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Regulation of Phosphatase and Tensin Homolog Expression and Activity by Transforming Growth-Factor Beta in the Trabecular Meshwork Cells: Implications for Primary Open Angle Glaucoma

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology

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Abstract

Glaucoma is a multifactorial condition caused, in part, by fibrosis of the sieve-like trabecular meshwork (TM) tissue, which impedes drainage of aqueous humor (AH), leading to increased intraocular pressure and associated optic nerve damage and blindness. Fibrosis of the TM is mainly caused by the increased levels of active transforming growth factor- β 2 (TGF β 2) in the AH of glaucoma patients.

Previous reports have shown that TGF β decreases the expression of Phosphatase and Tensin Homolog (*PTEN*) gene and that PTEN is a major regulator of ECM deposition. In this study we investigate the regulation of PTEN protein expression and activity by TGF β 2 in TM cells to determine the mechanism by which excess ECM is deposited.

Here we show that TGF β 2 induces collagen deposition in TM cells by phosphorylation of PTEN, a mechanism that is known to inactivate PTEN. Phosphorylation of PTEN by TGF β is novel and has not been previously reported. We have found that PTEN protein expression and phosphorylation is regulated by the PI3 kinase pathway. We further show that transfection of TM cells to express enhanced-active PTEN decreases TGF β 2-induced expression of collagen type I (COL1) protein. Thus, regulation of PTEN activity could serve as a therapeutic target with high potential to prevent excess ECM deposition in the TM of glaucoma patients.

Keywords:

Glaucoma, trabecular meshwork (TM), Phosphatase and Tensin Homolog (PTEN), excess extracellular matrix (ECM) deposition, fibrosis, aqueous outflow resistance, transforming growth factor- β (TGF β 2)

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List of Abbreviations

AH	Aqueous humor
AKT	Protein kinase B
ALK5	Activin A receptor type II-like kinase (TGF β R I)
Amp	Ampicillin
ANOVA	Analysis of variance
BSA	Bovine serum albumin
C124S PTEN	PTEN with cysteine to serine substitution at position 124
C124SV	C124S PTEN vector
C2 domain	Conserved domain of Protein kinase C targeting protein to the plasma membrane
<i>COL1A1</i>	Collagen I gene
COL1	Collagen I protein
CTM	Corneoscleral trabecular meshwork
CV	Control vector
DMEM	Dulbecco's Modified Eagle Medium
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	Enhanced chemiluminescence
ECM	Extra cellular matrix
EDTA	Ethylene-diamine-tetra-acetic acid
ePTEN	Enhanced-active PTEN

Euk 18S	Eukaryotic 18S rRNA
eV	Enhanced-active PTEN vector
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSK	Glycogen synthase kinase
HRP	Horseradish peroxidase
hTM	Human trabecular meshwork
IgG	Immunoglobulin G
IOP	Intraocular pressure
IP	Immunoprecipitation
JCT	Juxtacanalicular trabecular meshwork
LAP	Latency associated peptide
LB	Lennox broth
LY20	LY294002 20 μ M
LY30	LY294002 30 μ M
mAb	Monoclonal antibody
MAP	Mitogen activated protein
MKK3/6	Map kinase kinase 3 and 6
<i>MMAC1</i>	Mutated in multiple advanced cancers 1

MMP	Matrix metalloproteinase
mRNA	Messenger RNA
NF- κ B	Nuclear factor kappa light-chain-enhancer of activated B cells
ng/mL	Nanograms per milliliter
pAb	Polyclonal antibody
P10	PP2 10 μ M
P20	PP2 20 μ M
pAKT	AKT phosphorylated at Ser473
pFAK	FAK phosphorylated at Tyr397
PBS	Phosphate-buffered saline
PI	Phosphatase Inhibitor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PIP ₃	Phosphatidylinositol 3,4,5-trisphosphate
pg/mL	Picograms per milliliter
POAG	Primary open angle glaucoma
pPTEN	PTEN phosphorylated at Ser380/Thr382/383
P/S	Penicillin/Streptomycin
pSMAD	Phosphorylated SMAD
<i>PTEN</i>	Phosphatase and Tensin Homolog gene

PTEN	Phosphatase and Tensin Homolog protein
RAK	Fly-related kinase (FRK)
RGCs	Retinal ganglion cells
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RTK	Receptor tyrosine kinase
ROCK	Rho-associated protein kinase
RT-PCR	Real time – Polymerase chain reaction
SARA	SMAD anchor for receptor activation
SB20	SB431542 20 μ M
SC	Schlemm's Canal
SDS-PAGE	Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis
Ser	Serine
Shc	Src homology 2 domain containing transforming protein
si-RNA	Small interfering RNA
SLT	Selective laser trabeculoplasty
SMAD	Homolg of SMA (small body size) and MAD (mothers against decapentaplegic) protein
SPARC	Secreted protein acidic and rich in cysteine
Src	Tyrosine protein kinase Src
T1	TGF β 2 1ng/mL

T5	TGF β 2 5ng/mL
T10	TGF β 2 10ng/mL
TAK1	Transforming growth factor β activated kinase
<i>TEP1</i>	TGF β regulated and epithelial cell enriched phosphatase 1
TGF β 2	Transforming growth factor β 2
TGF β R I	Transforming growth factor β receptor I
TGF β R II	Transforming growth factor β receptor II
Thr	Threonine
Tyr	Tyrosine
TIMPs	Tissue inhibitors of metalloproteinases
TM	Trabecular meshwork
TRAF6	TNF receptor associated factor 6
Z2	ZSTK474 2 μ M
Z5	ZSTK474 5 μ M

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Overview

Glaucoma is a complex and heterogeneous disease characterized by progressive degeneration of the optic nerve and vision loss. Glaucoma affects more than 70 million people worldwide and is currently the second leading cause of irreversible blindness (1). In Canada, primary open-angle glaucoma (POAG), the most common form of glaucoma, is expected to increase by 105% by 2031 (2).

It has been shown that fibrosis of the trabecular meshwork (TM) tissue in POAG patients (3, 4) is associated with increased levels of active of TGF β 2 present in the AH (5). Since the TM is responsible for filtering and draining aqueous humor (AH), fibrosis of the TM tissue have downstream physiological effects. Primarily, increased thickness of the TM decreases the size of pores within the tissue, which results in increased resistance to AH outflow (3, 4).

As the dynamics of AH outflow shift and drainage of AH decreases, intraocular pressure (IOP) increases. Elevated IOP is a hallmark of glaucoma and remains, to date, the only modifiable risk factor of the disease. As such, chronically elevated IOP can cause irreparable damage to the optic nerve and permanent vision loss (6). Therapies aimed at preventing increases in IOP should thus target fibrosis of the TM, however, this is yet to be achieved.

TGF β is known to modulate the expression of protein Phosphatase and Tensin Homolog (PTEN) deleted on chromosome 10, which is a major regulator of ECM deposition (7, 8,). However, no study has examined the role of PTEN in regulating the ECM turnover in the TM.

We hypothesize that TGF β 2 regulates ECM deposition in the TM by modulating the expression and activity of PTEN, and that a decrease in PTEN expression or activity causes fibrosis of the TM in glaucoma patients.

To test this hypothesis we proposed the following approach of investigation:

1. To determine how TGF β 2 modulates PTEN expression and activity.

2. To identify mechanisms by which PTEN is regulated by downstream TGF β signaling pathways.
3. To characterize the functional role of PTEN in the ECM of the TM.

Our data show that TGF β 2 regulates collagen I expression in hTM cells by phosphorylating PTEN. We found that TGF β 2-induced phosphorylation of PTEN, which is known to inactivate PTEN, is mediated by the PI3 kinase/AKT signaling pathway. Furthermore, we show that overexpression of PTEN with an eight-fold increase in enzymatic activity, compared to wild-type PTEN, prevented TGF β 2-induced collagen expression in TM cells.

These results support our hypothesis and provide insight into a novel mechanism by which PTEN regulates fibrosis of the TM. Since little is known about the development and progression of glaucoma, our findings have great potential to establish part of the pathogenesis of this disease and guide development of targeted therapies. Further investigation is crucial as PTEN has high clinical potential as a target to address excess ECM deposition in the TM, and ultimately mitigate the risk of vision loss for glaucoma patients.

Chapter 1

1 Introduction

1.1 Aqueous Humor and Outflow

AH is a clear fluid normally found within the anterior segment of the eye. It is comprised mostly of water and contains an extremely low protein concentration, which maintains optical clarity. Ions essential to cell function, including hydrogen, chloride, and bicarbonate, are present along with cytokines, growth factors, and low levels of circulating enzymes (9).

Production of AH occurs posteriorly by the inner epithelial cells of the ciliary body. AH is then actively secreted by the ciliary processes and flows around the iris into the anterior segment (4, 9). Of the many functions of AH, most notably it is required to:

1. Maintain IOP and sustain the spherical shape of the eye by filling the space between the cornea and the lens to avoid collapse.
2. Provide nourishment to the avascular structures of the anterior segment, particularly the lens and the cornea.
3. Serve as a means to rid cells in avascular structures of accumulated waste products.

Roughly 85% of total AH outflow occurs through the trabecular meshwork, or conventional outflow pathway. Traveling along the conventional pathway, AH drains through the progressively smaller pores of the TM to gain entry into Schlemm's canal (as illustrated in **Figure 1.2.1**). Once in Schlemm's canal, AH will exit the eye via the episcleral venous system (4, 9).

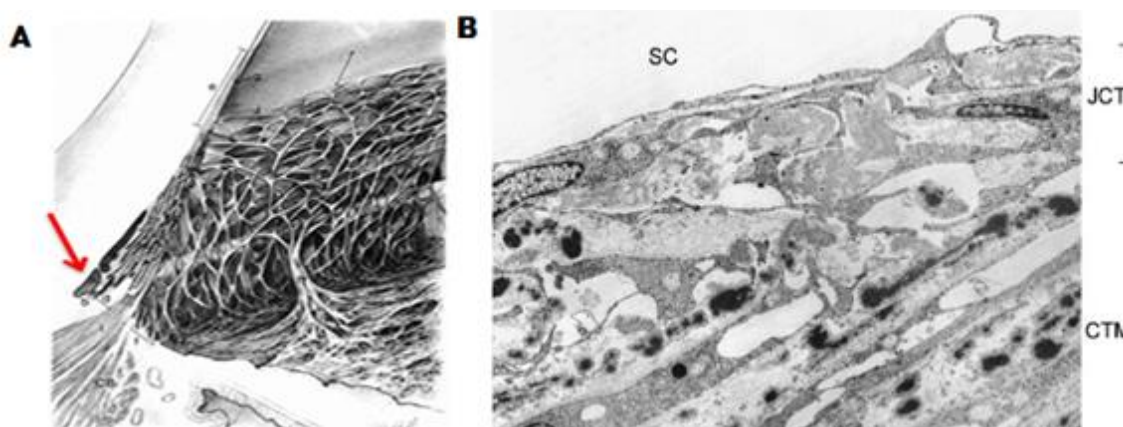


Figure 1.1.1 Schematic and electron-micrograph images of the TM.

(A) Schematic representation of the layers and meshwork structure of the TM. TM tissue lines the entrance to Schlemm's Canal (SC) (red arrow). Pore size decreases with increased proximity to Schlemm's Canal. Adapted from: Hogan, Michael J. (Michael John) & Alvarado, Jorge A & Weddell, Joan Esperson (1971). *Histology of the human eye: an atlas and textbook*. Saunders, Philadelphia. (10). (B) Electron-micrograph image of a cross-section of the TM. Large pores between beams are visible in the CTM region. In the JCT region beams are less obvious and pores are smaller. Adapted from: Tamm, E. R. (2009). The trabecular meshwork outflow pathways: structural and functional aspects. *Experimental eye research*, 88(4), 648-655. (9).

The remaining 15% of AH outflow occurs via the non-conventional uveal scleral outflow. Outflow through the non-conventional pathway occurs as AH diffuses through intercellular spaces among ciliary muscles fibers in the posterior chamber to enter suprachoroidal spaces (11, 12). Eventually AH exits the eye through vessels in the sclera.

Under physiological conditions, a homeostatic balance between production and drainage of AH must be maintained. Since AH is continuously produced, at a rate between 2.0-2.75 μ L/min (9, 13), efficient drainage at an equal rate is required to avoid changes in IOP.

1.2 The Trabecular Meshwork Tissue

1.2.1 Trabecular Meshwork Tissue Structure

The trabecular meshwork (TM) tissue is a structured network of cells and interconnected beams of extracellular matrix (ECM) that lines the entrance to Schlemm's Canal present at the angle where the cornea meets the iris (**Figure 1.1.1.A**). Cells of the TM tissue have an endothelial morphology (14) and exhibit contractile properties as well as phagocytic ability (4). The core of each beam is collagen and elastic-like fibers, surrounded by the TM cells themselves (3, 4). Together, the TM cells and beams form a multi-layered, porous structure, which serves as the major outflow pathway for the AH of the eye (14).

Anatomically, the TM is divided into three sections beginning with the corneoscleral TM, located most medially. The uveal TM is located immediately distal of the corneoscleral TM. Finally, the juxtacanalicular (JCT) TM is adjacent to Schlemm's canal. While there are no physiological markers to distinguish one section of the TM from another, the classification is based on anatomical position and morphology (4, 9, 10). The size of the pores between adjacent beams in the TM become smaller moving outward from the corneoscleral trabecular meshwork (CTM) to the uveal TM. In the JCT region, individual beams are less obvious as the surrounding ECM is compounded in a more irregular fashion, creating a denser layer with small pores (**Figure 1.1.1**). The successive decrease in pore size serves to filter AH as it drains from the anterior segment of the eye (4, 9, 10).

ECM is a major component of the TM tissue. The ECM of the TM tissue consists of collagens, proteoglycans, adhesive glycoproteins, and elastin (14). While fibrillary collagens type I and III are the most abundant, collagens type IV, V, VI, and VII are also expressed in the TM (14), as confirmed by immunostaining (15). In cultured TM cells, collagen I is the major type of collagen expressed (14). Proteoglycans expressed include chondroitin, dermatan, decorin, and versican, as determined by immunohistochemical analysis and RT-PCR (14, 16-18). Decorin is important for maintaining ECM structure as it binds collagen type I promoting

collagen fibrillogenesis and proper collagen fibril assembly (14, 19). Of the adhesive glycoproteins expressed in the TM, fibronectin and laminin are the most abundant. Fibronectin is insoluble and localized to the beams of the TM (17, 20). Laminin is associated with the basement membrane (17). Both are important for cellular adherence to the ECM.

Finally, elastin is also abundantly expressed in the ECM of the TM. High expression of elastin is consistent with a tissue type that must be able to reversibly distend in response to fluctuations of an outside force (14). Correlating tissue structure to function, we find a role for the TM in adapting to changes in IOP.

1.2.2 Trabecular Meshwork Tissue Function

Under normal physiological conditions, TM cells can detect changes in IOP and induce ECM turnover to maintain adequate outflow of AH and control IOP. Specifically, TM cells in the JCT region are able to sense changes in IOP through integrins, and alter signaling to remodel ECM accordingly (21). When the TM tissue becomes dysfunctional, signals to remodel the ECM are disrupted such that accommodations are not made to increase AH outflow, and IOP remains elevated. The perpetuating nature of this paradigm, with increased TM dysfunction (possibly due to increased cross-linking of the ECM (22)), causing decreased sensitivity to IOP changes creates a positive feedback loop to further increase IOP and the risk of damage caused by sustained elevated pressure (23, 24).

In regards to contractility and TM function, relaxation of the TM tissue increases space between the individual beams, widening pores to increase aqueous outflow. Contraction of the TM tissue decreases outflow by bringing pores closer together to decrease pore diameter. Outflow through the TM is pressure-dependent, moving along a gradient of high pressure in the anterior chamber to low pressure in the episcleral venous system, ensuring unidirectional flow of AH (4). In POAG thicker beams of TM tissue have been observed (**Figure 1.2.2**), such that excess ECM deposition narrows pores in the meshwork to increase resistance to aqueous outflow (3, 4, 24, 25). Downstream effects of increased outflow resistance are

increased IOP, and resultant degeneration of the optic nerve and eventual blindness.

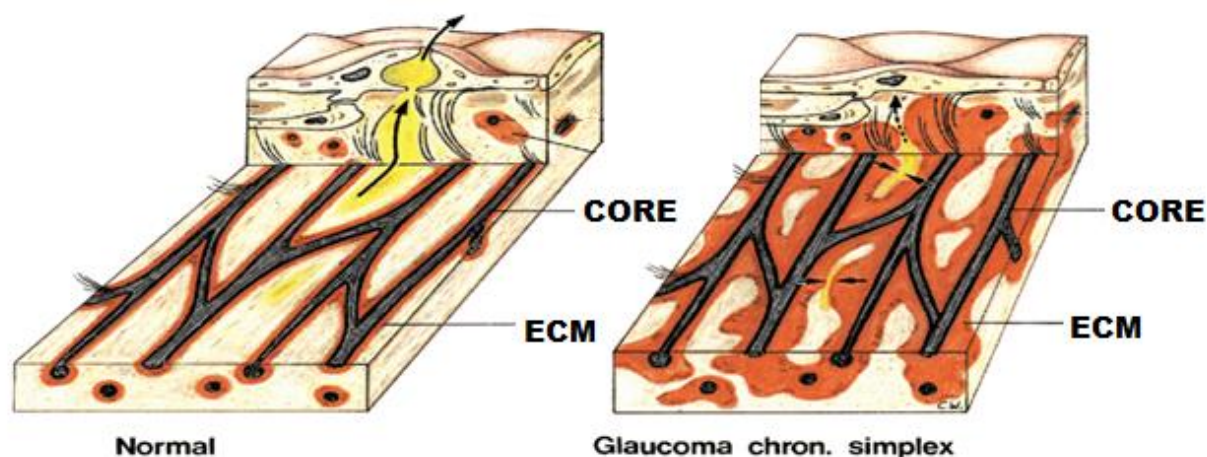


Figure 1.2.1 Schematic representation of increased ECM in glaucomatous TM tissue.

ECM surrounds core beams in the TM tissue. The TM tissue of normal eyes (left) has sufficient space between beams to facilitate drainage of AH. Increased ECM deposition found in the TM tissue of glaucomatous eyes (right) decreases spaces between adjacent beams, impeding drainage of AH (4). Adapted from Lutjen-Drecoll E., & Rohen JW, eds, (1996). *Morphology of Aqueous Outflow Pathways in Normal and Glaucomatous Eyes*. 2nd ed. St. Louis: Mosby.

Additional changes to the structure of the TM, with implications for efficient function, have been reported. Cellularity of the TM tissue decreases with age, such that TM cells detach from the ECM beams and fusion of adjacent cell-less beams increases (26-28). This further increases resistance to aqueous outflow. Similarly analysis of POAG patient samples, showed that while there are no differences in the rate of loss of cellularity between POAG patients and unaffected controls, the patients with POAG had lower levels of TM cells overall (27). Specifically, the TM of POAG patients was found to have thickened beams and an excess of collagen fibers, in addition to decreased cell number in the uveal TM. Finally the same study concluded that the lining of Schlemm's canal had indeed demonstrated increased ECM product deposition (28).

1.3 Glaucoma

1.3.1 Epidemiology and Classification

Age-related blinding ocular disease affects more than 4 million Canadians and in 2007 cost the Canadian healthcare system \$16 billion (2). Glaucoma, a progressive optic neuropathy with pathology occurring in the anterior segment of the eye, is currently listed as the second leading cause of irreversible blindness worldwide (1). As the baby-boomer generation progresses into retirement, an unprecedented percentage of the population will be over the age of 65. As a direct consequence, the overall burden of disease will increase substantially from the nearly 70 million currently affected, with global projections estimating upwards of 80 million people to be affected with glaucoma by 2020 (1). The burden of glaucoma is not restricted to the financial cost of treatments and surgeries. The impact of vision loss deeply affects the quality of life of patients, their caregivers, and family members (2, 29).

Two main types of glaucoma can be classified according to etiology: primary angle-closure glaucoma and primary open-angle glaucoma (POAG). Both types occur when AH is not able to drain from the anterior segment of the eye. Increased resistance to AH outflow leads to increased IOP resulting in optic nerve degeneration and blindness (29, 30). Primary angle-closure glaucoma involves forward movement of the iris that greatly impedes access to the trabecular meshwork (TM), interfering with AH drainage. Primary open-angle glaucoma occurs when aqueous outflow is impeded at the level of the TM due to tissue dysfunction. POAG is the most common type of glaucoma, affecting nearly 74% of all glaucoma patients (1). Secondary glaucoma can also occur, typically manifesting after ocular trauma and often as a result of prolonged corticosteroid use (30).

1.3.2 Pathogenesis

Although the pathogenesis of glaucoma has yet to be elucidated, there are multiple theories relating to disease development and progression. Since POAG is

complex and multifactorial, it is difficult to determine direct causation and it is more likely that effects are due to a complex interplay of many factors. Specifically, both a vascular and a mechanical theory exist to suggest a mechanism by which glaucomatous blindness occurs (31-33). The vascular theory suggests that damage to the retinal ganglion cells (RGCs) of the optic nerve occurs due to decreased blood flow to the area as a result of increased IOP (6, 31-33). The RGCs then become ischemic and are eventually damaged beyond repair, resulting in blindness. Alternatively, the mechanical theory suggests that RGCs are irreversibly injured by the mechanical force to which they are subjected when a patient has elevated IOP (29, 30, 34). The most likely scenario includes combinatory effects of both, such that IOP increases are able to disrupt vascular dynamics to decrease blood flow and viability of RGCs.

1.3.3 Risk Factors

Common risk factors for POAG include advanced age, African or Hispanic ancestry, family history of POAG, a high degree of myopia, and elevated IOP. While mostly affecting those 65 years of age and older, the prevalence of POAG has been shown to increase steadily with each decade after the age of 40 (35). POAG shows a strong racial predilection. This effect is most prominent among patients of African and Hispanic descent, with rates of blindness due to glaucoma reported to be six times higher than Caucasian glaucoma patients in the United States (29). Those with POAG are twice as likely to report a first-degree relative with a condition in comparison to those not affected by POAG (29, 30). High myopes are also at risk for POAG, as a lack of structural support from the sclera posteriorly makes the optic nerve more susceptible to damage (29, 32). Finally, elevated IOP is not only a risk factor for POAG, but also the most notable hallmark of the disease. Normal values for IOP range from 10-21 mmHg, with measurements above 21 mmHg considered to be pathologically high (29, 30). As IOP remains the only modifiable risk factor, clinical interventions are made with the intent to reduce IOP and prevent further damage to the optic nerve and resultant blindness. Studies have shown that a mean reduction of IOP by 25% considerably

slows progression of the disease, when considering vision loss as an end-point (29).

1.3.4 Diagnosis and Treatment Options

Since the development of POAG is poorly understood, few preventative measures (other than increased vigilance with screening and prophylactic reduction of IOP) are available to guard against the disease. Currently all treatment paradigms initially take a reactive approach aiming primarily to decrease IOP (the only modifiable risk factor), and subsequently take more aggressive approaches such as surgery to increase outflow facility. Patients labeled “glaucoma suspects” are referred to an ophthalmologist and a diagnosis of clinical glaucoma is made upon confirmation of at least two of the following findings: elevated IOP (above 21mmHg or in the upper limit of normal), increased cup to disc ratio of the optic nerve, or changes to the retinal nerve fiber layer, and decreased peripheral field of vision (29, 30). Due to the nature of the disease, vision loss progresses gradually such that early loss of vision is imperceptible to the patient. In fact, COS guidelines estimate that up to 50% of those with glaucoma are unaware of the condition (29).

Treatment options include pharmacological eye drops and surgery to reduce IOP and facilitate AH drainage. Prescription eye drops are often the first choice to control IOP. Prostaglandins and prostaglandin analogs assist with fluid drainage, whereas beta-blockers can be prescribed to decrease fluid production (29, 30). Selective laser trabeculoplasty (SLT) is recommended for patients unable to successfully control IOP with eye drops alone (29, 30). During an SLT procedure, a laser is aimed at the TM tissue, which improves the function of the TM and increases aqueous outflow. While SLT is usually successful, the effects are not permanent as the factors contributing to fibrosis of the TM are still present, and thus repeat SLT is often not useful. Finally, conventional surgery is performed as a last resort for patients who have been unresponsive to, or whose glaucoma has not been adequately controlled by previous measures (29, 30). A trabeculectomy is performed in which part of the TM tissue is removed and an opening in the sclera of the eye is created to facilitate AH drainage. Outcomes of trabeculectomy are

typically positive (80-90% effective at decreasing IOP (30)) and side-effects are usually treatable, although this procedure addresses the disease late in its progression. Procedures to insert stents as artificial means of drainage, can also be useful (29). Ultimately, there have not been any new developments in causal glaucoma treatments during the past two decades, despite an increasing number of glaucoma patients and a clear need for treatments to do more than simply assuage the symptoms. Most importantly, current treatments focus on reducing IOP while none are specifically targeted to treat and prevent TM tissue dysfunction.

1.4 Fibrosis

Fibrosis is an accumulation of excess extracellular matrix (ECM) that can result in scarring. The ECM is composed of many different components, including proteoglycans, structural fibers, and adhesion molecules; however, collagen and fibronectin are the most abundant and the most commonly implicated in disease (14). When present in excess quantities, especially with an increase of structural fibers, excess ECM can result in tissue dysfunction. The effects of increased ECM deposition causing organ failure and, often, death have been highly documented in a myriad of organs, including cardiac myofibroblasts, kidney, lung, and skin (7, 36-39) highlighting decreases in normal tissue function as a result.

The basic mechanisms for ECM maintenance and remodeling involve a balance between factors which promote ECM deposition, and factors which promote the degradation of existing ECM. Growth factors are common inducers of ECM deposition, whereas proteases are common ECM remodeling proteins. Primarily, increased matrix metalloproteinase (MMP) expression and activity acts to break down the ECM. The action of MMPs can be regulated by the expression of tissue inhibitors of metalloproteinases (TIMPs) and other tissue specific protease inhibitors (40, 41). Common MMPs found to act upon the ECM of TM cells are MMPs-1, -2, -13, and -14, with specific affinity for COL1 protein (40). It has also been reported that a variety of MMPs are normally expressed in unstimulated TM cells supporting the theory that TM cells have a crucial role in remodeling the ECM (40).

Furthermore, a study of hTM cells stimulated with TGF β 2 revealed increased expression of the MMP-2 proform as well as increased expression of plasminogen activator inhibitor-1, an endothelial cell specific protease inhibitor (42). The investigators found that plasminogen activator inhibitor-1 was acting to suppress activation of MMP-2 by maintaining MMP-2 in its proform, and therefore acting to inhibit ECM remodeling in the presence of TGF β 2 (42).

ECM is normally remodeled in response to mechanical stretch in order to maintain efficient aqueous outflow. Further importance is given to this role as upregulation of MMPs specific to COL1 occur after stimulation (particularly sustained exposure to increased pressure) (40). MMPs-2, and -14 have also been shown to be upregulated in TM cells in response to stretch of the TM tissue due to increased IOP (41). When the TM becomes fibrotic, cells may no longer be able to sense changes in pressure and may not respond appropriately resulting in further resistance to aqueous outflow (40).

A fine regulatory balance between factors affecting ECM deposition and turnover must be achieved to prevent fibrosis and tissue dysfunction. However, since pro-fibrotic growth factors are required for normal cellular function, targets downstream of growth factor signaling have the highest potential to successfully attenuate and prevent fibrosis.

1.5 Transforming Growth Factor β

Many factors are able to induce ECM deposition, and one such factor is transforming growth factor-beta (TGF β). TGF β is active in many different biological processes, among them cell differentiation, proliferation, migration, and survival (43-49). TGF β has also been identified as a key player in many disease processes including oncogenesis, autoimmunity, inflammation, and fibrosis (45, 50-54). Particularly, TGF β has long been implicated in the fibrosis of various tissues as well as multiple fibrotic conditions (55) including but not limited to renal and pulmonary fibrosis (39, 51).

TGF β s are secreted cytokines that belongs to the TGF β superfamily (43, 44). The latency-associated pro-region encodes a Latency Associated Peptide (LAP) that shields the protein to maintain an inactive state and assists in proper folding of the mature peptide. The LAP must be enzymatically cleaved from the remaining C-terminal sequence, for TGF β maturation, activation, and binding to its receptor, TGF β receptor (TGF β R) II (43, 44). Upon binding to TGF β , TGF β RII then dimerizes with, and phosphorylates, TGF β R I to initiate a signaling cascade.

In mammalian cells TGF β is found as three isoforms; TGF β 1, TGF β 2, and TGF β 3. Each of the three isoforms, while structurally similar, occupies a different niche function. For example, levels of TGF β 1 have been found to be elevated within the wound microenvironment (56). TGF β 1 assists in wound healing, but an increased amount can contribute to fibrosis (56). In addition, TGF β 1 has been dually implicated in cancer progression and suppression of tumor growth (54). TGF β 2 has been shown to be present within the AH of the eye (5, 57, 58). Conversely, TGF β 3 has been found to be highly expressed in fetal tissue and associated with *in utero* wound-healing without scar formation (59). Although the functions of the three TGF β isoforms may vary slightly according to cellular milieu, they are all activated and signal through TGF β Rs in a similar manner.

1.5.1 Ocular TGF β

We focus on the specific isoform TGF β 2, which is found to be significantly increased within the AH of POAG. The average level of TGF β 2 in normal eyes is roughly 1.48ng/mL, with about 37% of the TGF β 2 in active form. AH from the eyes of POAG patients, however, have an average concentration of 2.70ng/mL, up to 60.84% of which is activated at any given time (5, 57). In contrast, a recent investigation of TGF β 1 expression in human aqueous humor found total levels to be less than 0.1pg/mL in both control and POAG samples (60). Due to the low expression values, samples were not further analyzed to determine the ratio of mature to latent forms of TGF β 1 (60).

Expanding on previous work, Tripathi and colleagues found porcine TM cells to express the TGF β 2 mRNA, suggesting they are capable of producing the cytokine (58). Subsequent investigation showed that indeed TGF β 2 is secreted into the medium of porcine TM cells in culture, and that all secreted TGF β 2 is in latent form (58). Furthermore, hTM cells have also been found to express the TGF β receptor, both TGF β RI and TGF β RII, required for binding of TGF β and activation of the signaling cascade (61). Dual paracrine (58) and autocrine (61) modes of TGF β signaling are present in TM cells as evidenced by their ability to produce and respond to their own TGF β isoforms.

Physiologically, TGF β 2 is required to maintain the immune privileged status of the eye and is essential for normal ocular wound healing by stimulating production and deposition of ECM (62). TGF β accomplishes its role by activating multiple signaling cascades within the cell (43, 50, 55, 63-67).

1.5.2 TGF β Signaling

Canonical TGF β signaling involves intracellular signal transduction proteins, commonly known as SMAD effector proteins. The signaling cascade initiated by TGF β binding to its receptor results in transphosphorylation of TGF β RI by TGF β RII. TGF β RI phosphorylation of SMAD2 and SMAD3 is facilitated by SMAD anchor for receptor activation (SERA), which is able to bind to the plasma membrane via phosphatidylinositols (43, 44, 68). Next, pSMAD2/3 recruits the signal transducer SMAD4 and the complex then translocates to the nucleus. In combination with transcription factors, the SMAD2/3/4 complex is able to modulate transcription of various genes, including upregulation of *COL1A1* mRNA. Inhibitory SMADs, particularly SMAD7, are induced as part of a negative feedback loop to counteract the effects of TGF β signaling (43). SMAD7 is able to block TGF β signaling by recruiting ubiquitin protein ligases to degrade the receptors and inhibit propagation of the TGF β signal (44) (**Figure 1.5.1**).

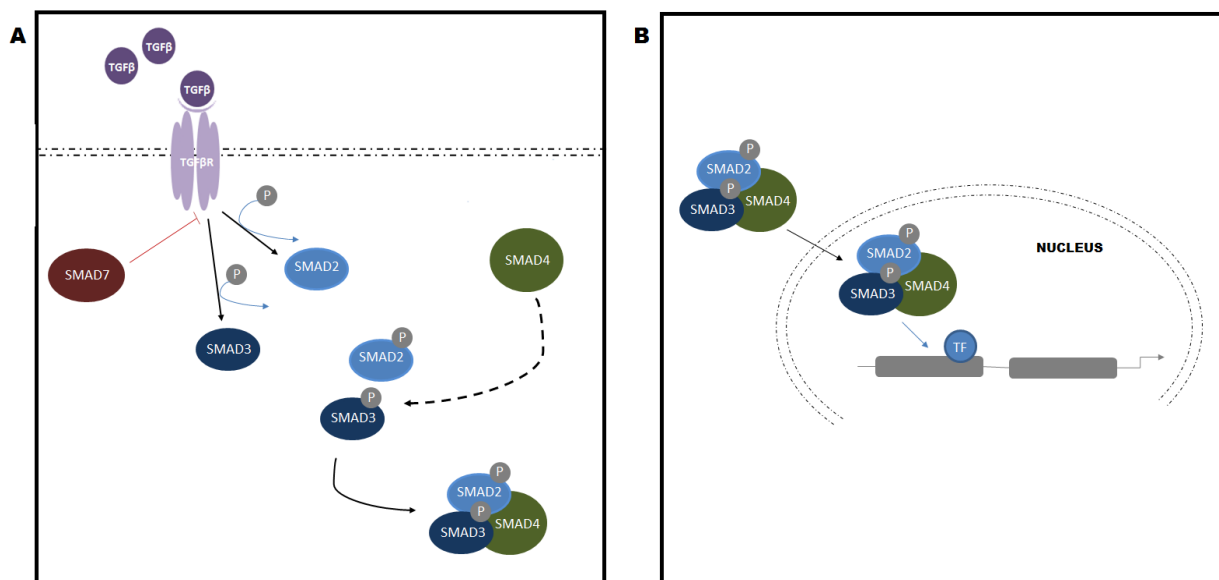


Figure 1.5.1 Canonical TGFβ signaling.

(A) Binding of TGFβ to TGFβRs causes phosphorylation of SMADs 2/3. Phosphorylated SMADs 2/3 recruit SMAD4 and form a complex of SMADs 2/3/4. **(B)** The SMAD complex translocates to the nucleus where it interacts with transcription factors (TF) to modulate gene transcription. SMAD7 is induced by SMAD3 to inhibit propagation of the TGFβ signal (43, 44, 68).

A more recent report outlines the importance of TGFβR trafficking for signal propagation and receptor turn-over (68). Internalization of TGFβRs in complex with SMAD7 occurs via the binding of caveolin-1 to TGFβRI (68). TGFβRs in caveolin-positive vesicles are then degraded in a ubiquitin-dependent manner (68). Alternatively, TGFβRs internalized in clathrin-coated pits, which form early endosomes, are sequestered from caveolin endosomes to prevent degradation (68). Endosome sequestering of TGFβRs may promote TGFβ signaling in more than one way, since phosphatidylinositols, a common substrate for many kinases and phosphatases, are enriched to the endosome (68).

Conversely, non-canonical signaling pathways involving AKT and FAK, which are responsible for controlling cell proliferation, migration, survival and ECM deposition, are inducible by TGFβ (43, 50, 63, 64, 69). The mechanisms of non-canonical TGFβ signaling are discussed in detail in Chapters 3 and 4.

Pathologically increased overall TGF β levels, and increased proportions of active TGF β isoforms can induce fibrotic changes. Most importantly, increased levels of TGF β 2 in the eyes of POAG patients correlate to increased ECM deposition within the TM (3, 4). Considering the function of the TM tissue and how tissue stiffness would negatively affect performance, fibrosis of the TM with decreased AH drainage is a reasonable mechanism to account for increased IOP.

1.6 Phosphatase and Tensin Homolog

1.6.1 PTEN Structure and Function

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) has recently emerged as a regulator of fibrosis. PTEN is a member of the protein tyrosine phosphatase family, and exhibits dual protein and phospholipid phosphatase activity (70). Alternatively, the gene that encodes *PTEN* is known as mutated in multiple advanced cancers 1 (*MMAC1*), and TGF β regulated and epithelial cell enriched phosphatase 1 (*TEP 1*).

The protein structure of PTEN is categorized into 3 major domains: a phosphatase region; a C2 domain; and a C-terminus (70). The active site of PTEN resides in the phosphatase region allowing for catalytic activity. The C2 domain is implicated in the intracellular localization of PTEN. When PTEN is bound to the plasma membrane it exhibits enhanced catalytic activity (70). The tail region contains various residues which can be modified by post-translational addition of functional groups, including glycosylation, ubiquitination, and phosphorylation (71). Several residues within the tail region can be phosphorylated, with phosphorylation patterns able to regulate the enzymatic ability and stability of PTEN (71). Overall, the stability, activity, and localization of PTEN within the cell are controlled by post-translational modifications.

The main enzymatic function of PTEN is its phospholipid phosphatase activity. By dephosphorylating phosphatidylinositol-3 (PIP3) into phosphatidylinositol-2 (PIP2), PTEN directly antagonizes phosphoinositide-3 kinase (PI3K) activity (70). PIP₃ is required for the activation of the AKT pathway, promoting cell growth,

proliferation, and survival. Therefore, by negatively regulating AKT, PTEN is responsible for cell cycle regulation, controlling cell growth, and initiating apoptosis.

Finally, PTEN also exhibits protein phosphatase activity by removing phosphate groups from tyrosine residues. Most importantly, two major targets involved in prominent cell signaling pathways are implicated with PTEN's protein phosphatase activity, including FAK (focal adhesion kinase) and Src homology 2 domain containing transforming protein (Shc). PTEN removes phosphate groups from tyrosine residues on the activated FAK molecule to decrease FAK signaling for cell migration and other parts of cell metabolism related to cytoskeleton (72, 73). Shc is implicated early in the mitogen-associated protein (MAP) kinase pathway that responds to various extracellular signals, including cellular stretch (72, 73).

Regulatory post-translational modifications in the C-terminal region are primarily phosphorylation events (70). Combinations of phosphorylation at multiple residues affect enzymatic activity by either increasing stability to decrease activity, or decreasing stability and increasing activity. Such combinations of phosphorylation are able to induce changes in conformation that affect cellular localization. PTEN's binding to the plasma membrane is crucial for enzymatic activity as PTEN is in closer proximity to its substrate PIP_3 . When localized to the cytosol, PTEN is less active (70). Analysis of PTEN activity is therefore more informative than consideration of expression levels alone. The specific effects of C-terminal phosphorylation are further discussed in Chapters 3, 4, and 5.

Phosphorylation of the C2 domain has also been reported, mostly by Src/RAK and ROCK in terms of stabilizing the molecule and promoting additional phosphorylation (70). Ubiquitination of PTEN is also observed in the C2 domain at lysine residues 13 and 289, dictating cellular stability and localization (70). With increased ubiquitination of PTEN, stability and activity decrease, but localization to the nucleus increases, where it is able to signal for apoptosis (70, 74).

Recent reports have also found evidence of a secreted version of PTEN. Discovery of a translational variant of *PTEN*, which is more than twice as long as the normal PTEN protein, yielded the classification of PTEN-Long (75). The PTEN-Long protein is a membrane-permeable lipid phosphatase. Secreted PTEN-Long can enter neighboring cells where it seems to function similarly to normal PTEN by restoring function and inhibiting the PI3K/AKT pathway in cells that have compromised, or lost, PTEN function (75).

Finally, emerging work shows that PTEN has a role in the modulation of ECM deposition. Specifically, increased PTEN expression decreases collagen deposition in the ECM, whereas decreased PTEN expression allows for an increase in collagen deposition (7, 38, 39). Most importantly, loss of PTEN causes fibrosis of dermal fibroblasts and lung fibroblasts in a mouse knockout model of *Pten* (7, 8). The specific function of PTEN in the TM of normal and glaucomatous eyes remains to be elucidated.

1.7 Hypothesis and Objectives

1.7.1 Hypothesis

We hypothesize that TGF β 2 modulates the deposition of ECM in the TM by regulating the expression and activity of PTEN protein, ultimately affecting aqueous outflow.

1.7.2 Objectives

1. To investigate the modulation of PTEN by TGF β 2 by studying *PTEN* mRNA expression, PTEN protein expression, and phosphorylation of PTEN.
2. To investigate the regulation of PTEN by studying the cell signaling mechanisms involved with the use of small molecule inhibitors of cell signaling pathways.
3. To investigate the functional role of PTEN in ECM regulation by inhibiting PTEN activity and by altering expression of PTEN.

Chapter 2

2 Materials and Methods

2.1 Human Donor Trabecular Meshwork Cell Culture

Human trabecular meshwork cells (hTM cells) (ScienCell, Carlsbad, CA, US) were cultured in DMEM (Life Technologies, Burlington, ON, CAN) supplemented with 10% FBS and 1% P/S (Life Technologies), and maintained at 37°C and 5% CO₂ in a humidified incubator. Cells were used at passage 4-5. All experiments were performed on 6-well tissue culture plates; either Nunc plastic (ThermoFisher Scientific, Waltham, MA, US), Bioflex™ pronectin-coated (Flexcell International Corporation, Burlington, NC, US) or Bioflex™ collagen type 1-coated (Flexcell). Once cells reached 90% confluency, the medium was replaced with DMEM supplemented with 0.5% FBS and 1% P/S (Life Technologies). After 5 hours cells were pretreated with specific small molecule inhibitors, either LY294002 (Selleck Chemicals), PP2 (Selleck Chemicals), SB431542 (Tocris Bioscience, Minneapolis, MN, US), VO-OHpic (Tocris Biosciences), or ZSTK474 (Selleck Chemicals) for one hour. Cells were then treated with either vehicle, human recombinant TGFβ2 (R&D Systems Inc), inhibitor alone, or a combination of TGFβ2 and inhibitor. Initially, concentrations of inhibitors were chosen based on commonly used concentrations cited in the literature. Concentrations were adjusted experimentally based on response of hTM cells. All treatments and concentrations used are listed in Table 2.1.

Table 2.1.1. List of Treatments Used for Cell Culture.

Treatment	Concentration s Tested	Concentration s Used	Function	Supplier
Human Recombinant TGFβ2	1ng/mL 5ng/mL 10ng/mL	1ng/mL 5ng/mL 10ng/mL	Growth factor	R&D Systems Inc, (302-B2- 002)

SB431542	12µM 20µM	20µM	ALK5 (TGFβ Receptor) inhibitor	Tocris Bioscience (1614)
LY294002	10µM 20µM 30 µM	20µM 30 µM	PI3K inhibitor PI3Kα/δ/β	Selleck Chemicals (S1105)
ZSTK474	2µM 5µM	2µM 5µM	PI3K inhibitor class I PI3K isoforms	Selleck Chemicals (S1072)
PP2	8 µM 10µM 16 µM 20µM 30 µM	10µM 20µM	Src inhibitor	Selleck Chemicals (S7008)
VO-OHpic	0.25µM 0.5µM 1µM 2.5µM 5µM	0.25µM 0.5µM 1µM 2.5µM 5µM	PTEN inhibitor	Tocris Bioscience (3591)

2.2 Cellular Transfection

hTM cells were maintained as described above. After plating on to Nunc plastic 6-well tissue culture plates (ThermoFisher Scientific), hTM cells were left to grow until slightly past confluent. At this point the medium in each well was replaced with 2mL DMEM supplemented with 0.5% FBS (Life Technologies). After 6 hours hTM

cells were transfected with 1.0µg DNA (either pcDNA3.1 + *IRES GFP*, *ePTEN*, or *C124S PTEN*; further discussed in Chapter 5) using the PolyMag Neo Magnetofection Kit (OZ Biosciences INC, CA, USA). 1.0µg of each type of DNA was dissolved into 200µL serum-free DMEM and then added to 1.0µL transfection reagent. The complex was incubated at room temperature for 20 minutes before adding drop-wise to the hTM cells. The 6-well plate was placed atop the PolyMag Neo magnet inside the incubator set at 37°C and 5% CO₂ for 4-5 minutes. Medium was removed immediately following transfection and replaced with 3mL DMEM supplemented with 0.5% FBS (Life Technologies). After 20 hours, culture medium was refreshed and select hTM cells were treated with human recombinant 5ng/mL TGFβ₂ (R&D Systems) dissolved in DMEM with 0.5% FBS (Life Technologies).

2.2.1 Subcloning and Purification of Bacterial Plasmids

Competent DH5α *Escherichia coli* (*E. coli*) (Life Technologies) were transformed as per supplier's protocol, with mutated *PTEN* DNA (*ePTEN* and *C124SV*) kindly donated by Dr. Miho Iijima (Department of Cell Biology at the John Hopkins School of Medicine, Baltimore, MD, US) and control vector pcDNA3.1 *IRES GFP* (Addgene, Cambridge, MA, US). Transformed *E. coli* were plated onto LB-agar plates with 100µg/mL ampicillin (Amp). Individual colonies were isolated and allowed to grow in 5mL of LB supplemented with 100µg/mL Amp for 8 hours at 37 °C. The culture was then diluted 1:500 in 50mL LB supplemented with 100µg/mL Amp, and incubated at 37 °C for 14 hours. Plasmids were then purified using the ZymoPURE™ Plasmid Midiprep Kit (Zymo Research Corporation, Irvine, CA, US).

2.3 RNA Isolation and RT-PCR

RNA was isolated using the RNeasy RNA extraction kit (Qiagen, Mississauga, ON, CAN) and quantified (Nanodrop 1000, ThermoFisher Scientific). Twenty five ng of RNA was used in each reaction of real-time polymerase chain reaction (RT-PCR). One-step RT-qPCR master mix (qScript XLT One-step RT-qPCR ToughMix, ROX; Quanta Biosciences, Beverly, MA, USA) was used along with TaqMan Assay-on-Demand primers (Applied Biosystems, Life Technologies) for *PTEN* and *COL1A1*.

Samples were run in triplicate and sequences were detected with ABI Prism 7900HT (Applied Biosystems). Target mRNA expression values were normalized to Euk 18S rRNA, and further analyzed using the $\Delta\Delta C_t$ method to determine fold change.

2.4 Protein Isolation and Immunoblotting

Cells were lysed in Pierce IP lysis buffer (ThermoFisher Scientific) supplemented with EDTA and PI cocktail (ThermoFisher Scientific) after 24 and 48 hours. Total protein concentration estimation was performed using the Micro BCA Protein Assay Kit (ThermoFisher Scientific). Equal amounts of protein (10-12 μ g) from each sample were prepared and run through SDS-PAGE, then transferred to a nitrocellulose membrane. Membranes were blocked in a solution of 5% BSA (Sigma, St. Louis, MO, US) dissolved in Tris-buffered saline (TBS) with 0.01% Tween-20 (TBS-T) for 1 hour, and incubated overnight at 4°C with anti-PTEN rabbit pAb (AF847) (1:2000; R&D Systems, Inc), anti-phosphoPTEN Ser380-Thr382/383 rabbit mAb (44A7) (1:2000; Cell Signaling Technologies, Danvers, MA, US), anti-AKT (pan) rabbit mAb (C67E7) (1:2000; Cell Signaling Technologies), anti-phosphoAKT Ser473 (D9E XP) rabbit mAb (1:2000; Cell Signaling Technologies), anti-collagen type 1 rabbit mAb (EPR7785) (1:2000; Abcam, Cambridge, UK) and anti-GAPDH mouse mAb (9B3: sc-66163) (1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, US). Blots were washed 3 times, for 5 minutes each, with TBS-T then incubated for 1 hour at room temperature with anti-rabbit IgG horseradish peroxidase conjugate (1:3000; BioRad, Mississauga, ON, CAN) or anti-mouse IgG horseradish peroxidase conjugate (1:3000; BioRad) secondary antibodies. Blots were imaged using an ECL detection method (WesternBright ECL HRP substrate (Advansta, Menlo Park, CA, US) and the Chemi Genius 2 Bio Imaging System (Syngene, Fredrick, MD, US)) to visualize proteins. Blots were stripped of bound antibodies using Restore Western Blotting Stripping Buffer (ThermoFisher Scientific), and then reprobed (to a maximum of two additional times) as described above. Residual signal was assessed between re-probing and stripping conditions were optimized to ensure adequate removal of primary and secondary antibodies.

Densitometric analysis of western blot bands was performed using ImageJ software.

Table 2.4.1. List of Antibodies Used for Immunoblotting.

Antibody	Concentration	Supplier
Anti-PTEN rabbit pAb	1:2000	R&D Systems Inc, (AF847)
Anti-phosphoPTEN (Ser380/Thr382/383) rabbit mAb	1:2000	Cell Signaling Technologies (44A7)
Anti-AKT (pan) rabbit mAb	1:2000	Cell Signaling Technologies (C67E7)
Anti-phosphoAKT (Ser473) rabbit mAb	1:2000	Cell Signaling Technologies (D9EXP)
Anti-collagen type I rabbit mAb	1:2000	Abcam (EPR7785)
Anti-FAK rabbit pAb	1:2000	ThermoFisher Scientific (PA5-16676)
Anti-phosphoFAK (Tyr397) rabbit mAb	1:2000	Cell Signaling Technologies (D20B1)
Anti-GAPDH mouse mAb	1:3000	Santa Cruz Biotechnology (9B3:sc-66163)

Anti-rabbit IgG Horseradish Peroxidase Conjugate	1:3000	BioRad (170-6515)
Anti-mouse IgG Horseradish Peroxidase Conjugate	1:3000	BioRad (170-6516)

2.5 Statistical Analysis

Data from RT-PCR experiments are represented as relative fold change in comparison to internal control (Euk 18S) and control treatment. Two-way ANOVA was used to determine whether the difference in means between treatment groups was statistically significant. Bonferroni's *post-hoc* test was then applied to determine the source of statistically significant effects detected by ANOVA. Protein expression levels were normalized to the internal control (GAPDH) for each protein sample, and then compared to the control level of expression for each donor to determine the relative fold change. One-way ANOVA was used to determine whether the means of different treatment groups were statistically significant. Tukey's *post hoc* test was applied to determine the source of statistical significance detected by ANOVA. Differences were considered statistically significant when $p < 0.05$. GraphPad Prism software (version 6.0, GraphPad Software, San Diego, CA) was used for all statistical analyses.

Chapter 3

3 Modulation of PTEN by TGF β 2

3.1 Rationale

In this chapter, we examine the effects of TGF β 2 on hTM cells with emphasis on PTEN and production of the ECM protein COL1. The pro-fibrotic role of TGF β has been widely documented in multiple organ systems, including the lungs, liver, and kidneys (43, 50, 51, 76). Furthermore, it has been established that elevated levels of TGF β 2 are present in the AH of POAG patients (5). Similarly, recent studies of PTEN support its role in fibrosis (39, 51, 52, 77, 78). While some mechanisms for their interaction have been suggested in other model systems (39, 51, 52, 77, 78), further work is required to determine how TGF β regulates PTEN in hTM cells. Moreover, regulatory control mechanisms are also of interest, particularly posttranslational phosphorylation of PTEN as it positively influences protein stability and negatively affects enzymatic activity (70).

We aim to examine *PTEN* and *COL1A1* mRNA expression and PTEN and COL1 protein expression in hTM cells to characterize the pro-fibrotic effects induced by TGF β 2.

3.2 Background

TGF β 2 is part of the TGF β superfamily, which is known to induce fibrosis and remodeling of the ECM (43, 44). Most importantly, TGF β 2 levels are significantly increased, with a greater ratio of active to latent TGF β 2 in the AH of glaucoma patients (5). TGF β 2 induces fibrosis of the TM tissue by inducing increases in expression of ECM proteins, including fibronectin (25), collagen type IV (79), and COL1 (80). Of particular interest is COL1, a component of the TM tissue (14, 17), and a marker of fibrotic conditions (55, 81). In fact, a mouse model of collagenase-resistant COL1 was shown to exhibit hallmarks of fibrosis and glaucoma with increased age (82, 83). As mutant COL1 was not degraded or remodeled, expression of COL1 increased throughout the anterior chamber of the eye (82).

Anterior chamber fibrosis was accompanied by significant increases in IOP as the mice aged (82, 83).

To identify a mechanism by which canonical TGF β signaling results in COL1 deposition, one study examined anterior segment fibrosis in secreted protein rich in acidic and cysteine (SPARC)-null mice (79). SPARC regulates the ECM, as overexpression of SPARC induces TM fibrosis in experiments using human cadaveric eyes (84). Using SPARC-null mice, it was determined that TGF β 2 is able to increase COL1 expression through a SMAD-independent pathway, primarily through the induction of connective tissue growth factor (79).

Finally, TM cells secrete their own TGF β 2 *in vivo* which could contribute to fibrosis (24, 58). The ability of cultured TM cells to produce and secrete TGF β 2 is maintained in *in vitro* experiments (58, 61). We aim to characterize the effects of TGF β 2 on COL1 expression of hTM cells.

It has been widely reported in the literature that TGF β is able to suppress the expression of *PTEN* mRNA and PTEN protein levels in various disease models of hepatocarcinoma, pancreatic and colon cancer, as well as in renal and pulmonary fibrosis (39, 51, 52, 77, 78, 85). These investigations detail a combination of post-transcriptional and post-translational mechanisms by which TGF β is able to suppress PTEN.

Initially, TGF β was shown to suppress *PTEN* mRNA and PTEN protein expression in a cell line of hepatocarcinoma. Specifically, TGF β increased degradation of the *PTEN* transcript in a post-transcriptional mechanism of suppression (85). The same study found decreases in total PTEN protein subsequent to TGF β treatment, which occurred at a rate that could not be explained by increased turnover of the *PTEN* transcript. Blockade of the ubiquitin-proteasome pathway rescued PTEN protein levels, suggesting TGF β is also able to control PTEN expression post-translationally by ubiquitination and degradation (85).

Investigation of a pancreatic cancer model has revealed that TGF β has a similar effect on PTEN expression. SMAD4-null pancreatic cancer cells were shown to have decreased PTEN protein and *PTEN* mRNA expression subsequent to treatment with TGF β (77). The mechanism of control in these cells occurs at the transcriptional level as TGF β was able to increase activity of the transcription factor NF- κ B to decrease transcription of the *PTEN* gene. When treated with TGF β in the presence of an NF- κ B super-repressor, sequestering NF- κ B in the cytoplasm, *PTEN* mRNA and PTEN protein expression was similar to the baseline expressed in control cells untreated with TGF β . Overexpression of SMAD4 had a similar effect to cytoplasmic sequestering of NF- κ B, decreasing NF- κ B activity even with TGF β treatment. Since NF- κ B activity increased after addition of TGF β in SMAD4-null cells, with further increases in NF- κ B activity observed in cells that had conditional knock-outs of SMAD2 and SMAD3, the transcriptional mechanism of *PTEN* repression utilizes the non-canonical TGF β signaling pathway (77).

In addition, a recent study on SMAD4-null colon cancer cells reported TGF β to decrease PTEN expression through increased tyrosine phosphorylation and activation of PI3K (52). Here, regulation of PTEN also involves non-canonical TGF β signaling. The investigators suggested total PTEN protein was decreased by a combination of suppressed *PTEN* gene transcription and increased PTEN protein degradation subsequent to PI3K activation (52).

Investigations of fibrotic conditions reveal similar results, characterizing an inverse relationship between TGF β and PTEN expression. Increased TGF β signaling induced after acute kidney injury caused a loss of PTEN protein in a subset of proximal tubule epithelial cells, resulting in renal fibrosis (51). Rescue of PTEN expression by inhibiting TGF β -signaling was able to decrease fibrosis (51). A similar model of TGF β -induced fibrosis in mesangial cells revealed the mechanism of PTEN downregulation to occur post-transcriptionally (78). TGF β induced expression of microRNAs 216a and 217, which bind the *PTEN* transcript to prevent translation (78). Translation of *PTEN* mRNA resumed following inhibition of these microRNAs, restoring PTEN protein expression (78). Furthermore, a study of

idiopathic pulmonary fibrosis discovered significantly decreased *Pten* mRNA and protein expression in fibrotic lung fibroblasts (39). Overexpression of *Pten* was found to be sufficient to reduce the fibrotic phenotype (primarily collagen deposition) of lung myofibroblasts treated with TGF β in a subsequent study (39). In the context of these findings, we aim to characterize the effects of TGF β 2 on *PTEN* mRNA and PTEN protein expression of hTM cells.

Finally, previous studies have shown that PTEN is post-translationally modified by many different molecules within the cell (70, 71). The most common and most reported upon modification is phosphorylation, particularly in the C-terminal region. Specifically, multiple studies have reported a triplicate site within the tail region of PTEN that increase protein stability and decrease activity when all three amino acid residues are phosphorylated in tandem (70, 71). This site is Ser380/Thr382/383 (pPTEN). The presence of these phosphorylation groups causes the tail region of PTEN to fold over the C2 domain. In this “closed” conformation, the C2 domain is not able to bind the plasma membrane where PTEN’s substrate, PIP3, is in abundance. pPTEN in its “closed” conformation then remains localized to the cytosol away from its substrate, and thus functionally inactive (**Figure 3.2.1**). In our study of hTM cells, we aim to investigate expression of pPTEN (Ser380/Thr382/383) as a marker of enzymatic inactivity.

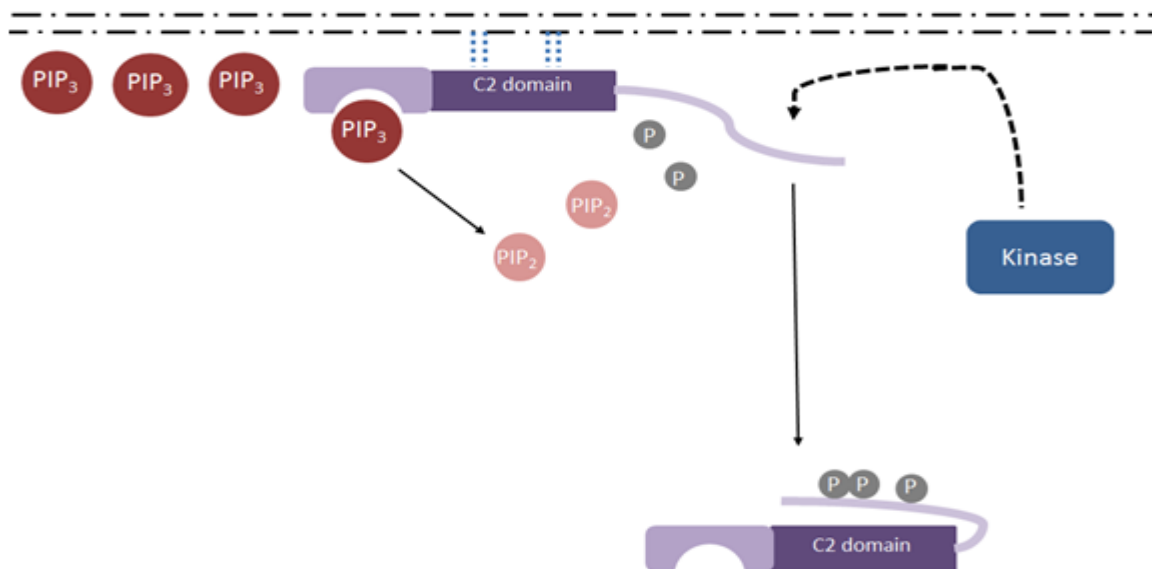


Figure 3.2.1. Open and Closed PTEN conformation.

When PTEN is in open conformation, the C2 domain is able to bind to the plasma membrane. Binding to the plasma membrane increases enzymatic activity of PTEN because the enzyme is localized to its substrate PIP₃. When PTEN is phosphorylated on its tail region at residues Ser380, Thr382, and Thr383, PTEN adopts a closed conformation with the tail region folding over the C2 domain. The closed conformation of PTEN prevents binding to the plasma membrane binding and as a result PTEN is localized to the cytosol.

3.3 Experimental Approach

1. To determine the effects of TGFβ2 on *COL1A1* and *PTEN* mRNA transcript levels, cultured hTM cells were treated with TGFβ2 (1ng/mL and 5ng/mL). RNA was extracted at 12 and 24 hours post-treatment and RT-PCR was performed.
2. To determine the effects of TGFβ2 on COL1 and PTEN protein, hTM cells were cultured on pronectin-coated plates with increasing concentrations of TGFβ2 and effect of TGFβ2 further confirmed using an inhibitor to the TGFβ receptor. Protein was isolated and immunoblotting was performed.
3. To indirectly determine the relative activity of PTEN protein, immunoblots were analyzed for pPTEN (Ser380/Thr382/382).

3.4 Results

3.4.1 TGF β 2 increases *COL1A1* mRNA expression.

Our first objective was to characterize the effects of active TGF β 2 on hTM cells. Here we treated hTM cells with TGF β 2 at 1ng/mL and 5ng/mL to cover a range of physiological to pathological levels of TGF β 2 present in the AH of glaucoma patients and measured changes in the mRNA transcript of *COL1A1*. At 12 hours post-treatment with TGF β 2 1ng/mL we found a significant increase in the expression of the *COL1A1* transcript ($p < 0.01$). Twenty four hours after treatment with TGF β 2 (1ng/mL and 5ng/mL) *COL1A1* mRNA was significantly elevated ($p < 0.0001$), roughly 4-fold in comparison to control (**Figure 3.4.1**).

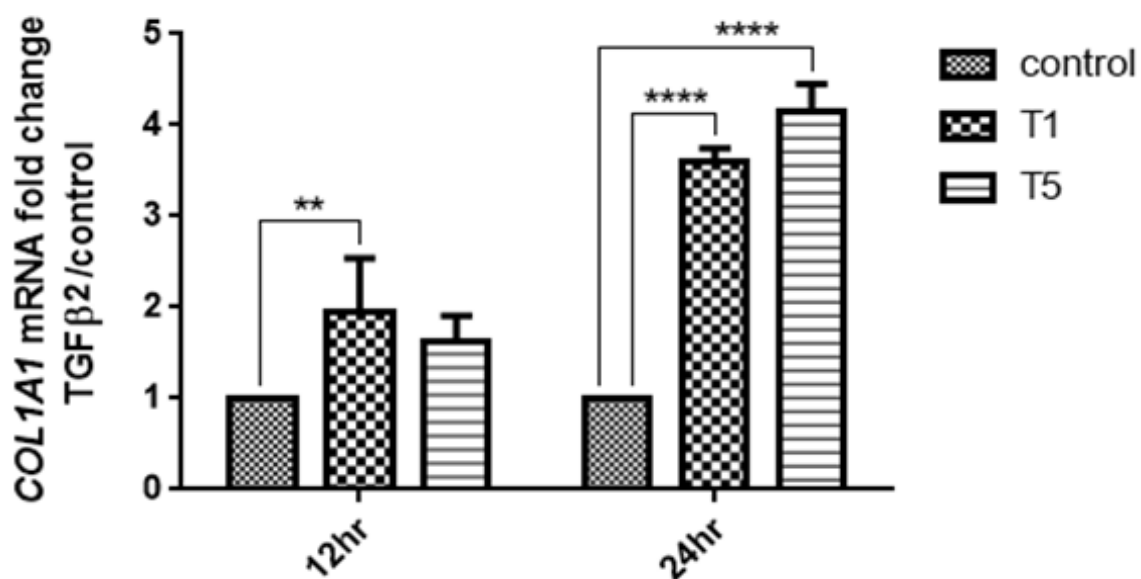


Figure 3.4.1 TGF β 2 increases *COL1A1* mRNA expression.

Real-time PCR results from hTM cells treated with TGF β 2 (1 or 5ng/mL). RNA was extracted at 12 and 24 hours. *COL1A1* mRNA expression levels were normalized to Euk18s. A two-way ANOVA was used to test whether the means of treatment groups were statistically significant. Bonferroni's *post-hoc* was then applied to determine the source of statistical significance detected by ANOVA. ** $p < 0.01$, **** $p < 0.0001$ in comparison to control; N=3.

3.4.2 TGF β 2 increases *PTEN* mRNA expression.

We investigated the effects of TGF β 2 on *PTEN* transcript levels, with the expectation that *PTEN* mRNA would be decreased as reported in the literature at similar time points. Instead, at 24 hours post-treatment with TGF β 2 5ng/mL, we observed a 2.15 fold increase in *PTEN* mRNA (statistically significant with $p < 0.05$) (**Figure 3.4.2**). Changes in the *PTEN* transcript induced by TGF β 2 after twelve hours were not significant.

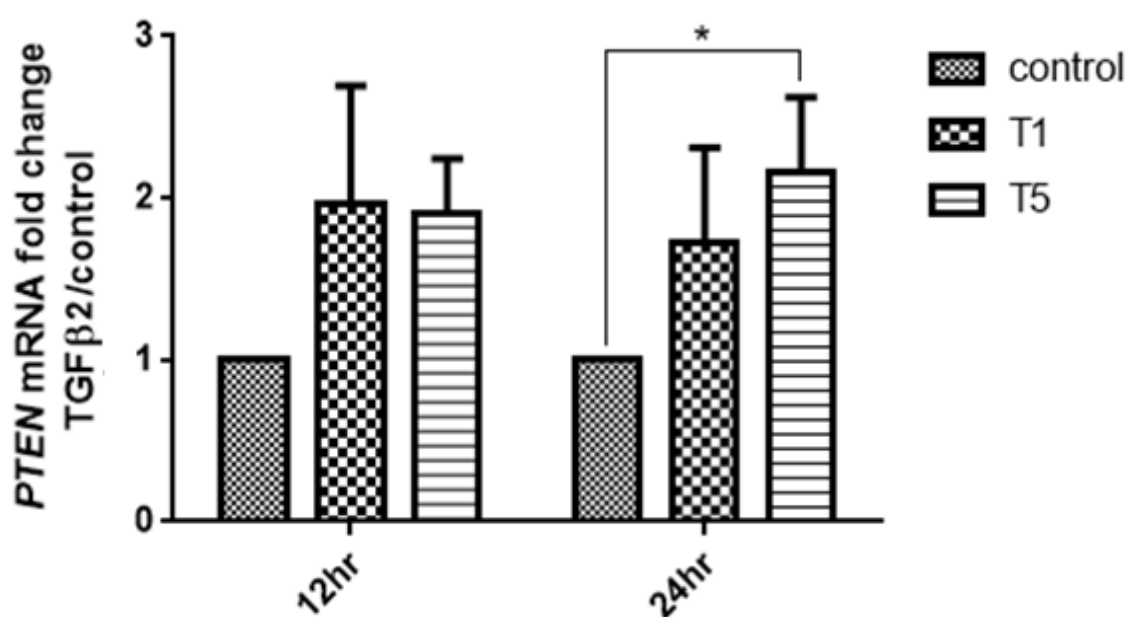


Figure 3.4.2 TGF β 2 increases *PTEN* mRNA expression.

Real-time PCR results from hTM cells treated with TGF β 2 (1 or 5ng/mL). RNA was extracted at 12 and 24 hours. *PTEN* mRNA expression levels were normalized to Euk18S. A two-way ANOVA was used to test whether the means of treatment groups were statistically significant. Bonferroni's *post-hoc* was then applied to determine the source of statistical significance detected by ANOVA. * $p < 0.05$ in comparison to control; N=3.

3.4.3 COL1 protein expression after TGF β 2 treatment.

Next, we wanted to verify that transcripts of genes upregulated by TGF β 2 were translated into functional proteins. To confirm that the increased *COL1A1* transcripts were translated into COL1 protein we cultured hTM cells on pronectin-coated plates using varying levels of TGF β 2 (1, 5, and 10ng/mL) and TGF β receptor inhibitor SB431542 (20 μ M). Protein expression of COL1 (**Figure 3.4.3**) after treatment with TGF β 2 is consistent with the upregulation of *COL1A1* mRNA seen in **Figure 3.4.1**. Addition of SB431542 decreased COL1 expression in comparison to TGF β 2-treated cells. COL1 expression was decreased expression below baseline levels at both 24 and 48 hours after the addition of SB431542.

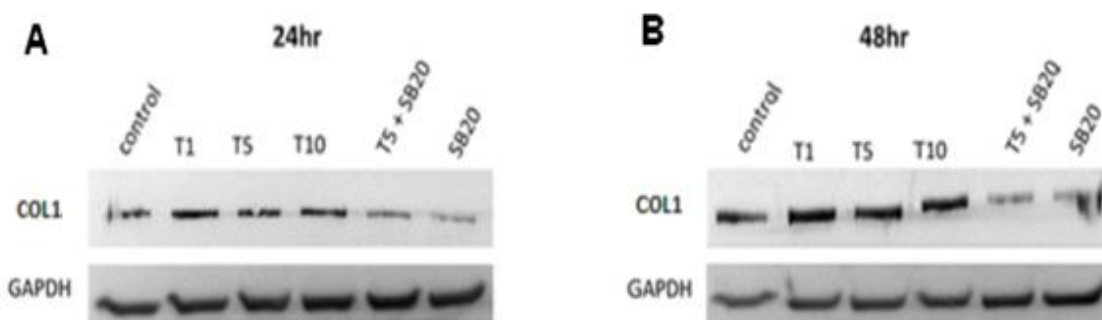


Figure 3.4.3 COL1 protein expression after TGF β 2 treatment.

Immunoblots from hTM cells treated with increasing concentrations of TGF β 2 (1, 5, or 10ng/mL), and TGF β receptor inhibitor SB431542 (20 μ M). Protein was extracted at 24 (**A**) and 48 hours (**B**). Immunoblots were probed with antibodies for COL1 and GAPDH. Western blot analysis shows increased COL1 expression with addition of TGF β 2 in hTM cells. N=1.

3.4.4 TGF β 2 increases PTEN protein expression and phosphorylation of PTEN.

To verify that *PTEN* mRNA upregulated 2.15 fold by TGF β 2 was further translated into PTEN protein, we cultured hTM cells on pronectin-coated plates using varying levels of TGF β 2 (1, 5, and 10ng/mL) and TGF β receptor inhibitor SB431542 (20 μ M). We found significant increases in PTEN protein compared to controls at 24 (p<0.05 for TGF β 2 10ng/mL) (**Figure 3.4.4**) and 48 hours (p<0.001 for TGF β 2 1, 5, and 10ng/mL) (**Figure 3.4.5**) post-treatment. Expression of PTEN protein was decreased upon pretreatment with SB431542, (significantly at 48 hours, p<0.001). Since TGF β is repeatedly reported to suppress PTEN expression (39, 51, 52, 77, 78, 85) we investigated post-translational modifications to reconcile our findings with those reported in the literature. We found that although TGF β 2 increased PTEN protein, TGF β 2 also increased phosphorylation of PTEN at Ser380/Thr382/383, which renders the molecule functionally inactive (70). Increases in pPTEN were decreased upon pretreatment with SB431542. Although TGF β 2 is able to induce significant increases in PTEN as shown over a range of concentrations, TGF β 2 can also increase phosphorylation of PTEN to inactivate the enzyme. Finally, the ratio of pPTEN to PTEN did not vary according to treatment suggesting the changes to PTEN expression are accompanied by simultaneous changes to PTEN's phosphorylation. Results were replicated on plastic and COL1-coated plates at the same time points (**Appendix A-B**).

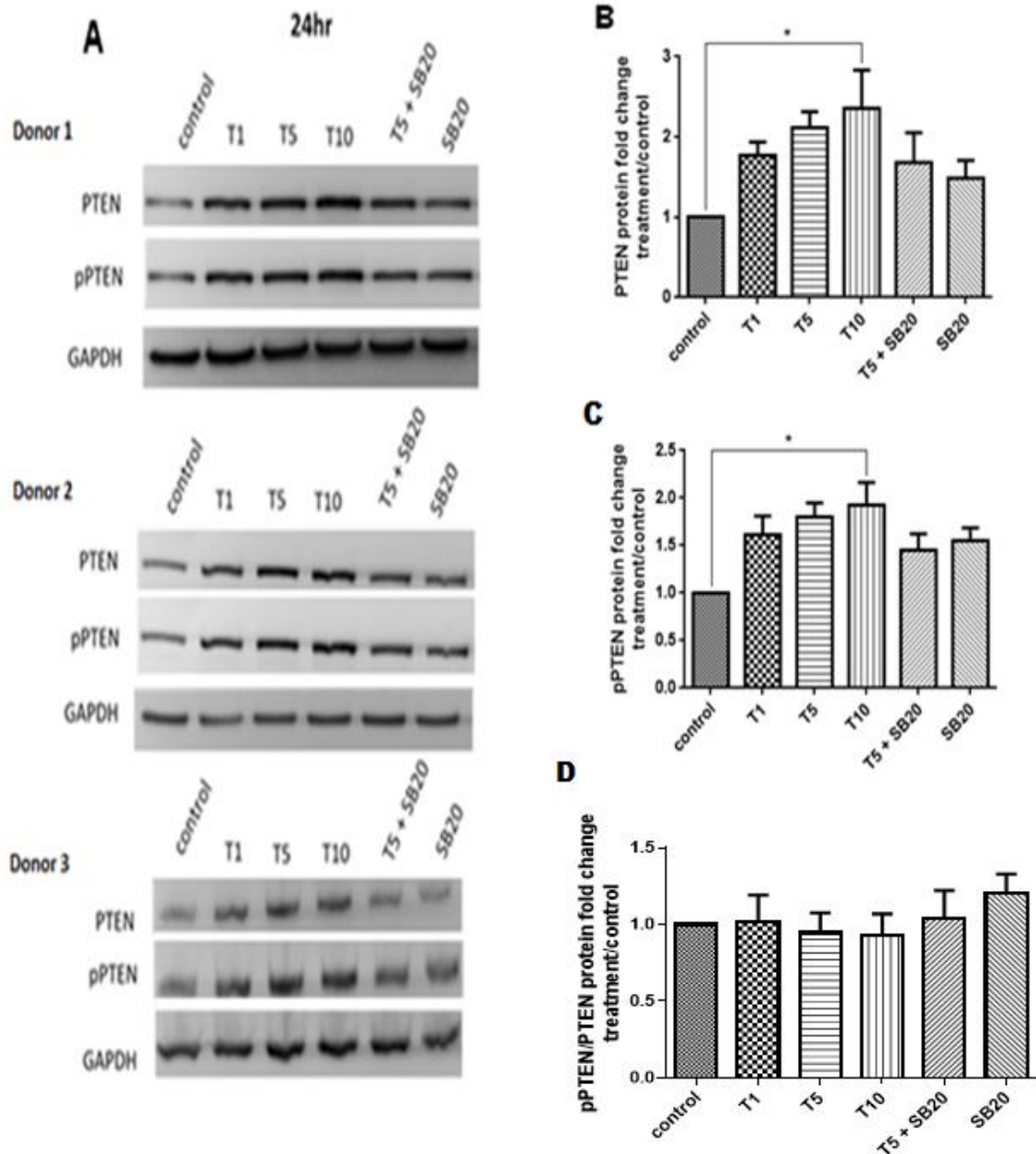


Figure 3.4.4 TGF β 2 increases PTEN protein expression and phosphorylation of PTEN at 24 hours.

Immunoblots from hTM cells treated with increasing concentrations of TGF β 2 (1, 5, or 10ng/mL), and TGF β receptor inhibitor SB431542 (20 μ M). Protein was extracted at 24 (A). Immunoblots were probed with antibodies for PTEN, pPTEN (Ser380/Thr382/383), and GAPDH. Levels of PTEN protein expression were increased with increasing amounts of TGF β 2 (1, 5, or 10ng/mL). Expression of PTEN protein was decreased upon pretreatment with SB431542 (20 μ M) prior to

addition of TGF β 2 5ng/mL. Equivalent increases in phosphorylation of PTEN seen with increasing amounts of TGF β 2 (1, 5, or 10ng/mL). Increases in PTEN phosphorylation were prevented by pretreatment with SB431542 (20 μ M) prior to addition of TGF β 2 5ng/mL. Densitometry for 24 PTEN (**B**), pPTEN (**C**), and relative pPTEN/PTEN (**D**) expression. Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test. (GraphPad Prism). * $p < 0.05$; N=3.

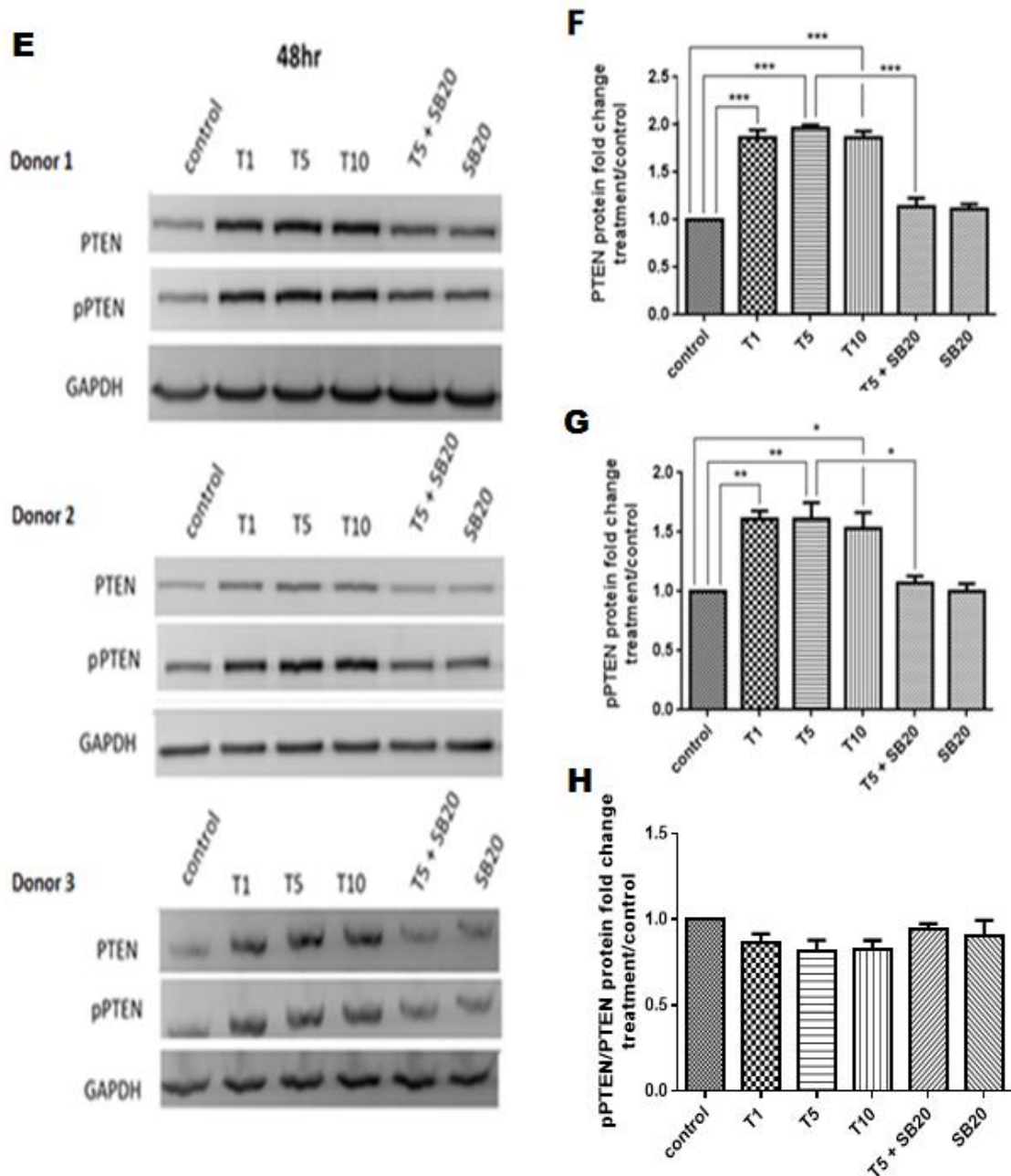


Figure 3.4.5 TGF β 2 increases PTEN protein expression and phosphorylation of PTEN at 48 hours.

Immunoblots from hTM cells treated with increasing concentrations of TGF β 2 (1, 5, or 10ng/mL), and TGF β receptor inhibitor SB431542 (20 μ M). Protein was extracted at 48 hours (**E**). Immunoblots were probed with antibodies for PTEN, pPTEN (Ser380/Thr382/383), and GAPDH. Levels of PTEN protein expression were increased with increasing amounts of TGF β 2 (1, 5, or 10ng/mL). Expression of PTEN protein was decreased upon pretreatment with SB431542 (20 μ M)

prior to addition of TGF β 2 5ng/mL. Similar increases in pPTEN were seen with increasing amounts of TGF β 2 (1, 5, or 10ng/mL). Increases in PTEN phosphorylation were prevented by pretreatment with SB431542 (20 μ M) prior to addition of TGF β 2 5ng/mL. Densitometry for 24 PTEN (**E**), pPTEN (**F**), and relative pPTEN/PTEN (**G**) expression. Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test (GraphPad Prism). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; N=3.

3.4.5 Summary of Results

1. TGF β 2 increased *COL1A1* mRNA and COL1 protein.
2. TGF β 2 increased *PTEN* mRNA and PTEN protein.
3. TGF β 2 phosphorylated PTEN at Ser380/Thr382/383.

3.5 Discussion

These findings establish the response of hTM cells to TGF β 2 stimulation. Consistent with previous studies, we show that TGF β 2 induces ECM protein production in hTM cells. To our surprise, we find that TGF β 2 is also able to induce increases in *PTEN* mRNA and PTEN protein, contrary to what has been reported in the literature.

In finding that PTEN expression is increased under the influence of TGF β 2, and that total PTEN protein expression is correlated with decreased PTEN activity (as measured by increased pPTEN expression) we propose a novel mechanism by which TGF β is able to regulate PTEN. Here, we show the previously unreported effect of TGF β 2 increasing phosphorylation of PTEN as a means of inactivating PTEN's enzymatic activity. The Ser380/Thr382/383 phosphorylation is associated with decreased PIP₃ enzymatic activity (86), such that phosphorylation at these sites causes the C-terminus of PTEN to fold over its C2 domain. Once the C2 domain is covered, PTEN is no longer able to bind to the plasma membrane and is then localized to the cytoplasm, away from its substrate PIP₃ (70). In this manner, PI3K signaling remains unopposed. Up to this point, TGF β has been

shown to only decrease expression of PTEN in various model systems (39, 51, 52, 77, 78, 85).

Decreased activity of PTEN by post-translational modification is consistent with the increased COL1 expression observed in our experiments. Even though the increase and subsequent inactivation of PTEN is a novel finding, the associated increase in COL1 is similar to other studies in which deletion of the *Pten* gene (7, 8) or inhibition of PTEN activity causes an increase in COL1 deposition (39).

One explanation for the novel regulatory mechanism of PTEN in hTM cells may be related to the nature of the TM cells and the niche role they occupy. As discussed earlier, TM cells are subjected to severe stress, in the form of mechanical stretch caused by fluctuations in IOP. TM cells must be able to quickly and efficiently respond to changes in stimuli to survive in this type of environment. To facilitate rapid remodeling of the ECM, changes in PTEN activity can be controlled via phosphorylation. The posttranslational mechanism of control would be favoured over a transcriptional control mechanism as it would allow for more rapid changes in activity. Since activity-induced degradation of PTEN is reported to occur (86) the increase in PTEN protein observed with the concomitant increase in its phosphorylation may serve as a compensatory feedback mechanism. Phosphorylation can quickly inactivate PTEN while retaining the protein product in a readily available state, which allows for continuous ECM remodeling to facilitate adequate AH outflow.

Alternatively, the phosphorylation of PTEN in response to TGF β stimulation, may be attributed to a TGF β RII isoform present on TM cells (61). Wordinger and colleagues identified mRNA of alternatively spliced TGF β RII isoforms in hTM cells (61). They postulated that the transcripts were subsequently translated into functional receptors, building on previous investigations that confirmed the expression TGF β Rs in porcine TM cells (87). Binding of TGF β to the TGF β R isoforms may slightly modify downstream signaling to give an altered response, although further investigations are required in this area.

Finally, we also observed decreases below baseline expression of COL1 protein after addition of SB431542. This phenomenon can be explained by consideration of endogenous TGF β 2 expression of hTM cells. Tripathi and colleagues determined that porcine TM cells in culture produce the mRNA transcript for TGF β 2, and also secrete TGF β 2 into the medium (58). Secretion of endogenous TGF β 2 was found to peak at 48 hours, with secreted TGF β 2 measured to be 20pg/mL (58). An earlier investigation comparing expression levels of TGF β in the aqueous humor of various animals found porcine aqueous humor to express 2.39 times less TGF β than human aqueous humor (490pg/mL compared to 1.17ng/mL) (88). The authors note that more than 90% of the total TGF β represented is the TGF β 2 isoform (88). With careful consideration of these findings, it is likely that the hTM cells employed in our model secrete endogenous TGF β 2 at a rate greater than 20pg/mL, although further investigation is required for confirmation. The decrease below baseline levels of COL1 protein with the addition of the TGF β R inhibitor could then be attributed to the additional blockade of endogenous TGF β 2 activity.

Overall, these findings demonstrate the novel response of hTM cells to TGF β 2 stimulation. We have confirmed that TGF β 2 increases COL1 protein expression, and that TGF β instead of decreasing the expression of PTEN as found in previous studies, is inactivated PTEN by phosphorylation. Our studies suggest that increased levels of active TGF β 2 likely contribute to fibrosis of the TM in POAG, and that PTEN is involved in this fibrotic process. Furthermore, we continue to investigate PTEN as a regulator of fibrosis with the potential to be used as a treatment target towards preventing and mitigating dysfunction of the TM in POAG. However, prior to developing PTEN-targeted treatments, the mechanism of action of TGF β signaling and its effect on PTEN must be deciphered.

Chapter 4

4 Signaling mechanisms regulating PTEN

4.1 Rationale

In the previous chapter, we described the effects of TGF β 2 stimulation on hTM cells *in vitro*. While there is evidence to support the TGF β -induced suppression of PTEN protein and *PTEN* mRNA (39, 51, 52, 77, 78 85), we have shown a different mechanism of regulation of PTEN activity in hTM cells. Although PTEN expression is increased, the concomitant phosphorylation of PTEN indicates its inactivation by TGF β . The phosphorylation of PTEN is accompanied by increased COL1 production induced by TGF β . In Chapter 4, we build upon these findings to investigate potential signaling pathways that may regulate PTEN to affect activity and ECM protein production.

PTEN is involved in various signaling pathways within the cell related to growth, differentiation, proliferation, and ultimately fibrogenesis (8, 39, 85, 89). Of particular interest is involvement of PTEN with the PI3K/AKT pathway and the Src/FAK pathway, usually reported in the context of oncogenesis and cancer development (90-92).

As introduced in Chapter 1, PTEN antagonizes PI3K activity by converting PIP₃ into PIP₂. Since PIP₃ is required for AKT activation (93), PTEN is able to negatively regulate the PI3K/AKT pathway (70). In turn, PI3K is also able to negatively regulate PTEN activity (94, 95). Similarly, PTEN is involved in the regulation of FAK (73, 96). Both pathways are able to initiate signaling cascades that increase ECM production, contributing to fibrosis (65, 67, 97, 98). Here we investigate TGF β -mediated activation of these pathways to determine their role in the regulation of PTEN. In addition, preliminary investigations on the role of the FAK pathway in PTEN-mediated collagen deposition were conducted because FAK has an important role in fibrosis (98) and PTEN is known to dephosphorylate FAK (72, 73) to downregulate FAK signaling.

4.2 Background

4.2.1 PI3K/AKT Pathway

Non-canonical TGF β signaling has been reported to involve the PI3K/AKT pathway, including evidence that TGF β is directly able to activate PI3K by inducing tyrosine phosphorylation (52). The PI3K/AKT pathway is highly involved in cell cycle regulation and various cellular processes, including downstream TGF β signaling and ECM deposition (55). We are particularly interested in this pathway because PTEN is a direct antagonist of PI3K, and PI3K is known to negatively regulate PTEN (94, 95) in a feedback mechanism that can enhance signaling through AKT.

There are a total of eight known PI3K isoforms, divided into three classes based on structure and substrate affinity (99). Class I PI3Ks use PIP₂ as a substrate and exist in association with a regulatory subunit. The main isoforms in this class are PI3K α , β , γ , and δ (100). PI3K $\alpha/\beta/\gamma/\delta$ are found in all mammalian cell types (101). PI3Ks $\alpha/\beta/\delta$ forms a complex with a p85 regulatory subunit, whereas PI3K γ binds with either a p101 or p87 regulatory subunit (100). Individual roles of the regulatory subunits have yet to be determined, but it is suggested they exist to stabilize PI3K and inhibit kinase activity in the absence of a stimulus, and recruit PI3K to activated receptors. Class I PI3Ks are the most studied and best understood.

Class II and III PI3Ks use phosphatidylinositol lipids as substrates (99). There are three isoforms classified as Class II PI3Ks, which have some role in signal transduction (102) although the mechanism and significance has not yet been clearly defined. The physiological role of Class III PI3Ks is still poorly understood, although some evidence has suggested they are involved in endosomal protein sorting and autophagy.

We focus on Class I PI3Ks as their main function, to phosphorylate PIP₂ into PIP₃, is directly antagonized by the action of PTEN (**Figure 4.2.1**). Deviations in PTEN signaling, or inactivation of PTEN by its phosphorylation (at residues Ser380/Thr382/383) leave PI3K relatively unopposed to increase signaling

through this pathway. Cancer associated mutations in Class I PI3K isoforms are associated with increased basal levels of kinase activity, resulting in increased AKT activation (103). Currently, clinical trials are underway using inhibitors to Class I PI3K isoforms to treat cancers with associated PI3K mutations (104). In terms of PI3K's regulation of PTEN, investigations by Tzenaki *et al.* have shown that the p110 δ subunit of PI3K is able inactivate PTEN (95). Tzenaki has shown that overexpression of the p110 δ PI3K subunit in prostate and breast cancer cells results in decreased PTEN activity and increased phosphorylation of AKT (95). PTEN activity is restored and phosphorylation of AKT is decreased upon inactivation of the p110 δ PI3K subunit (95).

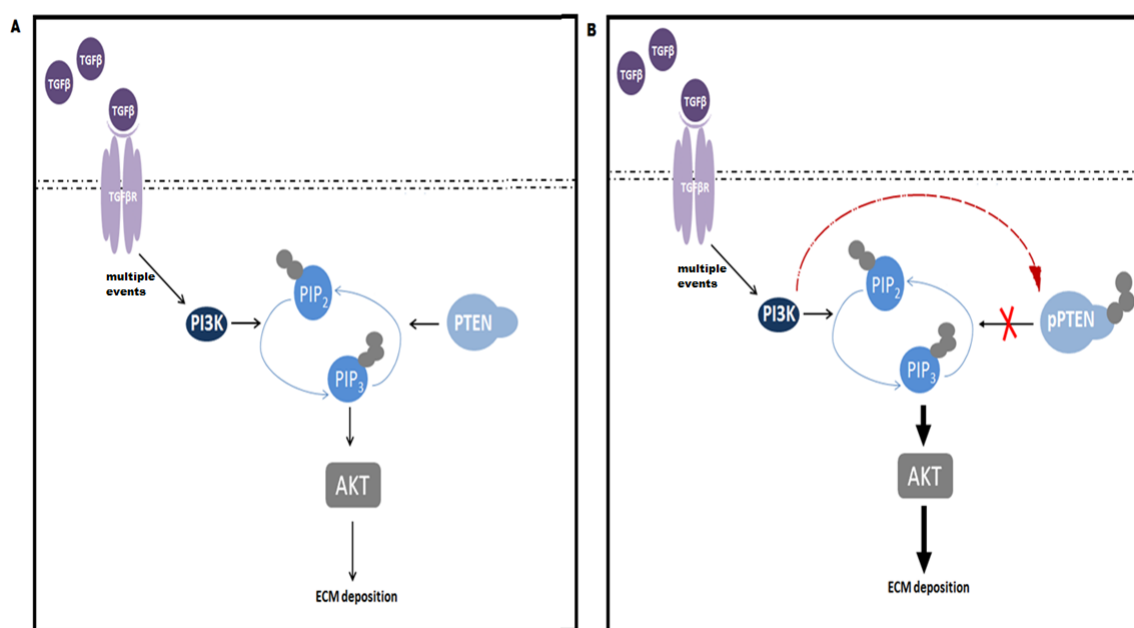


Figure 4.2.1 Schematic representation of PI3K/AKT pathway with active and inactive PTEN.

(A) TGF β is reported to signal through a non-canonical pathway involving PI3K/AKT to increase ECM production (85). Normally, PI3K phosphorylates PIP₂ into PIP₃, which is required for AKT activation. PTEN exerts its phosphatase activity to counteract the effects of PI3K, dephosphorylating PIP₃ into PIP₂. The actions of both PI3K and PTEN work to control AKT signaling in favour of maintaining homeostatic conditions. **(B)** When PTEN is phosphorylated to become inactive, PI3K activity is unopposed, leading to increased intracellular concentrations of PIP₃. AKT is then activated at an increasing rate and for a prolonged response. Alternatively, the p110 δ PI3K subunit

is also able to inactivate PTEN (as shown by the red arrow) (95). This mechanism also results in increased AKT activation.

AKT is a key mediator of downstream metabolic effects induced by cell stimuli including growth factors and hormones. PIP₃ is able to bind the pleckstrin homology (PH) domain of AKT to activate the molecule (105). Once activated, AKT localizes to the plasma membrane where it interacts with other kinases (93, 106). Kinases phosphorylate AKT to propagate cell signals and elicit downstream effects related to cell proliferation, migration, survival, and ultimately, fibrogenesis (93, 107). Increases in AKT activity are seen with phosphorylation of Thr308 (93, 107). Rapid and more intense activation is seen when Ser473 is phosphorylated along with Thr308 (93). Activated AKT can be confirmed by the phosphorylation of its Ser473 residue. Furthermore, activated AKT (pAKT) has been shown to be involved in ECM deposition and implicated in fibrotic processes (7, 8, 39, 55).

4.2.2 Src/FAK Pathway

The Src/FAK pathway is involved in cell adhesion, migration, and survival signals, and has also been implicated in fibrogenesis (65, 66, 97, 108, 109). Specifically, FAK protein is found in focal adhesions along with other structural components that connect the cellular cytoskeleton to the ECM (97). Activation of FAK can occur through integrin signaling, as activated integrins induce FAK to autophosphorylate at Tyr397 (110, 111). Alternatively, Tyr397 phosphorylation is mediated by growth factor signaling (64, 112). Most importantly, activation of FAK is necessary for binding of Src and subsequent signal amplification (110).

Src is ubiquitously expressed in all cells and involved in FAK signaling. Activation of Src can occur through external stimuli as sensed by receptors, particularly adhesion and cytokine receptors (113). Prior to activation, dephosphorylation of Src residue Tyr527 must occur (114). Src protein is then destabilized to reveal the kinase domain, which allows for autophosphorylation at tyrosine residue 416 to increase activity. Activated Src is recruited to sites of tyrosine phosphorylation (specifically Tyr397) on activated FAK. Once activated Src binds to activated FAK,

Src is able to phosphorylate additional residues on FAK to increase signal transduction and propagation (115).

As mentioned in Chapter 1, the protein phosphatase activity of PTEN allows for regulation FAK and Src signaling. PTEN removes phosphorylation of tyrosine residues, including phosphate groups on residues that are responsible for the activation of FAK (72, 73).

Tamura and colleagues used *PTEN*-deficient glioblastoma and breast cancer cells to determine the mechanism by which PTEN regulates FAK (72). Results showed that PTEN regulates FAK phosphorylation. In control cells that lacked PTEN expression, FAK phosphorylation was maintained even after FAK was no longer actively signaling because it was attached to the ECM (72). Increased levels of PIP₃, increased PI3K activity, and phosphorylation of AKT were also found in *PTEN*-deficient cells. Expressing PTEN in these cells reversed this phenotype. The phenotype was partially restored in cells that were exogenously expressing PTEN upon overexpression of FAK (72).

Furthermore, the investigators found Tyr397 phosphorylation of FAK was necessary for intracellular association of PTEN (72). When cells were detached from the ECM, FAK was quickly dephosphorylated in cells that expressed wild-type PTEN, while phosphorylation of FAK was retained in *PTEN*-deficient cells after detachment from the matrix, suggesting that PTEN regulates FAK by dephosphorylating it (72).

Several studies have established FAK as a major mediator of fibrogenesis (65, 66, 97, 98, 108, 109). In a study of idiopathic pulmonary fibrosis (109), immunohistochemical analysis of lung tissue samples from patients showed increased FAK expression and increased staining of pFAK (Tyr925) in comparison to healthy controls (109). The study also investigated a murine model of bleomycin-induced lung fibrosis and found that pharmacological and si-RNA mediated inhibition of FAK prevented progression of fibrosis. It was further found FAK-deficient fibroblasts did not undergo myofibroblast differentiation. Overall, the

investigators concluded that FAK seems to be mediating fibrosis in lung fibroblasts and has the potential to be involved in fibrogenesis of other tissues (109). We want to investigate the role of the Src/FAK pathway and its interaction with PTEN in the TGF β -induced fibrosis of hTM cells.

4.3 Experimental Approach

1. To determine involvement of PI3K/AKT pathway in the regulation of PTEN by TGF β -mediated signaling with the use of specific PI3K isoform inhibitors LY294002 and ZSTK474. Protein expression levels will be analyzed using immunoblots. Activity of PTEN will be assessed by analyzing levels of pPTEN.
2. To determine the involvement of the Src/FAK pathway in the regulation of PTEN by TGF β -mediated signaling with the use of Src/FAK inhibitor PP2. Protein expression levels will be analyzed using immunoblots. Activity of PTEN will be assessed by analyzing levels of pPTEN.

4.4 Results

4.4.1 PI3K/AKT Pathway

4.4.1.1 TGF β 2 increases phosphorylation of AKT.

Intracellular TGF β signaling is quite complex and involves cross-talk of multiple different pathways. We wanted to identify candidate pathways through which TGF β 2 may mediate its pro-fibrotic effects. After culturing hTM cells on pronectin-coated plates using varying levels of TGF β 2 (1, 5, and 10ng/mL) we observed at least 2-fold increases in the relative expression of pAKT in comparison to control at 24 (**Figure 4.4.1**) and 48 hours (**Figure 4.4.2**). The increase in pAKT was statistically significant with TGF β 2 1ng/mL and 5ng/mL ($p<0.05$ each) and TGF β 2 10ng/mL ($p<0.01$) at 24 hours. No relative change was observed in the total level of AKT at 24 or 48 hours. The fold change in relative pAKT expression with addition TGF β 2 was much higher at 24 hours suggesting early involvement of the PI3K/AKT signaling pathway. At both 24 and 48 hours, the level of pAKT returned to near control levels upon pretreatment with SB431542, a TGF β receptor inhibitor.

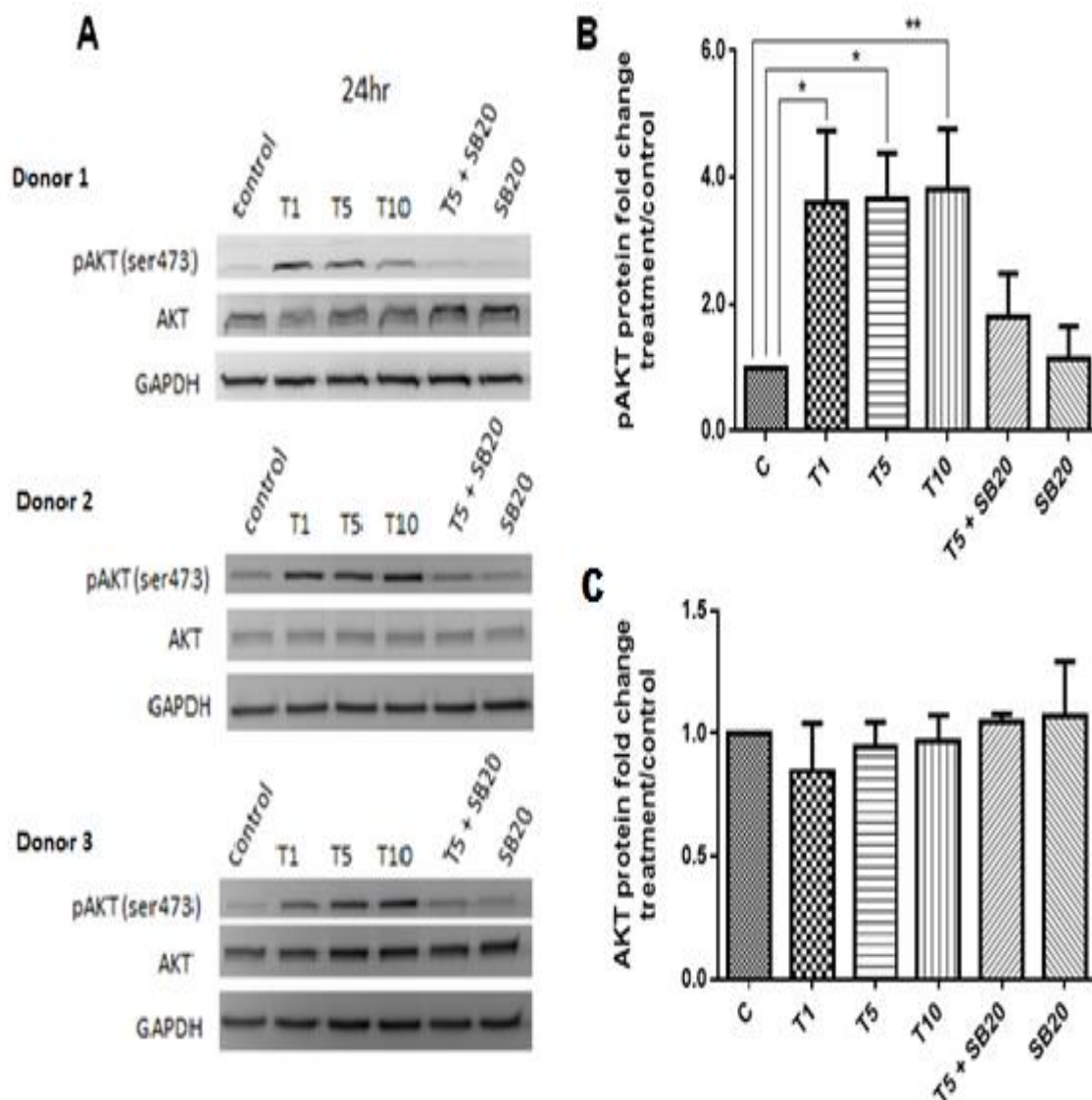


Figure 4.4.1 TGFβ2 increases phosphorylation of AKT at 24 hours.

Immunoblots from hTM cells treated with increasing concentrations of TGFβ2 (1, 5, or 10ng/mL), and TGFβ receptor inhibitor SB431542 (20μM). Protein was extracted at 24 hours (**A**). Immunoblots were probed with antibodies for pAKT (Ser473), AKT, and GAPDH. Levels of AKT protein expression did not vary drastically with addition of TGFβ2 (1, 5, or 10ng/mL). Increases in pAKT were seen with increasing amounts of TGFβ2 (1, 5, or 10ng/mL). Increases in AKT phosphorylation were prevented by pretreatment with SB431542 (20μM) prior to addition of TGFβ2 5ng/mL. Densitometry for 24 hour pAKT (**B**) and AKT (**C**). Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test (GraphPad Prism); N=3.

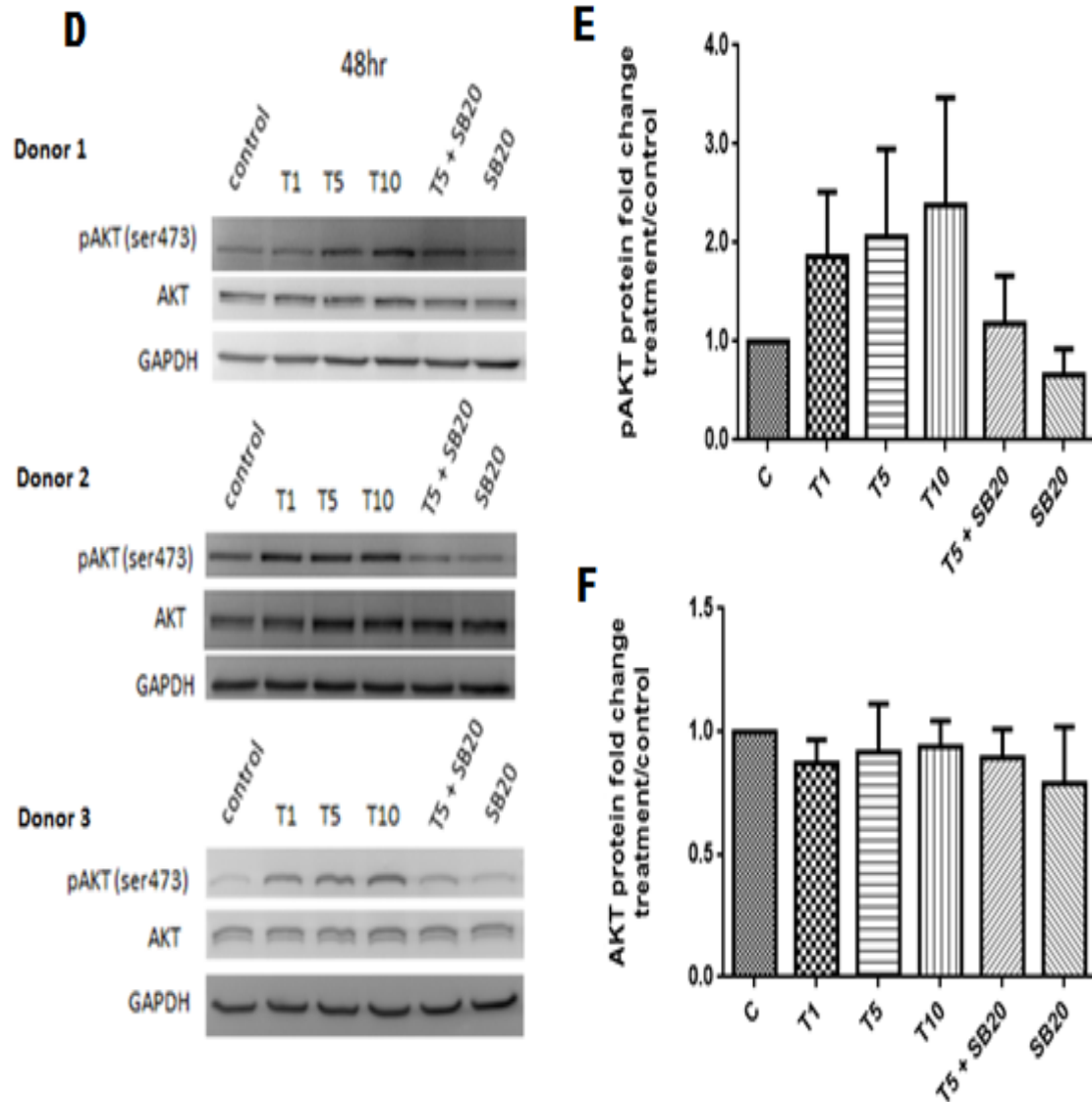


Figure 4.4.2 Expression of phosphorylated AKT 48 hours after TGF β 2 treatment.

Immunoblots from hTM cells treated with increasing concentrations of TGF β 2 (1, 5, or 10ng/mL), and TGF β receptor inhibitor SB431542 (20 μ M). Protein was extracted at 48 hours (D). Immunoblots were probed with antibodies for pAKT (Ser473), AKT, and GAPDH. Levels of AKT protein expression did not vary drastically with addition of TGF β 2 (1, 5, or 10ng/mL). Changes in pAKT (Ser473) expression were not statistically significant. Densitometry for 48 hour pAKT (E) and AKT (F). Data was analyzed using one-way ANOVA; N=3.

4.4.1.2 Inhibition of PI3K with LY294002 decreases COL1 protein expression.

To investigate downstream TGF β -signaling pathways we inhibited PI3K with LY294002, a pan-PI3K inhibitor with increased specific affinity for the PI3K $\alpha/\beta/\delta$ isoforms. hTM cells were cultured on plastic plates using TGF β 2 (1ng/mL) and LY294002 at 20 and 30 μ M. As expected, TGF β 2 significantly increased ($p<0.01$) COL1 protein expression in comparison to control (**Figure 4.4.3**). Upon addition of LY20 μ M to TGF β -treated cells, COL1 levels significantly decreased ($p<0.001$) in comparison to cells treated with TGF β 2 (1ng/mL) alone. Even greater decreases in COL1 expression were observed with higher concentrations of inhibitor ($p<0.0001$ for LY30 μ M), decreasing COL1 expression below baseline levels. TGF β 2 is able to induce increases in collagen, but these increases are prevented in the presence of a PI3K $\alpha/\beta/\delta$ isoform inhibitor suggesting that AKT signaling is involved in collagen expression.

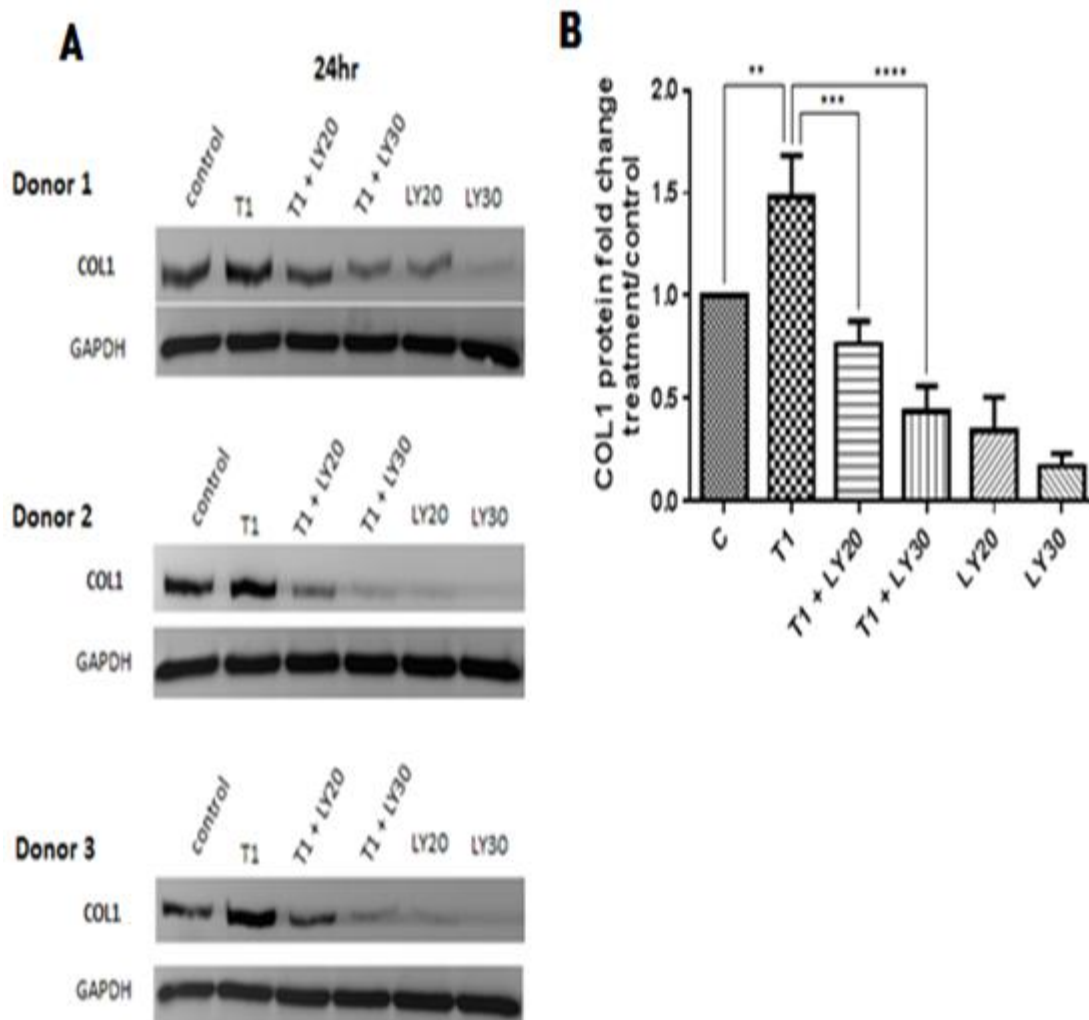


Figure 4.4.3 Inhibition of PI3K with LY294002 decreases COL1 protein expression.

Immunoblots from hTM cells treated with TGF β 2 (1ng/mL), and pan-PI3K inhibitor LY294002 (20 and 30 μ M). Protein was extracted at 24 hours (**A**). Immunoblots were probed with antibodies for COL1 and GAPDH. Levels of TGF β 2-induced COL1 protein expression were decreased following pretreatment with increasing concentrations of LY294002. Densitometry for 24 hour data (**B**). Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test (GraphPad Prism). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; N=3.

4.4.1.3 Inhibition of PI3K with LY294002 decreases PTEN protein expression and phosphorylation of PTEN.

To investigate the role of PTEN in downstream TGF β -signaling we inhibited PI3K with LY294002, a pan-PI3K inhibitor with increased specific affinity for the PI3K $\alpha/\beta/\delta$ isoforms. hTM cells were cultured on plastic plates using TGF β 2 (1ng/mL) and LY294002 at 20 and 30 μ M. Expression of both PTEN and pPTEN decreased 24 hours after treatment with a PI3K inhibitor to block PI3K/AKT signaling (**Figure 4.4.4**). Total PTEN expression significantly decreased ($p < 0.001$ for LY20 μ M and LY30 μ M) for cells pretreated before addition of TGF β 2, in comparison to cells treated with TGF β 2 alone. Phosphorylation of PTEN subsequently decreased significantly ($p < 0.05$ for LY20 μ M; $p < 0.01$ for LY30 μ M) in comparison to TGF β 2-treated cells. A comparison of the relative levels of pPTEN to total PTEN across various treatments showed no significant changes. TGF β 2 is able to induce increases in PTEN and pPTEN, but these increases are not as high in the presence of a PI3K $\alpha/\beta/\delta$ isoform inhibitor, suggesting a role for this pathway in the modulation of PTEN.

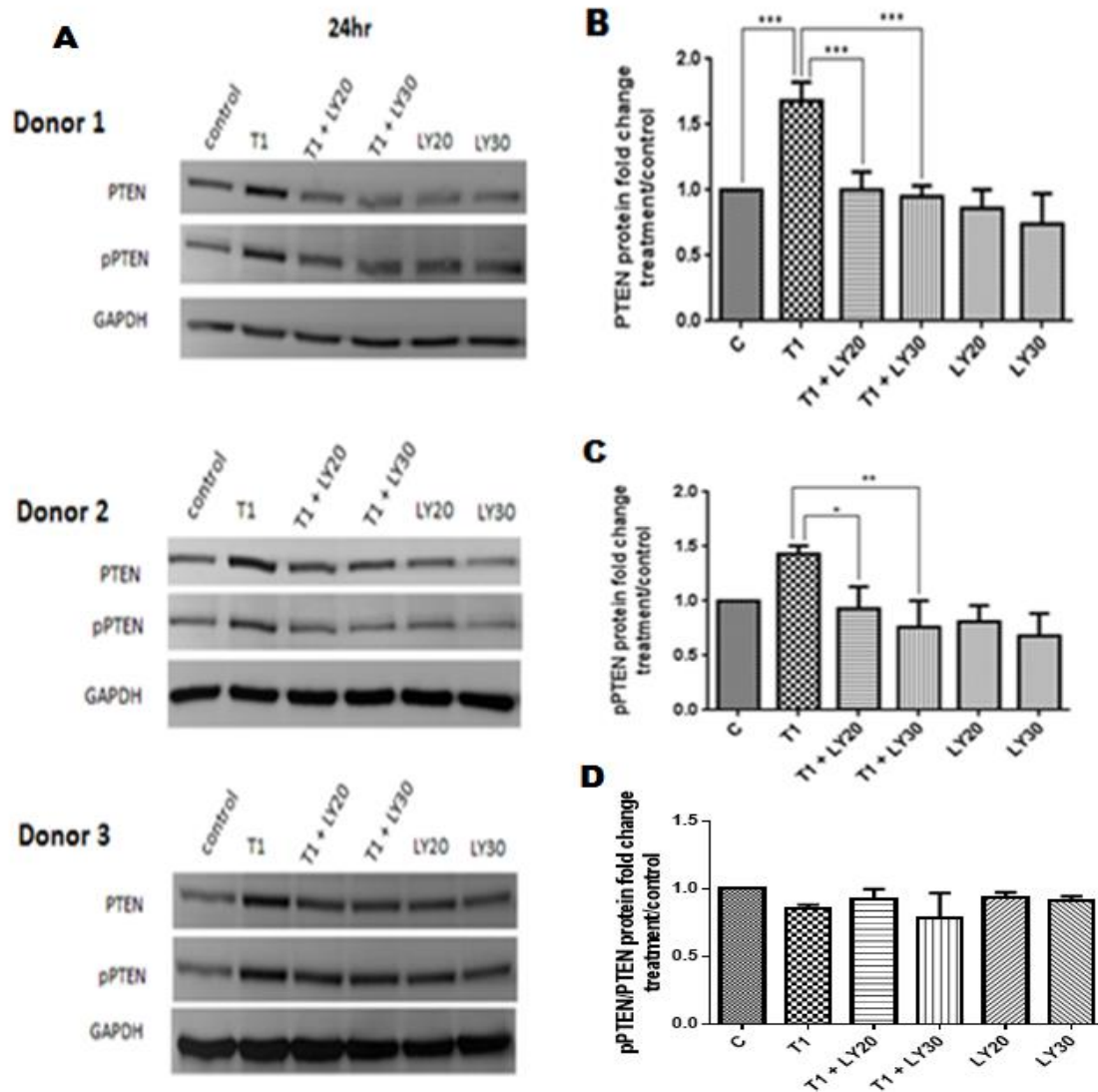


Figure 4.4.4 Inhibition of PI3K with LY294002 decreases PTEN protein expression and phosphorylation.

Immunoblots from hTM cells treated with TGF β 2 (1ng/mL), and pan-PI3K inhibitor LY294002 (20 and 30 μ M). Protein was extracted at 24 hours (**A**). Immunoblots were probed with antibodies for PTEN, pPTEN, and GAPDH. Levels of PTEN protein expression were decreased following pretreatment with increasing concentrations of LY294002. Decreases in pPTEN were also observed. Densitometry for 24 hour PTEN (**B**), pPTEN (**C**), and relative pPTEN/PTEN expression (**D**). Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test (GraphPad Prism). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; N=3.

4.4.1.4 Phosphorylation of AKT after inhibition of PI3K with LY294002.

To confirm that inhibition of PI3K prevents signaling through AKT, we probed for levels of pAKT 24 hours after treatment with TGF β 2 and with inhibitors. hTM cells cultured on plastic plates using TGF β 2 (1ng/mL) and pan-PI3K inhibitor (LY294002 at 20 and 30 μ M) show changes in pAKT (Ser473) at 24 hours in comparison to cells treated with TGF β 2 (1ng/mL) alone (**Figure 4.4.5**). All cells treated with LY294002 at either concentration have barely detectable levels of pAKT. pAKT expression after treatment with PI3K inhibitor decreased below expression levels seen in control cells. Immunoblot analysis is consistent with the results presented in **Figure 4.4.8**, supporting the hypothesis that TGF β signaling to induce COL1 expression requires PI3K activation of AKT. The absence of pAKT suggests inhibition of PI3K $\alpha/\beta/\delta$ isoforms successfully blocks AKT signaling for collagen expression.

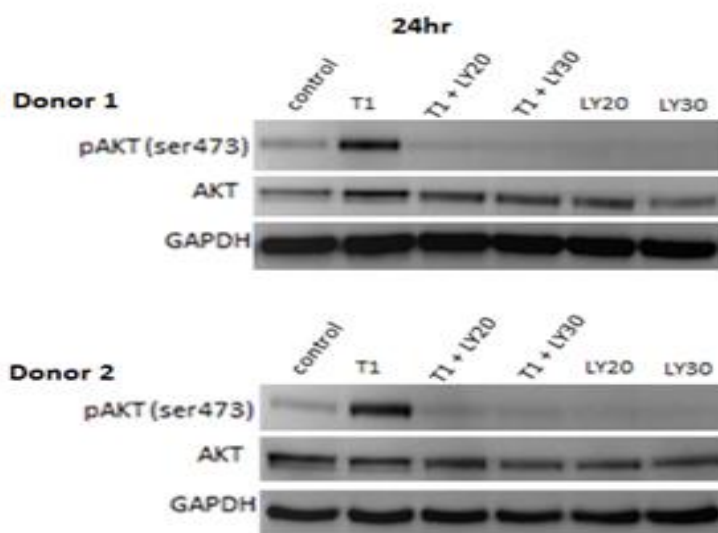


Figure 4.4.5 Phosphorylation of AKT after inhibition of PI3K with LY294002.

Immunoblots from hTM cells treated with TGF β 2 (1ng/mL), and pan-PI3K inhibitor LY294002 (20 and 30 μ M). Protein was extracted at 24 hours. Immunoblots were probed with antibodies for pAKT (Ser473), AKT, and GAPDH. Levels of AKT protein expression did not change with addition of PI3K inhibitors. Changes to expression of pAKT seen 24 hours after treatment with TGF β 2 and LY294002 are consistent with the hypothesis that COL1 expression induced by TGF β 2 requires activation of the PI3K/AKT pathway.

4.4.1.5 Inhibition of PI3K with ZSTK474 decreases COL1 protein expression.

To further confirm the role of PI3K signaling in PTEN-mediated collagen expression, we inhibited PI3K signaling in TGF β 2 treated hTM cells using another inhibitor- ZSTK474. hTM cells were cultured on plastic plates using TGF β 2 (1ng/mL) and ZSTK474 at 2 and 5 μ M. Although TGF β 2 treatment increased COL1 expression at 24 hours, pretreatment with increasing concentrations of ZSTK474 rescued this effect in a dose-dependent manner (**Figure 4.4.6**). Cells pretreated with ZSTK474 exhibited significantly decreased levels of COL1 ($p < 0.01$ for Z2, and $p < 0.001$ for Z5 μ M) in comparison to cells treated with TGF β 2 alone. Cells treated with inhibitor alone also exhibited decreases in COL1 expression below baseline levels. TGF β 2 is able to induce increases in collagen, but these increases are prevented in the presence of a PI3K inhibitor suggesting that signaling through AKT is involved in collagen expression.

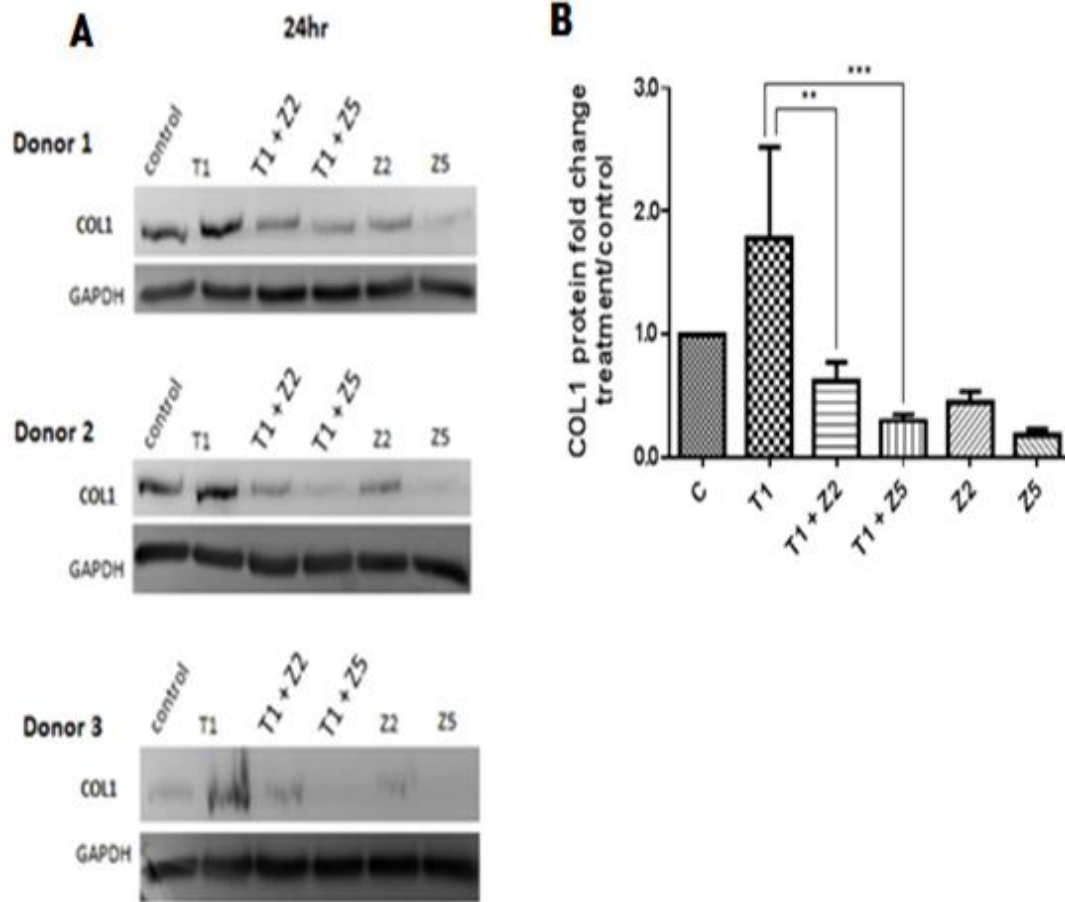


Figure 4.4.6 Inhibition of PI3K with ZSTK474 decreases COL1 protein expression.

Immunoblots from hTM cells treated with TGF β 2 (1ng/mL), and PI3K inhibitor ZSTK474 (2 and 5 μ M). Protein was extracted at 24 hours (**A**). Immunoblots were probed with antibodies for COL1 and GAPDH. Levels of TGF β 2-induced COL1 protein expression were decreased following pretreatment with increasing concentrations of ZSTK474. Densitometry for 24 hour COL1 (**B**). Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test (GraphPad Prism). **p<0.01, ***p<0.001; N=3.

4.4.1.6 Inhibition of PI3K with ZSTK474 decreases PTEN protein expression and phosphorylation of PTEN.

Next, we wanted to further investigate the role of PTEN in downstream TGF β -signaling when PI3K activity was inhibited by specific inhibitor ZSTK474. Cultures of hTM cells on plastic plates using TGF β 2 (1ng/mL) and ZSTK474 at 2 and 5 μ M show significant decreases ($p < 0.001$ for Z2 μ M; $p < 0.0001$ for Z5 μ M) in PTEN protein levels in comparison to cells treated with TGF β 2 alone (**Figure 4.4.7**). TGF β 2 significantly increased levels of pPTEN ($p < 0.05$). As seen with the PI3K $\alpha/\beta/\delta$ isoform inhibitor, pretreatment with the higher inhibitor concentration (Z5 μ M) prior to addition of TGF β 2 was sufficient to significantly decrease ($p < 0.01$) pPTEN in comparison to TGF β 2-treated cells. Overall, expression of both PTEN and pPTEN levels decreased upon pretreatment with PI3K inhibitors to block PI3K/AKT signaling. There were no significant changes to the relative ratio of pPTEN to total PTEN across various treatments. Although TGF β 2 is able to induce increases in PTEN and pPTEN, this response is dampened in the presence of a PI3K δ inhibitor suggesting a role for this pathway in the modulation of PTEN.

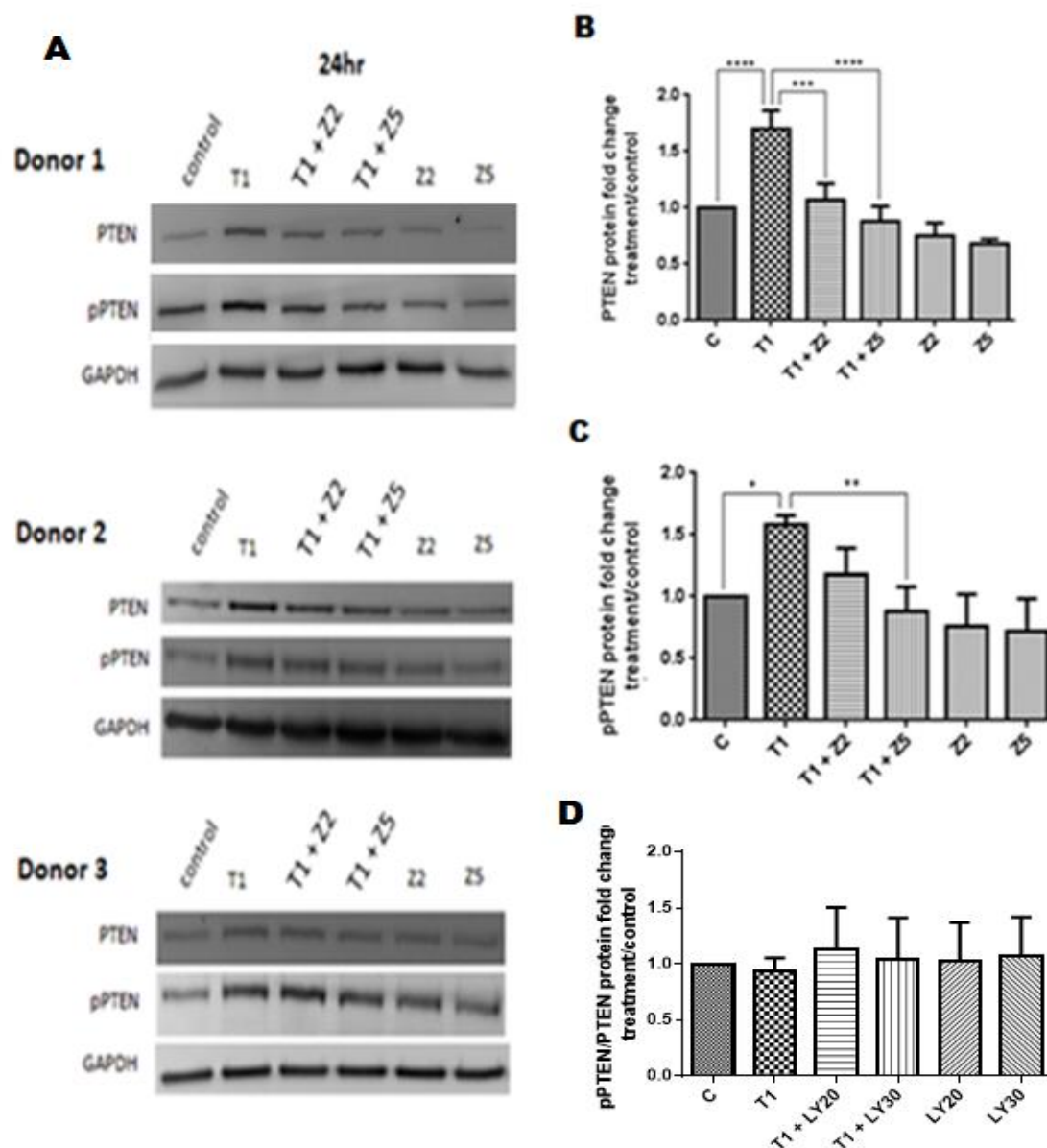


Figure 4.4.7 Inhibition of PI3K with ZSTK474 decreases PTEN protein expression and phosphorylation.

Immunoblots from hTM cells treated with TGF β 2 (1ng/mL), and PI3K inhibitor ZSTK474 (2 and 5 μ M). Protein was extracted at 24 hours (**A**). Immunoblots were probed with antibodies for PTEN, pPTEN, and GAPDH. Levels of PTEN protein expression were decreased following pretreatment with increasing concentrations of ZSTK474. Decreases in pPTEN were also observed. Densitometry for 24 hour PTEN (**B**), pPTEN (**C**), and relative pPTEN/PTEN (**D**) expression. Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test (GraphPad Prism). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; N=3.

4.4.1.7 Inhibition of PI3K with ZSTK474 decreases phosphorylation of AKT.

To further confirm that inhibition of PI3K prevents signaling through AKT, we checked for levels of pAKT 24 hours after treatment with TGF β 2 and inhibitors. hTM cells cultured on plastic plates using TGF β 2 (1ng/mL) and specific PI3K inhibitors (ZSTK474 at 2 and 5 μ M). All cells treated with ZSTK474 express very low levels of pAKT; even in comparison to control cells (**Figure 4.4.8**). Cells treated with ZSTK474 show decreases in pAKT (Ser473) at 24 hours ($p < 0.01$) in comparison to cells treated with TGF β 2 (1ng/mL) alone. Changes to expression of pAKT seen 24 hours after treatment with TGF β 2 and ZSTK474 are similar to the changes seen 24 hours after inhibition LY294002. The absence of pAKT suggests that inhibition of PI3K successfully blocks AKT signaling for collagen expression.

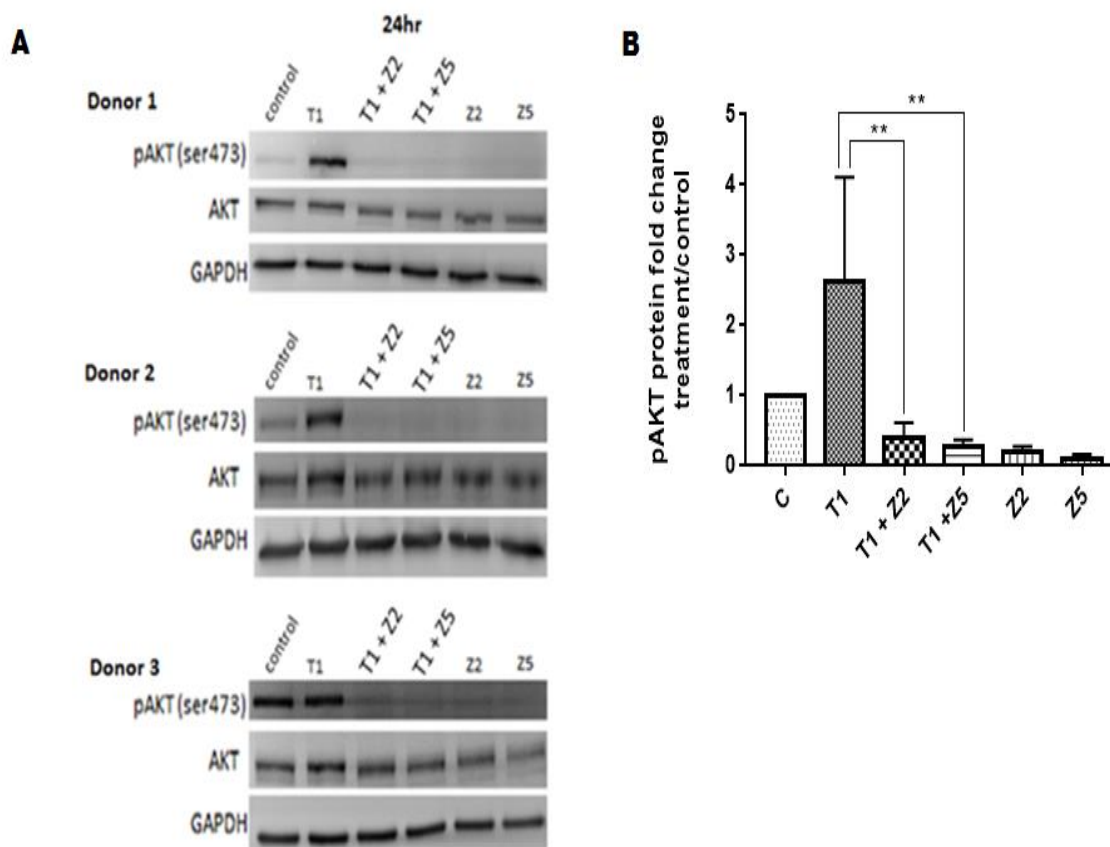


Figure 4.4.8 Inhibition of PI3K with ZSTK474 decreases phosphorylation of AKT.

Immunoblots from hTM cells treated with TGF β 2 (1ng/mL), and PI3K inhibitor ZSTK474 (2 and 5 μ M). Protein was extracted at 24 hours. Immunoblots were probed with antibodies for pAKT (Ser473), AKT, and GAPDH. Levels of AKT protein expression did not change with addition of PI3K inhibitors. Levels of pAKT expression were decreased following pretreatment with increasing concentrations of ZSTK474.

4.4.2 Src/FAK

4.4.2.1 Phosphorylation of FAK after TGF β 2 treatment.

After culturing hTM cells on pronectin-coated plates using varying levels of TGF β 2 (1, 5, and 10ng/mL) and TGF β receptor inhibitor SB431542 (20 μ M) we investigated involvement of the Src/FAK pathway. While visual analysis of the immunoblots at 24 (**Figure 4.4.9**) and 48 hours (**Figure 4.4.10**) seemed to indicate that pFAK (Tyr397) was increased with TGF β 2 treatment, subsequent densitometric and statistical analysis determined the changes to be non-significant.

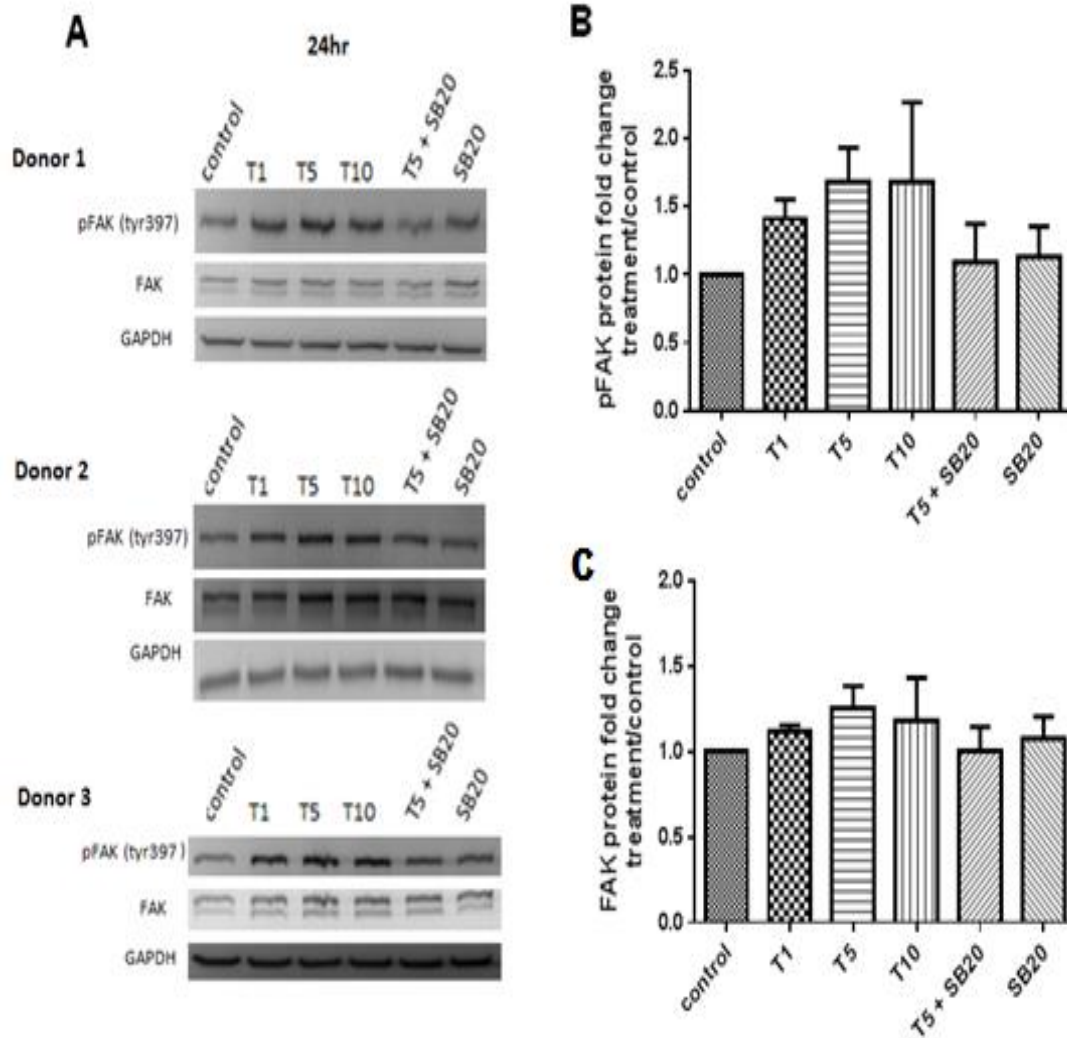


Figure 4.4.9 Phosphorylation of FAK 24 hours after TGFβ2 treatment.

Immunoblots from hTM cells treated with increasing concentrations of TGFβ2 (1, 5, or 10ng/mL), and TGFβ receptor inhibitor SB431542 (20μM). Protein was extracted at 24 hours (A). Immunoblots were probed with antibodies for pFAK (Tyr397), FAK, and GAPDH. Levels of FAK protein expression did not vary drastically with addition of TGFβ2 (1, 5, or 10ng/mL). Changes in pFAK with increasing amounts of TGFβ2 (1, 5, or 10ng/mL) were not determined to be statistically significant. Densitometry for 24 hour pFAK (B) and FAK (C). Data was analyzed using one-way ANOVA; N=3.

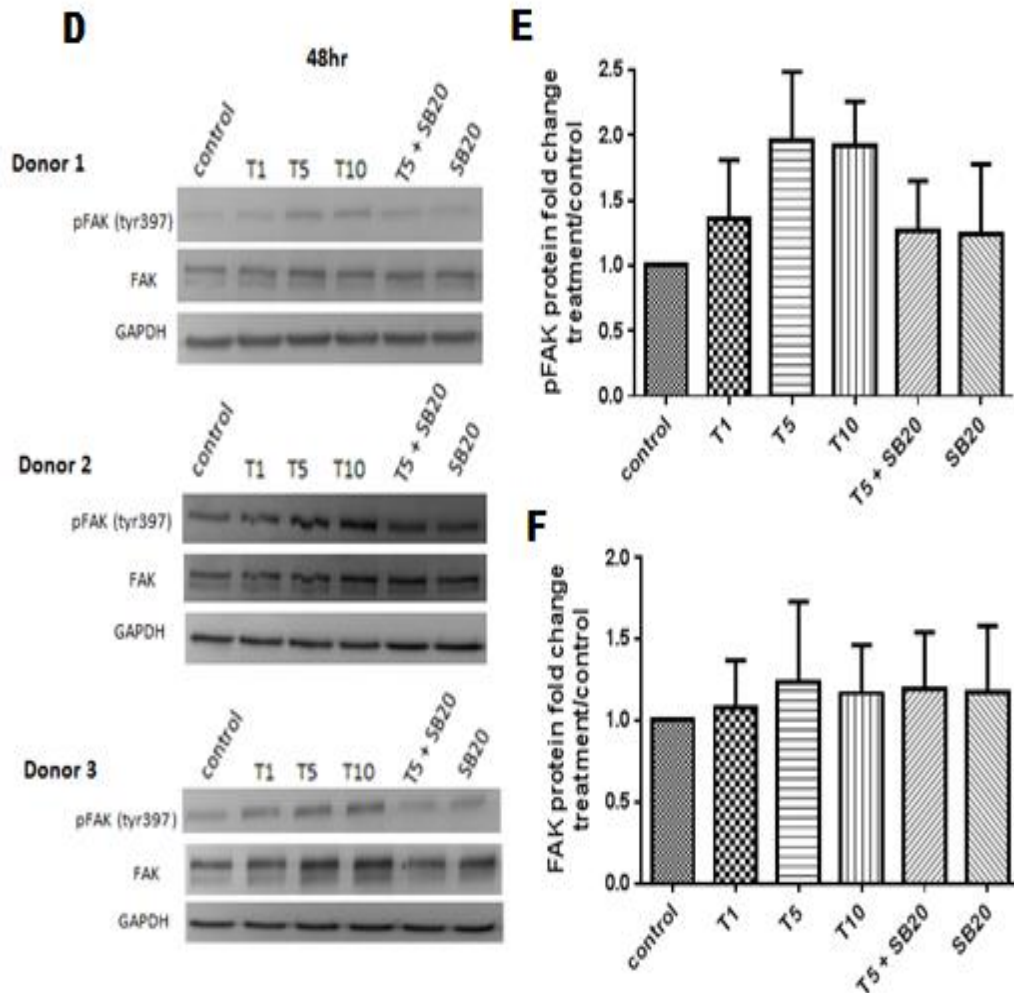


Figure 4.4.10 Phosphorylation of FAK 48 hours after TGFβ2 treatment.

Immunoblots from hTM cells treated with increasing concentrations of TGFβ2 (1, 5, or 10ng/mL), and TGFβ receptor inhibitor SB431542 (20μM). Protein was extracted at 48 hours (**D**). Immunoblots were probed with antibodies for pFAK (Tyr397), FAK, and GAPDH. Levels of FAK protein expression did not vary drastically with addition of TGFβ2 (1, 5, or 10ng/mL). Changes in pFAK with increasing amounts of TGFβ2 (1, 5, or 10ng/mL) were not determined to be statistically significant. Densitometry for 48 hour pFAK (**E**) and FAK (**F**). Data was analyzed using one-way ANOVA; N=3.

4.4.2.2 Inhibition of Src/FAK decreases COL1 protein expression.

To investigate the involvement of the Src/FAK pathway in TGF β -mediated collagen expression, we employed the Src inhibitor PP2. hTM cells were cultured on plastic plates using TGF β 2 (1ng/mL) and PP2 at 10 and 20 μ M. As expected, TGF β 2 increased COL1 protein levels significantly ($p < 0.01$) in comparison to control (**Figure 4.4.11**). Pretreatment with increasing concentrations of PP2 before TGF β 2-treatment resulted in significantly decreased ($p < 0.01$ for P10, and $p < 0.001$ for P20) COL1 expression in comparison to cells treated with TGF β 2 alone. Cells treated with inhibitor alone expressed less COL1 than control cells. Although TGF β 2 is able to induce increases in collagen, these increases are prevented by a Src/FAK inhibitor, suggesting that signaling through FAK may be involved in collagen production.

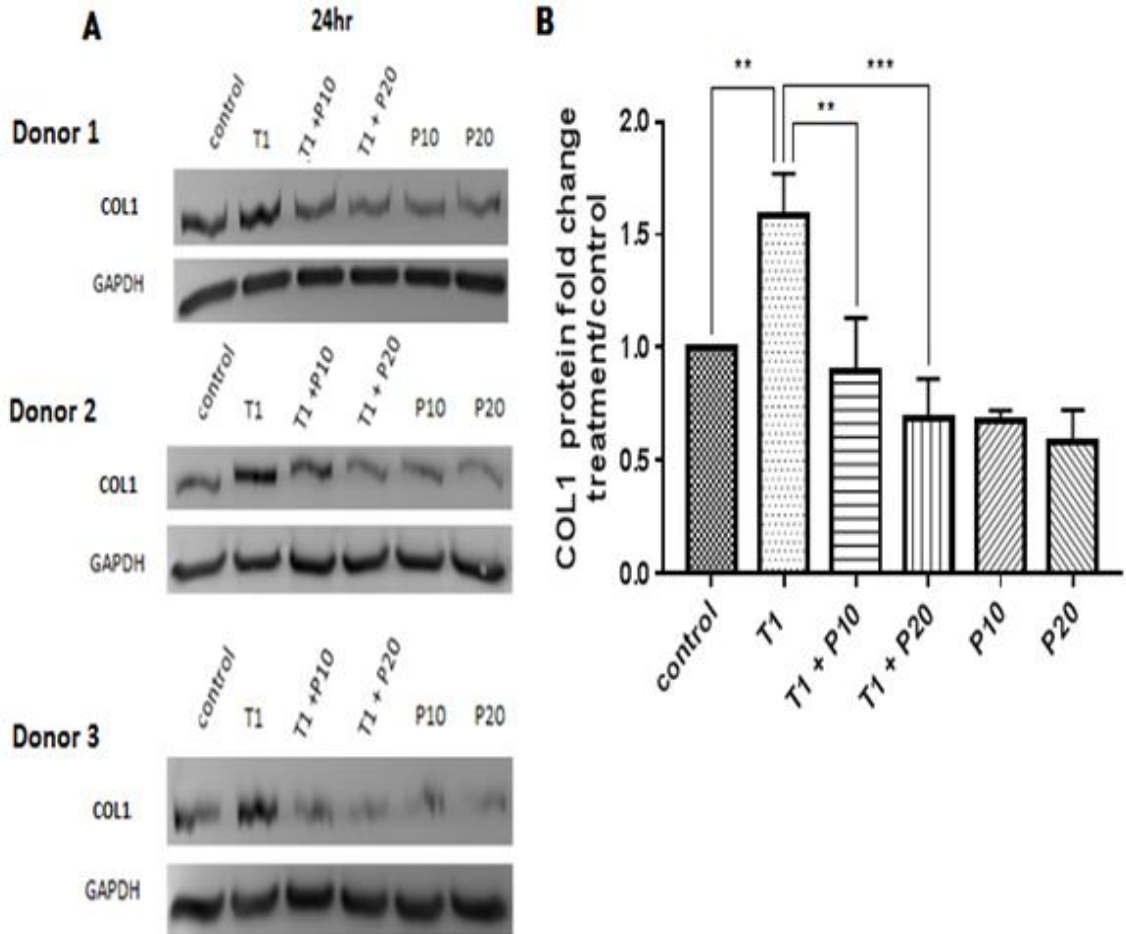


Figure 4.4.11 Inhibition of Src/FAK decreases COL1 protein expression.

Immunoblots from hTM cells treated with TGF β 2 (1ng/mL), and Src/FAK inhibitor PP2 (10 and 20 μ M). Protein was extracted at 24 hours (**A**). Immunoblots were probed with antibodies for COL1 and GAPDH. Levels of TGF β 2-induced COL1 protein expression were decreased following pretreatment with increasing concentrations of Src/FAK inhibitor. Densitometry for 24 hour data of hTM cells treated with Src/FAK inhibitor (**B**). Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test (GraphPad Prism). ** $p < 0.01$, *** $p < 0.001$; N=3.

4.4.2.3 Inhibition of Src/FAK decreases PTEN protein expression and phosphorylation of PTEN.

We wanted to investigate whether inhibition of the Src/FAK pathway leads to changes in PTEN. hTM cells were culture on plastic plates using TGF β 2 (1ng/mL) and Src/FAK inhibitor (PP2 at 10 and 20 μ M). Upon treatment with TGF β 2 we have a significant increase ($p < 0.05$) in PTEN, followed by a significant increase ($p < 0.01$) in pPTEN (**Figure 4.4.12**). Pretreatment with 10 μ M PP2 before TGF β 2 treatment significantly decreased PTEN and pPTEN ($p < 0.05$ for both) expression in comparison to TGF β 2-treated cells. This effect counteracting TGF β 2-induced increases in PTEN and pPTEN in comparison to TGF β 2-treated cells is stronger at the higher inhibitor concentration, as TGF β 2 with PP2 20 μ M decreased PTEN and pPTEN protein levels roughly 0.35-fold more that TGF β 2 and PP2 10 μ M treatment. Similar to experiments with the PI3K inhibitors, the ratio of pPTEN to total PTEN was not significantly different across various treatments. Again, since TGF β 2 is able to induce increases in pPTEN and PTEN, which can be prevented in the presence of a Src/FAK inhibitor, PTEN may be signaling through FAK to induce collagen expression.

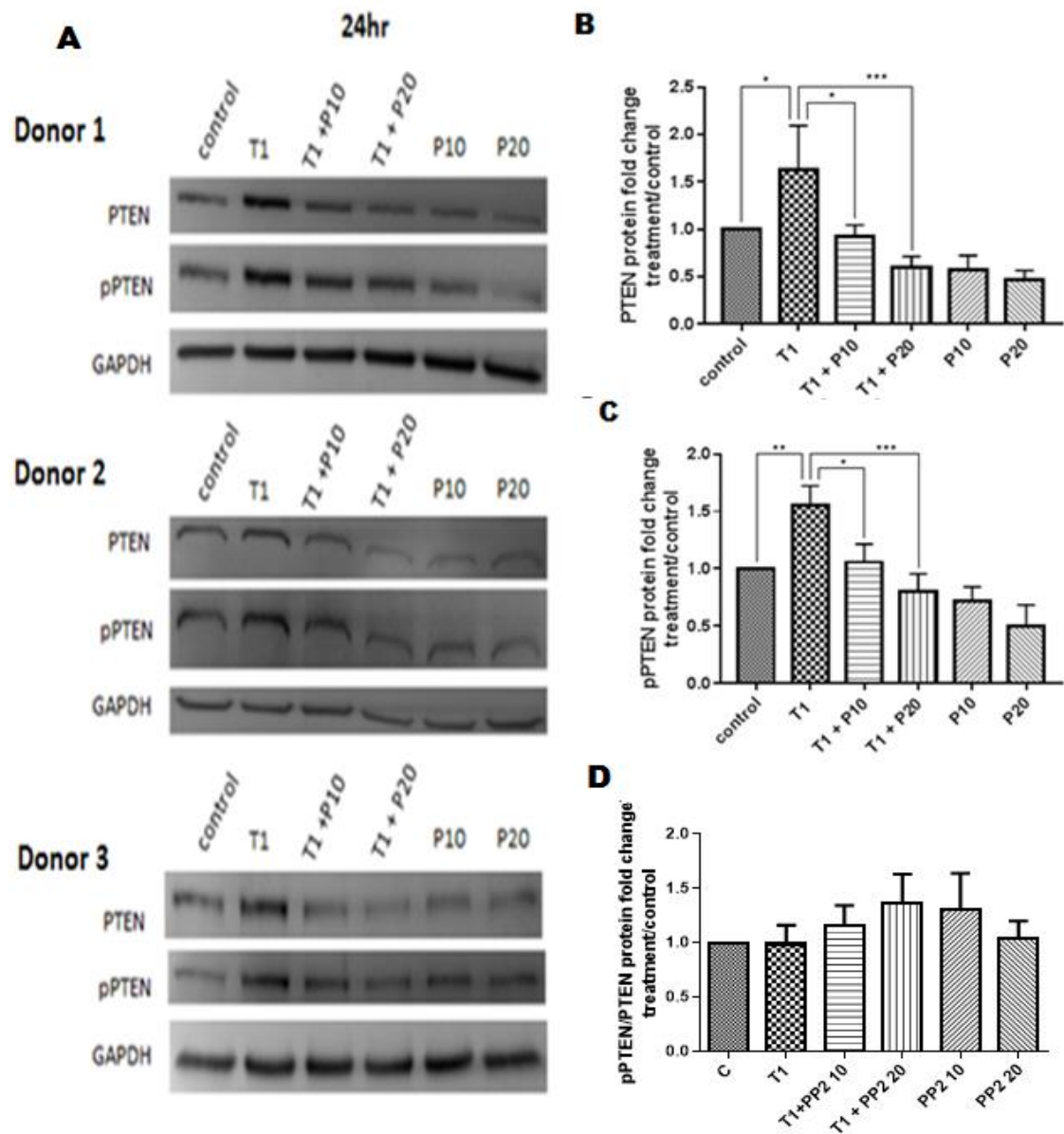


Figure 4.4.12 Inhibition of Src/FAK decreases PTEN protein expression and phosphorylation.

Immunoblots from hTM cells treated with TGF β 2 (1ng/mL), and Src/FAK inhibitor PP2 (10 and 20 μ M). Protein was extracted at 24 hours (**A**). Immunoblots were probed with antibodies for PTEN, pPTEN, and GAPDH. Levels of PTEN protein expression were decreased following pretreatment with increasing concentrations of Src/FAK inhibitor. Decreases in pPTEN were also observed. Densitometry for 24 hour PTEN (**B**), pPTEN (**C**), and relative pPTEN/PTEN (**D**) expression. Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test (GraphPad Prism). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; N=3.

4.4.2.4 Inhibition of Src/FAK shows variable changes in phosphorylation of FAK.

To investigate changes occurring in the Src/FAK pathway as a result of inhibition we cultured hTM cells on plastic plates using TGF β 2 (1ng/mL) and Src/FAK inhibitor (PP2 at 10 and 20 μ M). Consistent with the results presented in **Figure 4.4.9** we do not show significant increases in pFAK with TGF β 2 treatment after 24 hours. We also fail to see consistent changes in pFAK 24 hours after treatment with a Src inhibitor (**Figure 4.4.13**). Interestingly, some donors seem to decrease activation of the Src/FAK pathway after treatment with Src inhibitor PP2, while others seem to be insensitive to it, continuing to express pFAK.

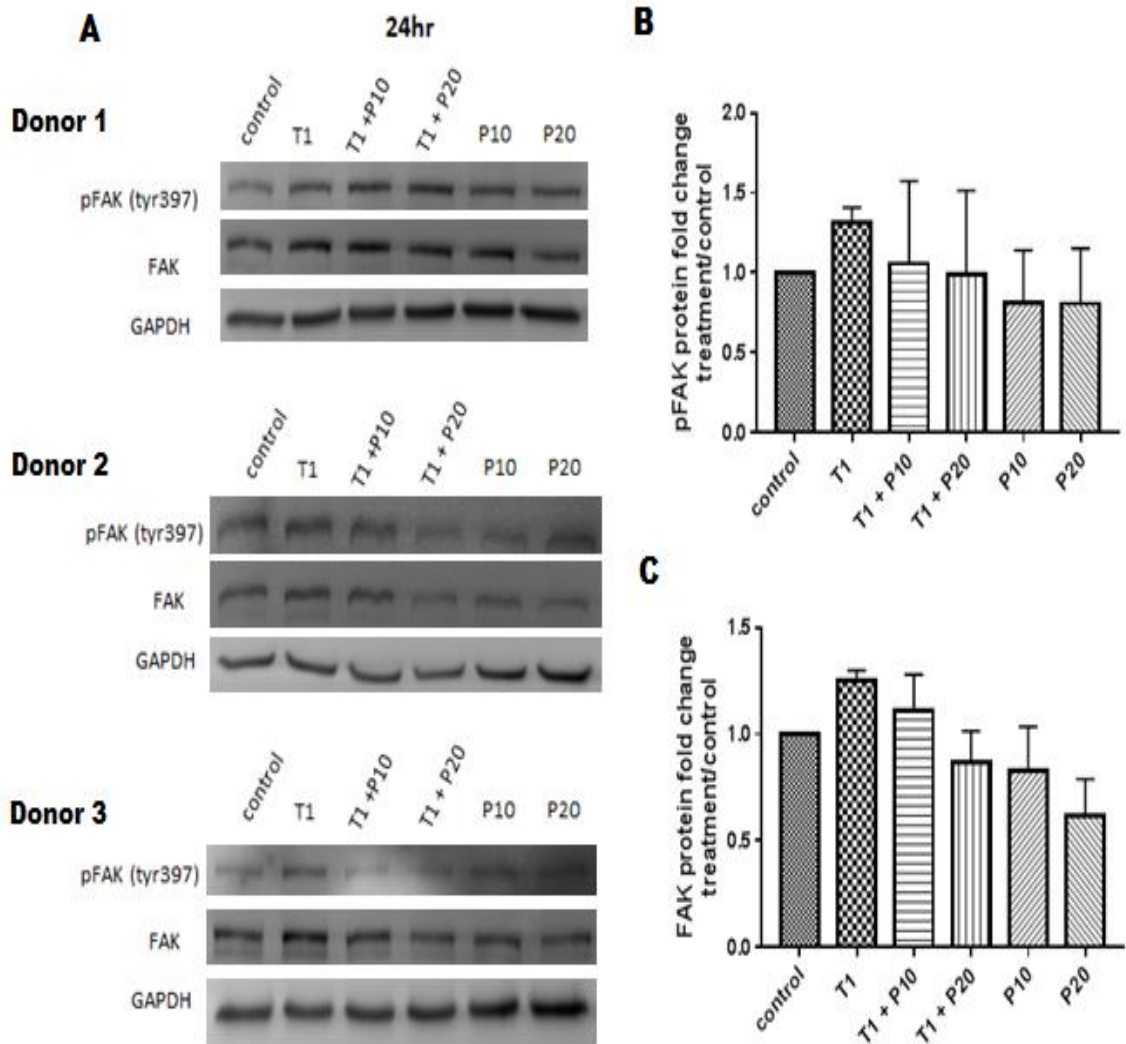


Figure 4.4.13 Inhibition of Src/FAK shows no consistent change in phosphorylation of FAK at 24 hours.

Immunoblots from hTM cells treated with TGF β 2 (1ng/mL), and Src/FAK inhibitor PP2 (10 and 20 μ M). Protein was extracted at 24 hours (**A**). Immunoblots were probed with antibodies for pFAK (Tyr397), FAK, and GAPDH. Levels of FAK protein expression remained relatively constant regardless of treatment applied. Changes in FAK phosphorylation (Tyr397) were not statistically significant. Densitometry for 24 hour pFAK (**B**) and FAK (**C**). Data was analyzed using one-way ANOVA (GraphPad Prism). N=3.

4.4.3 Summary of Results

1. TGF β 2 increased pAKT protein expression.
2. Inhibition of PI3K decreased COL1 expression.
3. Inhibition of PI3K decreased pPTEN expression.
4. Inhibition of Src decreased COL1 expression.
5. Inhibition of Src decreased pPTEN expression.

4.5 Discussion

Here we show that TGF β 2 utilizes a non-canonical signaling pathway involving PI3K/AKT to induce COL1 expression. Additionally, our preliminary investigations also show involvement of the Src/FAK pathway.

Treatment of hTM cells with TGF β 2 increased Ser473 phosphorylation of AKT, indicating activation of the AKT pathway. We find that activation of AKT (**Appendix C**) and phosphorylation of PTEN (**Appendix D**) occur at early time points under TGF β 2 stimulation, and after 24 hours the increased pAKT and pPTEN expression is seen concomitant to increases in COL1 expression. This further supports the role of PTEN phosphorylation in the inactivation of PTEN allowing for increased signaling through AKT and increased COL1 expression. This is consistent with other reports that AKT activation contributes to a fibrotic phenotype (7, 8, 39, 55).

Similar results were reported in *Pten*-deficient dermal fibroblasts, which showed increased pAKT expression along with increased expression of COL1 (7). COL1 expression decreased after *Pten*-deficient fibroblasts were treated with PI3K inhibitors LY294002 and wortmannin (an irreversible PI3K inhibitor), supporting the PI3K/AKT-dependent mechanism of fibrogenesis (7). In our study, we also found PI3K inhibitors LY294002 and ZSTK474 decreased COL1 expression induced by TGF β 2. The decrease in COL1 was accompanied by decreases in pPTEN and

pAKT, suggesting increased PTEN activity prevented activation of the AKT pathway. In fact, we show that inhibition of PI3K nearly ablates all expression of phosphorylated AKT. Decreased pAKT expression is also seen in our experiments with the TGF β R inhibitor SB431542, denoting that the effect is TGF β -specific activation of the PI3K/AKT pathway.

One interesting result is the decreased expression of total PTEN protein, which is observed along with decreased expression of pPTEN. Despite observing significant differences in levels of pPTEN and PTEN between treatments, the relative level of pPTEN to PTEN was not significantly different. This could occur for a number of different reasons that may require further investigation. The most likely explanation considers the stability and activity of PTEN, such that increased rates of enzymatic activity cause PTEN to become less stable and more quickly degraded (74, 85, 116). The active, non-phosphorylated, PTEN is subject to quick degradation and not measured by our methods. Examination of PTEN ubiquitination and degradation would further support this study. Maintaining a high turnover rate of active PTEN may serve as an internal control mechanism, preventing excess activation of a potent molecule that could promote apoptosis upon nuclear translocation (74).

Another noteworthy finding from our inhibitor experiments concerns the decreased expression of protein relative to our controls. Treatment with PI3K and Src/FAK inhibitors alone showed that expression of COL1, pAKT, and even PTEN and pPTEN in some cases, were expressed below baseline levels. The effect most likely could be attributed to blocking the action of endogenous TGF β 2. Endogenous TGF β expression is discussed in detail in **section 3.5**. Alternatively, this response may be due to non-specific effects of inhibitors. Since there is a great degree of cross-talk between the pathways that regulate ECM protein expression (63, 64, 85), and despite testing various concentrations of inhibitors to obtain the optimal dose, it is possible that the inhibitors alone may be affecting more than one target pathway to decrease protein expression levels below baseline.

Regarding the Src inhibition experiments, we failed to see consistent changes in pFAK expression. This may be due to a number of reasons. First, FAK could have been dephosphorylated at an early time point by PTEN activation upon Src inhibition and we have only tested FAK phosphorylation at 24 hours. Second, Src is known to phosphorylate FAK on an additional five tyrosine residues: Tyr407 in the amino terminal portion, Tyr576 and Tyr577 within the catalytic domain (phosphorylation of which is required for maximal enzymatic activity of FAK) (117), and Tyr861 and Tyr925 at the carboxyl terminal of FAK (118, 119). We have only investigated phosphorylation of FAK at Tyr397. Other phosphorylation sites have to be investigated. Finally, reports of additional FAK residues that can be phosphorylated under different conditions provide an alternate explanation for our results. While Tyr397 phosphorylation is indicative of activated FAK, the phosphorylation occurs specifically when FAK is clustered by integrins (96). When FAK is dissociated from focal adhesions, phosphorylation occurs at Tyr925 (96). It is possible that while FAK cycles through association with focal adhesions, another tyrosine residue was phosphorylated, most likely Tyr925.

Moreover, it has been suggested that FAK is further able to activate and signal through the PI3K/AKT pathway to promote cell migration and changes associated with the cytoskeleton and extracellular matrix (63). A study in fibroblasts examining the interaction between FAK and PI3K determined that the two molecules can associate *in vitro* (63). The same investigators found this association was mediated by the p85 regulatory subunit of PI3K and intracellular association was increased upon autophosphorylation of FAK at Tyr397 (63). Conclusions from the study suggest PI3K acts as an effector of FAK signaling.

Taking these points into consideration, it is possible that FAK and PI3K in hTM cells interact in a manner similar as proposed in **Figure 4.5.1**. Cellular association and signaling of PI3K and FAK could augment signaling through AKT. By blocking Src activity with inhibitor PP2 in our experiments, the drastic decrease in COL1 protein levels that we observed may be due to FAK's inability to bind PI3K and propagate signals through AKT. Furthermore, since active PTEN directly

antagonizes the action of PI3K, it may mitigate profibrotic signals coming from growth factor receptors, as well as RTKs and integrins (also shown to activate FAK (111, 115)) that signal through a combined PI3K and FAK pathway. Of course, the intermediary events required for TGF β to stimulate RTKs in hTM cells are not defined and further experiments are required to investigate whether this method of signaling occurs in hTM cells. This is further supported by the fact that Tamura and colleagues found FAK and the p85 subunit of PI3K to coimmunoprecipitate from *PTEN*-deficient cells. PI3K and FAK were not found to coimmunoprecipitate in cells expressing wild-type PTEN protein suggesting FAK and PI3K interact in the absence of PTEN activity (72). pAKT was also found to be down regulated in PTEN-expressing cells via a mechanism that may involve the dissociation of PI3K and FAK.

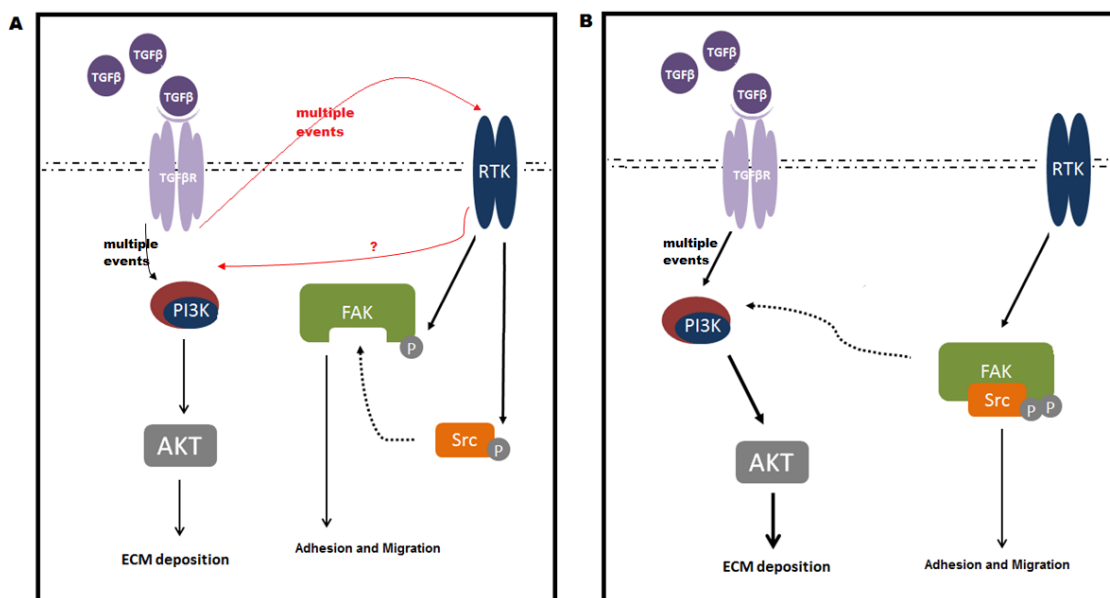


Figure 4.5.1. Schematic representation of proposed TGF β activation of Src/FAK and PI3K recruitment.

(A). Signals from TGF β binding to the TGF β R have been shown to activate receptor tyrosine kinase (RTK) activity through multiple intermediary events. Activation of RTKs can induce autophosphorylation of FAK at Tyr 397, as well as autophosphorylation of Src at Tyr 416 (64). RTKs may also be able to activate PI3K. **(B).** The Src/FAK complex has also been reported to associate with the p85 regulatory subunit of PI3K (shown here in dark red), such that PI3K acts as an effector of FAK signaling (63).

Furthermore, recent evidence indicates that FAK is able to regulate PTEN by phosphorylating tyrosine residue 336 to increase phosphatase activity, protein-lipid interaction, and overall stability of PTEN (120). Previous reports had suggested that PTEN Tyr336 was targeted only by Src (70). The reciprocal regulation of FAK and PTEN warrants further investigation, especially in models, such as TM cells, where there is a role for both of these pathways in continuous ECM remodeling.

The study indicates that TGF β 2 probably regulates PTEN through multiple signaling pathways. Interesting future directions could involve investigating the effects of other kinases on PTEN stability and activity and alternate conditions under which phosphorylation occurs. There have been reports that post-translation modification of GSK3 may be involved as well (70). Fundamentally, identification of pathways involved in the TGF β -PTEN signaling axis are crucial to enhancing understanding of this important biological process.

Chapter 5

5 The functional role of PTEN in the ECM of the TM

5.1 Rationale

As mentioned in Chapters 1 and 3, the role of PTEN has expanded beyond that of a tumor suppressor to now include the regulation of fibrosis. Investigations in multiple different models, including the liver, lungs, and skin, have shown that deletion of the *PTEN* gene, or inhibition of PTEN activity can induce excess ECM deposition to cause fibrosis (7, 8, 39, 121).

In Chapter 4, we investigated signaling pathways involved in the regulation of PTEN as part of the TGF β -PTEN axis. We found major involvement of the PI3K/AKT pathway as well as some involvement of the Src/FAK pathway. Now, with greater mechanistic understanding of PTEN signaling, we explore the effects of altered PTEN activity on COL1 expression. Specifically, we aim to determine whether increasing PTEN activity is able to mitigate the effects of TGF β 2 on COL1 expression.

5.2 Background

Loss-of-function mutations are common in many cancers (90-92). When PTEN is unable to maintain enzymatic activity to function as a tumor suppressor, growth and proliferation can proceed without restriction. To this effect, decreases in PTEN protein expression and indirectly PTEN activity, have been associated with fibrotic conditions (7, 8, 39, 121).

A study of hepatic fibrogenesis in rats, determined that hepatic stellate cells expressed decreasing levels of *Pten* mRNA and PTEN protein as *in vivo* hepatic fibrosis progressed secondary to biliary ductal ligation (121). Immunohistochemical analysis of liver tissue sections revealed increasing amounts of connective tissue disrupting normal hepatic architecture to be associated with decreasing PTEN protein expression levels (121). The

investigators concluded that external profibrotic stimuli were able to decrease PTEN expression levels in hepatic stellate cells, ultimately contributing to the progression of fibrosis in the liver (121).

In their 2006 study of idiopathic pulmonary fibrosis, White and colleagues correlated significant loss of PTEN expression to a myofibroblastic phenotype in human lung biopsy samples (39). A similar phenotype was confirmed in fibroblasts of *Pten*-deficient mice. Pharmacological inhibition of Pten in wild-type fibroblasts showed increased fibrotic phenotype, as characterized by α -smooth muscle actin expression and activation of AKT signaling in these cells. Overexpression of Pten in *Pten*-deficient fibroblasts rescued the fibroblast phenotype and reversed profibrotic changes (39). The investigation was clearly able to show that loss of PTEN, whether through decreased expression or decreased activity, was able to promote differentiation towards a fibrotic phenotype.

Most recently, PTEN protein expression was shown to be decreased in the dermal fibroblasts of patients with skin fibrosis in comparison to healthy controls (7). Dermal fibrosis was observed in *Pten*-deficient mice, which averaged a 1.5 fold increase in dermal thickness when compared to wild-type mice. The increased thickness of the skin was associated with significantly higher tissue expression of collagen and pAKT. Inhibition of the PI3K/AKT pathway decreased COL1 expression in *Pten*-deficient mice, as did overexpression of PTEN (7). This study provided evidence that PTEN is required to maintain normal ECM deposition and loss of PTEN causes fibrosis. A subsequent report stated that loss of PTEN in lung fibroblasts resulted in a similar fibrotic phenotype characterized by excess collagen production and increased phosphorylation of AKT (8).

PTEN is involved in regulation of the cell cycle and, by extension, growth and production of cellular products. Here we directly inhibit PTEN with a pharmacological inhibitor to determine the effects in hTM cells.

To manipulate PTEN activity by an alternate method we obtained vectors of mutated *PTEN* DNA from Dr. M. Iijima. Her lab has been successful in

characterizing some of the actions of PTEN with the involvement of various point mutations. Specifically, we employed *PTEN* vector *C124S*, designed with a mutation in the catalytic site replacing the cysteine residue at position 124, with a serine residue (122, 123). This mutation disrupts substrate binding such that the C124S PTEN mutant is unable to recognize and bind to PIP₂ so the mature mutant protein is functionally inactive (122). Dr. Iijima and colleagues have also been successful in engineering an enhanced-active version of PTEN (*ePTEN*). This particular vector contains mutations in the catalytic and C2 domains to increase localization and binding to the plasma membrane, as well as mutations in the C-terminus to prevent phosphorylation, specifically at Ser380 (123, 124). By preventing phosphorylation of Ser380, additional phosphorylation of the tail region (particularly Thr382/383) is prevented, and PTEN cannot become functionally inactivated by this method. *ePTEN* exhibits increased PIP₃ phosphatase activity making the synthetic PTEN enzyme 8-fold more active than wildtype PTEN (123, 125). In our study we investigate genetic mutants of *PTEN* to determine the effect of decreased and increased PTEN activity on the ECM of hTM cells.

5.3 Experimental Approach

1. To determine the role of PTEN in excess COL1 expression of hTM cells by inhibiting PTEN with a pharmacological inhibitor. Protein expression levels will be analyzed using immunoblots. Activity of PTEN will be assessed by analyzing levels of pPTEN.
2. To investigate the effect of alterations in PTEN activity on TGFβ₂-induced expression of COL1 by transfecting cells with mutated *PTEN* DNA. Protein expression levels will be analyzed using immunoblots. Activity of PTEN will be assessed by analyzing levels of pPTEN.

5.4 Results

5.4.1 Expression of COL1 protein after inhibition of PTEN.

To determine the functional role of PTEN in ECM remodeling we cultured hTM cells on plastic plates using the PTEN inhibitor VO-OHpic (concentrations from 0.25 μ M up to 5 μ M). Although visual representations of the images show differences in COL1 expression between the control and the highest dose of inhibitor (VO-OHpic 5 μ M) for each donor, when pooled together the results are not significantly different (**Figure 5.4.1**). The lack of statistical significance when reporting the COL1 protein fold increase can be attributed to variability in donor response to different concentrations of the compound. The variability in baseline collagen expression between different donors is another contributing factor. Overall, there is a nearly five-fold average increase in COL1 expression between controls and hTM cells treated with 5 μ M VO-OHpic.

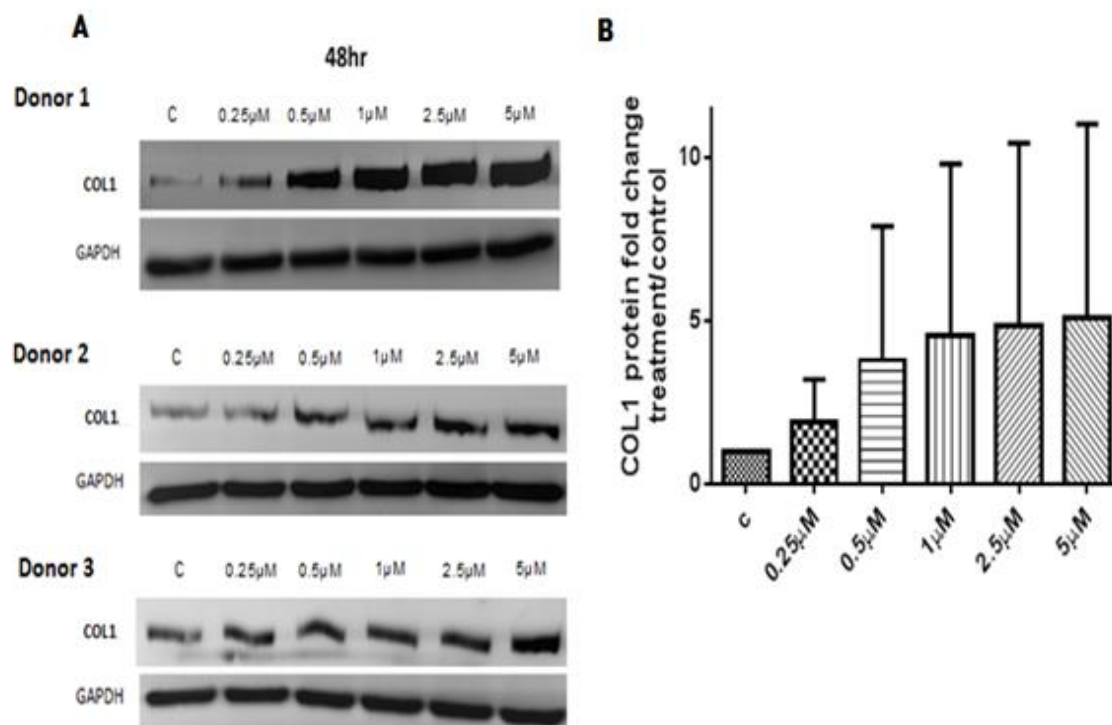


Figure 5.4.1 Expression of COL1 protein after inhibition of PTEN.

Immunoblots from hTM cells treated with increasing concentrations of PTEN inhibitor VO-OHpic (0.25 μ M - 5 μ M). Protein was extracted at 48 hours (**A**). Immunoblots were probed with antibodies for COL1 and GAPDH. Densitometry for 48 hour data (**B**). Data was analyzed using one-way ANOVA; N=3.

5.4.2 Expression of enhanced-active PTEN prevents increased COL1 protein expression.

To investigate the effects of increased PTEN activity on TGF β 2-induced COL1 expression we transfected cells with an enhanced-active *PTEN* (*ePTEN*; or eV in **Figure 5.4.2**) expressing vector. The *ePTEN* vector codes for a mutated version of PTEN that is easily able to localize to the plasma membrane and exhibits an eight-fold increase in enzymatic activity. We also used a vector for the expression of a mutant form of *PTEN* (*C124SV*) that was inactive, corroborating results from **section 5.4.1**. Addition of TGF β 2 to cells transfected with control vectors showed a significant increase ($p < 0.01$) in COL1 expression, as expected. We found that hTM cells transfected with *ePTEN* and then treated with a pathological dose of TGF β 2 (5ng/mL) exhibited a significantly decreased level of COL1 ($p < 0.01$) in comparison to TGF β 2-treated controls. Moreover, transfection with *C124SV* prior to TGF β 2 treatment caused cells to express significantly more COL1 than TGF β 2-treated controls ($p < 0.05$), as well as control-vector only cells ($p < 0.0001$).

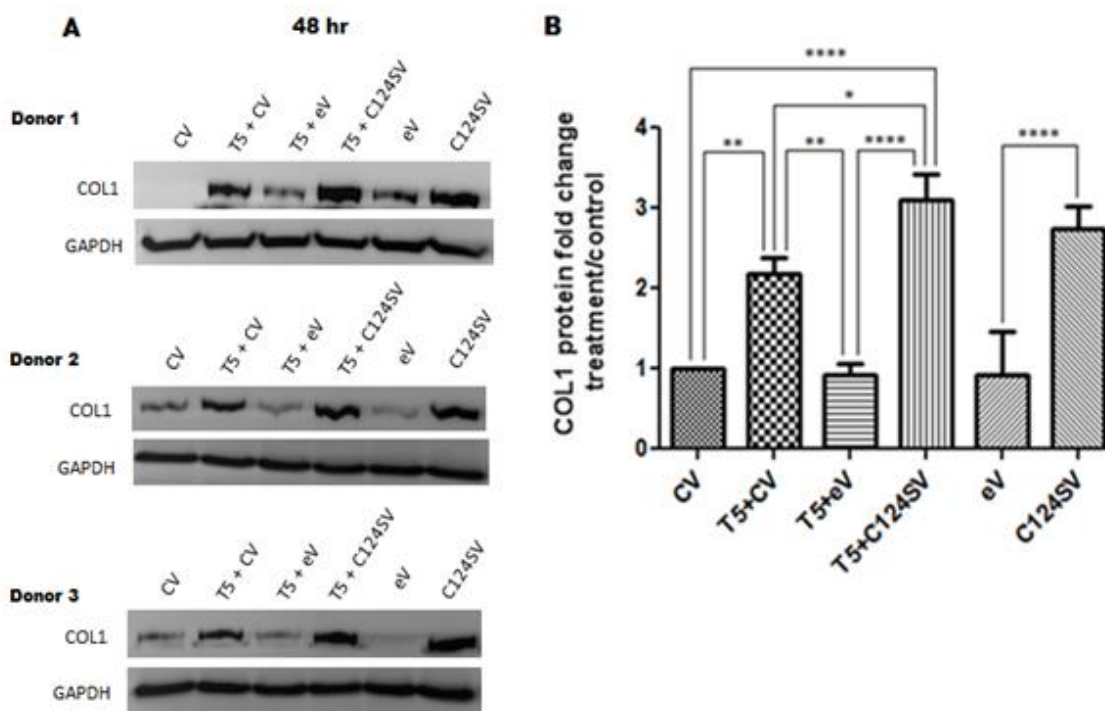


Figure 5.4.2 Expression of enhanced-active PTEN prevents increased COL1 protein expression.

Immunoblots from hTM cells transfected with 1.0 μ g DNA (either pcDNA3.1 + IRES GFP vector (control vector CV), ePTEN vector (eV), or C124S PTEN vector (C124SV) then treated with TGF β 2 (5ng/mL). Protein was extracted at 48 hours (**A**). Immunoblots were probed with antibodies for COL1 and GAPDH. Cells transfected with control vectors exhibited an increase in COL1 expression following addition of TGF β 2. Cells transfected with ePTEN vector were observed to have decreased COL1 expression. Increases in COL1 protein expression were observed in cells transfected with C124S vector. Densitometry for 48 hour data (**B**) was performed using ImageJ software. Protein expression levels were normalized to GAPDH. The relative fold change in protein expression levels was determined by comparing the normalized value to the normalized control for each donor. Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test (GraphPad Prism). * p <0.05, ** p <0.01, **** p <0.0001; N=3.

5.4.3 Expression of enhanced-active PTEN decreases phosphorylation of PTEN.

To confirm efficient transfection and investigate changes in post-translational markers of enzyme activity we checked expression levels of PTEN and pPTEN of transfected cells. Cells transfected with *ePTEN* did not have significantly higher levels of total PTEN when compared to cells transfected with control vector alone (**Figure 5.4.3**). As expected, TGF β 2 treatment increased PTEN expression levels ($p < 0.01$) in cells transfected with control vector. Transfection with *C124SV* prior to TGF β 2-treatment also caused significantly increased ($p < 0.01$) COL1 expression in comparison to control-vector only cells. No significant difference was observed in COL1 protein between TGF β 2-treated control and *C124SV* vector-only cells. Finally, *ePTEN* transfected cells treated with TGF β 2 expressed significantly less ($p < 0.05$) COL1 than TGF β 2-treated control and *C124SV* cells.

Changes in pPTEN expression followed a similar trend. Not surprisingly, *C124SV* transfected cells had significantly higher ($p < 0.01$) levels of pPTEN compared to *ePTEN* transfected cells in the absence of TGF β 2. Transfection with *ePTEN* prior to TGF β 2-treatment was sufficient to significantly decrease pPTEN ($p < 0.05$ and $p < 0.001$) in comparison to TGF β 2-treated control and *C124SV* cells, respectively. The ratio of pPTEN to total PTEN remained relatively unchanged across various treatments, most likely for reasons discussed in **section 4.5**. These findings further suggest that PTEN expression and activity are not directly correlated in TM cells.

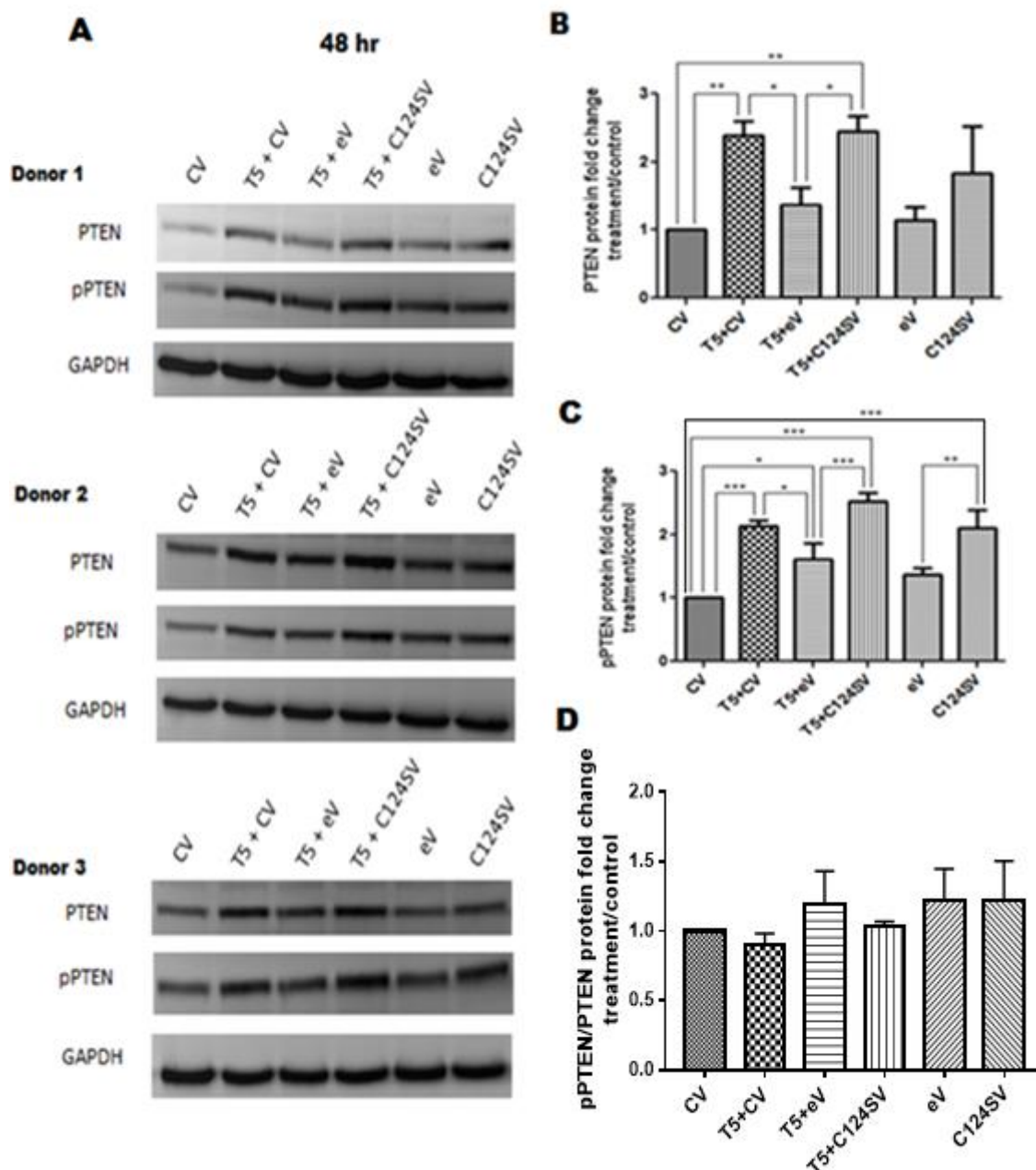


Figure 5.4.3 Expression of enhanced-active PTEN decreases TGF β 2-induced phosphorylation of PTEN.

Immunoblots from hTM cells transfected with 1.0 μ g DNA (either pcDNA3.1 + IRES GFP vector, ePTEN vector, or C124S PTEN vector) then treated with TGF β 2 (5ng/mL). Protein was extracted at 48 hours (**A**). Immunoblots were probed with antibodies for PTEN, pPTEN (Ser380/Thr382/383), and GAPDH. Cells transfected with ePTEN vector were observed to have decreased phosphorylation of PTEN (Ser380/Thr382/383). Increases in PTEN phosphorylation were observed in cells transfected with C124S vector. Densitometry for 48 hour data (**B**, **C**, **D**) was

performed using ImageJ software. Protein expression levels were normalized to GAPDH. The relative fold change in protein expression levels was determined by comparing the normalized value to the normalized control for each donor. Data was analyzed using one-way ANOVA. The source of statistical significance was determined by applying Tukey's *post-hoc* test (GraphPad Prism). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$, **** $p < 0.0001$; $N=3$.

5.4.4 Summary of Results

1. Pharmacological inhibition of PTEN with VO-OHPic shows a trend towards increased COL1 expression.
2. Expression of enhanced-active PTEN decreased COL1 expression.
3. Expression of PTEN with defective lipid phosphatase activity (C124S mutant) increased COL1 expression.

5.5 Discussion

Pharmacological inhibition of PTEN has been reported to increase COL1 deposition in the literature (39). Our findings did not show a statistically significant change in COL1 with different concentrations of VO-OHPic due to differences in individual donor response. Each donor showed a clear increase of COL1 with 5 μM VO-OHPic treatment with an average five-fold increase ($N=3$) compared to controls. Although increased COL1 expression is biologically significant, statistical significance could be achieved with additional donor samples to increase statistical power with a larger sample size.

Furthermore, hTM cells were not treated with exogenous TGF β 2 in order to examine the effect of PTEN inhibition on baseline COL1 expression. Since cultured TM cells have been shown to secrete endogenous TGF β (58) and are capable of responding to secreted TGF β in an autocrine manner (61), it is possible that differences in baseline endogenous TGF β activity for each donor resulted in variable COL1 expression. Endogenous TGF β activity is presented in more detail in the discussions for **Chapters 3** and **4**.

Finally, various models of induced *PTEN*-deficiency discovered increased accumulation of ECM proteins, and further noted increased expression of pAKT subsequent to loss of PTEN expression/activity (7, 8, 39). In fact, increased AKT phosphorylation was also seen in a macrophage model after administration of the same inhibitor used in our experiments, VO-OHpic, suggesting a mechanism by which the increased COL1 is seen as a result of increased PI3K signaling through AKT (94).

Next, we have successfully shown that the lipid phosphatase activity of PTEN is crucial to its role as a negative regulator of COL1 expression. Loss of lipid phosphatase activity as demonstrated by cells expressing the C124S PTEN mutant caused greatly increased expression of COL1. Transfection of C124S PTEN alone increased COL1 expression in comparison to controls. Since inactivation of PTEN is known to prevent the action of SMAD2/3 phosphatase (126), increased COL1 expression in C124S PTEN transfected cells might be due to increased SMAD signaling. Also it is to be noted that phosphorylation of PTEN is increased in C124S transfected cells. Increased phosphorylation (Ser380/Thr382/383) of C124S PTEN has also been reported in models of gastric epithelial cells (127) and pyramidal neurons (128). The mechanisms involved in the phosphorylation of the C124S mutant PTEN under normal conditions are not well-defined (127, 128), warranting further investigation.

Collectively, the results suggest that PTEN activity is required to maintain normal ECM remodeling, and that dysfunctional PTEN activity can have similar effects to pro-fibrotic factors present in the extracellular environment, such as TGF β 2 present in the AH, to increase fibrosis. Likewise, maintenance of normal PTEN expression and activity is required to prevent the development of cancer. PTEN-deficiencies have been associated with carcinogenesis (90-92), but it has recently been shown that as little as a 20% decrease in *PTEN* gene expression is sufficient to cause cancer, especially in breast and lymphoid tissue (129, 130). The specificity of tumor localization described in this study, and the previously

unreported response of PTEN to TGF β 2 seen in our hTM cell experiments warrant further investigation of tissue-specific PTEN expression and regulation.

Finally, with these experiments we have established that PTEN is highly regulated by post-translational modification of its C-terminus region and this is a predominant mechanism in hTM cells. Overall, an increase in PTEN activity, about eight-fold in this case, can have substantial downstream effects to prevent COL1 expression. hTM cells transfected with the *ePTEN* vector, harboring mutations to prevent inactivation of PTEN and increase its overall activity, significantly decreased TGF β 2-induced COL1 expression. The most likely mechanism by which this occurs, is decreased AKT signaling due to increased PTEN activity, as noted in other models that used *PTEN* overexpression or PI3K inhibitors to increase PTEN's relative activity (7, 39). Since PTEN is able to translocate to the nucleus in some cases and initiate apoptosis (70, 74), it is possible that increasing PTEN activity may cause apoptosis in some TM cells. During the course of our experiments, we were able to observe increased cell-death after transfection with the *ePTEN* vector. Although pharmacological activators of PTEN could avoid this problem by increasing its activity only minimally and still be useful in decreasing ECM production.

Chapter 6

6 Conclusions

6.1 Summary of Results

In Chapter 3 we set out to characterize the effects of TGF β 2 on COL1 and PTEN protein in cultured hTM cells. We found that TGF β 2 is able to increase *COL1A1* mRNA and COL1 protein expression, inducing a fibrotic phenotype. We further discovered that TGF β 2 induced increases in *PTEN* mRNA and PTEN protein, contrary to reports in the literature. Subsequent investigation of posttranslational modification of PTEN showed that TGF β 2 is able to phosphorylate PTEN to inactivate it in cultured hTM cells.

In Chapter 4, we investigated the signaling pathways involved with TGF β 2-induced fibrosis in hTM cells and found involvement of the PI3K/AKT and the Src/FAK pathways. Inhibition of PI3K decreased TGF β 2-induced collagen expression as well as levels of inactive pPTEN. Inhibition of Src had a similar effect, decreasing TGF β 2-induced collagen expression and decreasing expression of pPTEN. PI3K inhibition also decreased activation of AKT.

Finally, in Chapter 5, we set out to determine the functional role of PTEN in the COL1 expression of hTM cells. We showed that expression of a phosphatase activity-defective PTEN mutant increased collagen expression in hTM cells substantially. Alternatively, increasing PTEN activity via expression of an enhanced-active PTEN mutant was able to decrease TGF β 2-induced collagen expression. A summary of our study is presented in **Figure 6.1.1**.

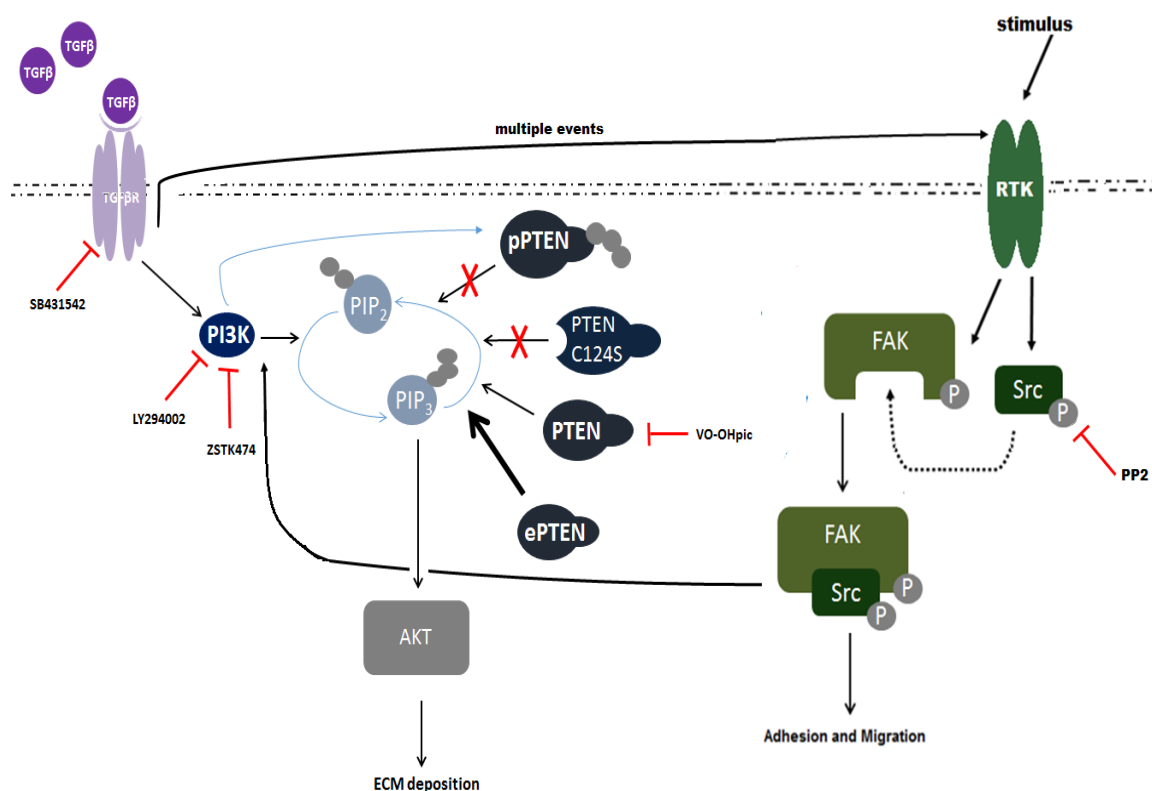


Figure 6.1.1. Summary of Experimental Study.

TGFβ signaling in hTM cells was investigated to determine the role of PTEN in hTM cell fibrosis. Pharmacological inhibition of key signaling molecules in a variety of pathways, including TGFβRI, PI3K, Src, and PTEN itself, were used. Inhibition of the TGFβ signal, via TGFβRI, and PI3K blockade, lead to decreased pPTEN expression and decreased COL1 expression. Similar results were observed with Src inhibition. Altered PTEN activity as investigated via transfection of PTEN mutants resulted in changes to COL1 expression, such that increased PTEN activity decreased COL1 expression and decreased PTEN activity increased COL1 expression.

Overall, we found that PTEN is involved in the pathways regulating ECM deposition in the hTM cells. Our findings contribute to the current understanding of TGFβ and fibrotic signaling pathways in hTM cells as demonstrated in **Figure 6.1.2.**

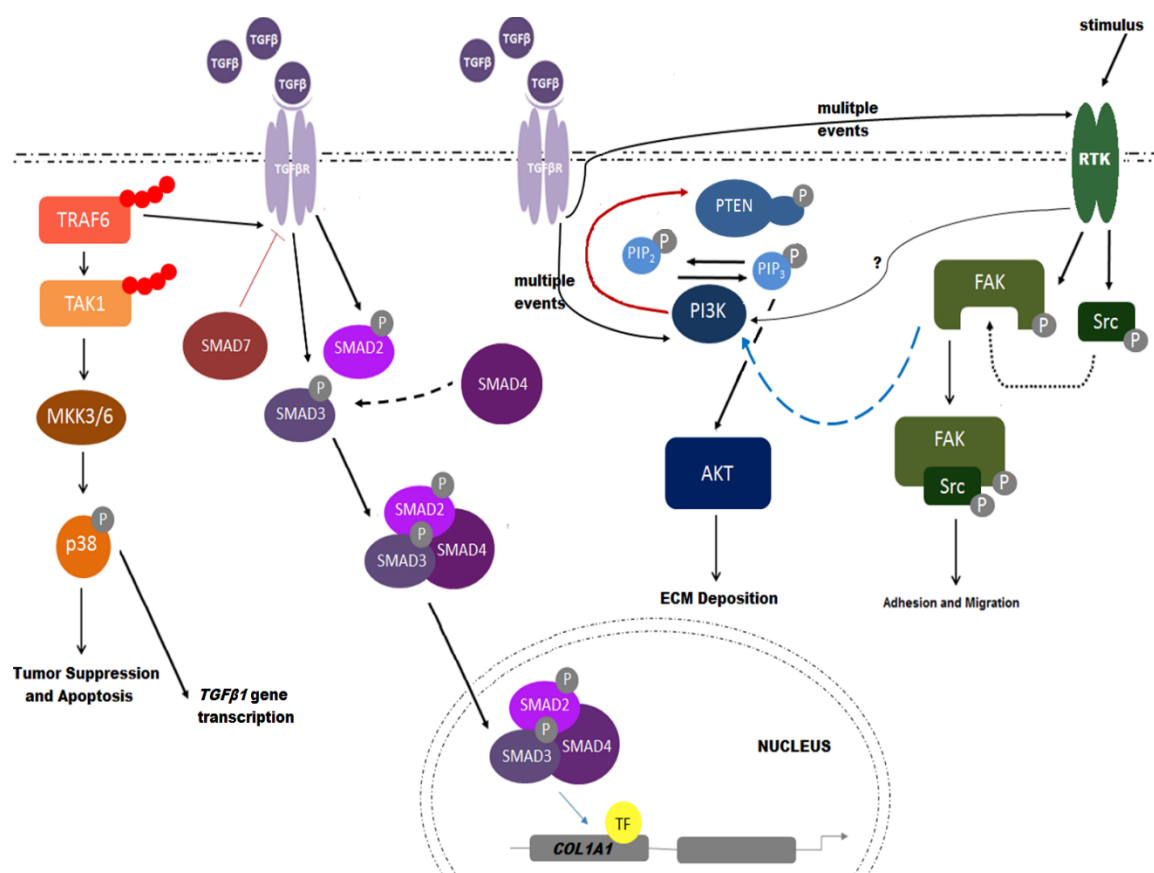


Figure 6.1.2 Summary Diagram of Fibrotic Pathways Involved in TGFβ signaling.

Canonical TGFβ signaling involves SMAD effector proteins, which are able to induce fibrosis by directly upregulating *COL1A1* transcription (43, 44). Inhibitory SMAD7 is induced by SMAD3 to inhibit propagation of the TGFβ signal (43, 44), and has been reported to act as an adaptor protein for the p38 pathway (131). p38 signaling is initiated in the presence of a TGFβ signal to activate TNF receptor associated factor6 (TRAF6) through poly-ubiquitination (131). Activated TRAF6 is able to activate transforming growth-factor β activated kinase 1 (TAK1), which initiates signaling to MAP kinase kinase 3 and 6 (MKK3/6) to phosphorylate and activate p38 (132). Activation of the p38 pathway can lead to tumor suppression, apoptosis, and increased propagation of TGFβ signaling, particularly by increasing transcription of the *TGFβ1* gene (132). Non-canonical TGFβ signaling through the PI3K/AKT and Src/FAK pathways have also been linked to fibrosis (55, 64, 85). Normally, PI3K phosphorylates PIP₂ into PIP₃, which is required for AKT activation. PTEN exerts its phosphatase activity to counteract the effects of PI3K, dephosphorylating PIP₃ into PIP₂. The actions of both PI3K and PTEN work to control AKT signaling in favour of maintaining homeostatic conditions. When PTEN is phosphorylated to become inactive, PI3K activity is unopposed, leading to increased intracellular concentrations of PIP₃. AKT is then activated at an

increasing rate and for a prolonged response. Alternatively, the PI3K is also able to inactivate PTEN (as shown by the red arrow) (95) to increase AKT activation. We have shown in hTM cells that treatment with TGF β 2 results in the phosphorylation and inactivation of PTEN. TGF β R binding can also activate receptor tyrosine kinase (RTK) activity through multiple intermediary events. Activation of RTKs can induce autophosphorylation of FAK and Src (64), to produce downstream fibrotic effects. Activated RTKs may also be able to activate PI3K, to employ PI3K as an effector of FAK signaling (63). Finally, PTEN has been shown by previous reports, as well as in our study, to be negatively regulated by TGF β signaling (39, 51, 52, 77, 78, 85). Since TGF β signaling employs various canonical and non-canonical pathways, which have been shown to have some degree of interaction with each other (63, 95, 131), PTEN is likely also involved in these pathways.

6.2 Limitations of the Study

Our investigation consisted of an *in vitro* cell culture model designed to investigate individual biological phenomena and simplify complex processes. While allowing us to clearly determine the effects of TGF β 2 in isolation on hTM cells, this model is not necessarily representative of the physiological or pathological milieu of hTM cells *in vivo*. As mentioned in **Chapter 1**, hTM cells are subjected to a variety of stresses, including TGF β 2 and mechanical stretch (5, 40, 41). Mechanical stretch activates different pathways including p38 (133) and MAPK pathways (72, 73, 133) in addition to Src/FAK signaling (134). Mechanical stretch may also utilize pathways predominately used for TGF β signaling, such as the PI3K/AKT pathway (133). For a better understanding of the complete effects of TGF β 2 on the trabecular meshwork, hTM cells also must be subjected to mechanical stretch to validate our current findings in a more complex model.

We are further limited by the availability of donor cells. TM cells are harvested from the TM tissue, which comprises a small area of the anterior segment of the eye. Unsurprisingly, only a limited quantity of cells can be obtained from the TM tissue and it is not necessarily feasible to perform multiple technical replicates of each experiment. Moreover, we have used TM cells from humans in our study, which further limits our sample size. Availability of TM tissue and the selection of different donors for biological replicates is limited by individual donation. We must

also exclude older donors to avoid confounding results with samples derived from a glaucomatous patient.

Finally, our investigation considers only one marker of fibrosis. We study COL1 as an indicator of fibrosis because its expression is commonly elevated in a wide array of fibrotic conditions (39, 55, 78, 81), and it has been shown to be expressed in the TM tissue (14, 15). Investigation of additional ECM components such as: fibronectin, which is found in the JCT region of the TM with increased expression in glaucoma patients (20, 135); collagen type III, which is abundantly expressed throughout the TM (14, 15); and the proteoglycan decorin, which is involved in collagen fibril assembly (14, 19) could also be beneficial.

6.3 Future Directions

In this section, future directions are presented to address identified limitations and expand upon the findings of the current study.

First, it is necessary to determine whether the increase in COL1 expression directly translates to increased ECM deposition. Immunocytochemistry of cultured cells could be performed to localize expression of COL1 and other ECM proteins. This should be followed up with an in-depth look at enzymes involved in ECM remodeling. MMPs, TIMPs, and tissue-specific proteases are of primary interest (40-42). Expression of MMP-2 should be investigated as it is reported to have specific affinity for COL1 (40) and to be upregulated under the influence of TGF β (42).

Second, the study should be expanded to more thoroughly investigate the mechanism of TGF β signaling, including the actions of different TGF β isoforms, as well as the expression of TGF β receptors. It would be interesting to determine to what degree the alternate mRNA splice variants identified by Wordinger and colleagues (61) are responsible for the mechanism of signaling through the TGF β -PTEN axis observed in our experiments.

Third, more direct methods of investigating PTEN activity should be employed. PTEN activity should be investigated directly by examining relative ratios of its substrate PIP₃ to the end product PIP₂ under various treatment conditions. Additionally, since increased activity of PTEN increases its rate of degradation (74, 85, 116) examining ubiquitination of PTEN would also be beneficial.

Fourth, it must be determined whether the mechanism of TGF β response is altered in hTM cells from glaucoma patients. Similar experiments to those presented in **Chapters 3-5** could be performed for comparison. It would be particularly interesting to determine whether increased PTEN activity is effective at reducing the fibrotic phenotype of these cells.

Fifth, since PTEN is a highly regulated molecule within the cell (70) we must ensure that changes to PTEN activity do not affect cell viability by promoting oncogenic or apoptotic pathways. Decreases in PTEN activity are usually associated with progression of cancer, whereas increased cell death is a concern with increased PTEN activity. Reports have stated that upon translocation to the nucleus PTEN has the ability to initiate apoptosis (70, 74). In fact, we noticed increased cell death in hTM cells transfected with enhanced-active PTEN mutant. Since the ePTEN mutant exhibited approximately 8-fold increased enzymatic activity, which very effectively decreased COL1 expression, the crucial next step would be optimize the level of activation such that COL1 expression remained low and cell viability was not affected. Pharmacological PTEN activators, including statins such as Simvastatin, Pravastatin and Fluvastatin (136) have been shown to increase PTEN activity by smaller increments (between 1.4-1.6 fold). Investigation of these compounds is necessary to determine whether PTEN activity can be safely increased without inducing apoptosis.

Finally, studies must move on to higher level models. Initially, employing an *in vitro* culture model with mechanical stretch is necessary to understand the combinatory effects of these two factors. This model would also allow for further investigation of the mechanisms that activate endogenous TGF β . Once target

pathways are identified, targeted compounds, including specific pathway inhibitors or PTEN activators, can be tested to determine their efficacy on reducing TM tissue fibrosis and increasing aqueous outflow facility in an organ culture model (137). Findings could hopefully be translated to a suitable animal-model of glaucoma. Ideally, an inducible model of ocular-specific PTEN activation would help determine the efficacy of a potential therapy.

6.4 Concluding Remarks

Recently, PTEN has emerged as a major regulator of fibrosis and many research groups have implicated dysfunction of PTEN as a key component of fibrotic disease. Our findings contribute to this emerging body of literature.

Here, we present a novel mechanism by which TGF β 2 is able to phosphorylate and inactivate PTEN to cause increased COL1 protein expression. The posttranslational mechanism of inactivation through phosphorylation has hitherto, to our knowledge, not been reported in TM cells or in any other model of fibrosis. Furthermore, we have shown that activation of PTEN has the potential to decrease excess ECM deposition in the TM. Taken together, our findings begin to establish PTEN signaling as a fundamental mechanism by which the TM regulates ECM turnover to maintain rapid and continuous remodeling.

Since little is known about the development and progression of POAG, our findings help to establish part of the mechanism of this complex disease process, and have the potential to be translated into targeted therapies. Despite an ever increasing number of glaucoma patients and a clear need for better and more effective treatments, there have not been any new developments in glaucoma therapies in the past two decades. Ultimately, current treatments focus on reducing IOP, while none are designed to treat and prevent TM tissue dysfunction. Here we propose PTEN as a druggable target that has high potential to emerge as an effective therapeutic for the treatment of glaucoma.

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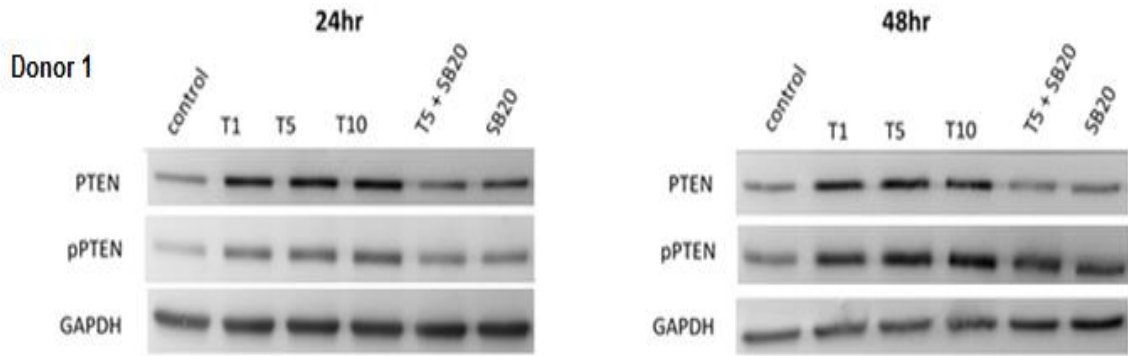
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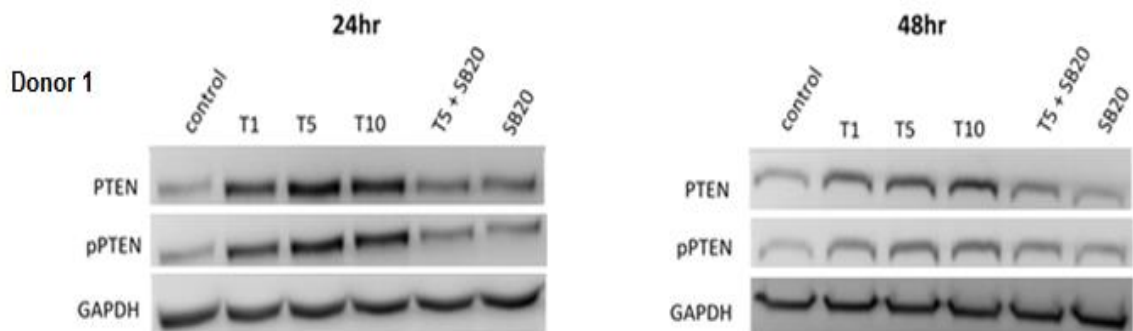
Appendices

Appendix A: TGF β 2 increases PTEN protein expression and phosphorylation of PTEN on plastic plates.



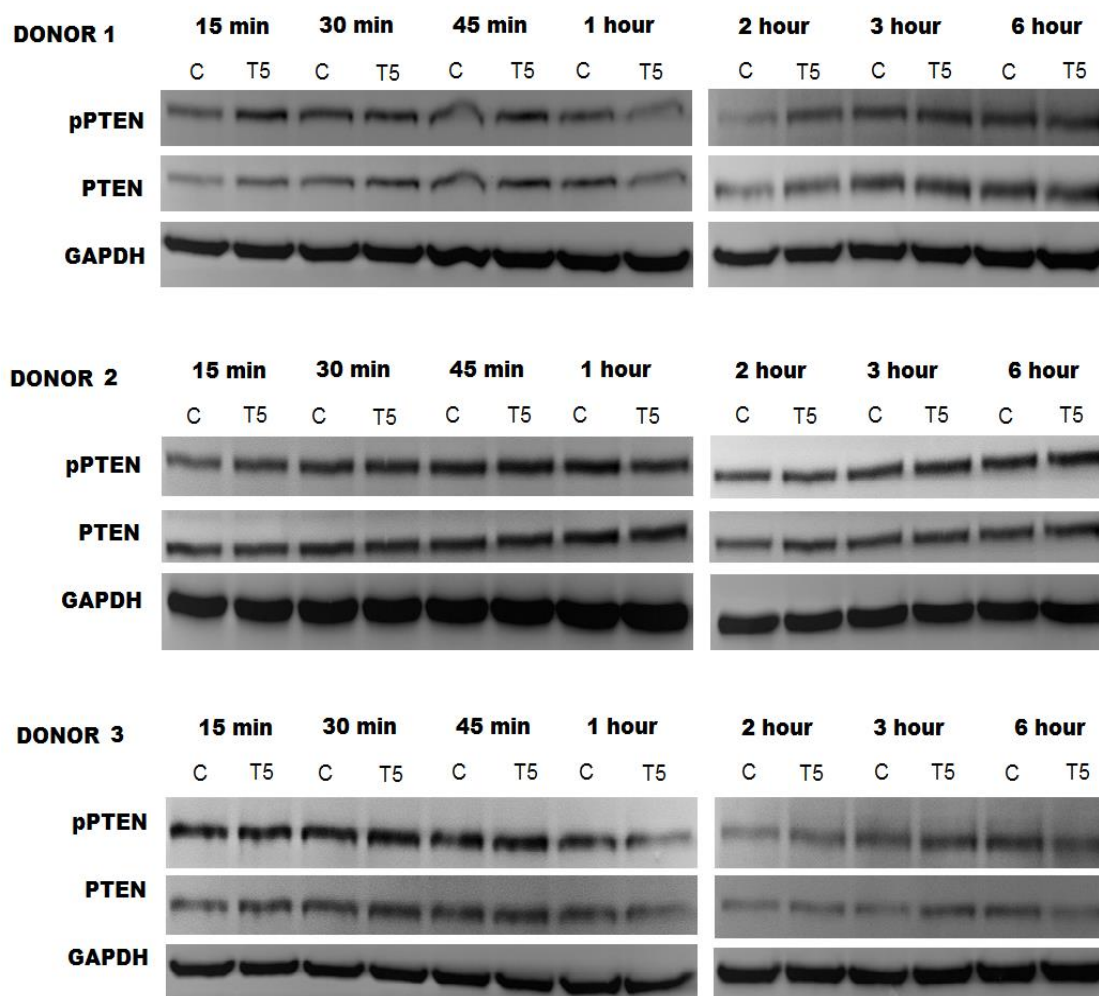
hTM cells cultured on plastic plates with TGF β and SB431542. Treatment with TGF β 2 increased expression of total PTEN and phosphorylation of PTEN after 24 and 48 hours.

Appendix B: TGF β 2 increases PTEN protein expression and phosphorylation of PTEN on COL1-coated plates.



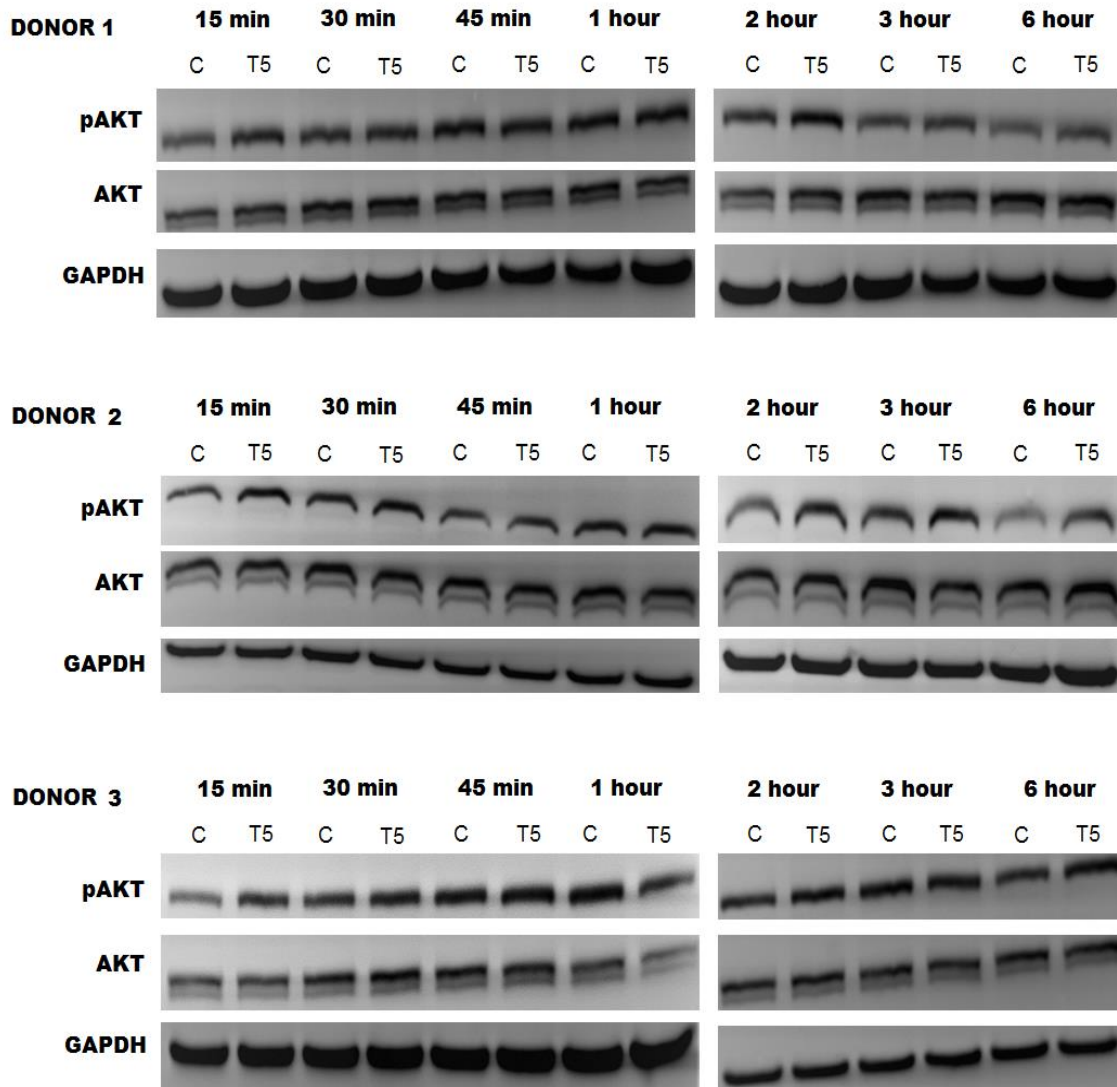
hTM cells cultured on COL1-coated Bioflex[™] plates with TGF β and SB431542. Treatment with TGF β 2 increased expression of total PTEN and phosphorylation of PTEN after 24 and 48 hours.

Appendix C: TGF β 2 increases PTEN protein expression and phosphorylation of PTEN at early time points.



Preliminary data from hTM cells cultured on plastic plates with TGF β 2 5ng/mL. Treatment with TGF β 2 increased phosphorylation of PTEN at 15 to 30 minutes. Decreases in total and phosphorylated PTEN are seen at 1 hour post- TGF β 2 treatment, with variable expression and phosphorylation after 2 hours. The early increase in PTEN phosphorylation may act as a feed-back mechanism, and may explain the increase in PTEN expression at 24 hours observed in this study.

Appendix D: TGFβ2 increases phosphorylation of AKT at early time points.



Preliminary data from hTM cells cultured on plastic plates with TGFβ2 5ng/mL. Increased expression of pAKT in comparison to control can be seen starting at 15 minutes post-TGFβ2 treatment. The effect is sustained through the time course to 6 hours post-treatment. The early increase in pAKT indicates that TGFβ2 may be directly affecting this pathway.

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Platform Presentations:

1. **Tellios N** & Parapuram S. *Mechanisms Underlying Excess Matrix Deposition in the Trabecular Meshwork in Glaucoma*. TOFS. April 30, 2016. London, Ontario, Canada.
2. **Tellios N** & Parapuram S. *Mechanisms Underlying Excess Matrix Deposition in the Trabecular Meshwork in Glaucoma*. Schulich School of Medicine Department of Ophthalmology Research Day. November 6, 2015. London, Ontario, Canada.

Poster Presentations:

1. **Tellios N**, Belrose J, Hutnik C, Tokarewicz A, Leask A, Motolko M, Iijima M, & Parapuram S. *TGF β regulates collagen deposition by modulating PTEN expression and activity in trabecular meshwork cells*. Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting. May 5, 2016. Seattle, Washington, USA.
2. **Tellios N** & Parapuram S. *TGF β regulates collagen deposition by modulating PTEN expression and activity in trabecular meshwork cells*. Western University Department of Pathology and Laboratory Medicine Research Day. Apr 7, 2016. London, Ontario, Canada.
3. Dang B, Liu H, Tellios V, **Tellios N**, Cronk A, Cejic T, & Hutnik C. *Bio-modulation of primary human tenon's capsule fibroblasts using a novel application of coated magnesium*. Western University Department of Pathology and Laboratory Medicine Research Day. Apr 7, 2016. London, Ontario, Canada.
4. **Tellios N** & Parapuram S. *TGF β regulates collagen deposition by modulating PTEN expression and activity in trabecular meshwork cells*. London Health Research Day. Mar 29, 2016. London, Ontario, Canada.
5. **Tellios N** & Parapuram S. *Mechanisms of ECM deposition in the trabecular meshwork in glaucoma*. Western University Department of Pathology and Laboratory Medicine Research Day. Mar 30, 2015. London, Ontario, Canada.
6. Shin A, **Tellios N**, & Parapuram S. *Effect of Mechanical Stretch on Extracellular Matrix of Human Trabecular Meshwork Cells*. Western University Department of Pathology and Laboratory Medicine Research Day. Mar 30, 2015. London, Ontario, Canada.