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Temporal Changes in MMP mRNA Expression in the Lens Epithelium during Anterior Subcapsular Cataract Formation

Zahra Nathu^{1,*}, Dhruva J. Dwivedi^{1,*}, John R. Reddan², Heather Sheardown³, and Judith A. West-Mays^{1,+}

¹Department of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada

²Oakland University, Rochester, MI, USA

³Chemical Engineering, McMaster University, Hamilton, ON, Canada

Abstract

Transforming growth factor beta (TGF β) has been known to play a role in anterior subcapsular cataract (ASC) formation and posterior capsule opacification (PCO), both of which are fibrotic pathologies of the lens. Several models have been utilized to study ASC formation, including the TGF β 1 transgenic mouse model and the *ex-vivo* rat lens model. A distinct characteristic of ASC development within these models includes the formation of isolated fibrotic plaques or opacities which form beneath the lens capsule. A hallmark feature of ASC formation is the epithelial to mesenchymal transition (EMT) of lens epithelial cells (LECs) into myofibroblasts. Recently, the matrix metalloproteinases (MMPs) have been implicated in the formation of these cataracts through their involvement in EMT. In the present study, we sought to further investigate the role of MMPs in subcapsular cataract formation in a time course manner, through the examination of gene expression and morphological changes which occur during this process. RT-QPCR and immunohistochemical analysis was carried out on lenses treated with TGF β for a period of 2, 4 and 6 days. Laser capture microdissection (LCM) was utilized to specifically isolate cells within the plaque region and cells from the adjacent epithelium in lenses treated for a 6 day period. Multilayering of LECs was observed as early as day 2, which preceded the presence of alpha smooth muscle actin (α -SMA) immunoreactivity that was evident following 4 days of treatment with TGF β . A slight reduction in *E-cadherin* mRNA was detected at day 2, although this was not significant until the day 4 time point. Importantly, our results also indicate an early induction of *MMP-9* mRNA following 2 days of TGF β treatment, whereas *MMP-2* was found to be upregulated at the later 4 day time point. Further experiments using FHL 124 cells show an induction in MMP-2 protein levels following treatment with recombinant MMP-9. Together these findings suggest an upstream role for MMP-9 in ASC formation.

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⁺Author for correspondence: Judith A. West-Mays, Ph.D., Dept. of Pathology and Molecular Medicine, McMaster University, Health Sciences Centre, Room 1R10, Hamilton, ON, Canada, L8N 3Z5, westmayj@mcmaster.ca, Tel.: (905) 525-9140 ext. 26237 FAX: (905) 525-7400.

^{*}Zahra Nathu and Dhruva J. Dwivedi contributed equally to the study

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INTRODUCTION

Cataract is a pathology of the lens that remains the leading cause of blindness worldwide (WHO, 1998; WHO, 2000). Anterior subcapsular cataract (ASC) and posterior capsule opacification (PCO) are two types of cataract, of a fibrotic nature, that share many cellular and molecular features. PCO, also known as secondary cataract, is a complication of primary cataract surgery that develops due to lens epithelial cells (LECs) remaining on the lens capsule after cataract surgery. These cells are triggered to proliferate and undergo a transition into myofibroblasts, through a process known as epithelial to mesenchymal transition (EMT) (Lovicu et al., 2002). PCO is characterized by cellular migration onto the posterior lens capsule, deposition of aberrant amounts of extracellular matrix (ECM) and capsular wrinkling, all of which can result in a loss of lens transparency (Bertelmann and Kojetinsky, 2001). ASC, unlike PCO, is a primary cataract that occurs when LECs in the lens epithelium, *in situ*, and are stimulated to transition into myofibroblasts. These fibroblasts form subcapsular plaques directly beneath the lens capsule, and similar to the transdifferentiated cells in PCO, they synthesize the contractile protein alpha smooth muscle actin (α -SMA) and secrete aberrant amounts of ECM (Font and Brownstein, 1974; Hay, 1995; Novotny and Pau, 1984).

A major cytokine implicated in mediating the EMT involved in ASC and PCO is transforming growth factor beta (TGF β) (Wormstone et al., 2002a; Wormstone et al., 2002b), a pleiotropic morphogen that modulates the tissue repair phenotype and also plays a role in numerous other fibrotic disorders such as cancer, lung disease and renal fibrosis (Margetts et al., 2005; Sternlicht et al., 1999; Willis and Borok, 2007). Multiple *in vitro* and *in vivo* models of TGF β -induced ASC and PCO have been developed, including an *ex-vivo* model in which excised rat lenses cultured with TGF β develop distinct ASC plaques within six days that closely mimic human ASC (Hales et al., 1995). In addition, TGF β signaling and its involvement in PCO as been extensively studied utilizing the human lens epithelial cell line, FHL 124 (Dawes et al., 2007; Wormstone et al., 2004). Another model includes a transgenic mouse with active TGF β ectopically expressed in lens fiber cells, under the control of the α -A crystallin promoter (Srinivasan et al., 1998). These mice also exhibit ASC plaques that closely resemble those observed in humans (Lovicu et al., 2002). Investigation of the progression of ASC plaque formation in these mice has revealed that LECs initially lose their cell-to-cell contacts due to reduced expression of the cell adhesion molecule, E-cadherin. This was followed by a multilayering of the cells and then a transdifferentiation into myofibroblasts through EMT. Continued growth of the plaque is provided by proliferation of the LECs flanking the plaque, which exit the cell cycle and subsequently undergo EMT (Lovicu et al., 2004).

The Matrix Metalloproteinases (MMPs) are a family of zinc-dependent matrix degrading enzymes, involved in multiple diseases including fibrosis, with emerging roles in a variety of cataract phenotypes, particularly ASC and PCO (West-Mays and Pino, 2007). Additionally, several MMPs have been identified throughout the lens including normal as well as cataractous tissues (Hodgkinson et al., 2007). In particular, the gelatinases, MMP-2 and MMP-9 have been shown to be induced by TGF β in excised rat lenses and in human capsular bags (Dwivedi et al., 2006a; Wormstone et al., 2002a). Furthermore, using the *ex-vivo* rat lens model, Dwivedi and colleagues showed that co-treatment of excised rat lenses with TGF β and either the broad-spectrum MMP inhibitor, GM6001, or a MMP-2/9 specific inhibitor, resulted in a significant suppression in the appearance of cataractous plaques typically observed in ASC (Dwivedi et al., 2006a). The mechanism by which the MMP inhibitors (MMPIs) suppress ASC formation is not known. However, it has been surmised that MMPIs may act through an attenuation of MMP mediated E-cadherin 'shedding' (Dwivedi et al., 2006a).

In order to further understand the involvement of MMPs in mediating the progression of ASC plaque formation, we sought to determine the relative changes in *MMP-2* and *MMP-9* gene expression in the subcapsular plaque cells relative to known phenotypic markers. These studies were conducted using the TGF β -induced *ex-vivo* rat lens model, in combination with laser capture microdissection (LCM) to capture cells from the cataractous plaques. Examination of gene expression from the epithelial cells adjacent to the plaque was also carried out to determine how these cells compare to the plaque cells and contribute to further growth of the subcapsular plaques. Finally, since *MMP-9* was found to be one of the early response genes, we further tested its ability to directly affect phenotypic markers of ASC, as well as MMP-2 expression.

MATERIALS AND METHODS

Ex-vivo rat lens cataract model

As previously described (Hales et al., 1995), lenses excised from male Wistar rats were cultured overnight in serum-free M199 medium (Gibco, Invitrogen, Burlington, ON, Canada) supplemented with 50 IU/ml penicillin, 50 μ g/ml streptomycin and 2.5 μ g/ml fungizone (Invitrogen, Burlington, ON, Canada). Lenses were subsequently treated with either TGF β 2 (R&D Systems, Minneapolis, MN) at a final concentration of 2 ng/ml or left untreated to serve as controls. Following 2 and 4 days of treatment, the lens epithelium (consisting of LECs and accompanying lens capsule) was removed and subjected to RNA isolation (explained below). Lenses treated for 6 days were prepared for LCM to isolate plaque cells (P) and adjacent cells (A). Untreated controls lenses were incubated for a period of 6 days as well.

Histology and immunohistochemistry

Lenses were fixed overnight in 10% neutral buffered formalin (Sigma-Aldrich, Oakville, ON, Canada), dehydrated, embedded in paraffin, and processed for routine histology. Serial sections were cut 5 μ m in thickness and stained with hematoxylin and eosin (H&E) or used for immunofluorescence analysis.

Sections were de-paraffinized, blocked with normal serum and incubated with primary antibody specific for α -SMA (1:100; Sigma-Aldrich, Oakville, ON, Canada) for 1 hour at room temperature. Bound primary antibodies were visualized with a fluorescein-isothiocyanate anti-mouse secondary antibody (FITC) (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA) and all sections were mounted in Vectashield mounting medium with 4',6-dia-minodino-2-phenylindol (DAPI) (Vector Laboratories, Burlington, ON, Canada). All staining was visualized with a microscope (Leica, Deerfield, IL) equipped with an immunofluorescence attachment, and images were captured with a high-resolution camera and associated software (Open-Lab; Improvision, Lexington, MA). Images were reproduced for publication with image-management software (Photoshop 7.0; Adobe Systems, Inc., Mountain View, CA).

Laser capture microdissection, RNA extraction, and cDNA synthesis

Lenses treated with TGF β for 6 days were fresh-frozen in Tissue-Tek OCT (Sakura Finetek, Torrance, CA) and stored at -70°C . In RNase-free conditions frozen sections were cut at 5 to 8 μ m in thickness, mounted on uncharged glass slides and stored again immediately at -70°C . Preceding LCM, frozen sections were thawed at room temperature for 10 seconds and then stained with Histogene LCM Frozen Section Staining kit (Arcturus, Mountain View, CA) using the protocol provided. LCM was performed on stained sections using the PixCell II (Arcturus) system as described by others (Bonner et al., 1997; Emmert-Buck et al., 1996). Cells were captured from the fibrotic plaque region and referred to as plaque cells

(P), and from the adjacent epithelial area and referred to as adjacent cells (A). Cells defined as adjacent cells were captured from monolayered lens epithelium regions flanking the plaque and care was taken to not capture early multilayering cells during this process. Cells from each region were captured onto CapSure Macro LCM Caps (Arcturus) and subjected to RNA isolation using the PicoPure RNA Isolation kit (Arcturus). Purified RNA was analyzed both qualitatively and quantitatively using the Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA) and subjected to standard reverse transcription reactions (SuperScript II; Life Technologies).

Quantification of gene expression using real time quantitative polymerase chain reaction (RT-QPCR)

E-cadherin, *α -SMA*, *Snail*, *MMP-2*, *MMP-9*, *MMP-14* and *Timp1* gene expression from recovered cDNA was analyzed with RT-PCR using a 96-well Taqman optical reaction plate format on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). RNA was normalized to *glyceraldehydes-3-phosphate dehydrogenase* (*GAPDH*) for each reaction. Each 25 μ l PCR reaction contained TaqMan Universal Master Mix (Applied Biosystems) and gene-specific 'TaqMan Gene Expression Assay on demand' mixtures containing forward and reverse primers as well as probes for target and endogenous control genes (Applied Biosystems). Serial dilutions (one- to five-fold) of standard samples were prepared in separate wells in duplicate for each gene including the endogenous control. Standards and samples were added in a volume of 5 μ l. Thermal cycling parameters consisted of the following: 2 minutes at 50°C, 10 minutes at 95°C, and 1 minute at 60°C. The number of target gene copies was calculated from a standard curve generated in parallel with each batch of samples. A linear relationship was detected over at least 5 orders of magnitude. The normalization of samples was performed by dividing the number of copies of *GAPDH*. PCR reactions for cDNA quantification were performed using standard cDNA dilutions curves. Quantitative data were analyzed statistically using one-way analysis of variance (ANOVA). The Tukey multiple comparisons test was used to compare the different treatment groups with each other. A probability value (P) = 0.05, indicating a 95% confidence interval, was considered significant.

Cell culture and MMP-9 treatment

Cells of the human lens epithelial cell line, FHL 124, (gift from Dr. Reddan) were utilized for the following study (Wormstone et al., 2000). These cells were routinely cultured up to passage 2–5 in minimum essential medium (MEM) (Gibco, Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS). When the cells were confluent, the medium was replaced with non supplemented MEM and cultured for another 24 hours prior to treatment with active recombinant MMP-9 (100 ng/ml; Calbiochem, San Diego, CA) for 3, 6, 12 and 24 hours. Untreated cells served as controls. FHL 124 cells treated with active recombinant human TGF β 2 at a final concentration of 2 ng/ml (R&D Systems, Minneapolis, MN) served as positive controls. After respective treatments, cells were washed with 1.5 ml of warm sterile phosphate buffered saline (PBS) solution and lysed on ice using the protein lysis buffer (50mM Tris HCL, 150mM NaCl, 5mM EDTA, 1% Triton 100X). Cells were then stored in lysis buffer at –20°C.

Western Blot Analyses

FHL 124 cells stored in lysis buffer were homogenized/sonicated and lysates were pre-cleared by centrifuging and the protein concentrations in the samples were determined by Bradford assay. Equal amounts of protein (30–40 μ g) per sample were electrophoresed on a 10% SDS-polyacrylamide gel. The resolved bands were electro-transferred onto a nitrocellulose membrane (Pall Corporation, Pensacola, FL). Membranes were then blocked with 5% skimmed milk powder in Tris-buffered saline (50 mM Tris base, NaCl pH 8.5) and

0.1% Tween-20 and then incubated overnight at 4°C with a primary antibodies generated against MMP-2 (1:500; Chemicon) or α -SMA (1:2000; Sigma-Aldrich). Following this incubation, membranes were probed with a horseradish peroxidase-conjugated secondary antibody (1:7500; Amersham Biosciences, Buckinghamshire, UK) and ECL detection reagents (Amersham Biosciences). Mean densitometry of the immunoreactive bands was assessed by image quantification software (ImageJ; NIH, USA). The relative density versus control ratio (RD/C) was estimated using the Graph Pad Prism Program. Quantitative data were analyzed statistically using a student's t-test and expressed as mean $\pm$ standard error mean (SEM). A value of $p < 0.05$ was considered significant.

RESULTS

Morphological and immunohistochemical alterations of LECs during ASC formation

Formation of ASC plaques in the cultured rat lens following TGF β 2 treatment was examined in a time course manner similar to studies conducted on the TGF β 1 transgenic mouse model (Lovicu et al., 2004). Compared to histological cross-sections of control lenses, which exhibited a monolayer of cuboidal LECs (Fig. 1A), lenses treated with TGF β for 2 days revealed regions of multilayering in the central epithelium (Fig. 1B). At this day 2 time point the cells in these multilayered regions were not immunoreactive for α -SMA (Fig. 1F). Lenses treated with TGF β for 4 days showed more extensive multilayering (Fig. 1C), and also showed strong immunoreactivity to α -SMA (Fig. 1G). Following 6 days of treatment, ASC plaques were evident (Fig. 1D) and many cells in the plaque region were immunoreactive to α -SMA, as were some of the cells adjacent to the plaque (Fig. 1H).

Early changes in LEC gene expression during ASC formation involve attenuated E-cadherin and increased α -SMA

It has been previously shown that TGF β -induced EMT results in downregulation of E-cadherin expression (Bhowmick et al., 2001) accompanied by an induction in α -SMA (Hales et al., 1995). In order to further determine the order and timing for the changes in *E-cadherin* and α -SMA mRNA expression in response to TGF β , RT-QPCR experiments were carried out on rat lenses treated with TGF β for 2, 4 and 6 days compared to untreated lenses. For these experiments, cryostat sections of lenses were subjected to LCM to specifically isolate cells from the plaque (at day 6) (P) or cells immediately adjacent to the plaque (A). For the control lenses and those treated with TGF β for 2 and 4 days, whole lens epithelium was isolated for analysis. RT-QPCR findings revealed that following 2 days of TGF β treatment, *E-cadherin* mRNA levels in the lens epithelium, relative to *GAPDH*, were slightly suppressed (1.3-fold) compared to untreated controls (Fig. 2A), however, this was not found to be significant. Following 4 days of TGF β treatment, a significant attenuation (2.3-fold) ($p < 0.01$) of *E-cadherin* mRNA levels was observed, and by day 6 of treatment cells captured from the subcapsular plaque region (P) exhibited a further, significant decrease (4.3-fold) ($p < 0.001$) in levels of *E-cadherin* mRNA compared to controls. Interestingly, cells adjacent to the plaque (A) in the TGF β treated lenses at 6 days expressed *E-cadherin* levels that were similar to that of untreated control lenses.

Examination of α -SMA mRNA expression was carried out for the same treatment groups outlined above and revealed a slight induction in α -SMA mRNA levels, relative to *GAPDH*, in the lens epithelium following 2 (2.1-fold) days of TGF β treatment (Fig. 2B), although this change was not statistically significant. In comparison, however, day 4 samples (2.6-fold) ($p < 0.05$) and cells from the plaque region (P) following 6 days of TGF β treatment exhibited a significant induction (4.4-fold) ($p < 0.001$) of α -SMA compared to untreated controls. Cells adjacent to the plaque (A) at day 6 also showed significantly higher levels (2.9-fold)

($p < 0.01$) of α -*SMA* mRNA than the epithelium of untreated lenses, and the levels were similar to that observed at day 4 of treatment (Fig. 2B).

Members of the Snail superfamily, including Snail and Slug, have been known to play a role in mediating EMT in cancer systems through its downregulation of E-cadherin (Blehschmidt et al., 2007). RT-QPCR findings showed that *Snail* mRNA was relatively undetectable in the lens epithelium from untreated lenses or lenses treated with TGF β for 2 days (Fig. 2C). However, following 4 days of TGF β treatment, a significant induction (6.7-fold) ($p < 0.01$) in *Snail* mRNA expression, relative to *GAPDH*, was observed and this was further induced (11.3-fold) ($p < 0.001$) in the plaque cells (P) at day 6 as compared to levels in untreated lenses. Cells adjacent to the plaque (A) in the 6 day treated lenses expressed detectable levels of *Snail* mRNA but these were not found to be significantly different than controls (Fig. 2C).

Progressive changes in MMP and TIMP expression in LECs during ASC formation

MMP-2 and MMP-9 have been implicated in cataract formation and therefore analysis of the temporal changes in expression of these genes is important to further understand their role in mediating ASC. To investigate the expression pattern and timing of these candidate genes, mRNA levels were analyzed using RT-QPCR on the same treatment groups mentioned above. RT-QPCR findings revealed that the lens epithelium from untreated lenses exhibited detectable levels of *MMP-9* mRNA, relative to *GAPDH* (Fig. 3B). Following 2 days of TGF β treatment the lens epithelium exhibited significantly higher (3.0-fold) ($p < 0.001$) levels of *MMP-9* mRNA compared to controls. Further inductions in *MMP-9* mRNA were observed in the lens epithelium following 4 days of TGF β treatment (3.7-fold) ($p < 0.001$) and in the plaque cells (4.5-fold) ($p < 0.001$) after 6 days of treatment (Fig. 3B). Adjacent cells (A) also showed significantly higher levels ($p < 0.001$) of *MMP-9* mRNA over controls with levels resembling that detected in lenses treated with TGF β for 4 days.

Similar to *MMP-9*, RT-QPCR findings showed that *MMP-2* mRNA levels are detectable, albeit low, in untreated rat lenses (Fig. 3A). However, unlike the findings for *MMP-9* mRNA, an induction in *MMP-2* mRNA levels following TGF β treatment was not observed until the day 4 time point (Fig. 3A). At both day 4 (10.3-fold) ($p < 0.001$) and day 6 (17.3-fold) ($p < 0.001$) *MMP-2* induction was significant compared to controls. Interestingly, the adjacent cells (A) also expressed an increased amount of *MMP-2* mRNA that resembled levels detected in lenses treated for 4 days (Fig. 3A).

Given that TIMP1 and MMP-14 have been shown to regulate MMP-9 and MMP-2 activity and expression respectively, we also examined their expression patterns in the *ex-vivo* rat lens model. *MMP-14* expression was found to be significantly higher (2.7-fold) ($p < 0.001$) in lenses treated with TGF β for 4 days relative to untreated lenses, as well as in plaque cells (P) (4.2-fold) ($p < 0.001$) and cells adjacent to the plaque (A) (3.2-fold) ($p < 0.001$) from lenses treated for 6 days compared to control samples (Fig. 3C). Significant elevation (1.7-fold) ($p < 0.01$) in *TIMP1* mRNA elevation was also observed following 4 days of TGF β treatment followed by a further significant increase (3.1-fold) ($p < 0.001$) in the plaque cells after 6 days of treatment (Fig. 3D). Interestingly, cells adjacent to the plaque (A) did not express significant levels of *TIMP1* mRNA compared to control levels (Fig. 3D).

Treatment with active recombinant MMP-9 show significant induction of MMP-2 protein in FHL 124 cells

Our RT-QPCR results which utilized the *ex-vivo* rat lens model revealed that *MMP-9* mRNA expression precedes that of *MMP-2*. In other systems, such as corneal wound healing and arterial remodeling, similar timing trends have been observed, in which MMP-9

is expressed earlier than MMP-2. To investigate the potential upstream role of MMP-9 in the cellular changes that occur during ASC formation, we utilized a human lens epithelial cell line, FHL 124. Cells were treated with active recombinant MMP-9 for 3, 6, 12 and 24 hours. Untreated cells served as controls, whereas cells treated with TGF β 2 served as positive controls. Cell lysates obtained from the above treatments were subjected to western blot analysis (Fig. 4). Immunoblots developed with an MMP-2 specific antibody revealed that unlike control cells, which did not exhibit detectable levels of MMP-2 protein, cells treated with recombinant active MMP-9 exhibited bands at 72 KDa corresponding to MMP-2 expression at 3, 6 12 and 24 hr time points (Fig. 4A). Densitometry of multiple immunoblots revealed a significant induction in MMP-2 protein at all time points examined as compared to the untreated control cells (Fig. 4C). The same samples above were also examined for α -SMA protein expression by western blot analysis and revealed the presence of bands at 42 KDa corresponding to α -SMA protein from cells treated with recombinant active MMP-9 at the 12 and 24 hrs time points, whereas low (negligible) levels of α -SMA were observed in untreated cells and cells treated with active recombinant MMP-9 for 3 and 6 hours (Fig. 4 AB). Together these findings show that treatment with recombinant MMP-9 results in a subsequent induction in MMP-2 protein expression that preceded an induction in α -SMA.

DISCUSSION

TGF β has been shown to play a key role in fibrotic pathologies, including ASC and PCO formation. Two models used to study ASC development include the transgenic mouse model and the *ex-vivo* rat lens model, both of which utilize TGF β to induce fibrotic lens opacities beneath the lens capsule (Hales et al., 1995; Srinivasan et al., 1998). Recent interest in the role of MMPs in ASC and PCO formation has developed due to their known involvement in fibrotic disease and in epithelial transdifferentiation and matrix-degradation (West-Mays and Pino, 2007). Previous research from our laboratory, using the *ex-vivo* rat lens model, has shown a role for MMPs in ASC formation, wherein an induction of MMP-9 and MMP-2 secreted protein occurs in the conditioned media of lenses treated with TGF β . Moreover, co-treatment of TGF β with the broad MMP inhibitor, GM6001 (Ilomastat) or the specific MMP-2/9 inhibitor, resulted in the suppression of ASC formation, highlighting the importance of MMPs in this pathology. However, the specific mechanism(s) by which these MMPs mediate ASC formation remains unknown.

In order to further understand the role that MMPs play in the progression of TGF β -induced ASC formation we sought to study their temporal gene expression patterns in the *ex-vivo* rat lens model, relative to markers known to be involved in ASC formation. This was accomplished using RT-QPCR on lenses treated with TGF β for 2, 4 and 6 days. These findings revealed that of the candidate genes examined, the only significant change in mRNA levels observed at the first time point (day 2) following TGF β treatment was that encoding for *MMP-9*. This preceded the induction in α -SMA mRNA and immunoreactivity in histological sections, as well as the induction in MMP-2 mRNA. These findings suggest that MMP-9 may play a more upstream role in TGF β induced ASC formation than MMP-2. Recent reports from our laboratory utilizing MMP-9^{-/-} null mice further support a critical role for MMP-9 in TGF β induced ASC formation. For example, while adenoviral gene delivery of active TGF β 1 (AdTGF β 1) to the anterior chamber of the eyes of wild-type mice produced ASC formation in almost all cases following 4 (98%) and 21 days (100%) of treatment, only a small proportion of MMP-9^{-/-} null mice treated with AdTGF β 1 for 4 days (10%), and 21 days (20%) exhibited visible ASCs (Dwivedi et al., 2006b). Thus, on the MMP-9 null background ASC formation is substantially reduced.

Our finding that treatment of the LEC line (FHL 124) with active recombinant MMP-9 resulted in an induction in MMP-2 and α -SMA protein levels provides further evidence for

an upstream role for MMP-9 in the EMT of LECs and in ASC formation. In other systems such as the corneal wound healing, MMP-9 is known to be involved in the initial stages of repair, including corneal re-epithelialization (Mohan et al., 2002), whereas MMP-2 has been shown to participate in the later stages of matrix degradation. Further reports focusing on MMP involvement in arterial remodeling have also shown that the induction of MMP-9 is an early occurrence preceding ongoing vascular wound healing response, while MMP-2 was found to play a role in subsequent arterial shrinkage (Godin et al., 2000).

The mechanism by which MMP-9 is acting upstream of EMT in ASC formation is not known. In addition, there is no evidence in the literature for direct regulation of MMP-2 by MMP-9. However, it is possible that MMP-9 acts upstream of ASC through its ability to activate certain growth factors (McCawley and Matrisian, 2001; Orlichenko and Radisky, 2008). For example, in tumour invasion studies, gelatinases like MMP-9 both have the ability to cleave latent TGF β , with further activation of TGF β mediated by MMP-9 when in complex with CD44 at the cell surface (Yu and Stamenkovic, 2000). In carcinoma cell culture studies (DU-145), MMP-9 has been shown to activate the insulin-like growth factor (IGF) triggered autocrine response. In addition, rat nerve injury studies have demonstrated a role for MMP-9 in TNF α activation (Manes et al., 1999; Shubayev and Myers, 2000). MMPs also have the ability to release certain growth factors from the surrounding ECM store. For example, long bone development was found to be delayed in MMP-9 null mice, where it plays a role in the release of VEGF, which acts as a chemoattractant for osteoclasts (Engsig et al., 2000). With regards to initiating EMT, MMPs have been shown to cleave cell-to-cell adhesion molecules and also play a role in activating ligands (West-Mays and Pino, 2007). For example, MMP-9 has been associated with EGF-dependent downregulation of E-cadherin in ovarian carcinoma cells, although MMP-9 treatment alone was also able to cause a disruption in E-cadherin structure (Cowden Dahl et al., 2008). Thus, based on these data it can be speculated that during ASC development, MMP-9 may be involved in the release and/or activation of certain growth factors, which may in turn regulate downstream markers of EMT and other MMPs.

Early changes in MMP-9 mRNA corresponded with the time at which multi-layering of LECs in the lens epithelium was observed. A distinct feature of EMT is the loss of cell-to-cell adhesion and the induction of mesenchymal cell markers such as α -SMA. The multi-layering observed during EMT is often attributed to an initial loss in cell-to-cell adhesion and increased motility of the cells (Lovicu et al., 2004). Similar to our findings, in the TGF β transgenic mouse model of ASC initial multi-layering of the epithelium was observed and found to precede the upregulation of α -SMA expression. In the transgenic model the early multi-layering was accompanied by a reduced E-cadherin mRNA expression, as detected by *in situ* hybridization (Lovicu et al., 2004). While we did observe a slight but non-significant repression in E-cadherin mRNA at day 2, a significant loss of E-cadherin mRNA in the rat lens was not observed until the 4 day time point, at the same time point that an induction in α -SMA mRNA and immunoreactivity was detected. One explanation for this discrepancy is that, unlike the TGF β transgenic mouse model in which changes in E-cadherin mRNA were examined at the individual cell level our analyses were carried out on the whole lens epithelium. Thus, while an overall significant change in E-cadherin mRNA was not found at day 2, changes within individual cells may have occurred at this timepoint. Studies have shown that alterations in E-cadherin junctions can occur prior to a reduction in E-cadherin expression during EMT progression. For example, mammary epithelial cells (NMuMG/E9) treated with TGF β 1 have demonstrated a similar pattern, in which E-cadherin junctions were disrupted prior to a reduction in E-cadherin expression (Maeda et al., 2005). This may also be the case for ASC formation, as we observe morphological changes in the lens epithelium following 2 days of TGF β (Maeda et al., 2005) treatment (Fig. 1), while a significant reduction in E-cadherin gene expression is not observed until day 4 (Fig. 2). Previous

research focused on cancer systems and development have established that some Snail family members act as suppressors of E-cadherin transcription during EMT (Peinado et al., 2004). In our study, we observed a significant suppression of *E-cadherin* mRNA following 4 days of TGF β treatment, which was correlated with a significant induction of *Snail* mRNA expression at the same timepoint. Thus, Snail signaling may have been responsible for the reduction of E-cadherin expression; however other family members of Snail, such as Slug or Snail2 may be involved in this process but were not assessed in this particular study.

Given that MMP-14 and TIMP1 are known to regulate MMP activity and expression, we examined their expression patterns in the TGF β induced ASC model. MMP-14, which is known to induce MMP-2 in other systems (Deryugina et al., 2001), was found to be significantly elevated following 4 days of TGF β treatment and further increased at the 6 day time point. Thus, MMP-14 induction occurred after MMP-9 induction, but in conjunction with the induction in MMP-2 expression. Similar to the MMP-14 results, TIMP1 was also found to be significantly induced at day 4 time point and further increased at day 6. Taken together, these findings suggest that MMP-9 expression precedes induction of regulatory factors, such as MMP-14 and TIMP1, which may further mediate EMT.

Our examination also involved a comparison of two distinct cell populations in the lenses treated with TGF β for 6 days; cells captured from the cataractous plaque region as well as cells situated adjacent to the plaque. This was of interest given that previous research using the TGF β transgenic mouse model had demonstrated the importance of these adjacent cells since they contribute to the body of the plaque through a pattern of proliferation and subsequent EMT (Lovicu et al., 2004). Our findings revealed that in almost all candidate genes examined (except for TIMP1 and Snail) the adjacent cell population in the day 6 treated lenses exhibited mRNA levels that were altered in a fashion similar to the plaque cells, albeit the changes were not as substantial as the plaque cells. Such findings suggest that these cells are on their way to becoming plaque cells. TIMPs are endogenous specific inhibitors of MMPs and inhibit their function by binding to the catalytic domain. Specifically, TIMP1 inhibits MMP-9 activity through this binding process (Van den Steen et al., 2002). Our results indicate an upregulation of TIMP1 in cells isolated from the cataractous plaques but no induction was observed in cells from the adjacent epithelium following 6 days of TGF β treatment. It has been demonstrated in other systems that TIMP1 regulates MMP-9 activity, although its induced expression is delayed in comparison to increased MMP-9 activity (Ichiyama et al., 2006). Therefore, TIMP1 may simply be delayed in expression, as compared to MMP-9, within this particular cell population during ASC development.

Overall, our results indicate an early induction in MMP-9 gene expression in rat lenses following TGF β treatment, which was accompanied by multilayering of LECs within the central epithelium and preceded the induction in MMP-2 mRNA and α SMA mRNA. These results lead to further investigation of an upstream role for MMP-9 in the EMT of LECs using FHL 124 cells. Cell culture studies demonstrated that active recombinant MMP-9 can induce myofibroblast differentiation and MMP-2 induction in this human lens epithelial cell line. Together, these results corroborate our previous studies demonstrating that the gelatinases, and specifically MMP-9, play a causative role in TGF β induced ASC formation.

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REFERENCES

- Bertelmann E, Kojetinsky C. Posterior capsule opacification and anterior capsule opacification. *Curr Opin Ophthalmol*. 2001; 12:35–40. [PubMed: 11150079]
- Bhowmick NA, Zent R, Ghiassi M, McDonnell M, Moses HL. Integrin beta 1 signaling is necessary for transforming growth factor-beta activation of p38MAPK and epithelial plasticity. *J Biol Chem*. 2001; 276:46707–46713. [PubMed: 11590169]
- Blechschmidt K, Kremmer E, Hollweck R, Mylonas I, Hofler H, Kremer M, Becker KF. The E-cadherin repressor snail plays a role in tumor progression of endometrioid adenocarcinomas. *Diagn Mol Pathol*. 2007; 16:222–228. [PubMed: 18043286]
- Bonner RF, Emmert-Buck M, Cole K, Pohida T, Chuaqui R, Goldstein S, Liotta LA. Laser capture microdissection: molecular analysis of tissue. *Science*. 1997; 278:1481–1483. [PubMed: 9411767]
- Cowden Dahl KD, Symowicz J, Ning Y, Gutierrez E, Fishman DA, Adley BP, Stack MS, Hudson LG. Matrix metalloproteinase 9 is a mediator of epidermal growth factor-dependent e-cadherin loss in ovarian carcinoma cells. *Cancer Res*. 2008; 68:4606–4613. [PubMed: 18559505]
- Dawes LJ, Elliott RM, Reddan JR, Wormstone YM, Wormstone IM. Oligonucleotide microarray analysis of human lens epithelial cells: TGFbeta regulated gene expression. *Mol Vis*. 2007; 13:1181–1197. [PubMed: 17679943]
- Deryugina EI, Ratnikov B, Monosov E, Postnova TI, DiScipio R, Smith JW, Strongin AY. MT1-MMP initiates activation of pro-MMP-2 and integrin alphavbeta3 promotes maturation of MMP-2 in breast carcinoma cells. *Exp Cell Res*. 2001; 263:209–223. [PubMed: 11161720]
- Dwivedi DJ, Pino G, Banh A, Nathu Z, Howchin D, Margetts P, Sivak JG, West-Mays JA. Matrix metalloproteinase inhibitors suppress transforming growth factor-beta-induced subcapsular cataract formation. *Am J Pathol*. 2006a; 168:69–79. [PubMed: 16400010]
- Dwivedi, DJ.; Robertson, J.; Fini, ME.; West-Mays, JA. Requirement for MMP-9 in TGFbeta-induced Anterior Subcapsular Cataract Formation; ARVO Annual Meeting Fort Lauderdale (FL); 2006b.
- Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA. Laser capture microdissection. *Science*. 1996; 274:998–1001. [PubMed: 8875945]
- Engsig MT, Chen QJ, Vu TH, Pedersen AC, Therkidsen B, Lund LR, Henriksen K, Lenhard T, Foged NT, Werb Z, et al. Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. *J Cell Biol*. 2000; 151:879–889. [PubMed: 11076971]
- Font RL, Brownstein S. A light and electron microscopic study of anterior subcapsular cataracts. *Am J Ophthalmol*. 1974; 78:972–984. [PubMed: 4140696]
- Godin D, Ivan E, Johnson C, Magid R, Galis ZS. Remodeling of carotid artery is associated with increased expression of matrix metalloproteinases in mouse blood flow cessation model. *Circulation*. 2000; 102:2861–2866. [PubMed: 11104745]
- Hales AM, Chamberlain CG, McAvoy JW. Cataract induction in lenses cultured with transforming growth factor-beta. *Invest Ophthalmol Vis Sci*. 1995; 36:1709–1713. [PubMed: 7601651]
- Hay ED. An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)*. 1995; 154:8–20. [PubMed: 8714286]
- Hodgkinson LM, Duncan G, Wang L, Pennington CJ, Edwards DR, Wormstone IM. MMP and TIMP expression in quiescent, dividing, and differentiating human lens cells. *Invest Ophthalmol Vis Sci*. 2007; 48:4192–4199. [PubMed: 17724206]
- Ichiyama T, Kajimoto M, Suenaga N, Maeba S, Matsubara T, Furukawa S. Serum levels of matrix metalloproteinase-9 and its tissue inhibitor (TIMP-1) in acute disseminated encephalomyelitis. *J Neuroimmunol*. 2006; 172:182–186. [PubMed: 16321449]
- Lovicu FJ, Ang S, Chorazyczewska M, McAvoy JW. Deregulation of lens epithelial cell proliferation and differentiation during the development of TGFbeta-induced anterior subcapsular cataract. *Dev Neurosci*. 2004; 26:446–455. [PubMed: 15855773]
- Lovicu FJ, Schulz MW, Hales AM, Vincent LN, Overbeek PA, Chamberlain CG, McAvoy JW. TGFbeta induces morphological and molecular changes similar to human anterior subcapsular cataract. *Br J Ophthalmol*. 2002; 86:220–226. [PubMed: 11815351]

- Maeda M, Johnson KR, Wheelock MJ. Cadherin switching: essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. *J Cell Sci.* 2005; 118:873–887. [PubMed: 15713751]
- Manes S, Llorente M, Lacalle RA, Gomez-Mouton C, Kremer L, Mira E, Martinez AC. The matrix metalloproteinase-9 regulates the insulin-like growth factor-triggered autocrine response in DU-145 carcinoma cells. *J Biol Chem.* 1999; 274:6935–6945. [PubMed: 10066747]
- Margetts PJ, Bonniaud P, Liu L, Hoff CM, Holmes CJ, West-Mays JA, Kelly MM. Transient overexpression of TGF- β 1 induces epithelial mesenchymal transition in the rodent peritoneum. *J Am Soc Nephrol.* 2005; 16:425–436. [PubMed: 15590759]
- McCawley LJ, Matrisian LM. Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol.* 2001; 13:534–540. [PubMed: 11544020]
- Mohan R, Chintala SK, Jung JC, Villar WV, McCabe F, Russo LA, Lee Y, McCarthy BE, Wollenberg KR, Jester JV, et al. Matrix metalloproteinase gelatinase B (MMP-9) coordinates and effects epithelial regeneration. *J Biol Chem.* 2002; 277:2065–2072. [PubMed: 11689563]
- Novotny GE, Pau H. Myofibroblast-like cells in human anterior capsular cataract. *Virchows Arch A Pathol Anat Histopathol.* 1984; 404:393–401. [PubMed: 6437072]
- Orlichenko LS, Radisky DC. Matrix metalloproteinases stimulate epithelial-mesenchymal transition during tumor development. *Clin Exp Metastasis.* 2008
- Peinado H, Portillo F, Cano A. Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol.* 2004; 48:365–375. [PubMed: 15349812]
- Shubayev VI, Myers RR. Upregulation and interaction of TNF α and gelatinases A and B in painful peripheral nerve injury. *Brain Res.* 2000; 855:83–89. [PubMed: 10650133]
- Srinivasan Y, Lovicu FJ, Overbeek PA. Lens-specific expression of transforming growth factor β 1 in transgenic mice causes anterior subcapsular cataracts. *J Clin Invest.* 1998; 101:625–634. [PubMed: 9449696]
- Sternlicht MD, Lochter A, Sympton CJ, Huey B, Rougier JP, Gray JW, Pinkel D, Bissell MJ, Werb Z. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell.* 1999; 98:137–146. [PubMed: 10428026]
- Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol.* 2002; 37:375–536. [PubMed: 12540195]
- West-Mays JA, Pino G. Matrix metalloproteinases as mediators of primary and secondary cataracts. *Exper Rev Ophthalmol.* 2007; 2:931–938.
- WHO. Ageing: a public health challenge. Fact sheet No135. 1998.
- WHO. Blindness: Vision 2020 - The Global Initiative for the Elimination of Avoidable Blindness. Fact sheet No213. 2000.
- Willis BC, Borok Z. TGF- β -induced EMT: mechanisms and implications for fibrotic lung disease. *Am J Physiol Lung Cell Mol Physiol.* 2007; 293:L525–L534. [PubMed: 17631612]
- Wormstone IM, Tamiya S, Anderson I, Duncan G. TGF- β 2-induced matrix modification and cell transdifferentiation in the human lens capsular bag. *Invest Ophthalmol Vis Sci.* 2002a; 43:2301–2308. [PubMed: 12091431]
- Wormstone IM. Posterior capsule opacification: a cell biological perspective. *Exp Eye Res.* 2002b; 74(3):337–347. [PubMed: 12014915]
- Wormstone IM, Tamiya S, Eldred JA, Lazaridis K, Chantry A, Reddan JR, Anderson I, Duncan G. Characterisation of TGF- β 2 signalling and function in a human lens cell line. *Exp Eye Res.* 2004; 78:705–714. [PubMed: 15106950]
- Wormstone IM, Tamiya S, Marcantonio JM, Reddan JR. Hepatocyte growth factor function and c-Met expression in human lens epithelial cells. *Invest Ophthalmol Vis Sci.* 2000; 41:4216–4222. [PubMed: 11095618]
- Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF- β and promotes tumor invasion and angiogenesis. *Genes Dev.* 2000; 14:163–176. [PubMed: 10652271]

Figure 1.

Progression of TGF β 2-induced anterior subcapsular cataract formation in the rat lens. H&E staining of untreated controls (A), lenses treated with TGF β (2 ng/ml) at day 2 (B), at day 4 (C) and day 6 (D). Immunolocalization of α -SMA (green) in untreated (E) and lenses treated for 2 days (F) revealed no immunoreactivity for α -SMA. Lenses treated for 4 days (G) did reveal strong immunoreactivity to α -SMA. Following a period of 6 days, strong positive immunoreactivity to α -SMA was detected within the plaque region, as well as in adjacent cells (H). All sections were mounted in a medium with DAPI to co-localize the nuclei (blue). Scale bars = 100 μ m

Figure 2.

Changes in expression of *E-cadherin*, α -SMA and *Snail* mRNA during ASC formation using RT-QPCR. *E-cadherin* mRNA expression following TGF β treatment following 2 (T2:n=5), 4 (T4:n=5) and 6 (T6:n=4) days of TGF β (2ng/ml) treatment compared to untreated control lenses (C:n=6) (A). Temporal changes of α -SMA mRNA expression following TGF β treatment at day 2 (T2:n=6), 4 (T4:n=6) and 6 (T6:n=4) compared to controls (C:n=8) (B). *Snail* mRNA expression pattern following a period of 2 (T2:n=3), 4 (T4:n=3) and 6 (T6:n=3) days of TGF β (2ng/ml) treatment compared to untreated controls (C:n=3) (C). Cells captured from regions adjacent to the plaque following 6 days of TGF β treatment were also analyzed for each gene (A) (*E-cadherin* : n=4; α -SMA: n=4; *Snail*: n=3). Statistical

analysis was conducted using one-way analysis of variance (ANOVA) with Tukey multiple comparisons test.

Figure 3.

Effect of TGF β on MMP and TIMP mRNA expression during ASC formation using RT-QPCR. *MMP-2* (A), *MMP-9* (B), *MMP-14* (C) and *TIMP1* (D) mRNA expression during ASC formation in the rat lens following 2, 4 and 6 days of TGF β (2ng/ml) treatment compared to untreated controls. Cells adjacent to the plaque at day 6 were isolated and also analyzed for each gene (A). Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Tukey multiple comparisons test. Sample sizes were as follows: for (A) C: n=6; T2: n=6; T4: n=6; T6(P): n=5; T6(A): n=5. (B) C: n=6; T2: n=5; T4: n=5; T6(P): n=5; T6(A): n=5. (C) C: n=4; T2: n=3; T4: n=3; T6(P): n=3; T6(A): n=3. (D) C: n=3; T2: n=3; T4: n=3; T6(P): n=3; T6(A): n=3.

Figure 4.

Effect of active recombinant MMP-9 on MMP-2 protein levels in FHL 124 cells. Representative Western blots. FHL 124 cells treated with active recombinant MMP-9 were examined by western blot analysis to determine MMP-2 and α-SMA expression (A). Cells treated with TGFβ (2 ng/ml) for 24 hrs served as positive controls. A significant upregulation in MMP-2 protein levels was observed following 3, 6, 12 and 24 hrs of MMP-9 treatment (n=3 for all treatments) (C). Following 12 and 24 hrs of MMP-9 treatment, FHL 124 cells exhibited a significant increase in α-SMA protein expression, which was comparable to cells treated with TGFβ (2 ng/ml) for 24 hrs, which served as controls (n=3 for all treatments) (A,B).