

THE ROLE OF CYTOSKELETAL SIGNALING IN LENS EMT

THE ROLE OF CYTOSKELETAL SIGNALING IN LENS EMT

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirement for the Degree

Doctor of Philosophy

McMaster University

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DOCTOR OF PHILOSOPHY

MCMASTER UNIVERSITY

Interdisciplinary, Biomedical Engineering

Hamilton, ON

TITLE: The Role of Cytoskeletal Signaling in Lens EMT

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NUMBER OF PAGES: xv, 144

ABSTRACT

Cataract is an opacification of the ocular lens and is the leading cause of visual impairment and blindness worldwide. The only form of treatment is removal of lens cells through cataract surgery, however, this procedure can lead to complications, the most common of which is the development of a secondary form of cataract known as posterior capsule opacification (PCO). PCO is caused by lens epithelial cells not removed during cataract surgery, which have the capacity to migrate to the previously cell-free posterior capsule. Here, these remnant cells can undergo epithelial-mesenchymal transition (EMT) into myofibroblasts that impair vision through the promotion of a fibrotic environment involving excessive deposition of extracellular matrix (ECM) and capsule wrinkling. This process is mediated by surgery-induced activation of transforming growth factor (TGF)- β . Remodeling of the actin cytoskeleton, mediated by the Rho family of GTPases, plays a key role in EMT, however, how actin dynamics affect downstream markers of EMT has not been fully determined. Our previous work suggests that myocardin related transcription factor A (MRTF-A), an actin-binding protein, and MMP-9 may be important mediators of lens EMT. The aim of the current study was to determine the requirement of RhoA/ROCK signaling in mediating TGF β -induced cell-cell contact disassembly and ultimate α -smooth muscle actin (α SMA) expression, key transdifferentiation events.

Using a rat lens epithelial explant model, my work demonstrated that Y-27632, a ROCK inhibitor, prevented TGF β -induced nuclear accumulation of MRTF-A, E-cadherin/ β -catenin complex disassembly, and expression of MMP-9 and α SMA. Furthermore, using a novel inhibitor specifically targeting MRTF-A signaling, CCG-

203971, my work further demonstrated that MRTF-A nuclear localization and activity is essential to the induction of α SMA expression. Lastly, using rat lens explants treated with recombinant human MMP-9 (rhMMP-9) and MMP-9 KO mouse lens explants I demonstrated a key role for MMP-9 in the proteolytic processing and regulation of membrane-associated E-cadherin.

Overall, the findings outlined in this thesis strongly suggest that ROCK-mediated cytoskeletal signaling is indispensable to lens EMT, and involved in several key aspects of the TGF β signaling pathway. The role of canonical TGF β signaling through Smads has been widely studied in the lens, however, only recently has the academic lens community begun to appreciate the involvement of other signaling pathways. As a result, our understanding of how actin dynamics are involved in lens EMT is still at an early stage. The work in my thesis progresses our knowledge of the interplay between cytoskeletal signaling and its specific downstream mediators that result in a sustained EMT response. This work also has implications beyond lens EMT, impacting our understanding of the pathogenic processes common to a broad array of fibrotic diseases. Importantly, my findings have implications for the therapeutic targeting of ROCK-mediated signaling in the prevention of fibrosis of the lens, and other tissues.

ACKNOWLEDGEMENTS

The image of a lonely scientist does not come to mind when I think on my years in the lab. I have been very fortunate to have many people around me that contributed to my experience and made it a positive one.

First and foremost, I would like to express my sincere gratitude to my supervisor, Dr. Judy West-Mays. Your guidance, expertise, and gentle encouragement to get writing and submitting those manuscripts has taught me a great deal about the academic world. But beyond science, you have been an inspiring role model for how to maintain a successful work-life balance.

I would like to thank my committee members, Dr. Heather Sheardown and Dr. Peter Margetts who have kindly advised my project over these years. Your meaningful input and genuine support have helped me throughout this process.

A very special thank you goes out to the most hard-working and selfless lab technician, Paula Deschamps. Your contribution to the lab and my project were immense and greatly appreciated.

The supportive environment in the West-Mays' lab is unmatched and this is a credit to the graduate students who have been a part of it. To my lab mates and colleagues over the years: Emily-Anne Hicks, Monica Akula, Mizna Zaveri, Vanessa Martino thank you for all the moral support and humour. To Dr. Christine Kerr and Dr. Madhuja Gupta for being the trio from day one, and sharing many great memories at ARVO. I would also like to thank my dear friends Dr. Elyse Rosa and Dr. Connie Cheng, you guys were always around to keep me sane and grounded.

Next, I'd also like to acknowledge the men in the lab, Dr. Aftab Taiyab for always having the time to chat about scientific and non-scientific matters. Scott Bowman, for being

my adversary in nearly every discussion, yet somehow managing to be my supportive teammate throughout. Working alongside both of you has been a pleasure.

A most special thank you to my absolute biggest fans: my parents, my brothers and my Marcel. You have all patiently watched me go to school all these years and supported me at every step. You have been my greatest source of love, strength and inspiration.

And last, but not least, I must acknowledge the little hairy being that kept me company while I wrote this thesis, my dog Ollie.

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LIST OF ABBREVIATIONS

ABP	actin-binding protein
AJ	adherens junction
ANOVA	analysis of variance
APC	adenomatous polyposis coli
ASC	anterior subcapsular cataract
CArG	CC(A/T) ₆ GG
CBP	CREB-binding protein
E-cadherin	epithelial cadherin
ECCE	extracapsular cataract extraction
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
F-actin	filamentous actin
FHL-124	human lens epithelial cell line
G-actin	globular actin
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GSK3β	glycogen synthase kinase
ICCE	intracapsular cataract extraction
IOL	intraocular lens
KO	knock-out
LAP	latency-associated peptide

LECs	lens epithelial cells
LEF	lymphoid enhancer factor
LIMK	LIM kinase
LLC	large latent complex
LTBP	latent TGF β -binding protein
M-SICS	manual small incision cataract surgery
mDia	mammalian diaphanous homolog
MLC	myosin light chain
MMP	matrix metalloproteinase
MRTF	myocardin-related transcription factor
MT-MMP	membrane-associated MMP
N-cadherin	neuronal cadherin
Nd:YAG	neodymium-doped yttrium aluminum garnet
NO	nitric oxide
PCO	posterior capsule opacification
pMLC	phosphorylated MLC
RBD	Rho-binding domain
RGD	Arg-Gly-Asp
rhMMP-9	recombinant human MMP-9
ROCK	Rho-associated coiled-coil-forming protein serine/threonine kinase
R-Smads	receptor-activated Smads
SBE	Smad-binding element

SDS	sodium dodecyl sulfate
SRF	serum response factor
TCE	TGF β control element
TCF	T-cell factor
TGFβ	transforming growth factor- β
TIMP	tissue inhibitors of MMPs
TJ	tight junction
TβRI	TGF β type I receptor
TβRII	TGF β type II receptor
αSMA	α -smooth muscle actin

CHAPTER 1: INTRODUCTION

1.1 Cataracts

1.1.1 Lens structure

The lens is a transparent, avascular tissue located in the anterior segment of the eye just behind the iris, which along with the cornea, transmits and focuses light onto the retina. The lens is encased by a lens capsule, the thickest basement membrane in the body, which protects the lens from its outside environment (Figure 1). The fluid surrounding the capsule at the anterior region of the lens is aqueous humour, while the posterior lens is bathed in vitreous humour. In contact with solely the anterior lens capsule lies a monolayer of cuboidal lens epithelial cells, while in contrast, the posterior capsule remains entirely cell-free. Apart from the anterior region of the lens, much of the tissue is made up of long, thin fibre cells that span the entire length from anterior to posterior pole. In a normal adult lens, it is the lens epithelial cells that differentiate into fibre cells, beginning their proliferation in the germinative zone, a region found slightly anterior to the equator of the lens, and continuing their migration downwards to the transitional zone at the equator. It is here the epithelial cells elongate and differentiate into fibre cells as they migrate inwards along the equatorial line (Tholozan and Quinlan 2007). The most superficial fibre cells, found closest to the lens equator, are metabolically active, and nucleated. As the fibre cells progress deeper towards the lens nucleus, a process known as terminal differentiation occurs

culminating in the loss of organelles, cytoskeletal elements and denucleation with the purpose of maintaining lens transparency (Tardieu, Veretout et al. 1992, Kenworthy, Magid et al. 1994, Michael and Bron 2011).

1.1.2 The global burden of cataract

A cataract is an opacification of the ocular lens that results in the obstruction and scatter of light leading to a gradual loss of lens transparency, and consequently, vision. Cataract continues to be the leading cause of visual impairment and blindness worldwide with an estimated 95 million people affected. According to the WHO, 82% of the population afflicted with blindness are greater than 50 years of age (WHO 2014). Thus, the number of individuals presenting with cataracts is expected to increase further as the population ages. Cataract surgery provides quick restoration of vision and is an effective form of treatment, however, it is not readily available in economically developing countries, while in developed countries it is the most frequently performed operative procedure. In the United States over 1.2 million cataract operations are performed per year, carrying a cost of \$3.4 billion (West 2000), while in Ontario alone 175,000 surgeries are performed per year, a number projected to rise by 43% in the next decade (Hatch, Campbell Ede et al. 2012).

Beyond steep costs, cataract surgery can lead to complications, the most common of which is the development of a secondary form of cataract known as, posterior capsule opacification (PCO). PCO is a significant eye health issue with 4-12% of patients

presenting with PCO within two years following uncomplicated cataract surgery in healthy adults (Findl, Buehl et al. 2010), while a much higher prevalence of PCO is seen in pediatric cases. These statistics demonstrate a need for the development of novel therapeutic strategies that can help ease the “global burden of cataract” (Rao, Khanna et al. 2011), both primary and secondary forms, that can be achieved through a deeper understanding of the underlying molecular mechanisms involved in cataract formation.

1.1.3 Types of cataract

Cataracts are classified based on age of onset and location within the lens. Congenital cataracts are visible at birth or soon after with mainly a genetic cause. Age-related cataracts can be nuclear, cortical or subcapsular. From birth the stiffness of the lens steadily increases until, after 40 years of age, the nucleus becomes denser than its surrounding cortex. Nuclear cataracts are characterized by an abnormally dense fibre cell nucleus with increased light scatter and nuclear discolouration. They are formed in a steady manner and result in uniform opacities. In cortical cataracts, opacities form in the superficial, nucleated lens fibre cell layers and occur in a discontinuous manner resulting in asymmetrical opacities. UV radiation, diabetes, and corticosteroid intake are all associated risk factors for cortical cataract (Michael and Bron 2011). Subcapsular cataracts form directly beneath the lens capsule at either the anterior or posterior poles. The present thesis will focus on the mechanisms involved in fibrotic lens pathologies, namely anterior subcapsular cataract (ASC) and post-surgical PCO discussed in detail later in this chapter (Chapter 1.3).

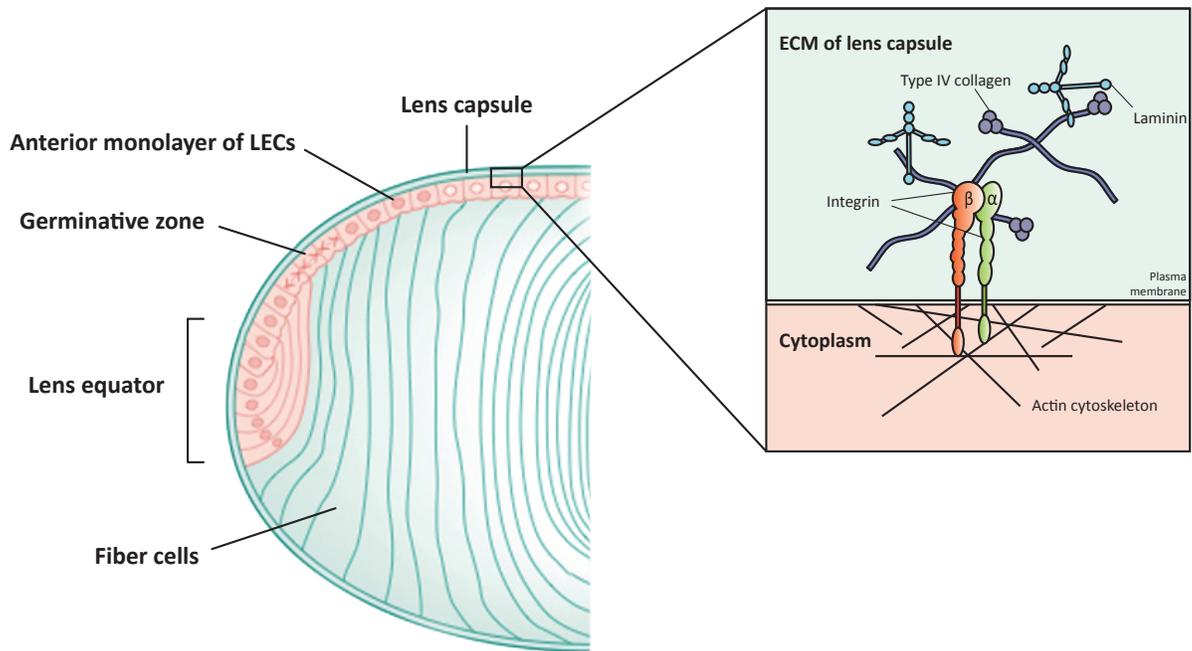


Figure 1. Lens structure. The lens is composed of two cell types, lens epithelial cells (LECs) and fibre cells, which are fully isolated from surrounding tissue by a thick basement membrane, the lens capsule. The lens capsule is primarily composed of collagen IV and laminin. LECs line the surface of the anterior central lens capsule. In the germinative zone, LECs begin to differentiate into fibre cells as they migrate downwards towards the lens equator, where they elongate and migrate inwards along the equatorial line. The integrins on the surface of LECs are responsible for adhering the cells with the ECM of the lens capsule. Figure from West-Mays and Korol (2014).

1.2 Cataract surgery

1.2.1 Primary cataract surgery

If a cataract is sufficiently deteriorating to a patient's vision, the clouded lens is removed and replaced with a synthetic intraocular lens (IOL). Initially, many decades ago, the most common cataract extraction surgery involved removal of the entire lens through a large corneal-scleral incision, cleaving zonular attachments. This technique, termed intracapsular cataract extraction (ICCE), required a week of hospitalization and was associated with numerous serious complications, such as vitreous loss, retinal detachment, chronic cystoid macular edema, and high astigmatism (Mohammadpour, Erfanian et al. 2012). The advent of extracapsular cataract extraction (ECCE) led to significant improvements where the lens material was now removed through an opening in the anterior lens capsule or "capsulotomy". The major strength of this technique is that most of the lens capsule is preserved, leaving a capsular bag still suspended by zonules into which an IOL is implanted (Lam, Rao et al. 2015). However, the use of ECCE did not gain immediate traction due to difficulty in adequately visualizing and removing lens material, which led to severe postoperative inflammation and PCO. In time, with improvements in aspiration, and capsulotomy techniques, ECCE became the standard where all modern cataract surgeries are now variations on this technique.

The emergence of phacoemulsification in the late 1960s is seen as the greatest leap in advancing the safety and efficacy of cataract surgeries. Developed by Charles Kelman

(Linebarger, Hardten et al. 1999), this method, in place of manual lens removal, uses an ultrasonic probe to fragment/emulsify the lens within the capsular bag, simultaneously aspirating the contents from the eye. Phacoemulsification requires a smaller corneal incision and improved control over the anterior chamber, providing a quicker and more controlled method for removing the cataractous lens, reducing the risk of complications. However, the high costs associated with acquiring and maintaining phacoemulsification equipment, as well as the steep learning curve that requires training, prohibit this technique from being used in developing countries where a manual small incision cataract surgery (M-SICS) is common. This technique is a variation on ECCE involving a smaller anterior capsule incision decreasing post-operative complications, and importantly, does not require costly equipment, training or consumables (Khanna, Pujari et al. 2011).

The latest technique, first described in 2010, is femtosecond laser-assisted cataract surgery, which automates the bulk of the surgery. A laser is able to carry out the corneal incisions, anterior capsulotomy, and lens fragmentation steps of the procedure, where a surgeon then continues with phacoemulsification and IOL implantation (Donaldson, Braga-Mele et al. 2013). The major cost associated with this technique and limited clinical benefits compared to pure phacoemulsification means it is currently limited in its use.

1.3 Posterior capsule opacification

1.3.1 Clinical presentation

Despite considerable advances in surgical technique and IOL design, cataract surgery can lead to secondary loss of vision through the development of PCO. PCO is the most common long-term postoperative complication and the second most common cause of vision loss (Apple, Escobar-Gomez et al. 2011). Development of PCO is attributed to the population of lens epithelial cells remaining in the capsular bag following surgery. Remnant lens epithelial cells not removed during the procedure are able to proliferate and migrate onto the normally cell-free posterior capsule, decreasing visual acuity (Figure 2). These cells exhibit aberrant behaviour and can take on one of two forms, differentiating into lens fibre-like or myofibroblast-like cells, in both cases hindering optical clarity of the implanted IOL. The two morphological forms of PCO consist of: (1) Elschnig pearls, which present as globular, swollen cells that express lens fibre cell-specific proteins (e.g. aquaporin 0, crystallins); (2) fibrous cells that deposit excess opaque, fibronectin- and collagen-rich ECM and lead to capsular wrinkling. It has been reported that PCO can consist of a mixture of both morphological forms of cells in the same eye (Raj, Vasavada et al. 2007). There is currently no treatment that fully eliminates these cells without causing further unwanted harm to the lens and surrounding tissues.

1.3.2 Incidence

Incidence of PCO varies widely, and causes are multi-factorial and include age, IOL material and design, and history of ocular inflammation. Intra-operative determinants of PCO include the size of anterior capsule incision, and crucially, the amount of residual lens material within the capsular bag (Bobrow 2000). Estimates of incidence of PCO are 11.8% after 1 year, 20.7% after 3 years, and 28.4% after 5 years (Schaumberg, Dana et al. 1998). These estimates differ in a pediatric population with a reported 40% rate 22 months after surgery where the significantly higher incidence of PCO in infants and children is thought to be due to the higher proliferative capacity of remnant lens epithelial cells (Jensen, Basti et al. 2002).

1.3.3 Secondary PCO surgery (Nd:YAG)

The procedure used to treat PCO is neodymium-doped (Nd): yttrium aluminum garnet (YAG) laser capsulotomy. Vision-threatening complications do occur, which include damage to the IOL (Durham and Gills 1985, Auffarth, Newland et al. 1994, Newland, Auffarth et al. 1994), increase in intraocular pressure (Fourman and Apisson 1991, Holweger and Marefat 1997, Ladas, Baltatzis et al. 1997), cystoid macular edema (Steinert, Puliafito et al. 1991, Smith, Moscoso et al. 1995, Magno, Datiles et al. 1997), and retinal detachment (Javitt, Tielsch et al. 1992, Powell and Olson 1995, Ranta, Tommila et al. 2004). On top of complications, Nd:YAG laser treatments carry a significant cost to the healthcare system with up to \$250 million spent annually in the US (Apple, Escobar-Gomez et al. 2011).

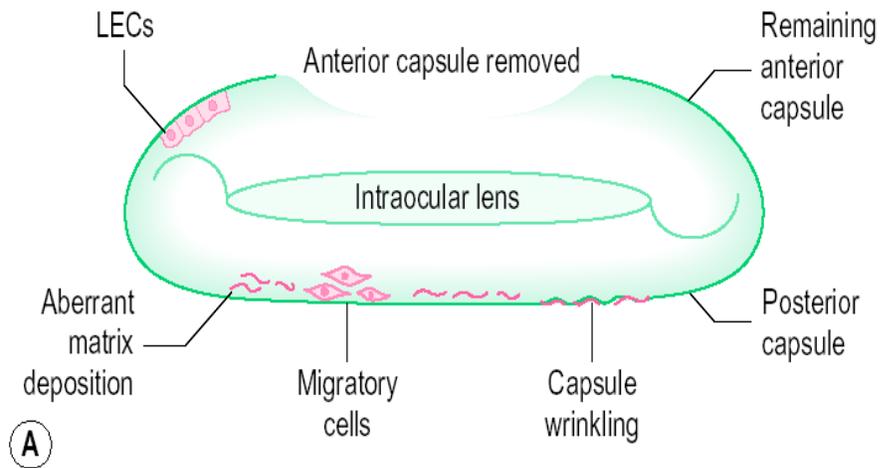


Figure 2. Development of PCO post-cataract surgery. Following removal of the native lens cells and implantation of an intraocular lens (IOL), remnant lens epithelial cells (LECs) can be stimulated to migrate towards the previously cell-free posterior capsule, where they can undergo epithelial-mesenchymal transition (EMT) into myofibroblasts resulting in aberrant matrix deposition and capsule wrinkling. Figure adapted from West-Mays (2010).

1.4 Epithelial-Mesenchymal Transition (EMT)

The focus of this dissertation is to study the mechanisms involved in post-surgical fibrotic PCO. This form of PCO results from remnant lens epithelial cells in the capsular bag that can undergo an epithelial-mesenchymal transition (EMT) into migratory, contractile myofibroblasts (Figure 3). Cells undergoing EMT gradually convert from an epithelial state to a mesenchymal one, losing epithelial markers, such as epithelial (E-) cadherin, cytokeratin, ZO-1, and expressing mesenchymal markers, such as neural (N-) cadherin, vimentin, and α -smooth muscle actin (α SMA) (Font and SA 1974, Novotny and Pau 1984, Hay 1995). Under normal physiological conditions, this conversion is critical during embryogenesis, as well as later in life to initiate a wound healing response. The purpose of myofibroblasts in wound healing is to secrete extracellular matrix (ECM) components that exert a contractile force to the local tissue, in an attempt to restore tissue homeostasis and function. However, if the presence of myofibroblasts persists, EMT has been implicated in pathological conditions, such as metastatic cancer and tissue fibrosis (Hay 1995). Persistent EMT is the key process involved in the formation of tissue fibrosis in the lung (Fabregat, Moreno-Caceres et al. 2016), liver (Albanis and Friedman 2001), kidney (Sato, Muragaki et al. 2003), skin (Darby, Laverdet et al. 2014), eye, (Bochaton-Piallat, Kapetanios et al. 2000), and, specifically, the lens (de Iongh, Wederell et al. 2005).

EMT can be classified into three types depending on the biological context (Kalluri and Weinberg 2009) (Kalluri 2009): (1) Type 1 occurs during embryogenesis and organ development; (2) Type 2 is involved in wound healing, inflammation and fibrosis; (3) Type

3 enables cancer and metastasis. Although EMT possesses distinct characteristics within these differing cellular contexts, the underlying mechanisms remain the same, namely:

- (1) Dissolution of epithelial cell-cell junctions leading to loss of apical-basal polarity
- (2) Reorganization of the actin cytoskeleton leading to a change in cell shape
- (3) Repression of epithelial and activation of mesenchymal/myofibroblast gene expression
- (4) Excessive remodeling and deposition of ECM components

In the current work, the lens was used as a model of fibrosis. As outlined in Chapter 1.1.3, the lens is a unique tissue that is completely isolated from its surroundings by the lens capsule. The epithelial cells that line the inner surface of the anterior capsule are responsible for lens fibrotic pathologies by undergoing EMT. And thus, in contrast to other fibrotic tissues where the origin of myofibroblasts is not clear, emergent myofibroblasts in the lens are of an epithelial origin.

1.4.1 Lens EMT

The EMT of lens epithelial cells occurs in the eye in response to ocular trauma (Chinwattanakul, Prabhasawat et al. 2006, Schnaidt, Schroder et al. 2009), implantable contact lenses (Khalifa, Moshirfar et al. 2010, Schmidinger, Lackner et al. 2010), as part of a pro-fibrotic wound-healing response (Lovicu, Ang et al. 2004), and in conjunction with diseases such as atopic dermatitis (Katsushima, Miyazaki et al. 1994, Brandonisio, Bachman et al. 2001) and retinitis pigmentosa (Cohen 1983). However, persistent EMT

and myofibroblast expression following cataract surgery leads to loss of lens transparency through the excessive production and deposition of ECM components and posterior capsule contraction, events typical of a fibrotic pathology.

The development of a primary form of cataract mentioned above, ASC, also shares many features with fibrotic PCO (Novotny and Pau 1984, Lovicu, Ang et al. 2004). In ASC, abnormally proliferating epithelial-turned-myofibroblast cells accumulate and manifest as plaques beneath the anterior lens capsule, which lead to the development of lens opacities and impaired vision (Hales, Chamberlain et al. 1999, Dwivedi, Pino et al. 2006). Therefore, understanding the mechanisms involved in lens EMT is critical to both the prevention of ASC, as well as the progression of secondary after-cataract impairment, PCO.

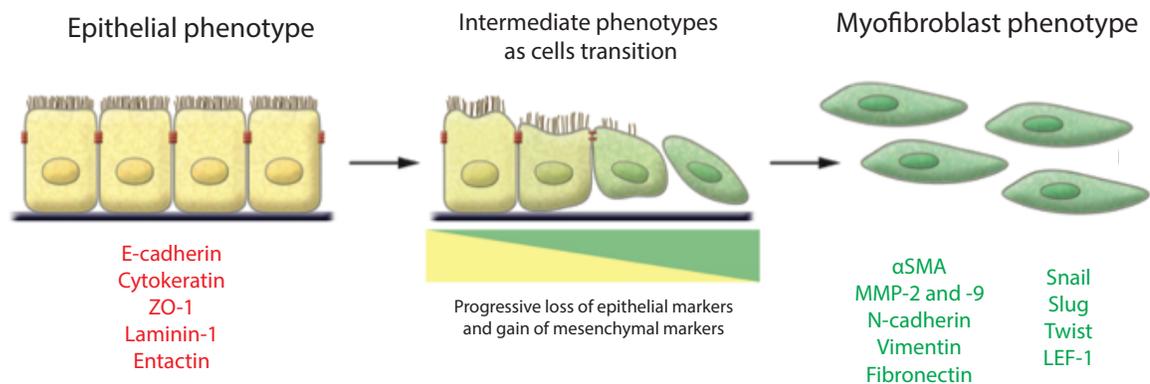


Figure 3. Epithelial-mesenchymal transition. EMT involves the transition of epithelial cells into migratory contractile myfibroblasts. Epithelial cells gradually lose epithelial markers and gain mesenchymal markers that result in the loss of cell-cell contact, a change in cell shape to an elongated morphology, and excessive remodeling and secretion of ECM components. Figure adapted from (Kalluri and Weinberg 2009).

1.5 TGF β signaling in EMT

Injury, accidental or surgery-induced, results in an inflammatory response that leads to elevated levels of a variety of cytokines and growth factors in the anterior chamber of the eye, as a means of tissue repair (Hinz 2016). In pathologies where EMT is a hallmark, one specific growth factor, transforming growth factor β (TGF β) has been identified as a primary mediator. TGF β is a multifunctional cytokine involved in both normal physiological, as well pathological processes inducing apoptosis, cell proliferation and differentiation, and morphogenesis (Roberts, McCune et al. 1992, Attisano and Wrana 2002, Shi and Massague 2003). TGF β levels are upregulated in response to injury as part of a wound healing response; however, unchecked, sustained TGF β signaling is implicated in numerous fibrotic disorders and is involved in the promotion of a fibrotic phenotype as a potent inducer of ECM synthesis and deposition (Ihn 2002, Schnaper, Hayashida et al. 2003, Huggins and Sahn 2004, Bataller and Brenner 2005, Gressner and Weiskirchen 2006, Liu 2006, Willis and Borok 2007).

1.5.1 TGF β in the anterior chamber

TGF β is one of many factors normally present in the aqueous humour of the eye, found in a latent, inactive form (Ohta, Yamagami et al. 2000, Schlotzer-Schrehardt, Zenkel et al. 2001). Levels of active TGF β are very low to begin with, while any activity is closely regulated/inhibited through proteins such as α 2-macroglobulin, a serum protein found in

the aqueous humour with a high affinity for active TGF β . This tight regulation controls its localization, preventing it from exerting effects at inappropriate sites (Schulz, Chamberlain et al. 1996, Munger, Harpel et al. 1997). In the eye, overexpression of TGF β is associated with cataract formation, proliferative vitreoretinopathy, and diabetic retinopathy (Kita, Hata et al. 2008, Gerhardinger, Dagher et al. 2009).

Following cataract surgery, TGF β signaling increases (Cousins, McCabe et al. 1991, Wallentin, Wickstrom et al. 1998, Saika, Miyamoto et al. 2002), and is heretofore the only factor known to induce lens epithelial cells to undergo EMT into myofibroblast cells, which are characterized by the expression of α SMA. It is thought that in response to injury physiological activators of TGF β are also increased in the anterior chamber, which are involved in activating latent TGF β . These include integrin α v β 6 (Munger, Huang et al. 1999), MMP-2 and -9 (Yu and Stamenkovic 2000), and thrombospondin-1 (Frazier 1991). The sustained treatment of excised whole rat lenses *in vitro* with TGF β 2 was shown to disrupt the normal architecture of the lens and resulted in characteristic changes associated with the conversion of lens epithelial cells into myofibroblasts, namely expressing α SMA (Dwivedi, Pino et al. 2006). Furthermore, *in vivo* overexpression of TGF β 1 specifically in the mouse lens led to the development of ASC, mimicking human cataractogenesis (Srinivasan, Lovicu et al. 1998). In the context of PCO, human capsular bags received from donors 1 month following cataract surgery revealed an increase in fibrotic markers, including α SMA, and matrix contraction of the posterior capsule (Wormstone, Tamiya et al. 2002). Additionally, treating *ex vivo* rat lens explants with TGF β triggered key features of EMT, namely loss of cell-cell contact, altered spindle-shaped morphology, and α SMA

expression (Gordon-Thomson, de Iongh et al. 1998). Therefore, *in vitro* and *in vivo* induction of ASC through TGF β overexpression in both mice and rats are practical models for the study of PCO, as well as ASC in humans.

1.5.2 TGF β structure and activation

Three isoforms of TGF β exist in mammals (-1, -2, -3), which are all secreted as a large latent complex (LLC) incapable of binding to cell surface receptors. The LLC consists of a TGF β dimer associated with latency-associated peptide (LAP) bound to latent TGF β binding protein (LTBP). Since the structure of LTBP is reminiscent of an ECM protein it gets incorporated into the ECM bringing with it the entire TGF β latency complex. TGF β activation occurs through proteolytic cleavage of the LTBP, which releases the TGF β -LAP complex from the ECM, and exposes sites on the LAP allowing for cell surface interaction. A second round of proteolytic cleavage is then necessary to release TGF β from LAP, leaving un-bound TGF β free to bind to cell surface receptors and activate the TGF β pathway.

Activation of the TGF β complex is context dependent, but can be induced by proteolytically active molecules such as integrin $\alpha v \beta 6$, MMPs, and thrombospondin-1 (Frazier 1991, Munger, Harpel et al. 1997, Yu and Stamenkovic 2000). Further, it has been demonstrated that TGF β 1 can be liberated from ECM-stores mechanically, requiring the generation of cell traction by the actin cytoskeleton against a mechanically resistant substrate, as would occur in a fibrotic tissue. It is proposed that this process is mediated

through integrin recognition and binding of Arg-Gly-Asp (RGD) sequences on the LAP, which when in conjunction with a rigid ECM leads to a conformational change that releases TGF β (Wipff, Rifkin et al. 2007). Integrin-/traction-mediated TGF β activation has been demonstrated *in vivo* (Munger, Huang et al. 1999) and *in vitro* (Annes, Chen et al. 2004).

All isoforms of TGF β signal through serine-threonine kinase receptors (Figure 4). TGF β binds to a homodimer of Type II receptors (T β RII), which induces the recruitment of a homodimer of Type I receptors (T β RI) forming a heterotetramer complex. The kinase activity of T β RII leads to the phosphorylation of T β RI resulting in its activation and signaling, the most well-described being TGF β canonical signaling through Smads. T β RI activation leads to the phosphorylation of receptor-activated Smads (R-Smads), Smad2 and Smad3, which then co-localize with Smad4 and translocate to the nucleus to activate transcription of genes under the influence of Smad-binding elements (SBEs). Importantly, non-canonical TGF β pathways exist and are critically involved in the context of EMT and fibrosis. TGF β has been shown to signal through RhoA/ROCK, ERK1/2, JNK, p38 MAPK, and PI3K/Akt (Mu, Gudey et al. 2012). The focus of the current work is on TGF β -induced cytoskeletal signaling through RhoA/ROCK, described in detail below (Chapter 1.6).

1.5.3 Non-canonical TGF β signaling

From their discovery nearly two decades ago, the Smads were identified as the transcription factors responsible for mediating intracellular TGF β signaling through their continuous shuttling in and out of the nucleus (Roberts et al 2006). However, it is now

appreciated that TGF β -mediated effects are due to more complex interactions and cannot only be attributed to Smad signaling, where it has been demonstrated that the use of inhibitors and disruptions to Smad signaling are not sufficient in preventing EMT. In fact, TGF β stimulates a wide range of responses and pathways, a number of which are Smad-independent; these include, RhoA/ROCK, ERK1/2, JNK, p38 MAPK, and PI3K/Akt (Mu, Gudey et al. 2012). Previous work in our lab revealed that lens-specific expression of TGF β 1 in the absence of Smad3 was still able to induce the formation of anterior lens opacities that were positive for α SMA (Banh 2006), specifically pointing towards the involvement of non-canonical signaling pathways downstream of TGF β -induced lens EMT.

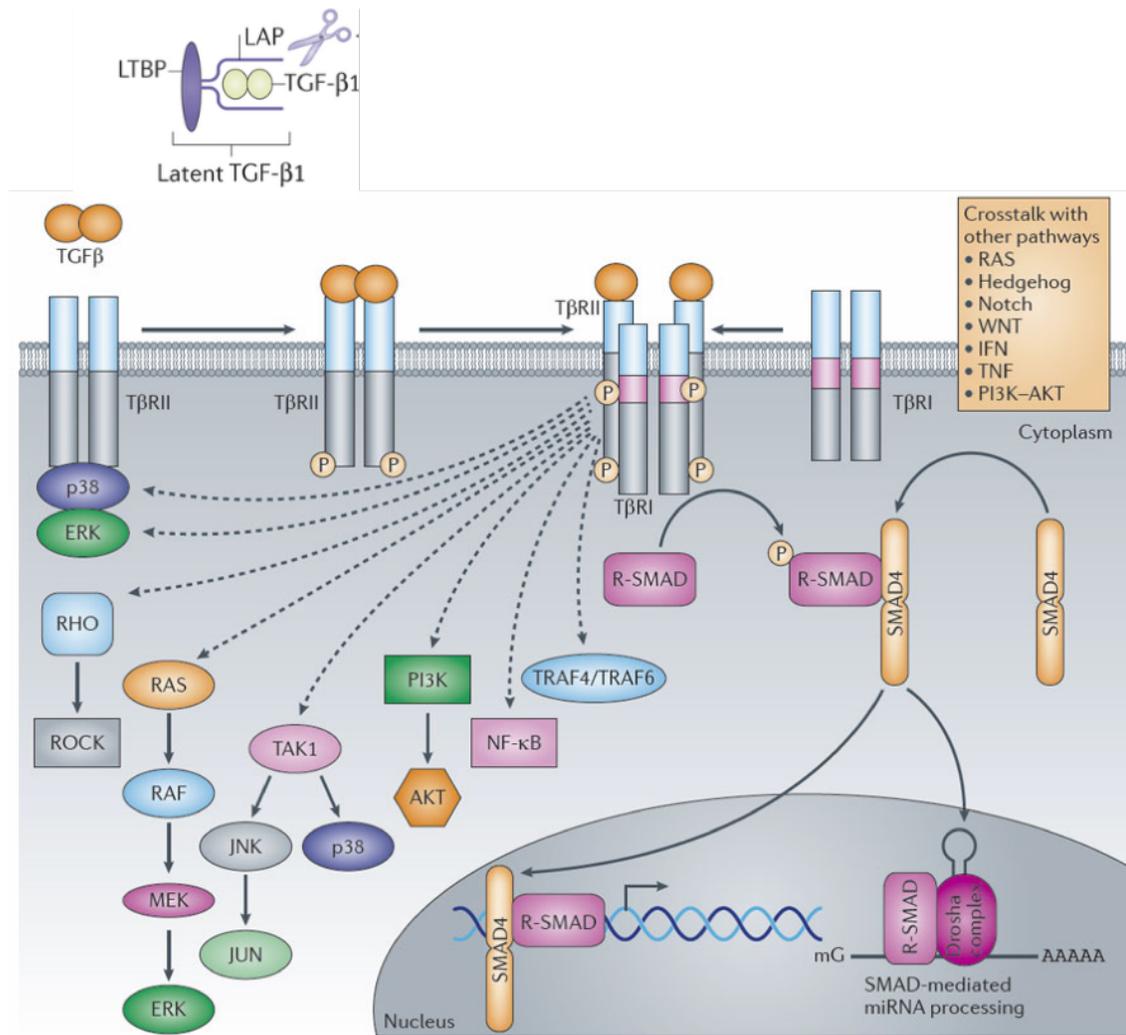


Figure 4. Canonical and non-canonical TGFβ signaling. Following latent TGFβ activation from the latency complex, TGFβ binds to a homodimer of Type II receptors (TβRII) that recruits a homodimer of Type I receptors (TβRI) to form a heterotetramer complex. TGFβ signals through the canonical Smad pathway, as well non-canonical pathways, including Rho/ROCK, PI3K/Akt, p38 MAPK, ERK 1/2 depicted. Figure adapted from Akhurst and Hata (2012).

1.6 Rho/ROCK-mediated cytoskeletal regulation

The TGF β signaling pathway affects the organization and regulation of the actin cytoskeleton, which has proven to be important for cell motility, differentiation and tissue organization (Moustakas and Heldin 2007). Key regulators of the actin cytoskeleton belong to the Rho family of small GTPases, which can be subdivided into three groups: Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2), and Cdc42. The activation of the prototypical members RhoA, Rac1, and Cdc42 by extracellular ligands leads to the formation of stress fibres, lamellipodia, and filopodia, respectively (Bishop and Hall 2000). RhoGTPases are essential for regulating actin polymerization and organization, and activating motor protein Myosin II involved in stimulating acto-myosin contractility; however, dysregulated actin dynamics can drive pathological conditions, such as fibrosis (Ivanov, Parkos et al. 2010). Myofibroblasts are defined by the *de novo* expression of α SMA, which is incorporated into actin-myosin contractile bundles, or stress fibres. In this manner, myofibroblasts exert tension on their surrounding ECM. This dissertation will focus on the role of Rho-mediated organization of the actin cytoskeleton and its involvement in pathological lens EMT.

1.6.1 RhoGTPase structure and activation

Similar to other GTPases, Rho GTPases act as molecular switches cycling between an inactive, GDP-bound form, and an active GTP-bound form. Inactive Rho-GTPase is activated through guanine nucleotide exchange factors (GEFs), while active Rho-GTPase returns to its inactive state through intrinsic GTPase activity catalyzed by GTPase

activating proteins (GAPs) (Van Aelst and D'Souza-Schorey 1997). Activated RhoA, bound to GTP, signals through its two effectors, Rho-associated coiled-coil-forming protein serine/threonine kinases (ROCK1 and 2) and mammalian diaphanous homologs (mDia1 and 2), which are both required for assembly of actin stress fibres and focal adhesions (Bishop and Hall 2000).

1.6.2 ROCK structure and function

ROCK consists of an N-terminal helix bundle domain, a kinase domain, central coiled-coil region, and C-terminal membrane-binding domains (Figure 5). The coiled-coil region contains the Rho-binding domain, while also separating the kinase domain from the membrane-binding domains. Activation of ROCK occurs through a conformational change, as it is normally autoinhibited (Amano, Chihara et al. 1999). It has recently been suggested by Truebestein, Elsner et al. (2015) that the function of ROCK within cells is dictated by its spatial positioning in relation to its substrate. The authors propose that the length, not the sequence, of its coiled-coil region is fundamentally important to ROCK-dependent signaling by acting as a 'molecular ruler' that is regulated by cytoskeletal tension.

ROCK is an important effector of RhoA-mediated regulation of the actin cytoskeleton, facilitating the interaction of myosin with filamentous (F-) actin. This promotion of acto-myosin contractile force generation is what enables alterations in cell shape, and is generated through myosin activity, activated both directly and indirectly

through ROCK (Turner 2000). The emergence of actin-myosin filaments is achieved by phosphorylation of two ROCK substrates: (1) through direct phosphorylation of the myosin light chain (MLC) regulatory units of Myosin II at Ser¹⁹, and (2) phosphorylation of myosin phosphatase leading to its inhibition and resulting in further phosphorylation of MLC (Tan, Ravid et al. 1992, Vicente-Manzanares, Ma et al. 2009). In addition, another substrate of ROCK, LIM kinase (LIMK), is responsible for the stabilization of actin filaments. Phosphorylation of LIMK leads to phosphorylation of cofilin on Ser³, which inhibits cofilin binding to actin subunits, thereby rendering it inactive. Phosphorylated (inactive) cofilin can no longer carry out its functions associated with binding and depolymerization of actin filaments (Shishkin, Eremina et al. 2016). Therefore, ROCK activity promotes cell contractility and formation of focal adhesion complexes, important for cell migration and polarity. Overexpression of ROCK, however, is associated with pathological conditions, such as metastasis (Bourguignon, Zhu et al. 1999), tumour invasion (Yoshioka, Nakamori et al. 1999), and hypertension (Masumoto, Hirooka et al. 2001).

1.6.3 ROCK-dependent signaling in epithelial cells

ROCK has also been implicated in the profibrotic responses of epithelial cells to tissue injury (Knipe, Tager et al. 2015). ROCK1-null mice exhibited protection from pathological induction of cardiac fibrosis with a decrease in fibrogenic cytokines, such as TGFβ₂ and CTGF mRNA, and collagen deposition (Zhang, Bo et al. 2006). The involvement of RhoA signaling through ROCK in TGFβ-induced EMT has been demonstrated in a number of *in vitro* and *in vivo* animal systems (Bhowmick, Ghiassi et al.

2001, Masszi, Di Ciano et al. 2003, Tian, Fraser et al. 2003, Tavares, Mercado-Pimentel et al. 2006, Zhang, Zhang et al. 2013). The first study to demonstrate these findings was carried out in mouse mammary epithelial cells, where the authors found that a 10 min TGF β treatment led to a 4-fold accumulation of activated RhoA when compared to control cells. In addition, in this system, dominant-negative RhoA cells blocked the acquisition of a mesenchymal phenotype following TGF β stimulation (Bhowmick, Ghiassi et al. 2001). In lens epithelial cells, TGF β induced RhoA activation with a corresponding increase in actin stress fibres and focal adhesions (Maddala, Reddy et al. 2003). Further, RhoA/ROCK signaling has been implicated in TGF β -induced α SMA expression in mouse lens epithelial cells cultures (Cho and Yoo 2007); however, the mechanism by which RhoA/ROCK signaling is linked to the expression of EMT-related markers has not been fully defined. In line with these studies, our own work showed a rapid increase in RhoA activity (GTP-bound form) in response to TGF β in a cultured human lens epithelial cell line (FHL 124), concomitantly demonstrating the presence of stress fibres, and overlapping expression of α SMA (Korol 2012).

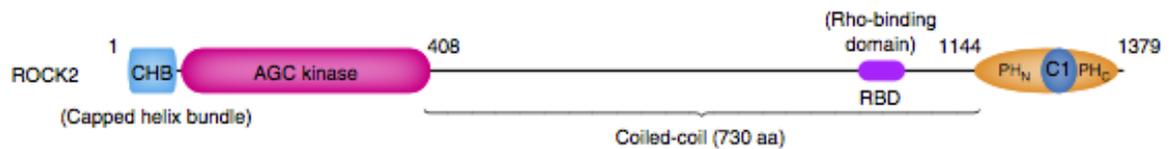


Figure 5. ROCK structure. ROCK consists of N-terminal kinase and C-terminal membrane-binding domains separated by 730 amino acids of a central coiled-coil, which also contains the Rho-binding domain (RBD). The N-terminal domain dimerizes to form a parallel coiled-coil. Figure adapted from Truebestein, Elsner et al. (2015).

1.7 Epithelial cell-cell adhesion

1.7.1 Adherens and tight junctions

As described above (Chapter 1.4), one of the earliest events in EMT is the loss of epithelial cell-cell adhesion. Epithelial cells adhere to one another through apical junctional complexes, which are composed of tight junctions (TJs) and adherens junctions (AJs) (Hartsock and Nelson 2008, Van Itallie and Anderson 2014). Through their cytoplasmic domains, both TJs and AJs are connected to the underlying actin cytoskeleton, which is important in maintaining epithelial cell morphology and function. While TJs are the primary determinants of epithelial barrier function, AJs are the principal structures involved in mechanical integration of cell-cell adhesion (Rodgers and Fanning 2011, Takeichi 2014, Lecuit and Yap 2015).

AJs consist of transmembrane cadherin receptors that anchor junctions by tethering with cytoplasmic components that are physically linked to actin networks (Takeichi 2014) (Figure 6). This organization both maintains cell adhesive properties and integrates intra- and intercellular signaling. Although mature epithelial junctions are stable, they are not static structures. AJs between cells are dynamic, essential to their ability to rearrange and remodel in response to external cues, such as during tissue morphogenesis and regenerative processes (ex. wound healing).

1.7.2 Interaction between AJs and actin cytoskeleton

It has been proposed that epithelial cell AJs can be divided into two forms: linear and punctate (Figure 6). The way in which E-cadherin interacts with actin filaments determines its organization and how it is visualized. Actin filaments within epithelial sheets are organized into linear actin bundles that run parallel to cell borders, outlining the plasma membrane of individual cells. This organization of actin, termed the circumferential actin belt, is localized at the apical ends of the cell, and it is along this belt that E-cadherin co-localizes in continuous clusters. This interaction results in stable epithelial architecture. Alternatively, at the edges of epithelial cell sheets or in cells such as mesenchymal cells, actin filaments are organized perpendicular to the plasma membrane. This orientation results in actin filaments exerting tension on AJs and when two neighbouring cells exhibit tense actin networks, E-cadherin gets pulled from both sides of the junction into a discontinuous, punctate form. This interaction leads to unstable, more mobile cell junctions, which are important for the remodeling of AJs for processes such as, morphogenesis and wound healing (Takeichi 2014).

The linear acto-myosin belt acts as a contractile F-actin ring that supports and stabilizes E-cadherin clusters at cell junctions. Conditional knockout of Myosin IIA in the mouse intestinal epithelium resulted in changes to the expression and localization of junctional complex components (Ivanov, Bachar et al. 2007, Smutny, Cox et al. 2010). However, excessive contractility of the acto-myosin apparatus is what results in the mechanical dissolution of cell junction complexes (Liu, Tan et al. 2010, Ramachandran

and Srinivas 2010). Mouse mammary epithelial cells undergoing EMT showed increased phosphorylation of Myosin IIA demonstrating the close association between acto-myosin contractility, stability of E-cadherin-based junctions and EMT (Beach, Hussey et al. 2011).

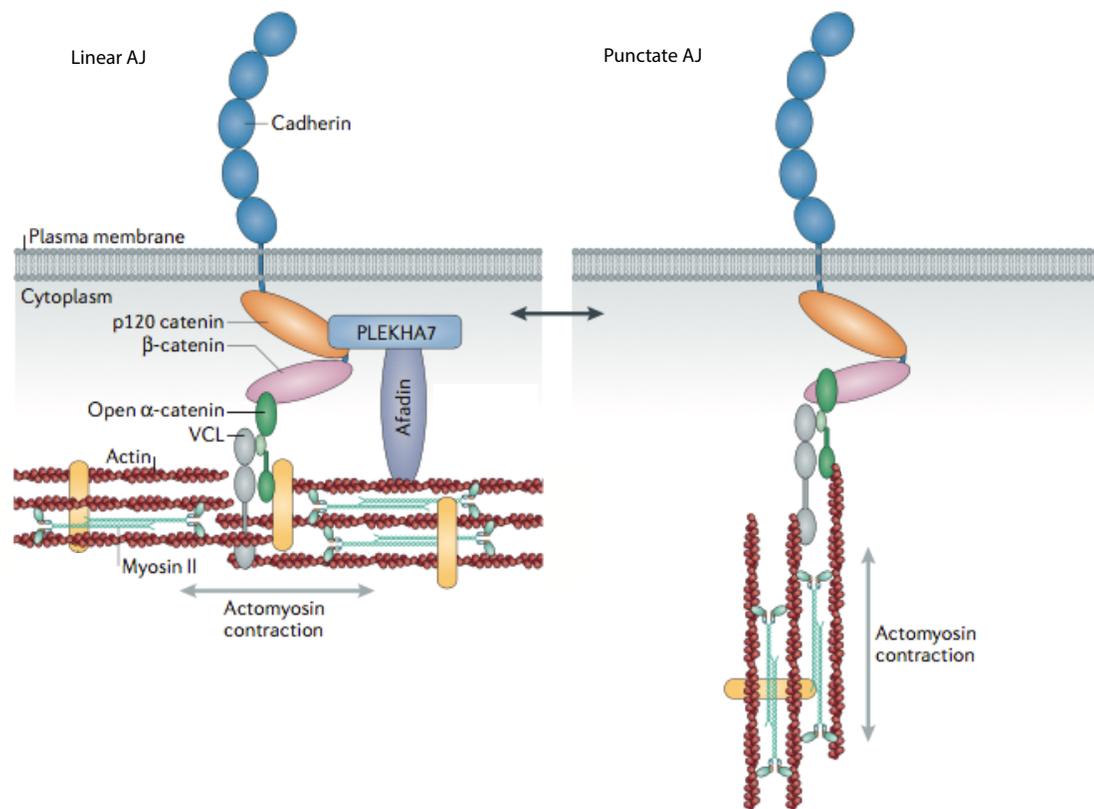


Figure 6. Proposed E-cadherin-based adherens junction (AJ) distribution. Linear AJ complexes interact with actin filaments that run parallel to cell borders, at the apical ends of epithelial cells. Punctate AJ complexes interact with actin filaments that run perpendicular to, and exert tension on, cellular junctions. Figure adapted from Takeichi (2014).

1.7.3 *E-cadherin structure and function*

The cadherin receptor superfamily is distinguished by a Ca^{2+} -binding amino acid motif. In vertebrates, there are 20 subtypes of ‘classic’ cadherins, the best characterized being E- and N-cadherin. Classical cadherins are single-pass transmembrane proteins with an extracellular region that binds to cadherins on the cell surface of adjacent cells. A unique and important feature of classic cadherins is their conserved cytoplasmic domain that interacts with intracellular catenin proteins, an interaction that is crucial to AJ formation and function (Takeichi 2014). Since E-cadherin downregulation and loss are hallmarks of EMT, and have previously been implicated in our lens explant system (Korol, Pino et al. 2014), the current work will focus on E-cadherin-mediated intercellular adhesion and its connection to the actin cytoskeleton.

E-cadherin is essential for proper formation and maintenance of epithelial cell junctions. The extracellular portion of E-cadherin consists of five extracellular cadherin domains (Figure 6). E-cadherins on neighbouring cells form Ca^{2+} -dependent homophilic *trans*-cadherin dimers through their first extracellular domain. On the cytoplasmic end of the adhesion complex, E-cadherin consists of two regions: (1) the juxtamembrane region regulates cadherin stability through binding to p120 and associated proteins; (2) the C-terminal catenin-binding domain directly binds γ - and β -catenins, and indirectly binds α -catenin. The cadherin-catenin interaction is critical to establishing proper cell-cell contact, as it is proposed that α -catenin binds F-actin, facilitating firm intercellular adhesion. The precise molecular mechanisms mediating cadherin-actin connections, and whether actin

binds directly to α -catenin or indirectly through vinculin, is still being debated and may be cell type-specific. However, the main point to highlight is the fact that cell-cell contact integrity is physically intertwined with actin filaments and thus, cytoskeletal dynamics regulate AJ stability, structure and function (Rodgers and Fanning 2011, Takeichi 2014, Lecuit and Yap 2015). Conversely, the state of AJs affects the organization of actin filaments and contractility (Izumi, Sakisaka et al. 2004, Cavey, Rauzi et al. 2008). The use of low Ca^{2+} cell medium to trigger disassembly of E-cadherin-based junctions led to strong RhoA activation, and TGF β -induced EMT (Masszi, Di Ciano et al. 2003, Fan, Sebe et al. 2007). Therefore, the level of acto-myosin contractility must be precisely balanced to maintain cell junction integrity, and thus, the epithelial phenotype, and any deviations can result in pathological EMT.

1.7.4 Role of cadherin-catenin complex

Beyond their structural role in cell adhesion, the dissolution of cadherin-catenin complexes is involved in activating key intracellular signaling networks. AJ dissolution results in punctate E-cadherin and, importantly, frees cytoplasmic components organized within the cadherin-catenin complex. Some of these components have dual roles as transcriptional cofactors, as in the case of β -catenin, described below, and myocardin-related transcription factor (MRTF), an actin-binding protein which will be discussed in the next section (Chapter 1.8).

β -catenin binds to the catenin-binding domain on the intracellular face of E-

cadherin, and plays an essential structural role in cellular junctions, connecting transmembrane E-cadherin to the actin cytoskeleton. Disruption to the E-cadherin/ β -catenin connection releases β -catenin from intracellular adhesion complexes. Cytosolic β -catenin is incorporated into a multiprotein destruction complex, which includes glycogen synthase kinase-3 β (GSK-3 β), axin, and adenomatous polyposis coli (APC). This complex facilitates β -catenin ubiquitination and targeted degradation (Aberle, Bauer et al. 1997, Orford, Crockett et al. 1997, Miller, Hocking et al. 1999). Alternatively, β -catenin is a central mediator of the canonical Wnt pathway, which when activated by Wnt receptors results in the inactivation of GSK-3 β , and release of β -catenin from the degradation complex. In this situation, β -catenin is now free and translocates to the nucleus, where it functions as a co-activator of T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors. This nuclear complex is involved in cell- and context-specific activation of genes, including those regulating cell proliferation, ECM remodeling, and cell-cell adhesion (Nelson and Nusse 2004, Klaus and Birchmeier 2008), cellular processes that are dysregulated in fibroproliferative disorders. Indeed, TGF- β -induced EMT involves cross-talk between other signaling pathways and their downstream components, such as the Wnt/ β -catenin pathway. In particular, during cataractogenesis, TGF β was found to promote the expression of Wnts, along with its receptor, Frizzled, involved in facilitating the translocation of β -catenin from the cell membrane to the nucleus (Chong, Stump et al. 2009). β -catenin has been shown to play an important synergistic role in TGF β -induced EMT in tubular epithelial cells (Masszi, Fan et al. 2004), as well as in the rat lens explant

system, in which a recent study demonstrated that β -catenin transcriptional activity, in complex with CREB-binding protein (CBP), is required for lens EMT (Taiyab, Korol et al. 2016).

1.8 MRTF-A

1.8.1 Actin-binding proteins

Having established that cadherin-based contacts are tethered to the actin cytoskeleton and can affect actin dynamics, the question that remains is how transcriptional machinery senses these changes in actin. The key to relaying cytoskeletal rearrangements to the nucleus is through the release, nuclear translocation and transcription of actin-binding proteins (ABPs). ABPs serve a number of roles in regulating actin dynamics, such as organizing actin filaments, localizing actin at the membrane, regulating actin stability and state of polymerization, and acting as scaffolds for signaling (Arnold, Stephenson et al. 2017). The nuclear activity of transcriptional cofactors belonging to the myocardin protein family, the MRTFs, are currently the focus of numerous studies that are considering how Rho-induced actin polymerization is involved in EMT. MRTFs have a dual function as ABPs, and, when not bound to actin, act as cofactors regulating the activity of serum response factor (SRF). MRTF/SRF-mediated transcriptional activity has been implicated in EMT (Miralles, Posern et al. 2003, Fan, Sebe et al. 2007, Morita, Mayanagi et al. 2007, Busche, Kremmer et al. 2010, Fintha, Gasparics et al. 2013) (33–38); however, little is

known about their role in the lens (Gupta, Korol et al. 2013).

1.8.2 MRTF structure and activity

Two isoforms of MRTF exist, -A and -B, which are widely expressed in numerous embryonic and adult tissues (Wang, Li et al. 2002). The subcellular localization of MRTF-A and -B is uniquely regulated by the relative concentrations of globular (G)- and F-actin. MRTF contains three N-terminal RPEL domains, which recognize and bind G-actin monomers, and when bound to MRTF, sequester it in the cytoplasm and prevent its activity (Figure 7). G-actin monomers physically block importin α/β from binding the nuclear localization signal of MRTF, preventing its nuclear import. Upon actin polymerization, G-actin is incorporated into F-actin polymers, thereby decreasing the ratio of cytoplasmic G- to F-actin, freeing MRTF and promoting nuclear accumulation by increasing nuclear import and blocking Crm1-mediated nuclear export (Mouilleron, Langer et al. 2011). The shuttling of MRTFs between the cytoplasm and the nucleus is a dynamic and reversible process dependent upon the mechanics of the plastic actin network. In this manner, MRTFs act as sensors for cellular concentrations of G-actin.

As a transcription factor, MRTF accumulates in the nucleus where it serves as a cofactor for SRF, activating transcription of cytoskeletal genes associated with EMT, including α SMA (Fan, Sebe et al. 2007, Miano, Long et al. 2007, Zhao, Laschinger et al. 2007, Sebe, Masszi et al. 2008). Indeed, the transition of dermal fibroblasts into α SMA-positive myofibroblasts in a mouse wound healing model was dependent upon the MRTF-

A/SRF transcriptional complex, downstream of TGF β (Tomasek, Gabbiani et al. 2002, Crider, Risinger et al. 2011). This makes MRTF-A essential in relaying changes in the actin cytoskeleton into changes in gene expression, promoting an SRF-mediated contractile phenotype. As key regulators of the actin cytoskeleton, it is not surprising that RhoA is the best-characterized mediator of MRTF-A/SRF complex formation and activity. RhoA signaling through ROCK results in actin polymerization and F-actin polymer stabilization, which depletes cellular G-actin pools, manifesting in MRTF nuclear accumulation and SRF-dependent transcription of EMT target genes.

1.8.3 MRTF-A in EMT

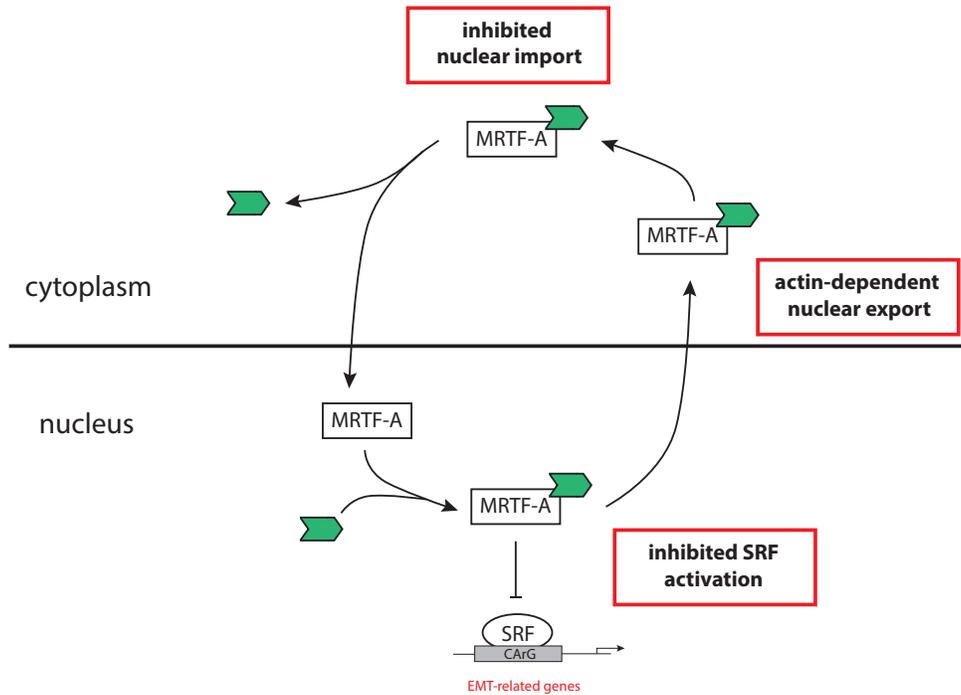
TGF β has also been linked to nuclear accumulation and activity of MRTF-A in several organ fibrosis models (Fan, Sebe et al. 2007, Hinson, Medlin et al. 2007, O'Connor, Riley et al. 2015) through its involvement in EMT. In tubular epithelial cells, nuclear accumulation of MRTF-A occurred in response to loss of cell-cell contacts, while silencing MRTF-A prevented TGF β -induced α SMA expression (Fan, Sebe et al. 2007). These observations suggest that MRTF-A is essential in promoting EMT and the myofibroblast phenotype. Work performed previously in our lab in lens explants demonstrated that, under control conditions MRTF was retained in the cytoplasm, while TGF β treatment triggered the nuclear localization of MRTF-A, but not MRTF-B (Gupta, Korol et al. 2013). Further, this study showed that subcellular localization of MRTF-A was responsive to changes in actin dynamics where the disruption of actin polymerization through cytochalasin-D and latrunculin-B blocked TGF β -induced nuclear accumulation. However, further studies in

the lens are required to determine the requirement of MRTF-A downstream of TGF β and its contribution to lens fibrosis.

1.8.4 MRTF/SRF induces α SMA expression

The MRTF-A/SRF transcriptional complex binds with high affinity to promoters with one or more functional CArG [CC(A/T)₆GG] elements in their promoters (Treisman 1986, Pellegrini, Tan et al. 1995). The α SMA gene promoter contains multiple transcriptional regulatory elements including, E-boxes (Kumar, Hendrix et al. 2003), SBES (Hu, Wu et al. 2003), a TGF β control element (TCE), and importantly CArG elements (Hautmann, Madsen et al. 1997). Tomasek, McRae et al. (2005) determined that, for TGF β to induce α SMA expression in *in vivo* myofibroblasts, the promoter region required both TCE and CArG elements, suggesting an important role for MRTF-A/SRF activity. Indeed, MRTF-A overexpression in tubular epithelial cells was sufficient to induce α SMA protein expression (Fan, Sebe et al. 2007, Elberg, Chen et al. 2008). Therefore, this suggests that MRTF-A acts as a link between actin dynamics and cytoskeletal gene induction, important for regulating myofibroblast differentiation.

Untreated



TGFβ treatment

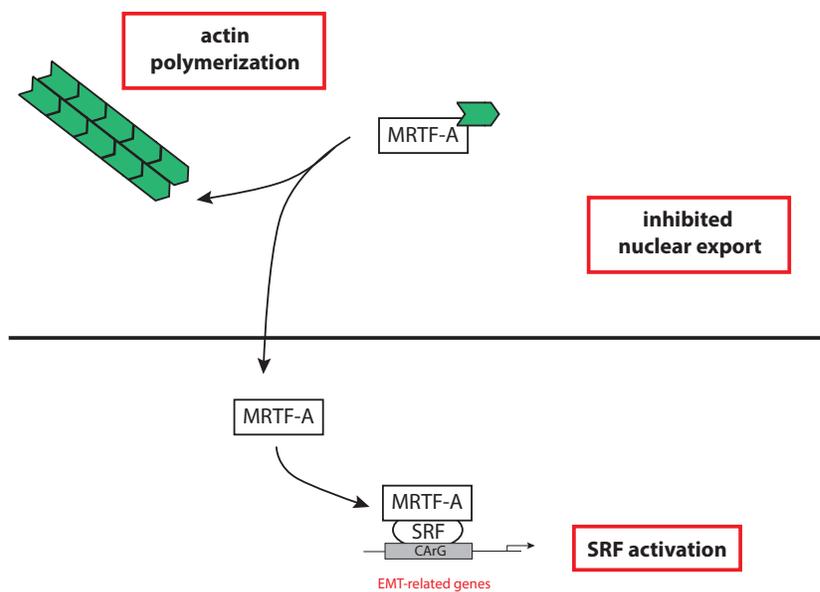


Figure 7. Regulation of MRTF-A localization. In untreated cells, MRTF-A is bound to monomeric G-actin, which maintains predominantly cytoplasmic MRTF-A localization through inhibited nuclear import and actin-dependent nuclear export. This prevents MRTF-A/SRF complex formation and activation of EMT-related genes. In contrast, TGF β depletes G-actin levels through the induction of actin polymerization, resulting in nuclear accumulation of MRTF-A, and inhibited nuclear export. This leads to MRTF-A/SRF-mediated transcription.

1.9 Matrix Metalloproteinases

TGF β -induced EMT events have been associated with increases in a family of zinc-dependent endopeptidases, the matrix metalloproteinases (MMPs). MMPs have historically been seen as enzymes responsible for the degradation of the structural components of the ECM, necessary for maintaining normal physiological processes such as, regulating tissue remodeling during embryonic development, and wound healing (Seomun, Kim et al. 2001). Recently, MMPs have also been shown to cleave circulating cell surface molecules and other extracellular non-matrix proteins, contributing to the regulation of cell behaviour (Nelson, Fingleton et al. 2000, Sternlicht and Werb 2001). Importantly, MMPs have been implicated in a number of ocular diseases including, retinal disease, glaucoma, corneal disorder, scleritis, uveitis, pterygium (Sivak and Fini 2002, Alapure, Praveen et al. 2012), and accumulating evidence suggests MMPs play an integral part in the mechanisms involved in cataract formation.

1.9.1 MMP family

The MMP family consists of over 20 structurally related members that can be classified as, membrane-bound (MMP-14, -15, -16, -17, -24, -25), or secreted proteins. These are further subdivided into four categories: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11, -12, -28), and matrilysins (MMP-7, -26) (West-Mays and Pino 2007) (Figure 8). The basic structural domains of MMPs are fairly conserved, each with a N-terminal signal peptide, for secretion, linked to a pro-

peptide domain, for sustaining enzyme latency. A catalytic domain, with a zinc-binding region, is attached to a hemopexin-like C-terminal domain functioning as a substrate recognition sequence, and determining substrate specificity (Nagase and Woessner 1999). The gelatinases, MMP-2 and -9, contain three fibronectin repeats, which facilitate the degradation of gelatinous substrates (Shipley, Wesselschmidt et al. 1996, Bode, Fernandez-Catalan et al. 1999). Uniquely, MMP-9 contains a central O-glycosylated domain that connects the active site to the hemopexin domain. This linker region has been demonstrated to be indispensable to MMP-9 function and confers the enzyme with a high degree of flexibility (Van den Steen, Van Aelst et al. 2006, Dufour, Sampson et al. 2008, Vandooren, Geurts et al. 2011).

1.9.2 MMP regulation and activation

MMPs are primarily regulated at the transcriptional level by growth factors, cytokines, cell-cell interactions, and their surrounding cellular environment. Most MMPs are constitutively secreted as latent enzymes, their pro-peptide domain containing an unpaired cysteine residue that associates with the active zinc site within the catalytic domain. Their activity is localized near the cell surface and depends upon the balance between MMP activators and endogenous inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs), in the surrounding extracellular space (Brew, Dinakarbandian et al. 2000). There are currently four TIMP isoforms identified (TIMP-1, -2, -3, -4), each with distinct affinities for different MMPs. TIMP-1 preferentially forms a complex with proMMP-9 influencing its proteolytic activity (Goldberg, Strongin et al. 1992).

1.9.3 MMP-regulated cell behaviour

Each MMP selectively degrades components of the ECM, leading to alterations in the surrounding microenvironment; however, MMP-driven proteolysis does not end in mere ECM digestion and remodeling, but continues in the indirect regulation of cell behaviour and activity in an “outside-in” manner. The ECM regulates cell shape, growth, and motility. By extension, MMPs are also responsible for regulating these cell processes by acting on cell matrices. Through the disruption of matrix barriers, MMPs can release and modify various growth factors and cytokines embedded within the ECM (Sivak and Fini 2002). In this manner, MMPs are controlling the bioavailability and bioactivity of growth factors and cytokines, and their receptors. These molecules are normally ECM-sequestered in an inactive state, and upon their release are free to activate signaling sequences, which are then propagated to the cell nucleus (Kessenbrock, Plaks et al. 2010). The gelatinases, MMP-2 and -9, have been shown to release and activate interleukin 8, interleukin 1 β , and TGF β from ECM constituents that are implicated in the pathways involved in angiogenesis, inflammation, and carcinogenesis (Schonbeck, Mach et al. 1998, Van den Steen, Proost et al. 2000, Yu and Stamenkovic 2000).

The mechanisms of aberrant MMP activity are highly studied in tumorigenesis and various types of cancer systems, where cancer cells express high levels of MMPs, as well as integrins correlating with a high degree of motility, EMT, and invasiveness (Bourboulia and Stetler-Stevenson 2010). Tumour growth and metastasis are less prevalent in mice with specific MMP gene disruptions, further indicating the involvement of MMPs in

uncontrolled cell growth and invasiveness (Itoh, Ikeda et al. 1997). This can occur through MMP-driven substrate cleavage, which can produce fragmented proteins with novel biological activities when compared to the intact molecule, leading to intracellular rearrangement of the cytoskeleton, and consequent cell migration (Lochter, Galosy et al. 1997, Noe, Fingleton et al. 2001). For example, MT1-MMP and MMP-2-induced cleavage of laminin-5 produces fragments, which through an exposed cryptic promigratory site, stimulate cell migration in mammary epithelial cells (Giannelli, Falk-Marzillier et al. 1997, Koshikawa, Giannelli et al. 2000).

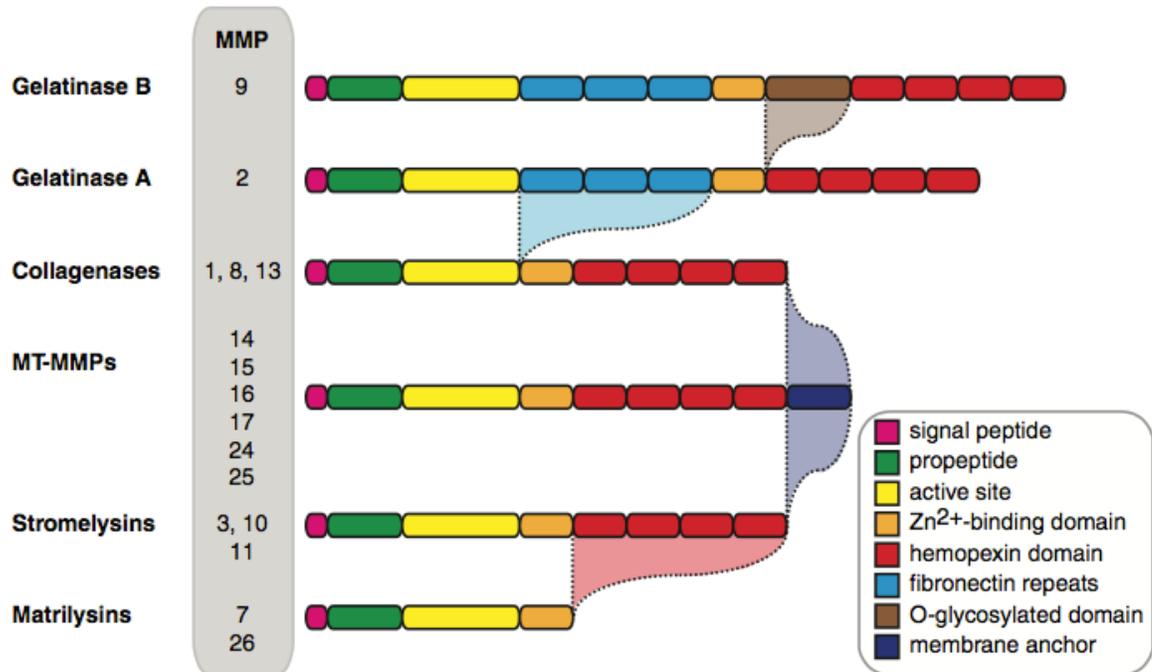


Figure 8. Structure of MMPs. MMPs are classified as membrane-associated (MT-MMPs) or secreted (collagenases, stromelysins, matrilysins, gelatinases). Generally, MMPs have: 1) an N-terminal signal peptide targeting zymogen secretion; 2) a propeptide domain maintaining enzyme latency; 3) a Zn²⁺ catalytic domain; 4) a C-terminal hemopexin domain determining substrate specificity. MMP-2 and -9 contain three fibronectin repeats that allow for degradation of gelatinous substrates, while uniquely, MMP-9 contains a central O-glycosylated region that links the active site to the hemopexin domain. Figure adapted from Vandooren, Van den Steen et al. (2013).

1.9.4 MMPs in the eye

In the eye, MMPs have been implicated in corneal wound healing, proliferative retinopathies, and macular degenerations (Fini, Cook et al. 1998, Sivak and Fini 2002). In the lens, lens epithelial cells synthesize and secrete MMPs following injury by ultraviolet irradiation, oxidative stress, or cataract surgery (Tamiya, Wormstone et al. 2000, Wormstone, Tamiya et al. 2002, Sachdev, Di Girolamo et al. 2004). MMPs are detected in the media of whole cataractous lenses compared to normal non-cataractous lenses, as well as capsular bags following sham cataract surgery. This indicates a role for MMPs in a wound healing process in response to injury or stress (Tamiya, Wormstone et al. 2000, West-Mays and Pino 2007). The most widely studied MMPs in the ocular tissue are MMP-2 and MMP-9 (Alapure, Praveen et al. 2012). Both MMP-2 and MMP-9 expression have been detected in the normal rat and mouse lens (John, Jaworski et al. 2004, Descamps, Martens et al. 2005, Dwivedi, Pino et al. 2006), and levels of both gelatinases were induced in response to TGF β . For example, Richiert and Ireland (1999) demonstrated that expression of both gelatinases was induced in primary chick lens cells following, and specific to, treatment with TGF β .

Previous studies in our lab have also demonstrated the enhanced secretion of both MMP-2 and -9 in the conditioned media of whole rat lenses following TGF β treatment. Furthermore, co-treatment of this *in vitro* lens cataract model with TGF β and a specific MMP2/9 inhibitor, suppressed anterior opacities that were otherwise visible on the lens when solely treated with TGF β (Dwivedi, Pino et al. 2006). This indicates a critical role

for the two gelatinases in the formation of ASC that is proposed to occur through the disruption E-cadherin. A loss of E-cadherin, and thus a loss of intercellular contacts, correlates with EMT, and research is focusing on the proteolytic activity of MMPs to explain this correlation (Zheng, Lyons et al. 2009). MMPs have been found to cleave the N-terminal extracellular domain of E-cadherin releasing a unique 72kDa fragment into the conditioned medium of *in vitro* lens epithelial cells, a phenomenon known as E-cadherin shedding (Dwivedi, Pino et al. 2006). Cleavage and downregulation of E-cadherin by MMPs eliminates both the link between cells at the cell junction, as well as the link between the ECM and actin cytoskeleton. Recent work in our lab using MMP-2 and MMP-9 knock-out (KO) mice sought to characterize the individual contributions of the gelatinases in lens EMT. Administering adenoviral TGF β 1 *in vivo* and TGF β 2 in *ex vivo* lens explants demonstrated that MMP-9, but not MMP-2, expression is necessary for TGF β -mediated α SMA expression and lens fibrosis (Korol, Pino et al. 2014). Further, overexpression of TGF β 1 in a transgenic mouse model bred onto an MMP-9 KO background showed resistance to ASC formation, whereas the MMP-2 KO mice did not. These studies identify MMP-9 as a critical participant in the EMT process, specifically in the lens; however, the nature of the role of MMP-9 in lens EMT has not been fully defined. The current work will elaborate on the involvement of MMP-9 in lens EMT, with a focus on cell contact dissolution and the involvement of the actin cytoskeleton.

CHAPTER 2: OBEJCTIVE, CENTRAL HYPOTHESIS AND AIMS

2.1 Objective

The overall objective of this work is to get a better understanding of the mechanisms involved in TGF β -induced lens EMT in the quest for the development of therapeutic strategies targeting fibrotic diseases, specifically the lens fibrotic pathology, PCO. Three strategies for preventing lens EMT in an *ex vivo* lens explant system are presented in this dissertation, targeting aspects of the TGF β signaling pathway that are critical to lens EMT.

2.2 Central Hypothesis

The central hypothesis around which the experiments in this thesis were designed can be summarized as:

Targeting components downstream of RhoA/ROCK-mediated cytoskeletal signaling will prevent TGF β -induced lens EMT

2.3 Specific Aims

The hypotheses of the thesis are addressed by the following specific aims:

Aim 1: To determine whether inhibiting ROCK-dependent cytoskeletal signaling using Y-27632 will prevent TGF β -induced lens EMT

Aim 2: To determine whether inhibiting nuclear accumulation of MRTF-A using CCG-203971 will prevent TGF β -induced lens EMT

Aim 3: To determine the specific involvement of MMP-9 in cytoskeletal remodeling and E-cadherin disruption in lens epithelial explants

Aim 1 & 2 resulted in the following publication:

Korol, A., et al. (2016). "RhoA/ROCK signaling regulates TGFbeta-induced epithelial-mesenchymal transition of lens epithelial cells through MRTF-A." Mol Med 22: 713-723.

Aspects of Aim 3 contributed to the following publication:

Korol, A., et al. (2014). "Matrix metalloproteinase-9-null mice are resistant to TGF-beta-induced anterior subcapsular cataract formation." Am J Pathol 184(7): 2001-2012.

CHAPTER 3: METHODOLOGY

3.1 Animal Studies

All animal studies were performed according to the Canadian Council on Animal Care Guidelines (*Guide to the Care and Use of Experimental Animals*, 2nd edition, 1993) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. MMP-9 KO mice (Jackson laboratories, Bar Harbor, ME) were bred on a C57BL/6 background and were generated by the removal of part of exon 2 and all of intron 2 of the *Mmp9* gene. In both cases these regions were replaced with a *pgk-neo* gene cassette. (Itoh, Ikeda et al. 1997, Vu, Shipley et al. 1998).

3.2 Genotype Analysis

DNA extraction and purification from mouse ear tissue was performed with a kit (DNeasy, Qiagen Inc., Toronto, ON). Genotypes were determined by polymerase chain reaction (PCR) analysis. *Mmp9* wild-type and KO alleles were detected using the following primers; The *Mmp9* wild-type allele was detected by using primers (5'-GTGGGACCATCATAACATCAC-3') and (5'-CTCGCGGCAAGTCTTCAGAGTA-3') amplifying a 277-bp fragment. The *Mmp9* KO allele was detected by using primers (5'-CTGAATGAACTGCAGGCAGA-3') and (5'-ATACTTCTCGGCAGGAGCA-3') to yield a 172-bp fragment. PCR reactions were performed for 35 cycles in the following

conditions: initial heating for 2 minutes at 94°C; denaturation for 30 seconds at 94°C; annealing for 30 seconds at 60°C; and extension for 2 minutes at 72°C. A final extension was performed for 10 minutes at 72°C. Agarose gel electrophoresis (1.5% agarose) with ethidium bromide detection was used to visualize the PCR reaction products.

3.3 Reagents

Recombinant human TGFβ2 and inhibitor CCG-203971 were obtained from R&D Systems (Minneapolis, MN). Active human recombinant MMP-9 (PF140) was purchased from EMD Millipore (Temecula, CA). Y-27632 was purchased from Calbiochem (La Jolla, CA). Primary antibodies: E-cadherin from BD Transduction Laboratories (Lexington, KY), active β-catenin (clone 8E7) and catalytic domain of MMP-9 from EMD Millipore, pMLC (phospho S20), pan actin [2Q1055], and GAPDH and β-tubulin from Abcam (Cambridge, MA), rhodamine phalloidin from Cytoskeleton (Denver, CO), MRTF-A from Santa Cruz Biotechnology (Santa Cruz, CA), αSMA fluorescein isothiocyanate (FITC)-conjugated and unconjugated from Sigma-Aldrich. All secondary antibodies for immunofluorescence staining were purchased from Invitrogen, while secondary antibodies for Western blots were obtained from LI-COR Biosciences (Lincoln, NE).

3.4 *Ex vivo* rat lens epithelial explants: preparation and treatment

Rat lens epithelial explants were obtained from 17- to 19-day old Wistar rats (Charles River Laboratories, Montreal, QC) (Figure 9). Rat lenses were isolated and placed in 35 mm culture dishes containing pre-warmed, serum-free medium M199, supplemented with antibiotics (all from Invitrogen). The posterior suture of the lens is located and a small incision is made to remove the posterior capsule. This reveals the fibre mass, which is then gently peeled off the anterior epithelium. Once separated, the epithelium is then pinned to the culture dish with a blunt tool, exposing lens epithelial cells to culture medium. After 24 hrs, confluent epithelial explants were left untreated in serum-free M199 or treated with TGF β 2 or rhMMP-9. For ROCK inhibitor studies, lens explants were pre-treated with Y27632 (20 μ M) (Uehata, Ishizaki et al. 1997) for 1hr, followed by a co-treatment with TGF β 2 (4 ng/ml) for 48 and 72 hrs. For MRTF-A inhibition studies, CCG-203971 was dissolved in DMSO and lens explants were pre-treated with CCG-203971 (5 μ M) for 1 hr, followed by a co-treatment with TGF β 2 (4 ng/ml) for 48 hrs. Untreated explants for CCG-203971 studies were incubated in DMSO (0.1%). For rhMMP-9 studies, lens explants were treated with either rhMMP-9 (100ng/ml) or TGF β 2 (4 ng/ml) for 1-4 days.

3.5 *Ex vivo* mouse lens epithelial explants: preparation and treatment

Similarly, to obtain lens epithelial cell explants, 6- to 8-week-old MMP-2 and -9 wild-type and KO mouse lenses were dissected and placed in 35 mm basement cell extract-

coated (Trevigen, Inc., Gaithersburg, MD) culture plates containing pre-warmed, serum-free medium M199, supplemented with antibiotics (Figure 9). The lens was placed posterior-side up, the posterior pole was gently torn, and the fibre mass slowly removed revealing the epithelium. Once separated, the epithelium is then pinned with a blunt tool to the bottom of the culture dish with the lens epithelial cells directly bathed by the medium, and the outer lens capsule facing downwards. Twenty-four hrs after explanting, confluent mouse epithelial explants were left untreated in serum-free M199 or treated with TGF β 2 at 500 pg/ml for 48 hours.

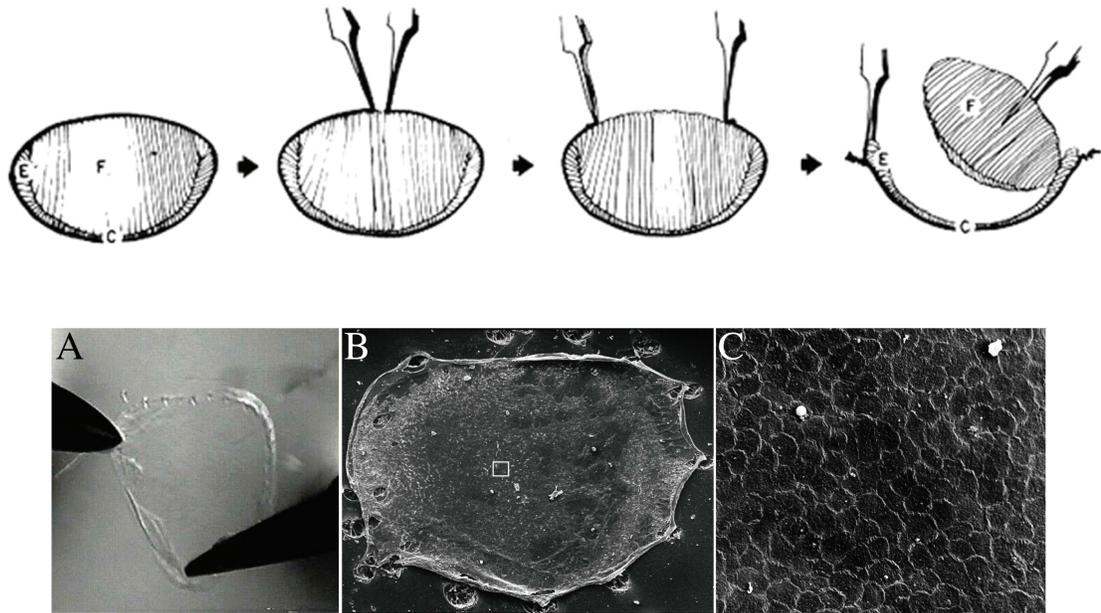


Figure 9. *Ex vivo* lens explant model. Rat and mouse lenses were isolated and placed in media-containing culture dishes. Lenses are first oriented with the central lens epithelial monolayer (top; C) in contact with the bottom of the dish. A small incision is made at the posterior lens capsule to reveal the fibre mass, which is then gently peeled off the anterior lens epithelial cells, importantly still left adhered to the lens capsule. The anterior epithelium is then flattened and pinned to the culture dish (A & B). After a 24 hr incubation, lens epithelial cells form a cobblestone monolayer that is ready for treatment (bottom; C). Figure adapted from West-Mays, Pino et al. (2009) and Belecky-Adams, Adler et al. (2002).

3.6 Immunofluorescence staining

Lens explants were fixed with 10% neutral buffered formalin for 15 mins, and washed with phosphate-buffered saline (Invitrogen). Explants were then detached from their culture dish and immersed in permeabilizing buffer (0.1% Triton X-100, 0.5% sodium dodecyl sulphate [SDS] and 5% donkey serum) for 1 hr at the bottom of their own 12x75 mm glass culture tube. Primary (overnight at 4°C) and secondary antibody (1.5 hr at RT) incubation, as well as PBS washes were all carried out in these tubes under gentle agitation, ensuring that explants are always immersed in a small volume of solution at the bottom of the tube. Following the last set of washes, explants were flattened onto a slide and mounted in Prolong Gold antifade reagent with DAPI (Invitrogen) to visualize nuclei. Immunostained explants were analyzed using a Zeiss Apotome inverted microscope (Carl Zeiss Canada, Toronto, ON) or a Leica DMRA2 fluorescence microscope (Leica Microsystems Canada, Richmond Hill, ON) equipped with a Q-Imaging RETIGA 1300i FAST digital camera (Q-Imaging, Surrey, BC). All immunofluorescence images are taken from the centre of the explant and are representative of the entire explant. All images in figures labeled “Control” are representative of both untreated and inhibitor-only-treated explants. Images were captured using OpenLab software 4.0 (PerkinElmer LAS, Shelton, CT) and minor adjustments in brightness/contrast were made using Photoshop CS3 (Adobe Systems, Mountain View, CA). MRTF-A nuclear localization was analyzed using Fiji image processing software (Schindelin, Rueden et al. 2015), where nuclear MRTF-A fluorescence intensity was measured and normalized to cytoplasmic fluorescence.

Nuclear/cytoplasmic ratios were reported relative to untreated control ratios.

3.7 Western Blot

Lens explants ($n \geq 5$ per sample per experiment) were pooled and lysed in Triton lysis buffer (50mM Tris, pH 8.0, 150mM NaCl, 1% Triton X-100) with Complete Mini, EDTA-free Protease Inhibitor cocktail (Roche, Laval, QC). Equal amounts of total protein were loaded on a gel, as determined by DC Protein Assay (Bio-Rad, Mississauga, ON) and SDS-PAGE was performed. Resolved bands were transferred onto a nitrocellulose membrane. Membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences) for 1 hr and incubated with unconjugated α SMA, E-cadherin, pMLC or MMP-9, always with GAPDH or β -tubulin primary antibodies as loading controls, overnight at 4°C. Blots were washed in Tris-Buffered Saline with Tween-20 (1%) and probed with corresponding secondary antibodies. Densitometry on visualized bands was performed using Fiji image processing software where each band was normalized to its corresponding GAPDH band. GAPDH-normalized values were reported as a fold reduction in α SMA, E-cadherin or MMP-9 expression relative to TGF β -treated controls (equal to 1).

3.8 E-cadherin ELISA

Levels of E-cadherin were measured in the cultured media of untreated and treated, wild-type and MMP-9 KO lens explants by using a mouse E-cadherin-specific ELISA

assay (R&D Systems Inc., Minneapolis, MN). Media from explant dishes (4-6 explants/dish) was collected and concentrated. Standards and samples were applied to a microplate coated with rat anti-mouse E-cadherin recognizing the N-terminal domain of E-cadherin, and the assay was carried out according to the manufacturer's protocol. The optical density of standards and samples was determined at 450 nm using a microplate reader and E-cadherin protein concentrations were extrapolated from a standard curve.

3.9 Statistical Analysis

Data are expressed as the means \pm SEM and statistical significance was determined by Student's *t*-test or two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test performed post-hoc (GraphPad Prism version 6.0, La Jolla, CA). Exact *p*-values are reported and considered significant when $p < 0.05$.

CHAPTER 4: RESULTS

All experiments in this thesis were carried out using rat and mouse lens epithelial explants as outlined above (Figure 9). Isolating and explanting of the lens epithelium is an effective *ex vivo* model that is well suited for studying the mechanisms involved in the transformation of epithelial cells into myofibroblasts. This system maintains the lens epithelium attached to its native basement membrane, the lens capsule, and separates it from the fibre cells of the remainder of the lens, allowing for ease of accessibility for microscopic visualization and treatment of lens epithelial cells with various factors. It is particularly interesting to note that, in contrast to most cell types, lens epithelial cells are able to survive and sustain their phenotype in the absence of serum, as long as they are in contact with their native matrix. Studies have demonstrated that isolated human capsular bags can be maintained in culture for over a year in protein-free medium and are capable of *de novo* protein synthesis throughout this period through an autocrine mechanism (Wormstone et al, 2001). This indicates the appropriateness of the lens explant model for use in the experiments outlined in this thesis. The drawback to the rodent lens explant model is the low number of cells isolated from small lens epithelia for protein quantification, which requires pooling at least 5 explants per sample, per experiment. This means that the Western blot analyses below encompassing three separate experiments across four treatment groups require an absolute minimum of 60 explants.

4.1 Aim 1

4.1.1 Inhibition of RhoA/ROCK signaling prevents TGF β -induced actin polymerization and expression of α SMA

To investigate the role of RhoA/ROCK signaling in TGF β -induced EMT in the lens, rat lens explants ($n \geq 5$ per treatment) were treated with TGF β for 72 hrs in the presence and absence of a ROCK inhibitor, Y-27632 (Figure 10). This compound targets the ATP-dependent kinase domain of ROCK1 and 2. One of the primary roles for ROCK, and the role of interest, is its regulation of actin-myosin cytoskeletal organization mediated through the phosphorylation/activation of MLC (pMLC). Immunofluorescence staining revealed that TGF β -treated lens explants were rich in cytoplasmic actin filaments that stained positively for pMLC, a mark of acto-myosin contractility and stress fibre formation (Figure 10A). In contrast, control and TGF β +Y-27632-treated explants showed a distinct absence of actin filaments, and instead exhibited diffuse cortical actin and pMLC staining indicating an inhibition of ROCK signaling. Accordingly, Western blot analysis revealed a decrease in pMLC expression in the presence of Y-27632, as compared to TGF β -treated explants (Figure 10B).

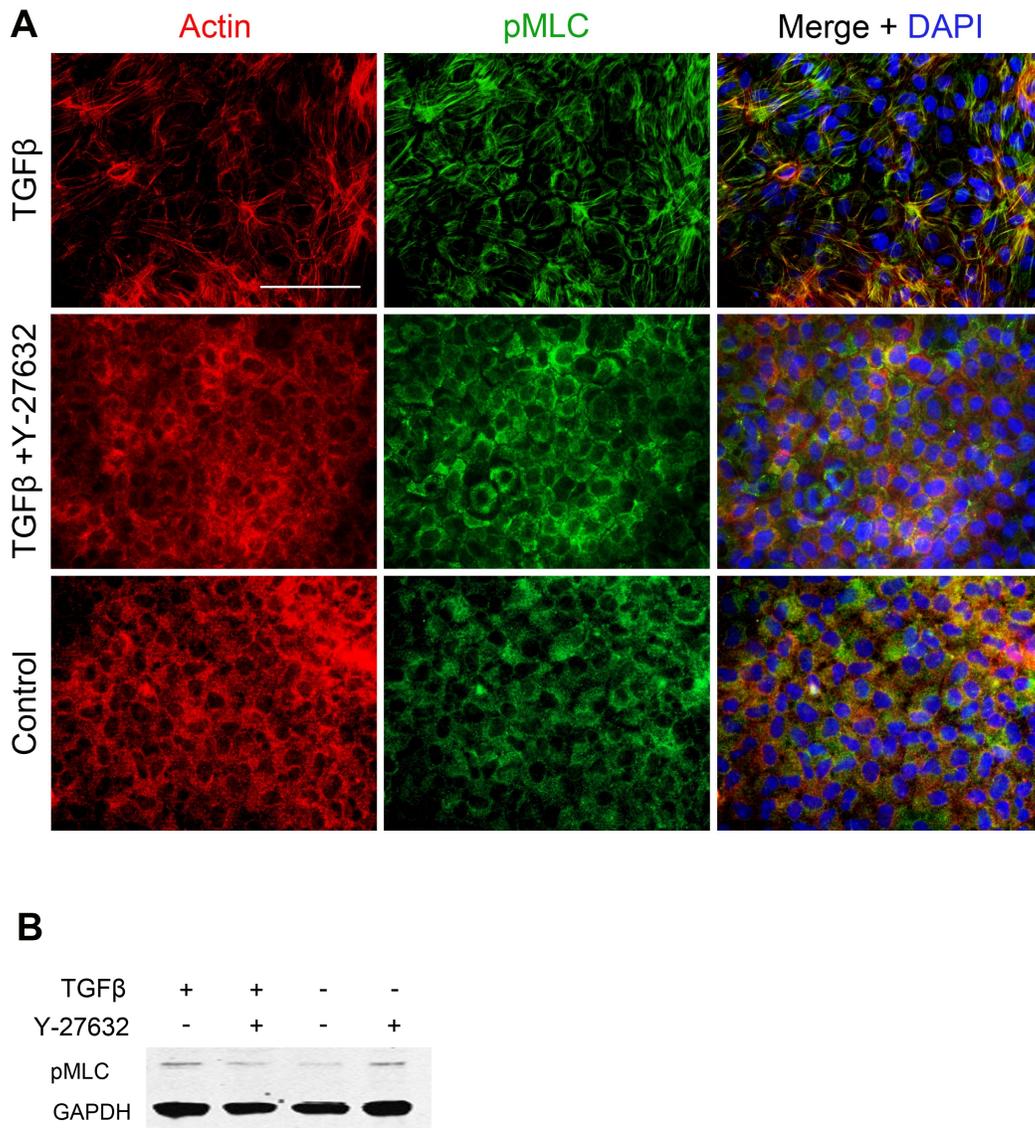


Figure 10. Y-27632 prevents TGF β -induced acto-myosin contractility. (A) Lens explants were treated with TGF β (4 ng/ml) in the presence and absence of Y-27632 (20 μ M) for 48 hrs. Fixed explants were co-stained for pan-actin and phosphorylated myosin light chain (pMLC), and mounted in medium with DAPI to co-localize nuclei. Scale bar = 100 μ m. (B) Corresponding Western blot analysis confirmed a TGF β -induced increase in pMLC protein that was prevented in the presence of Y-27632.

Next, explants were immunostained for rhodamine-phalloidin, which selectively labels F-actin. Our results revealed that TGF β treatment triggered the polymerization of actin into F-actin-containing stress fibres, positive for α SMA (Figure 11A; $n \geq 5$ per treatment). Not all F-actin fibres within lens cells co-localized with α SMA, however the large proportion that did are suggestive of a contractile, myofibroblast phenotype. In the presence of Y-27632, these effects of TGF β were prevented, where lens epithelial cells did not exhibit detectable F-actin nor α SMA staining. To quantify the previous finding, Western blot analyses were carried out on pooled explant lysates ($n \geq 5$ per sample per experiment) following a 72 hr treatment period. A significant 5.81 ± 0.07 -fold reduction in α SMA protein was confirmed in the TGF β +Y-27632-treated explant cells, as compared to explant cells treated with TGF β alone ($p < 0.0001$; $n=4$; Figure 11B&C). Similar results were obtained with a 48 hr time-course (data not shown).

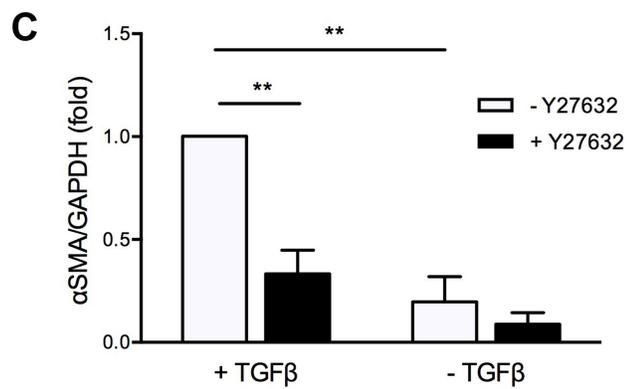
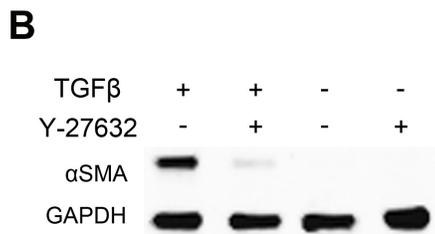
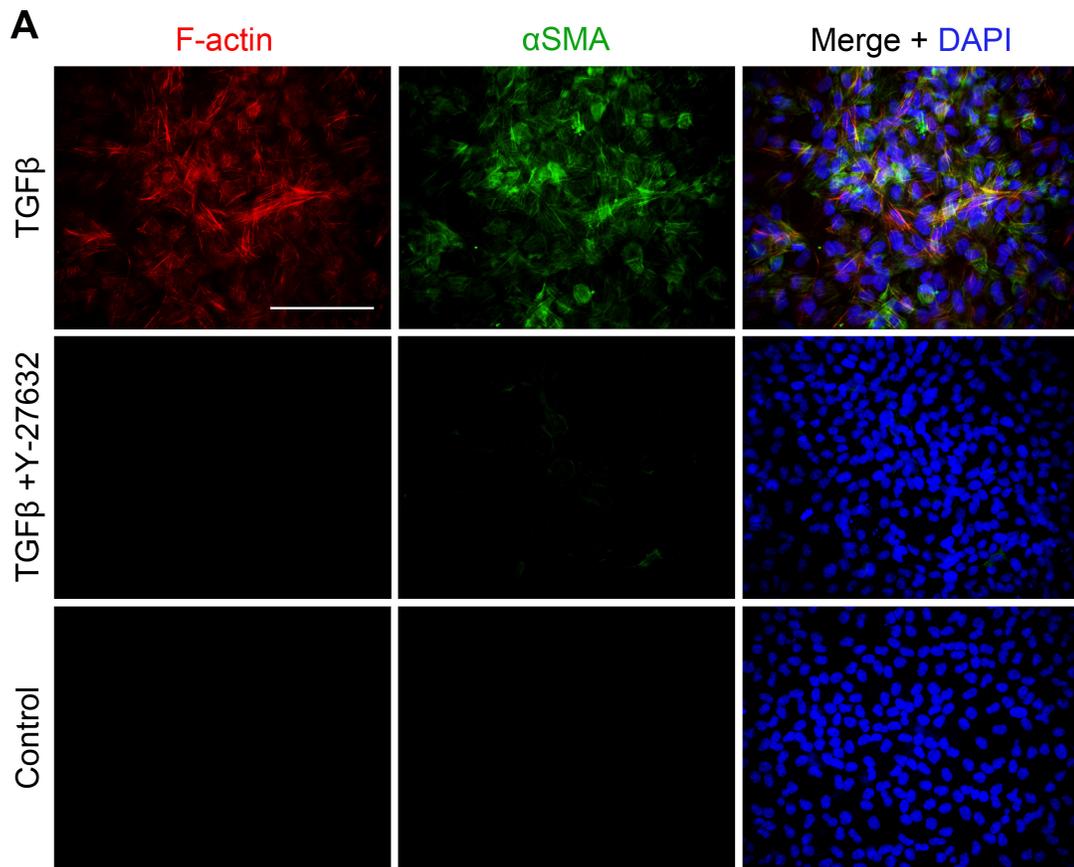


Figure 11. TGF β -induced actin polymerization and induction in α SMA expression are ROCK-dependent. (A) Lens explants were treated with TGF β (4 ng/ml) in the presence and absence of Y-27632 (20 μ M) for 72 hrs. Fixed explants were co-stained for F-actin and α SMA, and mounted in medium with DAPI to co-localize nuclei. (B) Western blot of pooled lens explant lysates ($n \geq 5$ explants per sample) were probed for α SMA, with GAPDH as loading control. (C) Densitometric quantification of α SMA/GAPDH protein indicating the fold reduction in α SMA expression normalized to TGF β -treated explants. Data expressed as the means \pm SEM from four separate experiments (** $p < 0.0001$). Scale bar = 100 μ m (A).

4.1.2 TGF β -induced disruption to E-cadherin/ β -catenin complex is ROCK-dependent

The E-cadherin/ β -catenin complex provides a direct link between cell-cell adhesion complexes and the actin cytoskeleton, therefore, E-cadherin and β -catenin localization were assessed in our lens explants. It was found that TGF β treatment resulted in a loss of E-cadherin staining from cell borders (Figure 12A), exhibiting a disorganized patterning that was associated with an increase in α SMA expression (data not shown). In comparison, untreated and Y-27632-treated explants exhibited E-cadherin staining that was localized to the cell membrane. Treating explants with TGF β in the presence of Y-27632 prevented membranous E-cadherin loss and disorganization, resembling control explants ($n \geq 5$ per treatment). Accordingly, TGF β treatment also led to the dissociation of β -catenin from the membrane resulting in its cytosolic/nuclear accumulation (Figure 12B) that coincided with an induction in α SMA expression (data not shown). In contrast, in the presence of Y-27632, TGF β treatment was unable to alter the localization of β -catenin, where it remained at the cell membrane, similar to control explants ($n \geq 5$ per treatment).

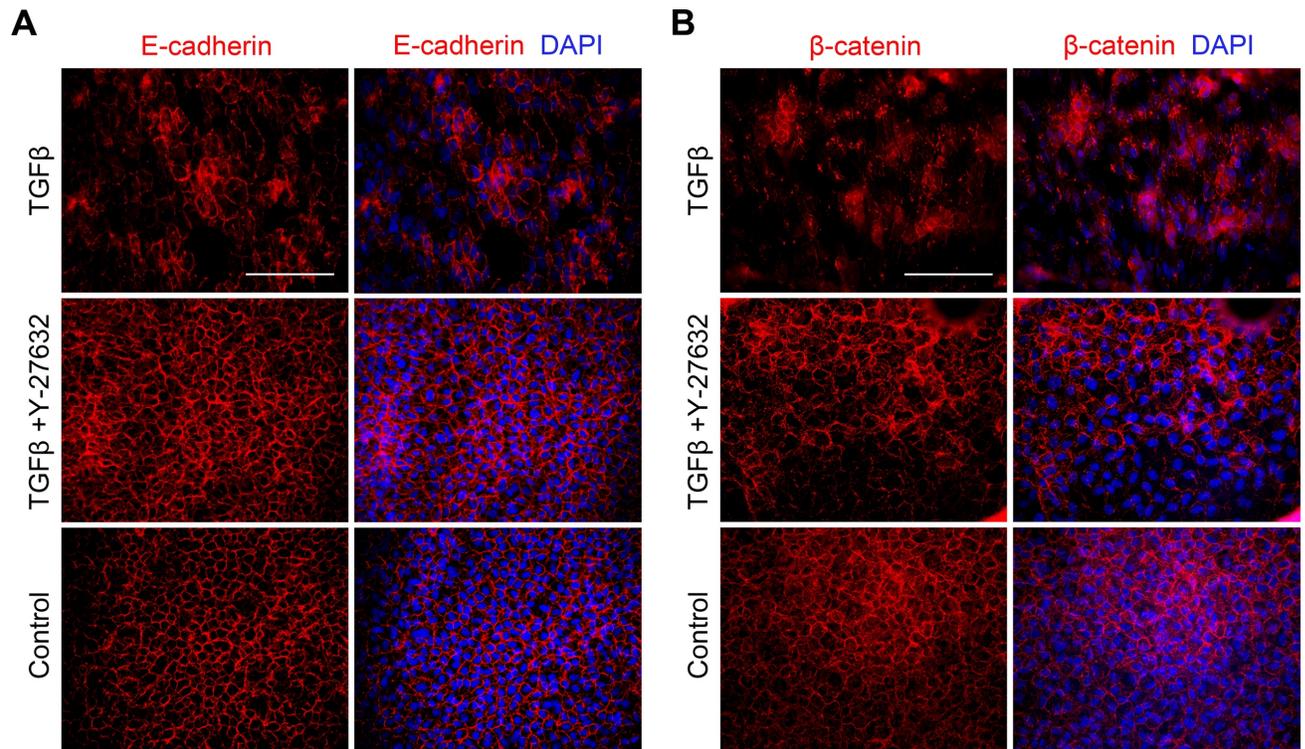


Figure 12. Y-27632 prevents TGF β -induced E-cadherin and β -catenin delocalization from the cell membrane. Lens explants ($n \geq 5$ per treatment) were fixed and stained for (A) E-cadherin or (B) β -catenin following TGF β (4 ng/ml) treatment in the presence and absence of Y-27632 after 48 hrs. All explants were mounted in medium with DAPI to co-localize nuclei. Scale bar = 100 μ m.

4.1.3 Nuclear accumulation of MRTF-A triggered by TGF β is ROCK-dependent

Previous work in the West-Mays lab on rat lens explants demonstrated the expression and nuclear accumulation of MRTF-A in response to TGF β treatment (Gupta, Korol et al. 2013). Therefore, next I sought to determine whether TGF β -induced MRTF-A nuclear localization in lens explants is ROCK-dependent. In control explants, MRTF-A was predominantly localized in the cytoplasm with no detectable α SMA expression (Figure 13A). Treatment with TGF β triggered distinct nuclear accumulation of MRTF-A (Figure 13A, inset), which was associated with increased expression of α SMA. In contrast, lens explants treated with TGF β in the presence of Y-27632 exhibited cytoplasmic MRTF-A staining corresponding with an absence of α SMA, similar to controls ($n \geq 5$ per treatment). Next, the ratio of nuclear-to-cytoplasmic fluorescence intensity of MRTF-A across the lens explant treatment groups was quantified. Accordingly, TGF β -treated explants exhibited a nuclear-to-cytoplasmic ratio that was 20.7 ± 3.2 -fold higher than untreated controls ($p = 0.0012$; Figure 13B). In contrast, MRTF-A nuclear-to-cytoplasmic fluorescence intensity with TGF β treatment in the presence of Y-27632 was 39.8 ± 3.2 -fold lower than explants treated with TGF β alone ($p = 0.001$), not differing from controls.

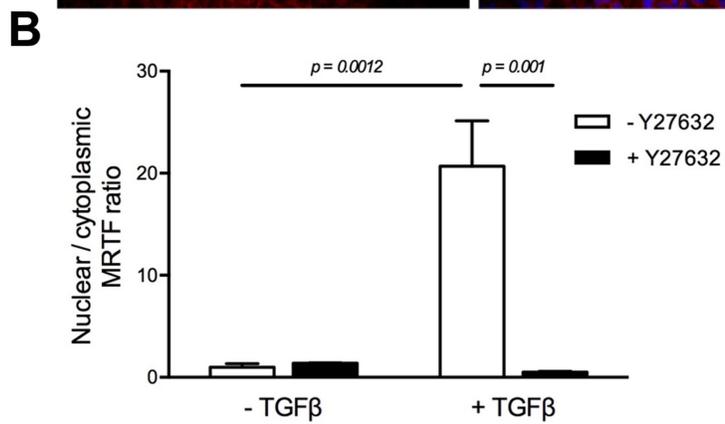
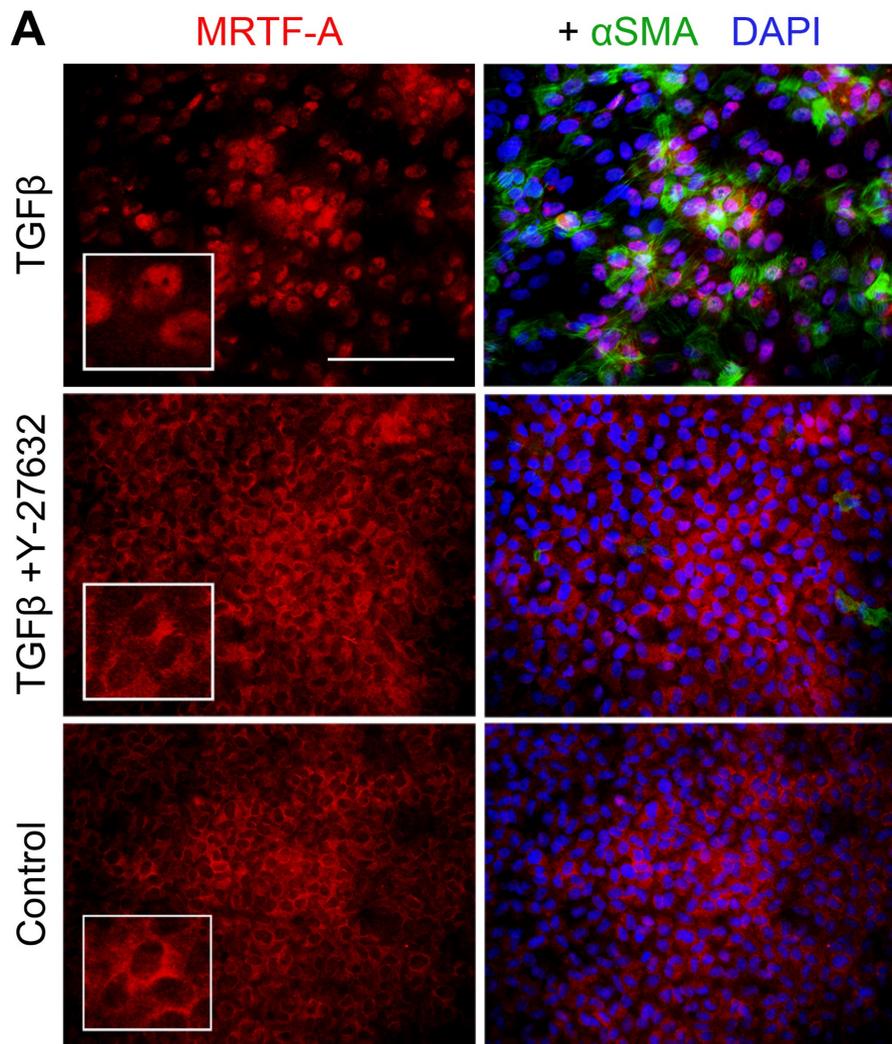


Figure 13. TGF β -induced MRTF-A nuclear localization is ROCK-dependent. (A) The presence of Y-27632 (20 μ M) prevented the nuclear localization of MRTF-A in response to TGF β (4 ng/ml) treatment for 48 hrs. Fixed explants were co-stained for MRTF-A and α SMA, and mounted in medium with DAPI to co-localize nuclei. (B) Quantification of the ratio of nuclear-to-cytoplasmic MRTF-A signal by measuring intensity of fluorescence in images as described in Chapter 3, Methodology. Data expressed as the means \pm SEM from three separate experiments. Scale bar and insets = 100 μ m (A).

4.2 Aim 2

4.2.1 CCG-203971 prevents TGF β -induced depletion of nuclear G-actin

Having established that TGF β triggers the nuclear accumulation of MRTF-A through RhoA/ROCK signaling, MRTF-A-mediated signaling was specifically targeted using a novel inhibitor, CCG-203971, a derivative of CCG-1423. The advantages of CCG-203971 over the original compound are its significant reduction in cytotoxicity with increased potency and selectivity (Bell, Haak et al. 2013). To better understand how CCG-203971 affects actin dynamics, lens explants were stained with DNaseI, which selectively binds unpolymerized, G-actin monomers (Figure 14). Under untreated and CCG-203971-treated conditions, the majority of G-actin appears to be localized in the lens cell nuclei. In contrast, TGF β led to a near depletion of G-actin pools from the nuclei with some non-selective staining of G-actin incorporated into filaments. Interestingly, treating explants with TGF β in the presence of CCG-203971 prevented G-actin pool depletion and staining resembled that of control explants ($n \geq 5$ per treatment).

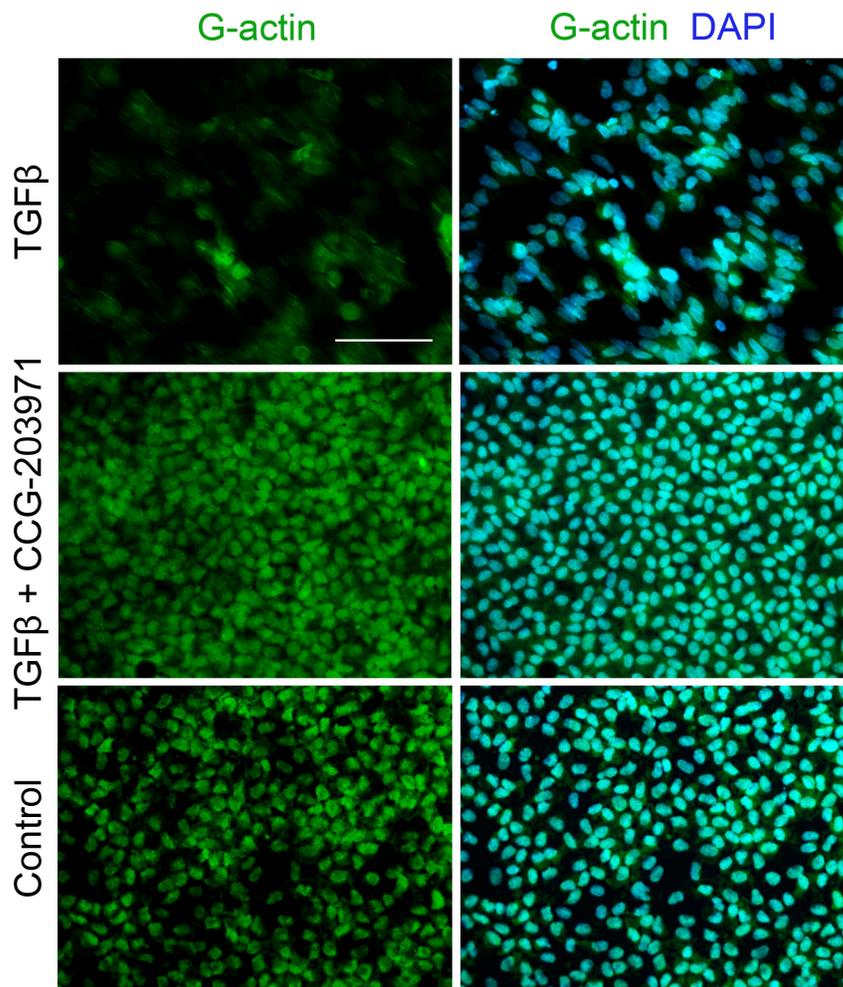


Figure 14. CCG-203971 prevents TGFβ-induced nuclear G-actin depletion. Lens explants were treated with TGFβ (4 ng/ml) in the presence and absence of CCG-203971 (5 μM). After 48 hrs, explants were fixed and stained with DNase I, which selectively binds G-actin monomers, then mounted in medium with DAPI to co-localize nuclei. Scale bar = 100μm.

4.2.2 CCG-203971 prevents TGF β -induced α SMA expression

Next, the effects of CCG-203971 on lens EMT were analyzed. Explants treated with TGF β in the presence of CCG-203971 revealed cytoplasmic localization of MRTF-A with an absence of α SMA expression (Figure 15A). This is in contrast to explants treated with TGF β alone, which exhibited nuclear accumulation of MRTF-A and expression of α SMA. Western blot analyses ($n \geq 5$ explants per sample per experiment) confirmed a significant 6.85 ± 0.05 -fold reduction in α SMA protein in the TGF β +CCG-203971-treated explant cells, as compared to explant cells treated with TGF β alone ($p < 0.0001$; $n = 3$; Figure 15B&C).

4.2.3 Nuclear accumulation of MRTF-A is required for TGF β -induced cell-cell contact dissolution

Further, the effects of CCG-203971 on E-cadherin and β -catenin localization were examined. It was found that treating explants with TGF β in the presence of CCG-203971 prevented TGF β -induced de-localization of E-cadherin (Figure 16A) and β -catenin (Figure 16B) from the cell membrane, where both adherens junction constituents exhibited distinct staining at sites of cell-cell contact ($n \geq 5$ per treatment). Corresponding Western blot analyses ($n \geq 5$ explants per sample per experiment) using an antibody that recognized the cytoplasmic domain of E-cadherin showed a 3.2 ± 0.9 -fold induction in E-cadherin in the TGF β + CCG-203971-treated group compared to TGF β -treated explants ($n=3$; Figure 16C&D).

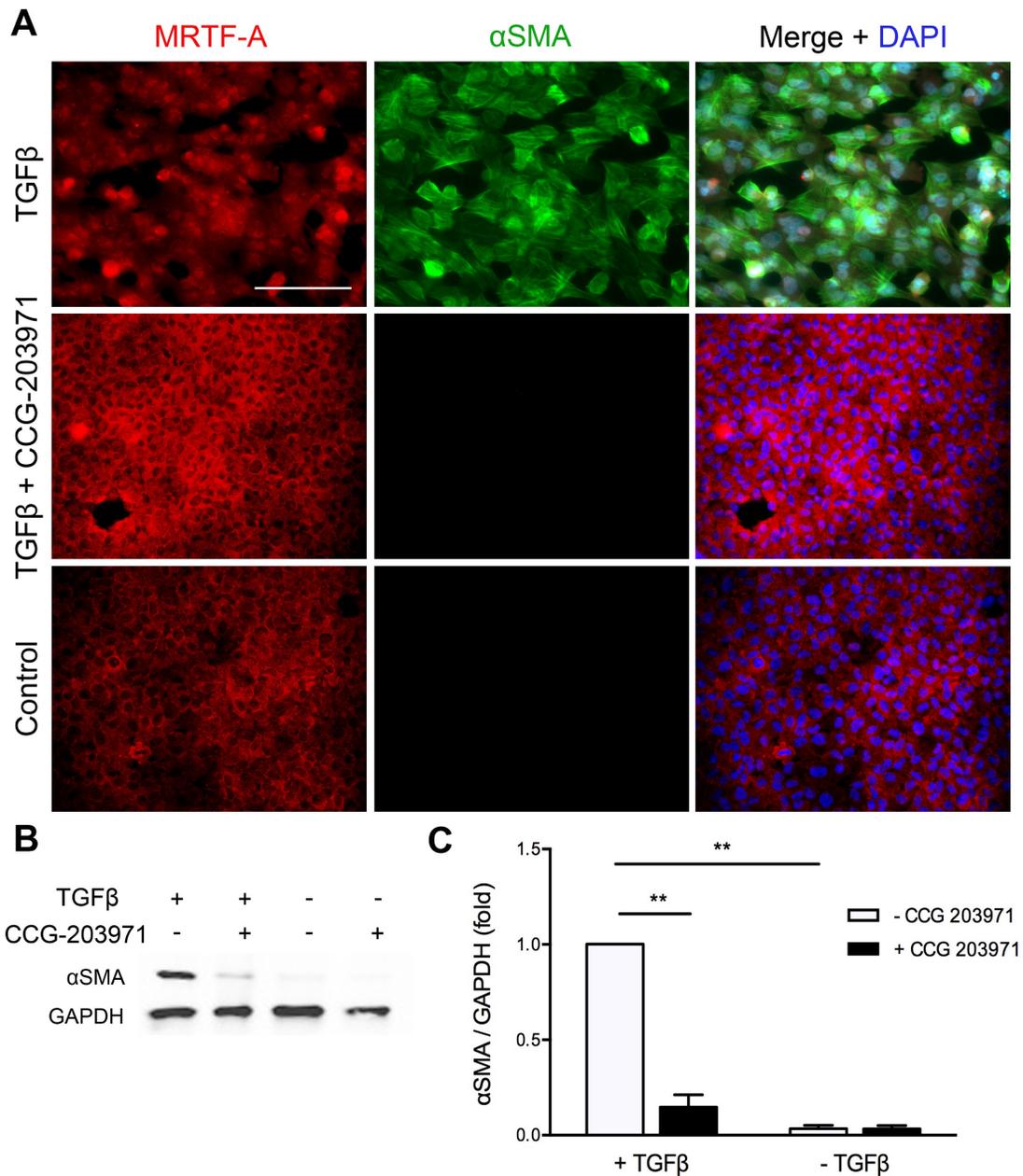


Figure 15. CCG-203971 prevents TGF β -induced actin polymerization and α SMA expression. (A) Lens explants were treated with TGF β (4 ng/ml) in the presence and absence of CCG-203971 (5 μ M). Fixed explants were co-stained for MRTF-A and α SMA, and mounted in medium with DAPI to co-localize nuclei. (B) Pooled lens explant lysates ($n \geq 5$ explants per sample) were probed for α SMA and GAPDH, as loading control. (C)

Densitometric quantification of α SMA/GAPDH indicating the fold reduction in α SMA expression normalized to TGF β -treated explants. Data are expressed as the means \pm SEM from three separate experiments (** $p < 0.0001$). Scale bar = 100 μ m (A).

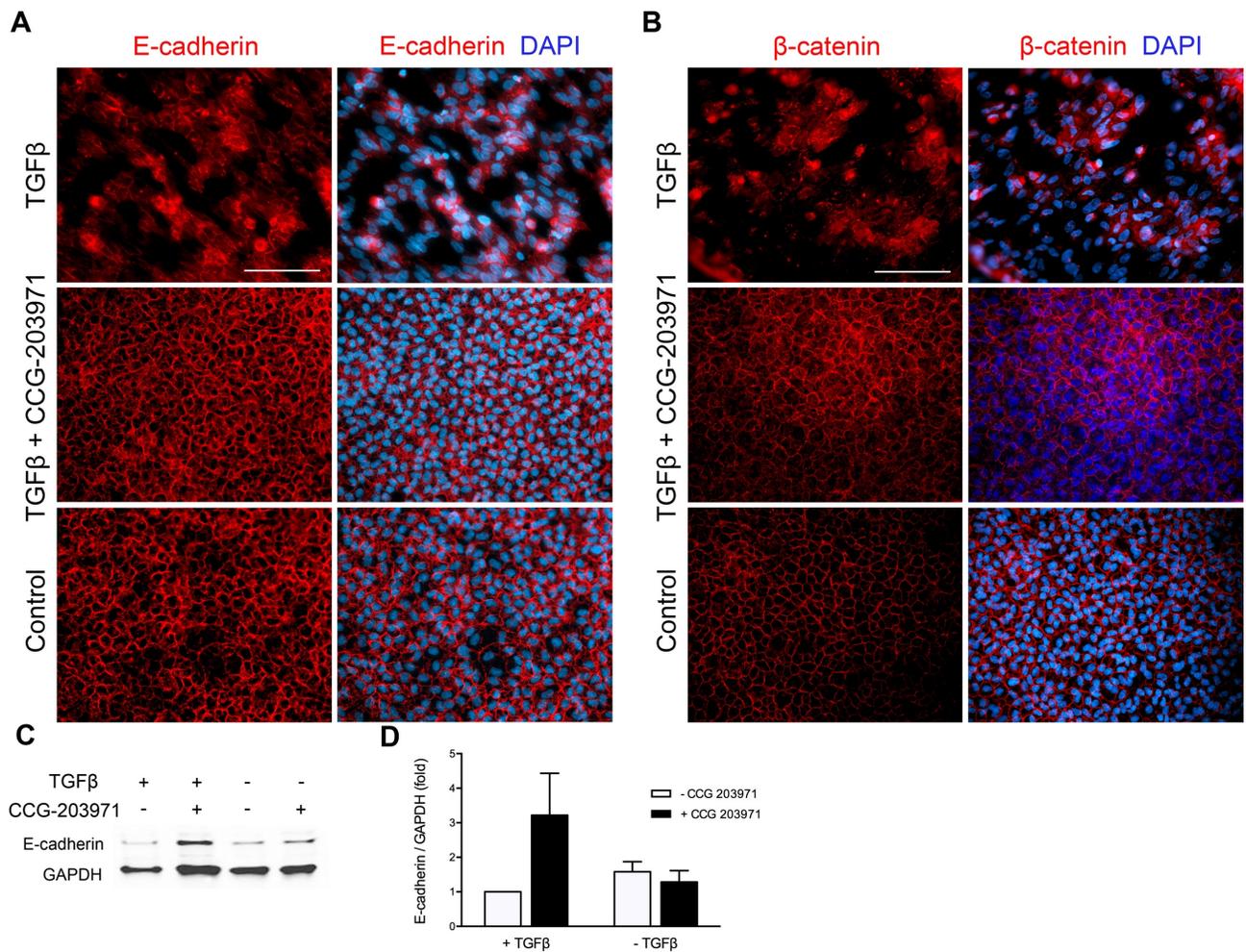


Figure 16. CCG-203971 prevents TGFβ-induced E-cadherin and β-catenin delocalization from the cell membrane. Lens explants ($n \geq 5$ per treatment) were fixed and stained for (A) E-cadherin or (B) β-catenin following TGFβ (4 ng/ml) treatment in the presence and absence of CCG-203971 (5 μM) after 48 hrs. All explants were mounted in medium with DAPI to co-localize nuclei. (C) Pooled lens explant lysates ($n \geq 5$ explants per sample) were probed for the cytoplasmic domain of E-cadherin and GAPDH, as loading control. (D) Densitometric quantification of E-cadherin indicating the fold reduction in E-cadherin/GAPDH expression normalized to TGFβ-treated explants. Data are expressed as the means \pm SEM from three separate experiments. Scale bar = 100μm (A).

4.3 Aim 3

4.3.1 MMP-9 expression is ROCK-dependent

Previous work using MMP-2 and -9 KO mouse lines suggested that MMP-9, but not MMP-2, was critical to TGF β -induced α SMA expression demonstrated both *in vivo* and in *ex vivo* lens explants (Korol, Pino et al. 2014). To gain a better understanding of the role MMP-9 plays in EMT, rat lens explant lysates (n \geq 5 explants pooled per sample per experiment) were collected after a 72 hr treatment period and MMP-9 levels were quantified. Treating explants with TGF β resulted in a 6.23 ± 0.13 -fold induction in the expression of MMP-9 compared to untreated explants ($p < 0.0001$; n=3; Figure 17A&B). In the presence of Y-27632, expression of MMP-9 was reduced 3.11 ± 0.13 -fold compared to explants treated with TGF β alone ($p < 0.0001$; n=3; Figure 17A&B).

4.3.2 MMP-9 is involved in TGF β -induced F-actin polymerization and E-cadherin/ β -catenin complex dissolution

To further study the resistance of MMP-9 KO lens epithelial cells to TGF β -induced EMT, MMP-9 KO mouse lens explants were used to analyze cytoskeletal remodeling and cell-cell contact dissolution in response to TGF β treatment. Mouse lens explants treated with TGF β (Figure 17C) exhibited pronounced F-actin filaments with a radial patterning that contrasts with untreated lens cells that displayed a cortical arrangement of cytoplasmic actin (Figure 18A). In the absence of MMP-9, TGF β was unable to stimulate actin

polymerization and actin organization remained cortical (Figure 18B), resembling untreated explants (Figure 18D).

Previous studies in the West-Mays lab demonstrated that untreated MMP-9 KO explants (without TGF β treatment) showed a decrease in E-cadherin expressed at the cell membrane (Korol, Pino et al. 2014). Therefore, the state of E-cadherin-based cell-cell contacts in these MMP-9 KOs was further assessed. Western blot analyses on lens explant lysates were performed to determine the overall level of E-cadherin protein in MMP-9 KO explants compared to wild-type littermates (Figure 19A). These experiments demonstrated that total E-cadherin protein in MMP-9 KO explants did not differ from wild-type controls (Figure 19B).

Since earlier studies from our group using whole rat lenses revealed the appearance of a 72 kDa E-cadherin fragment in the conditioned media of lenses treated with TGF β that was not detected in media from untreated lenses (Dwivedi, Pino et al. 2006), the conditioned media of explants from wild-type and MMP-9 KO mice were assayed using an ELISA specific to the extracellular domain of E-cadherin. These experiments revealed that unlike TGF β -treated wild-type explants that exhibited induced E-cadherin levels in the media compared with untreated controls ($p = 0.074$), the TGF β -treated MMP-9 KO explants did not (Figure 19C). In fact, the levels of E-cadherin in the TGF β -treated MMP-9 KO media were lower than all untreated explants and significantly lower than that of wild-type TGF β -treated explants ($p = 0.019$).

Finally, to further investigate the state of epithelial cell-cell contacts the localization of β -catenin was assessed. β -catenin is important in linking E-cadherin to the cell

cytoskeleton and its loss from the cell junction often precedes EMT in many systems. In lens epithelial cells, TGF β is able to augment the localization of β -catenin from a junctional patterning to a cytosolic and/or nuclear expression (Stump, Lovicu et al. 2006). In lens explants, immunofluorescent staining revealed that wild-type explants treated with TGF β exhibited predominantly cytosolic expression of β -catenin compared with untreated controls, which exhibit staining localized at the cell junction and outline the cobblestone arrangement of cells (Figure 20). Importantly, the MMP-9 KO explants treated with TGF β resembled untreated controls with β -catenin remaining at cell borders.

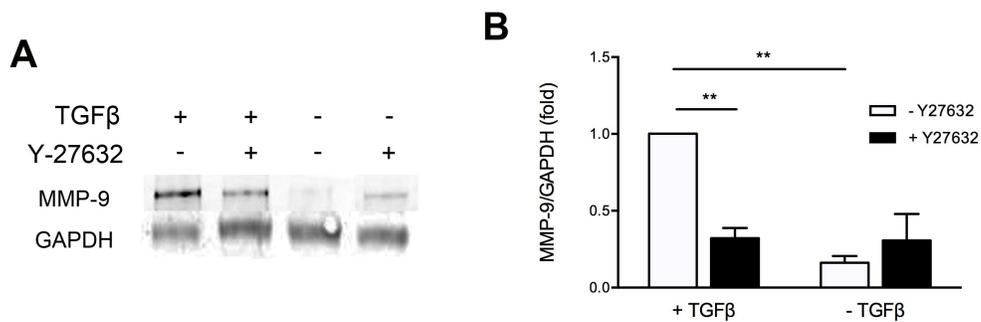


Figure 17. Y-27632 prevents TGF β -induced MMP-9 expression. (A) Pooled lens explant lysates ($n \geq 5$ explants per sample) were probed for the catalytic domain of MMP-9 and GAPDH, as loading control. (B) Densitometric quantification of MMP-9 indicating the fold reduction in MMP-9/GAPDH expression normalized to TGF β -treated explants. Data are expressed as the means \pm SEM from three separate experiments (** $p < 0.0001$).

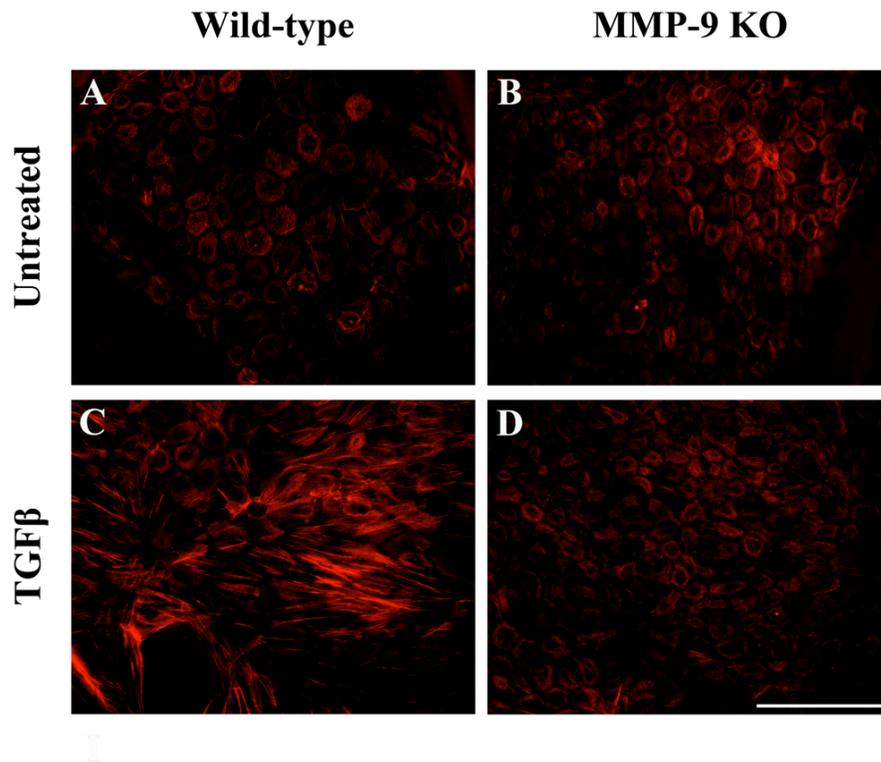


Figure 18. The absence of MMP-9 prevents TGFβ-induced radial F-actin distribution and polymerization. Mouse lens explants ($n \geq 5$ per treatment) were fixed and stained with rhodamine phalloidin to visualize cellular F-actin following TGFβ (4 ng/ml) treatment in wild-type (C) and MMP-9 KO (D) mice. Scale bar = 100μm.

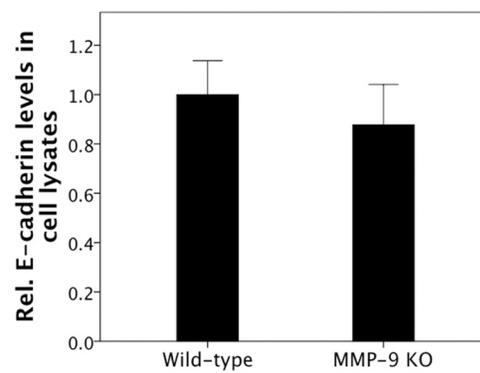
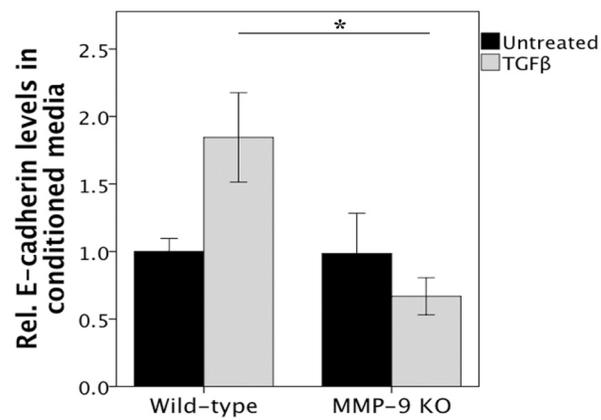
A**B****C**

Figure 19. E-cadherin protein levels in MMP-9 KO lysates and conditioned media.

(A) Representative Western blot reveals E-cadherin protein (120 kDa) in MMP-9 KO lens explant lysates compared to their wild-type littermates. (B) Densitometric quantification of E-cadherin indicating no significant difference in total E-cadherin protein between untreated MMP-9 KO ($n=8$) and wild-type ($n=5$) explants. Data normalized with β -tubulin control and expressed as mean \pm SEM. (C) ELISA measuring E-cadherin in conditioned media of untreated and TGF β -treated (500pg/ml for 48 hours) wild-type and MMP-9 KO lens explants. In the media, TGF β treatment leads to an increase in E-cadherin protein in wild-type explants when compared to untreated wild-type controls ($P = 0.072$). Levels of E-cadherin in the media of MMP-9 KO explants following TGF β treatment are significantly different from wild-type TGF β -treated levels ($P = 0.019$), and comparable to all untreated explant levels. Data expressed as mean \pm SEM ($n=3-6$; $*p < 0.05$).

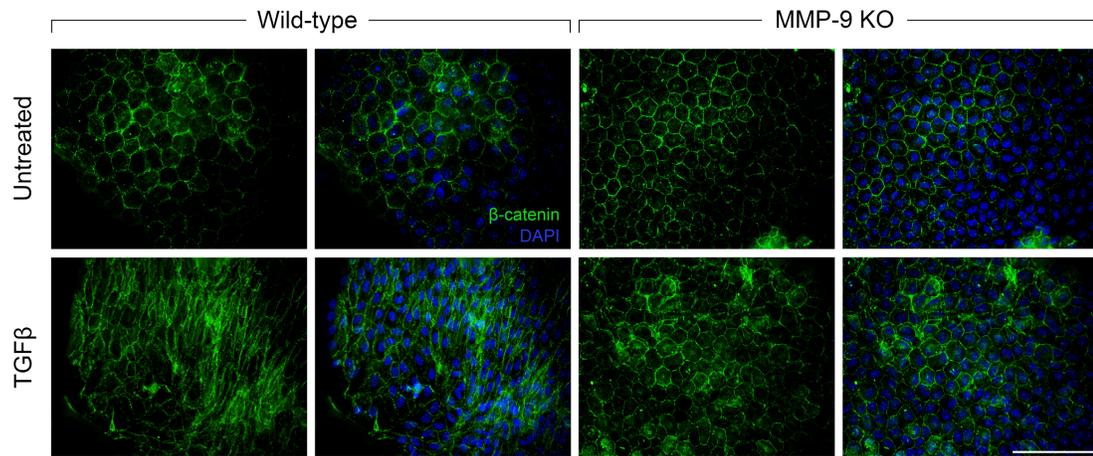


Figure 20. Effects of TGF β treatment on β -catenin localization in MMP-9 KO mouse lens epithelial explants. In untreated wild-type and MMP-9 KO explants, β -catenin is predominantly localized at the cell borders outlining a cobblestone arrangement. Upon TGF β treatment (500pg/ml) for 48 hours, wild-type lens cells exhibit primarily cytosolic expression of β -catenin, in contrast to TGF β -treated MMP-9 KO explants, which resembled controls with β -catenin localizing at cell borders. All explants were mounted in medium with DAPI to co-localize the nuclei (blue). Scalebar = 100 μ m.

4.3.3 MMP-9 is necessary but not sufficient to induce EMT

To directly assess the role of MMP-9 in the lens explant system, active recombinant human (rh) MMP-9 was applied to explants to determine its effects on the EMT process. Explants were treated with rhMMP-9 alone for 1-4 days and immunofluorescent staining for E-cadherin was carried out. Compared to control explants, which exhibited well-defined E-cadherin staining at cell borders, rhMMP-9 treatment resulted in regions of de-localized and punctate E-cadherin staining (Figure 21A; arrowheads). This patterning was seen after a 24 hr rhMMP-9 treatment and persisted until day 4. However, the discontinuity of membranous E-cadherin staining did not result in a change in cell shape and appeared to maintain a mostly cobblestone arrangement. These observed changes in E-cadherin distribution differ from that seen in TGF β -treated explants, which showed a loss of E-cadherin staining at the membrane, however, also exhibited an elongated morphology with a distinct orientation. Furthermore, the localization of β -catenin resembled that of E-cadherin across treatment groups, albeit to a less severe extent. While rhMMP-9 treatment led to regions of punctate, jagged-edged β -catenin staining (Figure 21B; arrowheads), the cobblestone arrangement seemed to remain intact. In contrast, TGF β treatment resulted in drastic de-localization of β -catenin from cell borders along with a clear loss of cell-cell contact and change in cell morphology.

Finally, given that TGF β was unable to stimulate radial F-actin patterning in the MMP-9 KO explants, the requirement of MMP-9 and whether it is needed to feed forward in activating cytoskeletal signaling was assessed. The effects of rhMMP-9 treatment on

MRTF-A localization and α SMA expression was determined. Treatment with rhMMP-9 alone did not induce the nuclear accumulation of MRTF-A, nor did it induce the expression of α SMA fibres (Figure 22). This is in contrast to TGF β -treated explants, which showed distinct nuclear accumulation of MRTF-A coinciding with α SMA expression.

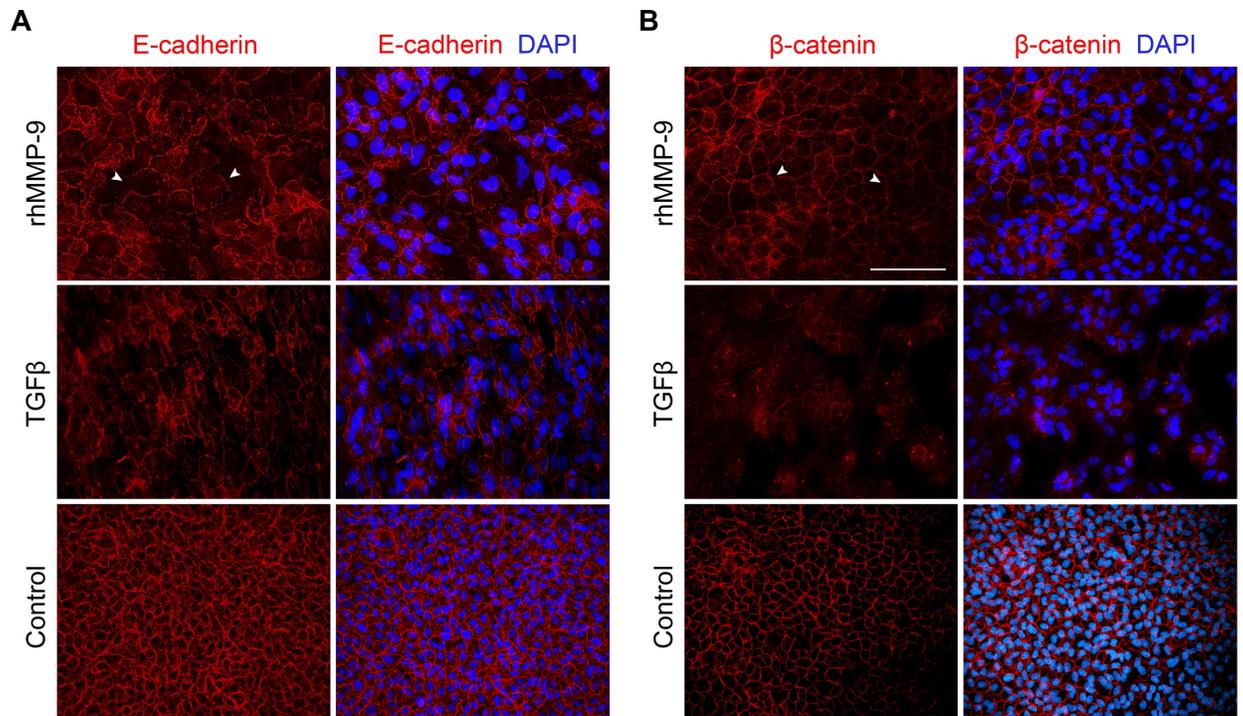


Figure 21. rhMMP-9 induces E-cadherin and β-catenin delocalization from the cell membrane. Lens explants ($n \geq 5$ per treatment) were fixed and stained for (A) E-cadherin and (B) β-catenin following rhMMP-9 (100 ng/ml) or TGFβ (4 ng/ml) treatment after 48 hrs. RhMMP-9 lead to jagged, punctate staining of E-cadherin and β-catenin at the cell membrane (arrowheads). All explants were mounted in medium with DAPI to co-localize nuclei. Scalebar = 100μm.

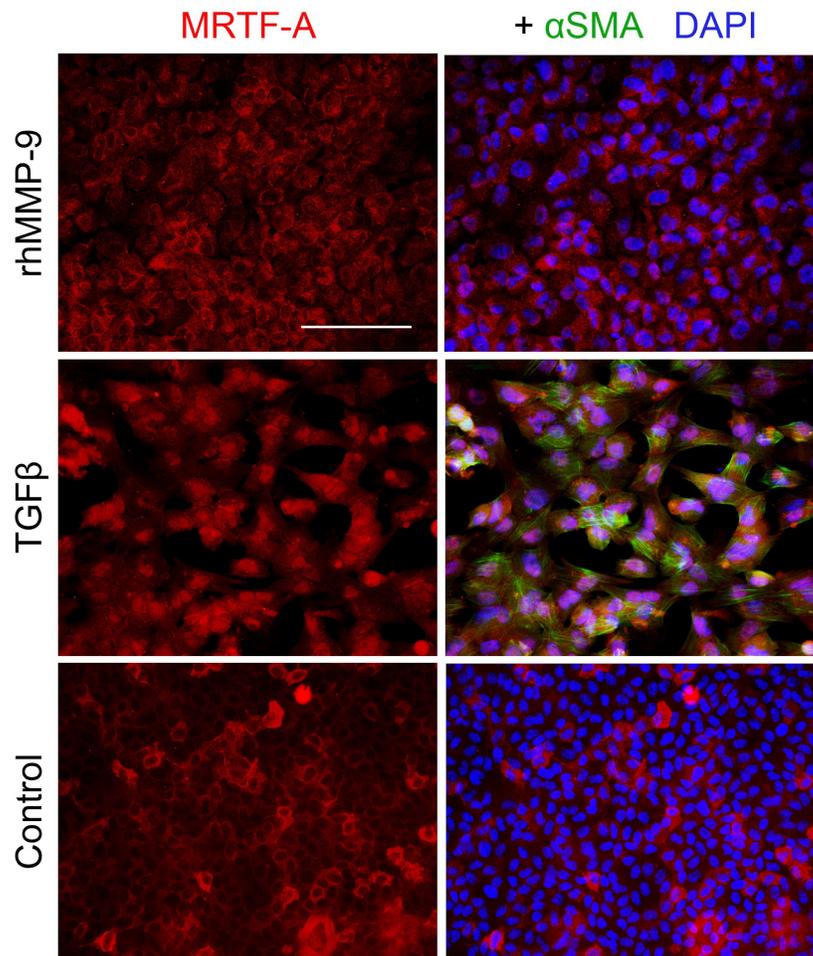


Figure 22. rhMMP-9 is not sufficient to induce nuclear MRTF-A accumulation nor α SMA expression. Lens explants ($n \geq 5$ per treatment) were fixed and co-stained with MRTF-A and α SMA following rhMMP-9 (100 ng/ml) or TGF β (4 ng/ml) 48 h treatment. All explants were mounted in medium with DAPI to co-localize nuclei. Scale bar = 100 μ m.

CHAPTER 5: DISCUSSION

Actin cytoskeletal reorganization is a phenotypic change widely associated with TGF β -induced EMT. Understanding how actin dynamics affect downstream expression of EMT-related targets is critical to furthering our knowledge of the pathophysiology of fibrosis of the lens and other tissues. The central aim of this work was to characterize the contribution and requirement of RhoA-mediated cytoskeletal signaling in TGF β -induced EMT. The studies and results outlined in this dissertation have identified three potential targets associated with RhoA signaling that affect and are required for the sustained EMT response in lens epithelial explants (see proposed mechanism outlined in Figure 23). Through the combined use of pharmacological inhibitors, Y-27632 and CCG-203971, this work demonstrated that TGF β -induced EMT is ROCK- and nuclear MRTF-A-dependent, respectively. Further, the requirement of MMP-9 downstream of cytoskeletal signaling was revealed and shown to be involved in cell surface E-cadherin/ β -catenin complex disruption. Overall, the results of the current work provide novel insights into the mechanisms that link cytoskeletal signaling with critical regulators of epithelial cell-cell adhesion and α SMA expression.

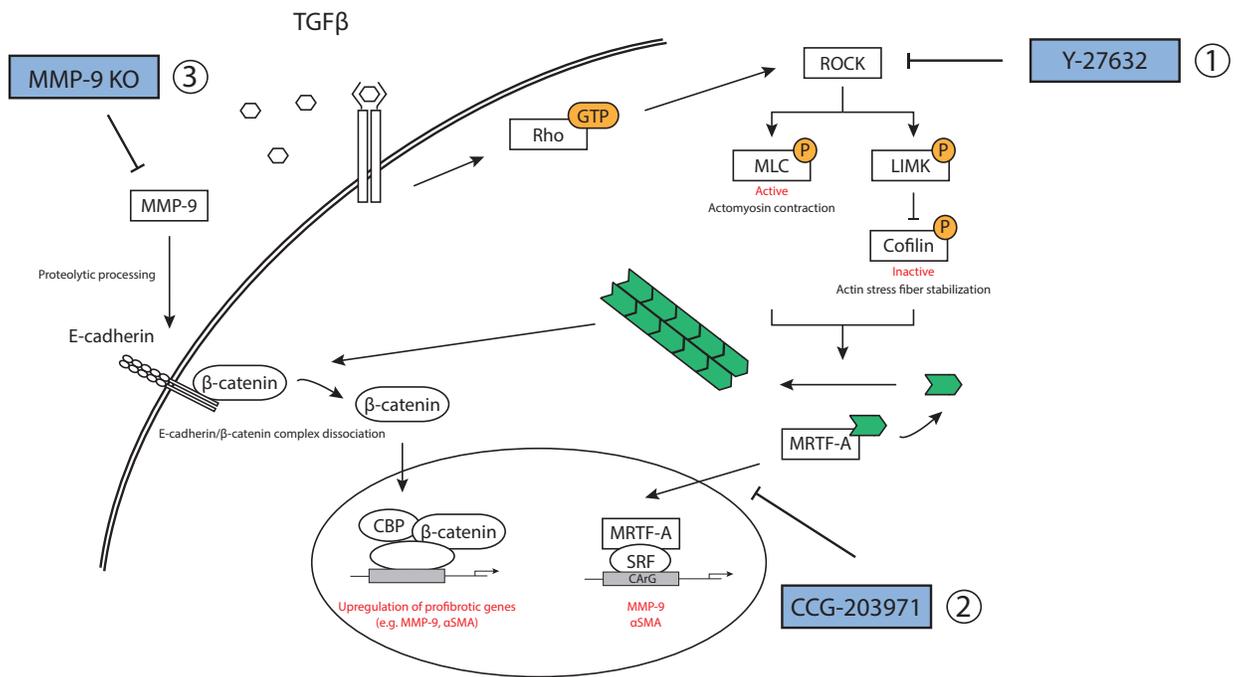


Figure 23. Proposed mechanism of ROCK-dependent cytoskeletal signaling in TGFβ-induced lens EMT and modes of intervention. (1) TGFβ activation leads to Rho-GTP-regulated ROCK activation. ROCK leads to actin stress fibre formation and actomyosin contractility through: phosphorylation of both MLC and LIMK, the latter of which phosphorylates cofilin, rendering it inactive. (2) Incorporation of G-actin monomers into contractile stress fibres leads to the nuclear accumulation of MRTF-A, which when in complex with SRF, can activate the transcription of EMT-related targets, such as MMP-9 and αSMA. (3) The upregulation of MMP-9 may then participate in the proteolytic processing of E-cadherin/β-catenin junctional complexes, which can amount in nuclear transcriptional activity of β-catenin, specifically through CBP.

5.1 ROCK/MRTF-A-mediated actin organization and α SMA expression

The first study of this thesis (Chapter 4.1) utilized the ROCK inhibitor, Y-27632, which has been widely employed in the study of ROCK-dependent signaling as a potential therapeutic target in a number of diseases including cancer (Rath and Olson 2012), asthma (Kume 2008), hypertension (Duong-Quy, Bei et al. 2013), corneal endothelial dysfunction (Koizumi, Okumura et al. 2014), and glaucoma (Rao, Deng et al. 2001, Waki, Yoshida et al. 2001, Murphy, Morgan et al. 2014). Using lens epithelial explants as a model of lens EMT, as occurs in PCO and ASC, TGF β stimulation was shown to induce assembly of actin into pMLC-positive stress fibres, which along with co-expression of α SMA, clearly indicates a transition into a myofibroblast phenotype. In the presence of Y-27632, TGF β -induced stress fibre formation and acto-myosin contractility was entirely prevented, and corresponded with an absence of α SMA expression. This finding indicates that polymerization of actin is required in order for α SMA to be incorporated into filaments.

Work by Menko, Bleaken et al. (2014) have shown that following mock cataract surgery pMLC2 is required to regulate the coordinated migration of lens epithelial cells on the lens capsule indicating a role for acto-myosin contractility in lens epithelial migration. These findings are in line with those carried out in other epithelial systems reporting that RhoA signaling was indispensable in TGF β -induced cytoskeletal remodeling (Bhowmick, Ghiassi et al. 2001, Masszi, Di Ciano et al. 2003), and α SMA expression (Masszi, Di Ciano et al. 2003), as well as in mouse lens epithelial cell cultures demonstrating that α SMA expression is ROCK-dependent (Cho and Yoo 2007). Work in tubular epithelial cells

demonstrated that constitutively active ROCK alone induced the formation of thick F-actin fibres, but was not able to induce significant α SMA promoter activity (Masszi, Di Ciano et al. 2003), suggesting the involvement of other TGF β signaling components. Importantly, this thesis has extended the findings mentioned to show that ROCK-mediated stimulation of α SMA expression occurs via the actin-binding protein, MRTF-A.

As outlined in the introduction, the transcriptional activation and expression of α SMA is regulated by SRF along with its co-activators, the MRTFs. The subcellular localization of MRTF-A is tightly regulated by the ratio of G- to F-actin in the cell, as determined by the state of Rho-mediated actin polymerization. G-actin binds to the RPEL motifs on MRTF-A and, when bound, G-actin has been proposed to inhibit nuclear import, enhance nuclear export, and repress transcription of target genes (O'Connor and Gomez 2013) (refer to mechanism depicted in Figure 7 in Chapter 1.8). Thus far, work in proximal tubular epithelial cells has been pivotal in determining the involvement of MRTF-A subcellular localization in the context of TGF β -induced EMT (Fan, Sebe et al. 2007, Morita, Mayanagi et al. 2007, Elberg, Chen et al. 2008). Morita, Mayanagi et al. (2007) demonstrated that the presence of constitutively active MRTF-A was sufficient to induce the expression of α SMA. The current work (Chapter 4.13) demonstrated that the presence of Y-27632 prevented TGF β -induced nuclear accumulation of MRTF-A, which corresponded with the absence of α SMA suggesting that in the lens explants, TGF β signals through Rho/ROCK/MRTF-A to induce the expression of α SMA. As such, subcellular localization of MRTF-A may act as an indicator of EMT.

To further corroborate these findings, the experiments outlined in Aim 2 used a novel, specific inhibitor of the MRTF-A pathway, CCG-203971. This inhibitor is a derivative of CCG-1423, which was identified to inhibit an MRTF-A/SRF transcriptional reporter downstream of RhoA (Evelyn, Wade et al. 2007). CCG-1423 has been shown to decrease mesenchymal protein expression, including α SMA in podocyte EMT in diabetic nephropathy (Zhao, Wang et al. 2016), while both CCG-1423 and CCG-203971 blocked matrix-stiffness- and TGF β -induced α SMA expression in intestinal fibroblasts (Johnson, Rodansky et al. 2014). The precise mechanism of action of these inhibitors has not been definitively established, however, it is suggested that they retain MRTF-A in the cytoplasm through: (A) binding of the nuclear localization signal of MRTF-A preventing nuclear import (Hayashi, Watanabe et al. 2014), (B) inhibiting the depletion of G-actin pools in the nucleus resulting in nuclear export of MRTF-A (Lundquist, Storaska et al. 2014). The observed cytoplasmic MRTF-A staining in our explants (Chapter 4.2.1) in the presence of TGF β and CCG-203971 coincides with a substantially higher level of G-actin localized to the nucleus when compared to the near depletion of G-actin pools observed in explant cells exclusively treated with TGF β . This finding suggests that one of the ways in which CCG-203971 acts in the lens explant system is by retaining G-actin in the lens cell nuclei, preventing the emergence of polymerized cytosolic filaments and, thus, nuclear accumulation of MRTF-A, similar to mechanism (B) outlined above. The presence of CCG-203971 prevented TGF β -induced α SMA expression, which demonstrates the critical nature of the balance of G- to F-actin in the MRTF-A-mediated expression of α SMA. By

preventing nuclear accumulation of MRTF-A, CCG-203971 is thereby preventing the transcriptional activation of MRTF-A/SRF-regulated target genes.

5.2 E-cadherin/ β -catenin complex regulation

Previous work in mouse lens epithelial cell explants in the West-Mays group demonstrated that TGF β led to the de-localization of adherens junction constituents, E-cadherin and β -catenin, from the cell membrane resulting in their disorganized patterning (Korol, Pino et al. 2014). The results outlined in the current work using rat lens explants, furthered these studies and demonstrated that this TGF β -induced E-cadherin and β -catenin delocalization is ROCK/MRTF-A-dependent, where both remained intact at the cell membrane in the presence of Y-27632 (Chapter 4.1.2), as well as CCG-203971 (Chapter 4.2.3). Similarly, in renal epithelial cells, the presence of Y-27632 following TGF β treatment prevented TGF β -induced adherens junction disassembly, including β -catenin delocalization and phosphorylation (Tian, Fraser et al. 2003). As well, activated RhoA has been shown to mediate the disassembly of epithelial cell contacts, preferentially signaling through ROCK, rather than mDia (Sahai and Marshall 2002). In terms of the role of MRTF-A, in tubular epithelial cells, inhibiting the function of endogenous MRTF-A through use of dominant-negative MRTF-A mutants prevented TGF β -induced decreases in E-cadherin and β -catenin (Morita, Mayanagi et al. 2007). In this model, MRTF-A in complex with Smad3 bound to the GCCG-like motif on the *slug* promoter and led to E-cadherin transcriptional repression and dissociation of cell-cell contacts. The upregulation of *slug*

expression and MRTF-A activity occurred after 12-24 h and up to 3 days after TGF β treatment, suggesting that they are involved in the sustained EMT response. From the reverse point of view, Ca²⁺ depletion-mediated E-cadherin-based AJ dissociation was sufficient to trigger nuclear accumulation of MRTF-A and SRF-reporter activation (Busche, Kremmer et al. 2010). Moreover, in the absence of the C-terminal β -catenin binding domain on E-cadherin, which was still able to localize to epithelial junctions, Ca²⁺ depletion no longer led to MRTF-A nuclear re-localization, nor SRF activity.

Based on the emerging role of E-cadherin-based adherens junctions as mechanosensors (le Duc, Shi et al. 2010), it is possible that a RhoA/ROCK-mediated increase in cytoskeletal tension is sensed through cadherin-based contacts. A compromise in cell-cell adhesion integrity can then lead to the dissociation of β -catenin, which is involved in regulating the transcription of EMT-related genes, and in this manner, E-cadherin complexes are translating cellular mechanics into a transcriptional response (Ladoux, Nelson et al. 2015). *In vivo*, conditional expression of active ROCK2 in mouse skin led to the redistribution of β -catenin from cell surface E-cadherin to the cytoplasm/nucleus (Samuel 2012). Indeed, novel, concurrent work by Taiyab, Korol et al. (2016) in the West-Mays lab demonstrated that β -catenin-mediated signaling, specifically when in complex with CBP, is required for TGF β -induced EMT, outlining a manner in which the effects of EMT are propagated through a feed-forward mechanism. Therefore, the findings in Aims 1 and 2 demonstrated a key role for RhoA/ROCK and MRTF-A signaling in regulating the integrity of adhesion complexes in response to TGF β , essential in determining cell phenotype in addition to their role in the induction of α SMA expression.

In this dissertation, the results of the studies outlined in Aim 3 also demonstrated the requirement of MMP-9 in the stability of E-cadherin/ β -complexes. Previous work by the West-Mays group suggested that MMP-2 and/ or MMP-9 may facilitate EMT of lens epithelial cells by acting on the non-matrix substrate E-cadherin (Dwivedi, Pino et al. 2006). Proteolytic cleavage of the N-terminal extracellular domain of E-cadherin by MMPs, referred to as E-cadherin shedding, resulted in the formation of a soluble E-cadherin extracellular domain fragments with reported sizes ranging from 50 to 84 kDa, compared with the intact 120-kDa protein (Grabowska and Day 2012). Indeed, as mentioned in the introduction, our group detected a shed 72-kDa E-cadherin fragment in the conditioned media of lenses treated with TGF β 2 that was not detected in the media from untreated lenses (Dwivedi, Pino et al. 2006). Importantly, the appearance of E-cadherin fragments in the TGF β -treated rat lenses was attenuated by co-treatment with an MMP-2/MMP-9-specific inhibitor, implicating these two MMPs in the shedding process. Further, my own previous work in MMP-2 and MMP-9 KO mouse lens explants specifically examined E-cadherin membranous localization (Korol, Pino et al. 2014). Explants from both wild-type and MMP-2 KO mice, after treatment with TGF β , exhibited cell-cell detachment with a distinct loss in E-cadherin, an elongation in cell shape, and increased expression of α SMA. Interestingly, the MMP-9 KO explants, without TGF β treatment, exhibited irregular localization of E-cadherin compared with their wild-type explants, and in some cells it appeared reduced. Despite this staining pattern, the current work (Chapter 4.3.2) revealed that overall E-cadherin protein levels in the lens explant lysates from untreated MMP-9 KO mice versus untreated wild-type mice were found to be

the same. This finding suggests that the absence of MMP-9 does not affect the transcription of E-cadherin protein, but rather leads to its mis-localization from cell membrane complexes, implicating a role for MMP-9 in the homeostatic regulation of E-cadherin junctions. A report in astrocytes found that MMP-9 KO mice exhibited an altered actin cytoskeleton and perturbed cell migration (Hsu, Bourguignon et al. 2008). The actin cytoskeleton is known to regulate E-cadherin localization and stability (Cavey, Rauzi et al. 2008) suggesting that MMP-9 KO lens epithelial cells may have an altered cytoskeletal arrangement that may be contributing to the reduced E-cadherin staining observed in the MMP-9 KO lens cells.

Importantly, the results presented in Chapter 4.3.2 also demonstrated the involvement of MMP-9 in a pathological, TGF β -stimulated state. TGF β treatment in the absence of MMP-9 resulted in significantly decreased E-cadherin protein levels in conditioned media when compared with wild-type TGF β -treated explants. Similarly, in a model of peritoneum membrane injury, the TGF β 1-induced level of E-cadherin fragments measured in peritoneal effluent was greatly reduced in MMP-9 KO mice when compared to TGF β 1-treated MMP-2 KO and wild-type mice (Padwal, Siddique et al. 2017). Further, MMP inhibition was able to regulate fibroblast cell adhesion and this was dependent on tampering with the normal stoichiometric ratio of 1:1 between MMPs and their endogenous inhibitors, TIMPs. The downregulation of TIMP-1, tipping the MMP:TIMP axis in favour of MMP activity, led to reduced cell-cell contact and multilayering through a reduction in cadherin levels, and a disruption in the assembly of focal adhesion complexes involved in

the formation of cell-ECM contacts (Ho, Voura et al. 2001). Mechanically, changes in the density and stiffness of the ECM led to re-localization of E-cadherin from cell-cell contacts (Kumar, Das et al. 2014). In lens EMT, the profile of ECM proteins shifted to an increase in expression of fibronectin, collagen I, and tenascin-C (Lee and Joo 1999, Mamuya, Wang et al. 2014, Li, Yuan et al. 2015). During EMT of a tumour cell line, MMP-9 has specifically been shown to induce the accumulation of fibronectin, which is involved in inducing the expression of the transcription factor Snail, a well-known E-cadherin repressor (Lin, Tsai et al. 2011). Overexpression of Snail resulted in decreased E-cadherin and an increase in EMT-related markers, including vimentin, fibronectin and MMP-9 itself. The phenomenon of MMP-dependent E-cadherin ectodomain shedding is not only specific to TGF β , but is also seen in nitric oxide (NO)-treated epithelial cells, where NO-induced E-cadherin cleavage and subsequent increase in cytosolic β -catenin were reversed upon broad-spectrum MMP inhibition (Mei, Borchert et al. 2002). Taken together, the results of this thesis and those demonstrated in other models suggest that TGF β -induced E-cadherin disruption is mediated by proteolytic processing of the ectodomain of E-cadherin by MMP-9.

To directly test whether MMP-9 itself can affect E-cadherin/ β -catenin complexes at the cell membrane, in Aim 3 rat lens explants were treated with active rhMMP-9 alone. The results obtained from these experiments (Chapter 4.3.3) demonstrated E-cadherin and β -catenin de-localization from regions of cell-cell contact, which present as jagged, disjointed membrane edges. However, this phenotype differed from that of TGF β -treated

explants in that rhMMP-9 did not seem to induce an elongated cell morphology. Additionally, the loss of E-cadherin from the cell membrane was also more apparent than the loss of β -catenin. These discrepancies may be explained by the idea that E-cadherin homophilic contacts are differentially regulated by two distinct F-actin pools (Cavey, Rauzi et al. 2008). Small F-actin patches regulate E-cadherin stability, while linear, cortical actin is involved in regulating dynamic E-cadherin contacts with rapid turnover. It is possible that MMP-9 acts on a subset of the E-cadherin population not involved in dynamic changes in cell shape. These findings combined with those in the MMP-9 KO explants suggest that MMP-9 may be specifically involved in E-cadherin-based contact dissolution in lens EMT. In line with these results, in human ovarian carcinoma cells MMP-9 alone was sufficient to cause E-cadherin loss, and immunohistochemical staining of human ovarian tumours revealed that MMP-9 colocalized with regions of reduced E-cadherin (Cowden Dahl, Symowicz et al. 2008). Immunofluorescence and Western blot of *in vitro* tubular epithelial cells treated with recombinant MMP-9 revealed a loss of membrane-associated E-cadherin and β -catenin, and acquired expression of α SMA, N-cadherin, vimentin (Tan, Zheng et al. 2010). Thus, based on these data it can be speculated that one of the critical roles of MMP-9 lies in its proteolytic processing of adherens junction constituents. Discussion on why MMP-9-mediated delocalization of E-cadherin is not sufficient to induce α SMA expression is presented below (Chapter 5.4).

5.3 MMP-9 is downstream of cytoskeletal signaling

Work in other systems has demonstrated that ROCK-mediated acto-myosin contractility affects the synthesis and turnover of ECM components (Wozniak, Desai et al. 2003, Huang and Ingber 2005, Provenzano, Inman et al. 2008, Butcher, Alliston et al. 2009). With this idea in mind, the current work investigated whether cytoskeletal signaling in lens explants works upstream of MMP-9 expression. The results in Chapter 4.3.1 of this dissertation demonstrated that, indeed, TGF β -induced MMP-9 protein expression in lens explants occurs in a ROCK-dependent manner. The presence of Y-27632 decreased MMP-9 expression following TGF β treatment when compared to TGF β -treated explants. A link between Rho-GTPase activation and MMP expression has been studied in different cell types. For example, in a human monocytic cell line the inhibition of Rho blocked MMP-9 expression, which was otherwise secreted in control cells upon stimulation (Wong, Lumma et al. 2001), while in a fibrosarcoma cell line Rac1 was sufficient to induce the activation of MMP-2 (Zhuge and Xu 2001). In nontransformed epithelial cells, it has been demonstrated that forced expression of MMP-7 resulted in a loss of E-cadherin and β -catenin immunofluorescent localization at the membrane correlated with enhanced migration and RhoA activation (Lynch, Vargo-Gogola et al. 2010). Only one study has been carried out in the context of TGF β -induced EMT, where Y-27632 inhibited the transcription of fibronectin and MMP-2 stimulated by TGF β in retinal pigment epithelial cells (Zhu, Nguyen et al. 2013). Collectively, these studies reveal that RhoA may be a very important link in determining how MMPs, specifically MMP-9, may contribute to the

formation of lens fibrotic pathologies. Indeed, the *MMP-9* promoter was shown to be a direct target of the MRTF-A/SRF complex (Gilles, Bluteau et al. 2009), and future work in lens explants can be carried out to determine whether TGF β -induced MMP-9 expression is also dependent on MRTF-A activity.

Beyond the role of cytoskeletal tension in regulating the makeup of the ECM in an inside-out manner, the ECM, in turn, regulates internal cellular forces. In the experiments carried out in mouse lens explants (Chapter 4.3.2), the absence of MMP-9 prevented the formation of radial F-actin patterning in response to TGF β treatment, instead maintaining a cortical actin arrangement. Taken together with results discussed above (Chapter 5.2), this suggests that in our system MMP-9 is both downstream of RhoA/ROCK signaling, and is also required to perpetuate the sustained EMT response, in the absence of which lens cells are not sensitive to TGF β stimulation. The requirement of MMP-9 in TGF β -induced EMT may be explained by its ability to proteolytically process and activate growth factors and other MMPs. As outlined in the introduction, one of the key pathways for regulating the activity of TGF β is through its sequestration in the ECM. MMP-9 has been found to cleave and activate latent TGF β releasing it from ECM-bound stores (Dallas, Rosser et al. 2002), specifically when in complex with CD44 at the cell surface (Yu and Stamenkovic 2000). CD44 is a cellular adhesion receptor that is upregulated following tissue injury. In the EMT of alveolar epithelial cells, it was demonstrated that TGF β 1 enhanced the expression of CD44 in a ROCK-dependent manner (Buckley, Medina et al. 2011). Further, the expression of a constitutively active RhoA mutant in human microvascular endothelial

cells induced the expression of MMP-9 and was demonstrated to co-localize with RhoA and CD44 at the cell surface (Abecassis, Olofsson et al. 2003). Collectively, these studies suggest that CD44 may concentrate active MMP-9 at the cell surface localizing it near potential substrates, such as E-cadherin complexes, latent TGF β , or MMPs themselves (Nagase and Woessner 1999). Taken together, it is feasible that downstream of ROCK-mediated lens epithelial cytoskeletal reorganization, CD44 localizes active MMP-9 at the cell surface where MMP-9 acts to further propagate TGF β signaling during early wound healing events, associated with ASC and PCO. The role of CD44 in lens EMT has not been studied and would be of interest in future investigations outlined in Chapter 6.1.

5.4 MMP-9 necessary, but not sufficient to induce α SMA expression

Previous rat lens explant studies led by the West-Mays group demonstrated that inhibiting MMP-2 and -9 prevented the TGF β -mediated MRTF nuclear accumulation (Gupta, Korol et al. 2013). As a result, the current work investigated whether application of active rhMMP-9 itself could stimulate MRTF-A-mediated α SMA expression (Chapter 4.3.3). However, the results revealed that rhMMP-9 alone did not lead to MRTF-A nuclear accumulation, which coincided with an absence in α SMA expression and resembled control explants. This suggests that while MMP-9 is required for actin remodeling and E-cadherin-based cellular junction dissolution, it alone is not sufficient to induce α SMA-expressing EMT, and instead may work in concert with other TGF β -stimulated

components. For example, it is possible that MMP-9, in conjunction with TGF β signaling, acts upstream of MMP-2. Quantitative RT-PCR analysis of ASC plaque cells from rat lenses revealed an early induction of MMP-9 mRNA after 2 days of TGF β treatment, whereas MMP-2 was found to be up-regulated at the later 4-day time point (Nathu, Dwivedi et al. 2009). A similar coordinated expression pattern of MMP-9 and MMP-2 has also been described in other models of fibrosis. For example, in corneal wound healing MMP-9 was shown to be involved in the initial stages of repair, such as corneal re-epithelialization, whereas MMP-2 was involved in later stages involving matrix degradation (Mohan, Chintala et al. 2002).

Although there is no evidence that MMP-9 directly regulates MMP-2 expression, there are indirect mechanisms by which MMP-9 may influence MMP-2. For example, MMP-14, which is known to induce MMP-2 in other systems (Sato and Takino 2010), was found to be up-regulated in rat lenses 4 days after TGF β treatment and 2 days after MMP-9 induction (Nathu, Dwivedi et al. 2009). Thus, the fact that induction in MMP-9 expression precedes MMP-14 suggests that MMP-14 may act as an intermediate for regulating MMP-2 expression. However, the current work demonstrated that rhMMP-9 alone is not sufficient to propagate the EMT response, which suggests that other downstream effectors of TGF β signaling are required. Activation of MMP-14 by MMP-9 could occur through the dissociation and activity of β -catenin from cell adhesion complexes. Evidence for this claim comes from a study by Takashi et al (2002) that found a TCF/LEF binding sequence in the *MMP-14* gene, and demonstrated that this motif is

recognized and bound by active β -catenin. As outlined in the introduction, β -catenin dissociated from cell junctions is degraded unless canonical Wnt signaling is activated. This may explain why MMP-9-mediated proteolytic processing of E-cadherin-based cell junctions was not enough to induce α SMA, where cross-talk with components of other signaling pathways, such as the Wnt/ β -catenin pathway, are required. Indeed, recent work in lens explants has shown that inhibition of β -catenin transcriptional activity prevents TGF β -induced stress fibre formation and α SMA expression (Taiyab, Korol et al. 2016).

CHAPTER SIX: FUTURE DIRECTIONS AND CONCLUSIONS

6.1 Future Directions

The current work has exposed ROCK and its downstream mediators, MRTF-A and MMP-9, as novel profibrotic agents. Beyond expanding our current knowledge this work has opened avenues for future experimentation.

Firstly, as outlined in the Discussion (Chapter 5.3), based on data from other systems it is possible that extracellular adhesion molecules, such as CD44, are involved in localizing MMP-9 at the cell membrane near cell junction complexes. CD44 has not been studied in the lens. Experiments in CD44-null mouse lens explants can be carried out to determine whether it is required in TGF β -induced EMT, and whether the phenotype observed is similar to that shown in MMP-9 KO mice. Immunofluorescent detection of EMT markers, such as E-cadherin, fibronectin, and α SMA following treatment with TGF β , as well as rhMMP-9 will reveal the requirement of CD44, specifically in the context of MMP-9-mediated E-cadherin proteolysis.

All cytoskeletal experiments carried out in this thesis were performed on *ex vivo* lens epithelial explants that are an excellent model for lens EMT in that lens epithelial cells remain adhered to their native basement membrane, the anterior lens capsule. Thus, this system has the *in vitro* advantage of ease of accessibility for visualization and treatment of lens epithelial cells, while maintaining an *in vivo* ECM profile, which is crucial in the study

of fibrosis. However, our system is not subject to all aspects of the normal *in vivo* environment, and therefore, it would be of great benefit to extend our cytoskeletal findings using an *in vivo* mouse model.

To that effect, the use of a conditional mutant mouse model of cofilin 1 would directly assess the contribution of ROCK-mediated signaling *in vivo*. As outlined in the introduction (Chapter 1.6), actin stress fibre formation through ROCK activity is mediated through two essential streams: (1) through the phosphorylation of MLC; and (2) through the inactivation of cofilin. Cofilin is an actin-binding protein that severs F-actin leading to actin depolymerization. Current unpublished work in the West-Mays lab has demonstrated that TGF β -treated MMP-9 KO lens explants have a defect in the levels of key actin cytoskeletal remodeling proteins, one of which is cofilin 1, work that compliments the findings outlined in this thesis. Therefore, in the future, using mice with a loss-of-function of cofilin 1 will reveal whether ROCK-mediated signaling is required for TGF β -induced lens fibrosis *in vivo*. With this model, an adenovirally delivered TGF β construct will be delivered to the lens as previously established in our lab (Robertson, Nathu et al. 2007). The lenses will then be assessed histologically, and along with immunofluorescent detection of EMT markers, such as F-actin, E-cadherin and α SMA.

6.2 Conclusions

Overall, the findings of this thesis demonstrate for the first time that the progression of TGF β -induced lens EMT is regulated by actin dynamics through a RhoA/ROCK/MRTF-A pathway. Specifically, the TGF β -mediated disassembly of the E-cadherin/ β -catenin complex and the subcellular localization of MRTF-A is ROCK-dependent and critical to TGF β -induced α SMA expression. Further, nuclear accumulation and activity of MRTF-A emerges as a novel sensor of the state of cellular actin in lens epithelial cells. My findings suggest that targeting MRTF-A activity, downstream of ROCK may serve as a promising means of preventing TGF β -induced EMT involved in fibrosis of the lens and other tissues.

The current work also implicates MMP-9 as the primary mediator of membrane-bound E-cadherin. The absence of MMP-9 in mouse lens explants prevented TGF β -induced E-cadherin fragments in conditioned media, while rat lens explants treated with rhMMP-9 alone facilitated the loss of E-cadherin and β -catenin at the cell periphery, exhibiting a punctate staining pattern. Furthermore, the findings that MMP-9 expression is also ROCK-dependent are novel to the literature on TGF β -induced EMT and fibrosis of other tissues. Therefore, based on the current results and together with previous work in other systems, I present MMP-9 as a key mediator of cell-cell contact dissolution in the lens epithelium, acting downstream of ROCK.

The results obtained in this thesis clearly delineate a critical role for ROCK-mediated cytoskeletal signaling in lens EMT, specifically implicating downstream MRTF-

A activity and MMP-9 expression. This work serves two key purposes: it advances the current understanding of how actin dynamics regulate TGF β -induced EMT, while simultaneously proposing novel therapeutic inhibitors and strategies for the prevention of fibrotic PCO, as well as fibrosis of other tissues. A ROCK inhibitor, Fasudil, has been in use in Japan since 1995 as a treatment for cerebral vasospasm and ischemia, importantly demonstrating no adverse effects. There are currently three ROCK inhibitors in human clinical trials for use in the treatment of glaucoma and ocular hypertension. In 2014, the ROCK inhibitor K-115 was approved in Japan as the first ophthalmic topical solution developed for the treatment of increased intraocular pressure (Kaneko, Ohta et al. 2016). The findings in this dissertation strongly suggest that targeting downstream elements of the TGF β pathway, specifically ROCK, MRTF-A and MMP-9, may prove to be an effective therapeutic approach that selectively targets profibrotic cells without affecting normally functioning cells and surrounding tissues.

PUBLICATIONS ARISING FROM THIS THESIS AND OTHER WORK:

Bowswell BA, **Korol A**, West-Mays JA, Musil LS. Dual function of TGF β in lens epithelial cell fate: implications for secondary cataract. *Molecular Biology of the Cell*, 2017 Feb 16. doi: 10.1091/mbc.E16-12-0865

Korol A, Taiyab A, West-Mays JA. RhoA/ROCK signaling regulates TGF β -induced epithelial-mesenchymal transition of lens epithelial cells through MRTF-A. *Molecular Medicine*, 2016 Sept 29;22. doi: 10.20119/molmed.2016.00041

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