Michela Montecchi-Palmer, TGFβ Signaling and the Formation of Cross-Linked Actin Networks (CLANs) in Human Trabecular Meshwork Cells, Doctor of Philosophy (Biomedical Sciences – Visual Sciences), April 2016, 127 pp., 24 illustrations, bibliography 164 titles.

Glaucoma is the leading cause of irreversible vision loss and blindness worldwide. One of the major risk factors for glaucoma is increased intraocular pressure (IOP); however there is little understanding of the initial causes of abnormal IOP. Actin cytoskeletal rearrangements known as cross-linked actin networks (CLANs) form at a higher incidence in the glaucomatous trabecular meshwork (TM) cells compared to non-glaucomatous TM cells. The incidence of CLANs is believed to increase the stiffness of TM cells and TM tissue, thereby increasing aqueous humor (AH) outflow resistance and IOP. Even though these actin formations are known to be present, the actual cause of their formation has not yet been elucidated. CLANs can be induced by either transforming growth factor-β2 (TGFβ2) or glucocorticoids such as Dexamethasone (Dex). The primary focus of this research is the TGFβ2 induced CLAN formation and the TGFβ pathways leading to these cytoskeletal rearrangements. Primary human TM cell culture was used to identify whether the Smad or non-Smad TGFβ pathways are the primary path to TGFβ2 induced CLAN formation by testing the ability of various inhibitors to prevent and resolve $TGF\beta_2$ induced CLANs. The results of this research will be a novel and critical source of information in the development of therapeutic strategies for the treatment of elevated IOP and glaucoma, and possibly a better understanding of additional factors contributing to the onset of this sight threatening disease.

TGFβ SIGNALING AND THE FORMATION OF

CROSS-LINKED ACTIN NETWORKS (CLANS) IN

HUMAN TRABECULAR MESHWORK CELLS

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DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the degree of

DOCTOR OF PHILOSOPHY

By

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Fort Worth, Texas

April 2016

ACKNOWLEDGEMENS

There are many people who have supported and mentored me along the path of my graduate career. Each has been invaluable in my success. Firstly, I would like to thank Dr. Abbot Clark for returning to academia and starting the Visual Sciences program. I was one of his first students in the program. He gave me a chance to pursue this degree knowing the difficulties of trying to juggle a graduate and professional career, in addition to having young kids and other commitments in addition to school. Dr. Clark has not only been a consistent source of guidance and knowledge, but also truly cares about each and every one of his students. There were times I felt I could not go on, but his encouragement and gentle pushes along the way kept me going. Thank you Dr. Clark for being more than just an expert in your field, but a true inspiration to all around you.

I would also like to thank my other advisory committee members Drs. Weiming Mao, Anuja Ghorpade, Iok-Hou Pang, Ann Schreihofer, and former members including Drs. Robert Wordinger, Michael Bergamini, and Robert Mallet. Your patience and expertise has been immensely appreciated throughout my graduate career. This has been a long and difficult road, but your support, guidance, and true caring nature have made what seemed to be the impossible, entirely possible. Weiming, your expertise and role as an Advisor has been invaluable and I will always be appreciative of your patience and guidance along the way.

I also want to thank my lab colleagues who always provided moral and technical support, as well as a lot of fun along the way. Even though I was not in the lab every day, I always felt the support and inclusion in the "lab family". Everyone was available to help whenever I needed it. You were always ready to exchange scientific ideas and discussions that I missed out on in lab meetings, etc. You are an impressive mix of professional and knowledgeable people who I will always admire.

I would also like to thank my Alcon colleagues who supported me through this journey. This would not have been possible without their understanding and willingness to provide me the freedom to adjust my schedule as needed in order to fulfill my professional and graduate obligations. In particular, I would like to thank Dr. Michael Brubaker for starting me down the graduate school path. He listened to my career goals and desire to return to school, and pushed me to begin the program right away and not delay. He always somehow knew when to contact me and provide me words of encouragement at the times I needed it most. Mike, without you I would never have ventured to pursue such an immense goal such as completing a Ph.D. program at this stage in my life.

And a tremendous appreciation goes to my friends and family. Thank you for your love, patience and support throughout my graduate career. You have molded me into who I am today and I would not be here if it was not for you. My parents and brother have instilled in me the stubbornness and persevering nature to push through the deepest valleys and struggles. To my incredible husband Michael and my two amazing boys Massimo and Stefano, thank you for your patience through the tough schedules, missing soccer games

and events, and falling asleep on the couch in 5 minutes or less. You never complained and always supported me to keep pushing forward. Massimo and Stefano were very young when I started this program so it is all they remember. But now I look forward to catching up and focusing on my family again.

And most importantly, I thank God for putting the people and events in my life that have made me successful. I truly have felt God's presence along the way in making everything possible.

TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF ABBREVIATIONS

Abbreviation	**Definition**
ADP	Adenosine diphosphate
AFM	Atomic force microscopy
AH	Aqueous humor
AP-1	Activator protein 1
ATP	Adenosine triphosphate
BMP	Bone morphogenic protein
CAI	Carbonic anhydrase inhibitor
cAMP	Cyclic adenosine monophosphate
CLAN	Cross-linked actin network
CPC	Cross-linked actin network positive cell
CTGF	Connective tissue growth factor
DBD	DNA-binding domain
Dex	Dexamethasone
ECM	Extracellular matrix
FN	Fibronectin
GAG	Glycosaminoglycan

Abbreviation	Definition
GC	Glucocorticoid
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HBD	Hormone binding domain
HSP	Heat shock protein
IOP	Intraocular pressure
JCT	Juxtacanalicular
LOX	Lysyl oxidase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MYOC	Myocilin
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nGRE	Negative glucocorticoid response element
ONH	Optic nerve head
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PGA	Prostaglandin analogue
POAG	Primary open angle glaucoma

Abbreviation	Definition
TGFR	Transforming growth factor beta receptor
TGFβ2	Transforming growth factor beta 2
TGM2	Transglutaminase 2
TM	Trabecular meshwork
αSMA	Alpha smooth muscle actin

CHAPTER 1

INTRODUCTION TO THE STUDY

1.

1.1. The Human Eye

The human eye is a small, yet complex organ of many components which work together in a delicate balance to create and process visual stimuli that are later perceived as clear images. The structure of the eye is compartmentalized into the anterior and posterior segments. These segments are then further compartmentalized into three chambers called the anterior chamber, posterior chamber, and vitreous chamber. As shown in Figure 1.1.1, the *anterior chamber* is marked by the posterior aspect of the cornea, the anterior aspect of the iris, and the lens. This chamber is primarily responsible for collecting and refracting light as it passes to the back of the eye. Adjacent to the anterior chamber, is a small space called the *posterior chamber* marked by the posterior aspect of the iris, the anterior aspect of the zonule fibers, the lens, and the ciliary processes. These two chambers make up the anterior segment of the eye. The posterior segment includes the *vitreous chamber* delineated by the posterior aspect of the zonule fibers and ciliary body, the posterior aspect of the lens, and the back of the globe with the optic nerve and retina.[1] The retina and optic nerve create neuronal signals that are sent to the brain via the optic nerve to be processed into actual images by the brain.

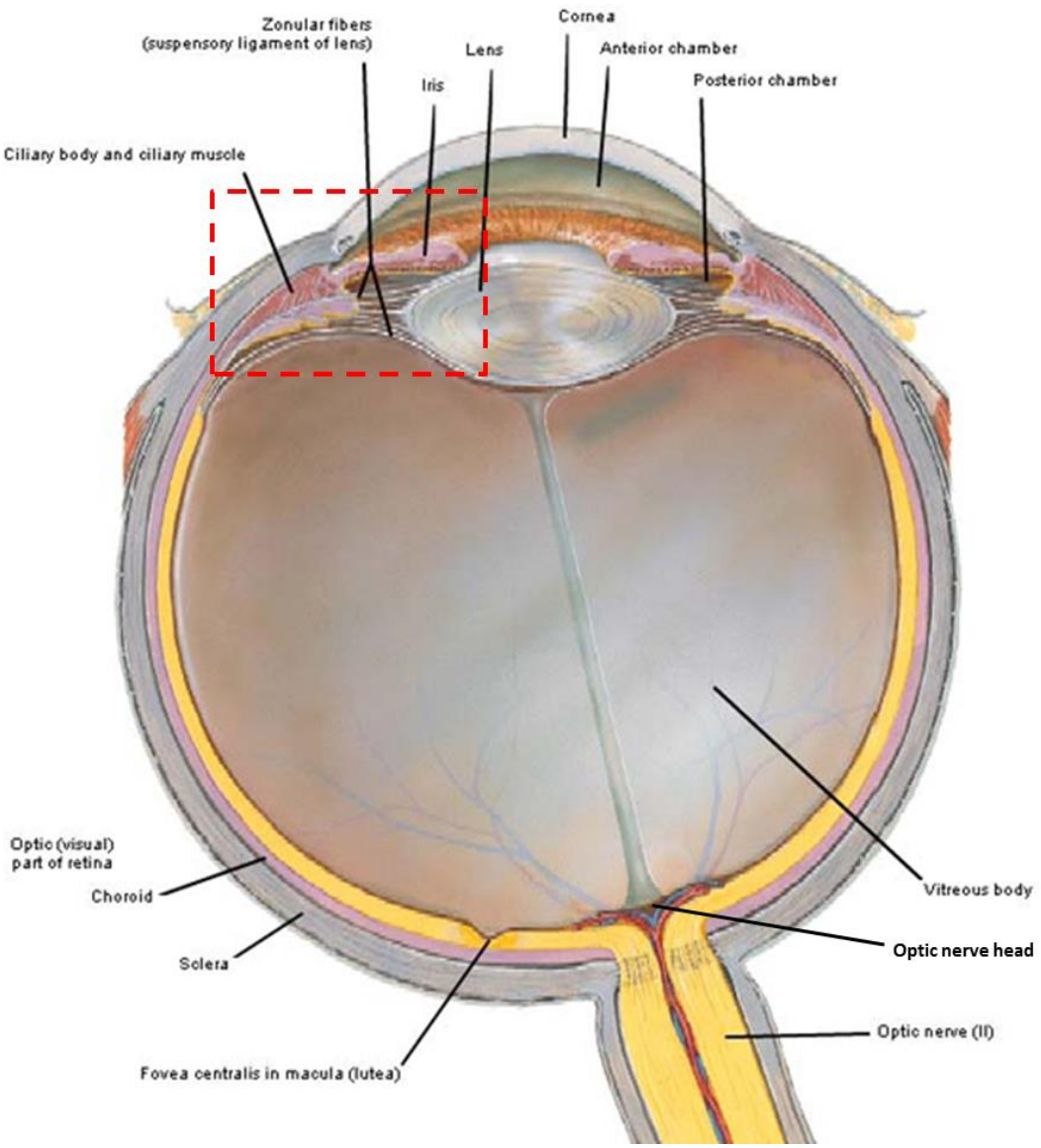

Figure 1.1.1. Anatomy of the Human Eye

A diagram of the human eye and its major components. Red dashed box indicates area for the location of the trabecular meshwork which is the primary focus of this dissertation. (Figure modified from image available at www.outlanderanatomy.com.)

Because the lens, cornea, and trabecular meshwork (TM) are devoid of blood vessels, nourishment and removal of waste products must be accomplished by another method. The aqueous humor (AH) is a clear fluid which contains nourishing components needed for maintaining the health of the ocular tissues, as well as building blocks for healing and recovery after insult. Since the AH is not stagnant but rather is constantly produced and flowing through the eye, the AH also transports oxygen and facilitates removal of waste products.[2, 3]

At the junction of the cornea and iris there are two structures called the ciliary body and the TM. The ciliary body produces AH which is released to the posterior chamber of the eye, then passes to the anterior chamber via the pupil, and later exits the eye through outflow pathways at the TM. This outflow of AH is accomplished through two pathways, and is a critical aspect to maintaining a healthy eye.

One outflow pathway referred to as the *unconventional pathway*, contributes approximately 25% of the overall AH drainage (Figure 1.1.2).[4, 5] In this pathway, AH enters the iris root in the anterior chamber and exits through the supraciliary space, through the sclera, and into the vortex veins or choroidal vessels and episcleral tissue.[6, 7] The major outflow pathway known as the *conventional pathway*, accounts for approximately 75% of the AH outflow [4, 5] and occurs primarily through the highly fenestrated TM, into Schlemm's canal, and then to the episcleral venous plexus.[6]

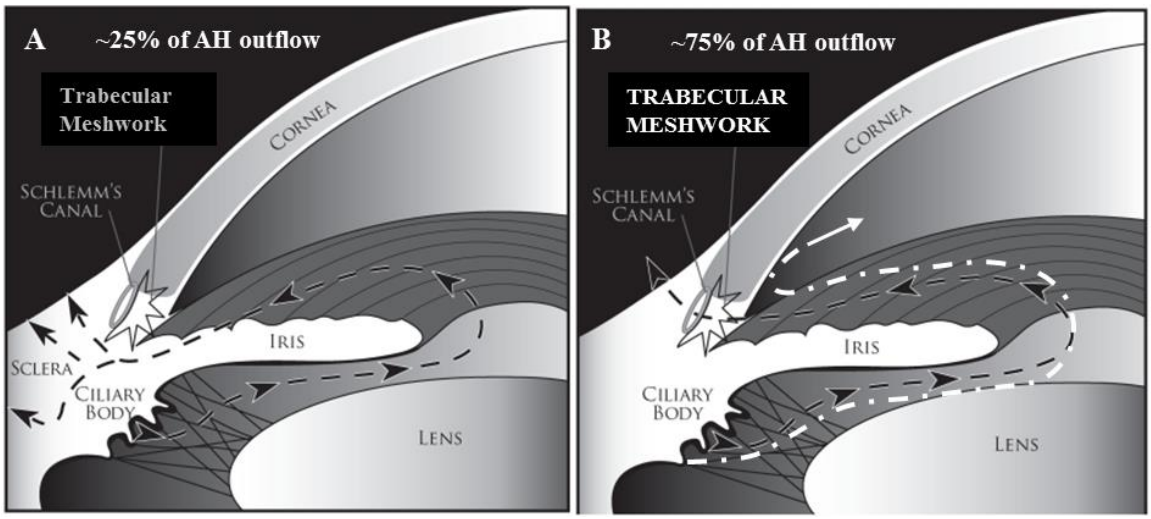

Figure 1.1.2. Aqueous Humor Outflow Pathways

AH is produced by the ciliary body, then flows through the pupil to the anterior chamber. **A)** Diagram depicting the *unconventional* AH outflow pathway. AH in the anterior chamber flows to the ciliary muscle and exits through either veins or through scleral pores to episcleral tissue. **B)** Diagram depicting the *conventional* AH outflow pathway. AH in the anterior chamber flows through the trabecular meshwork, into Schlemm's canal, and into the episcleral veins. Black dashed lines and arrow heads represent AH flow. White dashed line represents the disruption of outflow as AH outflow resistance increases at the TM. [6] (Figure modified from Goel et al, Open Ophthal Journal, 2010.)

The constant production of AH and a natural resistance to outflow at the TM, maintains a healthy intraocular pressure (IOP) which is necessary to maintain the proper shape, structure and function of the eye. Although the path of AH outflow is fairly simple, the outflow resistance is highly dependent on the structures through which the AH passes.

1.2. Glaucoma and the Trabecular Meshwork

Glaucoma is a progressive ophthalmic disease affecting both the anterior and posterior segments of the eye, and is the leading cause of irreversible vision loss and blindness around the world. It is estimated that glaucoma will affect approximately 80 million people by the year 2020.[8-10] The most common form of glaucoma is called primary open-angle glaucoma (POAG) with the primary risk factor being sustained elevated IOP. Multiple changes to the TM structure, extracellular matrix (ECM) composition, and cellular cytoskeletal rearrangements contribute to increases in IOP,[11-18] which correlates with axonal loss at the optic nerve head (ONH).[19] Damage at the ONH may be due to strain and stretch as a result of increased IOP, as well as possible inhibition of retrograde transport of neurotrophins.[20] With continued insult and loss of retinal ganglion cells and loss of supportive connective tissue, there is increased excavation of the optic nerve. Eventually these conditions result in cupping of the ONH and progressive visual field loss.[19] Since POAG has no obvious symptoms, most people do not know they have the disease until damage to the optic nerve has already occurred. To date, the only known treatments for POAG are methods of lowering IOP, but no treatments are currently available that target the mechanisms which lead to elevated pressure.

A key structure in regulation of a healthy IOP is the TM. The TM is actually composed of four regions referred to as the uveal meshwork, corneoscleral meshwork, cribiform or juxtacanalicular (JCT) meshwork and inner wall of Schlemm's canal (Figure 1.2.1). Schlemm's canal is just posterior of the TM and is lined with a single layer of vascular endothelial-like cells. Due to incomplete tight junctions as well as transcellular pores, AH flows through the inner wall into the canal.[21] The inner wall cells of Schlemm's canal then attach to the JCT meshwork which is the outer most part of the TM and represents the major source of AH outflow resistance. The JCT consists of connective tissue embedded with cells and lacks the large fenestrated openings. Instead, it contains a network of irregularly oriented fibrils in layers, with ECM between the layers. The inner layer of the JCT is continuous with the outer uveal and corneoscleral portions of the TM. The uveal and corneoscleral portions of the TM are highly fenestrated and made of TM beams covered by a continuous monolayer of TM cells.[11] The cells in this region are phagocytic and act as primary filters prior to the AH passing through the JCT and into Schlemm's canal.

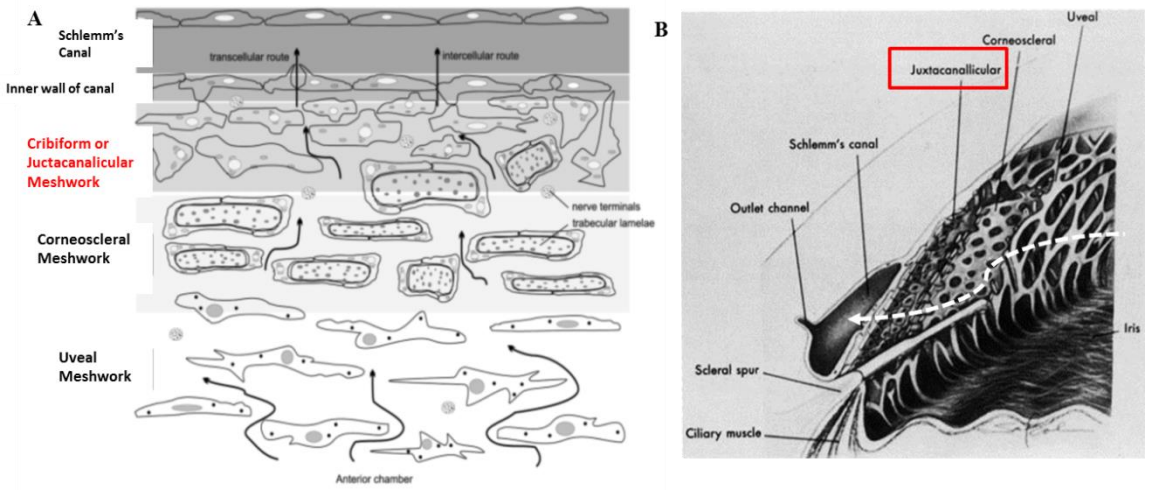

Figure 1.2.1. Structure of the Trabecular Meshwork

A) Four specific regions of the TM. Arrows indicate the direction of AH flow through intercellular spaces or intracellular pores from the anterior chamber through the uveal meshwork, followed by the corneoscleral meshwork, and the cribriform or juxtacanalicular meshwork toward Schlemm's canal. AH resistance progressively increases from the anterior chamber to Schlemm's canal as the TM becomes less fenestrated with greatest AH resistance occurring at the cribiform meshwork (indicated in red).[22] (Figure modified from Llobet et al, News Physiol Science, 2003.)

B) Representative diagram of the TM regions as they are found at the drainage angle of the eye. White dashed arrow line indicating direction of AH flow.[23] (Figure modified from Shields, Textbook of Glaucoma, 1987.)

The proper balance between AH production and outflow maintains a healthy IOP. However, there are multiple factors that can lead to alterations in the homeostatic AH flow. One requirement for maintaining proper AH outflow is the balance between ciliary muscle and TM contraction. The ciliary muscle connects to the TM by elastic fibers and is composed of smooth muscle cells. When contracted, the ciliary muscle pulls on the scleral spur attached to the TM, opening the TM and increasing AH outflow. Conversely, the cells of the TM also have muscle-like contractile properties due to the presence of alpha-smooth muscle actin (αSMA) and smooth muscle myosin which help regulate AH outflow.[24] The normal contraction of these tissues is regulated by phosphorylation and dephosphorylation of myosin light chain (MLC) by myosin light chain kinase (MLCK).[25, 26] When the TM cells are in a prolonged or abnormal contracted state, altered tensile integrity can determine cell shape, mechanical responsiveness of cells, and regulate cytoskeletal assembly and signal transduction.[17] These alterations can lead to decreased TM openings and subsequently increasing AH outflow resistance.

Additionally, there are molecular influences that result in increased AH outflow resistance. Several basement membrane proteins such as laminin and collagen are expressed throughout the outflow pathway, the basement membrane of TM beams, inner wall of Schlemm's canal, and the JCT.[11] Fibronectin (FN) is present abundantly in the basement membranes of TM beams, as well as the JCT and Schlemm's canal cells. The FN forms thick fibrous strands that are connected by integrins which trigger ECM and TM changes as a response to mechanical stretch stimuli such as in the presence of elevated IOP.[11] Collagens are another abundant ECM protein. Mechanical stretch can

again trigger increased expression of various collagens, as well as change the amount of lattice versus fibrillary collagens, and alter interactions of the collagens with FN or other ECM proteins thereby increasing outflow resistance.

Glycosaminoglycans (GAGs) within the TM have also been of particular interest for their potential role in increased AH outflow resistance. GAGs are long repeat disaccharide chains with high density negative charge at physiological pH, and line the apical surface of cells.[11] This negative charge attracts positively charged molecules and therefore can create obstruction through the TM if there is not adequate turnover of these molecules. The GAGs in the TM have been identified as 20-25% hyaluronic acid or hyaluronan, 40-60% chondroitin and dermatan sulfates, 5-10% keratan sulfate, and 15-20% heparin sulfate; all with varying concentrations in the different regions of the TM.[11] Anterior segment perfusion models have suggested a contribution of GAGs to outflow resistance, however their direct role particularly in the human eye, have not been fully demonstrated or understood.[11]

There are also various matricellular proteins involved in the reorganization of the ECM and cellular interactions.[27] The matricellular proteins interfere with cell-ECM communication to facilitate ECM organization. Specific matricellular proteins can be activators of other factors that also trigger abnormal TM changes. Likewise, several stimuli that alter outflow facility also alter the activity of key enzymes such as matrix metalloproteinases (MMPs). MMPs are responsible for initiating much of the ECM turnover. It has been shown that MMP activity, as well as expression of other genes and

ECM components, increase as IOP increases.[11, 28, 29] Over a few days, the TM adjusts and outflow resistance is decreased resulting in IOP returning to baseline values. This signifies the TM's ability to instantly employ a coordinated response to stimuli such as mechanical stretch.

Another important factor within the TM cells and contributing to elevated IOP, are ECM and cytoskeletal rearrangements. ECM cross-linking creates a stiffer TM cell by increased expression of enzymes such as lysyl oxidase (LOX)[30, 31] and transglutaminase 2 (TGM2).[32] The increased ECM cross-linking may create additional strain on the TM, and may also decrease some of the interactions that must occur to initiate signaling for the reorganization or degradation of ECM. Specific reorganization of the cytoskeleton resulting in cross-linked actin networks (CLANs) has also been studied over the years and is thought to further increase the stiffness of TM cells. CLAN formation will be discussed in detail later in this dissertation. Because of the various components and contributors to the TM homeostasis, all of these perturbations can create a vicious cycle ending in sustained elevated IOP, damage to the optic nerve, and eventual loss of vision.

Further instigating the detrimental effects within the TM are induction agents such as transforming growth factor-β2 (TGFβ2) or glucocorticoids such as Dexamethasone (Dex). Both are known to cause changes in ECM composition and altered gene and protein expression within the TM, and both induce elevated IOP (Figure 1.2.2).[30, 32-40]

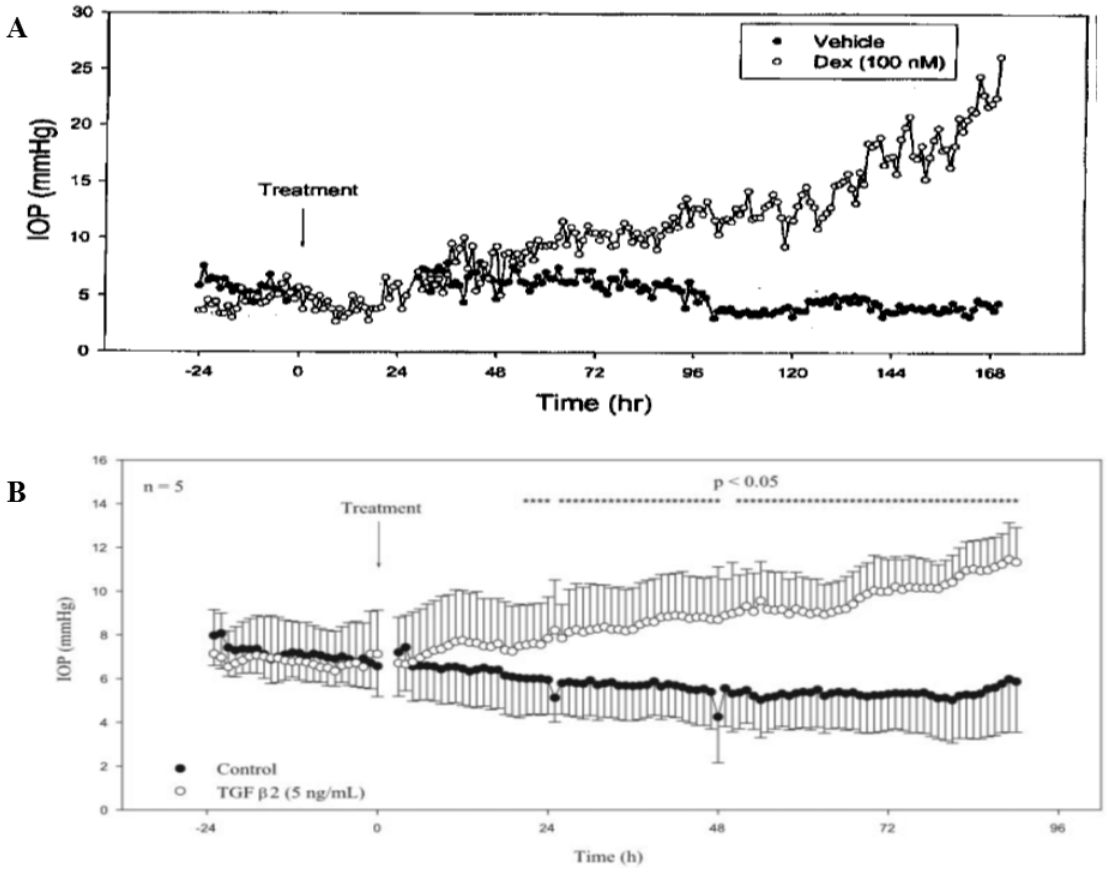

Figure 1.2.2. Effect of Dexamethasone and TGFβ2 on Intraocular Pressure

A) Effect of Dex on IOP versus vehicle in one pair of perfusion cultured Dex responder human eyes.[16] (Figure from Clark et al, Cell Motility and the Cytoskeleton, 2005)

B) Effect of TGFβ2 on IOP versus control in perfusion cultured human eyes.[37] Data expressed as mean ± SEM; (*) statistically significant ($p<0.05$) versus control. (Figure from Fleenor et al, IOVS, 2006).

External sources of glucocorticoids include Dex which is commonly prescribed to patients either systemically or as a topical regimen, as a potent anti-inflammatory agent for various diseases and conditions. One of the primary concerns among physicians when prescribing such treatments is the risk of significant increases in IOP that can occur after prolonged exposure to corticosteroids in 30-40% of the general population.[41] Dex induced ocular hypertension is associated with changes to the human TM including, but not limited to, thickened trabecular beams, decreased inter-trabecular spaces, and increased deposition of extracellular material in the JCT region with altered distribution of FN in the ECM.[42, 43] Dex induced inhibition of TM phagocytosis has also been observed.[44] The induced ocular hypertension is reversible after withdrawal of the medication; however irreversible damage to vision may have already occurred. Also of concern is those who have this abnormal response to steroids have a higher risk of developing POAG.[45-48]

Even though the prescription of glucocorticoids can in some cases be avoided, although somewhat controversial, an endogenous glucocorticoid called cortisol has also been thought to cause ocular hypertension as is seen in Cushing's syndrome. With this condition, patients have endogenously elevated levels of cortisol, and develop ocular hypertension which can lead to a condition similar to POAG.[49] The effects of cortisol further support the glucocorticoid impact on the TM and TM function.

Another ocular hypertension inducing factor is TGFβ2, a naturally occurring profibrotic cytokine found to be elevated in the AH of patients with POAG[50-53] and in glaucomatous

TM cells.[54] Several isoforms of TGFβ exist in mammalian cells, but TGFβ2 is considered the primary isoform present in the human eye.[55] Multiple measurements of aqueous humor samples have shown a significant increase in active TGFβ2 in POAG compared to non-glaucomatous eyes.[50-53] There are three possible explanations for increased TGFβ2 activity in glaucomatous eyes. The increased activity may be due to 1) accumulation resulting from decreased turnover because of a malfunctioning TM; 2) inflow of TGFβ2 due to breakdown of blood-ocular barriers; 3) increased secretion, decreased degradation, and increased activation of TGFβ2.[53] Since there has not been a significant association of increased active TGFβ2 with other forms of glaucoma, explanations 1 and 2 are less likely. However, there is reason to believe that increased secretion or activation of TGFβ2 is likely to be the case in POAG. The role of epigenetics in regulation of glaucoma-related growth factors within the TM has been recently studied.[56] It was found that histone acetylation is responsible for dysregulation of the expression of various growth factors including TGFβ2. Decreased ECM elasticity can also regulate and activate TGFβ2.[57, 58] Thrombospondin 1 expression, a matricellular protein and key activator of TGFβ2, has been found in high concentrations especially at the JCT region of the TM, and elevated compared to non-glaucomatous eyes in approximately one third of the POAG eyes tested.[59]

This increase in active TGFβ2 has significant ramifications. TGFβ2 is a key activator of plasminogen activator inhibitor (PAI-1) which is a potent inhibitor of MMPs thereby decreasing ECM turnover.[30, 37, 60] Due to the fibrotic nature of TGFβ2, similar changes to the TM are induced as has been discussed with exposure to Dex, including increased

expression of FN,[30, 61, 62] collagen, and laminin.[30, 37, 63] Active TGFβ2 has also shown to activate all five LOX genes[30, 64] as well as TGM2[32] increasing the ECM cross-linking and stiffness of the TM. The changes that occur within the ECM and TM are further amplified by changes that also occur within the TM cell, and in particular the cytoskeleton since the cytoskeleton is linked to the ECM via integrin receptors on the cell membrane.

1.3. The Cytoskeleton

The cytoskeleton is responsible for cell shape, motility, and intracellular transport. There are three components of the cytoskeleton which include microfilaments, intermediate filaments, and microtubules. Each cytoskeletal element is dynamic and able to assemble and disassemble based on the cellular needs for cell division, movement, shape, and structural integrity.

One of the major components of the cytoskeleton, and primary focus for this research, is the actin microfilaments. Actin monomers exist as single actin globules, also known as G-actin. When the concentration of actin monomers rises above the critical concentration, the monomers begin to bind to each other to form F-actin, or filamentous actin. This occurs in three phases (Figure 1.3.1).[65] The first phase includes ATP bound G-actin aggregating into oligomers. This is followed by the elongation phase where filaments are formed by addition of actin monomers with preferential addition of G-actin at the positive end of the actin filaments. Once the G-actin monomer concentrations decrease to equilibrium with the filament, ATP is hydrolyzed to ADP and a steady state

phase is reached. The steady state is regulated by the concentration of G-actin monomers. As the concentration increases above the critical concentration, actin filaments will again polymerize, while decreasing below the critical concentration will induce actin depolymerization.

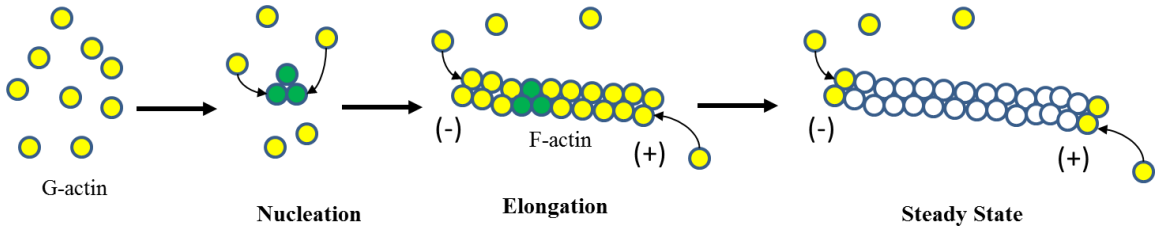

Figure 1.3.1. Actin Polymerization

Actin is present as globular actin (G-actin) and filamentous actin (F-actin). During the initial nucleation phase, ATP bound G-actin monomers (yellow) form complexes of actin (green). These actin globules form filaments in the elongation phase by addition of subunits to both ends of the filament, but preferentially at the positive end, resulting in actin polymerization. In the third phase, the ends of actin filaments are in a steady state and the subunits slowly hydrolyze ATP and become stable ADP F-actin (white). The steady state is regulated by the critical concentration of G-actin monomers.[65] (Figure modified from Lodish et al, Molecular Cell Biology, 2000.)

Certain actin binding proteins regulate the polymerization and depolymerization of actin filaments. One such protein is known as profilin. Profilin promotes the assembly of actin by binding to the positive end of the actin monomer. In doing this, it leaves the negatively charged ATP end of the monomer free to bind with the positive end of an actin filament. Profilin then dissociates leaving the end ready to accept another monomer. Actin binding proteins such as α-actinin serve as bundling proteins to organize filaments into bundles to form actin stress fibers which have contractile properties. Stress fibers form in the JCT region of the TM, and microfilaments heavily line the inner wall cells of Schlemm's canal which supports this region of the TM's particular contractile properties.

Another actin binding protein with the reverse effect of profilin is called cofilin. Cofilin is a ubiquitous actin binding protein required for reorganization of the actin cytoskeleton by its dephosphorylation and activation which results in actin severing and depolymerization.[66] Cofilin binds to G- and F-actin which increases actin depolymerization by interfering with the addition of new G-actin to the negative end of actin filaments. Cofilin can also sever actin filaments releasing new fragments which are ready to accept new G-actin and thereby also facilitating the continued remodeling of the actin cytoskeleton. Cofilin can also work in conjunction with another actin binding protein called Arp2/3 which binds to the side of F-actin. After dissociation from Arp2/3, cofilin and profilin then continue with polymerization and depolymerization to allow for actin filament branching and remodeling based on the cell's need.[67, 68]

There is evidence to support that the structure of the cytoskeleton in the AH outflow tract plays an important role in outflow facility.[69-73] The actin microfilaments form cell-cell and cell-ECM contacts by way of adherens junctions, other focal contacts, and integrins. Integrins are transmembrane glycoproteins responsible for cell-cell and cell-ECM interactions, and facilitate communication with factors that regulate the cytoskeletal structure.[74, 75]

The cytoskeletal interactions are regulated by extracellular calcium, activation of molecules, and mechanical tension induced by factors such as hydrostatic pressure.[69] F-actin structure has been shown to be affected by mechanical factors in the TM[76] and Schlemm's canal cells in situ.[3] In a study evaluating the actin architecture within glaucomatous and non-glaucomatous TM tissue, the authors observed the F-actin in non-glaucomatous eyes generally tended to be more organized in appearance. However, that of the glaucomatous eyes revealed a more disordered F-actin structure with many actin tangles most often seen in the JCT region.[18] This observation was consistent with what has been observed in TM cell culture as well.[3, 16] Similar differences in actin structure have been observed in response to mechanical stretch and sheer stress,[76] and by exposure to corticosteroids such as Dex,[16, 17, 33, 77-79] and exposure to TGFβ2.[37, 79] Since alterations of the actin architecture also result in changes in outflow resistance,[16, 17, 37, 69-73, 79-81] it is clear that the actin architecture plays an important role in AH outflow facility.

Another important restructuring of the actin architecture is in the observation of CLANs that form in the TM cells. These formations have been observed in greater frequency in

glaucomatous cells,[18, 42] with approximately 40% of glaucomatous cells versus 4% of non-glaucomatous cells containing CLANs.[42] The primary focus of this research is in regards to CLAN formation.

1.4. Cross-Linked Actin Networks (CLANs)

The actin cytoskeleton is normally well organized with elongated actin filaments throughout the cytoplasm. However, the actin fibers can also rearrange themselves into geodesic dome-like structures called CLANs.[16, 17, 33, 78, 79, 82] It is important to note that these CLAN formations have been observed in human,[16, 17, 77, 78, 82, 83] mouse,[84] and bovine[79, 85] cultured TM cells, and also occur in *ex vivo* models using human TM tissue.[16, 18] CLANs are also seen in situ at a higher incidence in glaucomatous eyes compared to non-glaucomatous eyes.[16, 18, 86] Therefore, CLANs are not simply an artifact generated by the biophysical cues inherent to certain cell culture.

The "normal" actin arrangement compared to CLANs is shown in Figure 1.4.1A CLANs are defined by approximately 5 distinct hubs connected by spokes to create a dome-like structure (Figure 1.4.1 B-D). In order to qualify as a CLAN, a minimum of 3 hubs linked to form at least one triangulated unit is required.[86]

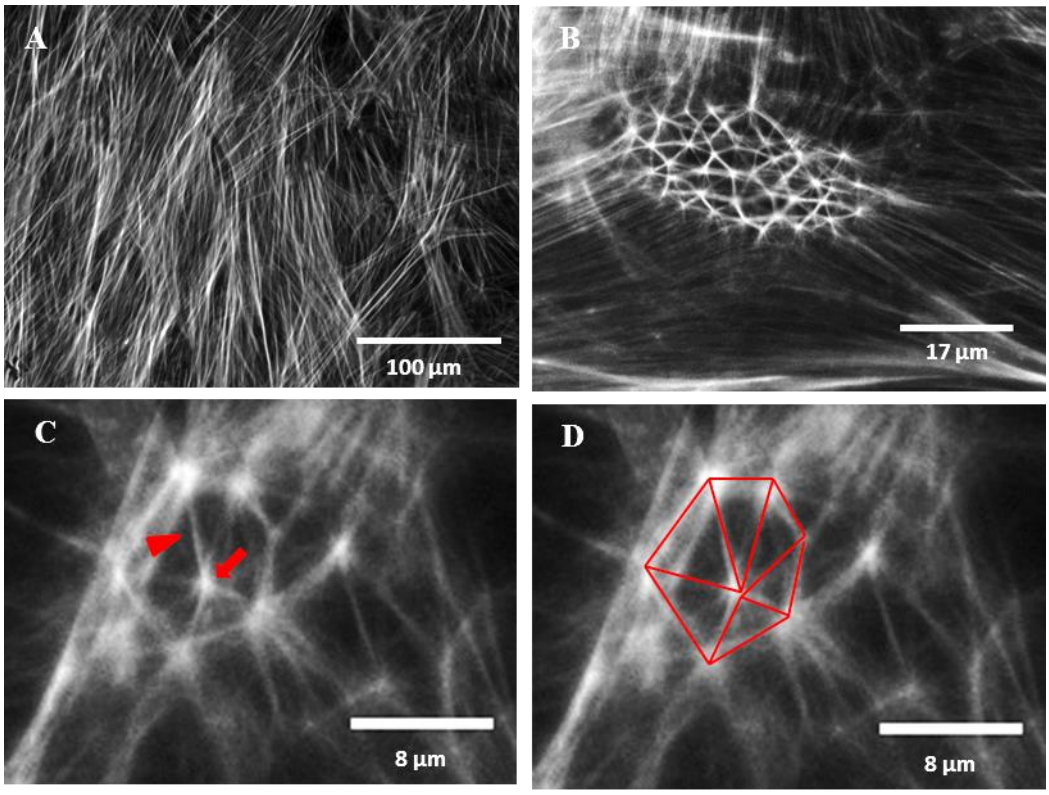

Figure 1.4.1. Normal cytoskeletal arrangement compared to CLANs

Human TM cells were permeabilized and actin fibers stained with Phalloidin conjugated with Alexa-488. **A)** "Normal" cytoskeletal arrangement with elongated stress fibers which are well organized. **B)** Multiple CLANs in the perinuclear region. **C)** Magnified image of a single CLAN. Distinct hubs are identified (red arrow) with connecting spokes (red arrow head) to form triangulated units. Typically CLANs will include at least 5 hubs. A minimum of 3 hubs with connecting spokes to form one triangulated unit is required to be considered a CLAN. D**)** Same image as that in (C) but with a red outline of the full CLAN to demonstrate the complete dome-like structure.

Geodesic actin structures have been observed in other tissues and under other conditions. It has been suggested that tension dependent structures gain stability by "triangulating" their internal support structures.[87] Although CLAN formation has been observed in cell types other than TM cells, those CLANs are usually found during cell attachment and cell spreading, and are thought to be transient in nature as precursors to stress fiber formation.[17, 88-92] Therefore, this rearrangement of the actin fibers may represent a mechanism to stabilize the cells during a highly dynamic process. Only in the TM[17] and lamina cribrosa[93], CLANs form in confluent cell cultures and are therefore not associated with cell attachment. However, the fact that CLANs are found at a higher incidence in glaucomatous eyes may represent a stabilizing response of TM cells to prolonged tension and mechanical stretch due to high IOP.

Even though CLAN formation may represent a protective mechanism of the cell, the natural response to stimuli in TM cells is impaired by the presence of CLANs resulting in a less functional cell and increased TM cell and tissue stiffness. The turnover rate of actin filaments in cultured fibroblast cells has been shown to be slowest when incorporated into actin polygonal networks compared to actin in stress fibers.[94] It has also been suggested that cells exposed to Dex were observed to be larger and rounder in shape, with large nuclei compared to control cells.[52] This may in part be due to the actin cytoskeleton being "locked in place" and unable to facilitate cell division even though DNA synthesis continues.[17] Likewise, a dynamic cytoskeleton would also be necessary for proper phagocytic activity and ECM turnover. Dex induced inhibition of TM phagocytosis has been associated with Dex induced reorganization of the TM

cytoskeleton.[95] Additionally, the observation of a time dependent Dex induced accumulation of FN[43] was similar to that of Dex induced cytoskeletal rearrangements. Therefore CLAN formation likely alters microfilament cycling, leading to more stable filament structures. These observations support the close relationship between the cytoskeleton and ECM and their ability to affect each other.

Further evidence for the potential impact of CLANs comes from the fact that CLAN formation is observed to increase over 10-14 days of exposure to each Dex or TGFβ2.[17, 79] Increases in IOP also initiate over this same timeframe, especially in the presence of TGFβ2 (Figure 1.4.2). Therefore, CLANs may be "locking" the TM cells into a more contracted state thereby contributing to the increase in AH outflow resistance.

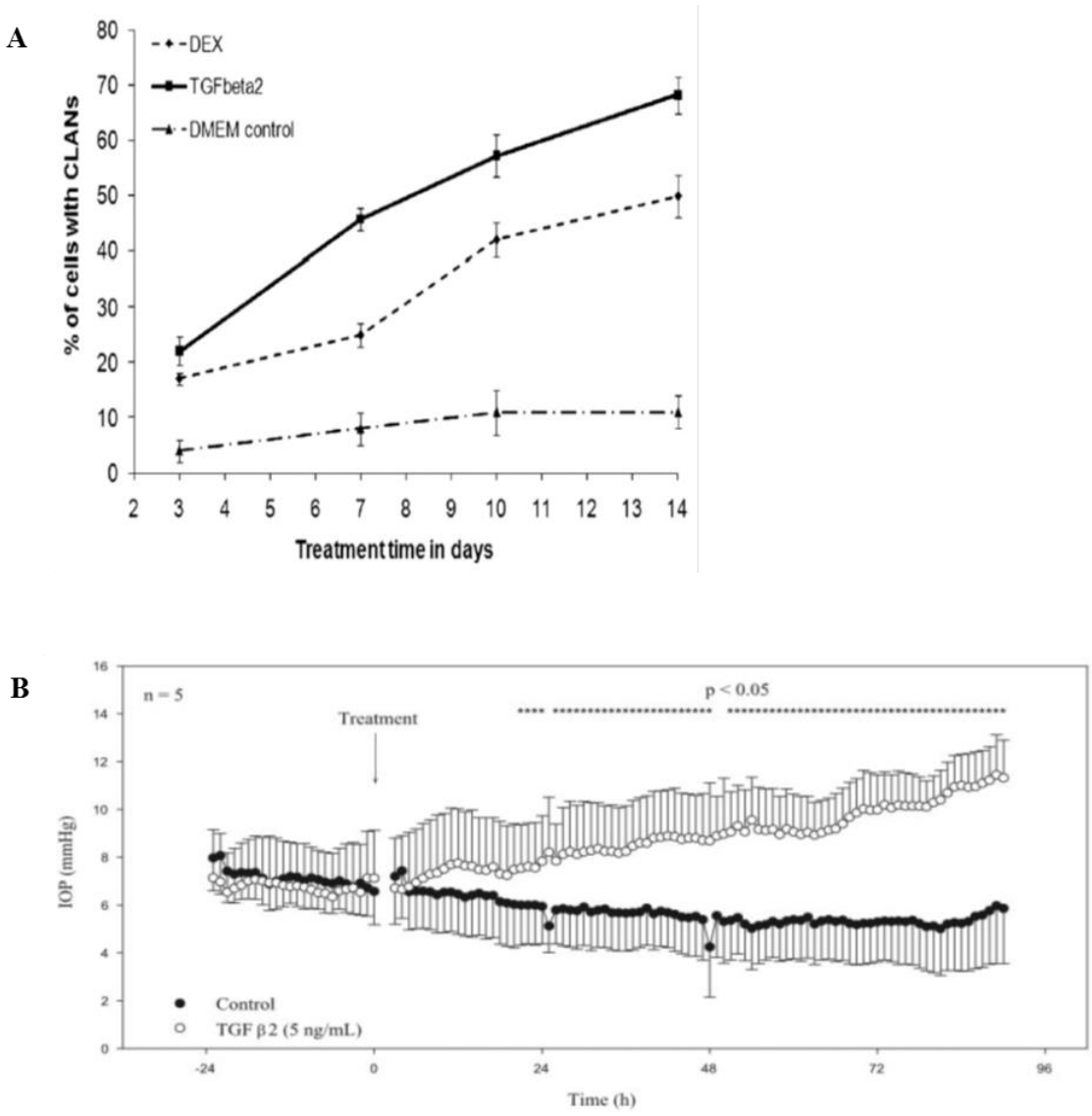

Figure 1.4.2. CLAN formation and IOP elevations

A) Effect of Dex or TGFβ2 on CLAN formation. Bovine TM cells were cultured to confluence, and then treated for 14 days with Dex (10^{-7} M), or TGFβ2 (2 ng/mL), or control medium containing 1% fetal calf serum only.[79] (Figure from O'Reilly et al, IOVS, 2011) **B)** Effect of TGFβ2 on IOP versus control in perfusion cultured human eyes.[37] Data expressed as mean ± SEM; (*) statistically significant ($p<0.05$) versus control. (Figure from Fleenor et al, IOVS, 2006).

The stiffness of the TM cells is affected by CLAN formation. One illustration of this utilized a mathematical model and the addition of a specific actin binding cross-linking protein called scruin to F-actin bundles.[96] Scruin is found in the sperm cell of horseshoe crab and is therefore not necessarily associated with the human TM. However, nonspecific scruin-scruin interactions cross-link and bundle with neighboring filaments which then form CLAN structures. Gardel and colleagues observed that CLAN formation resulted in a 2 order of magnitude increase in the stiffness of the actin fibers in their model.

When considering the effect of CLAN formation in the TM tissue, studies utilizing atomic force microscopy (AFM) have provided insight to the impact of the actin architecture.[97, 98] AFM utilizes application and monitoring of localized forces between a sharp tip and the surface of interest. The tip is mounted on a cantilever, and the deflection of the cantilever is detected in order to calculate the force required for its displacement. The position of a laser beam reflected from the backside of the cantilever onto a segmented photodiode is monitored to produce an elastic modulus by use of various mathematical equations.[87] The elastic modulus measured in kilo Pascals (kPa) is an indication of the stiffness of the surface being evaluated. Higher numbers indicate a greater degree of stiffness.

One study evaluating human TM tissue with the use of AFM, showed the non-glaucomatous TM tissue has an average stiffness measurement of approximately 4 kPa versus the glaucomatous TM of approximately 80 kPa.[97] Although Last and colleagues

did not specifically assess CLAN formation in relation to the stiffness of the TM tissue, it is possible that CLAN formation could be one factor contributing to the observed difference since CLAN formation can be found in non-glaucomatous TM cells at a rate of approximately 4% versus approximately 40% in glaucomatous TM cells.[42] Last and colleagues also observed a large range in TM tissue stiffness along a 50 μm segment of the glaucomatous TM tissue, which would be consistent with the fact that CLANs are not necessarily found in every TM cell.

Various groups have studied the genomics and proteomics in glaucomatous eyes compared to normal eyes,[38] as well as changes induced by Dex[34, 35, 99] or by TGFβ2.[36, 39] The results from these experiments are inconsistent and inconclusive. One group attempted to associate Dex induced genomic and proteomic changes with Dex induced CLANs.[100] However, a similar association has not been attempted with TGFβ2 induced CLANs. Prior to identifying specific genomic or proteomic candidates for CLAN formation, it is first important to understand the TGFβ2 signaling pathway which can proceed through two primary sub-pathways known as the Smad dependent (Smad pathway) and Smad independent (non-Smad pathway). Non-Smad TGFβ signaling can occur through various alternate pathways, each of which has demonstrated significant involvement in the TM.

CHAPTER 2

REVIEW OF RELEVANT SIGNALING PATHWAYS

2.1. Transforming Growth Factor-β2 (TGFβ2) signaling pathway

TGFβ2 is a profibrotic cytokine thought to be secreted primarily by the ciliary and lens epithelium.[101] TGFβ2 exists in a latent form which must be activated to elicit downstream effects.[53, 55] Exactly how TGFβ2 is activated is not well understood but likely involves proteases or MMPs.[102] The latent form of TGFβ2 has been reported in the AH as ranging from 1.6 ng/mL[53] to 8.1 ng/mL[50] with approximately 61% of the total TGFβ2 being in the active form.[50, 53] Once activated, the ligand binds to the receptor which forms a heterotetrameric complex of the TGFβ receptor type 1 (TGFR1) dimerized with TGFβ receptor type 2 (TGFR2). The TGFR2 actually phosphorylates and activates TGFR1.[103] The TGFβ signaling can then proceed either through the Smad pathway, or the non-Smad pathway. The delineation of the exact pathway responsible for a biological response is complex, and in many cases is not solely due to one pathway. Instead, there is often considerable interaction between them.

2.2. Smad signaling pathway

In the Smad pathway, the activated TGFβ receptor phosphorylates members of the Smad family (Figure 2.3.1). For this research we are primarily focused on the branch of Smad signaling including Smad 2/3. Phosphorylated Smad 2 complexes with phosphorylated Smad 3 and 4, followed by translocation of the complex to the nucleus, where it binds with DNA and transcription factors to regulate the expression of various genes.[103] In TM cells, inhibition of the Smad pathway with a Smad 3 phosphorylation inhibitor (SIS3) blocked secretion of ECM proteins as well as FN.[30] Additionally, TGFβ2 induces upregulation of the *protein, acidic, and rich in cysteine* (*SPARC*) gene; a gene regulating the expression of the matricellular protein SPARC that is highly expressed throughout the TM, and especially at the JCT meshwork.[104, 105] TGFβ2 induced expression of SPARC was shown to be inhibited by SIS3 in human TM cell culture.[105] Inhibition of the Smad pathway with SIS3 also resulted in complete inhibition of CLAN formation in bovine TM cell culture.[79]

2.3. Non-Smad signaling pathway

Alternatively, the activated TGFβ receptor can activate other non-Smad signaling pathways in a variety of ways. There are non-Smad signaling events that can occur through the TGFβ receptor complex activating proteins which then can modulate the activity of the Smads. Alternatively, the phosphorylated Smads can activate other proteins, which then transmit further signals into the cell. Further, the receptor complex can activate proteins, which then transmit signals without direct interaction with the Smads (Figure 2.3.1).[106]

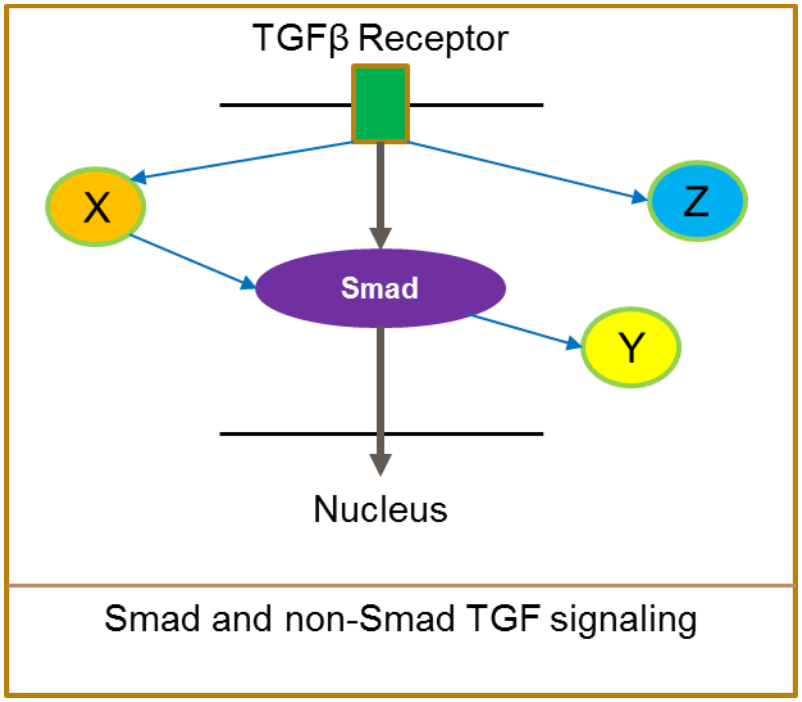

Figure 2.3.1 Smad and Non-Smad signaling pathways

The *Smad* pathway is represented by thick black arrows starting from the ligand-receptor complex and ending in the nucleus. Various *non-Smad* signaling mechanisms are shown by thin blue arrows. The receptor complex can activate protein X, which then modulates the activity of the Smad. Alternatively, protein X can activate Smad or the phosphorylated Smad can activate protein Y, which then transmits further signals into the cell. Thirdly, the receptor complex can activate protein Z, which then transmits signals without direct interaction with the Smad.[106] (Figure recreated from Moustakas et al, Cell Science, 2005).

The research in this dissertation tested various non-Smad pathways including branches of mitogen-activated protein kinase (MAPK) pathways such as extracellular signal regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and P38 kinases, as well as Rho-like guanosine triphosphatase (GTPase) signaling pathways.[107]

2.3.1. Extracellular signal regulated kinase (ERK) signaling pathway

TGFβ2 activation of the ERK signaling pathway is a result of the dual specificity of the receptor acting as both tyrosine and serine/threonine kinases. Phosphorylation of tyrosine residues in the activated receptors stimulates recruitment of various adaptor proteins such as Ras, Raf, and MEK1/2 (Figure 2.3.2).[106] ERK activation is critical for many cellular processes including cell migration and epithelial/endothelial-mesenchymal transitions (EMTs). The EMT is characterized by disruption of the cell-cell and cell-matrix adhesions, increased MMP activity for degradation of the surrounding matrix, and rearrangement of the actin cytoskeleton.[106, 107] Importantly, in some cell cultures, ERK can also phosphorylate receptor-activated Smads, including Smad2 and Smad3, to regulate their activities. Phosphorylation of Smads by ERK can also inhibit Smad activity.[107] ERK was found to decrease TGFβ activation of Smad3 potentially by decreasing the cell-surface concentrations of TGFR1.[103] Therefore the cross-talk between pathways is always an important, and sometimes necessary, factor in any biological process.

The ERK signaling pathway has demonstrated importance specifically to the TM. In TGFβ2 as well as Dex treated primary human TM cells, ERK activation is increased approximately 2 fold.[81, 100, 108] When exposed to ERK inhibition, αSMA and FN

expression are significantly decreased compared to control cells.[81] Additionally, changes in TGFβ2 induced HTM cell transdifferentiation, αSMA expression, and changes in cadherin and β-catenin expression appeared to be dependent on the ERK signaling pathway.[109] ERK activation has also been observed to increase with increasing rigidity of the substrate on which TM cells are grown which was thought to mimic the more rigid ECM of the glaucomatous TM. This increased ERK activation was again associated with increased αSMA expression and increased FN deposition.[110] Similar results are also seen with Dex induced TM changes. After 3 days or 4 weeks of exposure to Dex, primary human TM cells exhibited increased ERK phosphorylation, αSMA expression, and increased stress fiber formation. These changes were associated with a 4-fold increase in TM cell stiffness, and a 3.5-fold increase in TM tissue stiffness in the rabbit eye.[108]

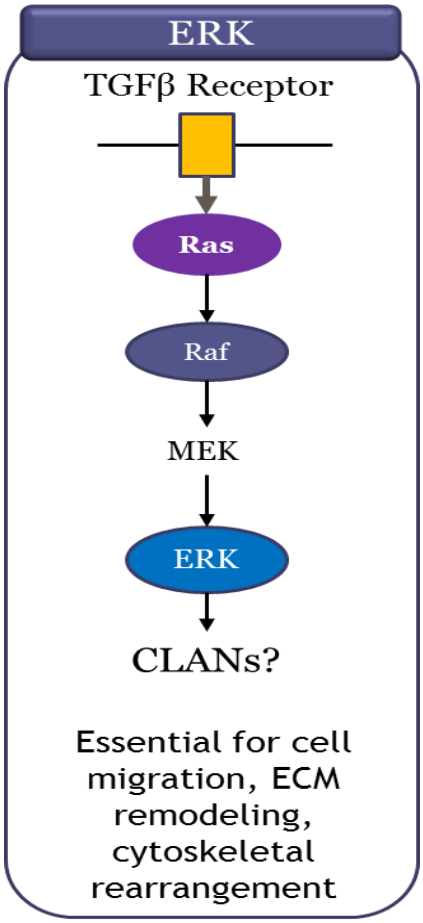

Figure 2.3.2. The ERK non-Smad signaling pathway

TGFβ can induce phosphorylation of TGFR1 and TGFR2. The phosphorylated tyrosines then recruit Grb2/Sos to activate ERK through Ras, Raf, and their downstream MAPK cascades. ERK then regulates target gene transcription through its downstream transcription factors.[107] (Figure modified from Zhang, Cell Research, 2009.)

2.3.2. c-Jun N-terminal kinases (JNK) and P38 signaling pathways

TGFβ2 activation of the JNK and P38 signaling pathways are also of interest. JNK and P38 are activated by the TGFβ receptor recruiting MAP kinase kinases (MKK) 4 and MKK3/6, respectively (Figure 2.3.3).[107] In certain cell lines, JNK and P38 activation is thought to be independent of Smad activation. This was demonstrated by utilization of a mutant TGFR1 which inhibited Smad binding and activation, but still retained its intact kinase activity and activation of JNK and P38.[111] Other potential mediators of JNK or P38 activation include TGFβ-activated kinase 1 (TAK1) and TNF receptor associated factor 6 (TRAF6).[107] Although TGFβ can induce JNK or P38 activation independently of Smads, the TRAF6-TAK1-JNK/P38 cascade is thought to function in conjunction with the Smad-dependent pathway to regulate downstream cellular responses to TGFβ, particularly in processes of apoptosis and EMT.[107]

In the TM, TGFβ2 can activate both JNK and P38 signaling pathways.[30] When the JNK or P38 signaling pathways were blocked using selective inhibitors (SP600125 or SB203580, respectively), the induction of several LOX proteins was also inhibited[40] which is important in the cross-linking of the TM cell ECM. Additionally, P38 was shown to mediate TGFβ2 upregulation via the *SPARC* gene.[104, 105]

The role of JNK and P38 pathways has also been studied in regards to their association with oxidative stress. Oxidative stress is a common pathological process associated with age-related conditions. Oxidative stress markers are found to be elevated in the AH of POAG patients.[112, 113] Deleterious effects due to oxidative stress are seen in

glaucomatous eyes including increased oxidative DNA damage,[114, 115] and other TM malfunctions including decreases in F-actin formation.[116] Inhibition of the P38 pathway demonstrated greater impact on the oxidative stress induced changes such as cell morphology while not affecting cell viability. The JNK signaling pathway seemed to be less impactful with less activation by oxidative stress.[116]

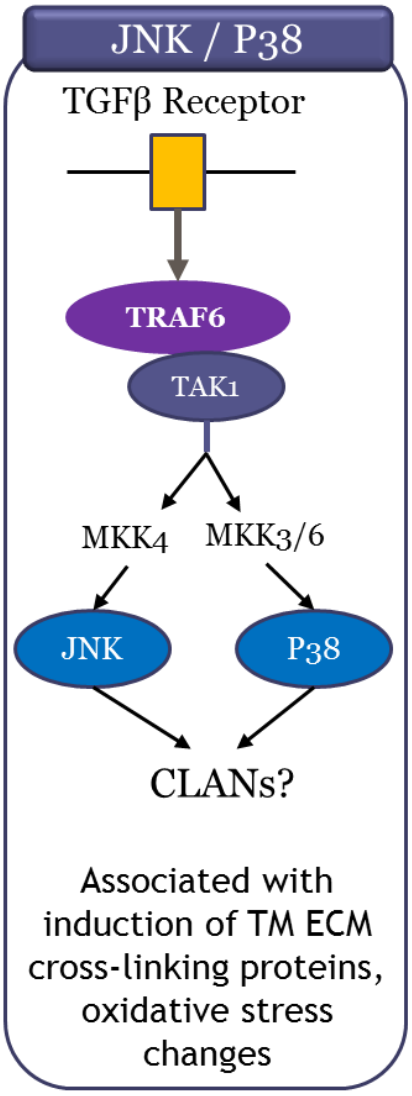

Figure 2.3.3. The JNK/P38 non-Smad pathway

TGFβ receptors interact with TRAF6 which then recruits TAK1 and MKK4 or MKK3/6 to activate JNK/P38 to then control the activities of downstream transcription factors.[107] (Figure modified from Zhang, Cell Research, 2009.)

2.3.3. Rho-like guanosine triphosphatase (GTPase) signaling pathway

Additionally, there are other non-Smad pathways that may be activated by the TGFβ receptor, such as the Rho pathway. Rho GTPases function as a molecular switch cycling between an inactive GDP-bound state and an active GTP-bound state. These Rho GTPases are believed to be involved in multiple cellular functions through phosphorylation of various substrates.[117, 118]

One of the effector molecules of Rho GTPases is known as Rho-associated coiled-coil-containing protein kinase (ROCK). ROCK is present in two isoforms called ROCKI and ROCKII. The isoforms are structurally conserved with approximately 64% overall amino acid identity, and over 90% conserved at the kinase domain suggesting they have similar substrate specificity.[117, 119, 120] ROCK I is thought to be essential for stress fiber formation while ROCK II is thought to mediate cytoskeletal rearrangements, cell motility, and cell contraction.[117] The activity of ROCKs is regulated by GTPase activating proteins (GAPs), while guanine nucleotide-exchange factors (GEFs) catalyze the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP). Furthermore, the guanine nucleotide-dissociation inhibitors (GDIs), sequester GTPases in the cytosol in the inactive, GDP-bound state.

The catalytic domain of ROCK is located at the amino terminus, followed by a coiled-coil-forming region and a pleckstrin-homology domain with a cysteine-rich repeat at the carboxyl terminus (Figure 2.3.4). In the case of ROCK, the carboxy-terminal coiled-coil region also encompasses the Rho-binding domain (RBD).[119] There is an auto-inhibitory

regulation of ROCK in that the C-terminal domain folds back onto the kinase domain. This forms an auto-inhibitory loop that maintains ROCK in an inactive state. The exchange of GDP to GTP bound Rho at the RBD results in activation of ROCK in response to extra cellular signals.[119] Rho activates ROCK, which then phosphorylates and activates or inactivates other molecules. The regulation of these molecules results in changes in cell contractility, cell shape, focal adhesions, and actin polymerization.[107] For example, ROCK phosphorylates MLC phosphatase (MLCP) keeping it inactive thereby maintaining MLC in its active state to facilitate contractile properties. Likewise, ROCK phosphorylates LIM kinase which phosphorylates and inactivates cofilin thereby reducing actin depolymerization and shifting the equilibrium toward actin polymerization.[66]

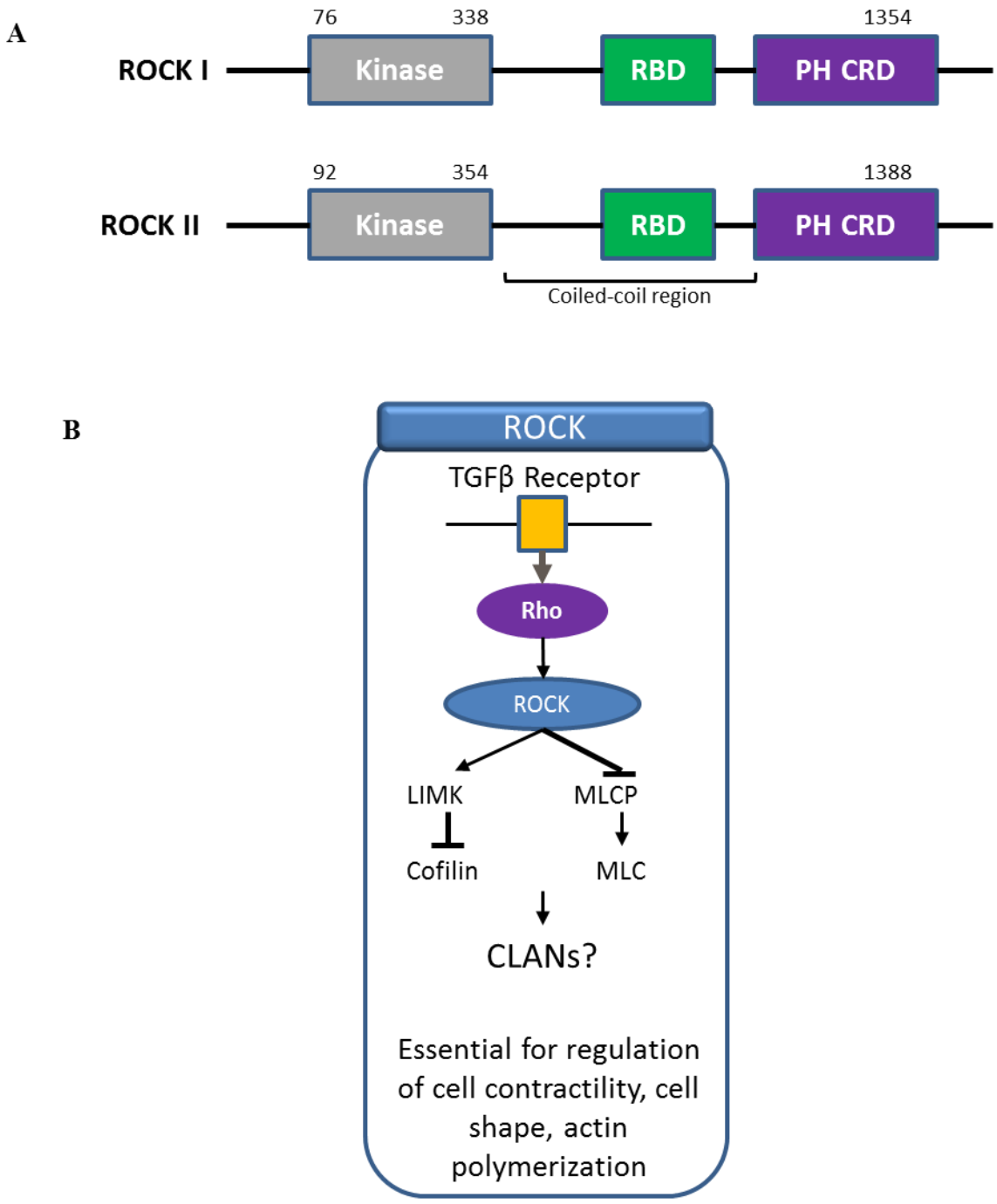

Figure 2.3.4. The ROCK signaling pathway

Figure 2.3.4. The ROCK signaling pathway

A) Molecular structure of ROCK I and II. The catalytic domain is located at the amino terminus, followed by a coiled-coil-forming region including the Rho-binding domain (RBD), and a pleckstrin-homology domain (PH) with a cysteine-rich repeat domain (CRD) at the carboxyl terminus. **B)** GTP-bound Rho activates ROCK which then leads to phosphorylation of various target proteins, especially those that regulate actin stress fiber formation and cellular contractility. ROCK phosphotylates LIM kinase (LIMK) which phosphorylates and thereby inhibits cofilin, resulting in actin polymerization. ROCK also phosphorylates myosin light chain phosphatase (MLCP) thereby inhibiting its activity which minimizes MLC inactivation therefore allowing for MLC induced cellular contraction.[119] (Figures modified from Mueller et al, Nature Reviews Drug Discovery, 2005.)

ROCKII is preferentially expressed in the brain, whereas ROCKI shows the highest expression levels in non-neuronal tissues, including heart, lung and skeletal muscles. Both ROCK I and ROCK II are expressed in the TM and ciliary muscle of humans and monkeys, with more abundant expression in the TM.[25] Even though Rho and ROCK expression was found in the TM, JCT and in Schlemm's canal, there does not appear to be a difference in expression of Rho or ROCK in the AH outflow tissues among glaucomatous and non-glaucomatous eyes.[121] However, Rho and ROCK are also expressed in the ONH with increased expression of Rho GTPases and ECM proteins in the ONH and ONH astrocytes of glaucomatous eyes compared to non-glaucomatous eyes.[121] Therefore, the effect of the Rho signaling pathway in glaucoma may go beyond just the TM itself.

ROCK inhibitors are currently in clinical trials to evaluate their safety and efficacy in reducing IOP in glaucomatous patients. Only the ROCK inhibitor named Ripasudil (also known as K-115) has recently received marketing approval in Japan, as an adjunct to prostaglandin analogues.[122] The effect of ROCK inhibitors on the TM is primarily due to relaxation of the TM by disruption of actin stress fibers, as well as decreasing inhibition of MLCP which regulates the activity of MLC in regards to cellular contraction.

Y27632 is another commonly used ROCK inhibitor in non-clinical settings. Y27632 is highly selective in blocking the effects of ROCK I and ROCK II.[118] Experiments have shown that treatment with 10μM Y27632 for 3 hours increased aqueous outflow facility

in porcine eyes by 40%.[72] This was further supported in non-glaucomatous human TM and Schlemm's canal cell culture which confirmed ROCK inhibition caused decreases in actin stress fibers and morphological changes to the cells[123] as soon as 30 min to 1 hour after initiating treatment.[72, 118] Cells exhibited a contracted appearance and significant decreases in actin stress fiber formation and focal adhesions, however the cells remained viable[124] suggesting the increase in outflow facility is not due to TM cell death. These effects of ROCK inhibition were reversible after 15-24 hours of withdrawal of the Y27632.[72, 125] Similar IOP lowering effects have been shown with the use of a dominant negative mutant of Rho-kinase to inhibit the activation of ROCK.[73] Y39983, another ROCK inhibitor thought to be more potent with the same selectivity as Y27632, has also demonstrated the ability to decrease IOP in monkey eyes with an effect similar to that of latanoprost, a commonly prescribed prostaglandin analogue IOP lowering medication.[126] Consistent with these findings with ROCK inhibitors, experiments conducted with constitutively active RhoA (RhoAV14) have shown an increase in AH resistance and increases in IOP.[127-129]

Because of the significance of each of the mentioned signaling pathways in the TM, each must be evaluated to determine their level of involvement in CLAN formation.

2.4. Hypothesis and Aims

Various groups have studied potential key proteins or genes that are modulated in glaucomatous eyes compared to non-glaucomatos eyes,[38] as well as changes induced by

Dex[34, 35, 99] or TGFβ2.[36, 39] The results from these experiments are inconsistent and inconclusive.

This research took the available information and extended it to further explore CLAN formation and its potential role in increased aqueous outflow resistance and IOP elevation by exploring the contribution of each TGFβ associated pathway. Since much focus in CLAN research has been on CLAN induction with Dex, this research focused primarily on the effect of TGFβ2 which would more closely mimic the natural POAG scenario.

The overall goal for this research was to determine whether the Smad or non-Smad TGFβ pathway is primarily involved in TGFβ2 induced CLAN formation. Therefore, **it was hypothesized that TGFβ2 induced CLAN formation is a result of both the Smad and non-Smad TGFβ pathways in human trabecular meshwork cells.**

To test the hypothesis, the study included two specific aims:

Specific Aim 1: Demonstrate the effect of Smad or non-Smad TGFβ pathway inhibition on TGFβ2 induced CLAN formation.

1.1 *Determine if inhibition of the Smad or non-Smad TGFβ pathway prevents TGFβ2 induced CLAN formation.*

Non-glaucomatous primary human TM cell cultures were treated for up to 10 days in the presence of both TGFβ2 and various inhibitors, and quantification of the resulting incidence of CLAN formation. This was done in 3 cell strains.

1.2. *Determine if inhibition of the Smad or non-Smad TGFβ pathway is able to resolve already formed TGFβ2 induced CLANs*.

Non-glaucomatous primary human TM cell cultures were treated for up to 10 days in the presence of TGFβ2, followed by 1 hour exposure to the various inhibitors. The incidence of CLANs was evaluated to determine the extent of CLAN resolution. This was done in 2 cell strains.

Specific Aim 2: Determine the effect of ROCK inhibition on Dex induced CLAN formation.

2.1 *Determine if ROCK inhibition prevents or resolves Dex induced CLAN formation*.

Similar methodology as described in Specific Aims 1.1 and 1.2 was used, however with the inclusion of Dex (10^{-7} M) for CLAN induction.

CHAPTER 3

TGFB2 INDUCES THE FORMATION OF CROSS-LINKED ACTIN NETWORKS (CLANS) IN HUMAN TRABECULAR MESHWORK CELLS THROUGH THE SMAD AND NON-SMAD DEPENDENT PATHWAYS

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Abbot F. Clark, Weiming Mao

Submitted to Investigative Ophthalmology & Vision Science (IOVS)

3.1. Abstract

Purpose

Increased intraocular pressure results from increased aqueous humor (AH) outflow resistance at the trabecular meshwork (TM) due to pathological changes including association with cross-linked actin networks (CLANs) formation. TGFβ2 is elevated in the AH and TM of primary open angle glaucoma (POAG) patients, and induces POAG-associated TM changes, including CLANs. We determined the role of individual TGFβ2 signaling pathways in CLAN formation.

Methods

Cultured non-glaucomatous human TM (NTM) cells were treated with control or TGFβ2, with or without the inhibitors of TGFβ receptor, Smad3, JNK, ERK, P38 or ROCK. NTM cells were co-treated with TGFβ2 plus inhibitors for 10 days, or pretreated with TGFβ2 for 10 days followed by 1 hour inhibitor treatment. NTM cells were immunostained with phalloidin-Alexa-488 and DAPI. Data were analyzed using one-way ANOVA and Dunett's post-hoc test.

Results

TGFβ2 significantly induced CLAN formation ($n=6-12$, $p<0.05$), which was completely inhibited by TGFβ receptor, Smad3, and ERK inhibitors, as well as completely or partially inhibited by JNK, P38, and ROCK inhibitors, depending on cell strains. One hour exposure to ROCK inhibitor completely resolved formed CLANs ($p<0.05$), while TGFβ receptor, Smad3 inhibitor, and ERK inhibitors resulted in partial or complete

resolution. JNK and P38 inhibitors showed partial or no resolution. Among these inhibitors, the ROCK inhibitor was the most disruptive to the actin stress fibers, while ERK inhibition showed the least disruption.

Conclusions

TGFβ2-induced CLANs in NTM cells were prevented and resolved using various pathway inhibitors. Apart from CLAN inhibition, some of these inhibitors also had different effects on actin stress fibers.

3.2. Introduction

Glaucoma is a progressive optic neuropathy affecting both the anterior and posterior segments of the eye, and is a leading cause of irreversible vision loss and blindness worldwide. It is estimated that glaucoma will affect over 80 million people by the year 2020.[8-10] The most common form of glaucoma is primary open-angle glaucoma (POAG), which is characterized by painless, progressive, and irreversible vision loss. Although the exact disease mechanism of POAG is not fully understood, elevated intraocular pressure (IOP) is the primary risk factor for the development and progression of POAG. In these patients, IOP elevation is caused by increased aqueous humor (AH) outflow resistance at the trabecular meshwork (TM). This increased resistance has been associated with loss of TM cells, excessive extracellular matrix (ECM) composition, and cytoskeletal reorganization.[11-16, 18, 42]

We are particularly interested in the actin cytoskeletal reorganization in TM cells. We first reported the formation of cross-linked actin networks (CLANs) in both HTM cells and tissues.[16-18] CLANs are three dimensional, geodesic dome-like structures formed primarily around the nucleus, although they can also be found throughout the cell. In two dimensional microscopic views, CLANs appear to be web-like structures and composed of numerous "hubs and spokes". Although cultured cell types other than TM cells form transient CLAN-like structures during the process of cell attachment and spreading,[88-92] only TM cells form and retain CLANs when they are confluent.[17]

In cultured TM cells, CLAN formation can be induced by glucocorticoids as well as by transforming growth factor β2 (TGFβ2). TGFβ2 is elevated in the AH and TM of POAG patients, and induces ocular hypertension in perfusion cultured human and porcine anterior segments as well as in mouse eyes.[14, 32, 37, 52, 53, 63, 130] In cultured TM cells, TGFβ2 induces the expression of ECM proteins such as fibronectin (FN) and factors that suppress proteolytic degradation of the ECM.[61, 62, 131] Additionally, TGFβ2 increases the expression of ECM cross-linking enzymes such as lysyl oxidase (LOX)[30] and transglutaminase-2.[54]

We have previously identified an association between CLANs and POAG by comparing glaucomatous human TM (GTM) cells to non-glaucomatous human TM (NTM) cells.[42, 86] In that study, we observed that approximately 40% of the GTM cells contain CLANs while only 4% of the NTM cells have CLANs. Specific mathematical models predict that CLANs increase the stiffness of actin filaments by 2 orders of magnitude.[96] Other studies showed that GTM tissues are stiffer compared to NTM tissues.[97, 98] Although those studies did not evaluate the direct correlation between CLAN formation and TM stiffness, it is believed that CLANs increase AH outflow resistance and IOP by increasing TM stiffness as well as disturbing TM homeostasis.[17]

TGFβ signaling has two primary pathways for signal transduction: the Smad dependent (Smad pathway) and independent (Non-Smad pathway) pathways. Both pathways are activated by TGFβ2 binding to the TGFβ receptor complex, which is then autophosphorylated. In the Smad pathway, the activated receptor complex

phosphorylates Smad 2 and Smad 3, which then binds with Smad 4 prior to translocation to the nucleus. The non-Smad pathway includes various branches of mitogen-activated protein kinase (MAPK) pathways such as extracellular signal regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and P38 kinases, as well as Rho-like guanosine triphosphatase (GTPase) signaling pathways.[107]

Rho-associated protein kinase (ROCK) inhibitors have the ability to reduce trabecular outflow resistance and IOP.[72, 73, 80, 125, 126, 132] The Rho/ROCK signaling pathway acts as a molecular switch in the regulation of focal adhesions, cellular contraction, cellular motility, cytokinesis, and the formation of actin stress fibers.[117] It is believed that the ocular hypotensive effect of ROCK inhibitors is due to "relaxation" of the TM cytoskeleton.[80, 117, 123, 129] Inhibition of ROCK decreases actin polymerization, relaxes the TM cells, and decreases outflow resistance. However, whether ROCK inhibition affects CLAN formation is not clear.

Because of the complexity of the TGFβ signaling, the delineation of which exact pathway is responsible for a particular biological response is difficult to predict. In the present study, we utilized NTM cells and various pathway inhibitors to determine the role of individual TGFβ signaling pathways in the formation of CLANs.

3.3. Methods

NTM cell cultures

Two primary NTM cell strains (NTM1022-02, and NTM30A) were used for these experiments and were generated and maintained as previously published.[16, 17, 42] Cells were plated onto 12 mm glass coverslips and grown to 100% confluency in low glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco Life Technologies, Grand Island, NY) containing 10-20% fetal bovine serum (FBS; Atlas Biologicals Products, Fort Collins, CO) and penicillin (100 units/ml), streptomycin (0.1 mg/ml), and L-glutamine (0.292 mg/ml; Invitrogen-Gibco Life Technologies).

Cell Treatments

Treatments were administered in high glucose DMEM with 1% FBS. High glucose medium was used to improve cell survival, and a low concentration of FBS minimized confounding effects of endogenous growth factors that may be present in the serum. Confluent NTM cells were treated with TGFβ2 (5 ng/ml) (RD Systems, Minneapolis, MN) to induce CLAN formation, or DMEM with or without DMSO (vehicle control for MAPK inhibitors). To inhibit the Smad pathway, NTM cells were co-treated with TGFβ2 and TGFβ receptor type I inhibitor SB431542 (5 µM; Sigma, Saint Louis, MO)[30] or the Smad3 phosphorylation inhibitor SIS3 (10 µM; Sigma).[30] To inhibit the non-Smad pathways, NTM cells were co-treated with TGFβ2 and the JNK inhibitor SP600125 (10 µM; CalBioChem, San Diego, CA),[30] MEK/Erk inhibitor U0126 (25 µM; Promega, Maddison, WI),[81] P38 inhibitor SB203580 (5µM; Tocris BioSci, Ellisville, MO),[30] or ROCK inhibitor Y27632 (10 µM; Sigma).[72, 80, 81, 124] All these inhibitors

were used in previous TM studies and have demonstrated successful pathway inhibition at the concentrations described previously. Cells were co-treated with TGFβ2 together with pathway inhibitors for 10 days for studying the prevention of CLAN formation, or pre-treated with TGFβ2 for 10 days followed by treatment with individual inhibitors for 1 hour to study CLAN resolution. Each treatment group consisted of 6-12 coverslips (n=6-12). Medium was changed every 2-3 days.

Epifluorescent staining of CLANs

NTM cells were fixed with 2% paraformaldehyde in phosphate buffered saline (PBS), washed with PBS, permeabilized using 0.5% Triton X-100, and blocked with Superblock (Thermo Scientific). F-actin was stained with Phalloidin conjugated with Alexa-488 (1:100; Life Technologies, Eugene, OR) for 1 hour at room temperature. After PBS washes, cover slips were mounted onto slides using ProLong Gold Anti-Fade with DAPI (Life Technologies) for nuclear counter staining.

Evaluation of CLANs

CLANs were visualized using the Nikon Eclipse Ti inverted fluorescence microscope (Nikon, Inc., Melville, NY) with 600X magnification. Cytoskeletal images were taken using the Nikon Eclipse Ti inverted fluorescence microscope equipped with the Cri Nuance FX Camera System (Perkin-Elmer, Inc., Waltham, MA).

CLANs were defined as an F-actin-containing cytoskeletal structure with at least one triangulated actin arrangement consisting of actin spokes and at least 3 identifiable

hubs.[86] Representative images of CLANs are shown in Figure 3.3.1 A-C. Each coverslip was assessed at 10 locations (Figure 3.3.1 D) with approximately 100-150 cells per coverslip. 6-12 coverslips were per treatment group.

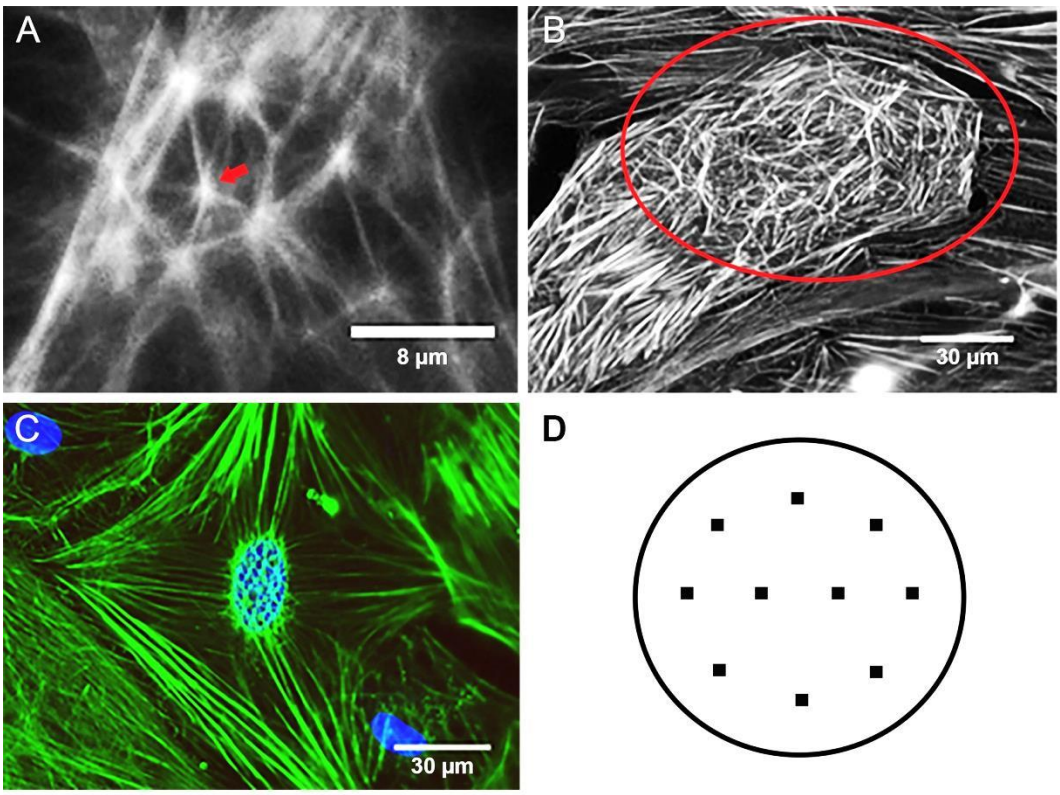

Figure 3.3.1. Morphology and evaluation of CLANs

(A) Representative image of a single CLAN in an NTM cell. CLANs consist of distinct hubs (red arrow) and F-actin "spokes", forming a dome-like structure. A minimum of 3 hubs creating at least one triangulated actin arrangement can be counted as a CLAN. **(B)** Representative image of a multiple CLANs within a single cell (600X magnification). **(C)** Representative image of multiple CLANs at the perinuclear region (600X magnification). Blue: DAPI. **(D)** CLAN formation (percentage of CPCs) in ten representative areas (dots) of each coverslip was evaluated.

All CLAN counting was done in a masked manner. CLAN positive cells (CPCs) were defined as any cell containing at least one CLAN, or multiple CLANs. The formation of CLANs was compared by using the percentage of CPCs, which is calculated by dividing the number of CPCs by the number of DAPI positive cells.

Statistical Analysis

The percentage of CPCs was compared using one-way ANOVA followed by Dunnett's multiple comparisons post-hoc test (GraphPad Prism 6.02; GraphPad Software, Inc., La Jolla, CA). Data are presented as mean ± standard error of means (SEM), with the significance level set at $p<0.05$.

3.4. Results

Smad and non-Smad pathway inhibitors prevented CLAN formation

We first studied whether inhibition of Smad and/or non-Smad pathways would inhibit CLAN formation. We treated human NTM cells with TGFβ2 together with inhibitors against the TGFβ pathways (SB431542), the Smad pathway (SIS3), the ERK pathway (U0126), the JNK pathway (SP600125), the P38 pathway (SB203580), or the ROCK pathway (Y27632). Because CLAN formation has been shown to peak after 10-14 days of TGFβ2 exposure,[79] we treated NTM cells for 10 days to ensure CLAN induction. Data are presented as the percentage of CPCs.

In NTM30A cells receiving vehicle controls (medium alone or medium with DMSO), the percentage of CPCs was 1.44% (±0.19%, SEM) and 1.62% (±0.14%), respectively

(Figure 3.4.1 A). These data are similar to our previous reports.[42] In contrast, TGFβ2 treated TM cells had 28.40% (±1.87%) CPCs ($p<0.0001$ vs. controls), confirming that TGFβ2 is a potent CLAN inducer.

Co-treatment with TGFβ2 and TGFβ receptor inhibitor (SB431542) or inhibitor of the Smad signaling pathway (SIS3) decreased the percentage of CPCs to 0.68% (±0.24%) and 2.7% (±0.65%), respectively ($p<0.0001$ vs. TGFβ2), showing their complete inhibition of TGFβ2-induced CLAN formation (Figure 3.4.1 A).

Different from the Smad pathway, inhibition of the non-Smad pathway had different effects on CLAN formation (Figure 3.4.1 A). The ERK pathway inhibitor (U0126) and ROCK pathway inhibitor (Y27632) resulted in 3.84% (±0.74%) and 5.33% (±1.66%) CPCs ($p<0.0001$ vs. TGFβ2), respectively, which demonstrated a complete inhibition of TGFβ2-induced CLAN formation. However, the JNK pathway inhibitor (SP600125) and P38 pathway inhibitor (SB203580) resulted in only partial inhibition of CLAN formation with 21.90% (±1.72%) and 8.17% (±1.29%) CPCs ($p<0.001$ vs. TGFβ2, and $p<0.001$ vs. control). Treatment with inhibitors alone did not significantly change CLAN formation at the basal level ($p>0.05$, Figure 3.4.1 A).

The formation of CLANs was mostly consistent in the two NTM cell strains studied. The exceptions in NTM1022-02 cells are 1) the ROCK pathway inhibitor showed partial inhibition of CLAN formation; and 2) the P38 pathway inhibitor alone slightly elevated

CLAN formation ($p<0.05$ vs Control) (Figure 3.4.1 B). These data suggest that the response of TM cells to individual pathway inhibitors may be strain-dependent.

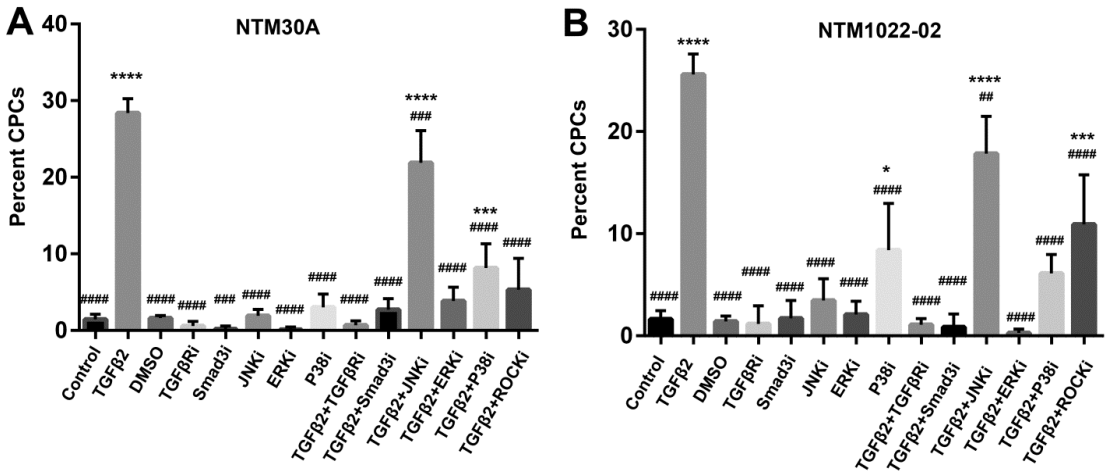

Figure 3.4.1. Prevention of CLAN formation in NTM cells by TGFβ pathway inhibitors

NTM30A **(A)** and NTM1022-02 **(B)** cells cultured on glass coverslips (n=6-12) were treated with control or TGFβ2 with or without indicated TGFβ Smad or non-Smad pathway inhibitors for 10 days. Percentage of CPCs was compared using one-way ANOVA with Dunnet's multiple comparisons post-hoc test. Columns and bars: means and SEM. *: $p<0.05$ the group of interest vs. control, ***: $p<0.001$, ****: $p<0.0001$; ##: $p<0.01$ the group of interest vs.TGFβ2, ###: $p<0.001$, ####: $p<0.0001$. TGFβRi: TGFβ receptor inhibitor (SB431542; 5μM); SMAD3i: Smad3 phosphorylation inhibitor (SIS3; 10 μM); JNKi: JNK pathway inhibitor (SP600125; 10 μM); ERKi: ERK pathway inhibitor (U0126; 25 μM); P38i: P38 pathway inhibitor (SB203580; 5 μM); ROCKi: ROCK pathway inhibitor (Y27632; 10 μM).

TGFβ pathway inhibitors resolved already formed CLANs

Although we found that TGFβ pathway inhibitors prevented TGFβ2-induced CLAN formation, it is still unclear whether they can also resolve already formed CLANs. Therefore, we pretreated NTM cells with TGFβ2 for 10 days to induce CLANs, and then treated them with individual inhibitors for 1 hour. Co-treatment of TGFβ2 and those inhibitors for 10 days was also incorporated as a positive control.

In both TM cell strains, we found that 1 hour treatment with ROCK inhibitor completely removed already formed CLANs, resulting in 0.00% (±0.00%, NTM30A) and 2.25% (±0.72%, NTM1022-02) CPCs ($p<0.0001$ vs. TGFβ2) (Figure 3.4.2 A and B).

In contrast, the effects of the inhibition of non-ROCK pathways are more diverse. In NTM30A cells, the TGFβ receptor inhibitor (SB431542), Smad3 inhibitor (SIS3), and ERK inhibitor (U0126) partially resolved preformed CLANs, with 19.05% (±4.64%), 16.61% (±2.83%), and 23.51% (±2.36%) CPCs (all $p<0.05$ vs. TGFβ2, and $p<0.0001$ vs. control), respectively (Figure 3.4.2 C). However, JNK or P38 inhibition did not show significant resolution of formed CLANs ($p>0.05$ vs. TGFβ2). The results from NTM1022-02 cells are similar, except that the TGFβ receptor inhibitor showed complete inhibition and the JNK inhibitor showed partial inhibition (Figure 3.4.2 D).

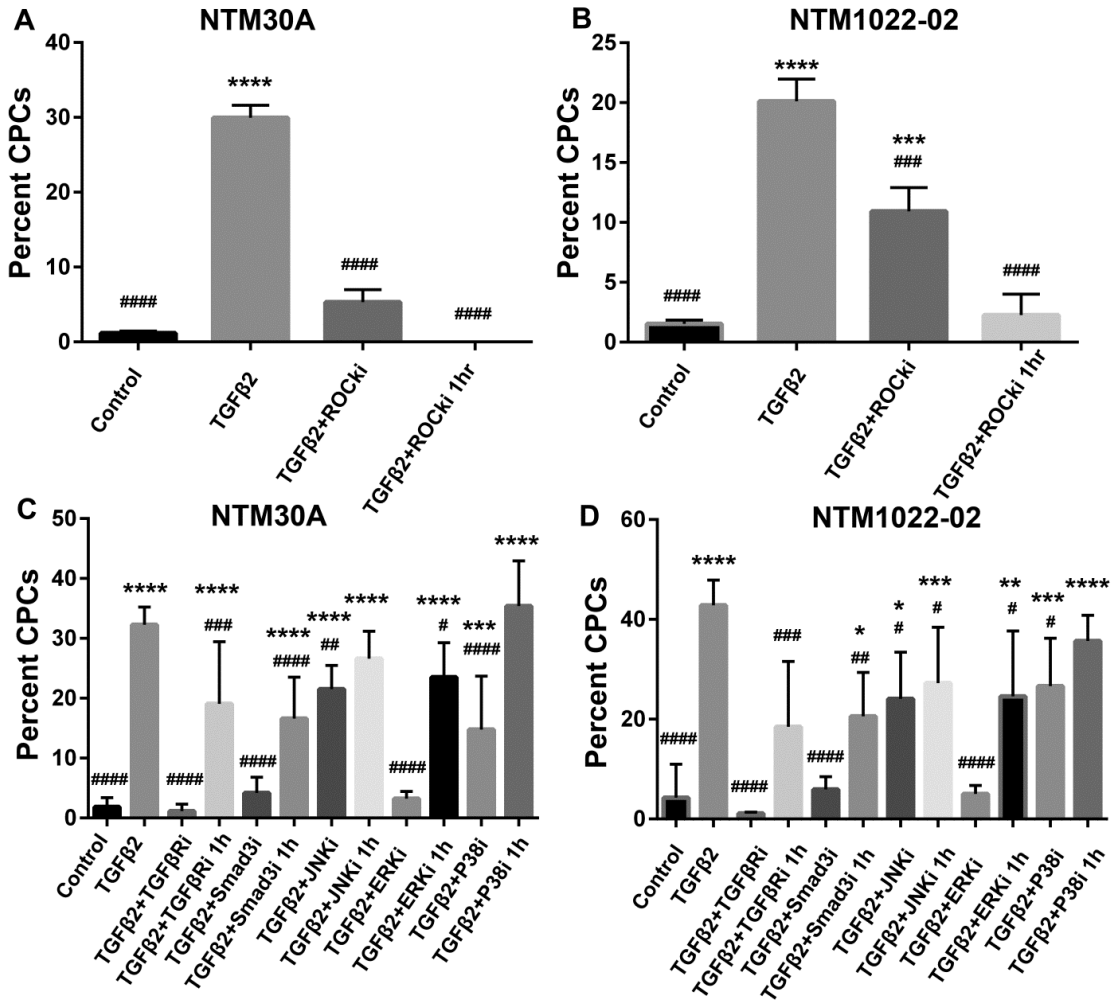

Figure 3.4.2. Resolution of CLAN formation in NTM cells by TGFβ pathway inhibitors

Figure 3.4.2. Resolution of CLAN formation in NTM cells by TGFβ pathway inhibitors

NTM cells cultured on glass coverslips (n=6) were pretreated with TGFβ2 for 10 days, followed by 1 hour treatment of the indicated inhibitors. Some cells were also co-treated with TGFβ2 and the indicated inhibitor for 10 days as a positive control. Percentage of CPCs was compared using one-way ANOVA with Dunnet's multiple comparisons post-hoc test. Columns and bars: means and SEM. *: $p<0.05$ group of interest vs. control, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$; #: $p<0.05$ for the group of interest vs. TGFβ2, ##: $p<0.01$, ###: $p<0.001$, ####: $p<0.0001$. TGFβRi: TGFβ receptor inhibitor (SB431542; 5μM); SMAD3i: Smad3 phosphorylation inhibitor (SIS3; 10 μM); JNKi: JNK pathway inhibitor (SP600125; 10 μM); ERKi: ERK pathway inhibitor (U0126; 25 μM); P38i: P38 pathway inhibitor (SB203580; 5 μM); ROCKi: ROCK pathway inhibitor (Y27632; 10 μM).

Differential effects of Smad and non-Smad pathway inhibition on actin stress fiber formation

It is unclear whether the TGFβ pathway regulates CLANs and actin stress fibers in the same manner. Therefore, we evaluated the effects of individual TGFβ pathway inhibitors on actin stress fibers in TM cells. It is our belief that a compound that is able to prevent/remove CLANs but has minimal impact on normal actin cytoskeleton structures such as stress fibers will treat one of the glaucomatous pathologies (CLANs), while potentially preserving/restoring the normal actin cytoskeleton.

As previously reported,[81] TGFβ2 increased and reorganized actin fiber formation compared to control (Figure 3.4.3). TGFβ receptor and Smad3 inhibitors restored actin stress fibers to baseline levels (vs. control). JNK or P38 inhibitors had little effect on TGFβ2-induced stress fibers. The ERK inhibitor, although it did not block TGFβ2-induced stress fibers, seemed to preserve the normal arrangement of those fibers. Different from these other inhibitors, 1 hour treatment with the ROCK inhibitor almost completely eliminated of stress fibers. However, some stress fibers reformed after 10 day co-treatment with TGFβ2 plus ROCK inhibitor.

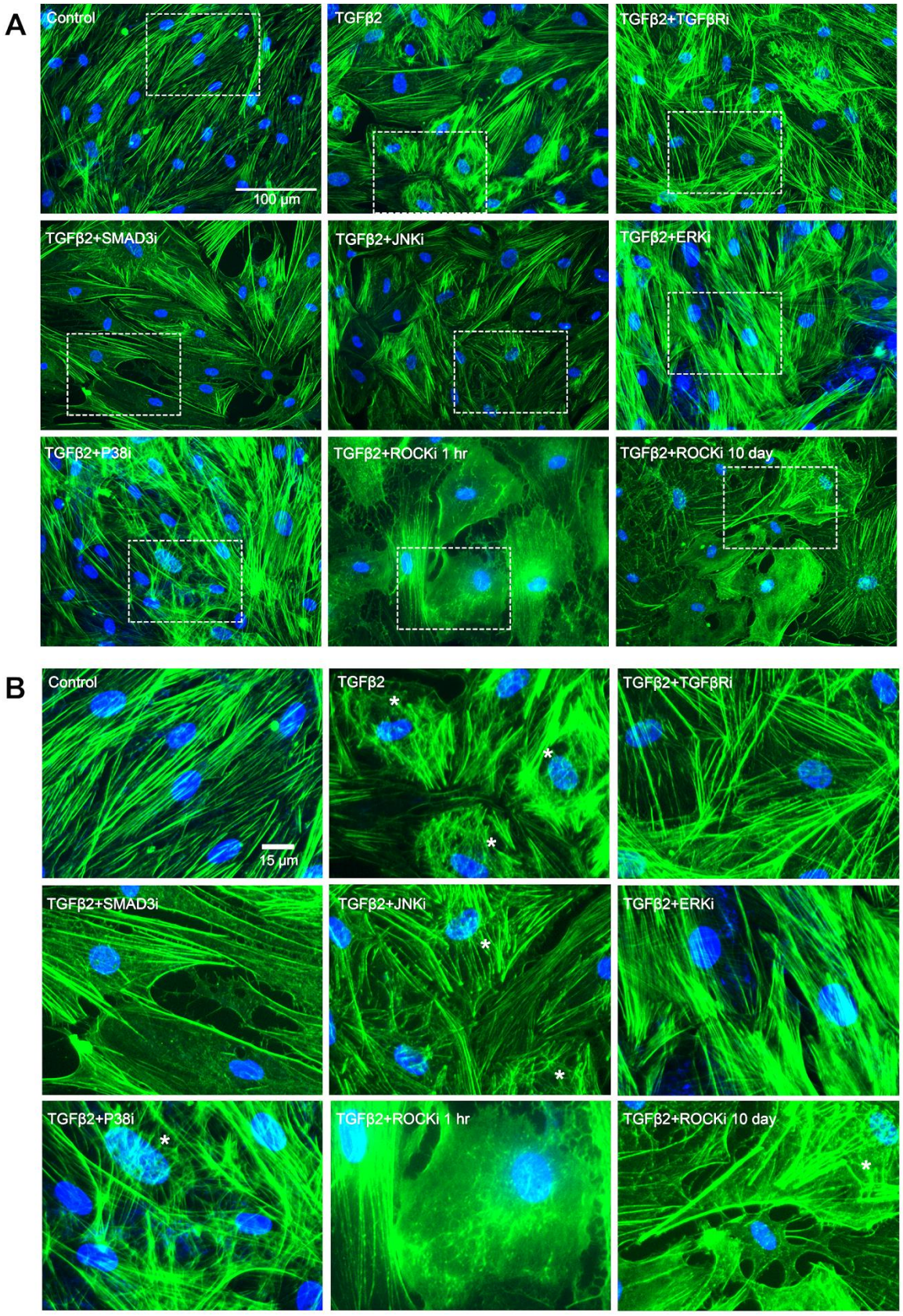
A
Control
TGFβ2
TGFβ2+TGFβRi
100 μm
TGFβ2+SMAD3i
TGFβ2+JNKi
TGFβ2+ERKi
TGFβ2+P38i
TGFβ2+ROCKi 1 hr
TGFβ2+ROCKi 10 day
B
Control
TGFβ2
TGFβ2+TGFβRi
15 μm
TGFβ2+SMAD3i
TGFβ2+JNKi
TGFβ2+ERKi
TGFβ2+P38i
TGFβ2+ROCKi 1 hr
TGFβ2+ROCKi 10 day

Figure 3.4.3. The effects of TGFβ pathway inhibitors on stress fiber formation in NTM cells

NTM cells cultured on glass coverslips (n=6) were co-treated with TGFβ2 and the indicated inhibitors for 10 days. Some cells were treated with TGFβ2 for 10 days, followed by 1 hour ROCK inhibitor treatment. Cells were fixed and used for immunofluorescent staining. **A)** Representative images of actin stress fibers upon inhibitor treatment. **B)** Enlarged images from corresponding areas in A. Asterisks: CLANs. TGFβRi: TGFβ receptor inhibitor (SB431542; 5μM); Smad3i: Smad3 phosphorylation inhibitor (SIS3; 10μM); JNKi: JNK pathway inhibitor (SP600125; 10μM); ERKi: ERK pathway inhibitor (U0126; 25μM); P38i: P38 pathway inhibitor (SB203580; 5μM); ROCKi: ROCK pathway inhibitor (Y27632; 10μM).

3.5. Discussion

In this study, we found that TGFβ2-induced CLANs in NTM cells could be both prevented and resolved using different TGFβ pathway inhibitors. These inhibitors had different effects on actin stress fibers, which did not completely match their effects on CLANs.

Our results suggest that TGFβ2 induced CLAN formation occurs via both the Smad and non-Smad TGFβ pathways (summarized in Figure 3.5.1). However, the contribution of individual TGFβ2 signaling pathways to CLAN formation seems to be different. In the two NTM cell strains studied, both the Smad pathway and non-Smad ERK pathways seemed to play major roles in TGFβ2-induced CLAN formation. The JNK and P38 pathway only partially participated in CLAN induction, while the contribution of the ROCK pathway was cell strain dependent. However, the overall trend was consistent.

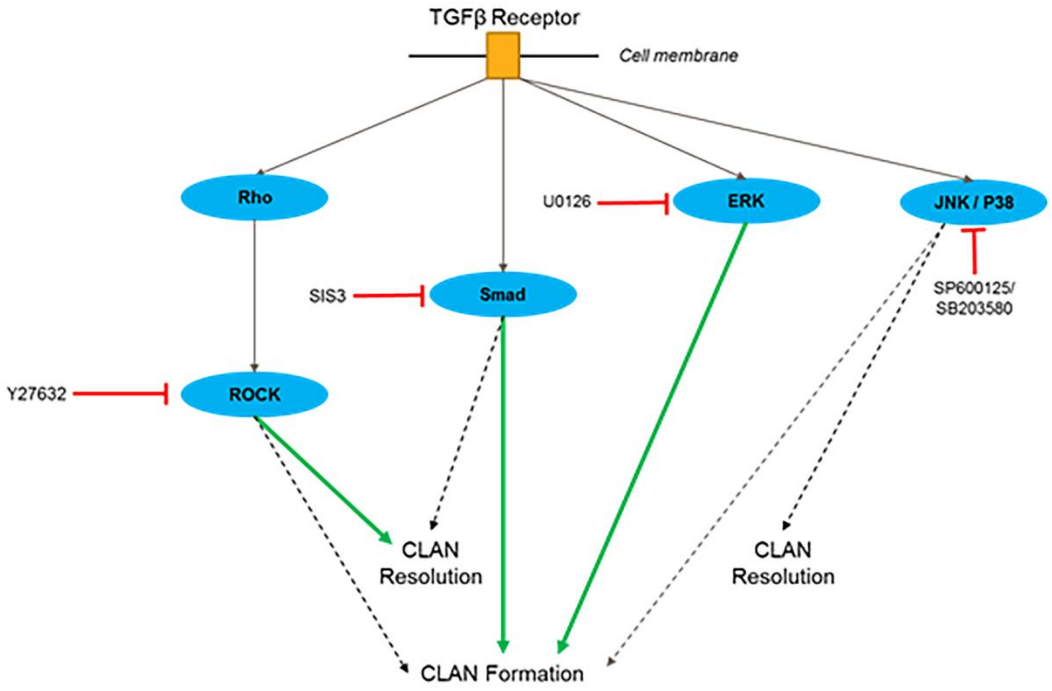

Figure 3.5.1. Hypothesized roles of the TGFβ2 pathway and inhibitors in CLAN formation

Solid green lines: pathways that may play major roles in CLAN formation or maintenance; Dotted lines: pathways that may play minor roles in CLAN formation or maintenance.

O'Reilly and colleagues previously showed that in bovine TM cells, the TGFβ receptor inhibitors LY-364997 and SB431542 partially inhibited TGFβ2-induced CLAN formation,[79] which is different from our findings where we found total inhibition. However, the Smad3 inhibitor SIS3 completely inhibited CLAN formation, which is consistent with our observations in NTM cells. The difference between the two studies may be due to the difference in species, suggesting that for results generated in non-human TM cells/tissues, a confirmation in human TM is necessary.

We also studied whether different TGFβ pathway inhibitors could remove already formed CLANs, which to our best knowledge, has not been previously determined. It surprised us that only 1 hour treatment with TGFβ receptor inhibitor, Smad, ERK and ROCK inhibitors completely or partially removed already formed CLANs. Since 1 hour is generally believed to be too short to affect both transcription and translation, these changes are very likely to be due to alterations in protein modification, especially protein phosphorylation. Alternatively, CLAN formation may be a more dynamic process than initially thought, and TGFβ signaling is required to maintain CLANs. Therefore, even a short-term disruption of the TGFβ pathway is able to disrupt CLANs.

Whether the formation of CLANs and actin stress fibers share a similar mechanism is not clear. Our findings suggest that each TGFβ signaling pathway had different impacts on actin stress fiber formation, which was different from their impact on CLANs. Although it is known that relaxation of the actin cytoskeleton (disassembly of actin stress fibers) is a valid approach to lower IOP (as seen with ROCK inhibitors), it still remains unknown if

long-term disruption of normal actin stress fibers will impair TM function. Also, the most common side effect with the use of ROCK inhibitors is ocular hyperemia because they also "relax" conjunctival blood vessels similar to their effect on the TM. Therefore, if a compound is able to remove CLANs with minimal or no changes in actin stress fibers, it may have ocular hypotensive effects with less side effects such as hyperemia.

This is our first attempt to dissect the role of individual TGFβ signaling pathways in CLAN formation. There are still many unanswered questions.

Firstly, it is still unclear whether these individual pathways work independently or in a synergistic manner. We found that inhibition of either the Smad or Non-Smad pathways was able to completely inhibit CLAN formation. Therefore, we believe that these pathways are more likely to work synergistically. If they do work together, it will be important to determine at which molecules this cross-regulation occurs.

Secondly, the downstream molecules of the TGFβ pathway that mediate CLAN formation are not entirely unclear. Peters and colleagues performed a proteomic study using spreading TM cells.[100] The authors first treated confluent TM cells with dexamethasone (DEX) to induce CLAN formation or with ethanol as a vehicle control. Then they dissociated and seeded TM cells into fibronectin coated dishes. TM proteins were fractionated and compared using mass spectrometry. They observed a change in 318 cytoskeletal proteins, some of which contained phosphorylated residues suggesting DEX affects the expression of these proteins at the transcriptional and translational levels.

However, many adherent cell types form transient CLANs when they are first attaching and spreading, while only TM cells form and retain CLANs when they are confluent. Therefore, it is still unclear whether the CLANs formed during cell spreading share the same signaling pathway and biological components as the CLANs formed in confluent cells.

Thirdly, although both glucocorticoids and TGFβ2 are able to induce morphologically similar CLANs via different pathways, it is unknown if TGFβ2-induced CLANs and DEX-induced CLANs are biochemically and/or mechanically identical. If the mechanisms are the same, there must be a set of common mediator molecules shared by the two pathways.

And finally, the role of connective tissue growth factor (CTGF) in CLAN formation has not yet been determined. CTGF is a profibrotic cytokine that mediates TGFβ2 signaling in the TM.[133] CTGF is among the most highly expressed genes in the TM.[134] It is elevated in the AH of glaucoma patients[133, 135] and plays a critical role in cell migration, adhesion, proliferation, matrix production, and mediates several of the downstream actions of TGFβ.[136] Overexpression of CTGF in a mouse model induces ocular hypertension and optic nerve damage.[137] ROCK inhibitors also affect both TGFβ2 and CTGF-induced cellular and cytoskeletal changes in a similar manner.[137, 138] Therefore, more research is needed to determine the effect of CTGF and its inhibitors on CLAN formation.

In summary, we found that different TGFβ signaling pathways play different roles in the inhibition and disassembly of CLANs as well as in actin stress fiber formation. This information may help in the development of novel ocular hypotensive agents.

Acknowledgement

This research was supported by National Eye Institute 5R21EY023048 (W.M.).

CHAPTER 4

EVALUATION OF DEXAMETHASONE INDUCED CLAN FORMATION

4.1. Role of Glucocorticoids in Glaucoma and CLAN formation

Glucocorticoids (GC) play an important role in the treatment of some diseases and conditions, yet also appear to play an important role in development of ocular hypertension and POAG.[45-48] Human TM cells contain GC receptors and are therefore able to respond to endogenous corticosteroids such as cortisol, as well as exogenous GCs such as Dex. The TM response to GCs occurs in a dose, time, and potency dependent manner.[21, 42, 45, 139, 140] Patients who experience an ocular hypertensive response to GCs are referred to as "steroid responders", and are thought to occur in approximately 30-40% of the general population.[41]

With the use of GCs, several TM changes have been observed including morphological changes to the TM and TM cells. The effects of prolonged Dex treatment (approximately three weeks or more) include:

- Proliferation and activation of the endoplasmic reticulum and Golgi apparatus resulting in an increase in TM cell nuclear size and DNA content[141]
- Average TM cell size increased by 50-100%[21]

- Increased ECM deposition including expression of ECM collagen, laminin, elastin, GAGs, and FN[21, 45]
- Reduced expression and activity of extracellular proteinases which are involved in ECM turnover[142]
- Decreased phagocytic activity of TM cells[44, 95]
- Decreased cellular motility/migration[17]
- Impaired cytokinesis[21, 45]
- Altered integrin expression[78, 143, 144]
- Realignment of gap junction complexes[21, 141]

Gene and protein expression are also altered by GCs.[34, 35, 99] One of importance is induction of the glaucoma gene myocilin (MYOC). This induction is of importance because of its association not only in GC induced changes, but also because MYOC mutations are found in approximately 4% of POAG patients.[45]

In addition to the general cellular effects discussed above, exposure to GCs also induces CLAN formation[16, 17, 42, 78, 100] in approximately 30-70% of human TM cells after exposure to Dex.[17] The appearance of Dex induced CLANs can range from the traditional geodesic dome-like structure to "actin tangles".[45] Dex induced CLAN formation steadily increases over 10-14 days, and resolves within 5-7 days after withdrawal. This CLAN formation is also associated with a gradual increase in IOP over several weeks of Dex exposure.

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The GC induced changes summarized in Figure 4.1.1, are similar to TM changes seen in POAG, and all contribute to increases in AH outflow resistance and therefore ocular hypertension.[21] Dex induced CLAN formation and ocular hypertension is also summarized in Figure 4.1.2.

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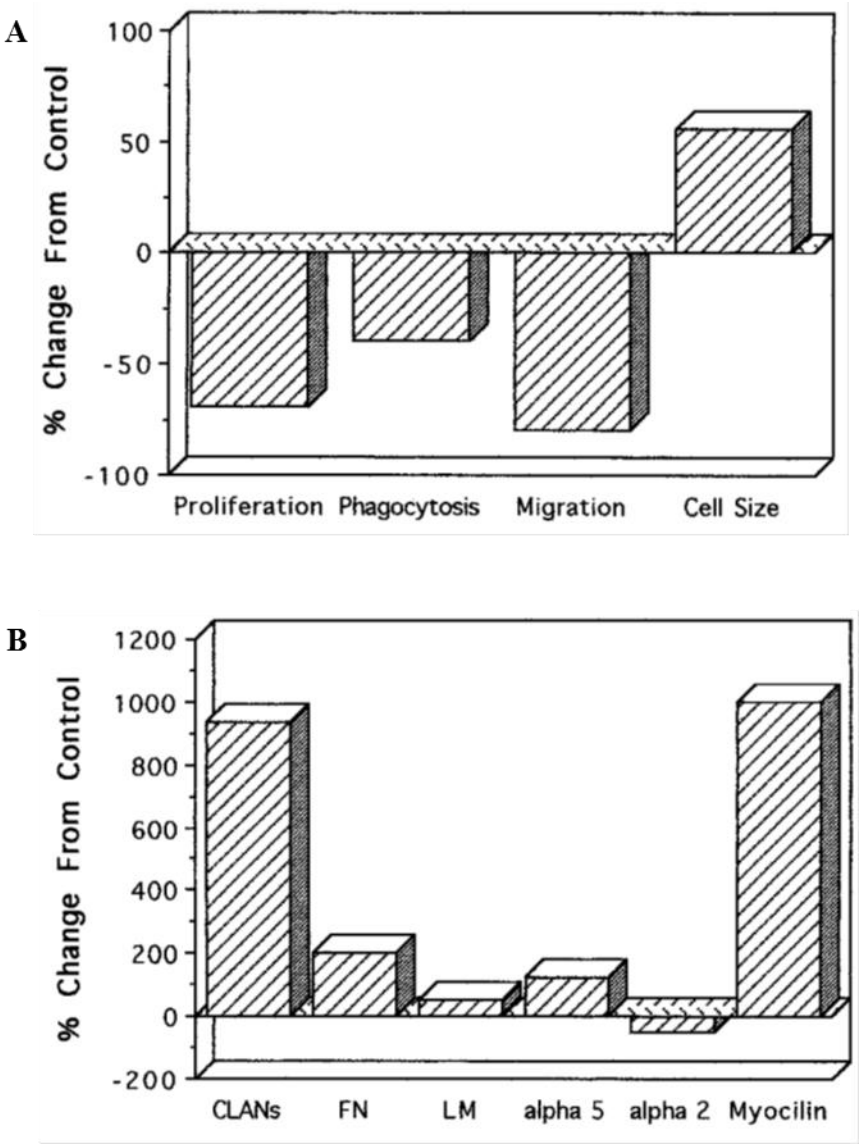

Figure 4.1.1. Summary of glucocorticoid induced changes in cultured TM cells

A) Representative quantified inhibition of cellular proliferation, phagocytosis, migration, and cell size after GC exposure. **B)** Representative quantification of alterations in CLAN formation, expression of fibronectin (FN), laminin (LM), integrins (α5 and α2 subunits), and myocilin.[21] (Figure from Wordinger et al, Progress in Retinal and Eye Research, 1999).

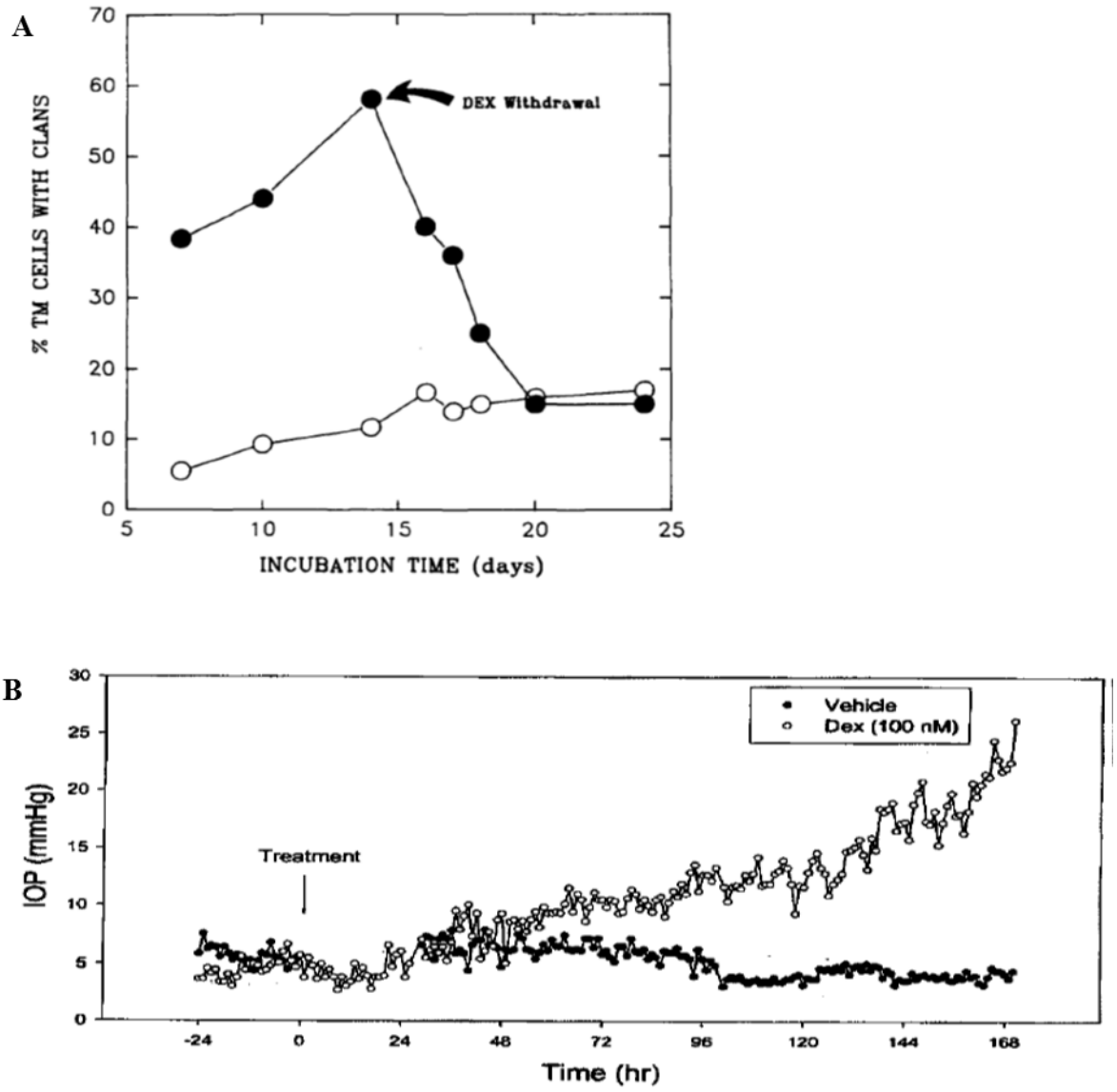

Figure 4.1.2. Dexamethasone induced CLAN formation and IOP elevation

A) Effect of Dex on CLAN formation, followed by resolution of CLANs after withdrawal of Dex. Human TM cells were cultured to confluence, and then treated with Dex (10^{-7} M) for 14 days prior to removal of Dex from the media.[17] (Figure from Clark et al, IOVS, 1994) **B)** Effect of Dex on IOP versus vehicle in one pair of perfusion cultured Dex responder human eyes.[16] (Figure from Clark et al, Cell Motility and the Cytoskeleton, 2005).

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4.2. Glucocorticoid Signaling Mechanisms

The biological effects of GCs are mediated through the GC receptor referred to as glucocorticoid receptor alpha (GRα). In the absence of ligand, GRα is in a heterocomplex with heat shock proteins (HSP) and immunophilins which play a significant role in protein folding and trafficking within the cell.[145] The heterocomplex facilitates a three dimensional folding that maintains the hormone binding domain (HBD) at the C-terminus in a high affinity state to attract ligand when present. Additionally, there are two zinc fingers surrounding the DNA-binding domain (DBD) which facilitate and help maintain the DNA binding to the target gene.[21]

Ligand binding to the GR induces a conformational change that allows the receptor to detach from the remaining complex of HSPs. This dissociation exposes the nuclear localization signals within the ligand binding domain in order to facilitate nuclear translocation. The GR dimerizes prior to interacting with the glucocorticoid response element (GRE) in the promoter region of the target genes. Transcription factors then coordinate altered transcription of target genes, leading to translation of the target genes' specific proteins.[21, 146]

Alternatively, GCs can also inhibit gene activation. In this case, the ligand-activated GR may bind to negative glucocorticoid response elements (nGRE) that result in inhibition of transcription of a gene. The activated GR is then translocated to the nucleus but forms a trimer rather than a dimer, and blocks messenger ribonucleic acid (mRNA) transcription

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by inactivation of transcription factors, and thereby inhibiting protein translation.[21] Both GC signaling mechanisms are illustrated in Figure 4.2.1.

The potential mechanism of POAG-like alterations in the TM of some patients upon exposure to GCs may be through transrepression. The activated GR binds directly to the activator protein-1 (AP-1) transcription factor, which inhibits expression of collagenase and other MMPs, resulting in decreased ECM turnover. Additionally, Dex may suppress specific growth factors that are normally regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and are essential for proper TM function.[21] GCs may also activate other signaling mechanisms that have not yet been fully elucidated that result in cytoskeletal rearrangements and CLAN formation.

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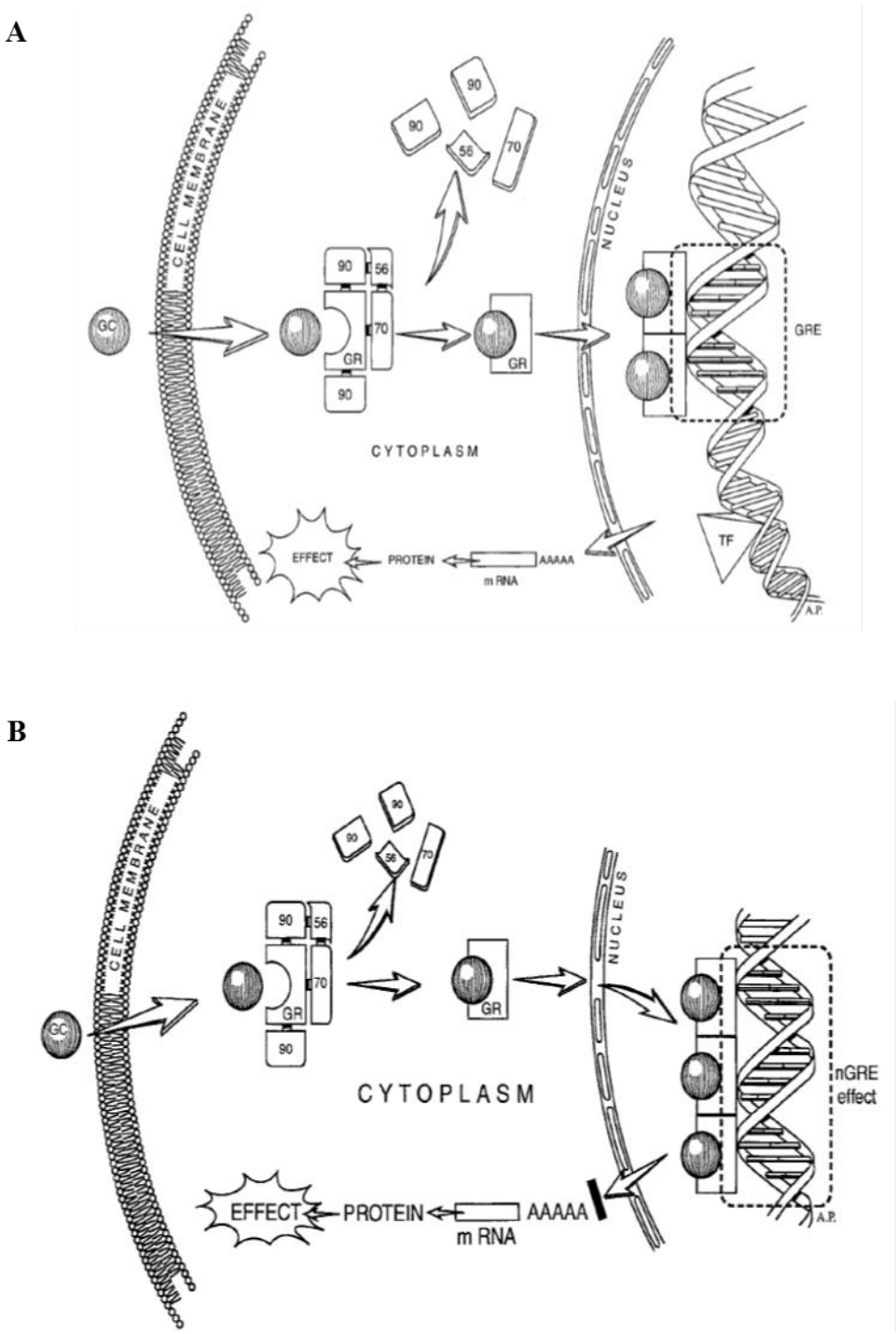

Figure 4.2.1. Illustration of glucocorticoid signaling mechanisms

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Figure 4.2.1. Illustration of glucocorticoid signaling mechanisms

A) The GC passes through the cell membrane and binds to the GC receptor (GR) heterocomplex in the cytoplasm. The heat shock proteins (HSP) are released and the activated GR complex is translocated to the nucleus. After dimerization, the GR binds to the glucocorticoid response element (GRE) located within the promotor region of the target gene. Transcription factors (TF) then dictate altered transcription of the target gene and translation of a specific protein. **B)** GC interaction can also lead to inhibition of transcription and translation by the activated GR translocating to the nucleus and forming a trimer. The GR then binds to the negative GRE (nGRE) of a specific gene. This binding then blocks mRNA transcription and protein translation.[21] (Figure from Wordinger et al, Progress in Retinal and Eye Research, 1999).

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4.3. Dexamethasone Induced CLAN Formation

Dex induced CLAN formation has been evaluated in TM cell culture as well as TM tissue.[16-18, 77-79, 82-85] Even though the effects of CLAN formation are well established, the mechanism of their formation is not well understood, and may differ based on the inducing agent. It has been suggested that CLAN hubs contain α-actinin, as well as proteins that regulate actin polymerization including phosphatidylinositol 4,5-bisphosphate (PIP_2), and syndecan-4.[78, 147] Tropomyosin-1 which is involved in actin filament contraction, and filamin which is an actin cross-linking protein, were also suggested to be components primarily of CLAN filaments, even though some localization at the hubs was also observed.[78] Of note, these components of CLANs have been identified with Dex induced CLAN formation in non-confluent human TM cell culture when cells were in the process of spreading. CLAN formation has been observed in other cell types during the process of cell spreading and attachment.[17, 88-92] However only in TM cells[17] and lamina cribrosa,[93] a high incidence of CLANs can be seen in confluent cells and tissue. It is unknown if all CLANs, regardless of the inducing agent or confluency of the cells, are initiated by the same mechanism or made of the same components.

Filla and colleagues suggested Dex induced CLAN formation may be associated with the Rac1 GTPase pathway through β-integrin signaling.[77, 144] Rac1 is a small GTPase known to regulate the polymerization of branched actin filaments during cell migration and cell spreading.[144] Even though Rac1 is a member of the family of Rho GTPases, there has been significant focus on the effect of ROCK inhibitors and their potential to

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decrease IOP.[72, 80, 125, 126, 132, 148] The association of this ROCK induced decrease in outflow resistance and IOP to Dex induced CLAN formation has not been established. Furthermore, whether CLAN formation and the effect of ROCK inhibition is the same in Dex induced versus TGFβ2 induced CLAN formation is not known. Therefore, an initial study was conducted to determine the effect of ROCK inhibition on prevention, as well as resolution of Dex induced CLAN formation.

4.4. Dexamethasone Induced CLAN Formation and Resolution

4.4.1. Methods

NTM cell cultures

Cells from a primary human TM cell strain (NTM176-04) were plated on 12 mm glass coverslips and grown to 100% confluency in low glucose DMEM containing 10% FBS and penicillin (100 units/ml), streptomycin (0.1 mg/ml), and L-glutamine (0.292 mg/ml).

Cell Treatments

Treatments were administered in high glucose DMEM with 0.5% FBS. Confluent NTM176-04 cells were treated with TGFβ2 (5 ng/ml) or Dex (10^{-7} M) to induce CLAN formation. TGFβ2 was included in this experiment as a positive control since ROCK inhibitors are known to lower IOP in glaucomatous eyes in which there are elevated levels of TGFβ2. Negative controls included DMEM alone or with 0.1% Ethanol (vehicle control for Dex). Inhibition of the ROCK pathway was done with Y27632 (10 μM).[72, 80, 81, 105, 124] Cells were treated with Dex or TGFβ2 for 8 days, then with ROCK inhibitor for 1 hour or 24 hours in order to study CLAN resolution. For studying

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the prevention of CLAN formation, some cells were co-treated with either Dex or TGFβ2 together with ROCK inhibitor for 8 days. Each treatment group consisted of 3 coverslips (n=3). Medium was changed every 2-3 days.

Epifluorescent staining of CLANs

NTM cells were fixed with 2% paraformaldehyde in phosphate buffered saline (PBS), washed with PBS, permeabilized using 0.5% Triton X-100, and blocked with Superblock. F-actin was stained with Phalloidin conjugated with Alexa-488 (1:100) for 1 hour at room temperature. After PBS washes, cover slips were mounted onto slides using ProLong Gold Anti-Fade with DAPI for nuclear counter staining.

Evaluation of CLANs

CLANs were visualized using the Nikon Eclipse Ti inverted fluorescence microscope with 400X magnification. Cytoskeletal images were taken using the Nikon Eclipse Ti inverted fluorescence microscope equipped with the Cri Nuance FX Camera System.

CLANs have been defined as an F-actin-containing cytoskeletal structure with at least one triangulated actin arrangement consisting of actin spokes and at least 3 identifiable hubs.[86] Each coverslip was assessed at the same 5 locations yielding on average approximately 300-400 cells evaluated per coverslip, resulting in approximately 900-1200 cells evaluated per treatment.

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All CLAN counting was done in a treatment masked manner. CLAN positive cells (CPCs) were defined as any cell containing at least one CLAN, or multiple CLANs. The formation of CLANs was compared by using the percentage of CPCs, which is calculated by dividing the number of CPCs by the number of DAPI positive cells.

Statistical Analysis

The percentage of CPCs was compared using one-way ANOVA followed by Dunnett's multiple comparisons post-hoc test (GraphPad Prism 6.02). Data are presented as mean ± standard error of means (SEM), with significance level set at $p<0.05$.

Differences in Methods

This study preceded the study described in Chapter 3 (Aim 1). Through the course of conducting multiple experiments with primary cell cultures for the assessment of CLANs, there were several learning points which slightly modified some of the methods. Therefore the differences in methods between this study and that in Chapter 3 are:

- This study used 0.5% FBS high glucose medium for administration of cell treatments. Increasing the concentration of FBS to 1% was noted to improve cell survival for the duration of the experiment while still avoiding potential confounding factors due to endogenous growth factors. Therefore, after this study was complete, 1% FBS high glucose medium was used for future experiments.

- There was some difficulty visualizing CLANs in some cases when using 400X magnification, especially when assessing TGFβ2 treated cells due to the notable

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increase in actin stress fibers. Therefore, after this study was complete, a 60X objective lens was obtained. Visualization of CLANs at 600X magnification allows for focusing at different depths and better visualization of the CLANs that may be underneath the abundant stress fibers.

- Because 400X was utilized in this study, there were more cells present within a single field location. Therefore, five locations were assessed for CLAN counting rather than the 10 locations used with 600X magnification.

- Induction of CLAN formation was done in this study for 8 days since CLAN formation is known to occur within at least 7-10 days of Dex or TGFβ2 exposure. In order to ensure full CLAN induction, subsequent experiments utilized 10 days of treatment.

4.4.1. Results

ROCK inhibition does not alter Dex induced CLAN formation

Since ROCK inhibitors have demonstrated IOP lowering capabilities and significant alterations to the actin cytoskeleton, the objective of this study was to assess the effect of ROCK inhibition on CLAN formation. Some cells were treated with Dex or TGFβ2 plus ROCK inhibitor (Y27632 10 μM) for 8 days in order to test prevention of CLAN formation. Other cells were treated with Dex or TGFβ2 for 8 days followed by either 1 hour or 24 hour ROCK inhibitor treatment in order to test the effect of ROCK inhibition on already formed CLANs. This short term ROCK inhibition was selected based on

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literature reports of reduced stress fiber formation within 30 min to 1 hour of exposure ROCK inhibitors.[72, 118] Data are presented as the percentage of CPCs.

NTM176-04 cells treated with control (DMEM alone or with 0.1% Ethanol for TGFβ2 and Dex controls respectively) resulted in similar percent CPCs as have been shown previously[42] with 3.13% (±0.65%, SEM) for Ethanol treated cells and 3.38% (±0.53%) for DMEM treated cells (Figure 4.4.1). CLAN induction was observed with both Dex and TGFβ2 with 17.17% (±3.36%; $p<0.0004$ compared to ethanol treated cells) and 7.49% (±1.90%; $p=0.0139$ compared to control) CPCs respectively.

Co-treatment with Dex and ROCK inhibitor for 8 days did not significantly prevent CLAN formation with 10.09% (±2.02%; $p>0.05$ compared to Dex alone) CPCs. However, 8 days co-treatment of TGFβ2 and ROCK inhibitor completely inhibited CLAN formation with 1.93% (±0.82%; $p<0.01$ compared to TGFβ2 alone) CPCs. A similar differential effect on resolution of Dex and TGFβ2 induced CLANs was observed. With 1 hour and 24 hour treatment, no significant reduction was seen in the Dex induced CLANs with 12.17% (±1.76%) after 1 hour ROCK inhibitor treatment, and 13.33% (±2.72%) CPCs ($p>0.05$ compared to Dex alone) after 24 hours. Conversely, ROCK inhibitor completely eliminated already formed TGFβ2 induced CLANs after both 1 hour and 24 hours of ROCK inhibitor treatment ($p<0.0001$ and $p=0.0004$ respectively compared to TGFβ2 alone).

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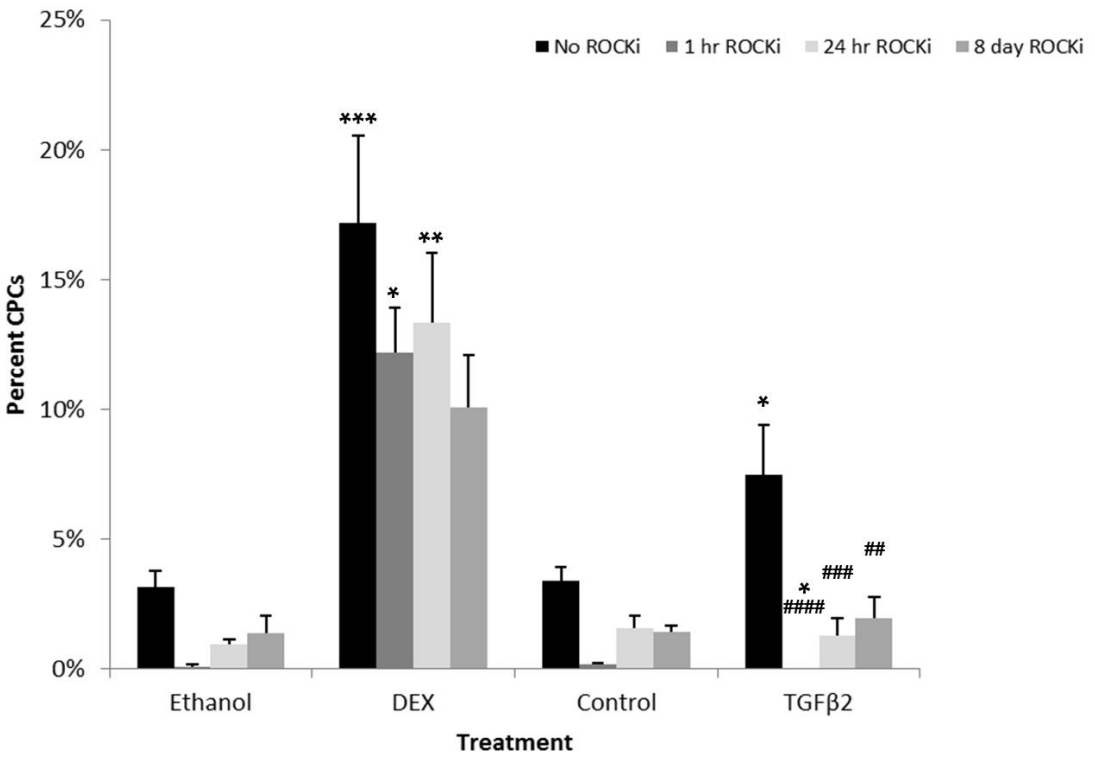

Figure 4.4.1. Effects of ROCK inhibition on Dex induced CLANs

NTM176-04 cells cultured on glass coverslips (n=3) were treated with control (DMEM alone, or with 0.1% Ethanol as Dex control), or Dex (10^{-7} M), or TGFβ2 (5 ng/mL) with or without ROCK inhibitor for 8 days. Some cells were treated with Dex or TGFβ2 for 8 days followed by 1 hour or 24 hour ROCK inhibitor. Percentage of CPCs was compared using one-way ANOVA with Dunnet's multiple comparisons post-hoc test. Columns and bars: means and SEM. *: $p<0.05$ the group of interest vs. the respective control, **: $p<0.01$ vs. control, ***: $p<0.001$ vs. control; ##: $p<0.01$ for comparisons between the group of interest and TGFβ2, ###: $p<0.001$ vs. TGFβ2, ####: $p<0.0001$ vs. TGFβ2. *Dex*: Dexamethasone; *ROCKi*: ROCK pathway inhibitor (Y27632; 10 μM).

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Effects of ROCK inhibition on stress fiber formation in Dex treated cells

During the evaluation of the effect of ROCK inhibition on CLAN formation, it was noted there was also an effect of ROCK inhibition on the actin stress fibers. Actin stress fiber formation has been documented as being altered with exposure to ROCK inhibitor (10 μM Y27632) within 30 minutes, and subsequent recovery of stress fiber formation with continued exposure.[118] In Dex treated cells, there was not a notable reduction in stress fibers as was seen in the Ethanol treated cells. Even after 1 hour or 24 hours (24 hour data not shown) of ROCK inhibitor treatment, Dex treated cells seemed to maintain abundant stress fibers although with notable rearrangement. On the contrary, TGFβ2 treated cells experienced a significant loss of stress fibers in the first hour of ROCK inhibitor exposure. This impact on stress fiber formation appeared to decrease with the longer exposures (Figure 4.4.2 and Figure 4.4.3). These observations regarding the actin stress fibers in TGFβ2 treated cells are consistent with the observed data previously discussed in Chapter 3.

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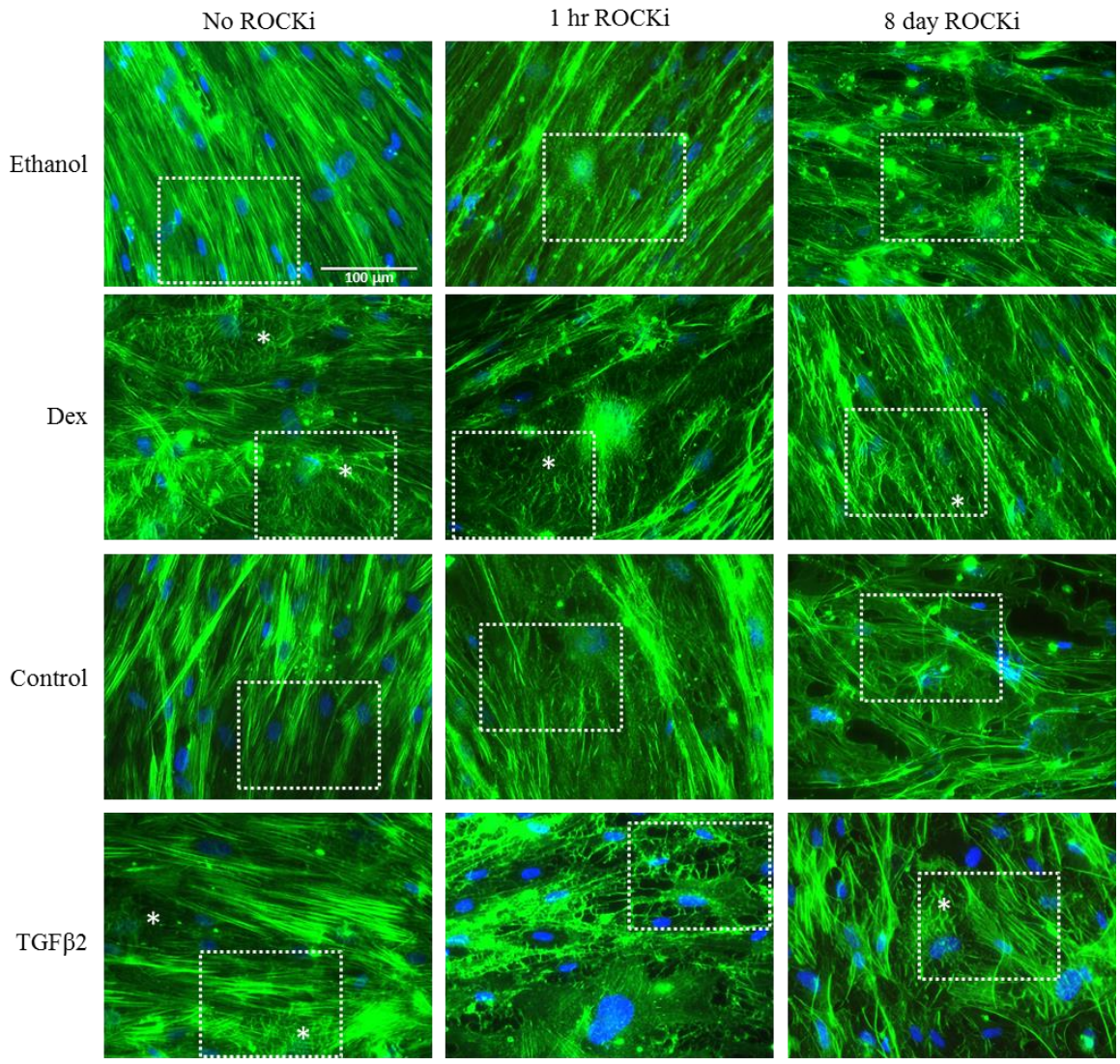

Figure 4.4.2. Effects of ROCK inhibitor on stress fiber formation in Dex and TGFβ2 treated cells

NTM cells cultured on glass coverslips (n=3) were co-treated with Dex or TGFβ2 with or without ROCK inhibitor. Some cells were treated with Dex or TGFβ2 for 8 days, followed by 1 hour ROCK inhibitor treatment. Cells were fixed and stained with Phalloidin conjugated with Alexa-488 with DAPI nuclear staining. *Asterisks*: CLANs. *ROCKi*: ROCK pathway inhibitor (Y27632; 10µM).

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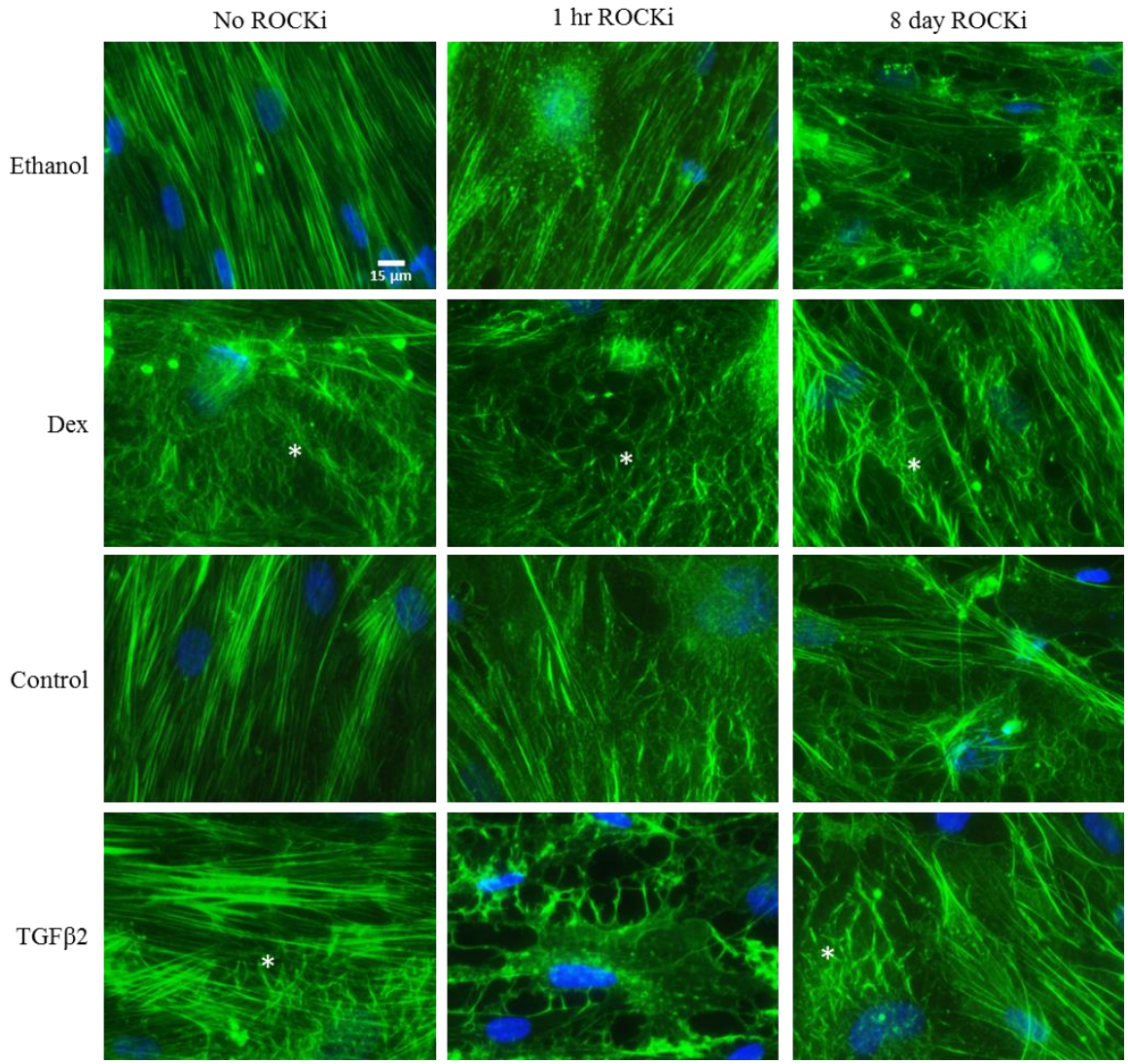

Figure 4.4.3. Enlarged images of effects of ROCK inhibitor on stress fiber formation in Dex and TGFβ2 treated cells

Same images as in Figure 4.4.2, but enlarged to show detail. Less stress fiber disruption is seen in Dex plus ROCK inhibitor treatment compared to controls, or TGFβ2 plus ROCK inhibitor. Dex induced CLANs can still be seen even after 1 hour of ROCK inhibitor treatment. *Asterisks*: CLANs. *ROCKi*: ROCK pathway inhibitor (Y27632; 10µM).

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4.4.3. Discussion

In this study, Dex induced CLANs were neither inhibited nor resolved with ROCK inhibitor treatment. Likewise, there was a minimal effect of ROCK inhibition on actin stress fiber formation in Dex treated cells. Conversely, ROCK inhibitor treatment resolved and prevented TGFβ2 induced CLAN formation, and had a more notable alteration to actin stress fibers.

Dex and TGFβ2 have very different mechanisms by which they cause their downstream effects. Dex must bind with its receptor and translocate to the nucleus to induce changes in gene transcription and translation. The lack of response to 1 hour ROCK inhibitor treatment with Dex treated cells would be consistent with the mechanism of action which would result in a "slow on" and "slow off" of steroid effect. TGFβ2 does not necessarily require nuclear translocation in order to induce its effects. The resolution of CLANs with only 1 hour of ROCK inhibitor treatment would indicate an event occurring that does not involve an alteration in transcription or translation. However, it does raise the question as to if Dex and TGFβ2 induced CLANs are formed by a similar mechanism and share common proteins that regulate their formation.

Alternatively, it may be possible that the Rac1 signaling pathway plays a role in Dex induced CLANs as has been suggested by Filla and colleagues.[77, 144] This signaling was suggested to be mediated through β-integrins. In those studies, they observed a 45-80% reduction in CLAN formation when using a Rac1 inhibitor (NSC23766) in human TM cell culture. Due to the fact the cells were not confluent and were tested in a cell

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spreading assay after exposure to Dex for 7 days, it is unknown if the outcome with Rac1 inhibitor would be the same when tested in a confluent cell culture model after longer Dex exposure. However, even though Rac1 can have downstream effectors that activate the ROCK signaling pathway, ROCK may not play a significant role in Dex induced CLAN formation.

In this study, both Dex and TGFβ2 CLAN induction was less than has been observed in other studies, although remaining statistically significant compared to controls. This may have been due to the use of the 400X magnification for CLAN counting. Using a higher magnification such as 600X, would likely have facilitated focusing through the abundant stress fibers to see additional CLANs that may be hidden underneath.

More research is needed to fully understand the mechanism of CLAN formation and what common converging points may exist between steroid and TGFβ2 induced CLANs. A better understanding of the regulating factors could lead to a more effective treatment for patients with ocular hypertension and glaucoma.

CHAPTER 5

OVERALL DISCUSSION

5.1. OVERALL DISCUSSION

Ocular hypertension is a primary risk factor for the development of glaucoma, and results from multiple TM cytoskeletal and ECM changes to increase AH outflow resistance.[11-16, 18, 42] The cytoskeleton and ECM have an important interplay which is critical for proper functioning of the cells. This interplay is potentially mediated through β-integrin signaling both in a glaucomatous eye,[149] as well as in cell culture.[77, 78, 109, 144] Changes to the TM cells cause cross-linking of the cytoskeleton and the ECM which alters the stiffness of the cells. The altered tensile integrity can determine cell shape, mechanical responsiveness of cells, and regulate cytoskeletal assembly and signal transduction.[17] All the cellular and extracellular alterations, as well as prolonged or abnormal contraction due to the muscle-like contractile properties of TM cells,[24] results in the TM's fenestrated structure being compromised which leads to increased AH outflow resistance.

Importantly, CLAN formation is associated with elevations in IOP. Mathematical models have predicted that CLANs increase the stiffness of actin filaments,[96] and TM cells and tissue of glaucomatous eyes have been shown to have a higher degree of

stiffness compared to non-glaucomatous eyes.[97] In order to better understand how CLANs contribute to ocular hypertension, it is important to identify how CLANs are formed.

Attempts at identifying alterations in gene or protein expression in glaucomatous eyes compared to non-glaucomatous eyes,[38] or upon Dex[34, 35, 99] or TGFβ2[36, 39] exposure have provided mixed results and are inconclusive. Only one group has explored gene and protein expression changes as they relate to Dex induced CLAN formation,[100] but the proteomics of TGFβ2 induced CLAN formation has still not been evaluated in a similar manner. However, a shotgun approach to identifying the genes or proteins of interest may not provide a clear path forward. In the work for this dissertation, there was an attempt to start elucidating the signaling pathways involved in TGFβ2 induced CLANs. This information may serve as a potential guide to eventually identifying a target factor contributing to TGFβ2 induced CLAN formation. There was one previous attempt at a similar strategy.[79] However, only the TGFβ Smad signaling pathway was investigated, primarily in bovine TM cells. There remained the question as to if the non-Smad signaling pathway was also involved in CLAN formation in human TM cells.

The work in this dissertation demonstrates that TGFβ2 induced CLAN formation is a result of both Smad and non-Smad signaling. Other events in the TM have also been associated with both the Smad and non-Smad signaling pathways. For example, Gremlin which is a bone morphogenic protein (BMP) antagonist, has shown to be elevated in glaucomatous eyes and associated with glaucomatous changes in the TM such as

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increased expression of FN, collagen, elastin, and contributed to increases in IOP.[61] These effects of Gremlin were shown to be a result of both Smad and Non-Smad signaling.[40]

In the current study, the Smad3 phosphorylation inhibitor was able to completely inhibit CLANs, as well as partially resolve CLANs within only one hour of exposure. The gradual increase in CLAN formation over several days, but rapid resolution of CLANs upon Smad inhibition would indicate that alterations of gene transcription and protein translation are needed for CLAN formation. Once formed however, without continued TGFβ2 signaling there may be phosphorylation or dephosphorylation of a particular protein which results in an interruption of the CLAN mechanism and therefore disassembly of CLANs.

There is still a question regarding the specificity of the Smad signaling in regards to CLAN formation. Smad3 inhibition resulted in altered stress fiber formation. In some cases cells had a similar appearance with lack of well-formed stress fibers as was seen with ROCK inhibitor treatment. This may be an important aspect to consider since Smad3 has been shown to cross-talk with the Rho signaling pathway in certain cell types. For example, RhoA dependent signaling regulates TGFβ1 induced cytoskeletal reorganization in the human retinal pigment epithelial cell line ARPE-19.[150] The *NET1* gene which encodes a specific GEF for Rho, was found to be critical for TGFβ1 induced cytoskeletal reorganization, N-cadherin expression, and RhoA activation. In cells expressing a dominant negative Smad3 or constitutively active Smad7 (inhibitory Smad),

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TGFβ1 failed to induce NET1 mRNA and protein expression, which was accompanied by decreased stress fiber formation. The authors therefore concluded that Smad3 regulates RhoA activation and cytoskeletal reorganization by controlling NET1. A similar cross-talk was observed in lung fibroblast cells where inhibition of Smad2 downregulated expression of RhoA and ROCK I.[151] Although these examples are not specific to TM cells or TGFβ2, it is plausible that similar cross-talk could be occurring in the outcomes observed in our study. The fact that cells treated with Smad3 inhibitor had fewer CLANs may be a result of the loss of stress fibers and may not necessarily mean that the actual CLAN mechanism was interrupted. This observation will need to be investigated further.

Conversely, ERK inhibitor also demonstrated complete inhibition of TGFβ2 induced CLAN formation. Interestingly, the reduction of CLANs was not associated with a loss of stress fibers. In fact, stress fiber formation was enhanced, in many cases even more so than with TGFβ2 exposure alone. The lack of CLAN formation with maintenance of stress fiber formation may indicate that ERK may play a more significant and specific role in the mechanism that leads to TGFβ2 induced CLANs. It is intriguing that one hour ERK inhibition did not resolve already formed CLANs. This may be an indication that longer exposure of ERK inhibitor would be needed to resolve already formed CLANs. Alternatively, it may indicate that without alteration in stress fiber formation, CLANs are still maintained by altered gene and protein expression and the phosphorylation state of a particular protein will not suffice for disassembly. Longer exposure to ERK inhibition

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after CLANs have formed should be investigated to determine the degree of involvement ERK may have in the CLAN mechanism.

When TM cells were treated with TGFβ2 and JNK or P38 inhibitors, there was not a significant impact on CLAN formation, nor was there a notable alteration of stress fiber formation. This would indicate these two signaling pathways do not play a significant role in TGFβ2 induced CLAN formation. What role these kinases play in Dex induced CLAN formation is unknown. Post-translational modifications such as phosphorylation are known to play an important role in GR signaling, and subsequently the regulation of protein function, cellular activity, and ultimately the resulting biological effect.[146, 152] The cross-talk between GR signaling and inflammatory kinase signaling pathways such as ERK, P38 and JNK, has been reported and is potentially driven by the fact that kinases can phosphorylate GRs and either up or down regulate their activity. For example, phosphorylation of JNK has been shown to down regulate GR activity whereas P38 can either up or down regulate GR activity in certain cell types, and such results can occur in a phosphorylation site and ligand dependent manner.[146] An example is an in vitro study utilizing a cell type with a specific mutant ligand binding domain on a constitutively active GR. This mutation decreased the cell responsiveness to P38 or ERK2 resulting in inhibition of apoptosis induced by Dex.[153] Similarly, JNK was shown to have an inhibitory effect on the rat GR, but mutation of the specific phosphorylation site diminished JNK's inhibition of the GR.[154] Therefore, site-specific phosphorylation and specific kinase pathways play an important role in GR regulation of specific target genes.[146] How the GR and kinase cross-talk functions within TM cells, and specifically

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in relation to CLAN formation, is not known. This cross-talk can also be further regulated by other signaling pathways such as the Rho GTPases which are known to regulate P38 and JNK activation. However, these kinases are thought to be preferential targets of the Rac1 and Cdc42 GTPases.[155] Therefore, if the Rac1 signaling pathway is in fact a significant contributor to Dex induced CLAN formation as suggested by Filla and colleagues,[77, 144] the JNK or P38 pathways may play a significant role in Dex induced CLANs, but may not contribute significantly to those induced by TGFβ2.

ROCK inhibitors have shown some promise in reducing IOP.[72, 73, 126] However, they also result in significant alterations in actin stress fibers. These changes to the actin stress fibers, downregulation of αSMA, along with loss of inhibition of MLCP which decreases the phosphorylation and activation of MLC[81] may support the concept of ROCK inhibitors relaxing the TM. Intriguingly, despite the relaxation effect, ROCK inhibitors have been reported to result in a "contracted" appearance of the TM cells. However, it may not be that the cells are truly contracted. Instead, the cells may have the contracted appearance as a result of a loss of actin structure which helps maintain the cell shape with contractile properties thereby providing the more uniform appearance to the cells. Last and colleagues[97] provide a possible explanation for this morphological change (Figure 5.1.1). They suggested that due to the known prolonged contraction of the ECM and cytoskeleton in glaucomatous cells, the cell becomes more flattened, less compliant, and more dysfunctional. Through cell culture conducted as a part of this dissertation, notable effects to the cell morphology were also observed upon treatment with ROCK inhibitor. When cells were treated with TGFβ2 plus ROCK inhibitor, there was a loss of

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actin stress fibers (as demonstrated in Chapters 3 and 4). Co-treatment with TGFβ2 and ROCK inhibitor also resulted in cells that appeared contracted (Figure 5.1.2). However, when cells were treated with TGFβ2 with other inhibitors such as ERK, JNK, or P38, the cells retained abundant stress fibers even though the microfilaments assumed a broken or disorganized appearance, and expressed significant CLAN formation (as demonstrated in Chapters 3 and 4). In this case, the cells did not have the same contracted morphology. These cells had a flatter appearance even as compared to control cells which would be consistent with the hypothesis that actin stress fibers may provide contraction within the cells thereby maintaining a certain cell shape. The "contracted" appearance of the TM cells may therefore be simply due to loss of shape resulting in a more rounded cell.

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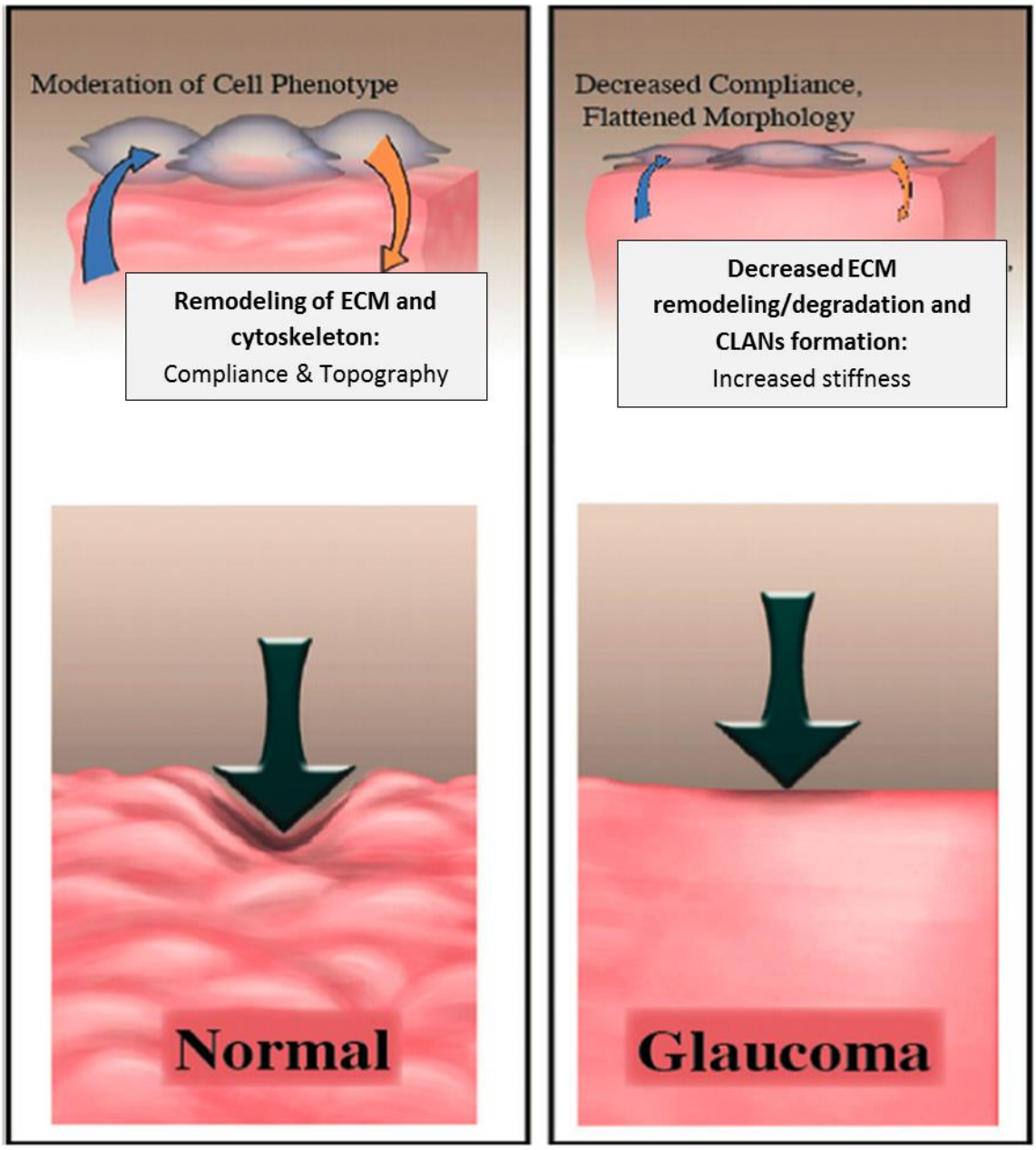

Figure 5.1.1. Cell stiffness and resulting change in cell morphology

Normal TM cells are less stiff and more compliant due to normal ECM turnover and cytoskeletal structure. Glaucomatous cells experience increased stiffness due to increased contraction of the ECM and cytoskeleton, as well as CLAN formation.[97] (Figure modified from Last et al, IOVS, 2011.)

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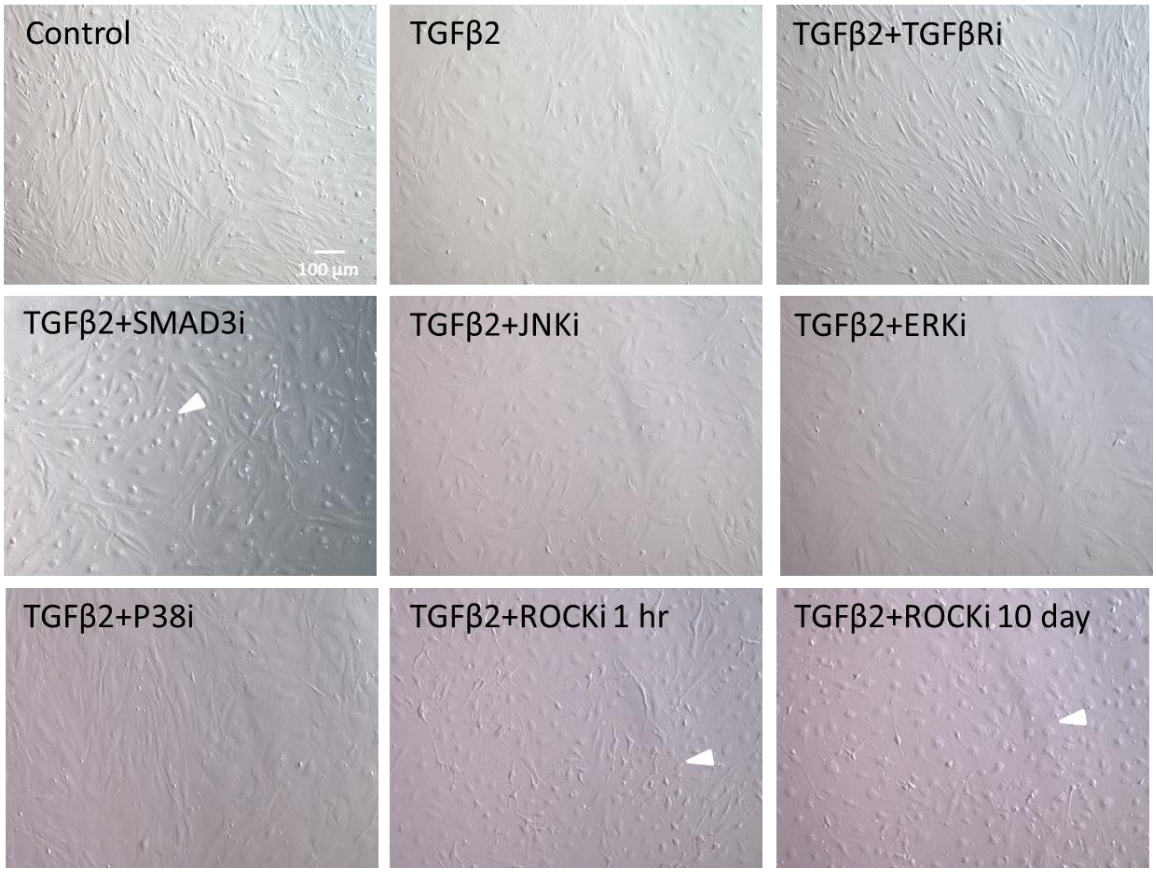

Figure 5.1.2. Cell morphology in the presence of TGFβ2 and pathway inhibitors

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Figure 5.1.2. Cell morphology in the presence of TGFβ2 and pathway inhibitors

NTM cells were treated for 10 days with TGFβ2 (5 ng/mL) with or without inhibitors. Some cells were treated with TGFβ2 for 10 days followed by 1 hour of ROCK inhibitor. ROCK inhibitor, as well as Smad3 inhibitor, reveals a much more "contracted" morphology of the cells (white arrow heads). Cells treated with TGFβ2 with or without JNK, ERK, or P38 inhibitors reveal a flatter cell shape. Considering previous data showing absence of CLANs and significant loss of actin stress fibers with Smad3 and ROCK inhibitors, the rounded or contracted morphology with ROCK or Smad3 inhibitors may be due to loss of cell shape rather than actual contraction of the cells. *TGFβRi*: TGFβ receptor inhibitor (SB431542; 5μM); *SMAD3i*: SMAD3 phosphorylation inhibitor (SIS3; 10μM); *JNKi*: JNK pathway inhibitor (SP600125; 10μM); *ERKi*: ERK pathway inhibitor (U0126; 25μM); *P38i*: P38 pathway inhibitor (SB203580; 5μM); *ROCKi*: ROCK pathway inhibitor (Y27632; 10μM).

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The current standard of care for treating patients with glaucoma includes pharmaceutical agents, laser trabeculoplasty, and glaucoma filtration surgery to lower IOP. Each of these options either decreases AH production, or increases AH outflow.[156] Prostaglandin analogs (PGAs) are one of the most commonly prescribed medications for decreasing IOP. PGAs work primarily on the unconventional outflow pathway, with the primary mode of action being through stimulation of MMP expression. MMPs hydrolyze excessive ECM which in turn decreases AH outflow resistance and IOP. PGAs also induce relaxation of the TM which decreases tension and facilitates opening of outflow pathways in the TM.[156]

Another commonly prescribed medication includes topical ocular β-blockers. The β-blockers are competitive antagonists of the β-adrenergic receptors. Blockage of the receptors leads to decreased cyclic adenosine monophosphate (cAMP) levels in the ciliary epithelium and therefore decreases AH production. Unfortunately, since the endogenous adrenergic activity is less during sleeping hours, β-blockers tend to be most effective during waking hours and minimally effective during sleeping hours.[156] There are also several systemic diseases such as lung disease and/or congestive heart failure that are contraindications for the use of β-blockers.[157] In fact, it is estimated that approximately half of POAG patients have some sort of contraindication to the use of β-blockers.[158, 159] Since POAG typically affects people later in life, the contraindicated conditions are not uncommon.

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Carbonic anhydrase inhibitors (CAIs) are also prescribed, but are usually only given as adjunctive therapy when PGAs or β-blockers alone do not suffice for adequate IOP control. CAIs reduce the production of bicarbonate ion which is critical for AH production.[156]

There are several other less commonly prescribed topical ocular medications available. However, none of them target the actual mechanism that leads to elevated IOP. Albeit that lowering IOP has demonstrated a two-fold decrease in the likelihood of developing glaucoma,[160] unfortunately in many cases one therapy is not enough to adequately control IOP throughout the entire day. In these cases patients require multiple topical ocular medications to avoid dangerous fluctuations in IOP. Even in some patients with multiple concurrent medications, the patient burden is significant and yet IOP may still not be consistently maintained at an acceptable level so they may require more invasive surgical procedures.

Since CLAN formation is believed to create a more stiff and less functional TM, if CLANs are still present despite the actions of PGAs or β-blockers or other medications targeted to decrease AH production or increase AH outflow, the TM may still create excessive AH outflow resistance. This may be one reason why monotherapy is not sufficient for many patients. This consideration is further supported by medications such as cytochalasins or Rho kinase inhibitors that have demonstrated efficacy in increasing AH outflow. However, these medications have significant impact on the cytoskeleton. Since the cytoskeleton is critical for proper intra- and extra-cellular communication, the

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long term effects of prolonged cytoskeletal disruption are not clear. Additionally, one of the key adverse events with these medications is ocular hyperemia due to relaxation of the conjunctival blood vessels. Even though hyperemia is not a significant health risk, it is a major complaint for patients. If a new therapy could inhibit CLAN formation while maintaining an intact cytoskeleton, it may provide a more efficacious option whether administered as monotherapy or as adjunctive therapy.

ERK inhibition resulted in complete inhibition of CLAN formation while maintaining the normal elongated, well-organized appearance of the stress fibers. This may indicate the feasibility of such a therapeutic target for reducing CLANs but maintaining the cytoskeletal structure. The ERK signaling pathway is critical to many biological processes. Therefore, prolonged inhibition of the ERK signaling pathway may not be advisable. However, if following this pathway can lead to a key protein or gene of interest, targeting that specific protein or gene may prove to be beneficial for targeting CLAN formation in a more specific mechanism, thereby decreasing the potential for other adverse effects. In order to fully explore this hypothesis, the mechanism of CLAN formation and the factors involved must be studied further.

CHAPTER 6

FUTURE DIRECTIONS

Much research has been conducted on CLAN formation in TM cells. However, the mechanism of their formation is still unknown. Our research has established the potential cooperation between the Smad and non-Smad signaling pathways that results in CLAN formation, as well as maintenance of CLANs.

The research in this dissertation utilized non-glaucomatous human TM cell culture and TGFβ2 induced CLAN formation. Since TGFβ2 is elevated in glaucomatous eyes and induces other TM cellular changes, this model is a good representation of what may be happening in the actual glaucomatous eye. To support the hypothesis, it would be interesting to see the effect of each of the Smad and non-Smad pathway inhibitors on CLAN formation in glaucomatous TM cells. Additionally, the effect of the inhibitors in a perfusion culture model to determine if the IOP lowering effect is aligned with the effect observed on CLAN formation.

Additionally, since Smad3 and ROCK, as well as ERK inhibition, appear to play a significant role in CLAN formation, further research needs to be done to determine which actual proteins or genes may be associated with, or responsible for, the observed

prevention or resolution of CLAN formation. It would be important to know if the Smad and non-Smad sub-pathways converge at a common point. The fact that resolution of already formed CLANs occurred within 1 hour of Smad3 inhibition suggests that a phosphorylation or dephosphorylation event occurs which releases the CLAN mechanism. Alternatively, the rapid disassembly of CLANs may be a result of actin disassembly and loss of actin stress fibers. Regardless, to determine proteins that may be associated with the CLAN mechanism, one could utilize 2D electrophoresis to identify proteins of interest after exposure to TGFβ2 and/or Dex, with and without the various inhibitors. Not only the protein itself, but also the phosphorylation status of the protein(s) should be evaluated. Likewise, a high through-put assay, or a chip assay can be used to identify changes in gene expression. Peters and colleagues did a proteomics and genomics analysis in relation to Dex induced CLANs[100] so a similar study should be done for TGFβ2 induced CLANs. Differential results in the presence or absence of the various inhibitors may facilitate elucidation of a common CLAN associated protein rather than a general approach which may only reveal changes that are inherent to TGFβ2 or Dex treatment. If a protein of interest is identified, this information can be used to upregulate or down regulate the expression and determine what effect that has on CLANs. Immunofluorescence to determine the location of the protein(s) of interest can also give further insight to the mechanism of CLAN formation, but perhaps also to what "locks" the CLANs in place. Since the CLANs formed during cell spreading and attachment may not be identical to the CLANs formed in glaucomatous tissue or confluent cell culture, the similarities or differences between these types of CLANs can also be investigated.

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For inhibition of the signaling pathways, more specific inhibition should be included such as the use of siRNA. There are limitations with the use of siRNA in studying kinases compared to their use in studying other proteins. There is an assumption that knockdown of expression would result in the same phenotypic response as with the kinase inhibitor. But, this may not necessarily be the case. The knockdown may result in different phenotypes due to the noncatalytic functions of the kinase and resulting signal transduction. There may also be a compensatory mechanism due to knockdown that elicits responses from other kinases.[161] Despite these limitations, the use of siRNA is commonly used and could contribute to the understanding of these signaling pathways and their relationship to CLANs.

It is well known that kinase inhibitors tend to be selective but not specific. Therefore, the resulting effects may be due to factors associated with but not directly related to the kinase itself. To attempt more specific inhibition, on could employ the use of compounds that bind to regions of protein kinases to regulate the kinase in ways other than ATP binding, etc. These compounds may be more specific since they would target less conserved residues outside of the ATP binding site. A similar approach is demonstrated by the use of a mutant variant of a Rho-kinase cloned into an adenoviral vector expressing the dominant negative Rho-binding domain of Rho-kinase. Multiple ROCK inhibitors, as well as use of a dominant negative Rho kinase, have shown promise in reducing IOP. The use of a dominant negative Rho-kinase in the CLAN experiments could provide more specific determination of the effect of the Rho/ROCK signaling pathway on CLAN formation.

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Since the Rac1 signaling pathway has been suggested as being associated with Dex induced CLANs during cell spreading, Rac1 should also be investigated for its role in TGFβ2 induced CLANs in a confluent cell culture model. It may be possible that the two pathways converge at a common TGFβ2 or Dex induced factor, or it may be possible that the Rac1 pathway may prove to not play a significant role in TGFβ2 induced CLANs. This in itself may be important information for determining if all CLANs are created equally. It may also help to rule in or out some potential target candidates.

There is still an open question as to if the CLAN reducing effect of ROCK inhibition, or even Smad3 inhibition, is due to an interruption of the CLAN mechanism, or solely to the fact that stress fiber formation is also compromised. Rho activation should be assessed in the presence of TGFβ2 as well as TGFβ2 plus the various inhibitors in order to better understand if the effect on stress fiber formation may be a result of Rho activation or inhibition. Rho activation can be measured using a pull down assay followed by Western Blots for quantification of the results. The same method can be used to evaluate Dex activation of Rho and Rac1, with or without the various inhibitors. Phosphorylation status of certain proteins such as MLC or cofilin after treatment with the inhibitors should also be evaluated. Alternatively, if a protein(s) of interest is identified as potentially being the orchestrator of CLAN formation, evaluating the expression or phosphorylation status of such a protein after treatment with the inhibitors may be important. Use of constitutively active Smads or Rho in the presence or absence of the inhibitors may also be an alternative to reveal important information that could lead to a better understanding of the role the signaling pathway plays in CLAN formation.

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Live cell imaging is another interesting aspect that should be evaluated to determine how CLANs are assembled, when does the assembly begin, and what potential morphological changes occur immediately prior to, during, or after CLAN formation. Live cell imaging to evaluate CLANs has historically been complicated by the low transfection efficiency in primary TM cells and therefore the limited incorporation of the green fluorescent protein (GFP) needed for such an analysis. However, a recent study was conducted by Fujimoto and colleagues evaluating the actin dynamics in dexamethasone treated porcine TM cells.[162] An actin-GFP fusion construct with a modified insect virus was transfected into porcine TM cells. The study yielded approximately 23% of cells expressing the GFP-actin. Of those cells, approximately 28% revealed CLAN-like structures after 72 hours of Dex treatment, and the lifetime of the CLAN-like structures increased after Dex treatment. They also observed that Dex treated cells were larger than control cells, and that cells with CLANs underwent less migration than control cells or Dex treated cells that did not contain CLANs. These data support the previously reported effects of CLANs, but in a dynamic model. A similar study could be done with TGFβ2 treated cells to determine if the actin dynamics leading to CLAN formation and maintenance of the CLANs is similar to what is seen with Dex treated cells. One could also determine if CLANs initiate from a rearrangement of already formed stress fibers, or from new localization of an initiating factor.

It is also unclear at this time if CLANs cause elevations in IOP, or if CLANs are a protective stabilization mechanism as a result of the stress and strain induced by elevated IOP, which then just further exacerbates the noncompliant TM to perpetuate AH outflow

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resistance. It may be of interest to utilize the perfusion organ culture model in a time course with various levels of pressure. The TM tissue could then be evaluated for CLAN formation. This may be technically challenging due to the variability that may accompany such an analysis, and the resulting number of eyes needed. However, if one can identify which comes first, CLANs or increased pressure, this could further lay the path to identifying the instigating proteins or genes related to CLAN formation. Likewise, already formed CLANs could be disassembled when the Smad TGFβ2 signaling was blocked, but this was in a stagnant cell culture assay. It would be interesting to determine if the stress and strain of elevated pressure is sufficient to maintain associated compensatory signaling that would prevent CLANs from disassembling even in the presence of various inhibitors. It could also further inform what proteins or genes are associated based on differential expression under these conditions.

Finally, there are other signaling pathways that may also be important to understanding the relationship to CLAN formation. There has been a link established between the POAG-associated cellular changes induced by TGFβ2 and those induced by CTGF. The main regulators of CTGF appear to be TGFβ2 and mechanical stress.[136] Therefore, there may be interplay between the Rho signaling and the TGFβ signaling pathways that converge at CTGF.[163] It is possible that the pathological effects may be initiated by TGFβ2, but ultimately are a result of downstream CTGF signaling. The Smad and non-Smad pathway inhibitors used for this thesis should be tested to determine the effect of neutralization of the CTGF signaling. Furthermore, the mechanical stress induction of

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CTGF may be dependent on the Rho signaling pathway.[164] ROCK inhibitors also affect both TGFβ2 and CTGF-induced cytoskeletal changes in a similar manner.[137, 138] Therefore, more research is needed to determine the effect of CTGF and various associated inhibitors on CLAN formation.

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