ROLE OF MMPS IN TGFβ INDUCED CATARACTOGENESIS

THE REQUIREMENT OF MATRIX METALLOPROTEINASE 2 AND 9 IN TRANSFORMING GROWTH FACTOR BETA INDUCED EPITHELIAL MESENCHYMAL TRANSITION OF LENS EPITHELIAL CELLS

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ABSTRACT

Fibrotic cataracts such as anterior subcapsular cataract (ASC) are induced by the cytokine transforming growth factor beta (TGFβ). The exact mechanism which governs TGFβ-mediated ASC has not been fully elucidated. What is known is that TGFβ is able to initiate the conversion of lens epithelial cells (LECs) to a myofibroblast phenotype, which expresses alpha smooth muscle actin (αSMA), through a process known as epithelial to mesenchymal transition (EMT). TGFβ-induced EMT leading to ASC has been associated with the upregulation of two specific matrix metalloproteinases (MMPs), MMP2 and MMP9. However, the specific roles for either of these MMPs have yet to be established in ASC.

To determine the involvement of MMP2 and MMP9 I used synthetic inhibitors in conjunction with an established ASC *ex vivo* rat lens model initiated by TGFβ. The results demonstrated that co-culturing rat lenses with TGFβ and the broad spectrum matrix metalloproteinase inhibitor (MMPI), GM6001 or an MMPI specific for MMP2/9 suppressed ASC and these lenses did not positively stain for the EMT marker αSMA. Additionally, studies conducted on the conditioned media from these treatments revealed that TGFβ induces the cleavage of E-cadherin ectodomain which is suppressed by co-culturing rat lenses with either MMPI. To further delineate a role for MMP9 *in vivo*, ASC formation was examined in two models of lens specific TGFβ overexpression in the absence of functional MMP9. Adenoviral delivery of TGFβ to the anterior chamber of the eye in the absence of functional MMP9 resulted in complete suppression of ASC and

did not correlate with αSMA expression. Similarly, lens specific TGFβ overexpression in the absence of MMP9 suppressed ASC in 75% of mouse lenses. Additional studies were conducted to determine other TGFβ-induced events that affect ASC. The results showed that connective tissue growth factor is able to mediate ASC, albeit to a lesser degree than TGFβ and the mitogen-activated protein kinases ERK1/2 and p38 are likely involved in this disease.

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αSMA	alpha smooth muscle actin
AdDL	adenovirus with no insert
AdLacZ	adenovirus with lacZ insert
AdTGFβ1	adenovirus with transforming growth factor beta 1 insert
ARPE-19	human retinal pigmented epithelial cell line
ASC	anterior subcapsular cataract
AP-1	activator protein 1
BVD	back vertex distance
CTGF	connective tissue growth factor
DAPI	4',6-diamidino-2-phenylindole
EMT	epithelial to mesenchymal transition
E	glutamic acid
E-cadherin	epithelial cadherin
ECM	extra cellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMEM	Eagle`s minimal essential medium
ERK	extracellular regulated kinase
EtBr	ethidium bromide

FCS	foetal calf serum
FITC	fluorscein-isothiocynate
FHL-124	human lens epithelial cell line
G	glycine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSK3β	glycogen synthase kinase 3 beta
H	histidine
HSP27	heat shock protein 27
IOL	intra ocular lens
JNK	cJun N-terminal kinase
LAP	latency associated protein
LCM	laser capture microdissection
LEC	lens epithelial cells
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
NaCl	sodium chloride
PBS	phosphate buffered saline
PCO	posterior capsule opacification
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pfu	plaque forming unit

PI3K	phosphoinositide 3-kinase
rhCTGF	recombinant human connective tissue growth factor
RTK	receptor serine/threonine kinase
RT-QPCR	real time quantitative polymerase chain reaction
SCp-2	mouse mammary epithelial cell line
SMAD	mothers against decapentaplegic
SV40	simian virus 40
Tris	Tris(hydroxymethyl)aminomethane
TβR	transforming growth factor beta receptor
TCF/LEF	T-cell factor/lymphocyte enhancer factor
TGFβ	transforming growth factor beta
TIMP	tissue inhibitor of matrix metalloproteinase
TSP	thrombospondin
X	variable amino acid
ZO1	zonula occludens 1

1.0 CHAPTER ONE:

General Introduction

1.1 Lens Development

The eye is a structure allowing organisms to interpret and interact with their environment and 540 million years of evolution has created only three simple eye types, and only eye with an ocular lens (the camera eye) (Parker 2011). The ocular lens develops from the surface ectoderm, as does the skin, and therefore it is not surprising that the lens continues to grow throughout life. Initiation of lens formation is marked by proliferation of the ectodermal cells which cover the optic vesicle, and is termed the placode. Development of the lens continues as the central part of the placode invaginates to form a lens pit. The lens pit deepens as growth carries on and is eventually sealed, resulting in an attachment to the ectoderm by a lens stalk. Upon degeneration of this stalk at the anterior most region, the lens becomes fully separated from the overlying surface ectoderm (Pei and Rhodin 1970; Lovicu and Robinson 2004; Lovicu and McAvoy 2005). In addition to creating an anterior/posterior axis, the invagination of the ectodermal cells confines the basal regions of these cells to the exterior where secreted basal lamina generates the lens capsule. This matrix encapsulation confers an immuno-privileged status to the lens (Coulombre 1979). The encapsulated cells at the posterior region elongate towards the anterior region of the lens where they undergo terminal differentiation into primary fiber cells. Terminal differentiation involves programmed organelle loss and expression of crystallin proteins which maintain lens transparency (FIG 1.1) (Cvekl and Piatigorsky 1996; Cvekl and Tamm 2004; Cvekl and Duncan 2007). Conversely, at the anterior region of the lens, cells undergo proliferation and generate a monolayer of cuboidal lens epithelial cells (LECs) which extend toward the equatorial region of the lens. At the

equatorial region, LECs are responsible for the continuous generation of secondary lens fibers cells throughout life (FIG 1.2).

In general, the lens fiber mass contains both a nucleus and a cortex and the delineation of this nuclear/cortical boundary is very specific even though it is dynamic. The cortex consists of superficial, intermediate and deep fibers. Superficial fibers are nearest to the lens equator and are continuously elongating whereas fully elongated fibers undergoing organelle elimination are characteristic of intermediate fibers (Blankenship, Hess et al. 2001). The deep fiber cells have eliminated organelles, lost certain cytoskeletal elements and exhibit cross-linking of crystallin proteins in a dehydration dependent mechanism (Tardieu, Veretout et al. 1992; Kenworthy, Magid et al. 1994). In contrast to cortical fibers, nuclear fibers are significantly denser and harder as a result of dehydration and the rearrangement of the lens specific membrane protein aquaporin0 into square arrays (Kuwabara 1975; Costello, McIntosh et al. 1985; Zampighi, Hall et al. 1989). Due to the nature of lens fiber growth beginning embryonically and as a result of a lack of fiber cell turnover the lens nucleus is comprised of tissue from embryonic, fetal, juvenile and adult stages of life.

1.2 Cataracts

Loss of lens transparency, or cataract, has a gradient of manifestations and according to the World Health Organization, accounts for nearly half of individuals who suffer from blindness (WHO 2000). Cataracts can affect any individual from birth until old age and are categorized by the age of onset as well as the location within the lens.

Congenital or infantile cataracts occur at birth or within the first year of life and require prompt surgical intervention to allow for proper development of visual processing pathways. Age-related cataracts can form within each of the lens layers. Nuclear cataracts occur when the lens fiber nucleus becomes denser than normal and turn yellow. This process is typically quite slow and commonly individuals lose their ability to see distant objects; these types of cataracts will eventually require surgical intervention. In contrast, cortical cataracts develop within the lens fiber cortex, progress at varied rates and could result in visual impairment if they are centrally located. The most common manifestation is night time glare from lights. Furthermore, cortical cataracts are also associated with diseases such as type II diabetes. Subcapsular cataracts are the most rapid progressing of the age-related cataracts and can occur at both the anterior or posterior capsular region of the lens. To correct these visual impairments, 12 million cataract surgeries are performed annually worldwide, where the opaque lens is extracted and replaced by a synthetic polymer lens known as the intra ocular lens (IOL) (WHO 2000). However, surgical intervention may not be sufficient to combat this problem as a phenomenon known as secondary cataracts or posterior capsule opacification (PCO) occurs in 35% of patients within two years, following primary cataract surgery. PCO, is caused by the response of remnant LECs to trauma as a result of the surgical procedure (Kappelhof and Vrensen 1992). These damaged LECs initiate a wound healing response and remain on the capsule where they proliferate, aberrantly deposit matrix molecules and express filamentous alpha-smooth muscle actin (αSMA), which induces light scatter (Frezzotti, Caporossi et al. 1990; Sappino, Schurch et al. 1990; Schmitt-Graff, Pau et al.

1990). Similarly, trauma to the LECs of an intact human lens, as a result of puncture, steroid exposure or following disease such as atopic dermatitis or retinitis pigmentosa also results in a wound healing response leading to spindle-shaped cell formation and capsular wrinkling (Schmitt-Graff, Pau et al. 1990). Furthermore, the wound healing response is localized to a region contained in the anterior epithelium of the lens, which expresses the contractile filament αSMA, forming what is known as an anterior subcapsular cataract (ASC).

Most of what is known about cataracts is limited to the physical protein-protein interactions which cause light scattering. However, developing effective therapies to treat and/or prevent cataracts will depend on a deeper understanding of the cellular and molecular signaling events preceding cataract formation.

1.3 Cataract Induction – Transforming Growth Factor Beta

Subcapsular cataract formation occurs as a result of damaged LECs, which become spindle shaped, exhibit wrinkling of the lens capsule, deposit matrix and characteristically begin to express αSMA. Studies of the ocular tissue in patients with ASC revealed high levels of transforming growth factor beta 2 (TGFβ2) in the aqueous humor. These findings putatively identified TGFβ as a candidate molecule for stimulating ASC induction (Jampel, Roche et al. 1990). To test this hypothesis, rat lens epithelial explants were co-cultured with TGFβ2 and it was confirmed that TGFβ2 was indeed a mediator of ASC (Liu, Hales et al. 1994). In fact, the same group demonstrated that all three TGFβ isoforms can mediate cataract formation albeit with TGFβ2 having the largest

potential and TGFβ1 the least (Gordon-Thomson, de Iongh et al. 1998). All isoforms of TGFβ (three in total) have the ability to cause capsular contraction, transform cuboidal LECs into spindle shaped cells, and cause the expression of filamentous αSMA (Liu, Hales et al. 1994).

Two other models of TGFβ delivery resulting in ASC have been generated and are employed for the study of ASC. Transgenic mice expressing a modified human TGFβ1 under the control of a lens specific promoter begin to develop ASC at three weeks of age. More specifically, these mice express a mutant form of TGFβ1 in which two cysteine residues (positions 223 and 225) have been replaced by serine residues, resulting in a self-activating form of TGFβ1. This self activating TGFβ1 is expressed embryonically, under the control of the lens specific promoter αA-crystallin and therefore becomes active at embryonic day 12.5 (Srinivasan, Lovicu et al. 1998). The resultant ASC in this model is similar in pathology to human ASC (Liu, Hales et al. 1994).

More recently, our laboratory has generated another model of ASC by employing an adenoviral vector to express TGFβ in the anterior chamber of the rodent lens. Gene transfer of active TGFβ1 to the anterior chamber of wildtype mice induces ASC formation beginning at four days post injection (Robertson, Nathu et al. 2007). These lenses showed distinct ASC plaques consisting of a focal multilayering of LECs beneath the intact anterior lens capsule. The resultant plaques exhibit reactivity to the myofibroblast cell marker, αSMA, and confirm that EMT events have occurred.

The delineation of the step-wise events involved in ASC formation begins with an understanding of how TGFβ is able to elicit its effects. TGFβ is activated by the

modification of the N-terminal latency associated protein (LAP) domain of the propeptide. This LAP domain is altered by a variety of factors including pH, thrombospondin1 (TSP1) and the cell adhesion receptor αvβ6 integrin (Munger, Huang et al. 1999; Murphy-Ullrich and Poczatek 2000; Yu and Stamenkovic 2000).

Activated TGFβ molecules initiate signal transduction pathways by inducing the heterodimerization of four transmembrane receptor serine/threonine kinases (RTK) (FIG 1.3). Specifically, a TGFβ ligand causes homodimerization of two TGFβ type II receptors (TβRII) which then recruit a homodimer of TGFβ type I receptor (TβRI). Activated TβRI then propagates the signal through both mothers against decapentaplegic homolog (SMAD), and SMAD-independent signaling (Massague 1998). The mammalian *SMAD* family is comprised of 9 genes which can be separated into three different categories; mediator, inhibitory and excitatory SMADS. The ubiquitous mediator SMAD, known as SMAD4, complexes with active SMADs to promote their translocation to the nucleus where they act as transcriptional regulators (Massague 1998). The inhibitory SMADs, which are SMAD6, 7, participate in the self-attenuation of TGFβ signals by acting as transcriptional repressors of TGFβ signaling. Excitatory SMADs (SMAD2,3) are activated by TβRI at specific sequences which results in cognate gene upregulation following nuclear translocation (Lo, Chen et al. 1998; Shi, Wang et al. 1998). Smad proteins are traditionally regarded as the primary mediators of TGFβ signaling, however TGFβ is also able to elicit signaling responses independent of Smad proteins (Yamaguchi, Shirakabe et al. 1995; Hanafusa, Ninomiya-Tsuji et al. 1999) (FIG 1.3). SMAD-independent TGFβ signaling can involve the mitogen activated protein kinases (MAPKs);

(i) p38, (ii) extracellular regulated kinase (ERK) and (iii) phosphoinositide 3kinase (PI3K). Taken together this would suggest that both SMAD-dependent and SMAD independent pathways co-operate to mediate the effects of TGFβ in cataractogenesis.

1.4 Effect of TGFβ on the Lens - Epithelial to Mesenchymal Transition

EMT results from a co-ordinated loss of cell-to-cell contacts, notably epithelial-cadherin (E-cadherin), and subsequent detachment from the basement membrane(Hay 1995). These actions result in a loss of cell polarity, and subsequent expression of contractile elements (αSMA), deposition of aberrant amounts of matrix and expression of a unique profile of proteases. The first report of lens epithelial cells undergoing EMT was carried out over three decades ago on chick LECs. Chick lens epithelial explants were stripped of their LECs and seeded into collagen gel matrices (Greenburg and Hay 1982). LECs seeded in native collagen began to develop morphologies identical to mesenchymal cells (Greenburg and Hay 1982). TGFβ was identified as the growth factor responsible for LEC conversion to mesenchymal cells and its direct ability to mediate EMT was first demonstrated in rat lens epithelial explants (Liu, Hales et al. 1994). Rat lens explants treated with TGFβ transformed to a mesenchymal phenotype with migratory ability and expressed filamentous αSMA concomitant with aberrant matrix deposition (Liu, Hales et al. 1994).

Since the discovery of TGFβ as a potent inducer of EMT, experiments have been aimed at elucidating the specific SMAD-dependent and independent signaling events that are responsible for the ensuing EMT. One study has determined that SMAD3, normally

sequestered in the cytoplasm of LECs, was found to localize to the nucleus in post-operative patients receiving cataract surgery (Saika, Miyamoto et al. 2002). Similarly, rat LEC explant cultures which undergo EMT also demonstrate nuclear translocation of SMAD3 (Saika, Miyamoto et al. 2002). These findings suggest a role for SMAD3 in TGFβ mediated EMT. Specific examination of SMAD3 in EMT which leads to ASC is possible through the use of a mouse model where functional SMAD3 has been ablated. In these mice, a stop codon was inserted following exon 7 of the gene, which resulted in a truncated SMAD3 protein (Yang, Letterio et al. 1999). This truncated SMAD3 lacks both the L3 loop and consensus SSVS sequence at the C-terminal domain necessary for phosphorylation by the TGFβ-receptor and subsequent activation (Lo, Chen et al. 1998). Exploring the role that SMAD3 plays in TGFβ mediated EMT of LECs has led to interesting findings.

To determine the individual role of SMAD3, cataract formation was assessed in the SMAD3 knockout mouse model, whereby EMT was elicited either through physical insult (lenticular puncture) or through the addition of exogenous TGFβ2 (Saika, Kono-Saika et al. 2004). The results demonstrated that mouse lenses lacking the TGFβ signaling transducer, SMAD3, failed to elicit EMT either through puncture or when cultured *ex vivo* with TGFβ2. These data suggest that the formation of ASC via EMT by TGFβ requires SMAD3. In contrast, work by Banh *et al* has directly challenged this notion. When mice with germline knockout of *Smad3* were bred with mice possessing a lens specific self-activating form of TGFβ1, ASC formation still occurred; although the cataracts formed in these mice were smaller than mice exposed to lens-specific TGFβ1

bred on a wildtype background (Banh, Deschamps et al. 2006). These data clearly suggest that SMAD-independent TGFβ signaling pathways are also involved in the EMT associated with ASC.

EMT resulting from SMAD-dependent TGFβ-signaling results in a number of molecular alterations. One such process is the transcriptional downregulation of the cell-to-cell contact protein, E-cadherin, resulting from increased transcription of the E-cadherin repressors *Snail1,2* and *3* (Barrallo-Gimeno and Nieto 2005). The Snail family of transcription factors all possess a conserved DNA-binding carboxy-terminal domain and C_2H_2-type zinc finger repeats which bind to CAGGTG E-box sequences in the promoter region of E-cadherin leading to transcriptional repression (Huber, Kraut et al. 2005; Przybylo and Radisky 2007). The ensuing loss of E-cadherin disrupts the E-cadherin/β-catenin complex, a complex that is essential for the polarization and function of epithelial cells and for the integrity of various cell strata (Tepass, Truong et al. 2000). The cadherin/catenin complex is maintained by the association of cytoplasmic the tail of E-cadherin to β-catenin which, in turn, binds α-catenin and associated with actin, and other actin-binding proteins that include vinculin, α-actinin, zonula occludens1 (ZO1) (Pokutta and Weis 2007). Under normal circumstances, E-cadherin is responsible for the sequestration of β–catenin at the membrane interface. β-catenin plays an important role in cell adhesion and is associated with the Wnt signaling pathway. The cytoplasmic/nuclear pool of β-catenin involved in Wnt signaling is regulated by a complex which contains AXIN, APC, and GSK3β proteins (Munemitsu, Albert et al. 1995; Nakamura, Hamada et al. 1998; Schwartz, Wu et al. 2003). GSK3β is able to

mediate the phosphorylation of the N-terminus of β-catenin, which targets its ubiquitination and degradation by proteasomes. This GSK3β-medaited degradation is only able to occur in the absence of Wnt signals and, the binding of Wnt to Fizzled-LRP (lipoprotein-receptor-related protein) receptors signals a pathway that inhibits GSK3β activity and therefore stabilizes β-catenin, preventing its degradation. Nuclear β-catenin is able to complex with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription regulator proteins to enter the nucleus and subsequently activate transcription of specific target genes (Nieto 2002; Huber, Kraut et al. 2005; Thiery and Sleeman 2006; Yook, Li et al. 2006). Some of the β-catenin/TCF/LEF regulated genes include VEGF, and MMP-7 (Brabletz, Jung et al. 1999; Zhang, Gaspard et al. 2001). More recently, matrix metalloproteinases (MMPs) have also been shown to increase the expression of Snail family of transcription factors, causing disruption of the E-cadherin/β–catenin axis and resulting in EMT (Radisky, Levy et al. 2005). This suggests that MMPs may play a role in ocular fibrosis.

1.5 Matrix Metalloproteinases and Their Role in Lens EMT and TGFβ Activity

MMPs were first discovered as enzymes of the tadpole tail which were capable of degrading gels of fibrillar collagen (Gross and Lapiere 1962). These enzymes were subsequently categorized as endopeptidases due to their ability to cleave internal peptide bonds (Stocker, Grams et al. 1995). Since then, research has determined that of the 25 vertebrate MMPs, 22 exist as human homologues (Nagase and Woessner 1999; Lohi, Wilson et al. 2001) (FIG 1.4**)**. MMPs are part of the larger metzincin superfamily, and

can be identified by their characteristic non-variable histidine (H), glutamic acid (E) and glycine (G) residues interspersed between variable (X) and bulky hydrophobic amino acid residues (B): HEBXHXBGBXHZ(Stocker, Grams et al. 1995). The final amino acid (X) designates family specificity, which for most MMPs is a serine.

Traditionally, MMPs were thought to share two key attributes: (1) their ability to degrade proteins of the extra cellular matrix (ECM) and (2) dependence on zinc and calcium divalent cations for catalytic activity (Stocker, Grams et al. 1995). However, upon further examination, the functional role of MMPs has expanded to include important processes in cell regulation. This is accomplished via cleavage of cell surface molecules as well as the liberation and activation of growth factors and receptors which are sequestered within the confines of the matrix (Sternlicht, Bissell et al. 2000). Through these processes, MMPs have been shown to regulate embryonic development, tissue morphogenesis, wound repair, inflammatory diseases and cancer (Nelson, Fingleton et al. 2000). The complex physiological roles that MMPs exert are briefly summarized in Table 1.1. This list illustrates that there are both specific and overlapping roles for MMPs.

In order to describe how MMPs elicit their physiological roles it is important to identify their structure. Generally, MMPs posses an N-terminal signal sequence that is cleaved following endoplasmic reticulum guidance and indicate that most MMPs are secreted enzymes. However, six MMPs possess transmembrane repeats and therefore localize to the cell surface. Downstream from this first domain is a propeptide domain,

which maintains latency until it is enzymatically removed, and a catalytic zinc binding domain that confers cleavage site specificity (Overall 2001).

For these modular zymogens to exert their biological functions, they must be regulated in a temporal and spatial manner which is mediated by a variety of cellular factors. Such regulation is evident at both the transcriptional and post-transcriptional levels in addition to specific endogenous peptide activators and inhibitors. Briefly, transcriptional activation of most MMPs is regulated by multiple growth factors including TGFβ (Fini, Cook et al. 1998). Induction from these stimuli generally induces the proto-oncogenes *c-fos* and *c-jun* which bind activator protein1 (AP1) sites on MMP regulatory regions. In addition, the MMP regulatory region also possesses; AP2, Sp1, Sp3 and p53 binding sites as well as retinoic acid response elements (Bian and Sun 1997; Fini, Cook et al. 1998; Lohi, Lehti et al. 2000; Ludwig, Basset et al. 2000). Post-transcriptional modification has also been reported in some MMPs. In such cases transcripts are stabilized by alternative polyadenylation, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and AU-rich sequences at the 3' untranslated region (Delany, Jeffrey et al. 1995; Sternlicht and Werb 1999; Vincenti 2001). The culmination of these regulatory processes leads to the synthesis of zymogens where latency is maintained as a result of an unpaired cysteine sulfhydryl group in the propeptide domain interacting with a zinc ion within the enzymatic active site (Van Wart and Birkedal-Hansen 1990). To activate MMPs, the zinc site must be liberated from contact with the unpaired cysteine and can be achieved by the removal of the propeptide domain (Nagase and Woessner 1999). Removal of the propeptide domain is accomplished by other activated MMPs or

serine proteinases. Interestingly, MMP2 is the only MMP which is not susceptible to serine proteinases and therefore requires activation through sequential cell surface activation (Strongin, Collier et al. 1995).

The *in vivo* activity of endogenous MMPs is balanced by both irreversible and reversible inhibition. Endogenous inhibition of MMPs results from interaction with the plasma protein α2-macroglobulin (Sottrup-Jensen and Birkedal-Hansen 1989). Complexes of α2-macroglobulin and MMPs are cleared by receptor-mediated endocytosis, leading to irreversible inhibition (Sottrup-Jensen and Birkedal-Hansen 1989). Conversely, four endogenous tissue inhibitors of matrix metalloproteinases, TIMP1 to 4, reversibly bind MMPs in a 1:1 stoichiometric relationship (Gomez, Alonso et al. 1997; Sternlicht and Werb 1999). Each TIMP contains two separated groups of six cysteine residues forming a two-domain, six looped structure when disulfide bridges are formed. Studies have also concluded that the six N-terminal loops interact with the MMPs, and as few as three loops are necessary to functionally inhibit MMPs (Murphy and Willenbrock 1995). Importantly, different TIMPs exhibit selective preference for the various MMPs they inhibit. For example, TIMP3 is a more potent inhibitor of MMP9 than the others, while TIMP1 is unable to bind and inhibit the membrane bound MT1-MMP (Nagase and Woessner 1999). Other non-endogenous factors that inhibit MMP activity include tetracyclines, chlorhexidine, ethylene diamine tetraacetic acid (EDTA) and GM6001 (Gendron, Grenier et al. 1999). In general, the effectiveness of these exogenous MMP inhibitors is attributed to their propensity to chelate calcium and zinc and thereby disrupt MMP function (Gendron, Grenier et al. 1999).

A link between MMPs and EMT events was first reported when 26 distinct tumor cell lines were characterized for expression of MMPs (Sato, Kida et al. 1992). It was determined that both MMP2 and 9, were present in a majority of these cell lines and that MMP9 correlated with tumorigenicity (Sato, Kida et al. 1992). Direct evidence to support MMP involvement in EMT was first suggested when mammary epithelial cells (SCp2) that were treated with recombinant MMP3 underwent EMT as a result of cell-to-cell contact loss and acquisition of the myofibroblast marker vimentin (Sternlicht, Lochter et al. 1999). In addition, co-treatment of SCp-2 cells with MMP3 and TIMP1 prevented EMT (Sternlicht, Lochter et al. 1999). In the lens, few studies have addressed the direct involvement of MMPs in TGFβ-mediated EMT resulting in cataract formation. What is known is that perturbation of lens cells with either TGFβ or H_2O_2 results in the increased expression of both MMP2 and MMP9 (Richiert and Ireland 1999; Tamiya, Wormstone et al. 2000). Additionally, over-expression of MMP2 in cultured LECs results in a change in cell morphology and polarity, increased cellular proliferation and expression of αSMA (Seomun, Kim et al. 2001). Furthermore, treatment with a synthetic inhibitor for both MMP2/9 is able to prevent the EMT associated with MMP2 over-expression (Seomun, Kim et al. 2001). Exactly, how MMPs mediate this EMT event in LECs has yet to be determined. However in other systems, MMPs participate in EMT via the disruption of the E-cadherin/β-catenin complex. For example, MMP9 has been shown to specifically exert EMT in a variety of epithelia via cleavage of the E-cadherin ectodomain, causing dissociation of β-catenin and subsequent nuclear localization leading to cognate gene regulation (Symowicz, Adley et al. 2007). Furthermore, disruption of the E-cadherin/β-

catenin axis involving MMP9 and TGFβ1 is a result of expression of Snail2, which transcriptionally represses E-cadherin (Zheng, Lyons et al. 2009). It remains to be determined if TGFβ mediated expression of MMP2 and MMP9 are involved in the associated EMT resulting in ASC.

1.6 Rationale for Study

Considerable efforts have already been made with respect to the underlying causes of ASC, namely the identification of TGFβ as a potent inducer of EMT leading to ASC. Additionally, growing evidence suggests that TGFβ induction of MMPs is correlated with the progression of this type of cataract. However, a direct requirement for MMPs in TGFβ-mediated ASC has yet to be established. **Therefore, I hypothesis that TGFβ's ability to induce MMP2 and MMP9 results in fibrotic cataracts such as anterior subcapsular cataract.**

The availability of commercial synthetic inhibitors for the spectrum of MMPs in conjunction with established models of ocular fibrosis are untapped sources of important information. Furthermore, hallmark features of EMT such as E-cadherin cleavage have not been investigated in a pathology which has become widely accepted as a direct result of the EMT process. *Therefore the first aim will be to use small molecule inhibitors of MMPs to determine if their inhibition affects the anterior subcapsular cataract phenotype. Furthermore experiments in this first aim will address if the phenomenon of E-cadherin shedding is in fact associated with anterior subcapsular cataracts.*

Examination of the role of MMPs in TGFβ-induced ASC may be facilitated by more recent developments in animal models pertaining to fibrosis. These models include the aforementioned transgenic mouse model expressing a lens specific self-activating form of TGFβ1 as well as direct adenoviral-mediated delivery of TGFβ1 to the anterior chamber via intracameral injection. Utilization of mice with germline ablation of *Mmp9* in conjunction with either or both of the TGFβ1 models will greatly improve our understanding regarding the role of MMPs in ASC. *Therefore the second aim of this study will be to determine what specific affect MMP9 plays in anterior subcapsular cataracts. In these experiments the induction of ASC by TGFβ will be assessed by two different in vivo models in conjunction with a mouse strain which lacks functional MMP9.*

In addition to the role that MMPs play in the formation of ASC, it is of critical importance to determine other signaling pathways elicited by TGFβ which assist in initiating and maintaining EMT. To this end, examination of the effects of MAPK such as p38 and ERK1/2 will extend our current understanding of the mechanism involved in ocular fibrosis. *Therefore the third aim of this study will be to determine which MAPK may be responsible for mediating TGFβ-mediated ASC as indentified by proteome arrays.*

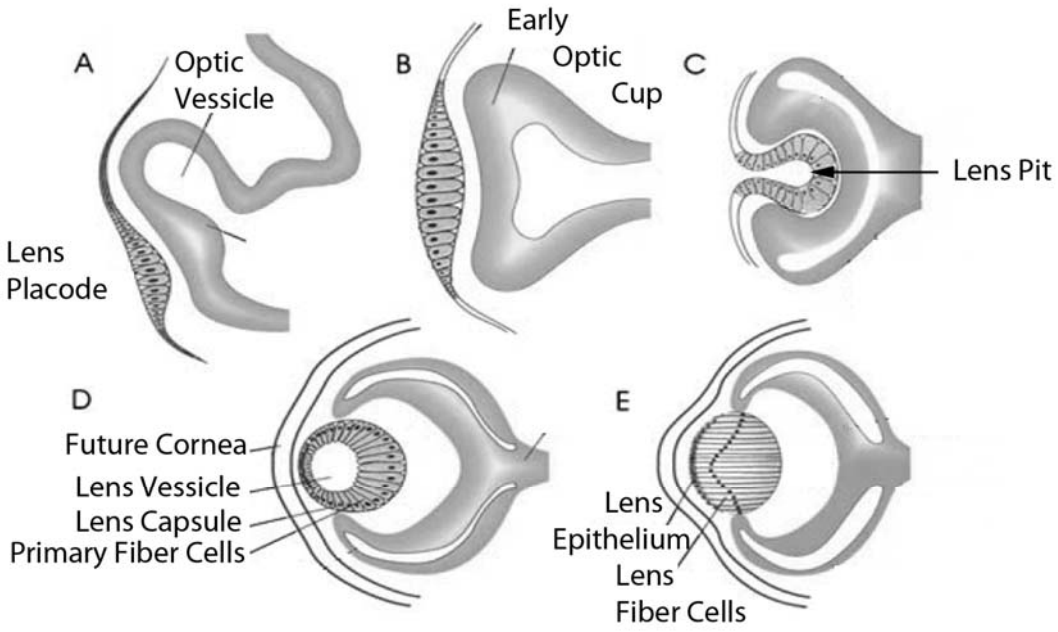

Fig 1.1 The lens develops from the ectoderm at the lens placode (A). The placode continues to thicken (B) and ultimately invaginates to generate the lens pit. Following this, the lens pit separates from the overlying ectoderm generating a vesicle with primary lens cells fibers at the posterior end which begin to elongate and subsequently lose all membrane bound organelles concomitantly expressing the lens crystallin proteins (D). The lens cells at the anterior region form a monolayer of nucleated LECss and are responsible for the generation of new fiber cells, which migrate to fill the lens vesicle (E). Adapted from (Cvekl and Piatigorsky 1996).

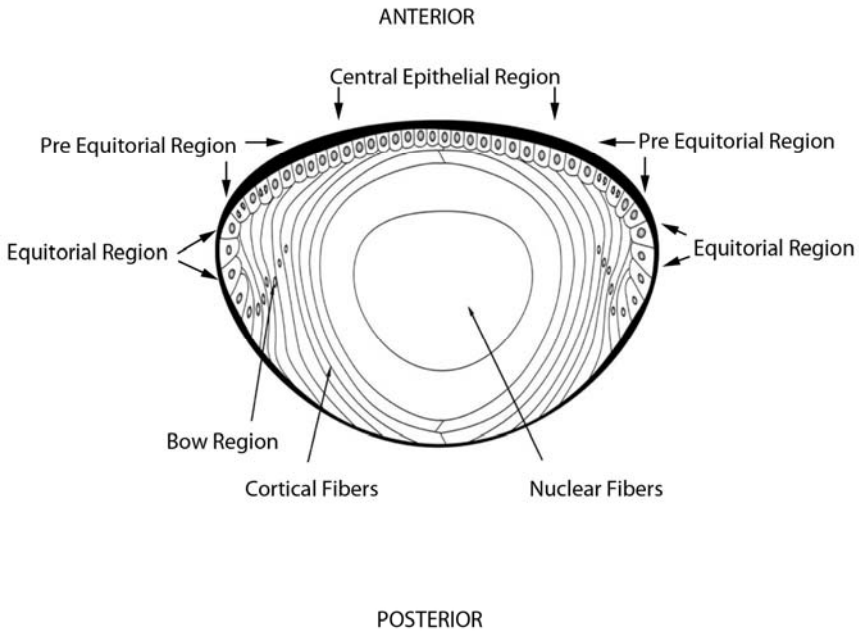

Fig 1.2 During development, the lens develops an anterior-posterior axis. The anterior region of the lens is divided into three distinct regions. The central lens epithelial region is defined as the region where the LECs are quiescent. LECs at the pre-equatorial region are non-quiescent and are responsible for the generation of new lens fiber cells. LECs elongate at the equatorial region and subsequently undergo programmed nuclear and organelle loss as they become cortical fibers, where they begin to express crystallin proteins and form the cortical lens fibers. The nuclear lens fibers at the centre of the lens are the primary lens fiber cells created at birth.

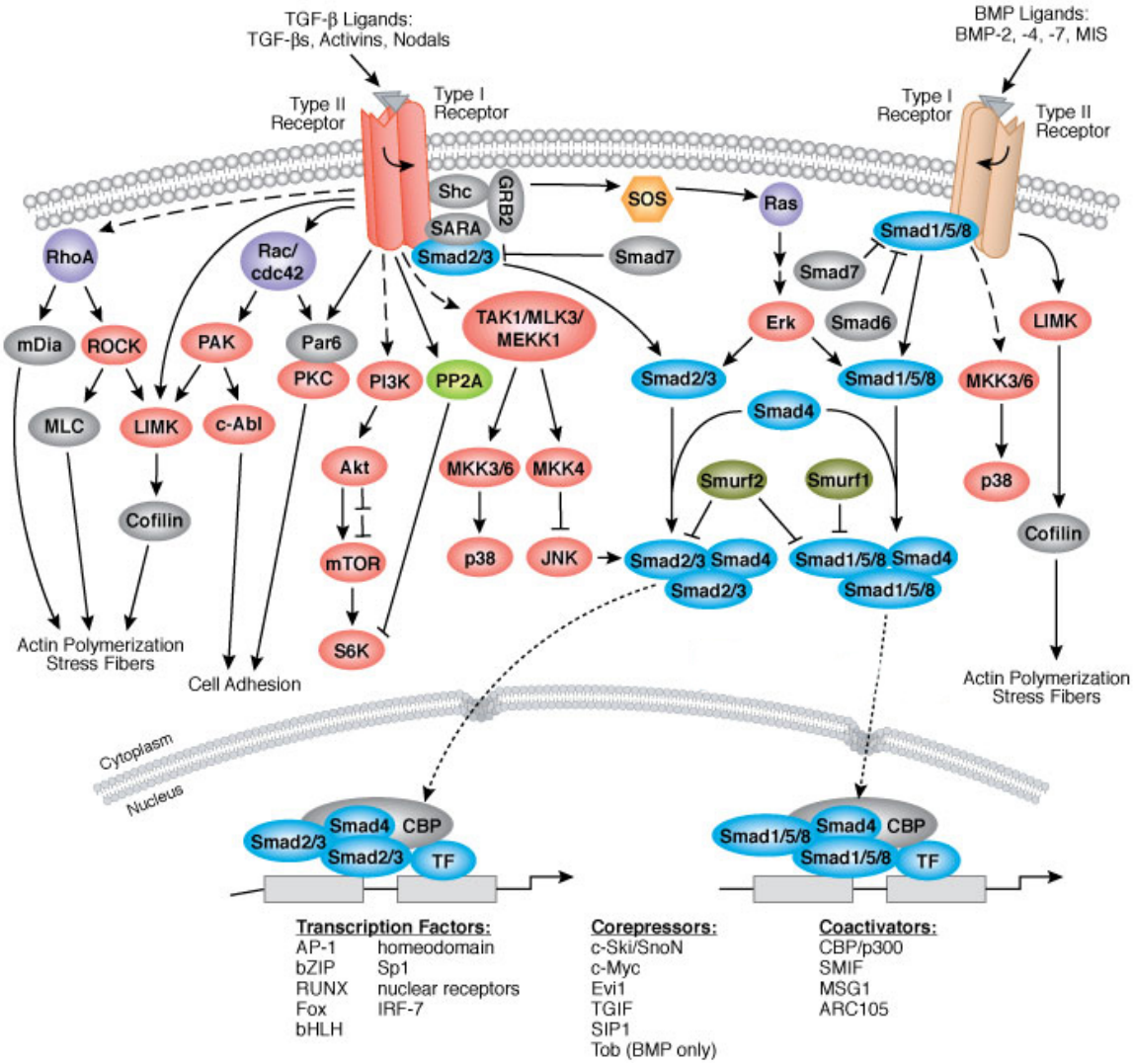

Fig 1.3 The TGFβ signaling cascade is initiated by binding of either TGFβ1, 2 or, 3 ligands causing the dimerization of two type II receptors. This dimerization then recruits, and activates via phosphorylation, two type I receptors to form an active tetrameric structure. An active TGFβ receptor is able to transduce signals through the activation of Smad2 and 3. These receptor activated SMADS complex with mediator SMAD4 to translocate into the nucleus where they are able to induce a variety of transcription factors. The activation of Smad2 and 3 is regulated by an inhibitory SMAD, SMAD7. In addition, TGFβ signaling occurs through the mitogen associated protein kinases (MAPKs), p38, cJun N-terminal kinase (JNK) or extracelluar regulated kinase 1/2 (ERK1/2). (Adapted from http://www.cellsignal.com/reference/pathway/TGF_beta.html)

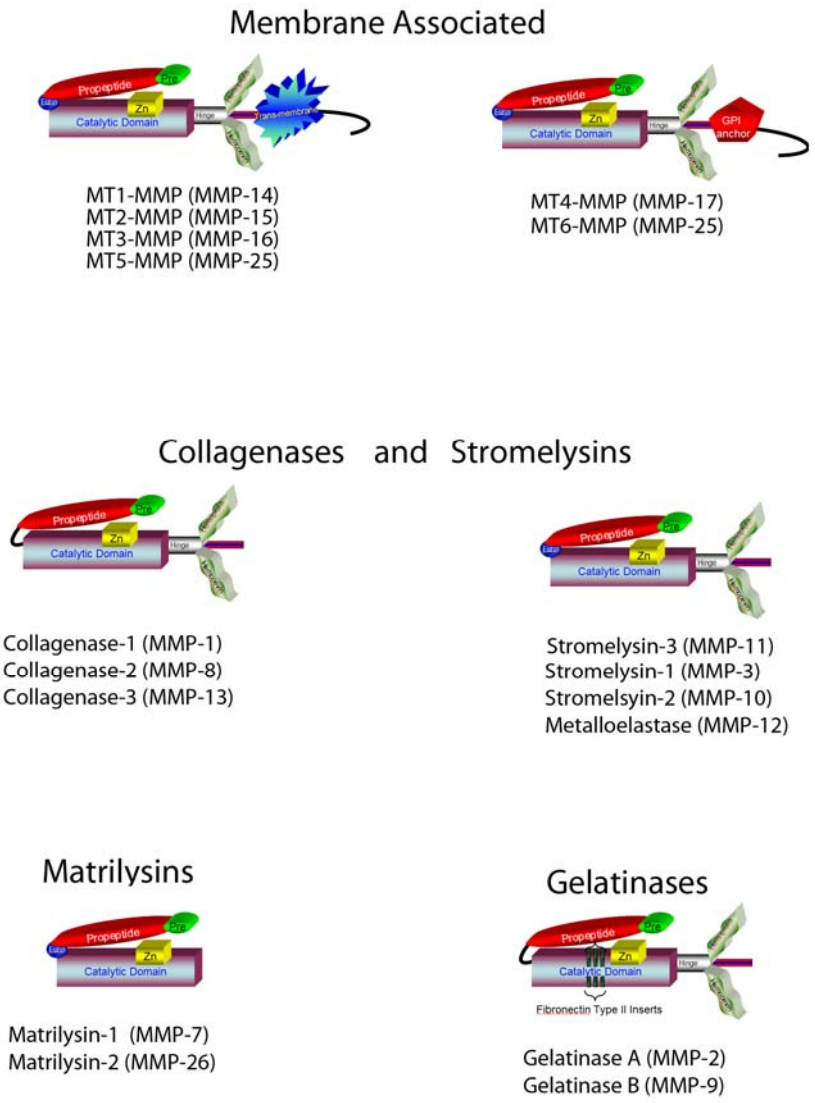

Fig 1.4 MMPs are classified based on their preferred matrix substrate cleavage ability. In addition, a group of MMPs are membrane associated and have roles including docking, receptor and zymogen activation. In general, MMPs have: 1) zinc catalytic domain; 2) a propeptide domain, which maintains latency; as well as 3) a pre-domain which is involved in targeting the zymogen for secretion.

MMP	Substrate Cleaved	Biological Effect
MMP-1	Type I collagen	re-epithelialization
	Fibronectin	cell migration
	IGFBP-3	increase IGF1/cell proliferation
	Processing IL1β from precursor	Pro-inflammatory
	IL1β degradation	Anti-inflammatory
	Monocyte chemoattractant protien3	Anti-inflammatory
	Protease activated receptor 1	PAR1 activation (Boire, Covic et al. 2005)
MMP-2	Chondroitinsulphate proteoglycan	Neurite outgrowth
	Fibronectin	cell migration
	BM40 (SPARC/Osteonectin)	Enhanced collagen affinity
	IGFBP3	increase IGF1/cell proliferation
	Laminin 5γ2 chain	Epithelial cell migration
	IL1β degradation	Anti-inflammatory
	Monocyte chemoatractant protein-3	Anti-inflammatory
	decorin	Increase bioavailability of TGFβ
MMP-3	Fibronectin	cell migration
	Basement membrane	Mammary epithelial cell apoptosis
	E-cadherin	Epithelial-mesenchymal conversion
	Plasminogen	Generation of angiostatin-like fragment
	BM40 (SPARC/Osteonectin)	Enhanced collagen affinity
	Perlecan	Release of bFGF
	IGFBP3	increased bioavailability of IGF1 and cell proliferation
	Processing IL1β from the precursor	Pro-inflammatory
	Monocyte chemoatractant protein-3	Anti-inflammatory
	decorin	Increase bioavailability of TGFβ
MMP-7	Fibronectin	Adipocyte differentiation
	BM40 (SPARC/Osteonectin)	Enhanced collagen affinity
	IGFBP3	increase IGF1/cell proliferation (Miyamoto, Yano et al. 2004)
	decorin	Increase bioavailability of TGFβ
	E-cadherin	Disrupted cell aggregation and increased cell invasion
	Fas ligand	Fas-receptor mediated apoptosis
	Pro-TNFα	Pro-inflammatory (Haro, Crawford et al. 2000)
MMP-9	BM-40 (SPARC/Osteonectin)	Enhanced collagen affinity
	IGFBP3	increase IGF1/cell proliferation
	Processing IL1β from the precursor	Pro-inflammatory
	IL1β degradation	Anti-inflammatory
	IL2Rα	Reduced IL2 response
	precursor of TGFβ	Bioavailability of TGFβ
MT1-MMP	CD44	cell migration
	Type I collagen	Kidney tubulogenesis
	Laminin 5γ2 chain	Epithelial cell migration
	Cell surface tissue transglutaminase	Reduced cell adhesion and spreading
Various MMPs	Type XVIII collagen	Plasminogen Generation of endostatin-like fragment
	CTGF	Activation of VEGF

Table 1.1 Biological activities mediated by MMP cleavage
Note: all references are from (Visse and Nagase 2003) unless otherwise stated.

2.0 CHAPTER TWO:

Matrix Metalloproteinase Inhibitors Suppress TGFβ-Induced Subcapsular Cataract Formation

I have carried out all of the studies outlined in this manuscript with the exception of the Back Vertex Distance Variability. These studies were carried out at the University of Waterloo in Waterloo, Ontario by Alice Banh.

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2.1 INTRODUCTION

Loss of transparency of the lens, or cataract, is the leading cause of blindness worldwide despite the availability of effective surgery in developed countries (McCarty and Taylor 1996). Currently there are no pharmacological agents to prevent the onset or inhibit the progression of cataract formation. Thus, an understanding of the cellular and molecular mechanisms regulating the normal and pathological differentiation of the lens may lead to the development of therapeutic strategies for the treatment and/or prevention of cataracts.

The lens is a relatively simple tissue composed of two cell types, epithelial cells and fiber cells. In the embryo, the lens consists of a highly proliferative monolayer of lens epithelial cells (LECs) covering the anterior half of the lens (Griep and Zhang 2004). At the lens equator these cells are stimulated to terminally differentiate into fiber cells by a gradient concentration of growth factors within the ocular media(Lang and McAvoy 2004). In adults, lens proliferation and differentiation occurs near the lens equator, albeit at a slower rate than in the embryo. However, in a pathological situation such as occurs following ocular trauma, surgery or systemically, as in diseases like atopic dermatitis and retinitis pigmentosa, the anterior LECs can be triggered to proliferate and multilayer beneath the lens capsule (Sasaki, Kojima et al. 1998; Lang and McAvoy 2004). A proportion of these cells also transform into plaques of large "spindle shaped" cells, or myofibroblasts, by a phenomenon known as epithelial-to-mesenchymal transition (EMT) (Font and SA 1974; Novotny and Pau 1984; Hay 1995).

The cytokine transforming growth factor β (TGFβ) has been shown to play a role in lens disease and to specifically induce these aberrant changes in LECs, including their conversion to myofibroblasts(Lang and McAvoy 2004). The resultant myofibroblasts express contractile elements like alpha smooth muscle actin (αSMA), and unlike epithelial cells, cease to produce type IV collagen (a component of the lens capsule) and the highly organized crystallin proteins and begin to secrete an abnormal accumulation of type I and III collagen (Lovicu, Schulz et al. 2002). Additional extracellular matrix proteins are deposited including tenascin and fibronectin and as a result, fibrous ASC plaques are formed that develop into distinct opacities in the lens. Similar to ASC, in secondary cataract, also known as posterior capsular opacification (PCO), LECs which remain within the capsule after cataract surgery are triggered to proliferate and migrate to the posterior lens capsule, where they can transition into myofibroblasts (Kappelhof and Vrensen 1992; Marcantonio, Syam et al. 2003).

Matrix metalloproteinases (MMPs) are a family of zinc endopeptidases that act as key regulators of tissue remodelling and have been shown to participate in a number of ocular diseases including retinal disease, glaucoma, and corneal disorders (Alexander and Werb 1989; Sivak and Fini 2002). MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs) have also been examined in the normal and cataractous lens. Although some MMPs, such as MMP9 and MT1-MMP have been found to be constitutively expressed in the lens, other MMPs such as MMP2 are typically expressed after treatment with growth factors or during cataract formation (Reponen, Sahlberg et al. 1994; Smine and Plantner 1997; Mohan, Rinehart et al. 1998). For example, Seomun and

colleagues reported an induction of *Mmp2* mRNA in a human lens epithelial cell line following treatment with TGFβ as well as in the subcapsular plaques of TGFβ-treated rat lenses (Seomun, Kim et al. 2001). TGFβ has also been shown to stimulate secretion of MMP2 and MMP9 in cultured annular pad cells of the chick lens and in human capsular bags (Richiert and Ireland 1999; Wormstone, Tamiya et al. 2002). Induction of the pro-forms of both MMP2 and MMP9 has also been reported during H_2O_2 induced-cataract formation and after sham cataract surgery (Tamiya, Wormstone et al. 2000). Multiple MMPs and TIMPs were also detected in the extracellular matrix and LECs from human capsules derived from post-cataract-IOL surgery tissue, whereas normal anterior lens capsules did not (Kawashima, Saika et al. 2000).

Evidence that MMP2 may have an active role in mediating the EMT which occurs in ASC has also been provided by findings in which overexpression of MMP2 within the human HLE B-3 lens cell line caused a conversion of the cells into a myofibroblastic phenotype (Seomun, Kim et al. 2001). The ability of other MMPs to induce LEC conversion however, has not been investigated. In addition, the requirement for MMPs in mediating the formation of the TGFβ-induced ASC plaques in the *ex vivo* lens and the mechanism(s) by which MMPs participate in ASC formation are not known. Investigations in other systems, including cancer, have shown that MMPs promote EMT by altering the E-cadherin/β-catenin pathway through a phenomenon known as E-cadherin shedding (Sternlicht, Lochter et al. 1999; Ho, Voura et al. 2001; Mei, Borchert et al. 2002; George and Dwivedi 2004). Specifically, the association between E-cadherin and β-catenin is vulnerable to enzymatic attack by multiple MMPs, including MMP9 and

MMP2, resulting in the formation of E-cadherin extracellular domain fragments with reported sizes ranging from 50 to 84 kDa (Hirai, Migita et al. 2001; Noe, Fingleton et al. 2001; Mei, Borchert et al. 2002). Induction of MMPs by TGFβ in the lens may lead to the EMT of LECs through a specific disruption in E-cadherin.

In the current study we directly tested the requirement of MMPs in ASC formation using a well established *ex vivo* rat lens model in conjunction with two different MMP inhibitors (MMPIs), the broad spectrum inhibitor GM6001 and a MMP2/9 specific inhibitor (MMPI2/9) (Hales, Chamberlain et al. 1995). Additional experiments examined the capacity of these MMPIs to prevent decreases in optical quality of the lens induced by TGFβ and to affect the cell adhesion molecule E-cadherin. Together these findings demonstrate that MMPs participate in TGFβ-induced E-cadherin shedding and degradation in the lens and that suppression of this phenomenon by an MMPI is the likely mechanism by which MMPIs inhibit ASC formation.

2.2 RESULTS

2.2.1 MMPIs Suppress Anterior Subcapsular Cataract Formation

In the following experiments, the broad spectrum MMP inhibitor GM6001 was used in the rat subcapsular cataract model to determine whether it could effectively suppress TGFβ–2 induced subcapsular cataract formation. To perform these experiments, excised rat lenses were treated with exogenous TGFβ2 for a period of 6 days. The TGFβ2 treated lenses exhibited multiple, distinct opacities on the anterior surface of the lens, as previously described (Fig 2.1B) (Hales, Chamberlain et al. 1995; Lovicu, Steven et al. 2004), whereas the untreated control lenses remained transparent and devoid of opacities (Fig 2.1A). In comparison with lenses treated with TGFβ–2 alone, lenses co-cultured with TGFβ2 and GM6001 (25μM) for the 6-day period did not exhibit visible subcapsular cataracts (Fig 2.1C) but resembled the control lenses. Histological cross-sections of the lenses treated with TGFβ2 revealed the presence of numerous plaques consisting of a multilayering of cells beneath the lens capsule (Fig 2.1F) in contrast to the simple cuboidal monolayer of epithelial cells observed in the control lens (Fig 2.1E). Strong immunoreactivity of αSMA was observed in a proportion of the cells of the subcapsular plaques in the TGFβ2 treated lenses (Fig 2.1F). In comparison, the lenses co-cultured with TGFβ2 and GM6001 (25μM) did not exhibit multilayering of the lens epithelium and no αSMA immunoreactivity was observed under the same immunolocalization conditions as outlined above (Fig 2.1G).

In parallel with the lenses above, lenses were co-treated with TGFβ2 and the specific MMP2/9 inhibitor at concentrations of 10 or 25μM for 6 days. Similar to the findings for GM6001, these studies revealed that co-treatment with the MMP2/9 inhibitor suppressed the appearance of ASC plaques and αSMA expression. Lenses co-treated with TGFβ2 and the MMP2/9 specific inhibitor (10μM) exhibited slight multilayering of the epithelium (two layers observed) in some regions of the lens with very faint αSMA immunoreactivity (data not shown). However, lenses co-treated with TGFβ2 and the MMP2/9 inhibitor (25μM) resembled control lenses and did not exhibit αSMA immunoreactivity (Fig 2.1, D and H). It should be noted that lenses treated with each of the MMPIs, in the absence of TGFβ2, resembled the control lenses (not shown).

A laser scanning system (ScanTox) was next used to determine quantitative differences in the optical quality of the lenses from three different treatment groups (control, TGFβ–2, and TGFβ–2 plus 25μM GM6001) at three time points: days 2, 4, and 6. Analyses of back vertex distance (BVD) errors showed that there was both a treatment and temporal effect of TGFβ–2 on the cultured rat lens (Fig 2.2). Although the BVD errors for the control lenses did not change significantly from day 0 to 6, treatment with either 1 or 2ng/mL of TGFβ–2 caused a significant increase in BVD error by day 6 of the time course. In contrast, the groups of lenses co-treated with TGFβ–2 (at 1 and 2ng/mL) and GM6001 (25μM) did not exhibit a significant change in BVD error over the 6-day period. Statistical differences between the treatment groups at each time point were also determined. At days 0, 2, and 4, no significant difference in BVD error was observed

between all groups (Fig 2.2). However, at day 6, both of the TGFβ–2 treated groups of lenses (1 and 2ng/mL) exhibited a significantly larger BVD error versus the control group and the GM6001 co-treated lenses (Fig 2.2). Lenses cultured with the inhibitor alone did not exhibit changes in BVD error relative to untreated lenses (not shown).

A dose-dependent effect of the broad spectrum inhibitor GM6001 in preventing the TGFβ–2 induced cataracts was also observed. Similar to earlier experiments, treatment with TGFβ–2 for 6 days resulted in BVD errors that were statistically greater than control lenses. Similarly, those lenses co-treated with TGFβ–2 and GM6001 (10 or 15μM) significantly differed from controls and exhibited cataracts. In contrast, lenses co-treated with higher concentrations of GM6001, such as 20 and 25μM, exhibited BVD errors that were very similar to the controls, further demonstrating the dramatic suppression in cataract formation (Fig 2.3). Treatment with an analog of GM6001, with no MMP inhibitory activity (negative control), exhibited a similar BVD error to the TGFβ–2 treated lens, indicating that the effect of GM6001 on maintaining lens optical quality was related to its MMP inhibitory activity.

2.2.2 TGFβ-induced ASC Formation is Accompanied by Enhanced Secretion of MMP2 and MMP9

To examine the timing and level of induction of MMPs in the rat lens after TGFβ2 treatment and subcapsular cataract formation, zymography was performed on conditioned media of lenses taken at the 6-day time point from the following treatment groups:

control, TGFβ2 (2ng/mL), TGFβ2 (2ng/mL) plus GM6001 (25μM), and TGFβ2 (2 ng/mL) plus the MMP2/9 specific inhibitor (25μM). Conditioned media from all treatment groups exhibited distinct bands on gelatin gels, indicating the presence of MMPs with gelatinolytic and/or collagenolytic activity (Fig 2.4A). Conditioned media from control lenses exhibited expression of a 92-kDa band, corresponding to the proform of MMP9 (Alexander and Werb 1989). In comparison with control lens media, media from lenses treated with TGFβ2 exhibited additional bands of 62, 65, and 72 kDa, corresponding to the active and proforms of MMP2 (Alexander and Werb 1989); MMP9 levels were also increased. Media obtained from lenses co-treated with TGFβ2 and either GM6001 or MMP2/9 inhibitor for 6 days exhibited reduced levels of all gelatinolytic bands relative to that of TGFβ2-treated lenses (Fig 2.4A).

Confirmation and quantification of MMP2 and MMP9 from all treatment groups at each time period was performed using Western blot analysis. Blots developed with an MMP2-specific antibody revealed the presence of latent and active species of MMP2 in conditioned media from lenses treated with TGFβ2, whereas the control lenses at all time points did not exhibit detectable levels of MMP2 protein (Fig 2.4B). In comparison, conditioned media from the lenses co-cultured with TGFβ2 plus GM6001 or TGFβ2 plus the MMP2/9 inhibitor showed undetectable levels of MMP2 similar to control lenses (Fig 2.4B). Blots probed with the MMP9 specific antibody revealed a band at 92kDa, corresponding to the proform of MMP9 (Fig 2.4B). Similar to the zymography results, constitutive MMP9 protein expression was evident in the conditioned media of control lenses. In comparison, media from lenses treated with TGFβ2 exhibited significantly

higher levels of MMP9 after 2, 4, and 6 days of treatment (Fig 2.4, B and C). Co-treatment with TGFβ2 and GM6001 revealed significant attenuation of MMP9 in conditioned media relative to TGFβ2 treatment alone at all three time points, whereas conditioned media from co-treatment of TGFβ2 and the specific MMP2/9 inhibitor showed a significant attenuation of MMP9 at days 2 and 4 only (Fig 2.4, B and C). It should be noted that lenses treated with either MMPI alone exhibited levels of MMP9 and MMP2 similar to that of control lenses (data not shown).

2.2.3 MMPIs Attenuate TGFβ-Induced E-cadherin Shedding in the Rat Lens

Because previous studies had shown the ability of MMPs to induce E-cadherin shedding, the following experiments were designed to determine whether TGFβ2 treatment of the rat lens results in an induction of E-cadherin shedding and whether this can be modulated by the MMPIs (Ho, Voura et al. 2001; Mei, Borchert et al. 2002; George and Dwivedi 2004). To accomplish this, previously concentrated conditioned media were obtained from the 2-, 4-, and 6-day treatment groups outlined earlier and subjected to Western blot analysis using an antibody specific for the extracellular domain of E-cadherin, which has been used in previous studies to detect the presence of soluble E-cadherin fragments(Banks, Porter et al. 1995; Noe, Fingleton et al. 2001). An E-cadherin fragment of approximately 72kDa was detected in the conditioned media from lenses treated with TGFβ2 for 6 days and was not observed in media from untreated lenses (Fig 2.5A). Media from lenses treated with TGFβ2 for shorter time periods (days 2 and 4) did not exhibit detectable levels of the fragment (not shown). Importantly, a

significant two-fold reduction in the levels of this fragment was detected in media from lenses co-treated with TGFβ2 and the broad spectrum inhibitor GM6001, whereas levels were undetectable in media from lenses co-treated with TGFβ2 and the MMP2/9 inhibitor (Fig 2.5A). Lenses treated with either of the MMPIs alone also exhibited undetectable levels of the E-cadherin fragment (data not shown). Thus, co-culture of lenses with either of the MMPIs tested resulted in attenuated levels of the TGFβ2-induced E-cadherin fragment.

MMP inhibitors have been shown to augment cell-cell adhesion and specifically increase expression of cadherins (Ho, Voura et al. 2001). We therefore examined the levels of *E-cadherin* mRNA using RT-QPCR. After 6 days of culture, we examined epithelial cells or plaque cells of lenses from the following treatment groups: control lenses, lenses treated with TGFβ2 (2ng/mL), TGFβ2 (2ng/mL) plus GM6001 (25μM), or GM6001 (25μM) alone. For these experiments, cryostat sections of lenses were subjected to laser capture microdissection (LCM) to specifically isolate the cells in the plaques. RT-QPCR findings revealed that whereas *E-cadherin* mRNA was detected in the normal lens epithelium (Fig 2.5B), its expression, relative to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)*, was suppressed nearly fourfold in the plaque tissue of TGFβ2 treated lenses. In comparison, *E-cadherin* levels in cells from the epithelial region of lenses co-treated with TGFβ2 and GM6001 (25μM) were significantly higher (6.9-fold) than those treated with TGFβ2 alone and significantly greater than that of controls. Treatment with 25μM GM6001 alone also produced a significant increase in *E-cadherin* when compared with control lenses ($P < 0.01$) (2.5-fold). When these experiments were

performed using the *18S* housekeeping gene, data exhibited the same trends seen with *GAPDH* (not shown). Thus, treatment of lenses with GM6001 prevented the attenuation of *E-cadherin* mRNA induced by TGFβ2 and further increased the constitutive levels of *E-cadherin* mRNA.

For comparison, we also examined the levels of α*SMA* mRNA in the same tissues used to perform the *E-cadherin* studies (Fig 2.5C). These experiments revealed little to no expression of α*SMA* mRNA in the cells obtained from the epithelial region of control lenses whereas its expression, relative to that of *GAPDH*, was significantly induced in the plaque tissue of TGFβ2 treated lenses. This corresponds with our earlier experiments, which showed increased αSMA protein immunoreactivity in the subcapsular plaques (Fig 2.1F). α*SMA* mRNA levels in the LECs co-treated with the MMPI were substantially reduced compared with the plaque cells of TGFβ2-treated lenses, demonstrating that co-culture with the MMPI resulted in suppression in induction of α*SMA* by TGFβ2. Addition of GM6001 (25μM) alone to lenses did not alter α*SMA* mRNA levels when compared with those of control lenses (Fig 2.5C).

2.3 DISCUSSION

Increasing evidence regarding the importance of MMPs in development and pathology has accumulated over the last 10 years. In the eye, MMPs have been shown to contribute to a number of ocular diseases and disorders including retinal disease, glaucoma, corneal ulcers and corneal postoperative haze(Sivak and Fini 2002). More recently, induction of MMP expression has been correlated with the formation of cataracts (Tamiya, Wormstone et al. 2000; Seomun, Kim et al. 2001; Wormstone, Tamiya et al. 2002). However, the role of MMPs and the mechanism by which they contribute to cataractogenesis are not well understood. In the current study, we used the previously established *ex vivo* rat lens subcapsular cataract model to directly test the involvement of MMPs in the initiation and progression of TGFβ2 induced ASC. We demonstrated that co-treatment with TGFβ2 and two different MMPIs, the broad spectrum inhibitor GM6001 and the MMP2/9-specific inhibitor, resulted in suppression of TGFβ2 induced ASC formation, including the appearance of αSMA expressing cells indicative of the EMT of LECs. Further evidence is provided to show that treatment of the rat lens with TGFβ2 resulted in appearance of proteolytic fragments of the cell-cell adhesion molecule E-cadherin, an event that was attenuated by co-treatment with either of the two MMPIs tested. Together, these data suggest that the suppression of TGFβ2 mediated E-cadherin shedding and degradation is a possible mechanism by which MMPIs reduce the appearance of ASC plaques.

Earlier work has shown that *Mmp2* expression is induced in the rat lens after treatment with TGFβ (Seomun, Kim et al. 2001). Furthermore, overexpression of *Mmp2* via stable transfection results in conversion of cultured human LECs into αSMA expressing cells (Seomun, Kim et al. 2001). Similar to these findings, we also report induced secretion of MMP2 in the rat lens after treatment with TGFβ2, accompanied by enhanced secretion of MMP9. A recent study revealed that *Mmp9* and *Mmp2* mRNA are expressed in the normal rat lens epithelium (John, Jaworski et al. 2004). This suggests that constitutive mRNA expression of these MMPs does not result in the EMT of LECs and cataract formation. However, induced levels of *Mmp2* and/or *Mmp9*, above those of constitutive expression, and accompanying secretion of MMPs could result in EMT in the lens. Induced levels of MMP2 and membrane-type 1-MMP have been correlated with EMT in the embryonic heart, and overexpression of MMP3 has been shown to cause conversion of mammary epithelial cells into mesenchymal cells (Sternlicht, Lochter et al. 1999; Song, Jackson et al. 2000). The fact that co-treatment with GM6001 and the specific MMP2/9 inhibitor resulted in suppression of TGFβ2 induced αSMA expression in the rat lens further corroborates these earlier findings and suggests that MMPs are important for mediating the EMT of LECs. We further show that treatment with MMPIs led to a substantial reduction in subsequent plaque formation in the *ex vivo* lens and maintenance of lens optical quality. Because MMP2 and MMP9 were induced after TGFβ2 treatment and the MMPIs used have inhibitory activity against both, it cannot be discerned whether one or both of these MMPs participate in ASC formation. Future studies that target the individual expression of MMP2 or MMP9, such as through gene

knockdown experiments or the use of specific *Mmp2* and *Mmp9* knockout mouse models, will help to further determine whether one or both of these MMPs is critical for ASC formation.

MMPs are principally known for their role in ECM remodelling (Alexander and Werb 1989). However, additional roles for MMPs have emerged, including their ability to regulate cell migration, invasion, and EMT (Sternlicht, Lochter et al. 1999; Song, Jackson et al. 2000). It has been suggested that MMPs may contribute to EMT by participating in the separation of epithelial cells from their basement membrane, thereby promoting their migration during cellular transformation (Hay 1995; Brown, Boyer et al. 1999; Steinhusen, Weiske et al. 2001). However, more recent findings suggest that MMPs participate in the initial activation stages of EMT through dissociation of the E-cadherin/β-catenin complex (Ho, Voura et al. 2001; Mei, Borchert et al. 2002; George and Dwivedi 2004). Proteolytic cleavage of the N-terminal extracellular domain of E-cadherin by MMPs, referred to as "E-cadherin shedding," results in the formation of an E-cadherin extracellular domain fragment with reported sizes ranging from 50 to 84kDa, compared with the intact 120kDa protein (Steinhusen, Weiske et al. 2001). Here, we report the appearance of a 72kDa E-cadherin fragment in the conditioned media of lenses treated with TGFβ2 that was not detected in the media from untreated lenses. To the best of our knowledge, this is the first report of E-cadherin shedding in a cataract model. The appearance of the E-cadherin fragment in the TGFβ2 treated rat lenses was also shown to coincide with enhanced levels of secreted MMP2 and MMP9, MMPs that have been implicated in E-cadherin shedding and degradation in other systems (Mei, Borchert et al.

2002; Nawrocki-Raby, Gilles et al. 2003). We further demonstrated that the TGFβ2 induced levels of the E-cadherin fragment were attenuated by co-treatment with either MMPI, broad spectrum GM6001, or MMP2/9 inhibitor, suggesting that TGFβ2 induced fragmentation of E-cadherin is mediated by MMP activity. Interestingly, the ability of GM6001 to suppress E-cadherin shedding has been shown in nitric oxide-treated murine colonic epithelial cells, and this led to further stabilization of E-cadherin junctions (Mei, Borchert et al. 2002; George and Dwivedi 2004).

Along with E-cadherin shedding, we also reported a significant decrease in the *E-cadherin* mRNA levels in the subcapsular plaque tissue of TGFβ2 treated lenses. Decreased *E-cadherin* expression has also been reported after dexamethasone-induced cataract formation in the cultured rat lens; however, evidence of E-cadherin shedding in this cataract model was not investigated (Lyu, Kim et al. 2003). Cadherins are known to act as cell signaling receptors by controlling the localization of β-catenin. Proteolytic shedding of E-cadherin causes dissociation of β-catenin from membrane-bound E-cadherin, resulting in increases in the levels of free β-catenin in the cytoplasm (Mei, Borchert et al. 2002). β-catenin can then localize to the nucleus where it associates with the T-cell factor/lymphoid enhancer factor(s) to activate or repress target gene expression (George and Dwivedi 2004). Thus, the changes in *E-cadherin* and α*SMA* mRNA levels that we observed may be associated with changes in β-catenin localization. The levels of *E-cadherin* mRNA in lenses co-treated with TGFβ2 and GM6001 were found to be well above that of control lenses, and treatment of lenses with GM6001 alone also resulted in a substantial elevation in the constitutive amount of *E-cadherin* mRNA. These findings

suggest that *E-cadherin* mRNA expression was either stimulated or stabilized by GM6001.

The findings of the current study also revealed that treatment with the broad spectrum inhibitor GM6001 suppressed TGFβ2 induced levels of both MMP2 and MMP9 secreted protein, whereas the MMP2/9 inhibitor specifically blocked MMP2 induced levels. The primary function of both MMPIs is to inhibit MMP enzymatic activity. However, MMPIs such as GM6001 have been shown to have an inhibitory effect on MMP expression in other systems, yet this has remained unexplained (Lei, Vieira et al. 2002; Kerkvliet, Jansen et al. 2003). Previous work has shown that the myofibroblast-like cells in the subcapsular plaques of rat lenses treated with TGFβ2 exhibited immunoreactivity for both αSMA and MMP2 (Seomun, Kim et al. 2001). Thus, the absence of MMP2-secreted protein in the media from lenses co-treated with either of the two MMPIs may simply be due to the fact that both MMPIs suppress the appearance of myofibroblasts, the cell type that expresses MMP2.

In summary, the findings of the current study have shown that treatment with the broad spectrum MMP inhibitor GM6001 and the specific MMP2/9 inhibitor significantly suppressed the formation of TGFβ2 induced ASC plaques in the rat lens. This suppression was associated with decreased αSMA expression and a significant decrease in the 72kDa soluble E-cadherin fragment, indicative of E-cadherin shedding. Together, these data suggest the novel finding that MMPIs suppress the EMT of LECs in ASC formation through inhibition in MMP-mediated disruption of E-cadherin. Based on these findings,

two possible therapeutic strategies for the prevention of ASC formation can be proposed: inhibition of MMP activity and/or attenuation of aberrant E-cadherin shedding.

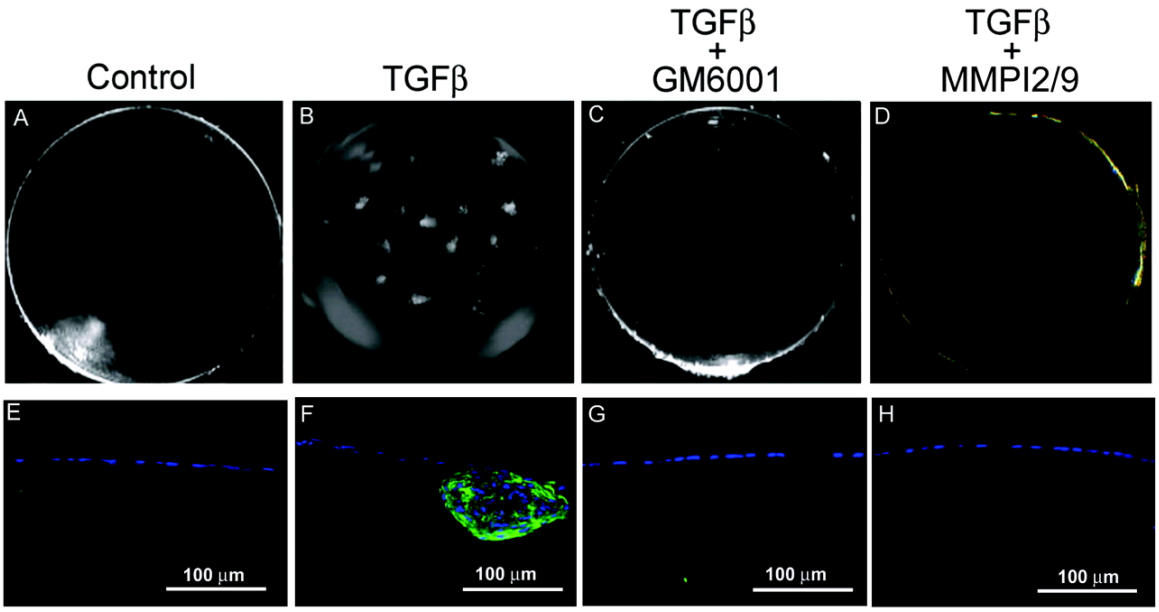

Fig 2.1 TGFβ2 induced anterior subcapsular plaque formation in the rat lens is inhibited by co-culture with GM6001 and specific MMP2/9 inhibitor. An untreated control lens (**A**), a lens treated with TGFβ2 (2ng/mL) (**B**), a lens co-cultured with TGFβ2 (2ng/mL) and GM6001 (25μM) (**C**), and a lens co-cultured with TGFβ2 (2ng/mL) and MMPI2/9 (25μM) (**D**) are shown after 6 days of culture. The TGFβ2 (2ng/mL)-treated lens (**B**) exhibited distinct subcapsular plaques unlike the untreated lens (**A**) or those co-cultured with GM6001 (25μM) (**C**) and MMPI2/9 (25μM) (**D**), which remained devoid of opacities. Immunolocalization of αSMA in cross-sections of lenses revealed strong immunoreactivity of αSMA (green) in sections of lenses treated with TGFβ2 (2ng/mL) (**F**), confirming the presence of subcapsular plaques. Control lenses (**E**), lenses co-cultured with TGFβ2 (2ng/mL) and GM6001 (25μM) (**G**), and lenses co-cultured with TGFβ2 (2ng/mL) and MMPI2/9 (25μM) (**H**) showed no observable immunoreactivity to αSMA. All sections were mounted in a medium with 4',6-diaminodino-2-phenylindol to co-localize the nuclei (blue). Scale bars = 100 μm.

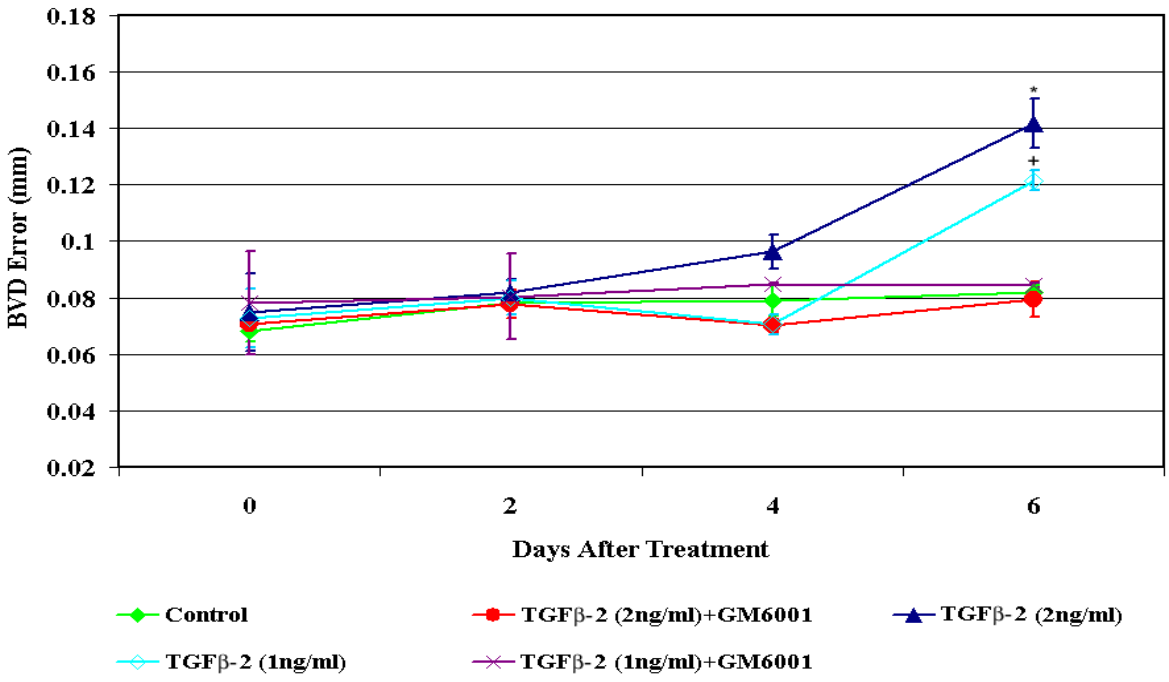

Fig 2.2 Back vertex variability for lenses left untreated, treated with TGFβ2, and treated with TGFβ2 and GM6001. The graph shows the changes in back vertex variability (BVD error; mm ± SEM) from day 0 (initial measurements before treatment) and 2, 4, and 6 days after treatment. Two concentrations of TGFβ2 (1 and 2ng/mL) were used in this experiment. Repeated-measures analysis of variance demonstrated that there are both treatment and temporal effects ($P \leq 0.05$). At day 6 after treatment, both concentrations of TGFβ2 ($^{+}$1 and *2ng/mL) showed significantly higher BVD error measurements than other treatment groups, including the control and the TGFβ2 (1ng/mL), GM6001 and TGFβ2 (2ng/mL), and GM6001 groups. Apparent differences between the TGFβ2 (1ng/mL) and TGFβ2 (2ng/mL) groups did not reach statistical significance.

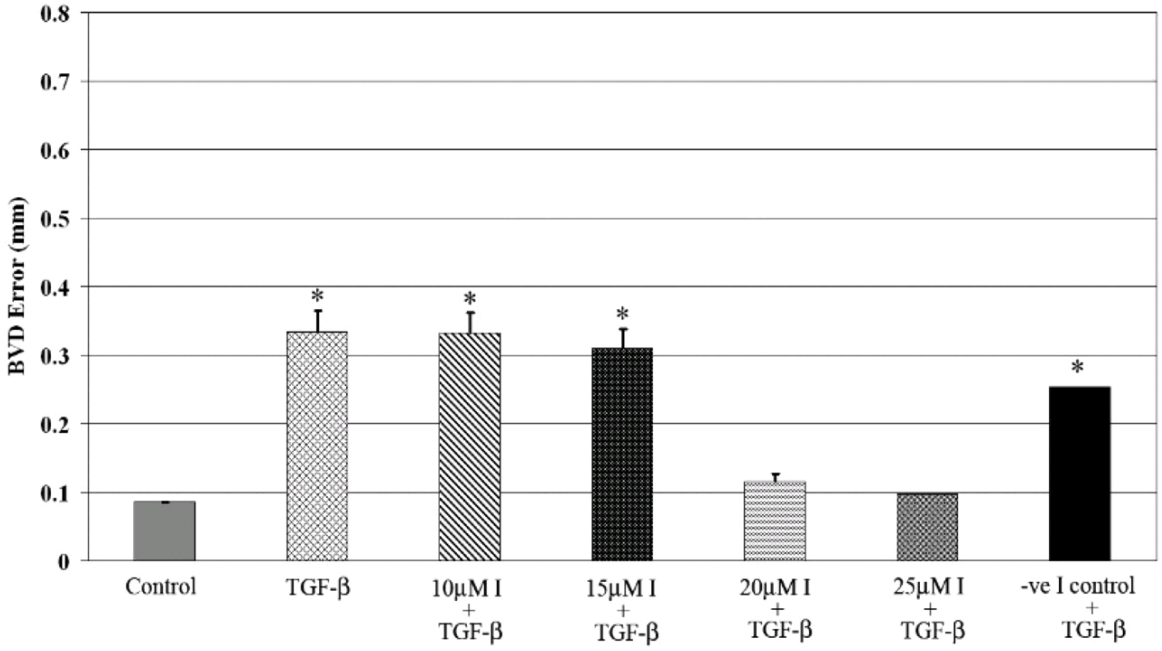

Fig 2.3 Dosage effect of GM6001 on TGFβ2 induced BVD variability. This bar graph represents the back vertex variability (BVD error, mm) ± SEM of lenses left untreated or treated with TGFβ2 (2ng/mL) or TGFβ2 (2ng/mL) plus four different concentrations of GM6001 (I) as indicated for 6 days. These measurements show a decrease in BVD error as the GM6001 concentration increases to 25μM. One-way analysis of variance was used to determine the dosage effect of GM6001 on TGFβ2 treated lenses ($P \leq 0.05$). BVD errors from TGFβ2 treated lenses co-treated with 20 or 25μM GM6001 were not significantly different from the control lenses. In contrast, lenses treated with TGFβ2 (2ng/mL) alone or with TGFβ2 (2ng/mL) plus GM6001 at 10 and 15μM had BVD errors that were significantly different from control lenses, as indicated by **asterisks**. Lenses treated with TGFβ2 and the negative control for GM6001 (25μM) also had BVD errors that were significantly different from the controls.

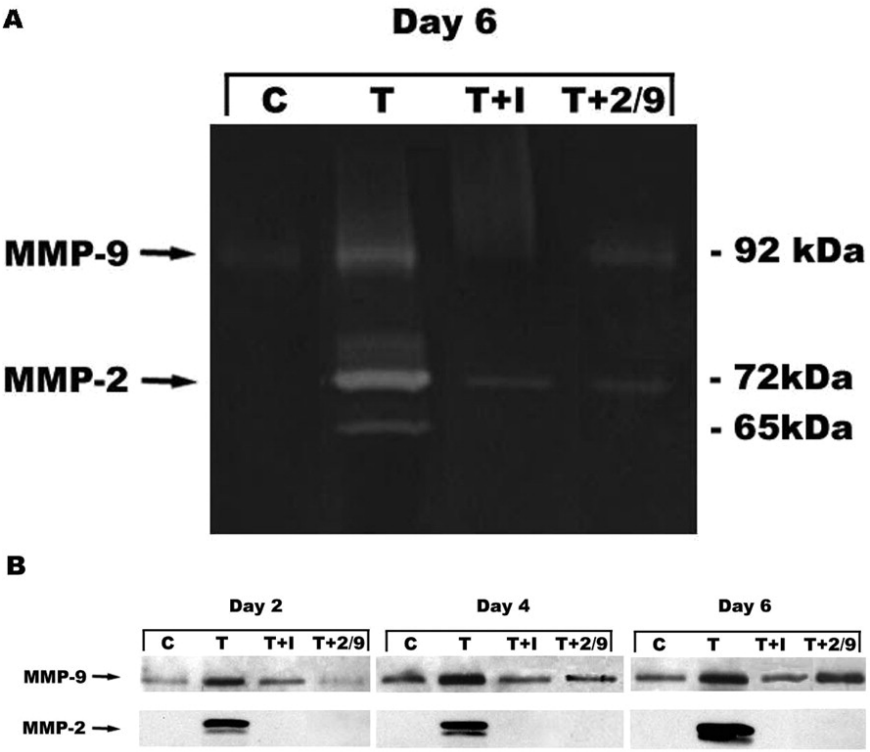
A
Day 6
C
T
T+I
T+2/9
MMP-9
MMP-2
- 92 kDa
- 72kDa
- 65kDa
B
Day 2
Day 4
Day 6
C
T
T+I
T+2/9
MMP-9
MMP-2
C
MMP-9
RD/C Ratio
3
2
1
0
Day 2
Day 4
Day 6
NS
C
T
T+I
T + 2/9
*P<0.05
NS = Non Significant

Fig 2.4 Effect of TGFβ2 and MMPIs on MMP protein levels. **A:** Gelatin zymography of conditioned media from cultured lenses after 6 days of treatment. MMP9 (92kDa) was detected in media from all of the untreated (C) lenses at day 6 of the culture period. After treatment with TGFβ2 (2ng/mL) (T), up-regulated levels of MMP9 were observed relative to controls. Conditioned media obtained from lenses treated with TGFβ2 (2ng/mL) and GM6001 (25μM) (T+I) exhibited attenuated bands and resembled levels secreted by control lenses. However, conditioned media obtained from lenses co-cultured with TGFβ2 (2ng/mL) and MMPI2/9 (25μM) (T+2/9) exhibited up-regulated levels of MMP9, relative to controls. Gelatinolytic bands for proMMP2 (72kDa) and active MMP2 (65kDa) were observed in conditioned media from the TGFβ2 treated (2ng/mL) (T) lenses but not in control (C) lenses. Media from TGFβ2 (2ng/mL) and GM6001 (25μM) (T+I) and lenses co-cultured with TGFβ2 (2ng/mL) and MMPI2/9 (25μM) (T+2/9) exhibited negligible activity for MMP2. **B:** Representative Western blot. Conditioned media obtained from control lenses (C) and lenses treated with TGFβ2 (2ng/mL) (T), TGFβ2 (2ng/mL) and GM6001 (25μM) (T+I), and TGFβ2 (2ng/mL) and MMPI2/9 (25μM) (T+2/9) at 2, 4, and 6 days were examined by Western blot analysis to confirm the identity of MMP2 and MMP9. A representative blot demonstrates that MMP9 was constitutively expressed in all of the control lenses (C) over 2, 4, and 6 days. An up-regulation in MMP9 protein was observed in media from lenses treated with TGFβ2 (2ng/mL) (T) at day-2, -4, and -6 time points compared with control lenses (C), whereas the lenses co-cultured with TGFβ2 and GM6001 (25μM) (T+I) exhibited levels similar to the controls at all three time points. Lenses co-cultured with TGFβ2 (2ng/mL) and MMPI2/9 (25μM) (T+2/9) exhibited an up-regulation in MMP9 protein at day 6 compared with control lenses (C), whereas at day-2 and -4 time points, the lenses exhibited levels similar to the controls. At all three time points, MMP2 protein was only detected in media from lenses treated with TGFβ2 (2ng/mL) (T), whereas the other treatment groups did not exhibit detectable levels. **C:** Densitometric analysis of MMP9 protein levels in conditioned media. The Western blot data for MMP9 from three separate experiments were analyzed by densitometry. Values are expressed as the relative density versus control ratio (RD/C) ± SEM of three blots. Note a significant up-regulation (*$P <$ 0.05) of MMP9 in the conditioned media of TGFβ2 (2ng/mL) (T)-treated lenses compared with control. A significant reduction (*$P < 0.05$) was observed in the expression of MMP9 in conditioned media of lenses co-treated with TGFβ2 (2ng/mL) and GM6001 (25μM) (T+I) compared with TGFβ2 (2ng/mL) (T). In the conditioned media of lenses co-cultured with TGFβ2 (2ng/mL) and MMPI2/9 (25μM) (T+2/9), a significant reduction (*$P < 0.05$) was observed in the expression of MMP9 at day-2 and -4 time points compared with TGFβ2 (2ng/mL) (T), whereas there was no significant reduction at day 6 compared with TGFβ2 (2ng/mL) (T).

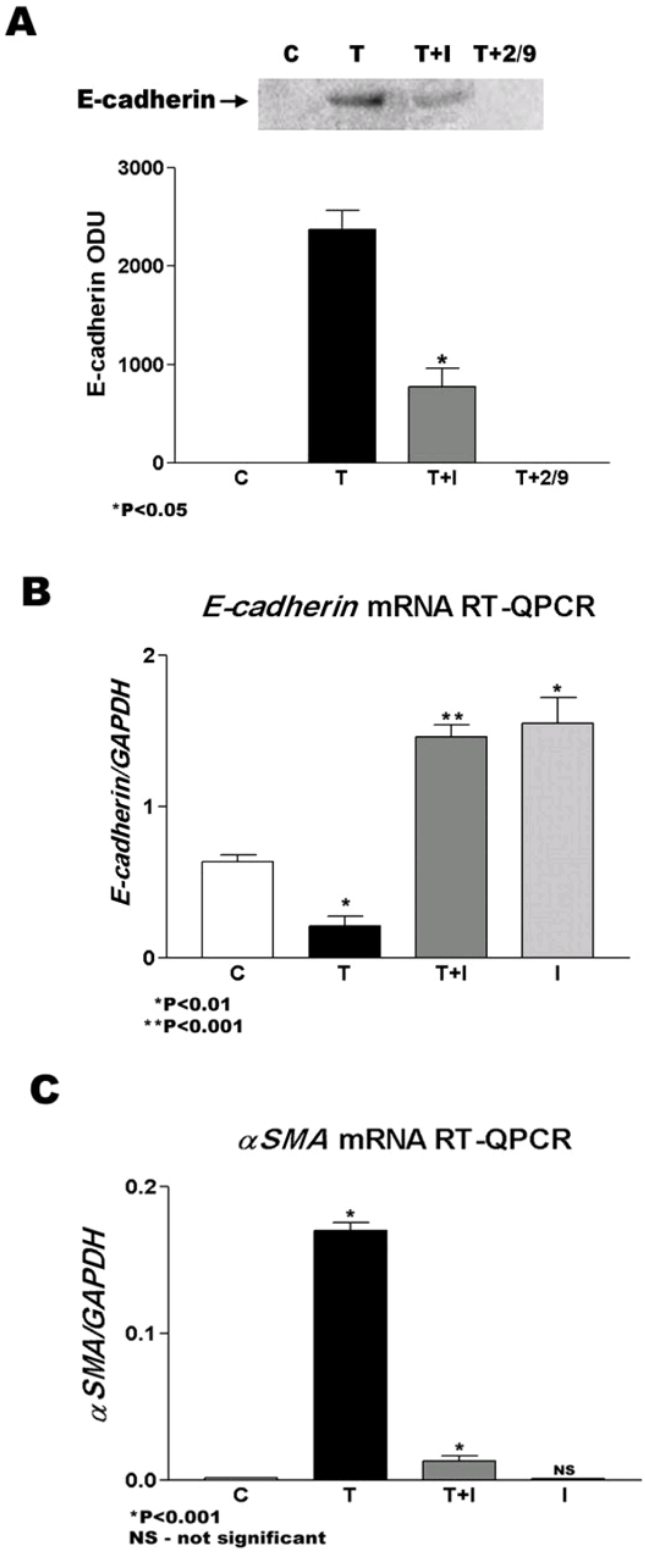
A
C
T
T+I
T+2/9
E-cadherin
3000
2000
1000
0
E-cadherin ODU
C
T
T+I
T+2/9
*P<0.05
B
E-cadherin mRNA RT-QPCR
2
1
0
E-cadherin/GAPDH
C
T
T+I
I
*P<0.01
**P<0.001
C
αSMA mRNA RT-QPCR
0.2
0.1
0.0
αSMA/GAPDH
C
T
T+I
I
NS
*P<0.001
NS - not significant

Fig 2.5 Effect of TGFβ2 and GM6001 on *αSMA* and *E-cadherin* mRNA expression and E-cadherin shedding. **A:** Representative Western blot and densitometric analysis of E-cadherin protein released into conditioned media. The Western blot revealed the presence of a 72-kDa E-cadherin fragment in the conditioned media from lenses treated with TGFβ2 (2ng/mL) (T) that was not detected in media from control lenses (C) and lenses co-treated with TGFβ2 (2ng/mL) and MMPI2/9 (25μM) (T+2/9). Levels of the E-cadherin fragment were suppressed in media from lenses co-cultured with TGFβ2 and GM6001 (25μM) (T+I), relative to that from TGFβ2 treated lenses. Values are expressed as optical density units (ODU) ± SEM of three blots. Note the presence of E-cadherin fragment in conditioned media of TGFβ2 treated (2ng/mL) (T) lenses compared with control (C). A significant (*$P < 0.05$) reduction in the levels of the E-cadherin fragment was observed in media from lenses co-treated with TGFβ2 (2ng/mL) and GM6001 (25μM) compared with TGFβ2 (T). **B:** *E-cadherin* mRNA expression using RT-QPCR. *E-cadherin* mRNA levels, relative to *GAPDH*, were also measured for the lenses treated in **A** using RT-QPCR, and the values are given as ±SEM of three separate experiments. All comparisons were two tailed. The RT-QPCR results revealed that cataractous plaques derived from lenses treated with TGFβ2 (2ng/mL) (T) for 6 days exhibited a significant suppression (*$P < 0.01$) in *E-cadherin* mRNA levels compared with epithelial cells from control lenses (C). Lenses co-cultured with TGFβ2 and GM6001 (25μM) (T+I) exhibited significantly higher levels (**$P < 0.001$) of *E-cadherin* compared with those treated with TGFβ2 (2ng/mL) (T) (nearly sevenfold). The GM6001 (25μM) alone group (I) exhibited significantly elevated levels of *E-cadherin* relative to control (C) lenses (*$P < 0.01$). **C:** α*SMA* mRNA expression using RT-QPCR. The α*SMA* mRNA levels were measured relative to *GAPDH*, and the values are given as ±SEM. All comparisons were two tailed. The RT-QPCR results revealed that the control lens epithelium (C) expressed minimal levels of α*SMA* mRNA. The cells isolated from the cataractous plaques of the TGFβ2 treated (2ng/mL) (T) lenses expressed significantly higher levels (*$P < 0.001$) of α*SMA* mRNA compared with epithelial cells from control lenses (C). Lens epithelium from lenses co-cultured with TGFβ2 (2ng/mL) and GM6001 (25μM) (T+I) exhibited significantly less (*$P < 0.001$) α*SMA* mRNA compared with those treated with TGFβ2 (2ng/mL) (T). Lenses treated with GM6001 alone (25μM) did not exhibit statistically significant elevated levels of α*SMA* when compared with control lenses (C).

2.4 MATERIALS AND METHODS

2.4.1 Ex-vivo rat lens cataract model

The previously established TGFβ-induced rat lens model was utilized for these studies. Briefly, lenses were obtained from adult male Wistar rats and cultured in 3.5ml of serum free M199 medium supplemented with 50 IU/mL penicillin, 50μg/mL streptomycin and 2.5μg/mL fungizone (Amersham Biosciences) overnight. The following day lenses were either left untreated or treated with TGFβ2 (n=8) (R&D Systems) at a final concentration of 1 or 2ng/mL. Lenses were also treated with TGFβ2 and the MMP inhibitor, GM6001 (Ilomostat) (Chemicon International) (n=8) at a concentration of 25μM or the MMP -2/-9 inhibitor ((2R)-[(4-Biphenylylsulfonyl)amino] –N–hydroxyl–3-phenylpropionamide) (Calbiochem) (n=5) at a concentrations of 10 and 25μM or the GM6001 negative control (N-t-Butoxycarbonyl-L-leucyl-L-tryptophan Methylamide) (Calbiochem) (n=3) at 25μM. Lenses were then harvested at subsequent time-points of 2, 4 or 6 days. The lenses were then photographed using a digital camera mounted to a dissecting microscope and either fixed for histology and immunofluorescence, or used for optical analysis. The conditioned media was also collected from each treatment group for zymography and western blot analysis.

2.4.2 Optical Analysis

Lens optical qualities (BVD) and sharpness of focus (BVD error) were assessed using the automated laser scanning system that was developed at the University of Waterloo. This system consists of a single collimated scanning helium-neon laser source

that projects a thin (0.05mm) laser beam onto a plain mirror mounted at 45^{o} on a carriage assembly. The mirror reflects the laser beam directly up through the scanner table surface and through the lens under examination. The mirror carriage is connected via a drive screw to a positioning motor. This positioning motor turns the drive screw and thereby moves the laser in user-defined steps across the lens in an automated fashion. A digital camera captures the actual position and slope of the laser beam at each step. When all steps have been made, the captured data for each step position is used to calculate the back vertex distance for each position and the difference in that measurement between beams. Lenses were placed in culture medium into a specially designed three-part chamber made of glass and silicone rubber. Each lens studied was suspended within the chamber on a bevelled washer, of inner diameter ranging from 3.0mm. Back vertex focal length (spherical aberration) was measured for 20 beam positions across each lens. BVD, in mm, is defined as the measurement of the distance from the surface of the lens to the focal point where the laser beam crosses the optical axis of the lens being scanned. The instrument first locates the optical centre of the lens, the position of zero or minimal deviation of the beam. BVD is determined for a set number of beam positions on either side of the centre. Normally, changes in BVD as a function of eccentricity from the centre indicate the spherical aberration of the lens. Change in this distance (BVD error) affect the sharpness of focus and are a result of spherical aberration, or morphological irregularities. BVD error (mm) was calculated as the standard error of the mean of BVDs measured for a single lens by the scanning laser.

When portrayed graphically the average BVD for the lenses is plotted for each eccentric position. If spherical aberration is minimal, the data points line up as a straight line. As already noted, spherical aberration is determined by measuring BVD focal length for laser beams of varying eccentricity about the lens optical axis. The aberration measured may be positive (under corrected) or negative (over corrected). In the case of under corrected spherical aberration, paraxial light rays (rays close to the optic axis of the lens) have longer BVD focal lengths than peripheral ones. This is in fact the case for a spherical lens with a homogeneous refractive index. In case of negative spherical aberration, as in a spherical lens in which the variation in refractive index between the centre and periphery is too great, paraxial BVD focal lengths are shorter than peripheral ones. The poorer the quality of the lens, the greater the variation in BVD (BVD error) is with eccentricity. Since BVD error is a more sensitive measure of lens damage, the results are expressed in terms of BVD error.

2.4.3 Statistical Analysis

The repeated-measures analysis of variance (repeated-measures ANOVA) (SPSS™ 11.0 statistical software) were used to assess treatment, concentration, and temporal effects on the back vertex variability for experiment 2: Concentration Effect of TGFβ-2. This is a two factor experiment with repeated measures on one factor: one within factor of lenses (time of optical scans) and one between factor of lenses (treatment group). Paired student's t-test is use for analyzing data in experiment 1 (TGFβ-2 and

Ilomastat Treatment) and 3 (Effect of Ilomastat Concentration). A probability value (p-value) ≤ 0.05, indicating a 95% confidence interval, was considered to be significant.

2.4.4 Histology and Immunofluorescence

Lenses were collected from different treatment groups and fixed overnight in 1:99 acetic acid:ethanol solution, dehydrated, embedded in paraffin, and processed for routine histology. For histological analysis, 5μm sections were stained with hematoxylin and eosin. Immunofluorescence was performed on 5μm thick paraffin-embedded sections. Sections were incubated with primary antibody specific for alpha smooth muscle actin (αSMA,1:100, Sigma) and bound primary antibodies were visualized with a fluorescein-isothiocyanate (FITC) anti-mouse secondary antibody, (1:50,Jackson ImmunoResearch Laboratories). All sections were mounted in Vectasheild mounting medium with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories) to visualize the nuclei.

2.4.5 Zymography

Conditioned media from all treatment groups was concentrated using 3.5mL 10K Microsep concentrating devices (Viva Sciences). Prior to concentration, refrigerated media was warmed to 37^{o}C. The media was centrifuged at 1000g (at room temperature) for 5 min to pellet any debris prior to loading. Each device was loaded with equal volume of supernatant and the concentration was performed by centrifugation at 25^{o}C for 30 min at 4000g. An equal volume of each concentrate was electrophoresed on 12% SDS-polyacrylamide gels containing either 1mg/mL gelatin or 2% beta-casein at a final concentration of 0.1% as the substrate. Following electrophoresis the gels were developed

as described previously and stained in 0.5% coomassie brilliant blue for 1hr followed by destaining with 10% isopropanol. Sites of gelatinase or caseinase activity were detected as clear bands against a background of uniform staining which was digitally photographed.

2.4.6 Western Blot Analysis

Concentrated samples derived from the conditioned media were also examined by western blot analysis. Equal volumes of sample were electrophoresed on a 10% SDS-polyacrylamide gel. The resolved bands were electro-transferred onto a nitrocellulose membrane (Pall Corporation). Membranes were blocked with 5% skimmed milk powder in Tris(hydroxymethyl)aminomethane-buffered saline (50mM Tris base, NaCl pH 8.5)+ 0.1% Tween-20 and then incubated overnight at 4^0C with a polyclonal antibody generated against MMP9 (1:500;Chemicon International) or MMP2 (1:500;Chemicon International) or E-cadherin (1:1500; BD Transduction Laboratories). Following this incubation, membranes were probed with an HRP-conjugated secondary antibody (1:7500;Amersham Biosciences) and ECL detection reagents (Amersham Biosciences). The western blots were scanned by a densitometer and analyzed by image quantification software (ImageJ, NIH, USA). The relative density versus control ratio (RD/C) was estimated using Graph Pad Prism Program (GraphPad). Quantitative data were analyzed statistically using a student's t-test and expressed as +/- standard deviation. A value of $p<0.05$ was considered significant.

2.4.7 Laser Capture Microdissection and Real-Time QPCR

Lenses were placed in a cryostat mould containing Tissue-Tek OCT (Sakura Finetek Torrance, CA), and frozen on dry ice then stored at –70 °C. The frozen tissue was then sectioned at 7 – 12μm in a cryostat, mounted on non-coated clean glass slides, and stored again at -70°C. Immediately before LCM, the frozen sections will be thawed for 10 seconds and then stained with Histogene (Arcturus, Mountain View, CA) using the protocol provided with strict adherence to RNAse-free conditions. The slides were then dried for 5 minutes after which LCM of the tissue should was completed within two hours. LCM was then performed using the PixCell II (Arcturus). The Histogene stain allowed for the identification of the general morphology of the epithelium. The tissue was then captured on ExtracSure HS caps (Arcturus) using the PixCell II LCM microscope (Arcturus) with a minimal beam diameter of 7.5μm. mRNA was then isolated from lifted tissue using a PicoPure RNA extraction kit (Arcturus Engineering Inc). Standard reverse transcription reactions were preformed (SuperScript II, Life Technologies). The quality of the recovered cDNA was measured using a microfluidic gel analyzer (Agilent Technologies). Recovered cDNA was analyzed using quantitative PCR (ABI Prism 7700, Applied Biosystems) using a probe (Applied Biosystems) and primer (Mobix, Hamilton) for rat E-cadherin.

3.0 CHAPTER THREE:

Matrix Metalloproteinase-9 Is an Important Mediator of TGFβ Induced Epithelial to Mesenchymal Transition in the Lens

3.1 INTRODUCTION

Fibrosis is a post-injury, reparative process common to various organs in the body such as the lung, liver, heart, and eye resulting in aberrant amount of matrix deposition secreted by myofibroblasts leading to impaired organ function. Myofibroblasts generated following injury may arise from the conversion of local fibroblasts and the transformation of resident epithelial cells through a process known as epithelial to mesenchymal transition (EMT) (Hay 1995). Study of EMT in the lens offers considerable advantages over other organs since it is a simple, avascular structure composed of a monolayer of lens epithelial cells (LECs), devoid of fibroblasts.

LECs experience EMT in a coordinated process involving the destruction of specialized epithelial cell-cell contacts, such as E-cadherin, and cell-basement membrane contacts resulting in a loss of polarity and acquisition of motility (Kalluri and Neilson 2003). The culmination of LEC EMT is termed cataract, which is the loss of transparency of the lens and can be categorized as either posterior capsule opacification (PCO) or anterior subcapsular cataract (ASC). LECs which are damaged, but not removed, in primary cataract surgery undergo EMT and migrate to the intact posterior lens capsule resulting secondary cataract or PCO. EMT of LECs in the anterior monolayer of the results in ASC, which are known to occur following injury, and in association with atopic dermatitis and retinitis pigmentosa (Dilley, Bron et al. 1976; Sasaki, Kojima et al. 1998).

The signaling molecule transforming growth factor beta (TGFβ) plays pivotal roles in pathological EMT induction in many organisms inducing lenticular fibrosis. In

the eye, latent TGFβ is a normal constituent of the aqueous humor which bathes and nourishes the lens (Cousins, McCabe et al. 1991). Activation of TGFβ ligands results in the recruitment of an active TGFβ-receptor tetramer capable of signal transduction which initiates EMT. In fact, active TGFβ2 added to rodent lenses induces EMT leading to ASC (Hales, Chamberlain et al. 1995; Hales, Chamberlain et al. 1999; Dwivedi, Pino et al. 2006). Furthermore, *in vivo* studies with direct intracameral injection of a self-activating from of porcine TGFβ1 and lens specific over-expression of TGFβ1 with a transgene both lead to ASC (Robertson, Golesic et al. 2010). Common to each of these models of TGFβ-induced ASC are the EMT hallmark features, including loss of E-cadherin expression, gain of a migratory phenotype, excessive accumulation of matrix molecules such as types I and III collagen, and the expression of the contractile element alpha smooth muscle actin (αSMA). Exactly how TGFβ mediates this E-cadherin loss and resultant EMT in ASC is only recently beginning to be understood and one possibility is the involvement of matrix metalloproteinases (MMPs).

MMPs are a family of structurally related zinc dependent zymogens with the ability to degrade matrix proteins and liberate as well as activate growth factors sequestered in the extra cellular matrix (ECM) (McCawley and Matrisian 2001). MMPs are categorized by their preferential ability to cleave ECM proteins and include membrane bound, matrilysins, collagenases, stromelysins and gelatinases. The gelatinases, MMP2 and MMP9, are known to be involved in the EMT of the lungs and skin (Kasai, Allen et al. 2005; Walsh, Xu et al. 2011). Earlier work has demonstrated that LECs treated with TGFβ undergo EMT and concomitant with this is the upregulation of

both MMP2 and MMP9 (Richiert and Ireland 1999). The role gelatinases play in TGFβ-mediated EMT of LECs has only recently been studied. Excised, TGFβ-treated rat lenses co-treated with a specific inhibitor for MMP2/9 did not develop ASC (Dwivedi, Pino et al. 2006). These data suggest that the gelatinases are involved in the progression of TGFβ-mediated, EMT in lenticular fibrosis.

MMP9 is known to induce loss of E-cadherin, either through proteolytic cleavage resulting in a truncated soluble E-cadherin fragment or through the upregulation of known E-cadherin repressors, and therefore supports an early role for this MMP in EMT (Symowicz, Adley et al. 2007; Zheng, Lyons et al. 2009). In fact, previous data from our research group has shown that E-cadherin disruption is a feature of lenticular fibrosis in TGFβ-induced EMT (Dwivedi, Pino et al. 2006). For example, rodent lenses cultured with TGFβ, which develop cataracts and demonstrate increased secretion of MMP2/9, also exhibit the formation of a soluble 72kDa fragment of E-cadherin in the culture media. Furthermore, the addition of an inhibitor for MMP2/9 not only suppressed cataract formation but also inhibited shedding of the E-cadherin fragment into the culture media. Since E-cadherin shedding is known to be an early EMT event mediated at least in part by MMP9, it is surmised that MMP9 expression and activity are essential to the formation of ASC. Support for a key role of MMP9 in TGFβ-mediated EMT in ASC is garnered by the temporal expression profile of the gelatinases (Nathu, Dwivedi et al. 2009). Our research group has previously shown that expression of MMP9 was induced prior to MMP2 in TGFβ-induced ASC. In addition, those studies also revealed that MMP9 was capable of

inducing MMP2 protein expression. Thus, of the two gelatinases, MMP9 appears to play a more upstream role in ASC formation.

The current study focused on the examination of the specific involvement of MMP9 in ASC using primary cell cultures and two *in vivo* models. Primary LEC cultures co-treated with TGFβ2 and an inhibitor specific for MMP9 prevented E-cadherin and β-catenin delocalization. Furthermore, addition of recombinant MMP9 alone was able to mediate disruption of both E-cadherin and β-catenin at the cell-cell borders. Finally, mice lacking functional MMP9 *in vivo* exhibit significant protection from EMT leading to ASC following adenoviral delivery of TGFβ1. The protective effect of MMP9 deletion was also observed in mice with lens specific over-expression of TGFβ1, albeit to a lesser degree.

3.2 RESULTS

3.2.1 *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice are protected from ASC following adenoviral gene transfer of TGFβ1

Adenoviral-mediated gene transfer of active TGFβ1 (AdTGFβ1) to the anterior chamber of wild-type mice induces ASC formation four days post injection which continued to progress into large fibrotic plaques by day 21 (FIG 3.1). These lenses showed distinct ASC plaques consisting of a focal multilayering of LECs beneath the intact anterior lens capsule. Each of the AdTGFβ1-treated eyes commonly exhibited one plaque, typically found in a central location of the anterior region of the lens. To confirm that the plaques of AdTGFβ1 treated eyes consisted of myofibroblasts, as previously reported in human ASC and multiple animal models of ASC, lens sections were prepared from day 4 and day 21 post-injected eyes and subjected to immunohistochemical staining for αSMA. In these AdTGFβ1 treated eyes, distinct expression of αSMA was observed in a substantial proportion of cells within the plaques (FIG 3.2). In contrast to wild-type eyes treated with AdTGFβ1, delivery of active AdTGFβ1 to the anterior eye chamber of *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice did not result in ASC (FIG 3.1) and LECs exhibited their characteristic monolayer, similar to that observed in untreated eyes. In addition, the undisrupted lens epithelial monolayer of *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice failed to stain positive for the myofibroblast cell marker, αSMA (FIG 3.1). Adenoviral control vectors with no insert within the deleted E1 region did not induce any features of ASC following injection into the eye at any timepoint examined and the eyes remained normal during the entire course of the study (data not shown).

3.2.2 *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice are partially protected from ASC formation induced by Tg(Craa-Tgfb1)853Ove mice

To corroborate the above findings, we assessed the involvement of MMP9 in a transgenic TGFβ1 model. This model involves the over-expression of self activating TGFβ1 driven by a lens specific alphaA-crystallin promoter (Tg(Craa-Tgfb1)853Ove) (Srinivasan, Lovicu et al. 1998). Tg(Craa-Tgfb1)853Ove mice develop one centrally localized fibrotic ASC beginning three weeks after birth. These Tg(Craa-Tgfb1)853Ove mice were crossed with *Mmp9*$^{tm(neo)abr/+}$ to obtain Tg(Craa-Tgfb1)853Ove/ *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice. All Tg(Craa-Tgfb1)853Ove mice on the *Mmp9*$^{+/+}$ background (n=10) developed focal multilayered opacities (FIG 3.3 and FIG 3.4). Interestingly, 75% (n=7) of lenses derived from Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice were devoid of multilayered plaques and were similar to control lenses without Tg(Craa-Tgfb1)853Ove (n=3) which display the characteristic monolayer of lens epithelial cells (FIG 3.3). Similar to one month old mice, three month old Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice did not exhibit ASC formation in 75% (n=12) of lenses (FIG 3.4). However, in both groups, the remaining 25% developed cataracts (FIG 3.3 and FIG 3.4).

All lenses were subjected to immunohistochemistry and monitored for matrix deposition to determine if they contained myofibroblasts, indicative of EMT. All of the lenses of Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{+/+}$ mice at both one and three months (n=6 and n=4 respectively) exhibited αSMA reactivity and collagen deposition, as monitored by Gomori's staining, in areas where focal opacities had developed (FIG 3.5 and FIG 3.6

respectively). Similarly, plaques which developed in 25% of younger and older Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ lenses (n=7 and n=12 respectively) expressed αSMA reactivity and collagen deposition (FIG 3.6 and FIG 3.7 respectively). Control (n=3), *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ lenses (n=3) and 75% of the Tg(Craa-Tgfb1)853Ove/ *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ lenses which did not exhibit ASC formation were also devoid of αSMA reactivity and did not show increased collagen deposition a (FIG 3.6 and FIG 3.7 respectively).

3.2.3 Inhibition of MMP9 prevents E-cadherin/β-catenin dissolution in Rat Lens Explants

The dissolution of cell-cell contacts, notably loss of E-cadherin, from epithelial cells is a pivotal role delineating that a transition of an epithelial cells phenotype to mesenchymal cells phenotype is occurring. Therefore, to visualize the effect of MMP9 on the dissolution of E-cadherin, rodent LEC explant cultures were employed. Rodent LEC explants allow the visualization of cell-cell borders due to their outstretched and therefore flattened nature. Rodent explants are derived from the isolation and subsequent flat mounting of the LEC monolayer on its native basement membrane the lens capsule. In the rodent explant, the anterior lens capsule is placed in direct contact with the culture dish, exposing the posterior aspect of the LEC monolayer to the surrounding media. In this way, the exposed LECs are amenable to treatment as well as visualization of the cell-cell borders. Lens explant cultures prepared from three week old Wistar rats were treated with TGFβ2 (4ng/mL) for 3 days. Following treatment the explants displayed a loss of both β-catenin and E-cadherin at the cell borders (FIG 3.8 C,D) whereas untreated control

explants retained β-catenin and E-cadherin at the cell periphery (FIG 3.8 A,B). To asses the involvement of MMP9 in TGFβ2 LEC disruption, co-treatment of explant cultures with TGFβ2 and an MMP9 specific inhibitor was carried out. Importantly, the inhibition of MMP9 alone was able to prevent TGFβ2 mediated β-catenin and E-cadherin loss (FIG 3.8 G,H respectively). We then examined the effect of human recombinant MMP9 on the E-cadherin/β-catenin complex in LECs. Lens explant cultures were treated with human recombinant MMP9 (2μg/mL) and showed loss of E-cadherin and β-catenin cell membrane localization, similar to that observed following treatment with TGFβ2 (FIG 3.8 E,F). Differences in immuofluorescence staining intensities between treatments further confirmed and quantified the findings out lined above (FIG 3.8 K,L).

3.3 DISCUSSION

The development of lenticular fibrosis is a pathology involving the transformation of lens epithelial cells to a mesenchymal phenotype which begin to accumulate aberrant amounts of matrix and induce light scatter. Our previous studies have determined that the matrix degrading enzymes known as MMP2 and 9 are functionally involved in this pathological process (Dwivedi, Pino et al. 2006). These current studies address the individual role of MMP9 in the EMT of lens fibrosis in two established *in vivo* models of ASC.

Our previous findings showed that MMPs are necessary for the TGFβ mediated EMT of LECs during ASC formation but did not determine the individual involvement of MMP2 and MMP9. However, results from the current study suggest that MMP9 is a critical mediator of TGFβ-induced ASC. The requirement of MMP9 in ASC formation is supported by results demonstrating that *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice fail to elicit plaque formation upon adenoviral gene transfer of TGFβ1 to the structures of the anterior chamber. In contrast, *Mmp9*$^{tm(neo)abr/+}$ mice developed TGFβ-mediated ASC to a similar degree as controls which received AdTGFβ1. To ensure that the loss of *Mmp9* was in fact involved in EMT of LECs, sections were monitored for the myofibroblast marker αSMA. These results indicate that plaques of AdTGFβ1 treated *Mmp9*$^{tm(neo)abr/+}$ mice, express the EMT marker αSMA. In contrast, the LECs of *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice which received AdTGFβ1 did not express αSMA. Thus, these findings suggest that a lack of *Mmp9* can prevent TGFβ-mediated EMT and ASC formation.

To further corroborate the specific *in vivo* role(s) of *Mmp9* in ASC, a transgenic mouse model with continuous lens specific overexpression of TGFβ1 (was established in the absence of *Mmp9* (Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$). The current study, examining mice at both one and three month time points revealed a suppression of cataract formation in 75% of Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ lenses. These mice also do not evidence expression of αSMA nor do they display aberrant matrix deposition characteristic of EMT. In contrast, all Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{+/+}$ lenses develop ASC and expressed both αSMA reactivity and matrix deposition. One possible explanation for the increased incidence of cataracts in the transgenic model versus the adenoviral delivery model may be a result of TGFβ1 expression. TGFβ1 in the transgenic model is continuously producing self activating TGFβ1 over the entire life-span of the transgeneic mouse whereas adenoviral delivery of TGFβ1 is transient. In fact, differences in the incidence of cataract formation between models with transient and continuous expression of TGFβ have been reported. In one study examining the lenses of *Smad3* null mice receiving either lenticular puncture or exogenous TGFβ2 and the results indicated that the absence of *Smad3* was sufficient in suppressing ASC (Saika, Kono-Saika et al. 2004). However, a separate study utilizing the lens specific TGFβ1 overexpressing Tg(Craa-Tgfb1)853Ove mice in conjunction with *Smad3* null mice indicated that ASC formation does occur albeit to a lesser degree (Banh, Deschamps et al. 2006). Interestingly, in the current study with Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ there was no gradational reduction in ASC formation; the fibrotic event either did not (75%) or did occur (25%). These results suggest that a critical threshold level of MMPs

or disruption is necessary to initiate the cascade of EMT events which leads to cataract formation.

Classifying MMP9 as a critical mediator of ASC with threshold properties that must be surpassed in inducing this pathological cascade is also supported by its known role in EMT as well as its temporal regulation with respect to cataract formation. E-cadherin has been recognized as a critical factor in maintaining the epithelial cell state and its disruption has been associated with the beginning of transition to a mesenchymal phenotype (Imhof, Vollmers et al. 1983; Thiery 2002). In fact, our previous studies in rat lens cultures have determined that the progression of ASC mediated by TGFβ involves the shedding of E-cadherin. We further established that the blockage of MMP2 and MMP9 using a specific small molecule inhibitor resulted in the concomitant suppression of ASC as well as E-cadherin shedding. This suggests that either MMP2 or MMP9 is able to mediate E-cadherin shedding leading to EMT and resulting in ASC. Our current results regarding the role of MMP9 in lenticular fibrosis are consistent with accumulating evidence suggesting that MMP9 may be an early mediator of transformation. It has been shown that epithelial differentiation to a mesenchymal cell type, via E-cadherin disruption, occurs as a direct result of MMP9 protease activity in epithelial ovarian carcinoma (Symowicz, Adley et al. 2007). Furthermore, studies regarding the temporal examination of MMPs expression during cataractogenesis have also been examined. Dwivedi *et al* determined that MMP9 expression precedes that of MMP2 by two days in whole rat lenses cultured with TGFβ2 which subsequently lead to cataract formation. Therefore, our current findings suggesting that MMP9 is an early mediator of TGFβ

induced EMT leading to ASC is consistent with previously established temporal expression pattern, highlighting its essential role during EMT.

Exactly how MMP9 contributes to EMT and ASC is still under investigation; however one possibility is that it could modulate the expression of *Mmp2*. In fact this relationship of *Mmp9* and *Mmp2* expression exists in other systems. Upon ligation of the carotid artery, rapid remodeling is reported and known to involve temporal expression patterns of both *Mmp2* and *9* (Godin, Ivan et al. 2000). Specifically, results demonstrated that MMP expression was significantly elevated above controls following ligation and that significant MMP2 expression commenced following peak MMP9 expression and lasted for the duration of the experiment (Godin, Ivan et al. 2000). Although no correlation between MMP9 modulation of MMP2 activity was examined in those studies, it nonetheless suggests that increase in MMP9 expression precedes that of MMP2 expression during remodelling events. Furthermore, a direct role for MMP9 as an inducer of MMP2 has been determined in human LECs. Stimulation of human LECs with human recombinant MMP9 evidenced an increase in expression of MMP2 and correlated with expression of the EMT marker αSMA (Nathu, Dwivedi et al. 2009). These results give rise to the possibility that MMP9 expression modulates MMP2 expression *in vivo*. In light of this, further experimentation regarding the role that MMP9 exerts on MMP2 are likely to enhance what the unique and cooperative roles of MMP2 and 9 are in TGFβ-mediated ASC.

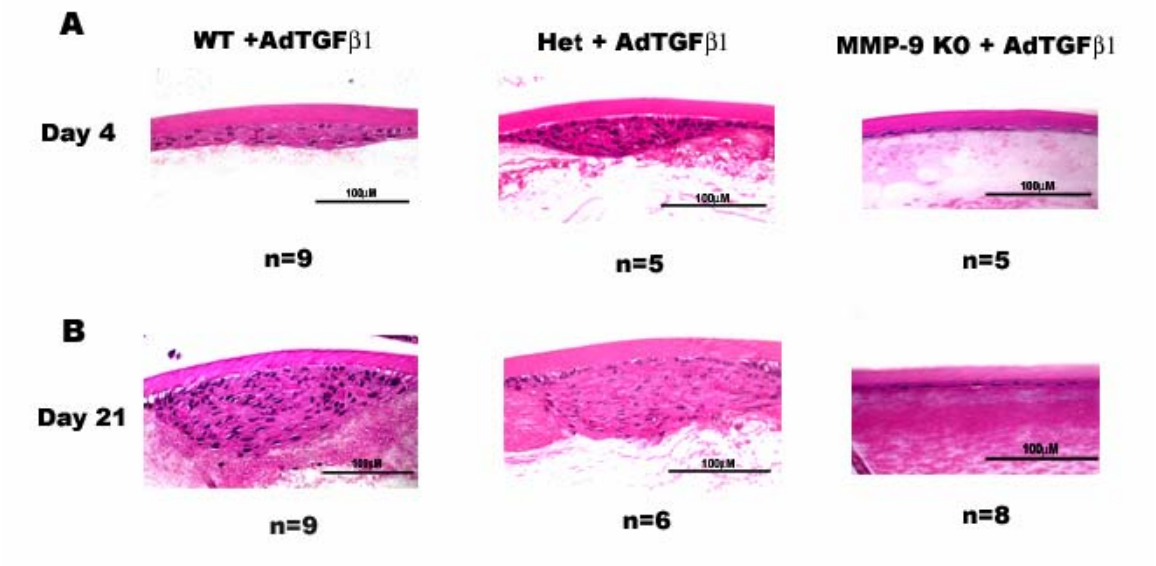

Fig 3.1 Histological sections from wildtype mice (WT), *Mmp9*$^{tm(neo)abr/+}$ mice (Het) and *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ (MMP-9 KO) following injection of Adenoviral TGFβ1 (AdTGFβ1) for 4 and 21 days (A and B respectively). Wildtype mice which received the transgene for TGFβ1 developed focal plaques starting at day 4 post injection and were sustained for 21 days (WT+AdTGFβ1). Mice with one functional copy of *Mmp9* also exhibit AdTGFβ1 mediated ASC similar to that of wildtype mice (Het+AdTGFβ1). Interestingly, *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice which received AdTGFβ1 were protected from ASC formation both at the 4 day and 21 day post injection timepoints (MMP-9KO+AdTGFβ1). Scale bars represent 100μM.

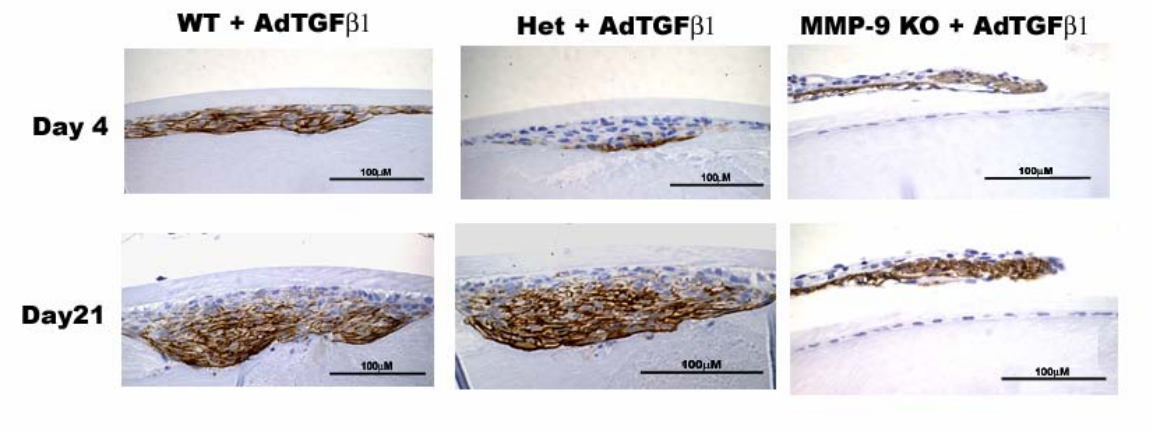

Fig 3.2 Immunohistochemical analysis was performed on lenses of mice across *Mmp9* genotypes following AdTGFβ1 treatment to assess EMT formation as evidenced by αSMA expression. The plaques of both $Mmp9^{+/+}$ and $Mmp9^{tm(neo)abr/+}$ mice following adenoviral delivery of TGFβ1 evidenced positive αSMA expression (WT+AdTGFβ1 and Het+AdTGFβ1 respectively) at both 4 and 21 days post injection. In contrast, the LECs of $Mmp9^{tm(neo)abr/tm(neo)abr}$ mice did not positively react for αSMA (MMP9 KO + AdTGFβ1). As a positive control, αSMA expression is evidenced in the irises of MMP9 KO + AdTGFβ1 mice. Scale bars represent 100µM.

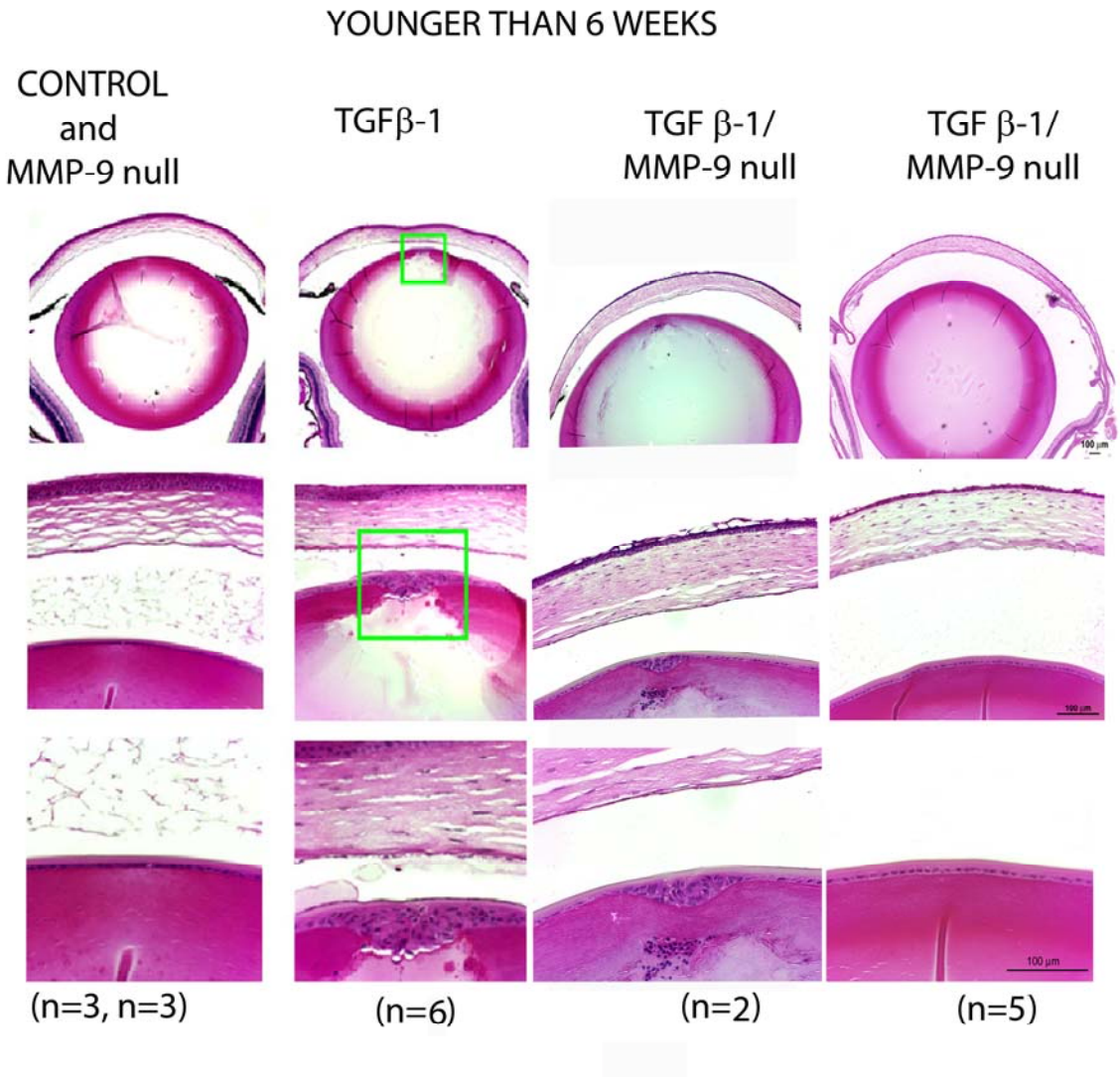

Fig 3.3 Histological sections from *Mmp9*$^{+/+}$ mice (CONTROL), Tg(Craa-Tgfb1)853Ove mice (TGFβ-1) and Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ (TGFβ-1/MMP9 null) upto 6 weeks of age. TGFβ-1 mice develop focal plaques starting from 3 weeks of age. Interestingly, nearly 75% (n= 5 of 7) of TGFβ-1/MMP9 null mice were protected from ASC formation while the remaining 25% (n= 2 of 7) developed ASC similar to TGFβ-1 mice .

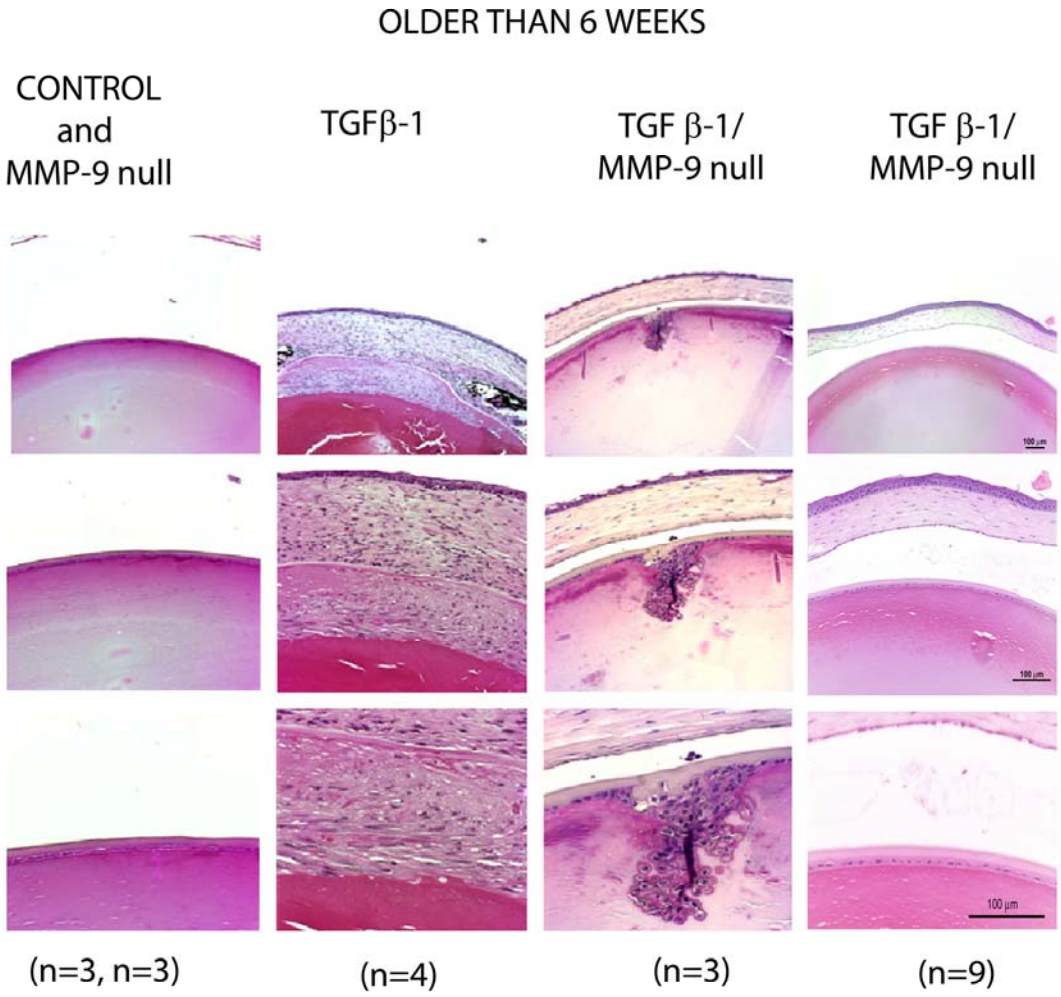

Fig 3.4 Histological sections from *Mmp9*$^{+/+}$ mice (CONTROL), Tg(Craa-Tgfb1) mice (TGFβ1) and Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice (TGFβ1/MMP9 null) at greater than 6 weeks of age. Tg(Craa-Tgfb1)853Ove mice at this timepoint exhibit a reorganization of cells in the plaque which exudes into the anterior chamber. ASC formation did occur in 25% of Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice (n=3 of 12) whereas the other 75% of mice did not display evidence of cataract formation (n= 9 of 12).

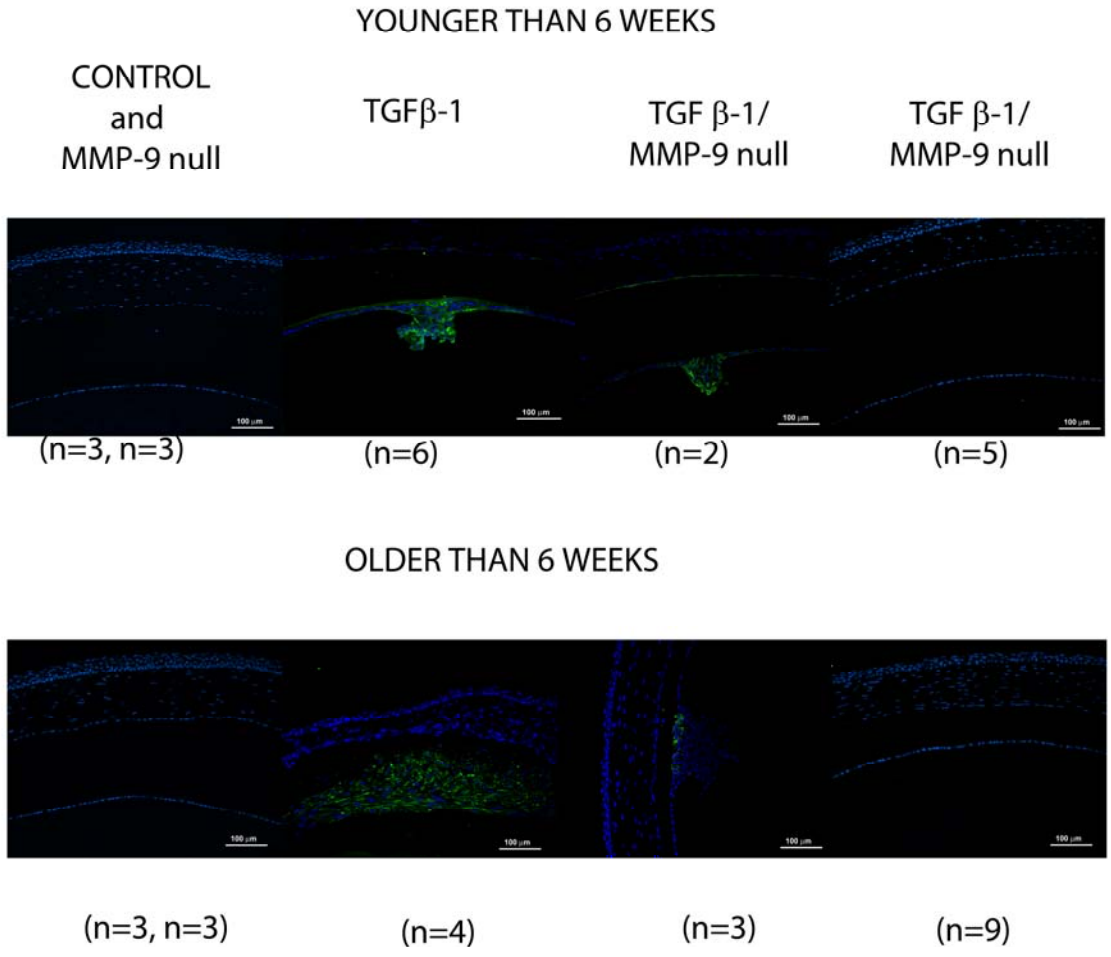

Fig 3.5 All *Mmp9*$^{+/+}$ (control) mice *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ (MMP9 null) mice do not positively react to immunostaining for the EMT marker αSMA in either age group. In contrast, Tg(Craa-Tgfb1)853Ove mice both plaque formation as well as αSMA reactivity in both age groups (TGFβ-1). Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ (TGFβ-1/ MMP-9 null) mice are protected from cataract formation (n=5 in younger and n=9 in older), do not stain positively for the EMT marker αSMA. However, the 25% of Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice which develop ASC demonstrate αSMA reactivity at both younger and older age groups.

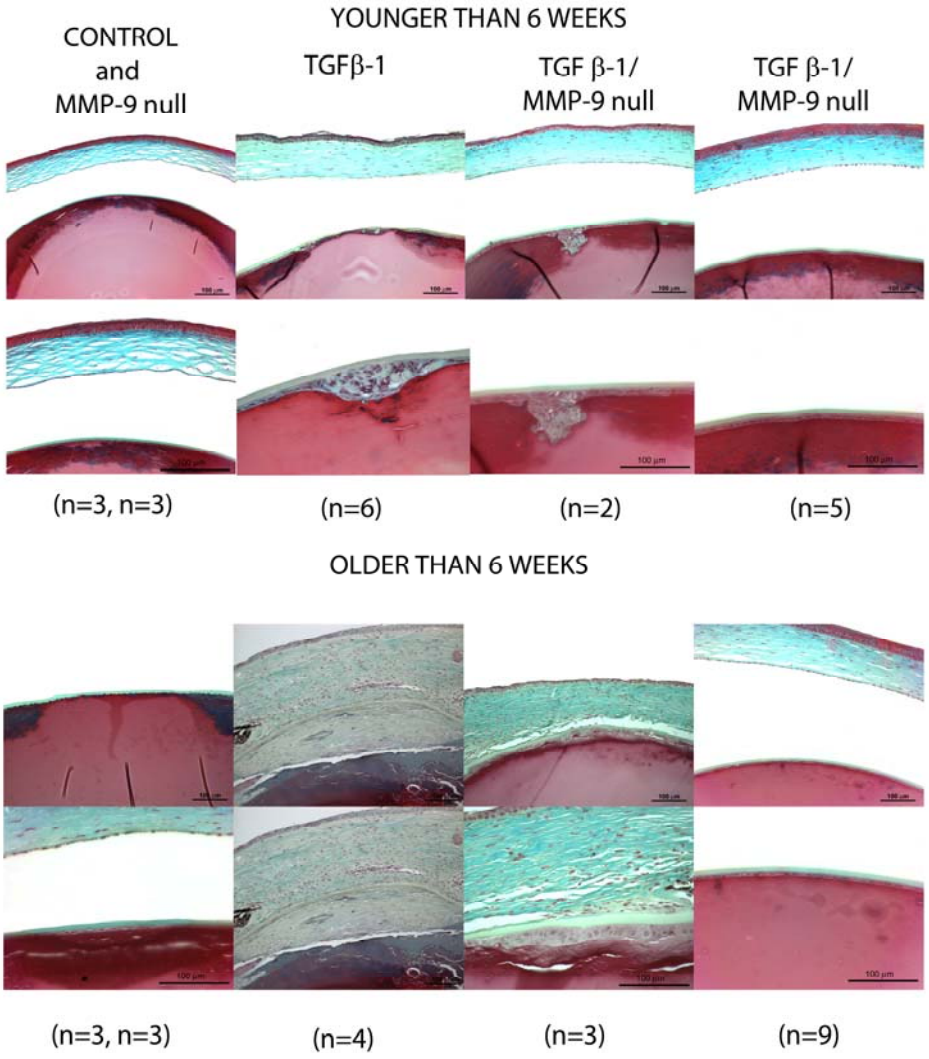

Fig 3.6 All *Mmp9*$^{+/+}$ and *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ do not exhibit aberrant collagen deposition in either age group (Control and MMP-9 null). In contrast, Tg(Craa-Tgfb1)853Ove mice exhibit both plaque formation as well as αSMA reactivity in both age groups (TGFβ-1). Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice which are protected from cataract formation (n=5 in younger and n=9 in older), also do not exhibit excessive matrix deposition. Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice which do develop ASC exhibit excessive matrix deposition in the resultant plaques.

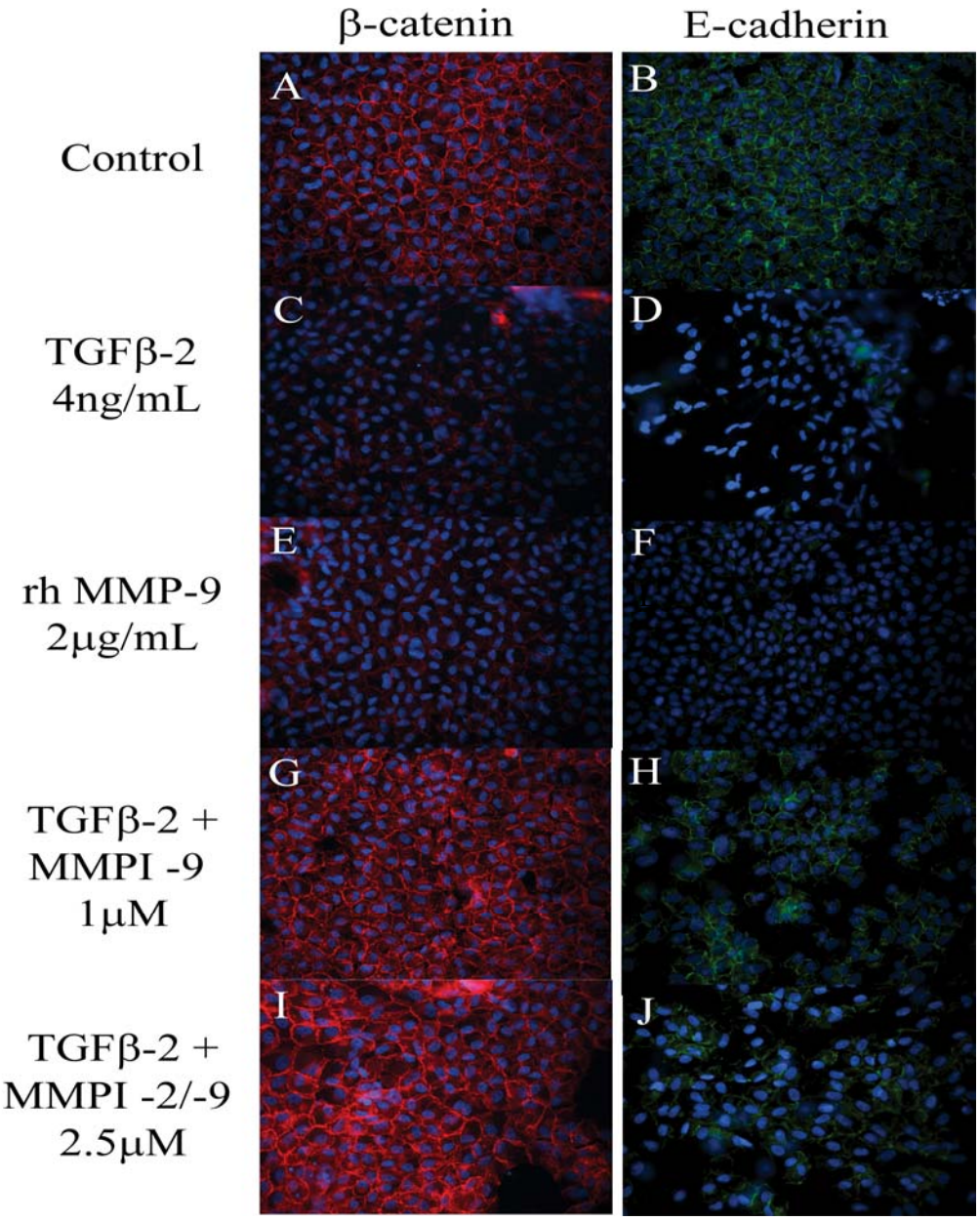

Fig 3.7 A: Immunofluorescence images showing β-catenin (A,C,E,G,I) and E-cadherin (B,D,F,H,J) in P21 rat lens explants (n=3 for each) corresponding to different treatments with TGFβ2 (C,D), active recombinant human MMP-9 (E,F), TGFβ2 and an inhibitor specific for MMP9 (G,H), TGFβ2 and an inhibitor of both MMP2/9 (I,J).

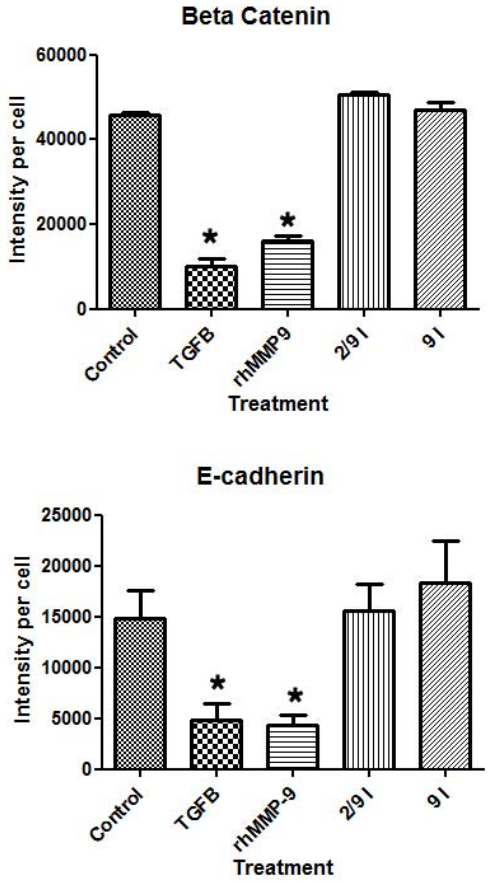

Fig 3.8 Quantification of immunofluorescence intensity of β-catenin (top) and E-cadherin (bottom). Treatment of rat explants with TGFβ alone or recombinant human MMP-9 results in a significant loss of both β-catenin and E-cadherin. Importantly, co-treatment with an inhibitor specific for either MMP2/9 or MMP9 alone is able to prevent the loss of both β-catenin and E-cadherin.

3.4 MATERIALS AND METHODS

3.4.1 Adenoviral Injection of TGFβ1 in *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice

AdTGFβ1 or AdDL or AdLacZ ($5x10^8$ pfu) were administered in a volume of 5 µl phosphate-buffered saline (PBS) to *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice with targeted disruption of *Mmp9* by replacement of a portion of exon 2 and all of exon 3 with a neomycin resistance cassette. Briefly, these mice were anaesthetized with isofluorane and placed under a dissecting microscope, in order to visualize general eye structures. A volume of no more than 5 µl of virus solution was injected into the anterior chamber using a 33 gauge needle affixed to a 10 µl Hamilton syringe. Eyes were covered with Lacri-lube® after injection and animals were allowed to recover before returning to their cages. Animals were sacrificed and enucleated 4 or 21 days after injection.

3.4.2 Propagation of Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ Mice

All animal studies were performed according to the Canadian Council on Animal Care Guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Tg(Craa-Tgfb1)853Ove mice contain a porcine TGFβ1 cDNA construct with an αA-crystallin promoter designed for lens-specific expression of active TGFβ1 on a FVB/N/C57BL/6J genetic background (Srinivasan, Lovicu et al. 1998). The *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ was generated by the replacement of part of exon 2 and all of intron 2 with a neomycin resistance cassette (Vu, Shipley et al. 1998). Tg(Craa-Tgfb1)853Ove mice were bred with *Mmp9*$^{tm(neo)abr/+}$ mice to generate mice with the

following genotypes: Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$, Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/+}$, Tg(Craa-Tgfb1)853Ove/*Mmp-9*$^{+/+}$, *Mmp9*$^{tm(neo)abr/+}$, and *Mmp9*$^{+/+}$. Wild-type littermates were used in order to account for the various differences between the strains of mice.

3.4.3 Genotype Analysis

DNA extraction and purification from mouse ear tissue was performed with a kit (DNeasy; Qiagen Inc., Toronto, ON, Canada). Genotypes were determined by polymerase chain reaction (PCR) analysis. The TGFβ1 transgene was identified by using primers specific for the simian virus 40 (SV40) sequences in the transgene: the sense primer (5'-GTGAAGGAACCTTACTTCTGTGGTG-3') and the antisense primer (5'-GTCCTTGGGGTCTTCTACCTTTCTC-3') yield a 300-bp fragment in the PCR reactions. PCR reactions were performed for 36 cycles using the following conditions: initial heating for 3 minutes at 94°C (only for cycle 1); denaturation for 30 seconds at 94°C; annealing for 1 minute at 57°C; and extension for 1 minute at 72°C. A final extension was performed for 10 minutes at 72°C. Agarose gel electrophoresis (1.5% agarose) with ethidium bromide (EtBr) detection was used to visualize the PCR reaction products.

Mmp9$^{+/+}$ (wild-type) and *Mmp9*$^{tm(neo)abr/tm(neo)abr}$ (knockout) alleles were detected using the following primers; For *Mmp9* wild-type allele, detected by using primers (5'-GTGGGACCATCATAACATCAC-3') and (5'-CTCGCGGCAAGTCTTCAGAGTA-3') amplifying a 277-bp fragment from wild-type and heterozygous knockout mice. The

Mmp9 null allele was detected by using primers (5'-CTGAATGAACTGCAGGCAGA-3') and (5'-ATACTTCTCGGCAGGAGCA-3)'to yield a 172-bp fragment, which is detected in both the heterozygous and homozygous MMP-null mice. PCR reactions were performed for 35 cycles in the following conditions: initial heating for 2 minutes at 94°C (only for cycle 1); 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. A 1.5% agarose gel electrophoresis with ethidium bromide detection was used to visualize the PCR reaction products.

3.4.4 Histology and Immunofluorescence

Lenses were collected from different treatment groups and fixed overnight in 10% neutral buffer formalin, dehydrated, embedded in paraffin, and processed for routine histology. For histological analysis, 5µm sections were stained with hematoxylin and eosin. Gomori's trichrome stain was performed on 5µm thick paraffin-embedded sections. Immunofluorescence was performed on 5µm thick paraffin-embedded sections. Sections were incubated with primary antibody specific for αSMA (1:100, Sigma) and bound primary antibodies were visualized with a fluorescein-isothiocyanate (FITC) anti-mouse secondary antibody, (1:50, Jackson ImmunoResearch Laboratories). All sections were mounted in Vectasheild mounting medium with 4',6-Diaminodino-2-Phenylindol (DAPI, Vector Laboratories) to visualize the nuclei.

4.0 CHAPTER FOUR:

Mitogen Activated Protein Kinases p38 and ERK1/2 and Connective Tissue Growth Factor Are Involved in Transforming Growth Factor β Mediated Lens Fibrosis

4.1 INTRODUCTION

The anterior region of the lens is defined by a quiescent monolayer of cuboidal lens epithelial cells (LECs) which give rise to the transparent, terminally differentiated fiber cells that comprise the remainder of the lens. Cataract or ocular fibrosis, is a repair response which results in LEC transition to myofibroblast-like cells in a process known as epithelial to mesenchymal transition (EMT). In fact, LEC EMT results in two distinct but related forms of human fibrotic cataract: (1) anterior subcapsular cataract (ASC); and (2) posterior capsular opacification (PCO). PCO is a complication of cataract surgery, while ASC develops as a result of physical, or chemical trauma or in conjunction with atopic dermatitis or retinitis pigmentosa (Schmitt-Graff, Pau et al. 1990; Kappelhof and Vrensen 1992; Liu, Hales et al. 1994). In either case, EMT is marked by the dissociation of cell to cell contacts and loss of cell adhesion molecules, including, among others, epithelial cadherin (E-cadherin), resulting in cellular depolarization and delamination (Hay 1995). Subsequently, changes in gene expression lead to myofibroblast formation include the expression of contractile elements such as alpha smooth muscle actin (αSMA) and excessive matrix deposition. Excessive deposition of types I and III collagen, tenascin, fibronectin and collagens induce the light scatter associated with cataracts.

Similar to a majority of other fibroses, the initiating factor of EMT in LECs is the activated cytokine transforming growth factor beta (TGFβ). The delivery of TGFβ either in *ex vivo* culture or *in vivo* using a self activating form of TGFβ1 results in the formation of distinct fibrotic lens plaques which closely resemble the human disease (Srinivasan, Lovicu et al. 1998; Robertson, Golesic et al. 2010). To mediate its fibrotic effects the

TGFβ ligand causes homodimerization of the type II receptor and subsequent activation by cross-phosphorylation of a type I receptor homodimer (Miyazono 1997). Activation of the type I receptor propagates the signal primarily through two pathways: (1) mitogen associated protein kinases (MAPK); extracellular signal-regulated kinase (ERK1/2), p38 and cJun N-terminal Kinase (JNK) or (2) via the SMAD signaling molecules, SMAD-2,-3 (Baker and Harland 1997; Miyazono 1997). Traditionally a greater amount of research has been focused on the SMAD dependent signaling events involved in TGFβ mediated EMT of the lens. For example, mice which lack functional Smad3 signaling are resistant to fibrosis induced either in culture with TGFβ or *in vivo* via a lenticular puncture, suggesting that ocular fibrosis requires Smad signaling (Saika, Kono-Saika et al. 2004). However, mice genetically modified to express lens specific TGFβ1 develop ocular fibrosis, although to less of a degree, even in the absence of Smad3 (Banh, Deschamps et al. 2006). These results suggest that Smad independent signaling mechanisms such as p38 and ERK 1/2 may also be involved in this pathologic process.

Recent studies examining the signaling events associated with TGFβ-induced EMT has determined an involvement of the MAPK p38 pathway. For example, normal murine mammary epithelial cells treated with TGFβ undergo EMT and exhibit increased levels of p38 activation. This EMT event initiated by TGFβ is dependent on β1 integrin rather than Smad signaling (Bhowmick, Zent et al. 2001; Yu, Hebert et al. 2002). Additionally, in these cells, addition of an inhibitor specific for p38 is able to prevent TGFβ-mediated transition of epithelial cells (Yu, Hebert et al. 2002). Similarly, retinal pigmented epithelial cells exposed to TGFβ have been shown to undergo cellular fibrosis,

accumulating extra cellular matrix (ECM) while gaining a migratory phenotype (Saika, Yamanaka et al. 2005). Furthermore, these cells also exhibit increased activation of p38 following TGFβ stimulation, suggesting that the fibrotic events in these epithelial cells may be mitigated in part by p38. In fact, specific inhibition of p38 either by a small molecule inhibitor or by dominant negative viral gene transfer is able to retard the migratory ability as well as suppress ECM accumulation of type I collagen and fibronectin (Saika, Yamanaka et al. 2005). Specifically in the lens, a role for p38 has not yet been established. However, significant similarities between p38 in other TGFβ-mediated fibroses have been established suggesting that p38 may also be involved in lens epithelial transition to myofibroblasts.

In addition to the p38 MAPK pathway, a role for the ERK1/2 MAPK in TGFβ-mediated EMT has been established in various epithelial tissues (Hartsough and Mulder 1995; Ellenrieder, Hendler et al. 2001; Zavadil, Bitzer et al. 2001). More specifically, in mammary epithelial cells, activation of ERK1/2 signaling occurs following TGFβ stimulation and specific inhibition of the ERK1/2 MAPK kinase, MEK1/2, was able to prevent the loss of epithelial phenotype induced by TGFβ (Xie, Law et al. 2004). However, understanding a role for ERK activation in TGFβ-induced EMT has only recently emerged. Treatment of quiescent renal tubular epithelial cells with TGFβ has been shown to lead to EMT and increased ERK1/2 activity; like LECs this transformation has also been shown to be correlated with decreased E-cadherin and increased αSMA (Li, Zhu et al. 2002; Rhyu, Yang et al. 2005). Interestingly, pre-treatment with an ERK inhibitor prior to TGFβ simulation was able to suppress both the loss of E-cadherin and

the increase in αSMA normally associated with TGFβ treatment (Rhyu, Yang et al. 2005). These findings directly suggest that ERK1/2 signaling is involved in mediating TGFβ-induced EMT in tubular epithelial cells. Tubular epithelial cells exhibit similar responses to TGFβ as those established for LECs, however, a role for ERK1/2 signaling during cataractogenesis remains to be elucidated.

In addition to activating MAPKs, signaling of TGFβ resulting in induction of EMT upregulates a down-stream fibrogenic co-factor, connective tissue growth factor (CTGF). CTGF belongs to a family of 6 proteins which are distinguished by an unusually high content of cysteine (11%) (Bradham, Igarashi et al. 1991). Its promoter region contains various binding sites, including two TGFβ-responsive elements, SMAD and Ets-1, supportive of its role as a downstream target of TGFβ (Grotendorst, Okochi et al. 1996; Holmes, Abraham et al. 2001; Holmes, Abraham et al. 2003; Leask, Holmes et al. 2003). CTGF is a critical factor in the formation of connective tissue surrounding muscle, the glomerulus, and hypertrophic chondrocyte cartilage of the growth plate (Yamamoto, Furukawa et al. 2002). In addition to its developmental role, CTGF is involved in mediating wound healing responses in organs including the kidney, liver, lungs, heart and skin, where it synergizes with TGFβ to promote sustained fibrosis (Mori, Kawara et al. 1999; Bonniaud, Margetts et al. 2003; Bonniaud, Martin et al. 2004; Dean, Balding et al. 2005; Ikawa, Ng et al. 2008; Liu, Fortin et al. 2008). Furthermore, subcutaneous injection of TGFβ alone in neonatal mice was able to only elicit transient fibrosis, while CTGF alone had little, if any, effect. However, co-administration of CTGF with TGFβ resulted in sustained fibrosis (Mori, Kawara et al. 1999). Additionally, the requirement of CTGF

for a sustained fibrotic response has also been shown in a model of pulmonary fibrosis (Bonniaud, Margetts et al. 2003; Bonniaud, Martin et al. 2004). Balb/c mice are protected from fibrosis following bleomycin challenge whereas this effect is not seen in other strains of mice. Balb/c protection from bleomycin is a result of impaired CTGF induction and the simultaneous delivery of CTGF with bleomycin resulted in fibrosis that was similar to that of susceptible mice with bleomycin alone (Bonniaud, Martin et al. 2004). Taken together, these data illustrate that CTGF is functionally involved in fibrotic events of both the skin and lungs. In contrast to other organs, relatively little is currently known regarding the role of CTGF in lens fibrosis. What has been shown is that clinical samples of ASC show levels of CTGF mRNA elevated to a similar degree as both the TGFβ ligand and receptor mRNA (Lee and Joo 1999). Furthermore, human clinical samples of both ASC and PCO exhibit elevated CTGF mRNA concomitantly with the expression of EMT markers αSMA, type I collagen and tenascin (Wunderlich, Pech et al. 2000). Collectively, these data suggest that CTGF is involved in ocular fibrosis and may be related to elevated levels of TGFβ.

In the current study, we examined the activation of MAPKs p38 and ERK 1/2 by TGFβ in a human lens epithelial cell line, FHL-124. We also tested the involvement of p38, using a specific p38 inhibitor SB202190, in both FHL-124 cells and an *ex vivo* rat lens culture model. Further evidence is provided suggesting the involvement of the TGFβ co-factor, CTGF in cataract development.

4.2 RESULTS

4.2.1 TGFβ induced EMT in ASC involves MAPK p38 and specific inhibitors for p38 are able to attenuate the EMT marker αSMA in lens culture.

To first assess the involvement of p38 in TGFβ-mediated signaling in the lens, enucleated rat lenses were cultured with TGFβ2. Following 3hrs of treatment with TGFβ2 (2ng/mL) rat lenses showed greater phosphorylation of p38 MAPK compared to untreated controls, as assessed by Western blot analysis (FIG 4.1A). Next, experiments were carried out in the human lens epithelial cell line, FHL-124, to assess the consequences of p38 inhibition when stimulated with TGFβ. FHL-124 cells were either treated with: (1) TGFβ2 (10ng/mL); (2) co-cultured with TGFβ2 (10ng/mL) and the specific p38 inhibitor SB202190 at 1μM or 10μM or (3) left untreated (negative control). After 48hr of culture, Western blot analysis showed that FHL-124 cells treated with 10ng/mL of TGFβ exhibit greater protein expression of the mesenchymal cell marker αSMA, as compared to controls (FIG 4.1B). Furthermore, specific inhibition of p38 by SB202190 at 10μM resulted in a less αSMA protein expression to levels below both control and TGFβ2 treated FHL-124. Co-culturing with 1μM of SB202190 did not affect protein expression of αSMA when co-treated with TGFβ2 (FIG 4.1B).

4.2.2 Inhibition of MAPK p38 in *ex vivo* rat lenses does not alter TGFβ mediated EMT leading to ASC

Based on the involvement of p38 in EMT, it was thought that p38 may also play a signficiant role in ASC. To directly test this hypothesis, experiments aimed at the specific inhibition of p38 in the *ex vivo* rat lens culture model were performed. Enucleated rat lenses were treated with TGFβ2 (2ng/mL) and p38 inhibitor SB202190 at concentrations ranging from 100nM to 100μM or were left untreated for 6 days. Lenses were subjected to routine histology and immunostaining for the EMT marker αSMA to assess the formation of ASC. Lenses treated with TGFβ2 alone (n=4) exhibited distinct opacities beneath the anterior surface of the lens which immunoreacted positively to αSMA. This is in contrast to untreated control lenses (n=3) which remained devoid of opacities and αSMA immunoreactivity (FIG 4.2 and FIG 4.3 respectively). Lenses co-cultured with TGFβ2 and p38I at concentrations ranging from 100nM to 100μM (n=3 for each) all exhibited visible subcapsular cataracts and exhibited immunoreactivity to αSMA (FIG 4.3 and FIG 4.3 respectively).

4.2.3 TGFβ induced EMT in ASC involves ERK1/2 MAPK

To determine the MAPKs that may be involved in TGFβ induced EMT, a proteome profile array with human lens epithelial cells (FHL-124) was performed. Arrays were probed with FHL-124 lysates from control or TGFβ2 (10ng/mL) supplemented cells following 3hr of treatment as outlined in the materials and methods section. The control array showed basal levels of ERK1, p38α, p38γ, and HSP 27 (FIG 4.4). An array probed

with FHL-124 cells treated with TGFβ2 at 10ng/mL for 3 hrs, exhibited increased ERK1 (2-fold), p38α (1.5-fold), and HSP-27 (2-fold) in comparison to controls. In contrast, p38γ was slightly decreased. Furthermore, TGFβ2 treatment also resulted in expression of activated ERK 2, which was absent from the control array (FIG 4.4). This experiment was performed once.

4.2.4 Induction of EMT marker αSMA in FHL124 cells treated with rhCTGF

Previous studies have implicated a role fro CTGF in TGFβ-mediated EMT of other tissues including the skin and lungs. Thus a role for CTGF in the EMT of LECs was also postulated. As such, FHL-124 cells were treated with recombinant human CTGF (rhCTGF) for 24hrs at various concentrations to determine whether it was possible to induce the expression of EMT associated markers. Treatment of FHL-124 cells with 1,5,50 or 100ng/mL of rhCTGF resulted in upregulation of αSMA protein expression above control levels (FIG 4.5). However, at higher concentrations, of 500 and 1000ng/mL, FHL-124 cells failed to exhibit upregulation of αSMA protein expression and resembled controls (FIG4.5).

4.2.5 Induction of EMT leading to ASC by rhCTGF

In order to assess the role of CTGF in ASC formation, whole exciecd rat lenses were either treated with rhCTGF (50ng/mL) or left untreated for 6 days. Sagital sections of rhCTGF treated rat lenses showed evidence of distinct fibrotic plaques within the anterior lens epithelium. This is in contrast with untreated control lenses which

maintained a distinct cuboidal LEC monolayer (FIG 4.6) Trichrome staining of rhCTGF treated lenses revealed the deposition of collagen within the fibrotic plaques which was not observed in control lenses (FIG 4.6). In addition, plaques from rhCTGF treated lenses exhibited positive immunostaining for αSMA protein expression (FIG 4.6). As expected, the LEC layer of untreated control lenses failed to exhibit detectable αSMA protein expression levels. The involvement of MMPs in CTGF-mediated ASC formation was next assessed through use of gelatin zymography of conditioned media obtained from rhCTGF treated lenses following the completion of the 6 day treatment period. Conditioned media from both treatment groups exhibited distinct bands on gelatin gels, indicating the presence of MMPs with gelatinolytic and/or collagenolytic activity (Fig 4.7) Conditioned media from control lenses exhibited expression of a 92-kDa band, as well as a 65-kDA band, corresponding to the pro-form of MMP9 and active form of MMP2 respectively (Alexander and Werb 1989). In comparison with control lens media, media from lenses treated with rhCTGF exhibited elevated levels of both gelatinolytic and/or collagenolytic bands.

4.3 DISCUSSION

A role for TGFβ-induced p38 and ERK1/2 MAPK signaling leading to transformation of epithelial to mesenchymal-like cells has been established for a variety of tissues (Yu, Hebert et al. 2002; Bates and Mercurio 2003; Xie, Law et al. 2004; Davies, Robinson et al. 2005; Compton, Potash et al. 2006). More specifically, in rat tubular proximal epithelial cells, TGFβ2 activation of p38 is associated with the increase of the EMT marker, αSMA, while specific inhibition of p38 significantly reduced αSMA expression (Rhyu, Yang et al. 2005). In the eye, the role of these MAPKs in TGFβ-mediated EMT is emerging. Our data suggest that the human FHL-124 cell line exhibited significant activation of both p38 and ERK 1/2 activity following stimulation with TGFβ2. To corroborate our *in vitro* cell culture findings, *ex vivo* rat lenses were treated with TGFβ2 for 3hr and examined for p38 activation. These results also showed activation of p38 and further support our claim that this MAPK may play a role in TGFβ-mediated EMT of LECs. This suggests that p38 mediates some of the transforming effects of TGFβ2. Therefore, studies in FHL-124 cells were carried out to determine if specific inhibition of p38 by SB202190 affects TGFβ-induced EMT and subsequent lens fibrosis.

Specific inhibition of p38 in TGFβ-stimulated FHL-124 cells resulted in decreased αSMA protein expression, suggestive of an involvement for p38 in lens fibrosis. To further explore the role of p38 in cataract development, whole rat lenses were co-cultured with TGFβ2 and SB202190. Interestingly, in contrast to our hypothesis, specific inhibition of p38 did not alter cataract formation, nor did it reduce αSMA

expression. It is possible that the lens capsule, which acts a selective barrier, prevented the SB202190 from entering the lens. In this study we did not assess whether the SB202190 did in fact reach the LECs. Therefore, future studies utilizing rat lens epithelial explant cultures which allow direct access to LECs should clarify the role of p38 in mediated TGFβ-induced EMT.

As an inducer of fibrosis, CTGF Maintenance of the fibrotic response generated from TGFβ signaling involves synergy with CTGF in the skin (Bonniaud, Martin et al. 2004). In contrast, very little is known about the role of CTGF in ocular fibrosis. What is known, is that FHL-124 cells treated with TGFβ exhibit significantly elevated levels of CTGF mRNA transcript and correlate with αSMA expression following epithelial differentiation (Wormstone, Tamiya et al. 2004). Abrogation of TGFβ signaling using a specific TGFβ antibody has been shown to prevent epithelial differentiation and its associated increase in CTGF expression (Wormstone, Tamiya et al. 2004). Although studies have not directly tested the requirement of CTGF in ASC, they do suggest that this cytokine may be involved in plaque formation.

Our current study is the first to implicate CTGF in cataract development. FHL-124 cells stimulated exclusively with rhCTGF were shown to express the myofibroblast marker αSMA, suggesting that CTGF mediates at least some component of LEC transformation. Importantly, treatment of whole rat lenses with rhCTGF was also shown to elicit formation of distinct focal opacities. Plaques resulting from CTGF treatment show both collagen deposition and positive αSMA immunoreactivity, suggesting a role for this cytokine in ocular fibrosis. One possible mechanism by which CTGF may be

able to mediate EMT leading to fibrosis in LECs may be through the involvement of MMPs. Our previous studies have implicated a role for MMPs, specifically MMP2 and MMP9, in the EMT associated with TGFβ-induced ASC formation (Dwivedi, Pino et al. 2006). In the present study, we have determined that treatment of FHL-124 cells with rhCTGF resulted in the increase of MMP2 protein expression. Furthermore, whole rat lenses exposed to CTGF for 6 days responded by increasing secretion of both MMP2/9 into the culture medium. These findings are consistent with other ocular epithelial ocular tissues treated with CTGF. Human retinal pigmented epithelial (ARPE-19) cells treated with rhCTGF have been observed to possess elevated levels of ERK1/2 associated with significant increases in both MMP2 mRNA and protein expression as well as increased secretion into the culture medium (Nagai, Klimava et al. 2009).

Our data show TGFβ signaling in LECs likely involves the co-operation of MAPKs p38 and ERK1/2 to increase known fibrotic modulators such as CTGF leading to increased MMP expression and activity and causing lenticular fibrosis. Further experimentation isolating the specific involvement of both p38 and ERK1/2 in MMP2/9 induction using the rat lens explant culture is necessary. Furthermore, future studies are aimed at the examination of the effect of how p38, ERK1/2, MMP2, and MMP9 on E-cadherin expression in both FHL-124 and rat lens explant culture, as this adhesion molecule has been shown to be directly implicated in the events leading to EMT.

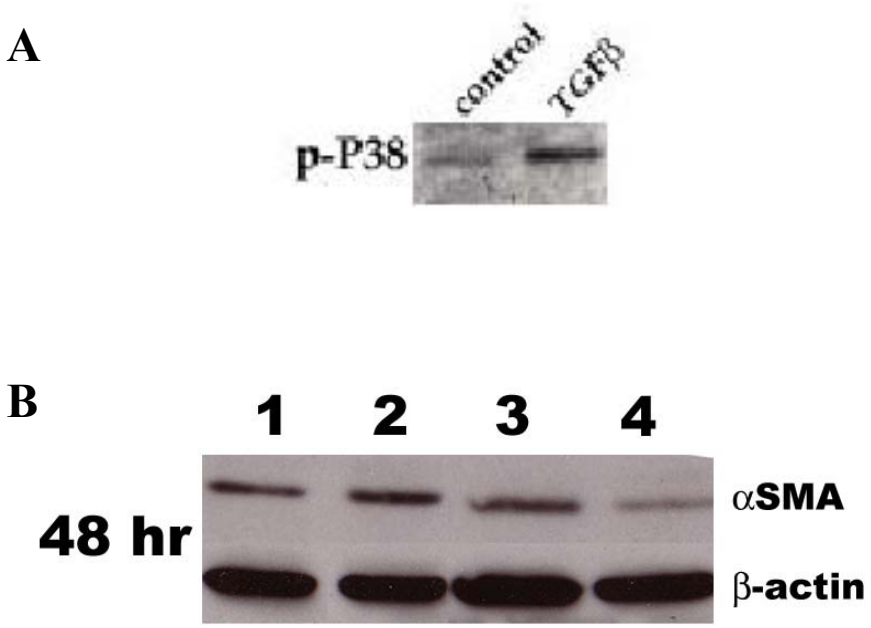

Fig 4.1 (A) Treatment of whole rat lenses with TGFβ (2ng/mL) for 3hrs exhibited increased phosphorylation of p38 when compared to control untreated whole lenses without TGFβ. (B) FHL 124 cells stimulated with TGFβ2 for 48hr (lane 2) exhibit increased αSMA as compared to untreated control cells (lane 1). Co-treatment of TGFβ2 with SB202190 at 1μM (lane 3) results in αSMA production which resembles TGFβ2 treatment alone. Importantly, Co-treatment of TGFβ2 with SB202190 at 10μM (lane 4) results in a reduction of αSMA production compared to both TGFβ-stimulated and untreated control FHL 124 cells.

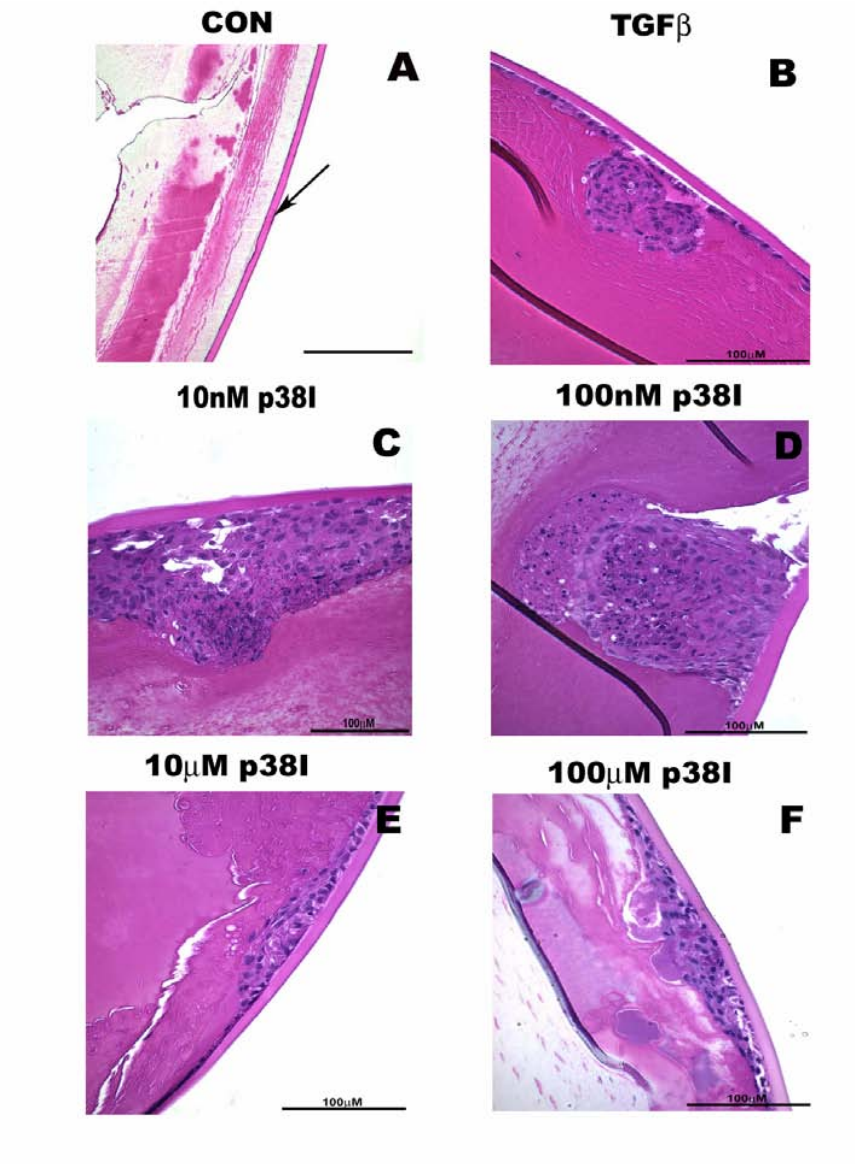

Fig 4.2 Sagital sections stained with hematoxylin and eosin of untreated control lenses (A) exhibit the characteristic lens epithelial monolayer directly beneath the lens capsule (arrow). Treatment with TGFβ2 results in ASC formation (B). Co-treatment with SB202190 at concentrations rangeing from 10nM to 100μM (C-F) results in plaque formation.

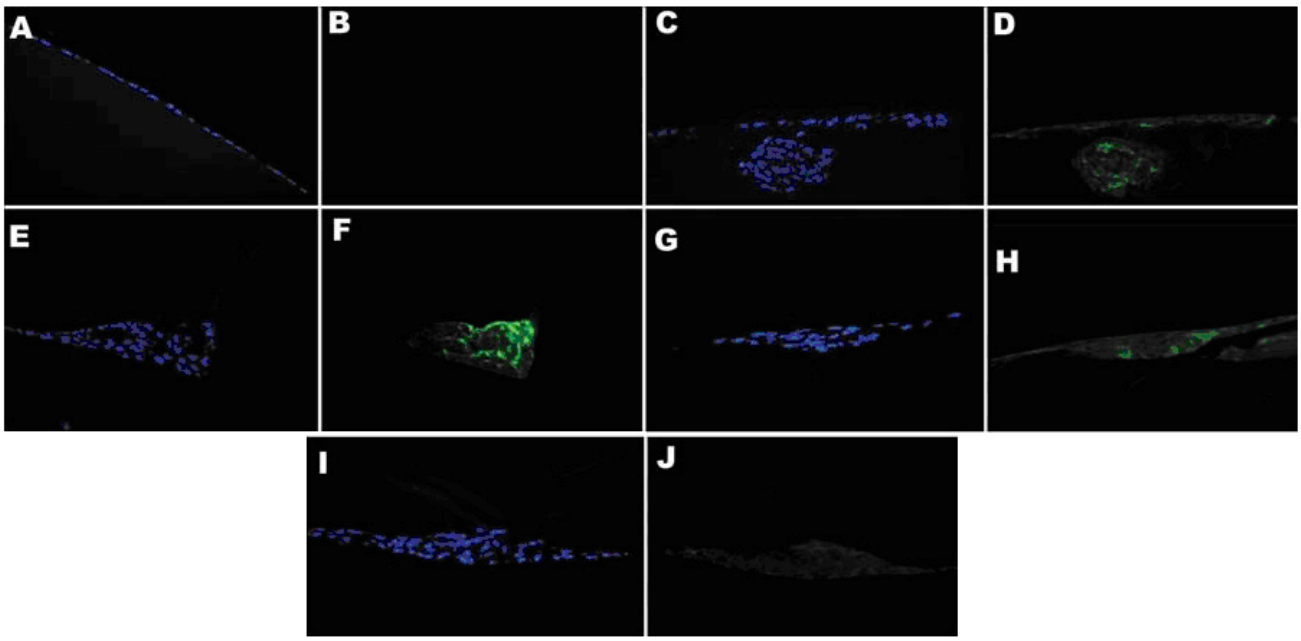

Fig 4.3 Sagital sections of excised rat lenses cultured in media alone exhibited the characteristic epithelial monolayer (blue) (A), which did not express αSMA (B). In contrast, lenses treated with TGFβ2 exhibited ASC formation (C), and positive reactivity for αSMA (green) (D). Co-culturing rat lenses with TGFβ2 and the p38 inhibitor, SB202190, at various concentrations (10nM (E,F), 100nM (G-H), 10μM (I,J) all resulted in sustained ASC formation along with positive αSMA immnoreactivity (green).

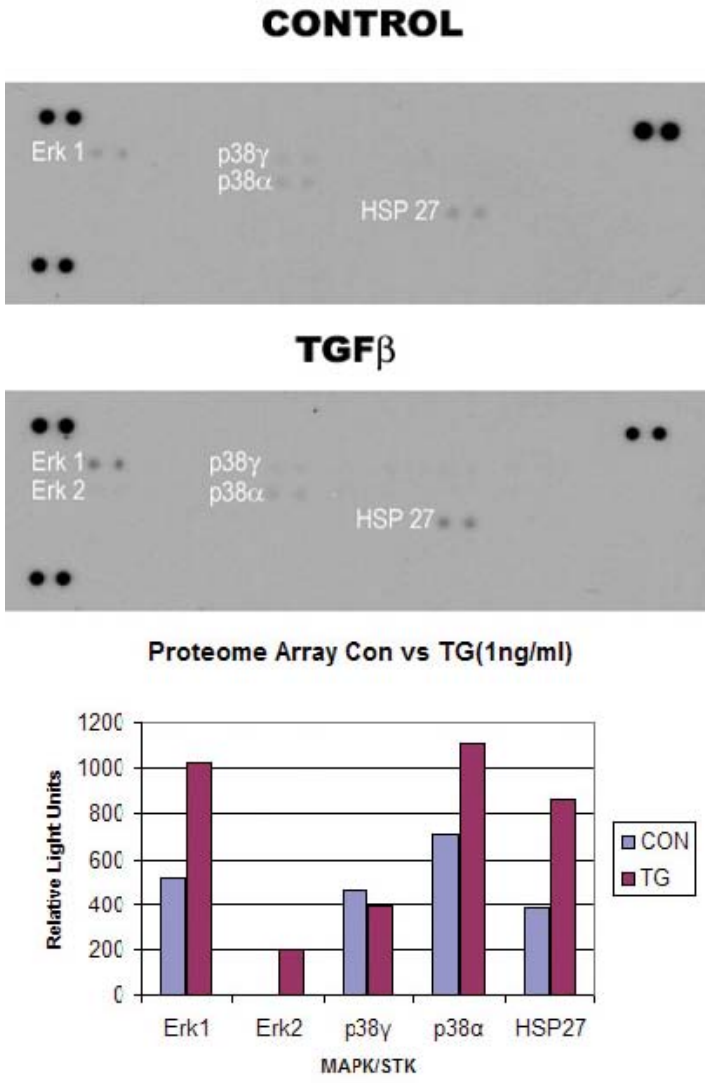

Fig 4.4 FHL-124 cells maintained in serum free conditions for 3 hours evidence active ERK1, p38α, p38γ, and HSP27 (CON). FHL-124 cells stimulated with TGFβ2 for 3 hours demonstrate phosphorylated ERK1, p38α and HSP27 above controls (TG). In addition, TGFβ-stimulated cells exhibit the presence of ERK2 and slight decrease in activation of p38γ. Quantification determined that ERK 1 was phosphorylated nearly 2-fold higher, p38α 1.5-fold higher and HSP27 just over 2-fold higher in TGFβ-stimulated vs untreated control cells.

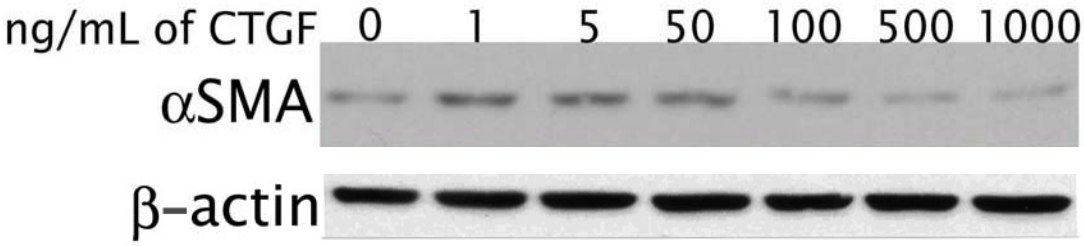

Fig 4.5 Treatment of FHL-124 cells with recombinant human CTGF (rhCTGF) for 24hrs resulted in dose dependant stimulation of the EMT marker αSMA. FHL124 cells not stimulated with CTGF (controls – 0 ng/mL) evidenced a basal level of αSMA protein expression. Importantly, treatment with 1, 5, 50, or 100 ng/mL of rhCTGF resulted in upregulation of αSMA above control levels. At higher concentrations, 500 and 1000 ng/mL, the FHL124 cells failed respond to CTGF-mediated EMT as monitored by αSMA, and resembled controls. Interestingly, a dose-dependent increase in MMP2 protein expression was evidenced. β-actin was used as a loading control and does not shown changes across treatments.

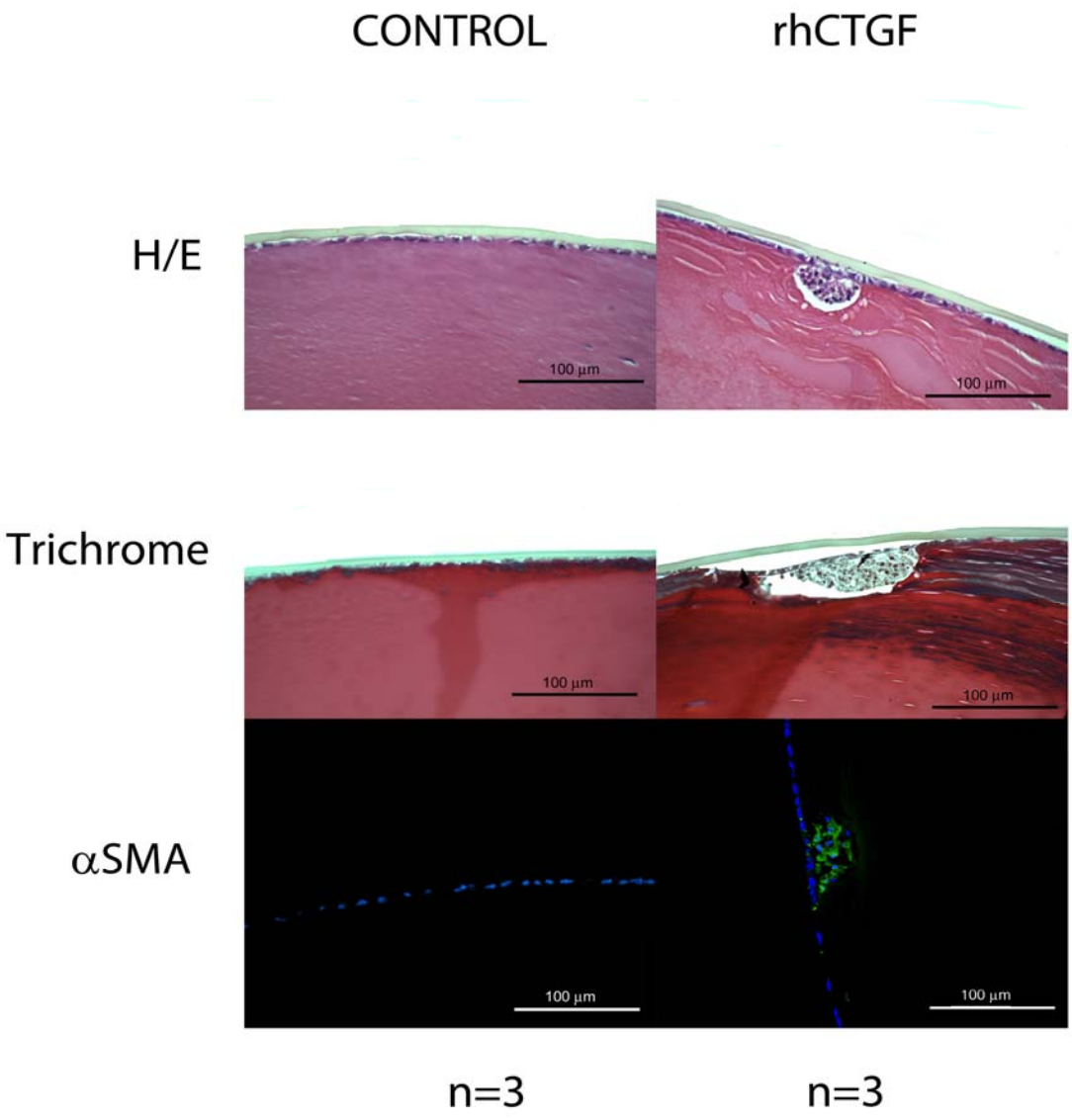

Fig 4.6 Excised rat lenses supplemented exclusively with rhCTGF (50ng/mL) exhibit ASC formation whereas control lenses without supplementation are devoid of opacities and retain their characteristic cuboidal epithelial monolayer (control). Consequent cataract formation is associated with matrix deposition of collagen (Trichrome) and positive αSMA immunoreactivity (green), which is absent in control lenses. Nuceli are stained with DAPI (blue)

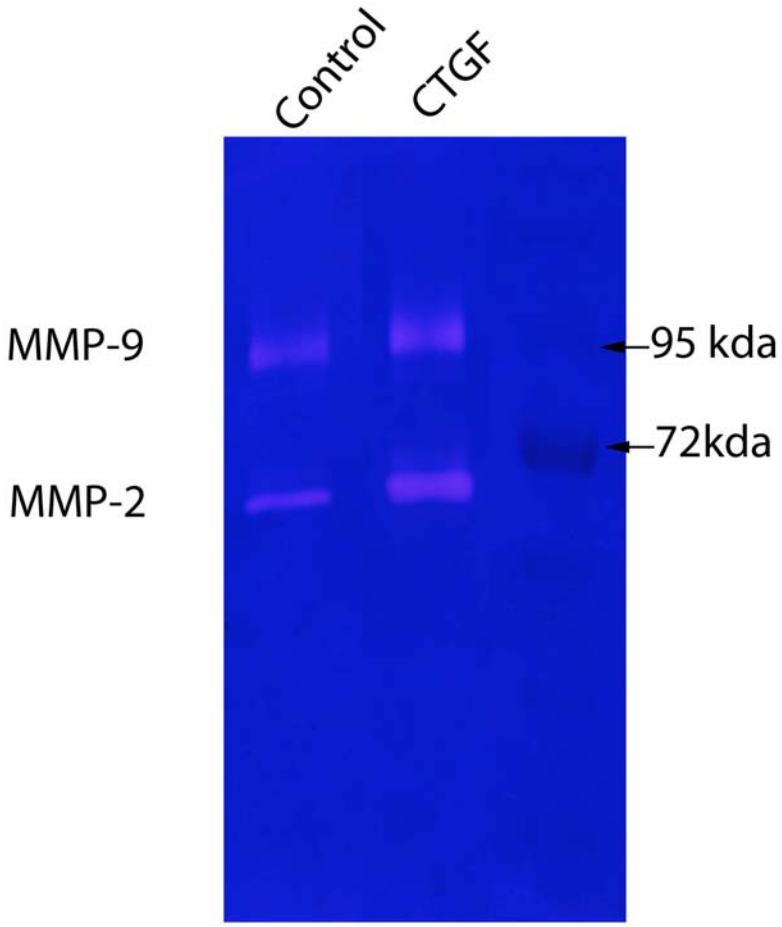

Fig 4.7 MMP9 (approximately 92kDa) and MMP2 (approximately 65kDa) were detected in media from the untreated (Control) (n=3) lenses at day 6 of the culture period. Following 6-days of treatment with rhCTGF (50ng/mL) (n=3)(CTGF), up-regulated levels of both MMP9 and MMP2 were observed relative to controls. Band were compared with Coomassie Brilliant Blue stained protein ladder to determine approximate sizes.

4.4 MATERIALS AND METHODS

4.4.1 Ex-vivo Rat Lens Cataract Model

The previously established TGFβ-induced rat lens model was utilized for the p38 inhibitor studies (Hales, Chamberlain et al. 1995). Briefly, lenses were obtained from adult male Wistar rats and cultured in 3.5ml of serum free M199 medium supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml fungizone (Amersham Biosciences) overnight. The following day lenses were either left untreated or treated with recombinant human TGFβ (R&D Systems) at a final concentration of 2 ng/mL. Lenses were also co-treated with TGFβ and the p38 inhibitor SB202190 at concentrations raging from 100nM to 100µM. Lenses were then harvested at time-points of 6 days and subsequently fixed for histology and immunofluorescence. The protocol was next modified in order to determine the effect of rhCTGF on ASC formation. Lenses from adult male Wistar rats wer cultured in 3.5 mL of serum free M199 medium supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml fungizone (Amersham Biosciences) overnight. The following day lenses were either left untreated or treated with rhCTGF (Peprotech) at a final concentration of 50 ng/mL. Lenses were then harvested at time-points of 6 days and fixed for histology and immunofluorescence.

4.4.2 FHL-124 Cell Culture

FHL-124 cells were cultured in six-well plates and maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% FCS, 1%

penicillin/streptomycin, 1% gentamycin and L-glutamine. FHL-124 cells were cultured until they reached 100% confluence, at which point they were serum starved overnight. Subsequently, four treatment conditions consisting of: (1) control (serum-free media); (2) rhTGFβ (10ng/mL) alone; (3) TGFβ (10ng/mL) + SB202190 (1μM); and (4) TGFβ (10ng/mL) + SB202190 (10μM) were examined at 48hr. At the end of the time point, the media was removed, the cells washed with PBS and 250μl Triton X lysis buffer was added to each well for 5 min and subsequently frozen for at least one hour at -80°C. Following this, the cells were allowed to thaw, scraped off each well, and subjected to sonic membrane disruption for 30 seconds.

4.4.3 Histology and Immunofluorescence

Lenses were collected from different treatment groups and fixed overnight in 10% neutral buffer formalin, dehydrated, embedded in paraffin, and processed for routine histology. For histological analysis, 5μm sections were stained with hematoxylin and eosin. Gormori's trichrome stain was applied to 5μm thick paraffin-embedded sections. Immunofluorescence was performed on 5μm thick paraffin-embedded sections. Sections were incubated with primary antibody specific for αSMA (1:100, Sigma) and bound primary antibodies were visualized with a fluorescein-isothiocyanate (FITC) anti-mouse secondary antibody, (1:50, Jackson ImmunoResearch Laboratories). All sections were mounted in Vectasheild mounting medium with 4',6-Diaminodino-2-Phenylindol (DAPI, Vector Laboratories) to visualize the nuclei.

4.4.4 Zymography

Conditioned media from all treatment groups were concentrated using 3.5-ml 10kDa Microsep concentrating devices (Viva Sciences, Hanover, Germany). The media were centrifuged at 1000 g (at room temperature) for 5 minutes to pellet any debris before loading. Each device was loaded with an equal volume of supernatant, and the concentration was performed by centrifugation at 25°C for 20 minutes at 3000g. An equal volume of each concentrate was electrophoresed on 10% SDS-polyacrylamide gels containing 0.1% gelatin as the substrate. After electrophoresis, the gels were developed as described previously(Heussen and Dowdle 1980) and stained in 0.5% Coomassie brilliant blue for 1 hour followed by destaining with 10% isopropanol. Sites of gelatinase activity were detected as clear bands against a background of uniform staining, which was digitally photographed.

4.4.5 Proteome Array

Cell lysates of FHL-124 from control and TGFβ (10ng/mL) treated were compared for differences in phosphorylation of MAPKs and other serine/threonine kinases. Cells were lysed at > $1*10^7$ cells/ml and the supernatant collected contained total protein at concentrations greater than 1µg/µl. Briefly, each array used was washed in Array Buffer 1 for 1 hr at room temperature. Following this, Array Buffer 1 and sample protein was added in a 5:1 ratio with the total protein added equaling nearly 300µg per array and allowed to incubate at 4^oC overnight on a shaking platform. The following day each array was washed 3 x 13 min in wash buffer on a rocking platform. Detection

antibody cocktail was diluted 1:1000 in Array Buffer 2/3 and allowed to incubate on each array for 2 hrs at room temperature followed by a wash as above. Streptavidin-HRP was diluted 1:2000 and allowed to incubate for 30 min at room temperature followed by a wash as previously described. Each array was then exposed to chemiluminescent reagents and exposed to X-ray film.

4.4.6 Western Blot Analysis

For samples from *ex vivo* lens culture, one lens from each treatment group was utilized. For cell lysates of FHL-124, confluent cells from one well of a 6-well dish were utilized. In all cases, the samples were homogenized in Trion-X 100 lysis buffer containing protease inhibitor cocktail (Roche Applied Science). A Bradford protein assay(Bradford 1976) was performed to ensure that equal amounts of total protein was electrophoresed on a 10% SDS-polyacrylamide gel. The resolved bands were electro-transferred onto a nitrocellulose membrane (Pall Corporation). Membranes were blocked with 5% skimmed milk powder in Tris-buffered saline (50mM Tris base, NaCl pH 8.5) and 0.1% Tween-20 and then incubated overnight at 4°C with antibodies generated against either αSMA (1:1000, Sigma), MMP2 (1:500, Chemicon), β-actin (1:1000, Abcam) was used as a loading control. Following this incubation, membranes were be washed 3x5min in PBS followed by probing with an HRP-conjugated secondary antibody (1:7000;Amersham Biosciences) for 2 hrs. Subsequently, membranes were washed 3x5 min in PBS and probed with ECL detection reagents (Amersham Biosciences) and finally exposed to x-ray film.

5.0 CHAPTER FIVE:

General Discussion

Cataract causes a clouding of the normally clear lens and is responsible for vision loss in nearly 18 million people worldwide. Although surgical intervention is available for individuals in developed countries, people in developing countries typically do not have access to surgical intervention and therefore are not able to generate a livelihood for their families. This, compounded with the fact that global cataract rates will drastically increase worldwide due to increased longevity, highlight the significant importance in understanding the disease mechanism in order to develop more cost-effective treatments. The current understanding of cataractogenesis is limited to our understanding of protein-protein interactions and significantly less is known about the molecular signaling cascades that are responsible for this disease. What has been studied in relation to molecular signaling events in cataracts has been determined by examining two related forms of fibrotic cataracts; posterior capsule opacification (PCO) and anterior subcapsular cataracts (ASC). Both PCO and ASC are similar in their disease progression in that they result from the transformation of epithelial cells to myofibroblast-like cells via a disease process known as epithelial to mesenchymal transition (EMT) (de Iongh, Wederell et al. 2005). The EMT that results in either PCO or ASC is similar to that which arises in fibrotic diseases and cancer which appear in other tissues like the lung, liver, kidney and skin (Zeisberg, Hanai et al. 2003; Yao, Xie et al. 2004; Han, Lu et al. 2005; Margetts, Bonniaud et al. 2005; Willis and Borok 2007). Therefore, it is reasonable *a priori* to postulate that there is potentially a significant amount of commonalities regarding the etiology of this disease mechanism across various tissues. In fact, the single largest deviation is perhaps only in the derivation of a sub-population of myofibroblasts. With

the exception of the lens, other tissues contain both fibroblasts and are vascularized and therefore receive nourishment from the systemic blood supply. As a result, a portion of the myofibroblasts derived from EMT in vascularized tissues is a result of resident fibroblast conversion (Abe, Donnelly et al. 2001; Iwano, Plieth et al. 2002; Kalluri and Neilson 2003). In contrast, the lens is a simple structure composed of only one nucleated cell type, the lens epithelial cells (LECs) and nourished by two distinct humors. The posterior aspect of the lens is bathed in the vitreous humor while the anterior region containing a monolayer of LECs, derives its nourishment from the aqueous humor. Furthermore, the lens contains no resident fibroblasts and as a result of its avascularity, also contains no circulating fibroblasts. Thus, one can reasonably assume that the entire myofibroblast profile in LEC EMT is a result of the direct conversion of epithelial cells to myofibroblasts. In essence, true EMT occurs in the lens unlike other tissues. Therefore, understanding the mechanisms associated with LEC EMT may be of significant benefit in determining the similarities and differences in the etiology of related EMT events of other tissues.

Typically, pathological EMT is the result of tissue injury (either mechanical or biochemical) leading to an increased release of cytokines, which result in ordered disassembly of the apical-basal polarity axis of epithelial cells via the release from both the basement membrane and surrounding epithelia. With this coordinated loss of polarity and detachment from the basement membrane as well as neighbouring epithelia, the cells acquire a myofibroblast phenotype and begin to migrate as well as aberrantly secrete extra cellular matrix and cytoskeletal molecules such as type I and type III collagen, as

well as alpha smooth muscle actin (αSMA) (de Iongh, Wederell et al. 2005). Irrespective of the tissue of origin, the summation of these events in fibrosis leads to impaired tissue function ultimately leading to failure while in cancer this leads to metastasis. One benefit of the lens is that its removal and replacement following failure is feasible, accessible and possible. However, secondary cataracts or PCO develops when LECs damaged during surgical intervention are not removed and undergo EMT (Kappelhof and Vrensen 1992). Similarly, when this EMT occurs in the anterior region of the lens as a result of topical steroid use and alongside other diseases such as atopic dermatitis and retinitis pigmentosa, it is called ASC. (Dilley, Bron et al. 1976; Carmi, Defossez-Tribout et al. 2006). Another similarity between fibrosis and cancer metastasis of other tissues as well as PCO and ASC is the EMT-inducing molecule, transforming growth factor beta (TGFβ) (Hales, Chamberlain et al. 1995; de Iongh, Wederell et al. 2005). Elevated levels of TGFβ have been found in clinical instances of both ASC and PCO. Furthermore, it has been established that TGFβ is responsible for the ensuing development of both PCO and ASC. In fact, all 3 distinct animal models which closely resemble ASC are initiated by TGFβ. These include: (1) culturing lenses in the presence of active recombinant TGFβ2; (2) direct intracameral injection of self activating form of TGFβ1; and (3) transgenic expression of self activating form of TGFβ1 under control of a lens specific promoter Tg(Craa-Tgfb1)853Ove (Hales, Chamberlain et al. 1995; Srinivasan, Lovicu et al. 1998; Robertson, Nathu et al. 2007). In each of these models, TGFβ is able to initiate a program where the cuboidal monolayer of LECs undergo an EMT event and develop into myofibroblasts which are responsible for diminished lens clarity and ultimate disruption

of vision. What has remained elusive in each of these models is the exact mechanism by which TGFβ is able to induce EMT leading to ASC.

Enzymes that degrade the extracellular matrix (ECM) have long been viewed as essential for diseases such as cancer and fibrosis where destruction of matrix barriers is responsible for EMT leading to pathology. In cancer, destruction of matrix barriers is critical in tumor metastasis where destruction of matrix barriers surrounding the tumor permitting invasion into surrounding connective tissues and entry and exit from blood vessels is necessary for metastasis. In fibrosis, ECM degrading molecules are necessary to release the polarized epithelial cells from the basement membrane allowing for EMT. Matrix metalloproteinases (MMPs) are endopeptidases and are prime candidates for these activities. This is because: (1) MMPs can collectively degrade all structural components of the ECM; (2) MMPs exert non-matrix degrading capacities including the propensity to act on cytokines, chemokines, cellular surface proteins, cellular receptors and junctional proteins (Levi, Fridman et al. 1996; McQuibban, Gong et al. 2000; McCawley and Matrisian 2001; McGuire, Li et al. 2003; Stamenkovic 2003; Page-McCaw, Ewald et al. 2007; Van Lint and Libert 2007); (3) MMPs are upregulated in virtually all human and animal tumors; and (4) in fibrotic diseases where TGFβ is a central mediator of development, deregulation of MMPs has been cited as a primary cause. Therefore it is reasonable to assume that MMPs are a root cause of an assortment of pathological contexts associated with EMT (McCawley and Matrisian 2001; Stamenkovic 2003; Parks, Wilson et al. 2004; Cauwe, Van den Steen et al. 2007).

In order to assess the involvement of MMPs in matrix degrading diseases, a significant number of inhibitors have been developed over the past thirty years, starting with broad spectrum MMP inhibition. In these early studies, MMP inhibition in tumor cell lines was able to block experimental metastasis in mouse models (Reich, Thompson et al. 1988). Those seemingly promising experiments gave way to clinical trials which revealed that prolonged treatment with MMPIs caused musculoskeletal pain and inflammation. These adverse side effects resulted from the inhibition of ubiquitous MMPs which are responsible for homeostasis (Coussens, Fingleton et al. 2002). Therefore significant efforts have been invested in determining which specific MMPs result in disease progression and which ones are necessary for cellular homeostasis. This is where experiments conducted in ocular tissue and more specifically in PCO and ASC are able to both benefit from previous research in ECM degrading diseases and perhaps advance it.

In the eye, MMPs have been shown to contribute to a number of ocular diseases and disorders including glaucoma, corneal ulcers and corneal postoperative haze (Sivak and Fini 2002). More recently, induction of MMP expression has been correlated with the formation of cataracts (Tamiya, Wormstone et al. 2000; Seomun, Kim et al. 2001; Wormstone, Tamiya et al. 2002). However, the role of MMPs and the mechanism by which they contribute to ASC is not well understood. What is known is that in the lens, gelatinases (MMP2 and MMP9) are upregulated during ASC formation driven by TGFβ. If MMPs are involved in TGFβ mediated ASC then the inhibition of MMP2 and MMP9 should lead to positive outcomes such as those evidenced in tumor studies. In fact, in Chapter 2 it was determined that using the established *ex vivo* rat model where TGFβ2

initiates cataractogenesis, co-treatment with the broad spectrum MMP inhibitor GM6001 resulted in suppression of ASC formation. Furthermore, this included suppressing the appearance of the known EMT marker αSMA. This important result is the first to identify that MMPs are involved in TGFβ mediated EMT leading to ASC. It is equally important to determine which specific MMPs are involved in ASC in order to determine what MMP inhibitors are likely to have the most beneficial clinical impact with the least amount of negative side effects. Therefore, the same strategy with a specific inhibitor for both MMP2 and MMP9 was employed. These results indicated that similar to the broad spectrum matrix metalloproteinase inhibitor (MMPI) GM6001, an MMPI for both MMP2 and MMP9 was able to abrogate all of the TGFβ2 mediated effects of ASC including the prevention of EMT.

In light of the fact that inhibition of both gelatinases results in suppression of ASC, it is of paramount importance to determine the specific roles that MMP2 and MMP9 each play in this disease. A recent study revealed that *Mmp9* and *Mmp2* mRNA are expressed in the normal rat lens epithelium (Nathu, Dwivedi et al. 2009). This suggests that constitutive mRNA expression of these MMPs does not result in the EMT of LECs and cataract formation. However, induced levels of *Mmp2* and/or *Mmp9*, above normal constitutive expression, along with their subsequent extracellular secretion of does result in EMT in the lens. In fact, Nathu *et al* have previously determined the temporal profiling of MMP expression in rat LECs treated with TGFβ. These studies revealed significant increases of *Mmp9* prior to *Mmp2* (Nathu, Dwivedi et al. 2009). Furthermore, the addition of recombinant human MMP9 to a human lens epithelial cell line, FHL-124,

demonstrated that MMP9 is able to induce both MMP2 as well as the EMT marker αSMA (Nathu, Dwivedi et al. 2009). These data suggest that in ASC, MMP9 is upstream of MMP2 in mediating ASC. Therefore, to assess the requirement of functional *Mmp9* in cataractogenesis, two distinct models of TGFβ-mediated ASC were examined.

The requirement of active MMP9 in ASC formation is supported by results in Chapter 3 where mice lacking proteolytically active MMP9 fail to elicit plaque formation upon adenoviral gene transfer of TGFβ1 to structures of the anterior chamber. In contrast, mice which retained at least one functional copy of *Mmp9* develop TGFβ-mediated ASC to a similar degree as controls which received AdTGFβ1. To ensure that the loss of *Mmp9* was in fact involved in EMT of LECs, sections were monitored for the expression of αSMA. These results indicate that plaques of AdTGFβ1 treated mice which contain at least one functional copy of *Mmp-9*, express the EMT marker αSMA. In contrast, the LECs of mice lacking proteolytically active *Mmp-9* which received AdTGFβ1 did not express αSMA. Thus, the data suggest that a lack of active *Mmp-9* is essential for TGFβ-mediated EMT and ASC formation.

To further corroborate the specific *in vivo* role(s) of *Mmp9* in ASC, a transgenic mouse model with continuous lens specific overexpression of TGFβ1 (Tg(Craa-Tgfb1)853Ove) was established in the absence of proteolytically active Mmp9 (Tg(Craa-Tgfb1)853Ove/$Mmp9^{-/-}$). These data revealed a suppression of cataract formation in 75% of Tg(Craa-Tgfb1)853Ove/$Mmp9^{tm(neo)abr/tm(neo)abr}$ lenses. These mice do not demonstrate the characteristic EMT marker αSMA nor do they display aberrant matrix deposition. In contrast, all Tg(Craa-Tgfb1)853Ove/$Mmp9^{+/+}$ lenses examined at both early and later

stages develop ASC and expressed both αSMA reactivity and matrix deposition. Interestingly, 25% of the Tg(Craa-Tgfb1)853Ove/*Mmp9*$^{tm(neo)abr/tm(neo)abr}$ mice were able to develop ASC. This suggests that MMP2, a MMP with similar structure, function and substrate specificity, may be able to compensate for the lack of active MMP9. Nevertheless, these data indicate that in fact *Mmp9* is a mediator of TGFβ-induced EMT leading to ASC. These significant findings are consistent with other reports in both malignant cancer and fibrosis. In cancer cells lines, MMP9 is the key biomarker that is able to accurately determine tumor metastatic ability and melanoma metastatic ability correlates positively with MMP-9 expression. (Bernhard, Muschel et al. 1990; van den Oord, Paemen et al. 1997). Furthermore, two distinct lung tumor cell lines derived from *Mmp9*$^{-/-}$ mice exhibited a 50% decrease in tumor metastasis when implanted into wildtype hosts and in renal fibrosis, tubular EMT was blocked in MMP9 deficient mice (Itoh, Tanioka et al. 1999; Wang, Zhou et al. 2010).

The exact roles which MMP9 may play during cataractogenesis remain to be determined. However, one strong possibility is that MMP9 is able to facilitate one of the primary events necessary for EMT; the dissolution of epithelial cell contacts. E-cadherin has been recognized as a critical factor in maintaining the epithelial cell state and its disruption has been associated with the beginning of transition to a mesenchymal phenotype (Imhof, Vollmers et al. 1983; Thiery 2002). Literature demonstrates that MMPs participate in the initial activation stages of EMT through dissociation of the E-cadherin/β-catenin complex (Ho, Voura et al. 2001; Mei, Borchert et al. 2002; George and Dwivedi 2004). Proteolytic cleavage of the N-terminal extracellular domain of E-cadherin

by MMPs, referred to as "E-cadherin shedding," results in the formation of an E-cadherin extracellular domain fragment with reported sizes ranging from 50 to 84kDa, compared with the intact 120kDa protein (Steinhusen, Weiske et al. 2001). Furthermore, in ovarian carcinoma it has been shown that epithelial differentiation to mesenchymal type, via E-cadherin disruption, occurs as a direct result of MMP9 protease activity (Symowicz, Adley et al. 2007). Interestingly, in Chapter 2 it was noted that E-cadherin shedding is a phenomenon associated with ASC. A shed 72kDa E-cadherin fragment appeared in the conditioned media of lenses treated with TGFβ2 that was not detected in the media from untreated lenses. The appearance of the E-cadherin fragment in the TGFβ2 treated rat lenses was also shown to coincide with enhanced levels of secreted MMP2 and MMP9. It was further demonstrated that the TGFβ2 induced levels of the E-cadherin fragment were attenuated by co-treatment with either GM6001, a broad spectrum MMPI, or an MMP2/9 specific inhibitor. This suggests that TGFβ2 induced fragmentation of E-cadherin is mediated by MMP activity. Furthermore, examination of E-cadherin mRNA within these treatments revealed that E-cadherin mRNA was significantly downregulated with treatment of TGFβ2 alone and significantly increased when TGFβ2 was co-cultured with the MMPI GM6001. This highlights the fact that either MMP2 or MMP9 is able to mediate transcriptional activity of E-cadherin. In fact, a recent study of a highly invasive tumor cell line determined that specific knockdown of *Mmp9* was able to suppress cell invasion and motility as well as decrease the E-cadherin transcriptional repressor, Snail (Lin, Tsai et al. 2011). The specific effect of *Mmp9* suppression on E-cadherin transcription or its transcriptional repressor Snail was not examined in this study.; yet,

other experimental data presented do suggest that *Mmp9* is a mediator of E-cadherin modulation leading to EMT in ASC. Support for the fact that MMP9 is the mediator of E-cadherin shedding is corroborated by data obtained from rat lens explants. In Chapter 3, rat lens explants treated with TGFβ2 alone displayed loss of E-cadherin at the cell-cell borders. In contrast, the co-treatment of rat lens epithelial explants with TGFβ2 and an MMP9 specific inhibitor was able to prevent E-cadherin loss. Furthermore, the treatment of lens explants with recombinant MMP9 alone was able to induce E-cadherin loss at the cell borders similar to TGFβ alone.

In light of these data presented above, MMP9 is emerging as a critical mediator of ASC. One might suspect that removing a factor which is responsible for matrix degradation would exacerbate a matrix-associated disease since it would prevent the turnover from occurring and therefore lead to increased fibrosis. This would be the case if the same molecule were not responsible for the formation of the disease itself at earlier stages. This is in fact what the data regarding MMP9 in ASC are beginning to indicate. Two important factors are necessary for progression of ASC: (1) the accumulation of enough cues to initiate ASC; and (2) the ability for these cues to enter the epithelial cells and initiate the molecular signaling cascade to allow for EMT; beginning with the dissolution of the cell-cell contacts. Both of these roles are established for MMP9 in diseases such as fibrosis and cancer. The data presented here clearly indicate a similar role for MMP9 in TGFβ mediated ASC. Furthermore, the failure of many MMPIs in clinical trials of cancer thus far may be attributed to two important features (Coussens, Fingleton et al. 2002). Firstly, that one specific MMP has yet to be isolated as a sole

mediator of malignancy, although very recent data have determined that MMP9 may be this mediator. Secondly, the EMT which occurs in other systems involves the conversion of resident or circulating fibroblasts to myofibroblasts. In contrast, the work presented here demonstrates that MMP9 is likely the necessary factor responsible for the EMT which occurs in ASC that is derived solely from a population of epithelial cells. Future experiments aimed at determining the exact mechanism whereby MMP9 mediates ASC are necessary. It is likely that determining the effects of MMP9 on E-cadherin repressor elements such as Snail will assist in specifying the mechanism. Furthermore, experiments aimed at isolating the physiological levels of increased MMP9 necessary to mediate both E-cadherin shedding and its other roles are important. Additionally, it will be equally as important to determine what effects the related MMP, MMP2 is able to exert in ASC. Assessing the *in vivo* role of MMP2 in cataractogenesis is possible though the use of a mouse strain which lacks functional MMP2 (Itoh, Ikeda et al. 1997). Thus, future studies which examine cataractogenesis in mice which lack functional MMP2 in the presence of lens specific TGFβ-overexpression will assist in determining the specific involvement of MMP2. It is only with this complete understanding of the cognate and overlapping roles of these related MMPs that effective therapeutics can be generated to avoid the pitfalls previously demonstrated in clinical trials of MMP inhibition.

An understanding of the cofactors necessary for TGFβ mediated EMT leading to ASC is essential in developing strategies to combat this disease. Given that the fibrosis in the lens is similar to other tissues it is possible that the cofactors in ASC are also in common with other EMT related fibrotic disorders. It has been reported that the fibrosis

initiated by TGFβ in tissues such as lung and skin is known to involve other growth factors. One specific growth factor associated with these fibroses is connective tissue growth factor (CTGF). CTGF in the lung, is able to promote EMT leading to fibrosis by synergizing with TGFβ (Bonniaud, Martin et al. 2004). This synergy allows CTGF to maintain a chronic fibrotic response as a result of ECM production, proliferation and migration. In the skin, CTGF itself is generally a poor inducer of fibrosis and subcutaneous injection of CTGF alone has negligible effect. In contrast, co-administration of CTGF with TGFβ results in sustained fibrosis (Mori, Kawara et al. 1999). Furthermore, studies in pulmonary fibrosis demonstrate that CTGF is necessary in restoring a profibrotic environment in mice which have developed resistance to fibrosis (Bonniaud, Martin et al. 2004). Although CTGF exerts a tissue specific effect, these data illustrate that CTGF is involved in fibrotic events of both the skin and lungs.

In contrast to other organs, relatively little is known regarding the role of CTGF in lens fibrosis. What is known is that the ocular tissue of patients with either ASC or PCO contains elevated levels of CTGF (Wunderlich, Pech et al. 2000). In addition, LECs cells treated with TGFβ exhibit significantly elevated levels of CTGF transcript and correlate with αSMA expression following epithelial differentiation. Furthermore, abrogation of TGFβ signaling using a specific TGFβ antibody is able to prevent epithelial differentiation and associated increase in CTGF (Wormstone, Tamiya et al. 2004). Although these data have not tested the direct requirement of CTGF in ASC, they do suggest that this cytokine may be involved in ASC formation. Data in Chapter 4 showed that treatment of FHL-124 cells with rhCTGF resulted in an increase of the EMT marker

αSMA as well as MMP2. The fact that CTGF was able to induce EMT associated molecules warranted experimentation to determine if this growth factor is able to initiate a fibrotic response in whole lenses. In fact, data presented in Chapter 4 showed that rat lenses treated with rhCTGF do develop ASC, which are also αSMA positive, suggesting that CTGF is able to initiate EMT. Furthermore, conditioned media from rat lenses treated with rhCTGF, which developed ASC, contained increased secretion of both MMP2 and MMP9. Taken together, these data indicate that in the lens, CTGF is able to mediate a fibrotic response that contributes at least in part to the elevation in expression of MMP2 and MMP9 and the induction of the EMT marker, αSMA. Our data regarding CTGF in the lens corroborate studies in other epithelial cell tissues such as kidney tubular epithelial cells, which undergo a partial EMT and increased αSMA production following treatment with either adenoviral CTGF of high doses of rhCTGF. Interestingly, in tubular epithelial cells, siRNA silencing of CTGF following stimulation with TGFβ was able to attenuate EMT (Burns, Twigg et al. 2006). These findings suggest that CTGF may also play an important role in TGFβ-mediated EMT and subsequent ASC. Elucidating the specific role of CTGF in TGFβ-mediated EMT is a primary goal for future experimentation. It is likely that CTGF is able to contribute to EMT in the lens by upregulating necessary factors such as MMP2 and MMP9. In order to clarify the mechanisms of CTGF involvement, future experiments are aimed at isolating the signaling factors initiated by TGFβ which modulate CTGF expression and lead to ASC. Furthermore, identifying the necessity of CTGF through its specific inhibition following

TGFβ stimulation will undoubtedly refine our understanding of this growth factor in cataractogenesis.

In addition to understanding the TGFβ cofactor profile, it is necessary to determine what specific TGFβ mediated signaling pathways are involved in ASC. TGFβ-induced MAPKs signaling leading to EMT has been established in a variety of tissues (Yu, Hebert et al. 2002; Bates and Mercurio 2003; Xie, Law et al. 2004; Davies, Robinson et al. 2005; Compton, Potash et al. 2006). In the eye however, the role of these MAPKs in TGFβ mediated EMT are inconclusive. Examination of HLE-B3 LECs for MAPK involvement during TGFβ-induced EMT determined that PI3K rather than p38 and ERK 1/2 were activated (Yao, Ye et al. 2008). A recent study has determined that treatment of the LEC line, FHL-124, treatment with TGFβ significantly increased both p38 and ERK 1/2 phosphorylation following 30 min of stimulation (Dawes, Sleeman et al. 2009).

To further elucidate the role that TGFβ signaling profile of stimulation in FHL-124 cells, a proteome screen was performed in Chapter 4. These results showed increased phosphorylation of ERK1, ERK2, p38α and HSP27, a downstream p38 target, above controls. These data further confirm that in FHL-124 cells treated with TGFβ, activation of p38 and ERK MAPK likely contributes to the ensuing EMT and ASC that develop.

In order to assess the role that p38 may exert in LECs, a p38 specific inhibitor, SB202190, was co-cultured with TGFβ2. These results identified that specific inhibition

of p38 was able to attenuate the upregulation of the myofibroblast marker αSMA when co-treated with TGFβ2 and suggests that inhibition of p38 by SB202190 is able to suppress the EMT of LECs mitigated by TGFβ. The ability of SB202190 to suppress TGFβ induced EMT has previously been demonstrated in murine mammary gland epithelial (NMuMG) cells. SB202190 treatment maintained normal epithelial morphology following TGFβ1 exposure, also preventing TGFβ1 mediated NMuMG cell elongation and actin stress fiber formation (Bakin, Rinehart et al. 2002). Since SB202190 is able to reduce the expression of the myofibroblast marker αSMA in FHL-124 cells, and SB202190 is able to prevent EMT in NMuMG cells, experiments in Chapter 4 examined the effect of p38 inhibition on ASC formation in cultured rat lenses. Inhibition of p38 by SB202190 was unable to reduce the appearance of ASC in TGFβ2 stimulated whole rat lenses. Furthermore, SB202190 was unable to reduce the *de novo* production of αSMA in TGFβ treated lenses. These results suggest that p38 inhibition may be independently insufficient in preventing TGFβ induced ASC. Alternatively, the lens capsule, which natively acts a barrier, prevented uptake of SB202190 by the LEC monolayer.

In addition to p38 MAPK, the proteome screen which was performed in Chapter 4 also identified HSP-27 as an activated target of TGFβ-induced LECs. The small heat shock protein HSP-27 is a well documented downstream target of p38 activity and has been shown to be an ideal candidate since it exerts cytoprotective functions (Doshi, Hightower et al. 2010). HSP-27 is a multifunctional heat shock protein capable of mediating distinct functions depending on the local cellular conditions. Under stress free conditions, unphosphorylated HSP-27 provides cytoskeletal support via its interaction

with actin (Bryantsev, Kurchashova et al. 2007). In contrast, during cellular stress, HSP-27 is upregulated, phosphorylated and acts as a chaperone protein whose function is to bind partially folded proteins, as well as inhibiting oxidative stress and apoptosis (Lavoie, Lambert et al. 1995; Mehlen, Schulze-Osthoff et al. 1996; Arrigo, Firdaus et al. 2005). In addition to its chaperone and cellular support functions, HSP-27 is able to mediate cell signaling, differentiation and proliferation (Alford, Glennie et al. 2007; Arrigo, Simon et al. 2007). Despite these pleiotropic effects, a direct role for HSP-27 in disease processes, such as EMT, remains to be elucidated. HSP-27 has been implicated in the EMT of tubular epithelial cells resulting from TGFβ stimulation. TGFβ-stimulated tubular epithelial cells, which develop EMT, showed increased mRNA, protein and phosphorylation of HSP-27 and αSMA concomitant with decreases in E-cadherin (Vidyasagar, Reese et al. 2008). Interestingly, in cells with the most pronounced myofibroblast like morphology, HSP-27 and E-cadherin co-localized in the cytoplasm, suggesting a possible interaction. In fact, Vidyasagar et al determined that overexpression of HSP-27 results in increased E-cadherin as a result of downregulation the E-cadherin repressor Snail (Vidyasagar, Reese et al. 2008). In LECs, stimulation with TGFβ resulted in a significant decrease of promoter activity of HSP-27 two days post-treatment (Sharma, Fatma et al. 2003). In addition, we show here, in Chapter 4, that HSP-27 is significantly upregulated following 3hrs post-treatment with TGFβ. From these experiments it is intriguing to infer that HSP-27 may in fact have a role in TGFβ induced EMT leading to ASC. One might speculate that the early increases in phosphorylated levels of HSP-27, known to induce dissociation of HSP-27 from oligomers to monomers

and dimers, gives way to the rearrangement of the cytoskeleton which is necessary for the acquisition of the motile myofibroblast phenotype (Kato, Hasegawa et al. 1994). Furthermore HSP-27 may assist in suppressing the epithelial cell phenotype in later stages of EMT, since it is has been shown that 2 days of TGFβ-treatment resulted in decreased promoter activity of HSP-27, which could in turn reduce repression of the E-cadherin modulator, Snail (Vidyasagar, Reese et al. 2008). Future experiments aimed at elucidating the involvement and function of HSP-27 in TGFβ-induced EMT leading to ASC may identify a new target of molecules necessary to combat this and other related fibrotic diseases.

Thus, the work presented above are the first to determine that MMPs, specifically MMP2 and MMP9 are necessary for TGFβ-induced ASC. Furthermore, this work is the first to implicate that E-cadherin shedding is a phenomenon in the EMT that leads to ASC. Also I have determined that the mechanism by which TGFβ induces E-cadherin shedding that leads to ASC is a direct result of MMP9. Therefore, I propose that development of fibrotic cataracts which are initiated by TGFβ, signals through a diverse set of pathways, including MAPKs, which ultimately leads to increases in MMP2 and MMP9. The accumulation of MMP9 is able to directly mediate the formation of ASC by the dissolution of E-cadherin/β-catenin complex. The dissociation of epithelial cell contacts and release from the basement membrane, in addition to other cellular cues, leads to myofibroblast generation through EMT. The myofibroblasts generated from EMT cause ocular fibrosis leading to ASC (Fig 5.1).

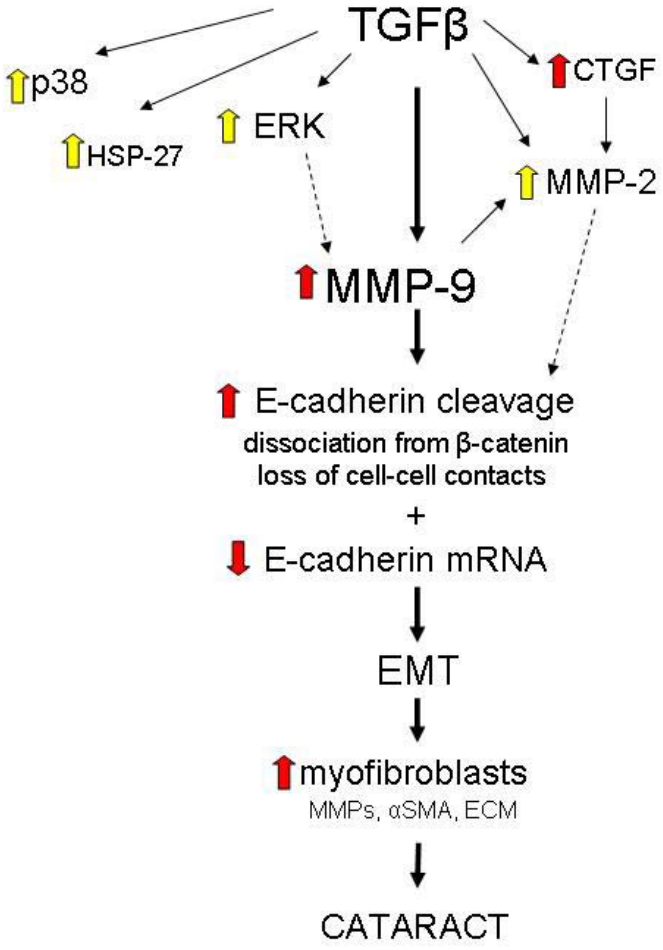

Fig 5.1 The ASC initiating factor TGFβ signals through a diverse set of pathways including MAPKs which ultimately leads to increases in MMP2 and MMP9. The accumulation of MMP9 is able to directly mediate the formation of ASC by the dissolution of E-cadherin/β-catenin complex. The dissociation of epithelial cell contacts and release from the basement membrane, in addition to other cellular cues, leads to myofibroblast generation through EMT. The myofibroblasts generated from EMT cause ocular fibrosis leading to ASC. Red arrows indicate critical factors necessary for ASC, while yellow arrows indicate that further research is necessary to determine what roles these specific factors play. Solid lines indicate experimentally confirmed relationships while dashed lines indicate putative relationships.

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