant growth factor to be assayed were included in each assay to generate a standard curve. Each sample was assayed in triplicate.

Statistical Analysis

The statistical significance, defined as $p<0.05$, was evaluated using a statistical software SPSS-10 for Macintosh. The mode of data distribution was determined before analysis of variance. For normally distributed data, student t-test was used for variance analysis between two groups; and one-way ANOVA was used for variance analysis among multiple groups. For the data sets that had skewed distribution, nonparametric analysis was used for variance comparison.

Results

BDNF Up-regulates TGFβ2 mRNA and Protein Levels in Cultured Normal Human TM Cells

BDNF (50 ng/ml) significantly up-regulated TGFβ2 mRNA levels as demonstrated by real-time PCR in a human TM cell line from a 54-year-old donor (Figure 1A). ELISA assays (Figure 1B, 1C) demonstrated that BDNF, at various concentrations, up-regulated

the levels of TGFβ2 in the serum-free medium from normal human TM cell lines. The serum-free medium was not acid-treated in order to detect native active TGFβ2 levels. It is noteworthy that the increase of mature TGFβ2 becomes statistically significant at as low as 2 ng/ml of BDNF in one normal TM cell line. While in the second normal TM cell line, mature TGFβ2 protein levels started to rise at 0.2 ng/ml but did not reach statistical significance until 10 ng/ml. Both cell lines showed induction of TGFβ2 by BDNF at 10 ng/ml. This probably reflects variation in different cell lines.

BDNF Does Not Alter the Levels of TGFβ2 mRNA or Protein in Glaucomatous Human TM cells

Three individual glaucomatous human TM cell lines were utilized. Messenger RNA levels of TGFβ2 were not altered by BDNF treatment of glaucomatous human trabecular meshwork cells. In Figure 2A, a cell line from a 72-year-old donor was treated with 10 ng/ml for 48 hours. Similar results were obtained when two additional cell lines (e.g. 75 and 79 year-old donors) were exposed to BDNF concentrations ranging from 0.2 to 50 ng/ml (data not shown). In addition, BDNF treatment did not alter TGFβ2 protein levels in serum-free medium from glaucomatous human TM (Figure 2B, 2C).

TGFβ2 Up-regulates BDNF mRNA and Protein Levels in Cultured Normal Human TM Cells

To examine the effect of TGFβ2 treatment on BDNF mRNA levels, three human TM cell lines were utilized. A representative graph from the treatment of an 81 year-old donor is demonstrated (Figure 3A). At 0.4 ng/ml level, TGFβ2 significantly up-regulated BDNF expression as demonstrated by real-time PCR. Also, TGFβ2 at low levels (<=0.4 ng/ml)

increased BDNF protein levels in the serum-free medium in two additional normal human TM cell lines (e.g. 71- and 77- year old donors) as shown by ELISA assay in Figure 3B and 3C. It is noteworthy that at a higher dosage (2 ng/ml), TGFβ2 was not able to increase the secretion of BDNF at least in one normal human TM cells (data not shown).

TGFβ2 Down-regulates BDNF mRNA and Protein Levels in Cultured Glaucomatous TM Cells

BDNF messenger RNA expression levels were downregulated in glaucomatous human TM cell lines following TGFβ2 treatment. In Figure 4A, a cell line from a 75 year-old donor was treated with 0.4 ng/ml TGFβ2 for 48 hours. Two additional cell lines (e.g. 72 and 79 year-old donors) were used to determine BDNF protein levels in serum-free medium. TGFβ2 concentrations greater than 0.4 ng/ml significantly decreased BDNF protein levels (Figure 4B, 4C).

Regulation of TGFβ Receptor Protein Levels by BDNF and TGFβ2 in Normal Human TM Cells

In one normal human TM cell line from a 71 year-old donor, BDNF at 10 ng/ml was able to down-regulate protein levels of TβRI without affecting TβRII levels as demonstrated by Western blot analysis (Figure 5A & 6A, lane 2). The TβRI was demonstrated at around 60 kDa as the most prominent band, and also a higher molecular weight band around 114 kDa. The identity of the higher band is unknown. The TβRII was demonstrated at around 85 kDa. TGFβ2 at low dosage (0.2 ng/ml) increased the protein levels of TβRI but not TβRII (Figure 5A & 6A, lane 3). BDNF and TGFβ2 antagonized

the regulatory effect of each other on TβRI protein levels when they were both present in the culture medium (Figure 5A & 6A, lane 4).

Regulation of TGFβ Receptor Protein Levels by BDNF and TGFβ2 in Glaucomatous Human TM cells

Consistent with normal human TM cells, TβRI protein showed up at around 60 kDa in a glaucomatous cell line from a 72 year-old donor as demonstrated by Western blot analysis (Figure 7A). The higher band at around 114 kDa as seen in normal human TM cells is also present. In contrast to the normal human TM cells, the higher band at around 114 kDa seems to be more prominent than the 60 kDa band. Also in accordance with normal human TM cells, TGFβ2 (0.2 ng/ml) was able to enhance intracellular TβRI protein levels. At 2 ng/ml, TGFβ2 further increased TβRI expression suggesting a dose-dependent up-regulation. However, BDNF at 10 ng/ml, augmented TβRI protein levels by glaucomatous human TM cells as opposed to its down-regulatory effect in normal human TM cells. As a result, the synergistic up-regulation of TβRI protein levels was obtained with a combination of both BDNF and TGFβ2 (0.2 ng/ml) treatment. Interestingly, when a higher dosage of TGFβ2 (2 ng/ml) was superimposed with BDNF in the treatment, TβRI protein expression levels remained the same as when TGFβ2 was applied alone. For TβRII, a band at around 85 kDa was identified as the receptor protein (Figure 8A). Neither BDNF at 10 ng/ml nor TGFβ2 at 0.2 or 2 ng/ml affected TβRII protein levels.

TGFβ Up-regulation of TrkB Protein Levels Can not be Abolished by BDNF in Normal Human TM Cells

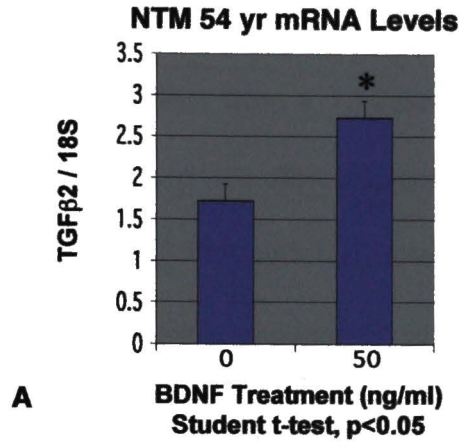
A normal human TM cell line from a 71 year-old donor was used to contrast the TrkB protein levels following growth factor treatment using Western blot analysis (Figure 9). The TrkB receptor protein was demonstrated at around 95 kDa. This is smaller than the previous reported full-length TrkB in neuronal tissues, suggesting the expression of a novel variant of this tyrosine kinase receptor. TGFβ2 at 0.2 ng/ml increased the protein levels for TrkB more than three fold, while BDNF at 50 ng/ml did not have an effect. Combination of BDNF and TGFβ2 induced TrkB at a level slightly lower than TGFβ2 alone.

TGFβ Up-regulation of TrkB Receptor Protein Levels Can be Abolished by BDNF in Glaucomatous Human TM Cells

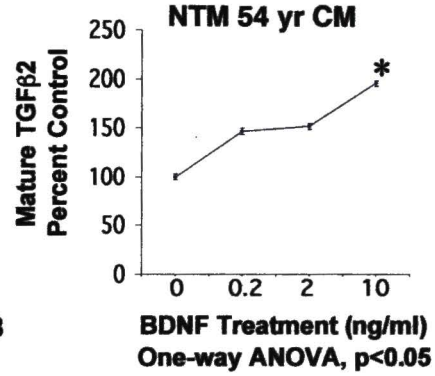
Similar to normal human TM cells, TrkB was demonstrated at around 95 kDa in a glaucomatous cell line from a 72 year-old donor as demonstrated by Western blot analysis (Figure 10). In contrast to the normal human TM cells, BDNF at 10 ng/ml inhibited the expression of TrkB more than two folds in glaucomatous human TM cells. In addition, TGFβ2 at a low level of 0.2 ng/ml failed to increase the protein levels of TrkB. At a higher level of 2 ng/ml, TGFβ2 induced TrkB expression. However, when both TGFβ2 and BDNF were applied in the treatment simultaneously, BDNF overrided TGFβ2 effects on TrkB protein levels.

Figure Legend

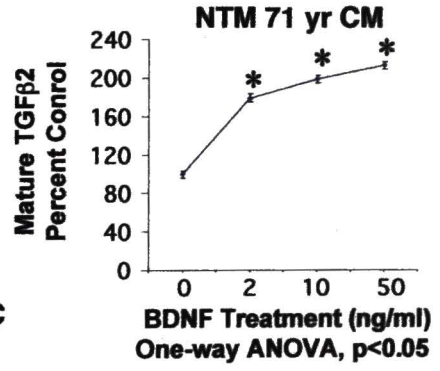
Figure 1. The effect of BDNF treatment on TGFβ2 levels in normal TM cells from human donors of 54 and 71 years old. (A) TGFβ2 mRNA levels is normalized to 18S rRNA levels using Real-time PCR in a normal TM cell line from a 54 year-old donor. (B, C) Mature TGFβ2 protein levels in serum-free medium were determined by ELISA assay from two normal human TM cell lines (donors of 54 and 71 years of age). Recombinant TGFβ2 was used for a standard curve plot for TGFβ2 protein estimation. For normal human TM 54 year old cells, BDNF at 0.2, 2, 10 ng/ml were used to treat the cells. For normal human TM 71 year old cells, BDNF at 2, 10, 50 ng/ml were used in the treatment. All treatments were performed in serum-free media for 48 hours with a non-treatment control included. * indicates statistical significance by one-way ANOVA, $p<0.05$, $n=4$. Error bar = 1 standard deviation.


NTM 54 yr mRNA Levels
TGFβ2 / 18S
3.5
3
2.5
2
1.5
1
0.5
0
*
0
50
BDNF Treatment (ng/ml)
Student t-test, p<0.05

A

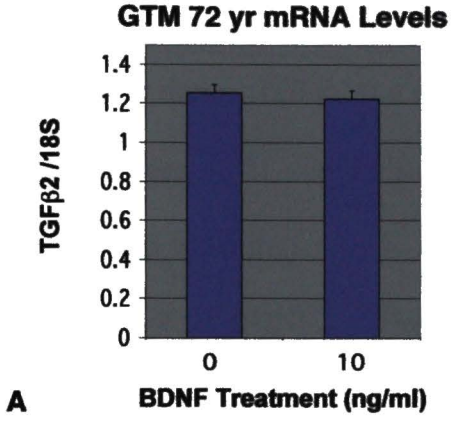

NTM 54 yr CM
Mature TGFβ2
Percent Control
250
200
150
100
50
0
*
0
0.2
2
10
BDNF Treatment (ng/ml)
One-way ANOVA, p<0.05

B

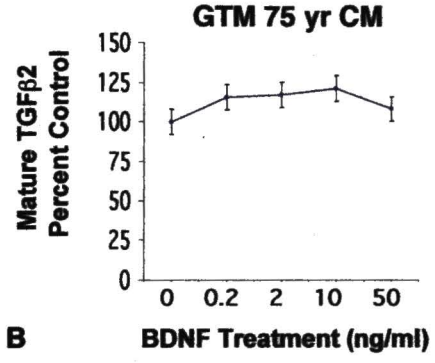

NTM 71 yr CM
Mature TGFβ2
Percent Conrol
240
200
160
120
80
40
0
*
*
*
0
2
10
50
BDNF Treatment (ng/ml)
One-way ANOVA, p<0.05

C

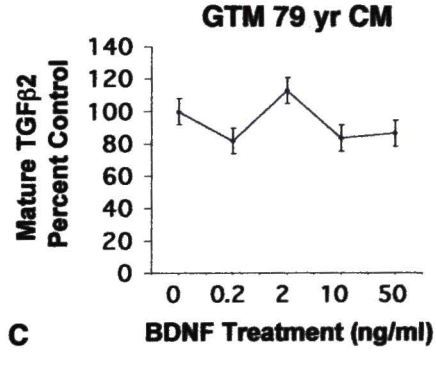
Figure 2. The effect of BDNF treatment on TGFβ2 message and protein levels in glaucomatous TM cell lines from human donors of 72, 75, and 79 years old. (A) TGFβ2 mRNA levels normalized to 18S rRNA levels were compared using Real-time PCR in a glaucomatous human TM cell line from a 72 year-old donor. BDNF (10 ng/ml) was administered to the cells. (B, C) In two other glaucomatous cell lines of 75 and 79 years old respectively, mature TGFβ2 intracellular protein levels were assayed by ELISA. Recombinant TGFβ2 was used for a standard curve plot for TGFβ2 protein estimation. For both glaucomatous human TM cells, BDNF concentrations of 0.2, 2, 10, 50 ng/ml were used to treat the cells. All treatments were performed in serum-free media for 48 hours with a non-treatment control included. The experiment was repeated three times. Error bar = 1 standard deviation.


GTM 72 yr mRNA Levels
TGFβ2 /18S
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
BDNF Treatment (ng/ml)

A

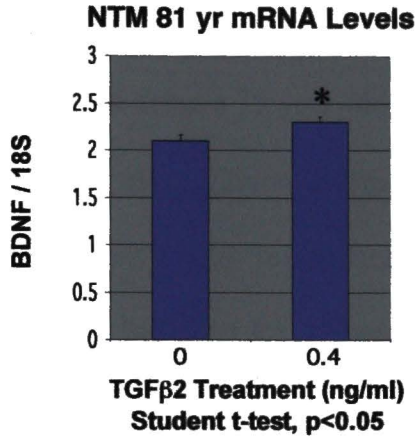

GTM 75 yr CM
Mature TGFβ2
Percent Control
150
125
100
75
50
25
0
0
0.2
2
10
50
BDNF Treatment (ng/ml)

B

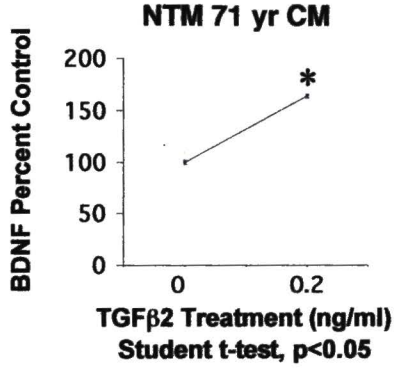

GTM 79 yr CM
Mature TGFβ2
Percent Control
140
120
100
80
60
40
20
0
0
0.2
2
10
50
BDNF Treatment (ng/ml)

C

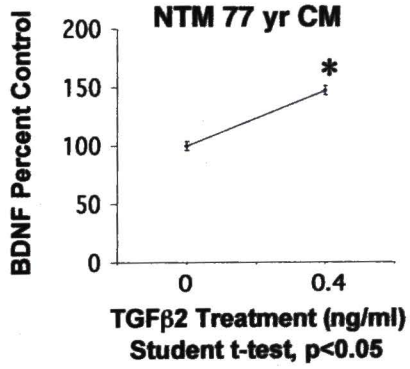
Figure 3. The effect of TGFβ2 on BDNF mRNA levels and protein secretion in normal human TM cells from donors of 77, 81, and 71 years old. (A) BDNF mRNA levels normalized by 18S rRNA levels were compared using Real-time PCR in a normal human TM cell line from a 81 year-old donor. TGFβ2 0.4 ng/ml were used to treat the cells. (B, C) In two other normal cell lines of 71 and 77 years old respectively, BDNF protein levels in the serum-free medium were shown by ELISA assay. Recombinant BDNF was included for a standard curve plot for BDNF protein estimation. In normal human TM 71 year old cells, TGFβ2 0.4 ng/ml was used to treat the cells. As for a normal human TM 77 year old cells, TGFβ2 0.2 ng/ml was used in the treatment. All treatments were performed in serum-free media for 48 hours with a non-treatment control included. * indicates statistical significance by one-way ANOVA, $p<0.05$, $n=4$. Error bar = 1 standard deviation.


NTM 81 yr mRNA Levels
BDNF / 18S
3
2.5
2
1.5
1
0.5
0
*
0
0.4
TGFβ2 Treatment (ng/ml)
Student t-test, p<0.05

A


NTM 71 yr CM
BDNF Percent Control
200
150
100
50
0
*
0
0.2
TGFβ2 Treatment (ng/ml)
Student t-test, p<0.05

B


NTM 77 yr CM
BDNF Percent Control
200
150
100
50
0
*
0
0.4
TGFβ2 Treatment (ng/ml)
Student t-test, p<0.05

C

Figure 4. The effect of TGFβ2 on BDNF mRNA levels and protein secretion in glaucomatous human TM cells from donors of 72, 75, and 79 years old. (A) BDNF mRNA levels normalized by 18S rRNA levels were compared using Real-time PCR in a glaucomatous human TM cell line from a 75 year-old donor. TGFβ2 0.4 ng/ml was used to treat the cells. (B, C) In two other glaucomatous cell lines of 72 and 79 years old respectively, BDNF protein levels in the serum-free medium were shown by ELISA assay. Recombinant BDNF was included for a standard curve plot for BDNF protein estimation. In both glaucomatous human TM cells, TGFβ2 concentrations of 0.2, 0.4, and 1 ng/ml were used in the treatment. All treatments were performed in serum-free media for 48 hours with a non-treatment control included. * indicates statistical significance by one-way ANOVA, $p<0.05$, $n=4$. Error bar = 1 standard deviation.

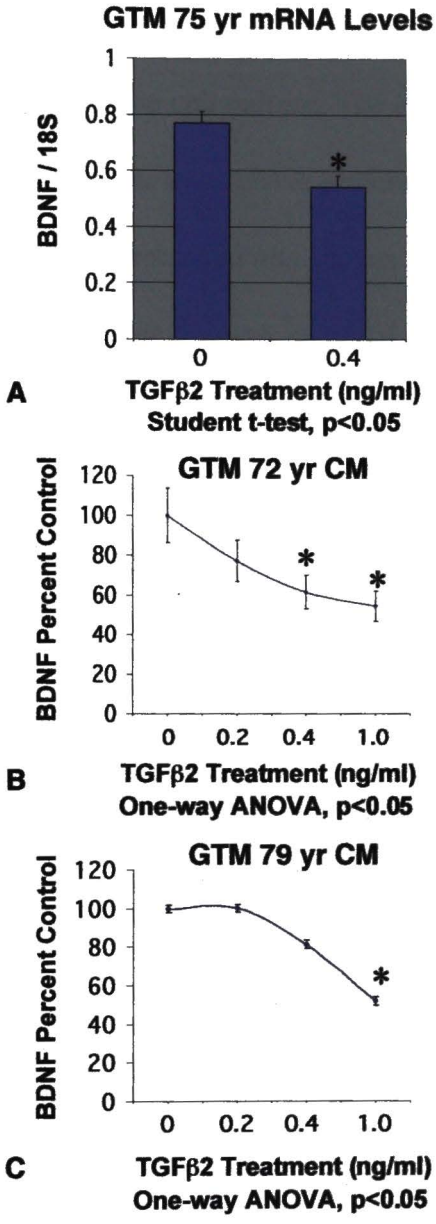

GTM 75 yr mRNA Levels
BDNF / 18S
1
0.8
0.6
0.4
0.2
0
0
0.4
*
A
TGFβ2 Treatment (ng/ml)
Student t-test, p<0.05
GTM 72 yr CM
BDNF Percent Control
120
100
80
60
40
20
0
0
0.2
0.4
1.0
*
*
B
TGFβ2 Treatment (ng/ml)
One-way ANOVA, p<0.05
GTM 79 yr CM
BDNF Percent Control
120
100
80
60
40
20
0
0
0.2
0.4
1.0
*
C
TGFβ2 Treatment (ng/ml)
One-way ANOVA, p<0.05

Figure 5. The effects of TGFβ2 and BDNF on TβRI protein levels in a normal human TM cell line from a 71 years old donor at the end of 48 hours under serum free condition. TGFβ2 at 0.2 ng/ml, BDNF at 50 ng/ml, and a combination of TGFβ2 0.2 ng/ml and BDNF 50 ng/ml were applied to the cell culture. The cell lysates were collected and equal amounts of protein were loaded per lane. (A) A picture of the Chemiluminescence detection of the western blot was recorded and shown. (B) A picture of the membrane stained with Sypro® Ruby after transfer was included for quantitation purpose. (C) The densities of each band normalized by the stained total protein were plotted. Lane 1= control; Lane 2 = BDNF at 50 ng/ml treatment; lane 3 = TGFβ2 at 0.2 ng/ml treatment; lane 4 = combination of BDNF 50 ng/ml and TGFβ2 0.2 ng/ml treatment. Human TM cells growing in serum-free medium without growth factor treatment for 48 hours was included as a control for the treatment. The experiment was repeated three times.

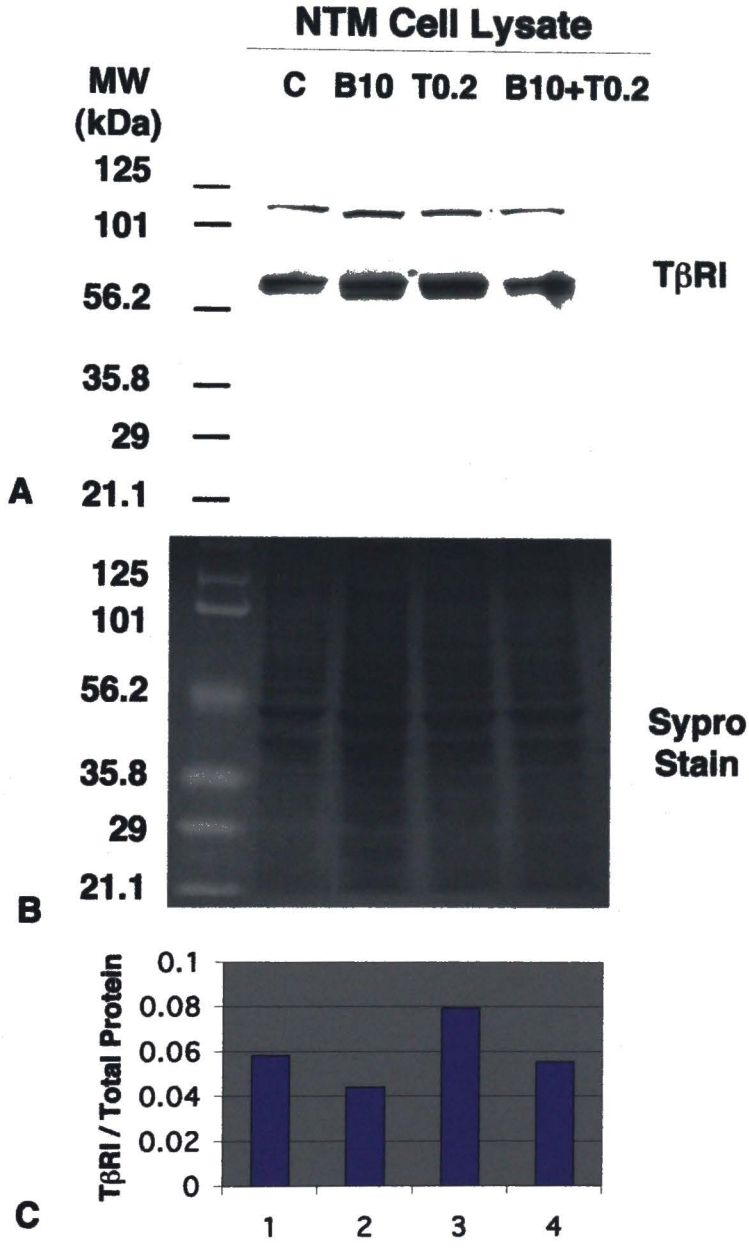

NTM Cell Lysate
C
B10
T0.2
B10+T0.2
MW (kDa)
125
101
56.2
35.8
29
21.1
TβRI
A
125
101
56.2
35.8
29
21.1
Sypro Stain
B
TβRI / Total Protein
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
C

Figure 6. The effects of TGFβ2 and BDNF on TβRII protein levels in a normal human TM cell line from a 71 years old donor at the end of 48 hours under serum free condition. TGFβ2 at 0.2 ng/ml, BDNF at 50 ng/ml, and a combination of TGFβ2 0.2 ng/ml and BDNF 50 ng/ml were applied to the cell culture. The cell lysates were collected and equal amounts of protein were loaded per lane. (A) A picture of the Chemiluminescence detection of the western blot was recorded and shown. (B) A picture of the membrane stained with Sypro® Ruby after transfer was included for quantitation purpose. (C) The densities of each band normalized by the stained total protein were plotted. Lane 1= control; Lane 2 = BDNF at 50 ng/ml treatment; lane 3 = TGFβ2 at 0.2 ng/ml treatment; lane 4 = a combination of BDNF 50 ng/ml and TGFβ2 0.2 ng/ml treatment. Human TM cells growing in serum-free medium without growth factor treatment for 48 hours was included as a control for the treatment. The experiment was repeated three times.

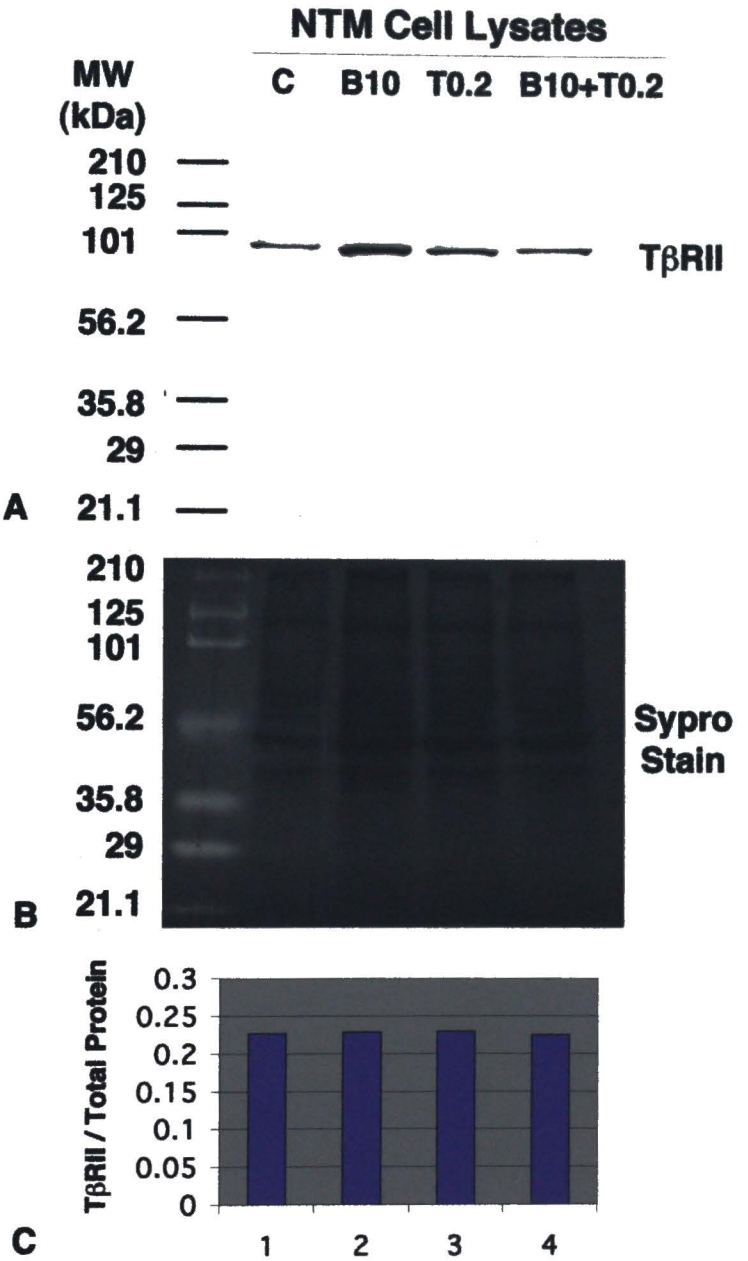

NTM Cell Lysates
MW (kDa)
C
B10
T0.2
B10+T0.2
210
125
101
56.2
35.8
29
21.1
TβRII
A
Sypro Stain
B
TβRII / Total Protein
0.3
0.25
0.2
0.15
0.1
0.05
0
1
2
3
4
C

Figure 7. The effects of TGFβ2 and BDNF on TβRI protein levels in a glaucomatous human TM cell line from a 72 years old donor at the end of 48 hours under serum free condition. TGFβ2 at 0.2 or 2 ng/ml, BDNF at 10 ng/ml, and a combination of TGFβ2 0.2 or 2 ng/ml and BDNF 50 ng/ml were applied to the cell culture. The cell lysates were collected and equal amounts of protein were loaded per lane. (A) A picture of the Chemiluminescence detection of the western blot was recorded and shown. (B) A negative picture of the membrane stained with Sypro® Ruby after transfer was included for quantitation purpose. (C) The densities of each band normalized by the stained total protein were plotted. Lane 1= control; lane 2 = BDNF at 10 ng/ml treatment; lane 3 = TGFβ2 at 0.2 ng/ml treatment; lane 4 = TGFβ2 at 2 ng/ml treatment; lane 5 = a combination of BDNF 10 ng/ml and TGFβ2 0.2 ng/ml treatment; lane 6 = combination of BDNF 10 ng/ml and TGFβ2 2 ng/ml treatment. HTM cells growing in serum-free medium without growth factor treatment for 48 hours was included as a control for the treatment. The experiment was repeated three times.

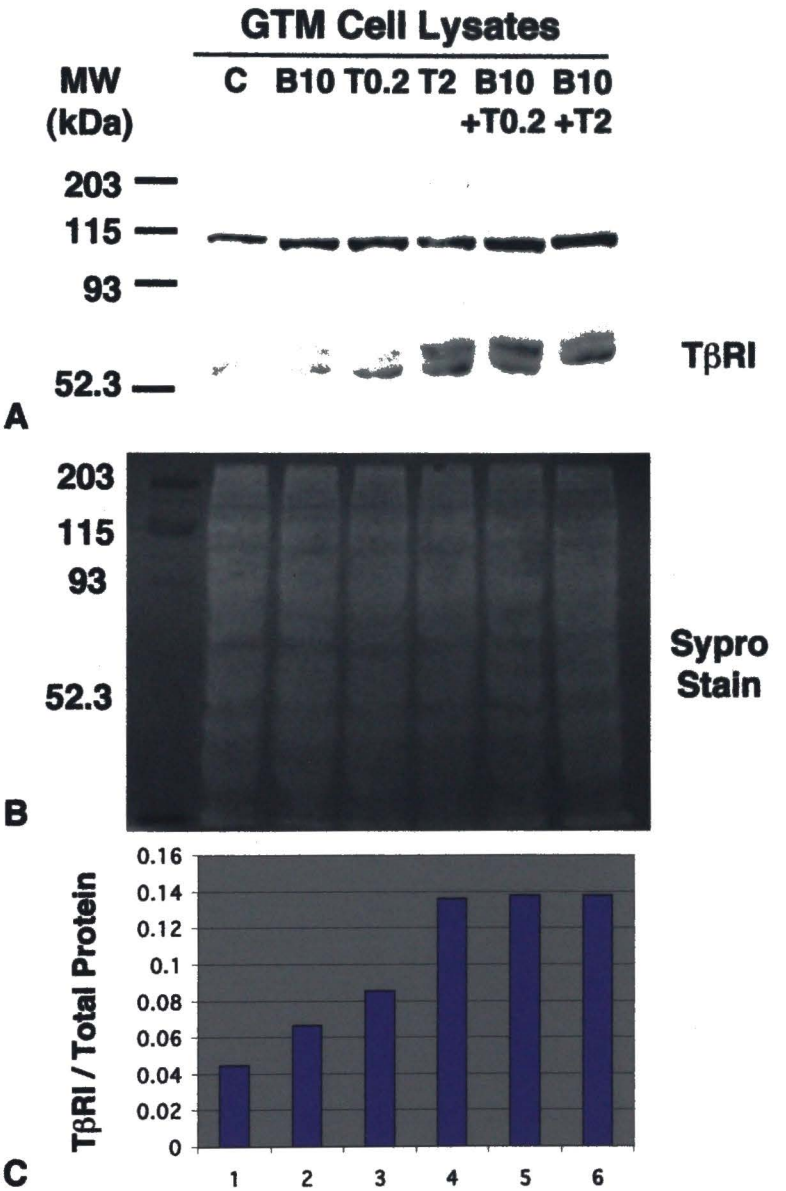
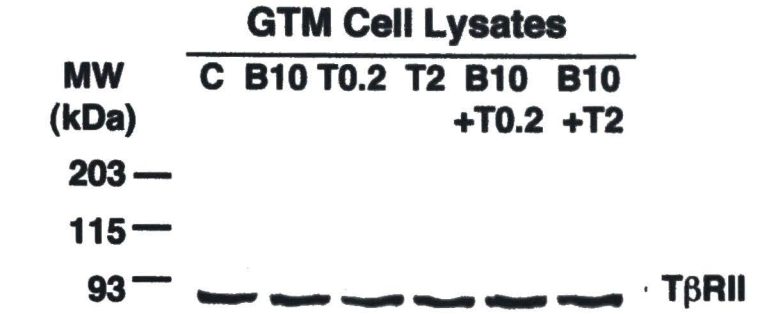
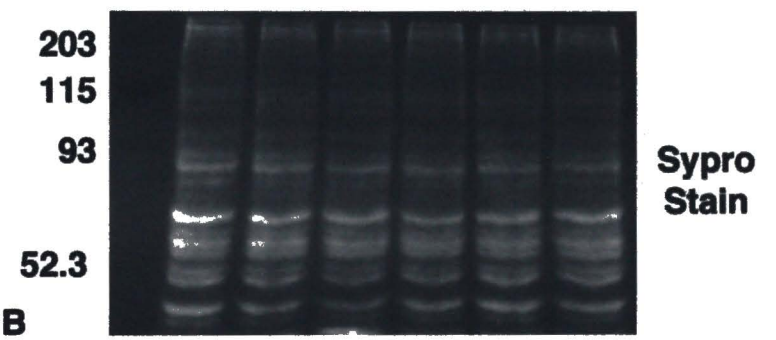

GTM Cell Lysates
MW (kDa)
C
B10
T0.2
T2
B10 +T0.2
B10 +T2
203
115
93
52.3
TβRI
A
203
115
93
52.3
Sypro Stain
B
TβRI / Total Protein
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
5
6
C

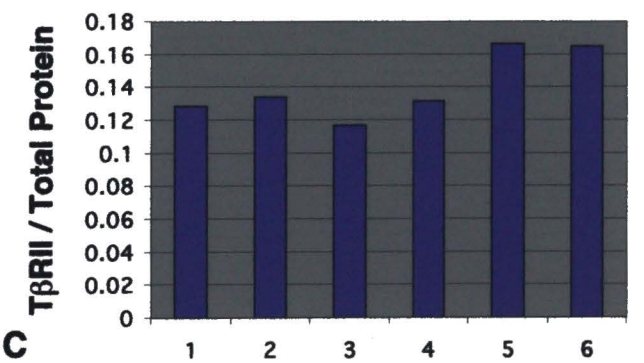
Figure 8. The effects of TGFβ2 and BDNF on TβRII protein levels in a glaucomatous human TM cell line from a 72 years old donor at the end of 48 hours under serum free condition. TGFβ2 at 0.2 or 2 ng/ml, BDNF at 10 ng/ml, and a combination of TGFβ2 0.2 or 2 ng/ml and BDNF 50 ng/ml were applied to the cell culture. The cell lysates were collected and equal amounts of protein were loaded per lane. (A) A picture of the Chemiluminescence detection of the western blot was recorded and shown. (B) A picture of the membrane stained with Sypro® Ruby after transfer was included for quantitation purpose. (C) The densities of each band normalized by the stained total protein were plotted. Lane 1= control; lane 2 = BDNF at 10 ng/ml treatment; lane 3 = TGFβ2 at 0.2 ng/ml treatment; lane 4 = TGFβ2 at 2 ng/ml treatment; lane 5 = a combination of BDNF 10 ng/ml and TGFβ2 0.2 ng/ml treatment; lane 6 = a combination of BDNF 10 ng/ml and TGFβ2 2 ng/ml treatment. Human TM cells growing in serum-free medium without growth factor treatment for 48 hours was included as a control for the treatment. The experiment was repeated three times.


GTM Cell Lysates
MW (kDa)
C
B10
T0.2
T2
B10 +T0.2
B10 +T2
203
115
93
TβRII

A


203
115
93
52.3
Sypro Stain

B


TβRII / Total Protein
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
5
6

C

Figure 9. The effects of TGFβ2 and BDNF on TrkB protein levels in a normal human TM cell line from a 71 years old donor at the end of 48 hours under serum free condition. TGFβ2 at 0.2 ng/ml, BDNF at 50 ng/ml, and a combination of TGFβ2 0.2 ng/ml and BDNF 50 ng/ml were applied to the cell culture. The cell lysates were collected and equal amounts of protein were loaded per lane. (A) A picture of the Chemiluminescence detection of the western blot was recorded and shown. (B) A picture of the membrane stained with Sypro® Ruby after transfer was included for quantitation purpose. (C) The densities of each band normalized by the stained total protein were plotted. Lane 1= control; Lane 2 = BDNF at 50 ng/ml treatment; lane 3 = TGFβ2 at 0.2 ng/ml treatment; lane 4 = a combination of BDNF 50 ng/ml and TGFβ2 0.2 ng/ml treatment. Human TM cells growing in serum-free medium without growth factor treatment for 48 hours was included as a control for the treatment. The experiment was repeated three times.

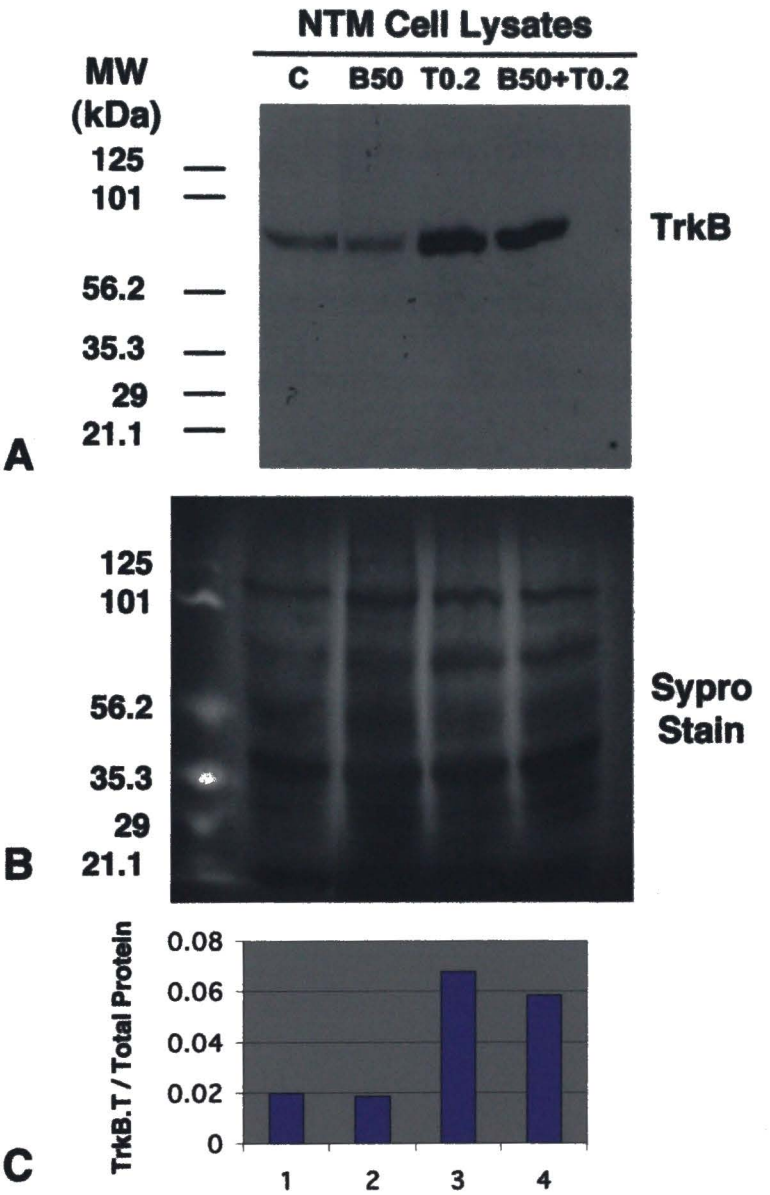
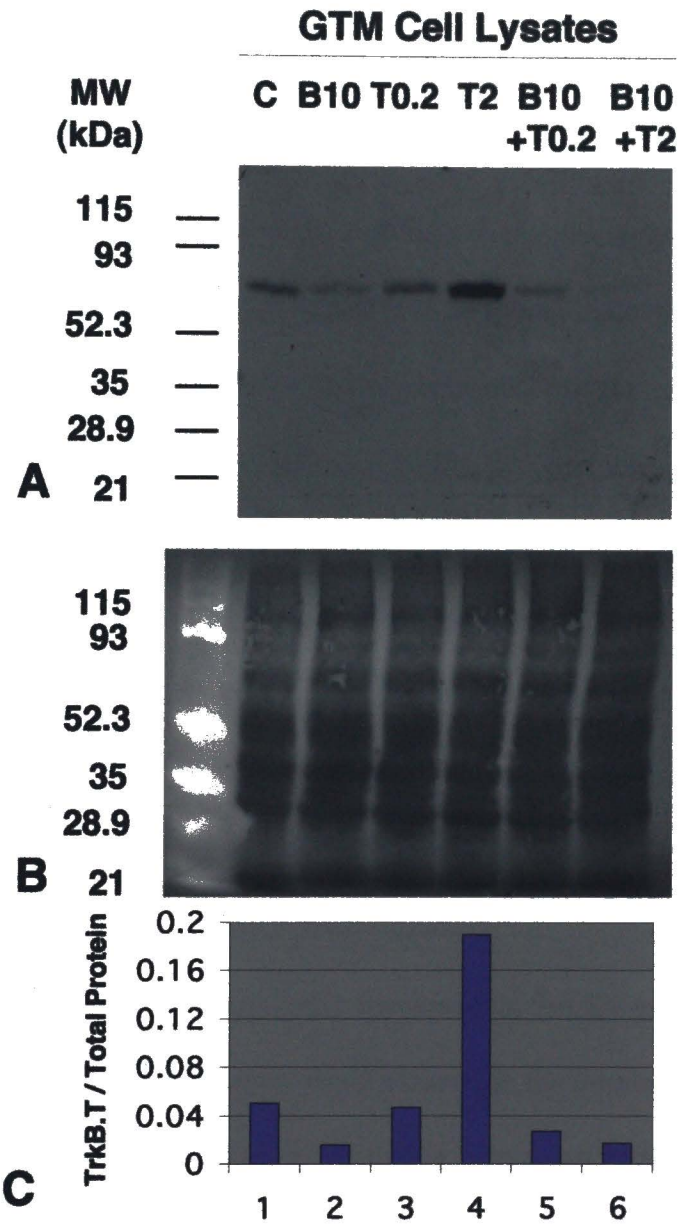

NTM Cell Lysates
C
B50
T0.2
B50+T0.2
MW
(kDa)
125
101
56.2
35.3
29
21.1
A
TrkB
125
101
56.2
35.3
29
21.1
B
Sypro
Stain
TrkB.T / Total Protein
0.08
0.06
0.04
0.02
0
1
2
3
4
C

Figure 10. The effects of TGFβ2 and BDNF on TrkB protein levels in a glaucomatous human TM cell line from a 72 years old donor at the end of 48 hours under serum free condition. TGFβ2 at 0.2 or 2 ng/ml, BDNF at 10 ng/ml, and a combination of TGFβ2 0.2 or 2 ng/ml and BDNF 50 ng/ml were applied to the cell culture. The cell lysates were collected and equal amounts of protein were loaded per lane. (A) A picture of the Chemiluminescence detection of the western blot was recorded and shown. (B) A picture of the membrane stained with Sypro® Ruby after transfer was included for quantitation purpose. (C) The densities of each band normalized by the stained total protein were plotted. Lane 1= control; lane 2 = BDNF at 10 ng/ml treatment; lane 3 = TGFβ2 at 0.2 ng/ml treatment; lane 4 = TGFβ2 at 2 ng/ml treatment; lane 5 = a combination of BDNF 10 ng/ml and TGFβ2 0.2 ng/ml treatment; lane 6 = a combination of BDNF 10 ng/ml and TGFβ2 2 ng/ml treatment. Human TM cells growing in serum-free medium without growth factor treatment for 48 hours was included as a control for the treatment. The experiment was repeated three times.


GTM Cell Lysates
MW (kDa)
C
B10
T0.2
T2
B10 +T0.2
B10 +T2
115
93
52.3
35
28.9
21
A
115
93
52.3
35
28.9
21
B
TrkB.T / Total Protein
0.2
0.16
0.12
0.08
0.04
0
1
2
3
4
5
6
C

Discussion

Growth factor signaling pathways are not isolated events but rather segments in a dense signaling network. It is through crosstalk and feedback links that the network oversees and modulates the activity of each constituent pathway. TGFβ2 normally inhibits human TM cell proliferation[9] and selectively promotes extracellular matrix synthesis and secretion, such as fibronectin[27]. A recent report by Lutjen-Drecoll et al.[33] demonstrated that TGFβ2 can decrease aqueous outflow and increase extracellular matrix deposition in perfusion cultured human TM. On the other hand, BDNF is a survival factor for both neuronal[28] and non-neuronal[29] cells in response to injurious insult. BDNF may normally regulate TGFβ2 signaling pathway via three mechanisms. First of all, BDNF can down-regulate TβRI, thereby decreasing the availability of TGFβ2 to signal. Secondly, BDNF can activate ERK1, which may in turn block the translocation of smad proteins into the nucleus. In addition BDNF can also up-regulate the expression of TGIF, which is an inhibitory factor for TGFβ2 signaling.

It may be through these regulatory mechanisms that the normal human TM maintains homeostasis. During primary open-angle glaucoma, the human trabecular meshwork displays a characteristic morphology including a decrease in the human TM cell number and an increase in extracellular matrix deposition[3], which may be a result of an uncontrolled TGFβ2 signaling pathway. The results of this study (summarized in Table 1 and Figure 11) suggests that in glaucomatous human TM cells, BDNF regulation of TGFβ2 signaling pathway is debilitated. BDNF increased the availability of TβRI, which

acts in synergy with TGFβ2. BDNF can also down-regulate its own receptor-TrkB. By doing so, BDNF removed the other two negative regulatory mechanisms mediated by TrkB. As a result, uncontrolled TGFβ2 signaling pathway may eventually lead to glaucomatous pathology.

In conclusion, our study suggested the presence of a regulatory loop between two endogenous growth factors (i.e. BDNF and TGFβ2) in normal human TM, which may be disrupted in the glaucomatous human TM. In addition, TGFβ2 has multiple effects on different types of cells or tissues via a variety of pathways. BDNF may be able to negatively modulate the effects of TGFβ2 under normal but not glaucomatous condition. Therefore, there is distinct possibility that innate differences exist in normal human TM cells and may play a role in POAG pathophysiology.

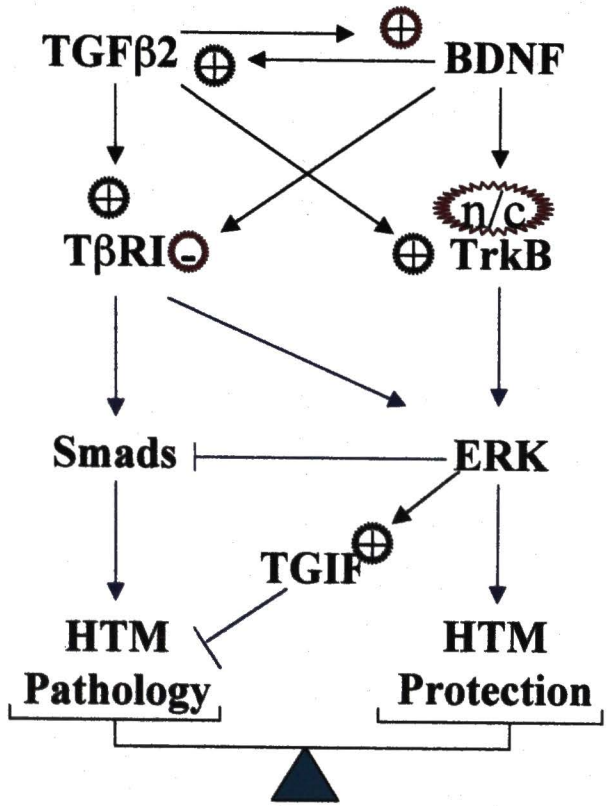
Table 1. Summary of the Results

Normal Human TM Cells					
Treatment	BDNF	TGFβ2	TβRI	TβRII	TrkB
BDNF (10, 50 ng/ml)	*-	+	-	N/C	N/C
TGFβ2 (0.2 ng/ml)	+		+	N/C	+
TGFβ2 (2 ng/ml)	N/C				
BDNF + TGFβ2 (0.2 ng/ml)			N/C	N/C	+
BDNF + TGFβ2 (2 ng/ml)					

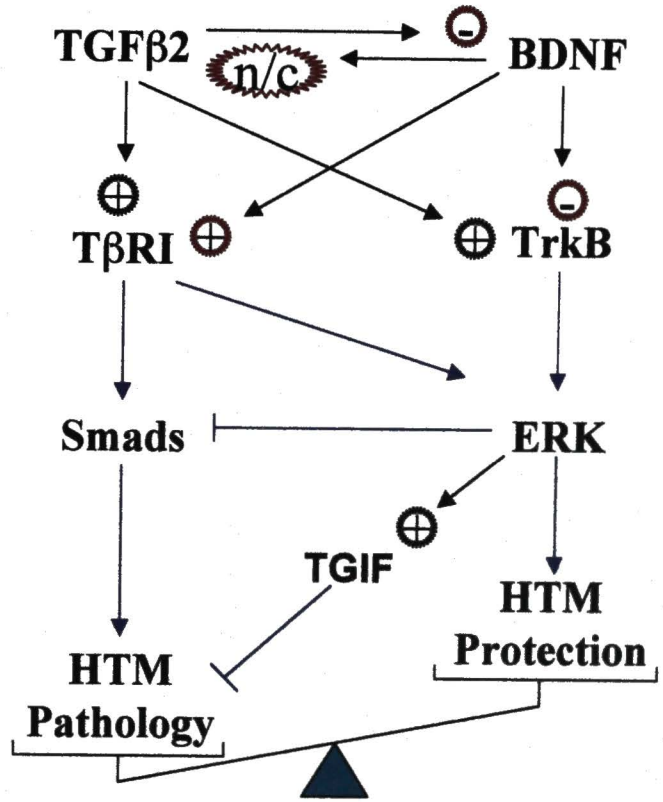
Glaucomatous Human TM Cells					
Treatment	BDNF	TGFβ2	TβRI	TβRII	TrkB
BDNF (10, 50 ng/ml)	*-	N/C or -	N/C	N/C	-
TGFβ2 (0.2 ng/ml)	N/C	*-	+	N/C	N/C
TGFβ2 (2 ng/ml)		*-	+	N/C	+
BDNF + TGFβ2 (0.2 ng/ml)			+	N/C	-
BDNF + TGFβ2 (2 ng/ml)			+	N/C	-

* indicates mRNA expression levels. Intracellular protein levels are summarized in the table unless specified. TβRI = TGFβ receptor type I; TβRII = TGFβ receptor type II; TrkB = tyrosine kinase B.

Figure 11. Comparison of possible crosstalk between BDNF and TGFβ2 signaling in normal versus glaucomatous human TM cells. BDNF = brain-derived neurotrophic factor; TGFβ2 = transforming growth factor-beta2; TβRI = TGFβ receptor type I; TrkB = tyrosine kinase B; Erk = extracellular response kinase; smad = small body size, mothers against decapentaplegic; TGIF = TG3-interacting factor; NTM = normal trabecular meshwork; GTM = glaucomatous trabecular meshwork.


TGFβ2
BDNF
TβRI
n/c
TrkB
Smads
ERK
TGIF
HTM
Pathology
HTM
Protection

NTM


TGFβ2
n/c
BDNF
TβRI
TrkB
Smads
ERK
TGIF
HTM
Protection
HTM
Pathology

GTM

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CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

This study has shown that BDNF can up-regulate myocilin mRNA levels and protein secretion, while TGFβ2 exerts opposing effects on myocilin expression by *in vitro* cultured normal human TM cells. Additionally, there is reciprocal up-regulation between BDNF and TGFβ2 mRNA levels and protein secretion in the cultured normal human TM cells. BDNF may also switch among multiple TGFβ downstream signaling pathways by regulation of TβRI expression and smad protein sequestration in normal human TM. It might be through the interaction between BDNF and TGFβ2 that human TM maintains a fine tuned balance *in vivo*. However, the reciprocal regulatory loop between BDNF and TGFβ2 is no longer intact in the cultured glaucomatous human TM cells. Variable results were found upon growth factor treatment. *In vitro* cultured human TM cells from normal and glaucomatous donors were thought to act the same way because the *in vivo* insult is no longer exist. However, our results suggested *in vitro* cultured glaucomatous human TM cells might conserve distinct phenotypic characteristics relevant to their roles in the pathogenesis of glaucoma. Consistently, Hernandez et al. (2002) demonstrated that normal and glaucomatous optic nerve head astrocytes display differential mRNA levels profile via cDNA microarray analysis.

The overall **objectives** of this research were to (a) examine the role that BDNF and TGFβ2 play in the regulation of myocilin expression; (b) determine the interaction between TGFβ2 and BDNF in trabecular meshwork cells; and to (c) demonstrate a relationship between paracrine signaling of BDNF and TGFβ2 in human TM cells.

This report elicited more questions than it answered. It is still unknown if BDNF levels are increased in the aqueous humor during intraocular hypertension. Future studies need to be done using human aqueous humor samples to demonstrate the change in BDNF levels during intraocular hypertension. The TrkB we identified is shorter than the full-length TrkB reported in the literature. This novel isoform of TrkB contains the kinase domain, and it is necessary to sequence the gene in order to elucidate its function in human trabecular meshwork. There is also a higher molecular weight, TβRI-immunoreactive protein identified on the western blot. Characterization of this probable TβRI isoform may direct us to a novel signal pathway in the human TM. Studies should be carried out to further dissect the signaling pathways of BDNF and TGFβ2 in the human TM. Pathway dissection can elucidate the common denominator in the signaling event involved in the BDNF and TGFβ2 interaction. Since we demonstrated that glaucomatous human TM lack the reciprocal induction of BDNF and TGFβ2, and hypothesized that it may contribute to the glaucomatous pathophysiology, it is essential to test this theory in *in vivo* systems. Perfusion culture of anterior segment of POAG eyes can be employed to test (1) if BDNF and TGFβ2 perfusion can induce IOP change; (2) if BDNF and TGFβ2 can induce extracellular matrix deposition and cell death similar to POAG eyes. To further clarify the mechanism that might lead to POAG pathology, the

mRNA levels profile of BDNF and TGFβ2 signaling pathway in normal versus glaucomatous human TM cells should be compared.

Chapter VI

APPENDICES

Studies that related to this project but not included in previous chapters are illustrated in the appendices. The presence of MYOC mRNA in multiple human normal TM cells from different donors are demonstrated in Figure 1. MYOC PCR cycle linear range is shown in Figure 2. Representative pictures of normal TM cells with or without BDNF treatment were compared (Figure 3). Additional data regarding the effects of TGFβ2 on MYOC expression are also included here (Figure 4 & 5).

Figure 1. MYOC gene expression by normal human TM cells and tissues. Normal human TM Cells from donors of 2, 5 days, 2 months, 2, 54, 71, 77, 81, 87 years old and tissue from donors of 83 and 85 years old were used to detect MYOC gene expression by reverse transcription followed by polymerase chain reaction (PCR). The PCR products electrophoresed on a 2% agarose gel is shown. Lane 1 = human TM cells from a 2 day-old donor; lane 2 = human TM cells from a 5 day-old donor; lane 3 = human TM cells from a 2 month-old donor; lane 4 = human TM cells from a 2 year-old donor; lane 5 = human TM cells from a 54 year-old donor; lane 6 = human TM cells from a 71 year-old donor; lane 7 = human TM cells from a 77 year-old donor; lane 8 = human TM cells from a 81 year-old donor; lane 9 = human TM cells from a 87 year-old donor; lane 10 = human TM tissue from a 83 year-old donor; lane 11 = human TM tissue from a 85 year-old donor; lane 12 = water control for PCR reactions.


1 2 3 4 5 6 7 8 9 10 11 12
MYOC
360 bp

Figure 2. MYOC PCR cycle linear range determination. A mRNA sample from a flask of non-treatment control normal human TM cells were reversely transcribed and the cDNA was used as template for the subsequent PCR reactions. Multiple PCR reactions using MYOC specific primers were assembled and loaded onto the PCR machine. A reaction tube was taken off the thermocycler every other cycle starting from cycle 25. At the end of 40 cycles, 20 μl of the PCR products from each tube were electrophoresed on a 2% ethidium bromide stained agarose gel. The intensity of the bands was measured using a densitometry and the OD values were plotted against cycle numbers as shown in the figure.

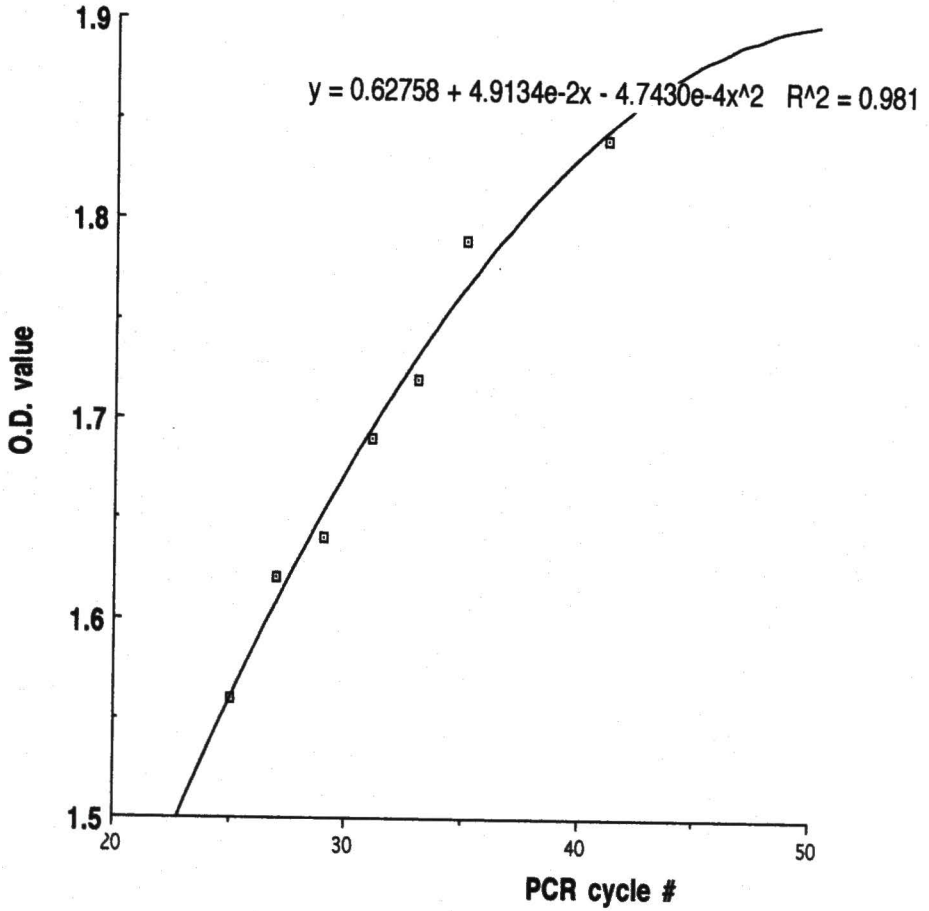
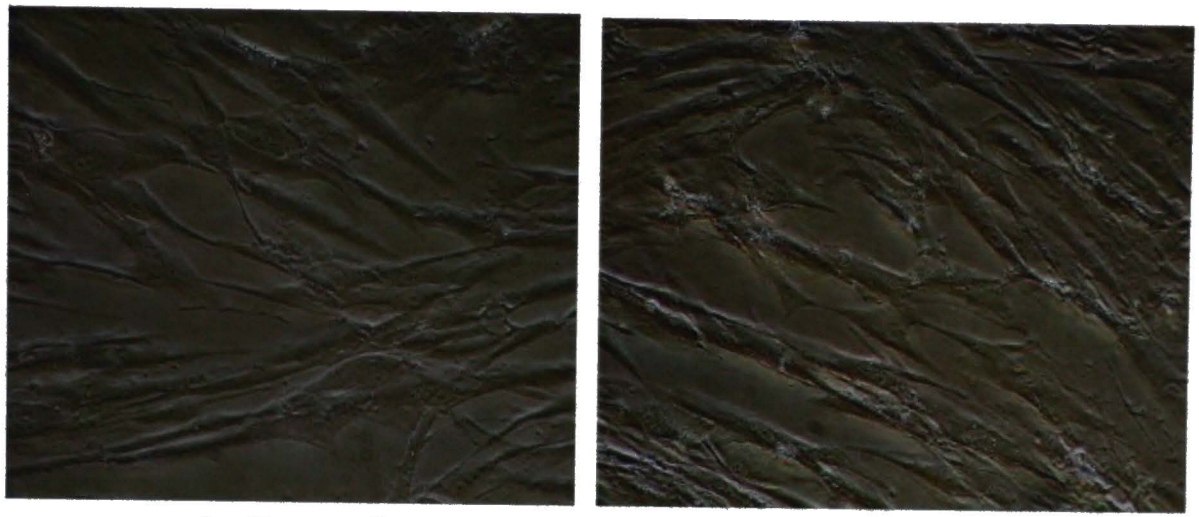

y = 0.62758 + 4.9134e-2x - 4.7430e-4x^2 R^2 = 0.981
1.9
1.8
1.7
1.6
1.5
O.D. value
20
30
40
50
PCR cycle #

Figure 3. Morphology of human TM cells from a normal donor of 71 years old with or without BDNF 50 ng/ml treatment in serum-free medium at the end of 48 hours.

Normal TM Cells from a 71 year old
Donor in Serum Free Media 48 hours



A. Control **B. BDNF**

Figure 4. The effects of TGFβ2 (0.4 ng/ml) on myocilin protein secretion in human GTM cells. (A) The result from a representative experiment using a non-transformed human TM cell line from a glaucomatous donor of 72 years old treated with TGFβ2 0.4 ng/ml is shown. (A) Secreted myocilin levels were compared by Western blot analysis. Serum-free medium was collected at the end of 48 hours and concentrated. An equal amount of total protein was loaded per lane. A representative blot is shown in (A). A sample of serum-free medium from a stably transfected CHO cell line was also included to serve as a positive control (PC) for myocilin protein. Sypro® Ruby staining of the same blot was also included for quantitation purposes. (B) Secreted myocilin protein levels normalized by Sypro® Ruby staining were used for statistical analysis. * indicates statistical significance by student t-test ($p < 0.05$), n=4. Error bar = 1 standard deviation.

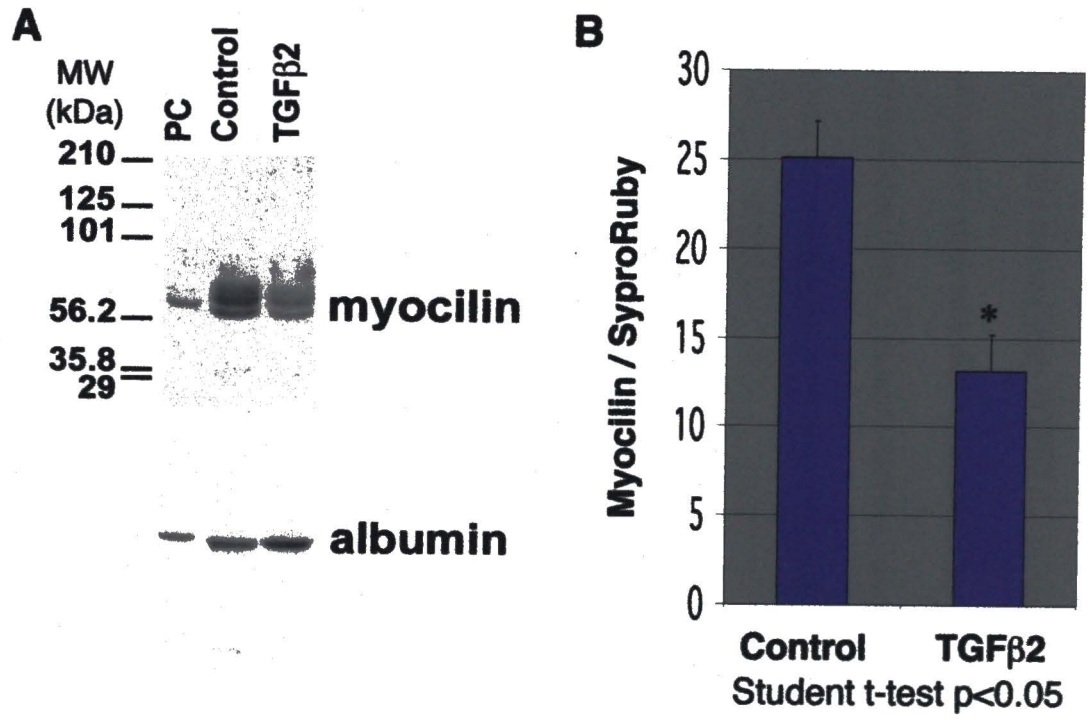
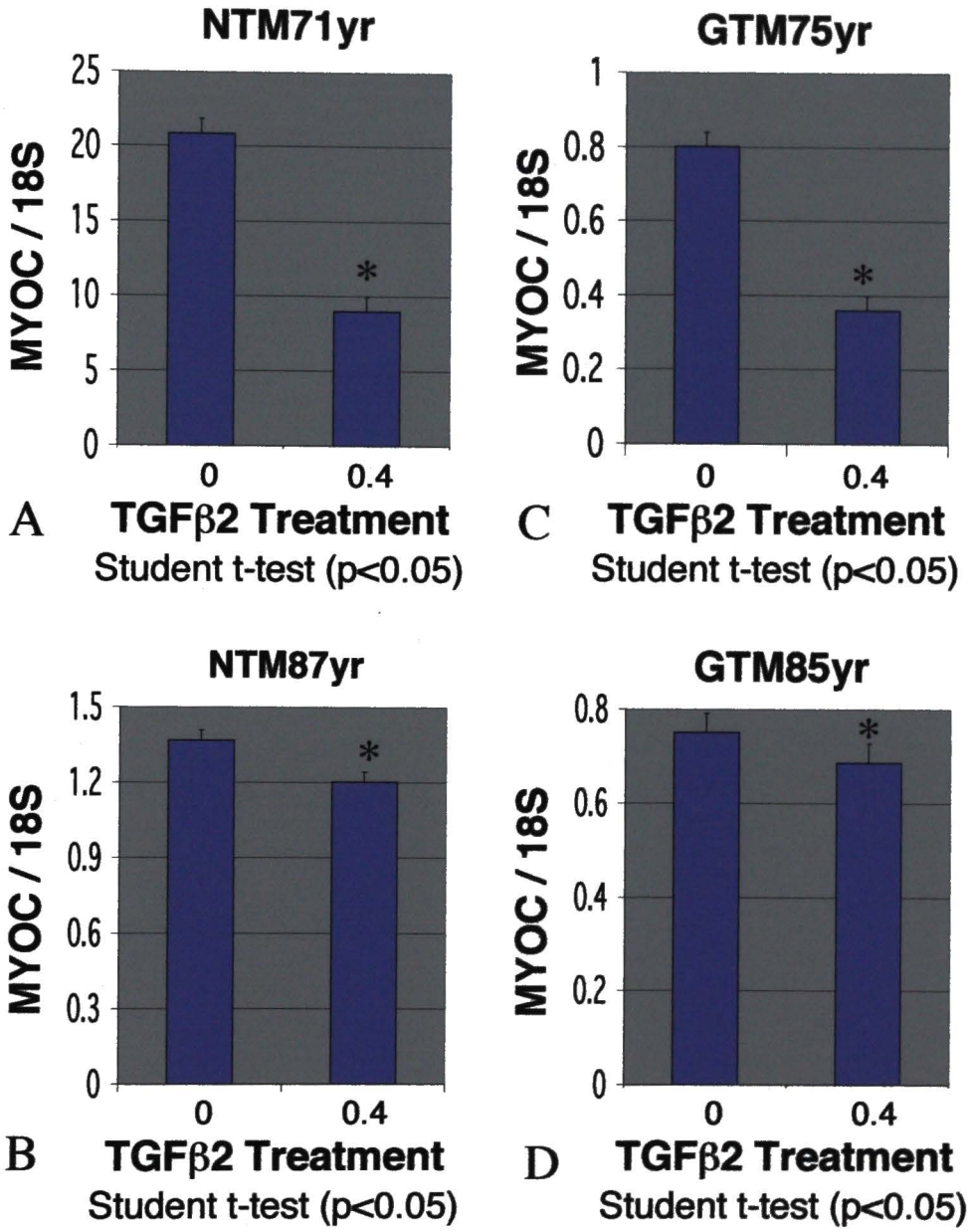

A
MW
(kDa)
PC
Control
TGFβ2
210
125
101
56.2
35.8
29
myocilin
albumin
B
Myocilin / SyproRuby
30
25
20
15
10
5
0
*
Control
TGFβ2
Student t-test p<0.05

Figure 5. The effect of TGFβ2 on MYOC mRNA levels in both normal and glaucomatous human TM cells. Myocilin mRNA levels normalized to 18S rRNA levels were compared using Real-time PCR. Two normal human TM cell lines from 71, 87 year old donors (figure 5A, 5B) and two glaucomatous human TM cell lines from 75, 85 year old donors (figure 5C, 5D) were treated with TGFβ2 0.4 ng/ml for 48 hours in serum-free medium, and a non-treatment control was also included. Error bars=1 standard deviation. The expriment was repeated four times. Student t-test was used for the statistical analysis. Statistical significance was determined by $p<0.05$ and indicated by * in the figure.


NTM71yr
MYOC / 18S
25
20
15
10
5
0
*
0
0.4
A
TGFβ2 Treatment
Student t-test (p<0.05)
GTM75yr
MYOC / 18S
1
0.8
0.6
0.4
0.2
0
*
0
0.4
C
TGFβ2 Treatment
Student t-test (p<0.05)
NTM87yr
MYOC / 18S
1.5
1.2
0.9
0.6
0.3
0
*
0
0.4
B
TGFβ2 Treatment
Student t-test (p<0.05)
GTM85yr
MYOC / 18S
0.8
0.6
0.4
0.2
0
*
0
0.4
D
TGFβ2 Treatment
Student t-test (p<0.05)