THE REGULATION OF TRANSFORMING GROWTH FACTOR BETA (TGFβ) SIGNALING IN POSTERIOR CAPSULAR OPACIFICATION

by

Mahbubul H. Shihan

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Sciences

Summer 2020

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by

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ACKNOWLEDGMENTS

First and foremost, I would like to thank my supervisor, Dr. Melinda K. Duncan. She is not only an excellent researcher and a dedicated educator, but also someone kind, compassionate, and empathetic. Dr. Duncan is someone who truly cares about her students' development. She pushed me out of my comfort zone to explore my potential.

Special thanks to Dr. Yan Wang for her constant support, help, encouragement, and being my lab mom. I want to thank all the previous and current Duncan lab members especially Dr. Yichen Wang and Samuel G. Novo for their help and participation in cheerful banter. Besides, I would like to thank Erin E. Jackson and Nicole M. Rossi who I mentored during my Ph.D. life.

I would like to thank my committee members Dr. Salil A. Lachke, Dr. Donna Woulfe, Dr. Ramona Neunuebel, and Dr. Xianqiao Jia for helping me becoming a scientist. I want to thank all our collaborators especially Dr. Ralph Michael and Justin Christopher D'Antin for arranging my summer training at Centro de Oftalmologia Barraquer, Barcelona, Spain. Special thanks to all the cataract surgeons who participated in my interview study.

I want to thank members from Lachke lab especially Shaili Patel and Sandeep Aryal and Dr. Justin Parreno from Fowler lab for their constant support and encouragement. I would like to thank everyone from the department of biological sciences especially Betty Cowgill for her sincere help during my stay at this department.

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Finally, I would like to thank my family members and my friends especially Keith Markowitz, Reetika Dutt, Vivek Puthussery, Tanjima Ferdous, Naim Ahmed, and Fazlur Rahman for their constant mental and emotional support during this rocky journey.

This work was supported by the National Institutes of Health grant (EY015279) to Melinda K. Duncan; NSF I-Corps Entrepreneurial Award to Melinda K. Duncan, Mahbubul H. Shihan, and Samuel G. Novo; Fight for Sight summer scholarship and Sigma XI Grant-in-Aid of Research to Mahbubul H. Shihan.

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ABSTRACT

Posterior capsular opacification (PCO), one of the major complications of cataract surgery, occurs when lens epithelial cells (LCs) left behind post cataract surgery (PCS) undergo epithelial to mesenchymal transition, migrate into the optical axis and produce opaque scar tissue. Despite preventive strategies such as modern cataract surgery and improved materials and shapes of intraocular lenses (IOLs), recent data suggests that about 28% of adults develop PCO at 5 years whereas 40% pediatric patients develop PCO by 2 years post cataract surgery (PCS). The only FDA approved treatment for PCO is YAG laser capsulotomy which is not devoid of side effects. Besides, YAG laser can be unsuitable for pediatric patients, while the availability of YAG lasers and technical expertise are limited in developing and underdeveloped countries, suggesting that understanding the molecular mechanisms of PCO to develop preventive therapeutics would improve the outcome of cataract surgery. Although it is well established that activated transforming growth factor-beta (TGFβ) signaling mediates fibrotic PCO, the initiation, activation, and bioavailability mechanisms of TGFβ signaling PCS are not well understood. Besides, if a preventive therapeutic against PCO is made available, at the start of my study, it was unclear whether clinicians treating cataract surgery patients would be interested in instituting it into their clinical practice. In total, four studies are covered here. The first one is a survey-based study on understating cataract surgeons' viewpoints on the clinical challenges they encounter in routine practice and the types of therapeutic interventions

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that would enhance the long-term efficacy of cataract surgery and PCO (Chapter 3). The surgeons surveyed agree that PCO/VAO (visual axis pacification) remains an unsolved problem in pediatric and veterinary cataract surgery while the long-term outcome of adult cataract surgery could be improved by additional attention to this issue. The next three studies are focused on understanding the molecular mechanisms of TGFβ signaling (the major mediator of PCO) regulation PCS. Chapter 4 focuses on understanding the ability of remnant LCs to express inflammatory cytokines leading to the infiltration of neutrophils and macrophages into the lens capsular bag, and the possible implications of these events in the initiation of TGFβ signaling PCS. Chapter 5 focuses on identifying an αV integrin heterodimer that is critical for the activation of TGFβ signaling PCS and characterizes the effects of an antibody which can block integrin function PCS. Chapter 6 focuses on elucidating the regulatory role of a fibrotic extracellular matrix (ECM) molecule, fibronectin, in relationship to latent TGFβ complex regulation PCS and its multifunctional roles in sustaining fibrotic PCO. All these studies fill the major knowledge gap, providing the regulatory mechanisms of initiation, activation, and the bioavailability of TGFβ signaling in PCO pathogenesis as well as novel molecular targets for PCO prevention.

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Chapter 1

INTRODUCTION

1.1 The lens: Structure & function

The lens is an epithelial tissue located behind the iris and in front of the vitreous humor. It is composed of two types of cells, a monolayer of cuboidal lens epithelial cells (LCs) which are found on the anterior surface of the lens whereas the vast majority of the lens is composed of concentric elongated layers of fiber cells. LCs differentiate into lens fiber cells at the peripheral transition zones throughout life. The entire lens is encapsulated by a basement membrane which is known as the lens capsule. Lens capsule compartmentalizes lens cells from the rest of the eye (Wormstone and Wride 2011) (Figure 1.1).

The lens fiber cells contain high concentrations of water-soluble crystallin proteins, important for the high refractive index and its transparency throughout life (Andley 2007). α and β/γ crystallins make up about 90% of the proteins found in the lens and their high intrinsic stability against stress and thermal stability are the reasons of the lifelong function (Andley 2007; Donaldson et al. 2009).

The lens capsule is a thickened basement membrane that surrounds the lens and provides structural support for lens cells. Lens cells produce the lens capsule extracellular matrix (ECM) proteins, and interaction between lens cells and the capsule is important for cell survival, proliferation, and appropriate cellular differentiation (Danysh and Duncan 2009). Indeed, lens cells cultured on inappropriate ECMs lose lens characteristics (Greenburg and Hay 1982; de Jong-Hesse et al. 2005)

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indicating that lens cell-capsule interactions provide a feed-forward mechanism that is essential for maintaining the lens phenotype.

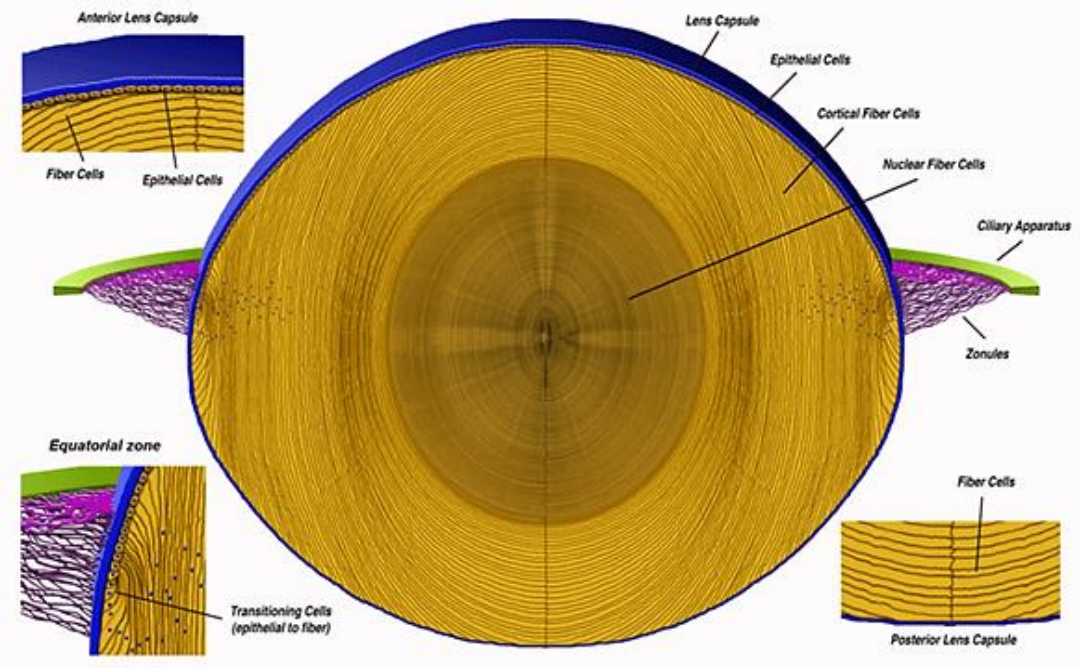

Figure 1.1: The anatomy of the lens as an epithelial tissue. (Image adapted from Danysh and Duncan 2009)

1.2 Cataracts and cataract surgery

Lens transparency is required to refract light onto the retina for vision to occur (I. Michael Wormstone and Wride 2011). However, the loss of lens transparency due to cataract is the most common cause of blindness worldwide (Asbell et al. 2005). According to the WHO, 65.2 million people are visually impaired worldwide due to cataracts (WHO 2019). Several factors have been found to cause cataracts ranging from aging, injury to the eye, oxidative stress, metabolic dysfunction, loss of ion/water

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balance, mutation of structural proteins, defects during eye development, as well as drug-induced changes (Asbell et al. 2005; Liu et al. 2017) (Figure 1.2).

Figure 1.2: The clouded lens of a person with cataracts (Image adapted from https://www.nei.nih.gov/about/news-and-events/news/nei-charts-clearer-future-cataract-prevention-and-treatment)

Although no confirmed method has been developed yet to prevent cataract formation (Asbell et al. 2005), it is treatable by phacoemulsification surgery (I. Michael Wormstone, Wang, and Liu 2009). The procedure begins by making a 2-3mm incision on the peripheral side of the cornea or sclera (Figure 1.3-1) followed by a gentle tearing of the lens capsule, a procedure referred to as capsulorhexis (Figure 1.3-2). A handset device that vibrates at ultrasonic frequency (40,000 Hz), is used to simultaneously emulsify and aspirate the opacified natural lens leaving behind an intact elastic lens capsule (Figure 1.3-2). The surgeon then vacuums the lens residue and inserts an artificial intraocular lens (IOL) and the patient's vision is restored

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(Figure 1.3-3&4). Although this procedure is very successful and has been used for years; it has its complications (I. Michael Wormstone, Wang, and Liu 2009; Kohnen 2015).

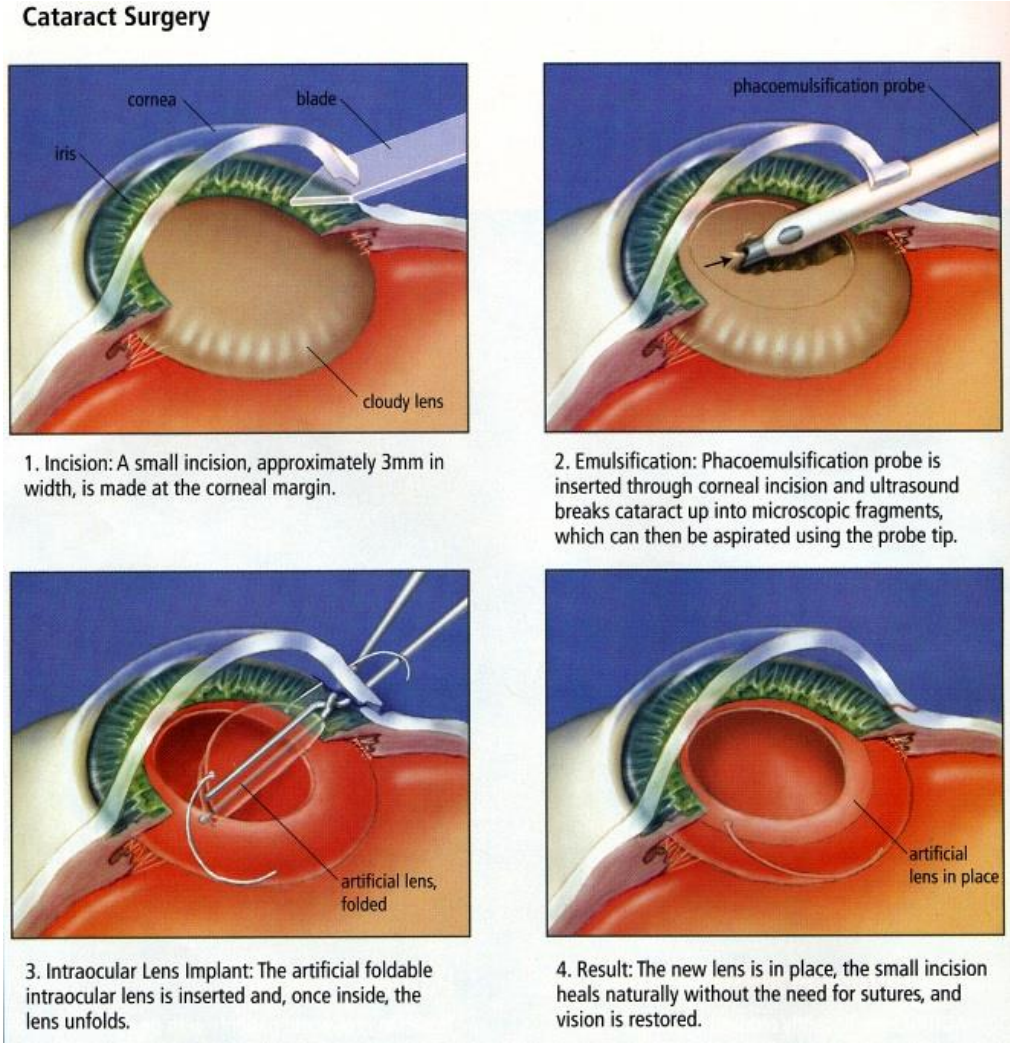

Figure 1.3: The steps of the modern cataract surgery procedure. (Image adapted from https://www.corkeyeclinic.ie/cataract-removal-surgery)

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1.3 Complications of cataract surgery

Cataract surgery has been demonstrated to improve the quality of life (Gray and Ackland, n.d.). However, this procedure has some consequences.

1.3.1 Post cataract surgical inflammation

Cataract surgery triggers acute ocular inflammation which can be painful and slows visual recovery (Liu et al. 2017; Chan, Mahroo, and Spalton 2010). Inflammation is currently treated by either anti-inflammatory eye drops which are plagued by low patient compliance (Juthani, Clearfield, and Chuck 2017) or installation of anti-inflammatories into the eye at the time of surgery ("drop-less" cataract surgery) (Lindstrom et al. 2017). While this acute inflammation usually resolves quickly in the absence of infection, low-level inflammation can persist for months post surgery and may exacerbate other ocular pathologies such as uveitis and glaucoma (Abbouda et al. 2016; Bhutto and Lutty 2012; 2012; Teh et al. 2017; Diagourtas et al. 2017) suggesting that the further understanding of the molecular mechanisms behind the development of post cataract surgery inflammation would improve the patient compliance and treatment regimen.

1.3.2 Posterior capsular opacification (PCO)

Months to years following cataract surgery, a significant proportion of cataract patients experience an apparent recurrence of their cataract as Posterior Capsular Opacification (PCO) (Figure 1.4) (Wormstone, Wang, and Liu 2009; Liu et al. 2017; Awasthi, Guo, and Wagner 2009). PCO develops due to the presence of a mixture of scar-producing myofibroblasts and aberrant lens fiber cells in the optical axis (Figure 1.5) (Wormstone, Wang, and Liu 2009). PCO is treated by a quick outpatient procedure using Nd-YAG laser (neodymium-yttrium-aluminum-garnet) (Awasthi,

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Guo, and Wagner 2009). However, it also results in side effects which include acute onset of macular hole, cystoid macular edema, retinal detachment, glaucoma, and increases in intraocular pressure (IP) (Awasthi, Guo, and Wagner 2009; Burq and Taqui 2008; Beale et al. 2006). Besides that, improved intraocular lenses (IOLs) have been developed to reduce the prevalence of PCO. However, it is not able to prevent the development of PCO completely (Beck et al. 2001; Nagamoto and Eguchi 1997; Peng et al. 2000). Therefore, understanding the molecular mechanism of PCO pathogenesis is required to develop preventive anti PCO therapies to improve the outcome of cataract surgery.

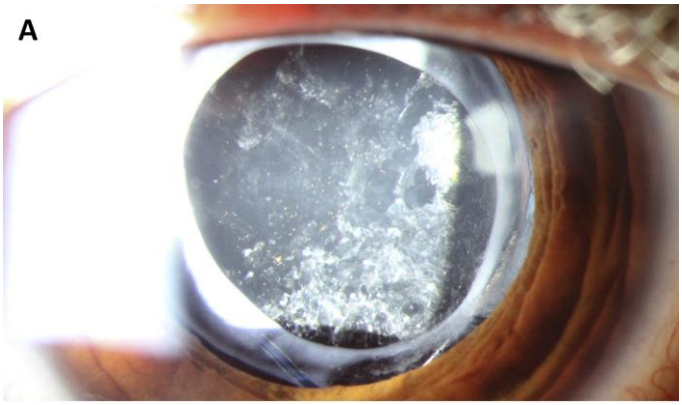

Figure 1.4: Human eyes with posterior capsular opacification (PCO). (Image adapted from Nibourg 2015)

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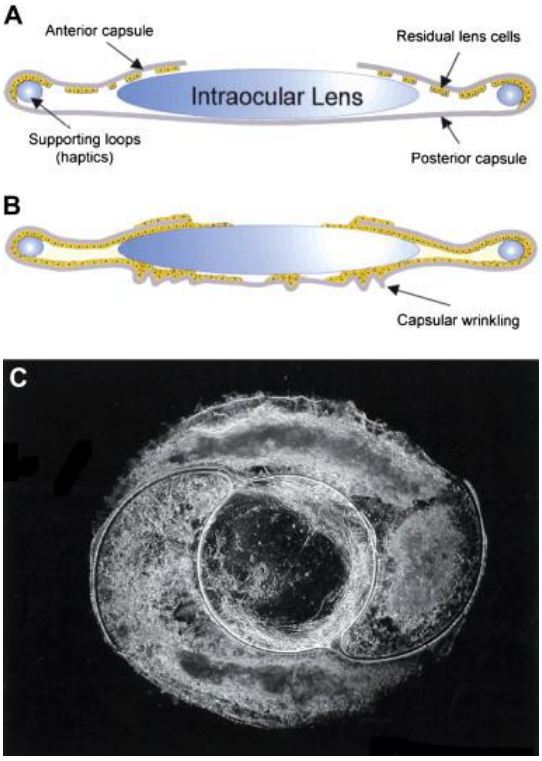

Figure 1.5: A schematic diagram showing the remnant lens epithelial cells following cataract surgery undergo epithelial to mesenchymal transition (A, B). C shows a dark field image of the development of PCO in an adult patient. (Image adapted from Wormstone, Wang, and Liu 2009)

1.4 Epithelial-to-mesenchymal transition (EMT) of lens epithelial cells (LCs)

Epithelial-mesenchymal transition (EMT) is defined as a biological process that allows polarized cells to undergo multiple biological changes including loss of epithelial characters such as apical-basolateral polarity, cell-cell communication mediated by tight and adherens junctions, and the ability to synthesize basement membranes. Besides that, these cells rearrange their actin cytoskeleton and become migratory by forming filopodia and lamellopodia, interact with stromal extracellular matrices (ECM) due to changes in cell surface matrix receptors such as integrins, begin direct synthesis of stromal ECM and become contractile myofibroblasts (Kalluri

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and Neilson 2003; Mamuya and Duncan 2012). A number of different cell signaling pathways such as growth factors, extracellular matrix components, matrix metalloproteinases, and integrin have been suggested to contribute to PCO development (Wormstone, Wang, and Liu 2009). However, among all these cell signaling pathways, activated TGFβ signaling has extensively been studied and found to be a known mediator of LCs EMT (de Iongh et al. 2005)

1.5 Transforming growth factor beta (TGFβ) signaling- a major mediator of PCO

While TGFβ is known to mediate EMT, it also regulates a wide array of other cellular processes including cell division, differentiation, motility, apoptosis, tumor suppression, and the suppression of inflammatory response (Taipale, Saharinen, and Keski-Oja 1998; Yue and Mulder 2001; Sanjabi, Oh, and Li 2017). There are three known isoforms of TGFβ in mammals, TGFβ1, TGFβ2, and TGFβ3 (Derynck et al. 1988; Massagué 2008). The expression and function of all three isoforms vary dramatically among tissues and can even vary from species to species (Massagué 2008). For example- in mouse, adult lens epithelial cells (LCs) express all three TGFβs (1-3) (30, 115, and 7 rpkm respectively) constitutively, while TGFβ1 expression upregulates 2 fold and TGFβ3 upregulates 3 fold in LCs by 48 hours post cataract surgery (PCS) (Shihan et al. 2020). Therefore, the mechanism by which TGFβ function is regulated is quite complex (Mamuya and Duncan 2012).

To initiate this TGFβ mediated signaling cascade, at first TGFβ, needs to be synthesized and secreted into the extracellular environment. TGFβ and latent TGFβ-binding protein (LTBP) are translated into the endoplasmic reticulum (ER) where pro-TGFβ dimerizes and is then disulfide-bonded to LTBP to form a ternary complex. The

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TGFβ dimer is cleaved from its pro-peptide (latency-associated peptide [LAP]) in the trans-Golgi network, but TGFβ and LAP remain strongly associated via noncovalent interactions forming the large latent complex (LLC). Once secreted, the LTBP may bind various ECMs that sequester latent TGFβ until it is released by an activator. The latent complex is then activated, by one of several potential mechanisms, releasing the mature TGFβ. Active TGFβ may bind to cell-surface receptors and initiate TGFβ mediated signaling cascade (Robertson and Rifkin 2016).

1.6 Regulators of TGFβ signaling activation and bioavailability

1.6.1 Integrins

Integrins are heterodimeric matrix receptors consisting of one α and one β subunit. Integrins are reported to mediate different cellular behaviors during development (Proctor et al. 2005; Mamuya and Duncan 2012). Besides that, integrins have been widely studied due to their potential roles in tissue fibrosis, cancer, and other pathological conditions, including eye diseases (Mamuya et al. 2014; Raab-Westphal, Marshall, and Goodman 2017). In other systems, matrix metalloproteinases (MMPs) can activate latent TGFβ by proteolytic cleavage of the latency associated peptide (LAP) and/or latent TGFβ binding protein (LTBP), liberating TGFβ from its ECM bound stores (Robertson and Rifkin 2016). MMPs can be tethered to the cell surface by interaction with αV integrins leading to site-specific MMP functions (Dwivedi et al. 2006). Notably, αV integrins are required for both the onset of PCO and the activation of TGFβ signaling in LCs following cataract surgery, indicating that αV integrins play an important role in the pathogenesis of fibrotic PCO (Mamuya et al. 2014). αV-integrins have the potential to also affect PCO pathogenesis via their

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ability to both direct extracellular matrix (ECM) assembly, and signal in response to ECM binding (Robertson and Rifkin 2016). Notably, αV-integrin forms functionally distinct heterodimers with a variety of β integrins, while four of the five possible β integrins were upregulated (β1, β5, β6, and β8) PCS (Mamuya et al. 2014). However, the identity of the specific β subunit whose heterodimerization with αV-integrin is required to drive TGFβ signaling in LCs PCS and by extension, PCO pathogenesis, was not known, and is the topic of Chapter 5 of this dissertation.

1.6.2 Fibronectin

Fibronectin, an important extracellular matrix (ECM) molecule, regulates cell migration, differentiation, proliferation, and survival during normal and pathological conditions by multiple signaling mechanisms and growth factors, especially latent TGFβ complex (Grinnell 1984; Blumenstock et al. 1986; Chen et al. 2015; Robertson and Rifkin 2016; George, Baldwin, and Hynes 1997; CLARK 1983; Muro et al. 2003). In the lens, fibronectin is produced throughout its development (Parmigiani and McAvoy 1991) and is essential for early lens morphogenesis (Huang et al. 2011). Fibronectin deletion permits ectoderm expansion which prevents lens placode formation. This failure of placode formation prevents the invagination that forms the lens pit (Huang et al. 2011). After early lens development, the only robust fibronectin expression by lens cells is detected during lens fibrotic diseases. Increased expression of fibronectin has been reported in human capsular bags cultured with TGFβ2 (Dawes et al. 2008). In a mouse cataract surgery model, fibronectin mRNA levels upregulate by 24 hours post cataract surgery (PCS), and fibronectin protein is deposited around fibrotic LCs by 48 PCS (Mamuya et al. 2014). Notably, disruption of fibronectin assembly attenuates LCs conversion to myofibroblasts in cell culture (Tiwari et al.

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2016), although fibronectin has also been proposed to be a negative regulator of posterior capsular wrinkling in PCO (Dawes et al. 2008). Thus, fibronectin function following cataract surgery *in vivo* was not well understood and investigation of this question is the topic of Chapter 6 of this dissertation.

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Chapter 2
MATERIALS AND METHODS

2.1 Interview methods

Institutional review board review was conducted at the University of Delaware and this project was deemed "not human subjects research" (see Appendix C) as it consisted of "Information-gathering interviews where questions focus on things, products, or policies". Practicing cataract surgeons (50) were recruited through direct email contacts (Figure 2.1), ARVO-connect, the online community for the Association for Research in Vision and Ophthalmology, and direct conference contacts at the American Association for Pediatric Ophthalmology and Strabismus (AAOPS) Annual Meeting-2018-Washington DC, USA, The Association for Research in Vision and Ophthalmology (ARVO) Annual Meeting-2018, Honolulu, Hawaii, USA, Centro de Oftalmología Barraquer, Investigación, Barcelona, Spain, the Ryan Veterinary Hospital - Penn Vet - the University of Pennsylvania, USA, and private practice offices in Newark, Delaware, and Plymouth, Pennsylvania, USA to participate in in-person, phone and e-mail interviews (Figure 2.1). Only people who self-identified as board-certified ophthalmologists who perform cataract surgery as the major portion of their clinical duties were surveyed for this study. Typical interview questions are listed below and the same questions were asked to each cataract surgeon who participated in this study.

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Questions

1. What are your top three concerns regarding post-surgical management after cataract surgery?

2. How do you manage post cataract surgical inflammation? Are you satisfied with the current standard of care for post cataract surgical inflammation treatment? Any alternatives that you would prefer?

3. How many Posterior Capsular Opacification (PCO) cases do you get per year? Do you still think PCO is a clinical problem?

4. How long does PCO take to develop after surgery in your patients (based on your experiences)?

5. How do you treat PCO? How many patients get subsequent consequences such as macular edema and retinal detachment? (you can use a percentage of patients out of the total number of PCO patients)

6. Are you satisfied with the treatment protocol for PCO? What do you desire or what changes would you do?

7. If there was a non-surgical approach to treating PCO/post-surgical ocular inflammation would you use it? What type of target would you prefer?

8. Which drug delivery system would you prefer? (Ointment/injection during surgery) –

This question was least discussed during the interview session.

● Do you have any colleagues that could help us out with our market research? Please leave contact information below either yours to reach out later or that of your colleagues interested in participating in this type of interview.

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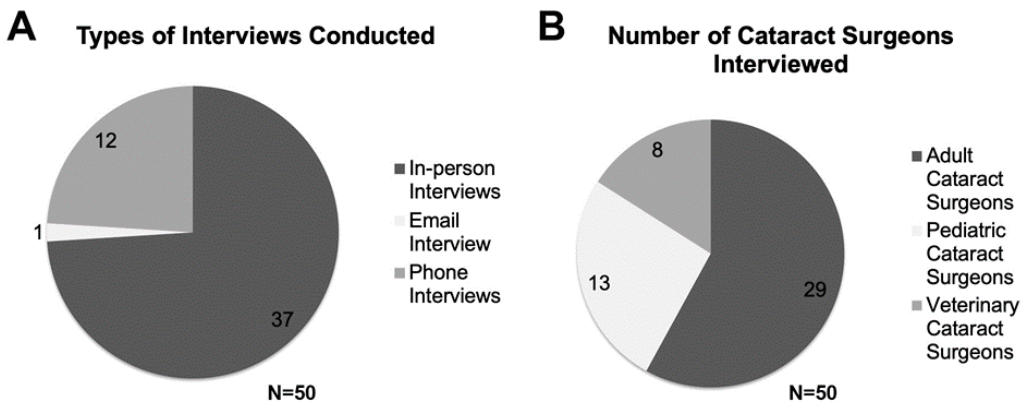

Figure 2.1: (A) 50 cataract surgeons were interviewed in person, by email, and over the phone and (B) included cataract surgeons treating human adults and children as well as animals.

2.2 Animals

All animal experiments for this study were performed per the Association for Research in Vision and Ophthalmology (ARVO) Statement on the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Delaware Institutional Animal Care and Use Committee (AUP- 1039- 2019-1, see Appendix C). All mice were maintained under pathogen-free conditions at the University of Delaware animal facility under a 14/10-hour light/dark cycle.

2.2.1 Fibronectin conditional knockout (FNcKO) mice

Mice lacking the fibronectin gene from the lens (FNcKO mice) were created by mating mice harboring an FN1 allele in which exon 1 is flanked by lox P sites (B6;129-Fn1tm1ref, originally created in Dr. Reinhard Fassler's lab (Sakai et al. 2001)

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and obtained from Dr. David Beebe, Washington University, St. Louis, St. Louis, Missouri) with MLR10-cre mice which express Cre recombinase in all lens cells from the lens vesicle stage onward (H. Zhao et al. 2004) (mice on an FVB/N background obtained from Dr. Michael Robinson (Miami University, Oxford, Ohio) and backcrossed to C57Bl/6<har> for over 10 generations at the University of Delaware).

2.2.2 β5 integrin null, β6 integrin null and β8 integrin conditional knockout (β8ITGcKO) mice

Mice homozygous for a null mutation of β5 integrin subunit were obtained from The Jackson Laboratory (Itgb5tm1Des, Mixed (C57BL/6J, 129/Sv) Donating Investigator- Dean Sheppard, University of California San Francisco (UCSF), CA, USA) and the deletion of β5 integrin gene from mouse embryonic stem (ES) cells was confirmed (X. Huang et al. 2000). Homozygous β6 integrin null mice were obtained from Dr. Xiaozhu Huang (UCSF, CA, USA) generated on a 129Svems genetic background as described (X. Z. Huang et al. 1996). β6 integrin null mice were crossbred back to establish lines of wild type, homozygous, and heterozygous animals. Mice lacking β8 integrin gene from the lens (β8ITGcKO) were created by mating mice harboring an integrin β8 allele in which exon 4 is flanked by lox P sites (Proctor et al. 2005) (Mixed (C57BL/6J, 129/Sv) ((obtained from Dr. Thomas D. Arnold, UCSF, CA, USA) with MLR10-cre mice which express Cre recombinase in all lens cells from the lens vesicle stage onward (H. Zhao et al. 2004) (mice on an FVB/N background obtained from Dr. Michael Robinson, (Miami University, Oxford, Ohio) and backcrossed to C57Bl/6<har> for over 10 generations at UD).

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2.3 DNA extraction, genotyping and genomic PCR for the gene deletion

DNA was isolated either from tail snips or whole lenses using the PureGene Tissue and Mouse Tail kit (Qiagen, Hilden, Germany). Briefly, 0.5cm length of mouse tail or one whole mouse lens was immersed in a microfuge tube containing 600µl of PureGene cell lysis solution cocktail and 5µl of 20mg/ml Proteinase K solution (Life Technologies, Carlsbad, CA). The microcentrifuge tube was inverted several times and incubated at 55°C overnight in a gentle shaking water bath. After incubation, 200µl of protein precipitation solution (ammonium acetate) was added to the cell lysate solution and vortexed at high speed for about 20 seconds followed by 6 minutes of centrifugation at 16,000 rpm. The supernatant containing the DNA was separated from the precipitated protein pellet and poured into a microcentrifuge tube containing 600µl of 100% isopropanol, mixed by inverting 25-30 times and centrifuging at 16,000 rpm for 2 minutes. The supernatant was carefully discarded and 600µl of 70% ethanol was added to the microfuge tube containing the DNA pellet, washed by inverting the tube several times followed by a 2 minutes centrifugation at 16,000 rpm. The ethanol was carefully discarded without disturbing the pelleted DNA. The microcentrifuge tube containing the pelleted DNA was left open to air dry at room temperature for 20 minutes and then the pelleted DNA was rehydrated with 20 µl of pureGene DNA hydration solution or by nuclease free molecular grade water and incubated overnight at room temperature. Finally, the DNA concentration and purity were examined by NanoDrop® ND 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and stored at 4°C until use.

Polymerase Chain Reaction (PCR) was performed using a Taq DNA Polymerase Kit (Qiagen, Hilden, Germany). Briefly, genomic DNA was quantified by nanodrop, then diluted down to about a 100 ng/µl final concentration. A 20µl PCR mix

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cocktail containing 1µl of the 100 ng/µl DNA, 1µl of each desired primer (Table 2.1), 10µl of Taq PCR Master Mix and 7µl of water was added to a 0.2ml PCR tubes and mixed gently by pipetting while kept on ice at all times. PCR was carried out with a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The cycling conditions for all tail genotyping and gene deletion primers are listed in Table 2.1. PCR product bands were analyzed by 2% agarose gel electrophoresis with ethidium bromide concentration of 0.5µg/ml and examined under UV gel imager (Carestream Gel Logic 212 Pro, Rochester, NY).

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Table 2.1: List of all primers and PCR conditions used for genotyping and gene deletion study.

Gene	**Forward Primer**	**Reverse Primer**	**PCR conditions (genotyping)**
FN1 F/F (tail)	5′-GTA CTG TCC CAT ATA AGC CT CTG-3′	5′-CTG AGC ATC TTG AGT GGA TGG GA-3′	1. Initiation/Melting- 95ºC (5 min.) 2, Denaturation- 94ºC (30 sec.) 3. Annealing- 58ºC (45 sec.) 4. Elongation- 72ºC (1 min. 30 sec.) Steps 2-3-4 cycle in sequence-33 cycles 5. Amplification- 72ºC (10 min.) 6. Hold- 4ºC
FN1 (lens)	5′-CTG AGC ATC TTG AGT GGA TGG GA-3′	5′-CGA GGT GAC AGA GAC CAC AA-3′	
MLR 10 cre (H. Zhao et al. 2004)	5′-CCT GTT TTG CAC GTT CAC CG-3′	5′-ATG CTT CTG TCC GTT TGC CG-3′	

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Gene	Forward Primer	Reverse Primer	PCR conditions (genotyping)
β6-integrin (Tail) (Mohazab et al. 2013)	5'-TAAGTGAGTGAACTCCCTGG- 3' (WT) 5'-CAGTAAATCGTTGTCAACAG-3' (β6-integrin null)	5'-CAGCAATGAGTGAAAGCCA- 3'	1. Initiation/Melting- 94ºC (2 min.) 2, Denaturation- 94ºC (1 min.) 3. Annealing- 60ºC (1 min.) 4. Elongation- 72ºC (1 min.) Steps 2-3-4 cycle in sequence-40 cycles 5. Amplification- 72ºC (5 min.) 6. Hold- 15ºC band size –wildtype- 450 kb; β6-integrin null- 250 kb; het- both bands.
β8-integrin (tail) (Lakhe-Reddy et al. 2014)	5'-GAGATGCAAGAGTGTTTACC-3'	5'-CACTTTAGTATGCTAATGATGG-3'	1. Initiation/Melting- 94ºC (5 min.) 2, Denaturation- 94ºC (15 sec.) 3. Annealing- 65ºC to 55 ºC (↓ 1ºC/cycle) (30 sec.) for first 10 cycles, next 30 cycles anneal at 55 ºC 4. Elongation- 72ºC (40 sec.) Steps 2-3-4 cycle in sequence 5. Amplification- 72ºC (5 min.) 6. Hold- 15ºC band size –wildtype- 250 kb; β8-integrin floxed – 370 kb; het- both bands. This PCR protocol is developed by MMRRC at the University of California, Davis, USA. https://mmrrc.ucdavis.edu/protocols/014108Geno_Protocol.pdf
β8-integrin (lens) (Lakhe-Reddy et al. 2014)	5′-GTGGTTAAGAGCACCGATTG-3′ (F1) 5′-GAGATGCAAGAGTGTTTACC-3′ (F2)	5′-CACTTTAGTATGCTAATGATG-3′ (R1)	

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2.4 Morphological Analysis

Lens clarity was determined by viewing isolated lenses using darkfield optics while lens optical properties were assessed by placing lenses on a 200-mesh electron microscopy grid as described previously (Shiels et al. 2007; Scheiblin et al. 2014). For histological analysis, eyes were isolated and immediately fixed in Pen-Fix (Richard Allan Scientific, Kalamazoo, Michigan) for two hours, then stored in 70% ethanol until paraffin embedding by the Histology Core Laboratory, College of Agriculture, University of Delaware. Six-micrometer sections were stained with hematoxylin and eosin (H&E) and photographed on a Zeiss Axiophot microscope fitted with a Nikon digital camera.

2.5 Mouse cataract surgery model

Surgical removal of lens fiber cells to mimic human cataract surgery was performed in adult mice as previously described (Mamuya et al. 2014; Call et al. 2004). Briefly, adult mice were anesthetized, a central corneal incision was made, and the entire lens fiber cell mass was removed by a sharp forceps, leaving behind an intact lens capsule. For analysis, mice were sacrificed at various time intervals PCS ranging from 24 hr to 5 days.

2.6 RNA sequencing

2.6.1 Adult mouse lenses

RNA sequencing of intact mouse lenses was performed by isolating RNA from 8 weeks old FNcKO and C57BL/6NHsd (wildtype) lenses (three biological replicates for each condition, two lenses per replicate) using the SV Total RNA Isolation System

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(Promega- Catalog number- Z3100, Madison, Wisconsin, USA). Sequencing libraries were produced using SMARTer Stranded Total RNA-Seq - Pico Input Mammalian (Takara Bio USA, Inc., Mountain View, CA, USA) and sequenced by DNA Link, USA (901 Morena Blvd. Ste 730 San Diego CA92117, USA) on an Illumina NextSeq500 (San Diego, CA, USA). Paired end 101 nucleotide reads were processed using the Tuxedo Suite tools TopHat and Cuffdiff for alignment and differential expression analysis (Trapnell et al. 2012). The UCSC Genome version GRCm38/mm10, and RefSeq GRCm38.p5 annotations were used as the reference for alignment and feature abundance estimates. Read pairs corresponding to RNA fragments were enumerated as FPKM (fragments per kilobase million) by Cuffdiff.

2.6.2 Post cataract surgical samples

RNA sequencing of lens cells (LCs) isolated from operated lenses was performed by removing the lens fiber cells from one eye of C57BL/6NHsd (WT) or FNcKO mice, then 48 hours later, the other eye was operated upon, followed by the immediate sacrifice of mice (chapter 6). In the case of chapter 4 where we studied to understand the early response of the remnant LCs to the injury, 24 hours post cataract surgery of WT was performed too. Lens capsular bags with attached cells were isolated, and samples from five individual mice were pooled, and flash frozen on dry ice, to create one, 0 hour, one 24, and one 48-hours, post cataract surgery (PCS) biological replicate. RNA was isolated using the RNeasy Mini Kit (50) from Qiagen (Cat No./ID: 74104, Germantown, MD, USA). One microgram of total RNA was processed using the Illumina TruSeq RNA Sample Prep Kit (Cat#FC-122-1001) to produce sequencing libraries. Three biological replicates from both WT and FNcKO LC at 0 hour and 48 hours PCS and three biological replicates from WT at 24 hours

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PCS were analyzed on an Illumina HiSeq 2500 by the Genotyping and Sequencing Center, Delaware Biotechnology Institute, the University of Delaware. Single end 51 nucleotide reads were processed using a modified MAP-Rseq pipeline. Read alignments were generated with Tophat against the UCSC mm10 genome build and annotations (Kalari et al. 2014). HTseq was used to quantify reads aligning to genomic features, and edgeR was used for differential expression analysis. Reads per kilobase million (RPKM) was calculated for each gene from count data based on library size and exon-union transcript length. Biologically significant differentially expressed genes (DEGs) are defined as those exhibiting statistically significant changes (False Discovery Rate-FDR < 0.05), a change in mRNA level greater than 2 RPKM (Reads Per Kilobase Million) or FPKM (Fragments per kilobase million) between conditions, Fold Change (FC) greater than 2 in either the positive or negative direction, and expression levels in either condition that were 2 RPKM/FPKM or greater (Manthey, Terrell, Lachke, et al. 2014; Audette et al. 2016).

By using the above mentioned *in vivo* mouse model of cataract surgery, samples from WT and β8ITGcKO (three biological replicates for each condition, five capsules per replicate) were harvested at 0 hr and 24 hr PCS, flash frozen on dry ice, and RNA was harvested using RNeasy Mini Kit (50) from Qiagen (Cat No./ID: 74104). RNA libraries were prepared for sequencing using standard Clonthech protocols for SMARTer® Stranded Total RNA-Seq Kit-Pico Input Mammalian (Takara Bio USA, Inc., Mountain View, CA, USA) and sequenced by DNA Link, USA (901 Morena Blvd. Ste 730 San Diego CA 92117, USA) on NovaSeq 6000 (San Diego, CA, USA). Read pairs corresponding to RNA fragments were enumerated as FPKM (fragments per kilobase million) by Cuffdiff. Bioinformatics was performed

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where biologically significant differentially expressed genes (DEGs) are defined as those exhibiting statistically significant changes (False Discovery Rate-FDR < 0.05), a change in mRNA level greater than 2 FPKM between conditions, Fold Change (FC) greater than 2 in either the positive or negative direction, and expression levels in either condition that were 2 FPKM or greater (Manthey, Terrell, Lachke, et al. 2014).

2.7 Pathway analysis

Pathway analysis was performed on all genes whose expression was called "present" (>1 cpm (counts per million) in at least two samples) with DEGs defined as those exhibiting FC ≥ |2| and FDR < 0.05 using iPathwayGuide (Advaita Bioinformatics, Plymouth Michigan, USA). This software package uses Impact Analysis, an approach that considers both whether DEGs participating in a particular pathway (as defined by the Kyoto Encyclopedia of Genes and Genomes, KEGG (Kanehisa et al. 2017), analysis performed with KEGG release 84.0+/10-26, Oct 17) are overrepresented in the gene list and their directional interactions within the pathway (Tarca et al. 2009).

2.8 Rescue experiments by active TGFβ1 & gremlin-1

Rescue experiments were performed by instilling either active recombinant human TGFβ1 protein (5 µl of 0.1ng/µl TGFβ1 in balanced saline solution (BSS); R&D systems, Minneapolis, MN, USA; catalog no- 240-B) or recombinant human gremlin-1 protein (5 µl of 1ng/µl gremlin-1 in BSS; R&D systems, catalog no- 5190-GR) into the lens capsular bags of FNcKO and β8ITGcKO mice immediately following removal of the lens fibers. BSS were installed into the lens capsular bags of controls (WT/ β8ITGcKO/ FNcKO) PCS.

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2.9 Tail vein injection of αVβ8 integrin blocking antibody

Tail vein injection of an αVβ8 integrin blocking antibody was performed as described (Resch et al. 2019). Briefly, 20 mg/kg of a single dose of ADWA-11 (humanized αVβ8 integrin blocking antibody- obtained from Dr. Dean Sheppard and Dr. Amha Atakilit, UCSF, USA) was administered to wildtype mice via the lateral tail veins immediately following removal of the lens fiber cells on one eye. Control isotype-matched antibody (humanized αVβ3 integrin that does not cross-react with the mouse) was injected into the tails of control mice (wildtype and β8ITGcKO).

2.10 Immunofluorescence & confocal imaging

Immunofluorescence was performed to assay protein expression at the cellular level as described previously (N. A. Reed et al. 2001). Briefly, eyes were embedded in Optimum Cutting Temperature Media (Tissue Tek, Torrance, CA, USA) immediately after harvest, and stored at -80°C. Frozen sections (16 μm) were obtained with a Leica CM3050 cryostat (Leica Microsystems, Buffalo Grove, IL, USA), and mounted on Color Frost plus slides (Fisher Scientific, Hampton, NH, USA). Sections were fixed either in 1:1 acetone-methanol for 15 minutes at -20°C or 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT). After washing with PBS, slides were blocked for 1 hour at RT, then incubated with either a primary antibody diluted in blocking buffer (Table 2.2 for specifics of the primary antibodies, blocking buffer compositions, incubation times and dilutions used in this study) or just blocking buffer to serve as a negative control to exclude nonspecific staining by the secondary antibodies or channel bleed through as previously described (N. A. Reed et al. 2001). Following primary antibody treatment, slides were washed, then incubated for 1 hour at room temperature with 1:200 dilution of species appropriate Alexa Fluor

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488/568/633 labeled secondary antibody (Thermofisher Scientific, Waltham, MA, USA) in PBS. DNA/cell nuclei were detected by adding either a 1:2000 dilution of Draq-5 (Biostatus Limited, Shepshed, Leicestershire, UK) or a 1:1000 dilution of DAPI (Fluoropure D21490, Thermofisher Scientific, Waltham, MA, USA) to the secondary antibody solution. Some experiments also included a 1:250 dilution of fluorescein-labeled anti-αSMA (Sigma-Aldrich, St. Louis, MO, USA) in the secondary detection solution to visualize myofibroblasts. During imaging, the negative control was used (N. A. Reed et al. 2001) to set the baseline so that the low levels of non-specific binding of the secondary antibody are subtracted from the images.

Co-localization of two proteins when the requisite antibodies were raised in the same species was performed using a three step blocking procedure as previously described (Lewis Carl, Gillete-Ferguson, and Ferguson 1993). Blocking step 1 was performed as described above, blocking step 2 used a 1:100 dilution of a rabbit polyclonal antibody against phospho FAK-Tyr 397 (cat-3283, Cell signaling, Danvers, MA, USA) that is unable to detect its target in indirect immunofluorescence assays, and blocking step 3 utilized a 1:20 dilution of an unlabeled pre-adsorbed goat F(ab')2 anti-rabbit IgG - (Fab)'2 (cat- ab6107, Abcam, Cambridge, United Kingdom). The co-localized samples were compared with samples where each primary antibody, as well as the non-binding blocking antibodies, were stained separately to ensure that the blocking was complete.

Each staining experiment/time point was replicated using at least three biologically independent specimens (3-5 mice, at least 2 sections per mouse). Fluorescently labeled slides were visualized either using Zeiss LSM780 or Zeiss LSM880 confocal microscopes (Carl Zeiss Inc., Gottingen, Germany), and

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comparisons of images were made between slides imaged using identical imaging parameters. In some cases, the brightness and contrast were adjusted to allow viewing on diverse computer screens; however, these adjustments were made identically for all images within a particular time course.

Table 2.2 Primary antibodies used in this study

Primary antibody	**Fixation**	**Blocking buffer**	**Primary antibody conditions**
Fibronectin (ab2413, Abcam)	4% PFA	2% BSA in PBS	1:200; 1 hour at RT
Collagen I (PA5-95137, Invitrogen)	4% PFA	5% goat serum and 2% BSA in PBS	1:100; overnight at 4°C
Tenascin C (T3413, Sigma-Aldrich)	4% PFA	2% BSA in PBS	1:200; overnight at 4°C
Aquaporin0 (AB3071, Millipore)	1:1 acetone-methanol	2% BSA in PBS	1:200; overnight at 4°C,
α-SMA (1A4 F3777 & C6198, Sigma-Aldrich)	1:1 acetone-methanol	2% BSA in PBS	1:250; 1 hour at RT,
Ki 67 (D3B5, Cell Signaling)	4% PFA	Blocking buffer-5% NGS with 0.3 % TritonX-100 in PBS, Antibody buffer- 2% BSA with 0.3% TritonX-100 in PBS	1:100; overnight at 4°C
α5-integrin (ab150361, Abcam)	4% PFA	2% BSA and 5% NGS in PBS	1:200; overnight at 4°C
β1-integrin (MAB 1997, Millipore)	1:1 acetone-methanol	2% BSA in PBS	1:100; 1 hour at RT,
Phospho- S423/S425 SMAD3 (ab52903, Abcam)	4% PFA	10 min. wash in 5% BSA followed by 5% NGS, 10% horse serum and 0.3% Triton X-100 in PBS	1:100; overnight at 4°C

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Gremlin-1 (PA5-13123, Invitrogen)	4% PFA	5% goat serum and 2% BSA in PBS	1:200; over night at 4°C
αV-integrin (AB1930, Millipore Sigma)	4% PFA	5% goat serum and 2% BSA in PBS	1:200; overnight at 4°C
pFAK (44-624G, Thermofisher)	4% PFA	5% goat serum and 2% BSA in PBS	1:100; overnight at 4°C
β8 integrin (ab80673, Abcam)	4% PFA	5% goat serum and 2% BSA in PBS	1:100; overnight at 4°C
E-cadherin (24E10) Rabbit mAb #3195, Cell Signaling)	4% PFA	5% goat serum and 2% BSA in PBS	1:100; overnight at 4°C
Collagen I (ab21286, Abcam)	4% PFA	5% goat serum and 2% BSA in PBS	1:100; overnight at 4°C
pSmad1/5/8 (sc-12353, Santa Cruz)	4% PFA	2% BSA and 0.3% Triton X-100 in PBS	1:200; overnight at 4°C
LTBP1 (ab78294, Abcam)	1:1 acetone-methanol	5% goat serum and 2% BSA in PBS	1:800; overnight at 4°C,
Aquaporin0 (AB3071, Millipore)	1:1 acetone-methanol	2% BSA in PBS	1:200; overnight at 4°C,
Cleaved caspase 3 (9661, Cell Signaling)	4% PFA	5% goat serum and 2% BSA in TBS	1:100; overnight at 4°C
Thrombospondin 1 (18304-1-AP, Proteintech)	4% PFA	5% goat serum and 2% BSA in PBS	1:50; overnight at 4°C
ECM1 (11521-1-AP, Proteintech)	4% PFA	5% goat serum and 2% BSA in PBS	1:50; overnight at 4°C
Periostin (ab14041, Abcam)	4% PFA	5% goat serum and 2% BSA in PBS	1:100; overnight at 4°C
CXCL1 (12335-1-AP, Proteintech)	1:1 acetone-methanol	2% BSA in PBS	1:100; overnight at 4°C
LCN2 (AB2267, EMD Millipore)	1:1 acetone-methanol	5% goat serum in PBS	1:100;over night at 4°C

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COX-2/PTSG2 (ab15191, Abcam)	1:1 acetone-methanol	5% goat serum and 2% BSA in PBS	1:200; 1 hour at RT
G-CSF/CSF3 (ab181053, Abcam)	4% PFA	10% goat serum, 2% BSA and 0.3% Triton X-100 in PBS	1:100; overnight at 4°C
S100a9 (ab203133, Abcam)	1:1 acetone-methanol	5% goat serum and 2% BSA in PBS	1:100; overnight at 4°C
CCL2 (ab25124, Abcam)	4% PFA	5% goat serum, 2% BSA and 0.3% Triton X-100 in PBS	1:200; overnight at 4°C,
HMOX1 (ab13243, Abcam)	1:1 acetone-methanol	5% goat serum and 2% BSA in PBS	1:100; overnight at 4°C
CD11b (550282, BD Pharmingen)	1:1 acetone-methanol	2% BSA in PBS	1:50;1 hour at RT
F4/80 (565409, BD Pharmingen)	1:1 acetone-methanol	2% BSA in PBS	1:50; 1 hour at RT
Ly6G (557445, BD Pharmingen)	1:1 acetone-methanol	2% BSA in PBS	1:50;1 hour at RT

2.11 Flow Cytometric Analysis of α smooth muscle actin (αSMA) post cataract surgery (PCS)

Single cell suspensions of the cells associated with the capsular bag PCS (three replicates of 0 hour and 5 days PCS from WT and FNcKO, 4 capsular bags per replicate) were prepared by modifying an established protocol (Maeda et al. 2009). Briefly, the PCS capsular bags were isolated from the eye and then treated with 0.25% trypsin at 37°C for 30 min, and cells were dissociated every 10 minutes using fine-tipped pipettes. Cells were fixed for 15 minutes at room temperature with 100 µl

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Medium A from a FIX & PERM™ Cell Permeabilization Kit (GAS-003, Invitrogen, Carlsbad, CA, USA). Cells were washed in 2ml cold D-PBS (Dulbecco's phosphate-buffered saline, Invitrogen, Carlsbad, CA, USA), supernatant aspirated. 100 µl Medium B from the FIX & PERM™ Cell Permeabilization Kit was added to each tube. αSMA (dilution 1: 200, cat-F3777, Sigma Aldrich, St. Louis, MO, USA) and isotopic control- IgG2a (FITC Mouse IgG2a, κ Isotype Ctrl Antibody, cat- 400207, Biolegend, San Diego, CA) were added to the appropriate tubes. Samples were stained in the dark at 4 °C for 15 minutes and then washed in 2ml D-PBS. Samples were aspirated and 500 µl D-PBS was added to each tube. 5 µl of 1mg/ml propidium iodide (cat- P4170, Sigma Aldrich, St. Louis, MO, USA) was added 10 minutes prior to acquisition for DNA content analysis. Data were acquired using a BD FACSAria Fusion 15-color flow cytometry with FACSDiva software (V8.0.3) and analyzed using FCS Express (V5.0, research version).

2.12 ImageJ quantification and statistical analysis

Immunofluorescence images were quantified by determining the mean fluorescence intensity (MFI) of lens capsule associated tissue viewed in three randomly chosen confocal images from biological independent samples using ImageJ (v1.52P, NIH). Average Number of Nuclei (ANN)/section at different PCS of WT, FNcKO, and β8ITGcKO (six randomly chosen immunofluorescence images from each time point of PCS of biologically independent samples) was counted by ImageJ as described (Grishagin 2015). All statistics were assessed using either Student's t-test (correct for multiple comparisons using the Holm-Šídák method) or one-way ANOVA with Tukey's post hoc test performed using GraphPad Prism 8.3.0 software. Data are

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presented as mean ± SE (SEM) and differences were considered significant at $P \leq 0.05$.

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Chapter 3

CATARACT SURGEONS VIEWPOINTS ON THE NEED FOR NOVEL PREVENTIVE ANTI-INFLAMMATORY AND ANTI-PCO THERAPEUTICS

All the contents included in this chapter are described in a manuscript entitled Shihan, Mahbubul H., Samuel G. Novo, and Melinda K. Duncan. 2019. "Cataract Surgeon Viewpoints on the Need for Novel Preventative Anti-Inflammatory and Anti-Posterior Capsular Opacification Therapies." Current Medical Research and Opinion 35 (11): 1971–81.

3.1 Introduction

Cataracts, a major cause of blindness worldwide (Khairallah et al. 2015; Liu et al. 2017; C. M. Lee and Afshari 2017), are efficiently treated by surgery followed by implantation of an artificial intraocular lens (IOL) (Liu et al. 2017). However, cataract surgery triggers acute ocular inflammation which can be painful and slows visual recovery (Liu et al. 2017; Chan, Mahroo, and Spalton 2010). Inflammation is currently treated by either anti-inflammatory eye drops which are plagued by low patient compliance(Juthani, Clearfield, and Chuck 2017) or installation of anti-inflammatories into the eye at the time of surgery ("drop-less" cataract surgery) (Lindstrom et al. 2017). While this acute inflammation usually resolves quickly in the absence of infection, low-level inflammation can persist for months post surgery and may exacerbate other ocular pathologies such as uveitis and glaucoma (Abbouda et al. 2016; Bhutto and Lutty 2012; Teh et al. 2017; Diagourtas et al. 2017). Then, months to years following cataract surgery, a significant proportion of cataract patients

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experience an apparent recurrence of their cataract as Posterior Capsular Opacification (PCO) (I. Michael Wormstone, Wang, and Liu 2009; Awasthi, Guo, and Wagner 2009).

While the rates of PCO in human patients as determined within the first year of surgery have greatly diminished over the past 10 years due to the widespread introduction of "square edge" IOLs made of hydrophobic materials, late-onset PCO still occurs in a significant number of adult patients which limits the long-term outcome of cataract surgery (Kent 2008; Bellucci and Bellucci 2013; Y. Zhao et al. 2017; Ying Li et al. 2013). Further, short term PCO rates in infants and animals undergoing cataract surgery are still high as the remnant lens cells have a higher proliferative and migratory potential than is typical in age-related human cataract patients (Y Li, Yan, and Wolf 1997; Dawes, Duncan, and Wormstone 2013; Cook 2008; Nasisse, Dykstra, and Cobo 1995). Animals also tend to develop relatively dense PCO plaques, perhaps due to the high prevalence of lens-induced uveitis in veterinary patients due to their more advanced cataract phenotypes at surgery (Cook 2008; Brookshire et al. 2015).

In adult humans, PCO is routinely treated non-invasively by YAG laser capsulotomy (Awasthi, Guo, and Wagner 2009; Liu et al. 2017). However, while YAG therapy for PCO is highly effective in this population, it can result in retinal detachment, macular edema, glaucoma, IOL damage, and IOL dislocation (Beale et al. 2006; Burq and Taqui 2008; Wesolosky, Tennant, and Rudnisky 2017; Kruijt and van den Berg 2012) especially when high laser power is used to treat dense PCO (Beale et al. 2006). Although these complications are relatively uncommon (0.5-2% of patients undergoing YAG), and could be resulting from the cataract surgery itself and not

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YAG capsulotomy per se, it has been pointed out that these potentially blinding sequelae still affect significant numbers of people as both cataract surgery and YAG therapy for PCO are common (Sabbagh 2018). Outside of clinical considerations, YAG laser capsulotomy is also a cost for these patients and their health insurers (Aaronson, Grzybowski, and Tuuminen 2019). Further, YAG laser therapy is problematic in young children both due to their inability to sit at the instrument for the procedure and a tendency to form dense PCO (Fan et al. 2006), while it is generally not performed on animals due to both their tendency to form dense PCO and cost (Cook 2008; Beale et al. 2006) suggesting that understanding molecular mechanisms to prevent PCO would improve the outcome of cataract surgery.

In recent years, we have gained a significant cell biological understanding of what pathways drive remnant lens epithelial cells towards fiber cell differentiation versus transdifferentiation into fibrosis producing myofibroblasts (Mamuya et al. 2014; Y. Wang et al. 2018; Shu, Wojciechowski, and Lovicu 2017; 2019; de Iongh et al. 2005). To date though, pharmacological antagonists of these pathways have shown limited success in preventing PCO, although most of the tested therapies only targeted one of the many signal transduction pathways likely involved in PCO pathogenesis (Christian Wertheimer et al. 2015; Dong, Tang, and Xu 2015; C. Wertheimer et al. 2013; Christian Wertheimer et al. 2018; Sureshkumar et al. 2012). However, the feasibility of continuing to develop our deepening understanding of PCO pathogenesis into FDA approved anti-PCO therapies is impaired by a perception by some that PCO is not still an issue of clinical concern (Sabbagh 2018).

Thus, we undertook a survey of cataract surgeons who treat a breadth of patients including adults, children, and animals to discuss the clinical challenges they

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encounter and the types of therapeutic interventions that would enhance the long term efficacy of cataract surgery.

3.2 Results

3.2.1 What are your top 3 concerns for post-surgical management after cataract surgery? *Post cataract surgical inflammation and PCO are major patient management concerns post cataract surgery (PCS)*

Cataract surgeons were asked about their major concerns with patient management post cataract surgery (PCS) (Figure 3.1). 41% of adult cataract surgeons have stated that PCO is one of their top three major post cataract surgical complications and 31% of adult cataract surgeons mentioned that inflammation is a major patient management concern PCS. Macular edema is a major concern of 24% of adult cataract surgeons while post-surgical infection (21%) and retinal detachment (17%) were also major concerns of some of adult cataract surgeons interviewed (Figure 3.1A).

92% of pediatric cataract surgeons surveyed reported that visual axis opacification (VAO) is their major concern PCS while 77% of pediatric surgeons felt that post-surgical glaucoma was among their top three major concerns. Post-surgical inflammation was a major concern for 69% of pediatric surgeons interviewed while fewer surgeons gave post-surgical infection (23%) and retinal detachment (7%) as major management concerns for pediatric cataract patients (Figure 3.1B).

In contrast to adult and pediatric cataract surgeons, 100% of veterinary cataract surgeons report that glaucoma is among their top three major concerns PCS while 88% mentioned inflammation among their major clinical concerns after cataract surgery. 63% of veterinary cataract surgeons report that PCO is a long term clinical issue

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affecting their patient's vision PCS, while 50% of them report that retinal detachment is a major PCS complication. In contrast to human ophthalmologists, 13% of veterinary ophthalmologists mentioned that corneal healing can be an issue PCS in their patients (Figure 3.1C).

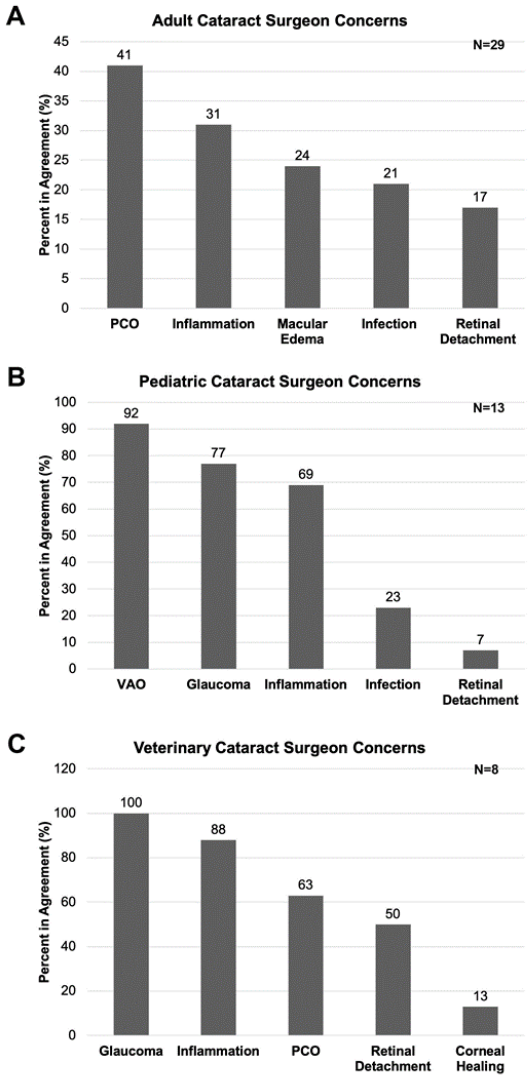

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Figure 3.1: Cataract surgeon opinion on their major concerns regarding post cataract surgical side effects. A- adult cataract surgeons; B- pediatric cataract surgeons; C- veterinary cataract surgeons.

3.2.2 How do you manage to post cataract surgical inflammation? Are you satisfied with the current standard of care for post cataract surgical inflammation treatment? Any alternatives that you would prefer?

Steroidal and nonsteroidal anti-inflammatory agents (NSAIDs) are the standard of care for the management of inflammation post cataract surgery

Steroidal and nonsteroidal anti-inflammatory agents (NSAIDs) are regularly administered to treat and/or prevent post cataract surgical inflammation. Cataract surgeons treating both adult and pediatric human patients are quite satisfied with the current standard of care for the management of post cataract surgical inflammation. In contrast, 62% of veterinary cataract surgeons are dissatisfied with this standard of care as animals, such as dogs, often need to be treated with anti-inflammatory agents for 3 to 6 months PCS for complete recovery as they often present at surgery with phacolytic uveitis (Coster 2019; Esson 2015). Thus, veterinary cataract surgeons expressed an interest in trying out alternatives if they are made available. (Figure 3.2).

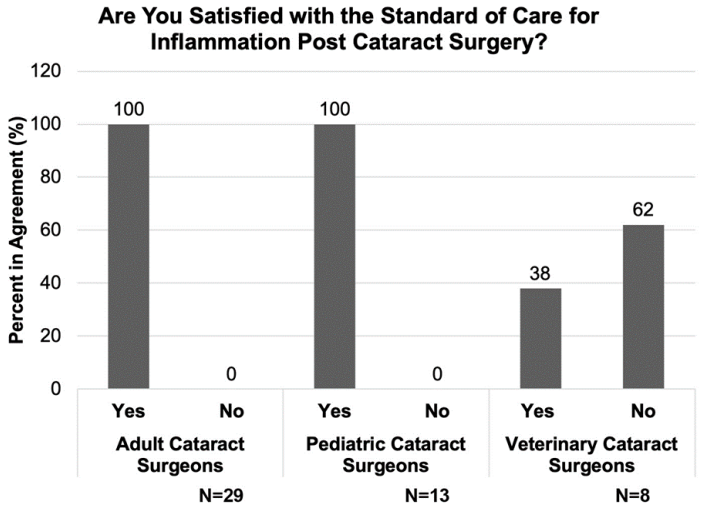

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Figure 3.2: Cataract surgeon opinion on the current standard of care for the management of post cataract surgical inflammation. Left- adult cataract surgeons; Middle- pediatric cataract surgeons; Right- veterinary cataract surgeons.

3.2.3 How many Posterior Capsular Opacification (PCO) cases do you get per year? *25% of adult and veterinary patients, and almost 100% of pediatric patients develop clinically significant PCO post cataract surgery (PCS)*

Most adult cataract surgeons surveyed estimated that approximately 25% of the cataract patients they treat return with clinically significant PCO within 10 years of cataract surgery. Cataract surgeons from developing countries such as South Africa and Peru have stated that 50% of their patients develop clinically significantly PCO in their clinical settings. In contrast, few cataract surgeons from developed countries experience 10% or fewer PCO patients per year. On the other hand, pediatric cataract surgeons said that almost 100% of pediatric patients develop PCO within a few months to years after cataract surgery. Some veterinary cataract surgeons report that 100% of their patients develop PCO, especially dogs. However, not all PCO impairs vision enough to hinder animals from performing regular activities like walking or finding food. Thus, about 25% of animal owners bring their animals to the veterinary cataract surgeon for behaviorally significant PCO (Figure 3.3A).

3.2.4 How long does PCO take to develop after surgery in your patients (based on your experience)? *Pediatric patients develop PCO quickly compared to adult and veterinary patients*

Surgeons who treat adult human patients feel that it takes between 2 to 5 years (average 3.5 years) to develop PCO PCS. Pediatric surgeons have informed us that it takes 12- 16 months for pediatric patients to develop PCO/VAO. However, several pediatric cataract surgeons have seen PCO/VAO development as early as 4 weeks PCS. Veterinary cataract surgeons report that behaviorally significant PCO develops

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in about 1-2 years PCS in younger dogs (Figure 3.3B), although many older dogs do not live long enough for this to be a concern.

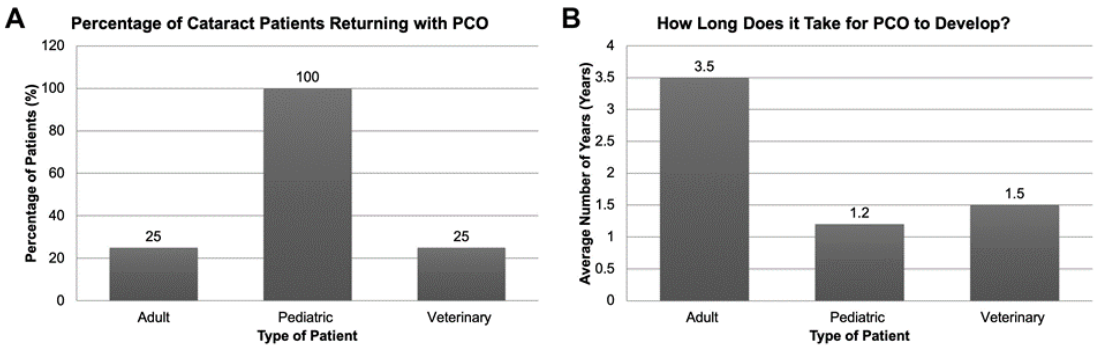

Figure 3.3: (A) Cataract surgeon estimates on the percentage of treated cataract patients returning with PCO (B) Cataract surgeon estimates of the time it takes to develop clinically significant PCO post cataract surgery.

3.2.5 Do you still think PCO is a clinical problem? *PCO is still clinically important*

Since all cataract surgeons report PCO in their practice, next we asked if they consider PCO as a significant, unsolved clinical issue and found that 100% of pediatric and veterinary cataract surgeons and 97% of adult cataract surgeons surveyed agreed with this statement. The sole disagreeing cataract surgeon who treats adult patients has told us that PCO is not clinically important since 1% or less of their patients develop PCO and this is easily treated by YAG when it occurs (Figure 3.4).

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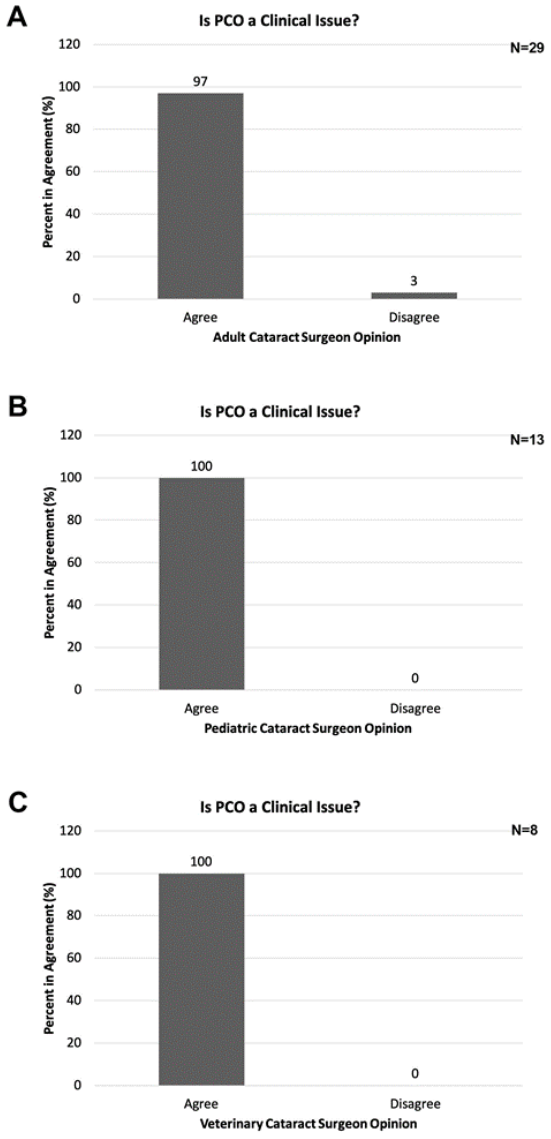

Figure 3.4: Percentage of cataract surgeons who report that PCO is still a clinical issue in their practice. A- adult cataract surgeons; B- pediatric cataract surgeons; C- veterinary cataract surgeons.

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3.2.6 How do you treat PCO? *YAG laser capsulotomy is the treatment of choice for PCO in adults while this is less used in pediatric and veterinary patients*

Cataract surgeons specializing in adult patients reported that YAG laser therapy is the treatment of choice for PCO. While pediatric cataract surgeons told us that YAG is sometimes possible in older children when the posterior capsule is intact, YAG is less feasible in younger or developmentally delayed children unable to sit still. Even when YAG is possible, children often develop dense PCO which cannot be removed by YAG and still require a posterior capsulotomy and anterior vitrectomy. Further, pediatric patients often develop PCO-like symptoms even if they underwent posterior capsulotomy during the initial cataract surgery due to the growth of cells across the anterior hyaloid membrane (visual axis opacification (VAO)). Due to the location of the aberrant cells, VAO must be treated by anterior vitrectomy (Vasavada, Trivedi, and Nath 2004; Shrestha and Shrestha 2014). In contrast, all veterinary cataract surgeons interviewed stated that there is no practical treatment for PCO in animals (Table 3.1).

Table 3.1: Cataract surgeons' opinion on the current treatment to manage PCO.

Question	**Response from cataract surgeons**
How do you treat PCO?	Adult- YAG laser therapy Pediatric- Sometimes YAG is possible when the posterior capsule is intact, but VAO may require anterior vitrectomy. Veterinary- There is no practical treatment for PCO in animals.

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3.2.7 How many patients get subsequent consequences such as macular edema and retinal detachment? (you can use a percentage of patients out of the total number of PCO patients). *Most adult cataract surgeons surveyed reported that they have not seen side-effects following YAG laser capsulotomy, while others stated that although the negative consequences of YAG laser capsulotomy are often minimal, important side effects still occur*

YAG laser is the treatment of choice for adult PCO among the cataract surgeons surveyed. As some negative consequences of YAG therapy have been reported (Beale et al. 2006; Burq and Taqui 2008; Wesolosky, Tennant, and Rudnisky 2017; Trinavarat, Atchaneeyasakul, and Udompunturak 2001), we asked adult cataract surgeons whether their patients have experienced any negative consequences after YAG laser therapy and found that 62% of adult cataract surgeons report that they have not seen any side effects following YAG laser therapy. However, 18% of adult cataract surgeons state that they have seen approximately 1% of patients experience retinal detachment following YAG laser therapy in their clinical practice, whereas 3% of adult cataract surgeons report a 2% retinal detachment rate, and a 1% rate of macular edema after YAG laser therapy. 14% of adult cataract surgeons surveyed reported that they have seen glaucoma after YAG laser treatment, but this occurs in 1% or less of all patients (Figure 3.5).

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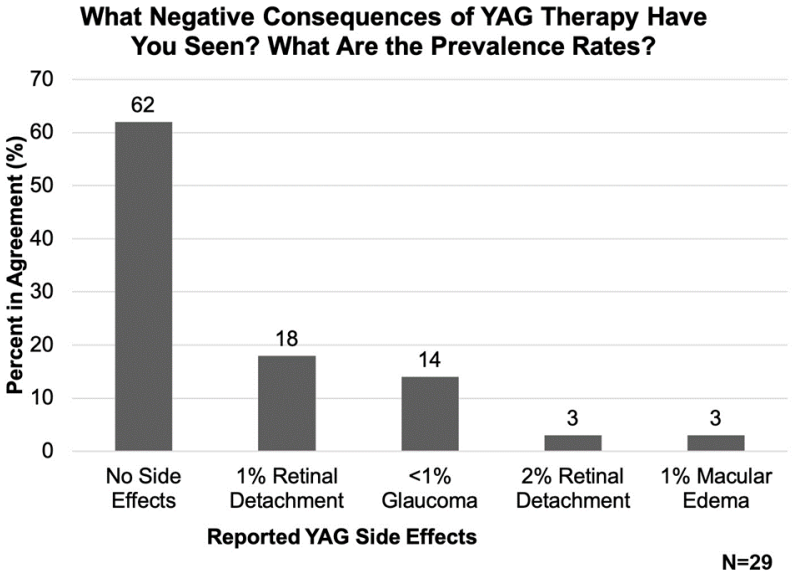

Figure 3.5: Adult cataract surgeon estimates of the prevalence of negative consequences of YAG therapy based on their clinical experience.

3.2.8 Are you satisfied with the treatment protocol for post cataract surgical inflammation and PCO? What do you desire or what changes would you do? If there was a new non-surgical approach to treating PCO/post-surgical ocular inflammation would you use it? *Most of the cataract surgeons surveyed are interested in new ways to prevent PCO while they are generally satisfied with the standard of care for inflammation prevention post cataract surgery*

Finally, we asked if cataract surgeons would be interested in incorporating anti PCO therapeutics into their clinical practice and found that all pediatric and veterinary cataract surgeons showed interest in preventive anti PCO therapeutics (Figure 3.6B and C). However, 14% of adult cataract surgeons think that YAG laser is adequate to treat PCO and thus do not feel that additional preventive anti PCO measures are necessary (Figure 3.6A). Overall, cataract surgeons treating humans feel that standard of care is sufficient for the treatment of inflammation associated with cataract surgery

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(Figure 3.2), however, those who treat animals would like additional therapeutic options for inflammation as this is not adequately controlled in their patients with the current standard of care (Figure 3.2).

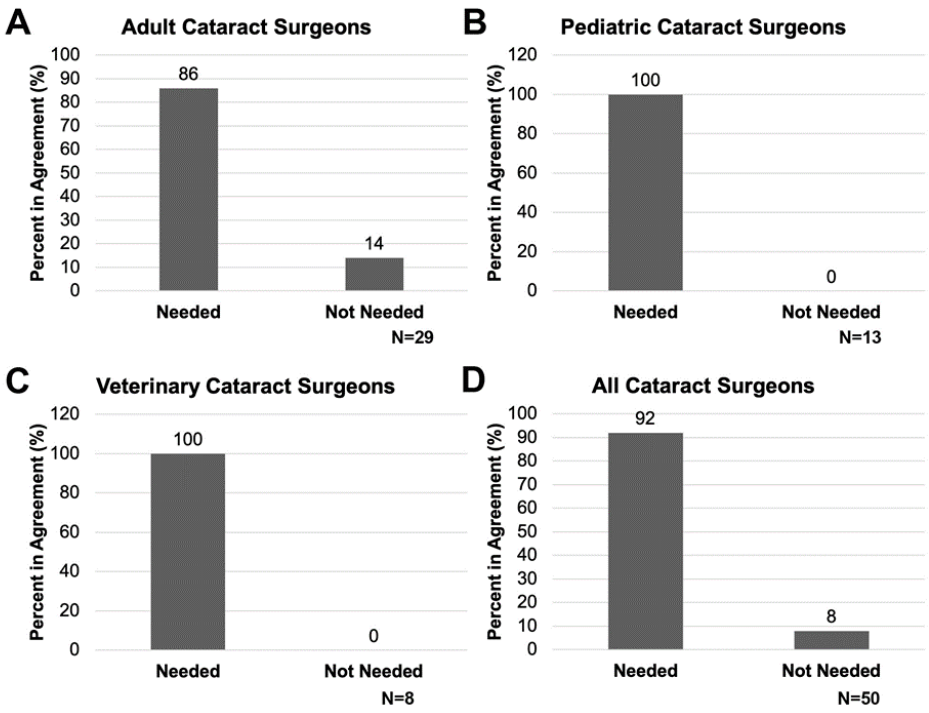

Figure 3.6: The percentage of cataract surgeons who report that new PCO therapies are needed. A- adult cataract surgeons; B- pediatric cataract surgeons; C- veterinary cataract surgeons; D- all cataract surgeons.

3.3 Discussion

The development of extracapsular cataract surgery followed by intraocular lens implantation is one of the most significant advances in modern medicine, taking cataract from being the major cause of human blindness and low vision that it was through most of human history, to a condition that can be treated with a quick

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outpatient procedure (Olson 2018). However, even the most robust surgical intervention has the potential for negative sequelae(Liu et al. 2017; I. Michael Wormstone, Wang, and Liu 2009; Awasthi, Guo, and Wagner 2009).

3.3.1 Most cataract surgeons surveyed are satisfied with the treatments available for ocular inflammation following cataract surgery

Cataract surgery is one of the most commonly performed surgeries in the world and has been highly successful for decades (Juthani, Clearfield, and Chuck 2017). However, cataract surgery results in acute ocular inflammation arising as a normal response to the surgical wound (Kohnen 2015). While high levels of inflammation can be an important sign of infection, uncontrolled "sterile" ocular inflammation is undesirable as it opacifies the ocular media and can lead to several other complications notably uveitis, secondary glaucoma, macular edema and even retinal detachment (Abbouda et al. 2016; Juthani, Clearfield, and Chuck 2017; McColgin and Heier 2000). Thus, its post cataract surgical management is of paramount importance. Currently, corticosteroids or nonsteroidal anti-inflammatory agents (NSAIDs), or a combination of both, are the treatment of choice in the management of inflammation post cataract surgery (Colin 2007; Juthani, Clearfield, and Chuck 2017). In general, steroids are more effective in managing inflammation than NSAIDs, however, in some cases, NSAIDs might be sufficient in routine patients undergoing cataract surgery (Grzybowski and Kanclerz 2018).

Due to the importance of inflammation management post cataract surgery, we first asked cataract surgeons if they see post-surgical inflammation in their clinical settings. All of the cataract surgeons have informed us that inflammation is a common side effect that they see following cataract surgery which is consistent with reports that

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most if not all cataract patients develop inflammation post cataract surgery (Colin 2007; Juthani, Clearfield, and Chuck 2017; McColgin and Heier 2000). Next, we have asked them about their preferred methods to manage post-surgical inflammation. Consistent with the literature, some of the cataract surgeons we surveyed prefer to use corticosteroids, while others apply NSAIDs or a combination of both (Juthani, Clearfield, and Chuck 2017). Most surveyed adult and pediatric cataract surgeons stated that they were satisfied with the current standard of care for inflammation management post cataract surgery. However, veterinary cataract surgeons were interested in additional ways to prevent inflammation post cataract surgery as it can take a few months to resolve inflammation PCS in dogs, which is time-consuming and not cost-effective. This observation is consistent with the literature which suggests that a high proportion of dogs receive cataract surgery after the onset of phacolytic uveitis resulting from mature cataract which must be treated to prevent side effects such as inflammation and glaucoma (Biros et al. 2000).

3.3.2 Most cataract surgeons surveyed feel that PCO is still an important clinical problem

As surgical therapies for cataract were being developed, intracapsular lens removal became the therapy of choice since extracapsular lens extraction was plagued by PCO as proliferation, migration, and differentiation/transdifferentiation of the remnant lens epithelial cells resulted in a rapid recurrence of visual symptoms which could only be treated with subsequent invasive surgery (Olson 2018). Later, attempts to develop intraocular lens prostheses were greatly slowed by high rates of PCO, however, the advent of YAG laser capsulotomy made this treatable by a non-invasive office procedure (Karahan, Er, and Kaynak 2014). This has been bolstered by the

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development of square edge IOLs which have reduced PCO rates in adults to the single digits when measured within the six months to a year after cataract surgery (Vasavada, Trivedi, and Nath 2004; Auffarth et al. 2003; Buehl and Findl 2008), allowing extracapsular lens extraction followed by IOL implantation to become the standard of care for cataract treatment. However, there is some controversy about the global use of square edge IOLs as their design results in pseudophakic dysphotopsia which is the most prevalent reason patients are dissatisfied with cataract surgery (Olson 2005; Masket et al. 2018). Further, it has been noted that the growing popularity of "premium" IOLs which correct vision at all distances make PCO more clinically important in adults, while YAG laser capsulotomy is not without side effects (MacRae, Zheleznyak, and Yoon 2013). A new surgical technique bag-in the-lens (BIL) has shown promising results preventing PCO in both adult and pediatric patients compared to the traditional lens-in-the bag (LIB) procedure. However, this approach has not widely adopted in the USA due to the lack of clinical studies in the USA, and the specialized technical expertise needed (Nystrom et al. 2018; M.-J. Tassignon et al. 2007; M.-J. B. R. Tassignon, De Groot, and Vrensen 2002; Altenburg, Ni Dhubhghaill, and Tassignon 2017; De Groot et al. 2006; De Groot, Tassignon, and Vrensen 2005; Gobin, Dhubhghaill, and Tassignon 2019).

These reports have led to some controversy as to whether PCO is a "solved" clinical problem or still should be the focus of both basic science and drug development efforts due to its high importance. Most adult cataract surgeons surveyed stated that about 25% of adult patients undergoing cataract surgery develop clinically significant PCO within 2- 5 years post cataract surgery. The remaining surgeon reports PCO rates below 1% due to their routine use of "posterior optic capture/bag in

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the lens" surgery in which a posterior capsulotomy is performed at surgery and the remanent lens cells are trapped at the capsule periphery by placing the IOL in Berger's space (Ocular Surgery News 2017). Surgeons practicing in developing countries reported higher PCO rates compared to developed countries due to lower access to improved IOLs and modern cataract surgical techniques. YAG laser therapy is the treatment of choice for adults presenting with PCO, and the surgeons surveyed are generally satisfied with the clinical outcomes of YAG laser therapy although they acknowledge that some patients do develop undesirable sequelae such as macular edema and retinal detachment. These opinions closely correlate with published reports that show that the long-term (1-10 years post surgery) PCO rates are still 20-70% in adults (Sabbagh 2018; Liu et al. 2017). The relatively low rates of severe negative outcomes observed after YAG are also borne out in the literature which reports rates of 1-3% (Karahan, Er, and Kaynak 2014; Liu et al. 2017). Several cataract surgeons informed us that YAG laser capsulotomy is not a financial burden for patients as Medicare and government health systems cover or subsidize YAG therapy, although this opinion does not consider the cost to these health care systems. For instance, YAG laser capsulotomy to treat PCO was the 10th most costly ambulatory procedure performed on Medicare patients in 2016 (Sabbagh 2018). The adult cataract surgeons surveyed are generally satisfied with YAG laser therapy as a treatment for PCO, although it is less available in developing countries (Findl et al. 2010). Overall, 86% of adult cataract surgeons surveyed would be interested in pharmacological methods to robustly prevent PCO, although some adult cataract surgeons emphasized the need for improved IOL designs and surgical techniques as other ways to improve the visual outcomes for their patients. However, the pediatric cataract surgeons interviewed told

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us that the reality is quite different for their patients. They stated that almost 100 percent of pediatric patients undergoing traditional extracapsular lens extraction followed by IOL implantation develop PCO within the first year following surgery which is supported by reports in the literature (Batur et al. 2016; Vasavada and Praveen 2014). Even in cases where prophylactic posterior capsulotomy is performed at the time of cataract extraction, the response of the remnant lens epithelial cells to surgery is still a problem because they can migrate onto the anterior hyaloid and/or cause phimosis of the anterior capsulotomy resulting in visual axis opacification (VAO) (Shrestha and Shrestha 2014; Khaja et al. 2011). While children over 6 years of age can often be treated with YAG laser capsulotomy, younger children or those with developmental delays can not sit still at the instrument (Batur et al. 2016). The pediatric cataract surgeons surveyed also noted that VAO/PCO treatment is problematic in young children as this requires general anesthesia, and YAG instruments optimized for treating anesthetized children are generally not available. Further, YAG is often counter-indicated when the PCO is dense or when VAO involving the anterior hyaloid is present (Batur et al. 2016; Vasavada, Trivedi, and Nath 2004). In these cases, invasive posterior capsulotomies are done, and/or anterior vitrectomies are required to remove the opacification, and even then VAO can re-occur (Vasavada, Trivedi, and Nath 2004; Batur et al. 2016; Petric and Lacmanovic Loncar 2004; Hutcheson et al. 1999). All pediatric cataract surgeons surveyed stated that they would be interested in exploring new approaches to prevent PCO and/or VAO, including the use of pharmacological agents to prevent these conditions, due to the need for increasing the safety and efficacy, while reducing the cost, for cataract treatment in children.

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The veterinary cataract surgeons surveyed noted that animals (dogs, cats, and horses) tend to get PCO earlier and progress faster than humans, with PCO rates approaching 100% which is consistent with the literature (Gift et al. 2009). However, veterinary surgeons also note that PCO is often less clinically significant as animals have shorter life expectancies and most pets only need sufficient vision to navigate their surroundings and find food, so have less need for excellent visual acuity. Even with that, 25% of their owners bring their animals back to the veterinary cataract surgeon reporting a recurrence of visual symptoms. However, few animals are treated for PCO. First, YAG laser therapy is not effective for most animal PCO cases due to both the tendency for animal PCO to be denser than that seen in humans, as it forms more aggressively and must be severe before the owner notes a reduction in their pet's vision (Gift et al. 2009). Second, as only 25% of veterinary patients develop clinically significant PCO by the end of their life, and as YAG lasers are costly, they are uncommon in veterinary practices. Thus, all veterinary cataract surgeons surveyed are very interested in preventive anti-PCO therapeutics.

These interviews suggest that most cataract surgeons are satisfied with the current standard of care for post-surgical inflammation while the majority surveyed felt that new approaches to prevent PCO would be clinically useful, although the relatively small number of surgeons interviewed here (50 total across three different practice types; adult, pediatric and veterinary) could mean the market for new PCO prevention strategies is not as large as suggested here. However, as major progress has been made in recent years towards understanding the pathophysiology of PCO (Mamuya et al. 2014; Jiang et al. 2018; de Iongh et al. 2005; I. Michael Wormstone, Wang, and Liu 2009; Y. Wang et al. 2018), there is likely sufficient information about

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PCO pathogenesis to identify new anti-PCO therapeutics which would be of particular use in veterinary and pediatric cataract surgery. Simultaneously, new surgical approaches such as “bag in the lens” and further refinements in IOL design have clinical promise in PCO prevention for all patient populations. Thus, the future looks bright for approaches to reduce the incidence of PCO, improving the long term effectiveness of cataract surgery.

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Chapter 4

LENS EPITHELIAL CELLS INITIATE AN INFLAMMATORY RESPONSE FOLLOWING CATARACT SURGERY

All the content included in this chapter are described in a manuscript entitled [Jiang, Jian, Mahbubul H. **Shihan (co-first author)**], Yan Wang, and Melinda K. Duncan. 2018. “Lens Epithelial Cells Initiate an Inflammatory Response Following Cataract Surgery.” Investigative Ophthalmology & Visual Science 59 (12): 4986–97

4.1 Introduction

Cataracts have traditionally been the most prevalent cause of human blindness, however, in recent decades, their impact has been greatly reduced by the adoption of extracapsular and/or phacoemulsification cataract extraction followed by intraocular lens (IOL) implantation into the lens capsular bag (Olson 2005; Liu et al. 2017; Khairallah et al. 2015; C. M. Lee and Afshari 2017). However, the long term outcome of cataract surgery is compromised when residual lens epithelial cells (LECs) begin proliferating concurrently with either epithelial-mesenchymal transition (EMT) leading to the formation of pro-fibrotic myofibroblasts, or the onset of a regenerative response where the remnant LECs convert to structurally aberrant lens fibers(I. Michael Wormstone, Wang, and Liu 2009). If these LEC derived cells remain at the periphery, they form Soemmering's ring which is largely benign (Bhattacharjee and Deshmukh 2017) or even beneficial for long term IOL stability (D. J. Spalton et al. 2014). However, Soemmering's ring can continue to expand many years post cataract surgery (PCS) compromising the function of advanced IOLs (Alio et al. 2009; D. J.

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Spalton et al. 2014) even leading to late IOL dislocation (Gimbel and Venkataraman 2008). If LEC-derived myofibroblasts migrate anteriorly PCS, they can cause anterior capsular fibrosis/phimosis which opacifies the visual axis and can de-centrate the IOL (Michael et al. 2010; Epstein et al. 2014). If myofibroblasts migrate onto the posterior lens capsule, they again form scar tissue in the visual axis leading to fibrotic posterior capsular opacification (PCO) (Awasthi, Guo, and Wagner 2009; Apple et al. 2011). Finally, even if the posterior lens capsule is ablated at the time of surgery, lens-derived myofibroblasts can opacify the visual axis by migrating from the lens capsular bag onto the anterior hyaloid membrane, particularly in pediatric patients(Khaja et al. 2011; Elkin, Piluek, and Fredrick 2016).

While there is controversy in the literature about the population-wide rates of these undesirable outcomes, PCO rates alone are reported to be 40% or higher in adult patients living 10 years or more PCS (Apple et al. 2011; Ronbeck and Kugelberg 2014), and approach 100% in children (Khaja et al. 2011; Elkin, Piluek, and Fredrick 2016). While these PCS side effects are generally treatable by either YAG laser ablation or surgery, poor outcomes can result due to ocular inflammation, difficulties ablating dense fibrosis, IOL displacement, and retinal complications (Chan, Mahroo, and Spalton 2010; Burq and Taqui 2008; Wesolosky, Tennant, and Rudnisky 2017). Thus, prevention of LEC EMT would improve the long term visual outcome of cataract surgery (Apple et al. 2011; Billotte and Berdeaux 2004).

TGFβ signaling can drive LEC EMT (de Iongh et al. 2005), while sustained TGFβ signaling has been observed in both fibrotic PCO (S. Saika et al. 2002) and the lens fibrotic disease, anterior subcapsular cataract (ASC) (Shizuya Saika et al. 2004; Ishida et al. 2005). However, while TGFβ concentrations are high in the eye even

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prior to surgery, most of this TGFβ is in an inactive form (Maier et al. 2006) and is thus unable to elicit fibrotic responses. This makes it likely that the induction of pathways that result in latent TGFβ activation (Nibourg et al. 2015; Srinivasan, Lovicu, and Overbeek 1998; Eldred, Dawes, and Wormstone 2011; Mamuya and Duncan 2012) are key steps in PCO pathogenesis.

We developed an *in vivo* mouse model of cataract surgery where the lens fiber cells are surgically removed, leaving behind the lens capsule and attached LECs (Desai et al. 2010; Manthey, Terrell, Wang, et al. 2014). In this model, the upregulation of mRNAs encoding fibrotic markers such as alpha-smooth muscle actin (αSMA), fibronectin, and tenascin-C are detected in remnant LECs 24 hours PCS, while the first induction of these proteins is seen 48 hours PCS(Mamuya et al. 2014). Notably though, it takes 48 hours for the first obvious upregulation of the pSMAD2/3 levels associated with TGFβ pathway activation, and up to five days for a maximal response (Mamuya et al. 2014). This lag between injury and TGFβ pathway activation thus makes the mouse an excellent model to study the mechanisms by which ocular trauma/surgery results in fibrotic PCO, and we have successfully used this mouse "cataract surgery" model to direct the power of mouse genetics to the study of PCO pathogenesis (Manthey, Terrell, Wang, et al. 2014; Mamuya et al. 2014). Here we use RNAseq to discover the gene expression changes that LECs undergo after cataract surgery but prior to the onset of TGFβ signaling. This analysis revealed that LECs robustly activate the innate immune response within hours of cataract surgery and support prior speculation that post-surgical inflammation is mechanistically related to lens capsular bag fibrosis PCS (Lewis 2013).

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4.2 Results

4.2.1 The lens epithelial cell transcriptome is drastically altered by 24 hours following cataract surgery

While it is accepted that fibrotic PCO results from the epithelial to mesenchymal transition of lens epithelial cells (LECs) driven by TGFβ signaling (I. Michael Wormstone, Wang, and Liu 2009; Shirai et al. 2018), we have previously shown that there is a 48 hour or longer lag between cataract surgery and the onset of robust Smad-mediated TGFβ signaling in LECs in a mouse model, likely due to the need to activate latent TGFα post cataract surgery (PCS) (Mamuya et al. 2014). Thus, we used RNAseq to gain insight into the initial response of LECs to cataract surgery by comparing the transcriptome of LECs isolated immediately following surgery, with that of LECs isolated 24 hours later. The resulting dataset which includes three biological replicates from both time zero and 24 hours PCS LECs was submitted to the Gene Expression Omnibus (GEO) under accession number GSE111430. This analysis revealed that 14,454 genes exhibited measurable expression in LECs, while 2251 were differentially expressed genes (DEGs) in LECs (1255 upregulated, 996 downregulated) isolated at 24 hours PCS compared to 0 hour PCS (FDR ≤0.05; more than 2 fold change in mRNA levels; expressed higher than 2 RPMK either immediately PCS or 24 hours later).

Analysis of the DEGs for disease associations using iPathwayGuide (Advaita Corporation) revealed that "cataract" was the most significant (FDR corrected $P < 9.1 X10\text{-}4$), with 19 of the 27 known cataract-associated genes in the KEGG database being differentially expressed in LECs by 24 hours PCS. Of these, 14 are downregulated (Sipa1l3, Gja3, Mip, Foxe3, Gja8, Bfsp2, Tdrd7, Maf, Cryab, Bfsp, Cryaa, Pitx3, Hsf4, and Pax6), while 5 are upregulated (Vim, Wfs154, Epha2, Ftl1,

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and Gcnt2) suggesting that the "lens" phenotype of LECs is perturbed by 24 hours PCS. Notably, seven of the other nine significant predicted disease associations (FDR corrected P <0.028-0.036) are chronic autoimmune/inflammatory/infectious conditions.

In order to predict which pathways are perturbed in LECs at 24 hours PCS, we used iPathwayGuide to perform impact analysis (Tarca et al. 2009) which takes into account both the overrepresentation of genes within a pathway and whether the later genes in a pathway are significantly more perturbed than the earlier ones. This analysis predicts that 132 KEGG defined pathways are significantly affected in LECs by 24 hours PCS (Figure 4.1A) with the top 10 overrepresented pathways including cell adhesion molecules, actin cytoskeletal regulation, and numerous KEGG pathways associated with inflammatory responses (Figure 4.1B). Notably, of these, 91 DEGs are known to be involved in cytokine/cytokine receptor pathway interactions (Figure 4.2), including CXCL1, the DEG most upregulated in LECs PCS at 3866 fold (FDR corrected $P \leq 1.6 \times 10^{-52}$). Consistent with this, the genes differentially regulated in LECs at 24 hours PCS are also highly enriched for Gene Ontology (GO) terms related to immune responses including; *response to cytokine* (270 of 590 genes associated with the term; FDR corrected $P=1X10^{-24}$), *cytokine production* (226 of 498 genes associated with the term; FDR corrected $P=3.5X10^{-20}$), and *the innate immune response* (186 of 447 genes associated with the term; FDR corrected $P=3.7X10^{-12}$).

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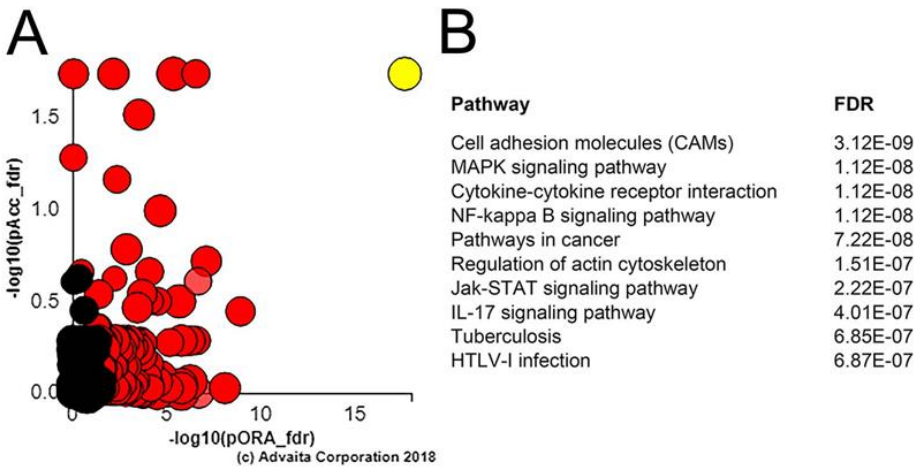

Figure 4.1 RNA Seq analysis revealed that LECs exhibit highly perturbed cell signaling at 24 hours PCS. A) Impact analysis (Tarca et al. 2009) of genes differentially expressed in LECs at 24 hours PCS revealed that 132 pathways (red or yellow circles) at defined by the Kyoto Encyclopedia of Genes and Genomes project (KEGG) were significantly impacted in LECs upon cataract surgery as calculated by an overrepresentation of genes within a pathway (horizontal axis) and/or significantly perturbed accumulation (vertical axis). One pathway (yellow) circle represents cytokine-cytokine receptor interactions, which are predicted to be highly perturbed by both criteria. Black circles represent pathways that were not significantly affected. Axis labels: -log10(pAcc_fdr) represents the -log10 of the FDR corrected P-value for the accumulated perturbation of the pathway;–log10(pORA_fdr) represents -log10 of the FDR corrected P-value for overrepresentation of pathway genes among the DEGs. B) The top 10 pathways that are significantly impacted in LECs 24 hours PCS reported along with their FDR corrected P values. Note that while the cell adhesion molecule (KEGG: 04514) pathway exhibits the lowest FDR corrected P-value due to the overrepresentation of genes in this pathway among the DEGs, it does not exhibit significantly accumulated pathway perturbation.

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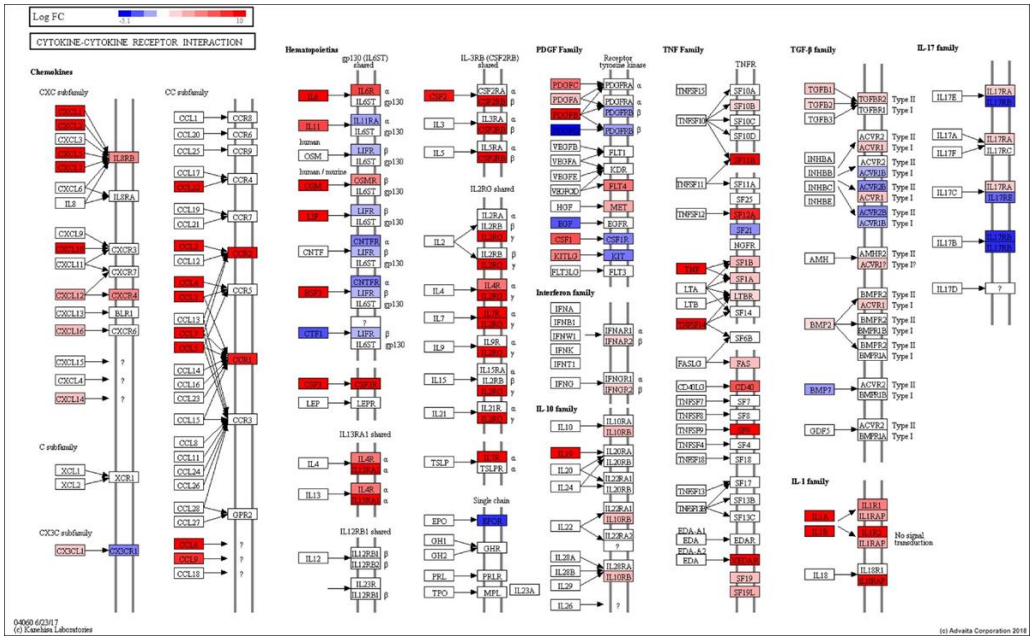

Figure 4.2: RNA Seq analysis revealed that LECs exhibit a highly perturbed cytokine-cytokine receptor pathway at 24 hours PCS. The cytokine-cytokine receptor pathway, as defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG:04060) (Kanehisa et al. 2017), is annotated to highlight all pathway genes which are differentially expressed in LECs at 24 hours PCS. Blue- genes downregulated in LECs at 24 hours PCS; Red- genes upregulated in LECs at 24 hours PCS.

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4.2.2 Lens epithelial cells upregulate diverse genes involved in the inflammatory response within the first 24 hours of cataract surgery

While cataract surgery is very effective, its short term outcome is hampered by the onset of ocular inflammation by 24 hours PCS(El-Harazi and Feldman 2001) which is usually attributed to surgically induced breaks in the blood-aqueous barrier which allows for plasma protein leakage into the aqueous humor and immune cell infiltration**.** Since RNAseq analysis revealed that the three genes most upregulated in LECs at 24 hours PCS were the mediators of innate immunity, CXCL1 (3866 fold), S100a9 (1505 fold) and CSF3/G-CSF (1119 fold), (Table 4.1), we sought to determine their protein expression dynamics in lens capsular bags between 0 hour and 10 days PCS (Figure 4.3). The expression of the chemokine CXCL1(Kobayashi 2008) was absent in capsular bags at 0 and 1 hour PCS but was detected in LECs at 3 and 6 hours PCS. CXCL1 protein levels peaked in LECs at 24 hours PCS, sharply downregulated in capsular bags by 48 hours PCS, and remained low between 3 and 10 days PCS.

The pro-inflammatory alarmin S100a9 (Austermann, Zenker, and Roth 2017) was not detected in capsular bags isolated at 0 or 3 hours PCS. S100a9 immunostaining was first detected in capsular bags at 6 hours PCS which became more intense at 24 hours PCS. S100a9 levels sharply downregulated in capsular bags by 48 hours PCS and remained low between 3 and 10 days PCS.

There was a weak immunolocalization signal for CSF3 (granulocyte colony-stimulating factor, G-CSF, an important cytokine in neutrophil development (Panopoulos and Watowich 2008)) in LECs immediately PCS. This staining became more intense at 1 hour PCS and continued to increase through 6 hours PCS, peaking at 24 hours PCS. G-CSF protein levels declined by 48 hours PCS and were nearly undetectable between 3 and 10 days PCS.

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In addition to the three most upregulated genes studied above, the RNAseq data revealed that a number of other genes that function in diverse pro-inflammatory pathways were also upregulated in capsular bags at 24 hours PCS. PTSG2, the gene encoding the enzyme cyclooxygenase 2 (COX-2) which catalyzes a key step in prostaglandin synthesis (Alexanian et al. 2014), was 248 fold upregulated in capsular bags at 24 hours PCS. COX2 protein was not detected in capsular bags immediately PCS (Figure 4.4), however, weak COX2 immunostaining was detected 1 hour PCS and continued to increase through 6 hours PCS, peaking at 24 hours PCS. COX-2 levels decline by 48 hours PCS and remain low, but detectable at 3 and 4 days PCS. However, significant COX-2 immunostaining was associated with capsular bags at 5 days PCS, although these levels again decreased by 10 days PCS.

CCL2 encodes the chemokine, monocyte chemoattractant protein-1 (MCP-1) (Yoshimura 2018), whose mRNA levels are 92 fold upregulated in lens capsular bags at 24 hours PCS. No CCL2 immunolabeling was detected in lens capsular bags either immediately, or 3-6 hours PCS. Modest CCL2 immunolocalization was detected in capsular bags from 6-48 hours PCS but its levels decrease thereafter. CCL2 protein was not detectable in capsular bags from 3 to 10 days PCS (Figure 4.4).

LCN2 (neutrophil gelatinase-associated lipocalin/lipocalin2), is a multifunctional protein often upregulated in stressed tissues, particularly following injury. It has antimicrobial activity via its ability to scavenge microbially derived siderophores (Moschen et al. 2017), it binds to and stabilizes MMP9 (Moschen et al. 2017)which is implicated in TGFβ mediated LEC EMT(Korol et al. 2014), while also inducing the synthesis of pro-inflammatory cytokines by neutrophils (Moschen et al. 2017). LCN2 mRNA levels upregulate 60 fold in LECs by 24 hours PCS. LCN2

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protein was not detected in capsular bags at the time of surgery but was found at modest levels at 1 and 3 hours PCS. LCN2 levels further increase in capsular bags at 6 hours PCS and are maximal at 24 hours PCS. LCN2 levels fall sharply by 48 hours PCS and are essentially undetectable between 3-10 days PCS (Figure 4.4).

Heme oxygenase (HMOX1) is an enzyme that catalyzes the degradation of hemoglobin into bilirubin and carbon monoxide which modulates innate and adaptive immunity while protecting cells from inflammation-induced oxidative stress (Espinoza, Gonzalez, and Kalergis 2017). RNAseq revealed that HMOX1 mRNA levels are 27 fold upregulated in lens capsular bags at 24 hours PCS compared to 0 hour PCS. No HMOX1 protein was detectable by immunolocalization in lens capsular bags between the time of surgery and 3 hours PCS. Modest HMOX1 staining was detected in lens capsular bags between 6 hours and 48 hours PCS, while HMOX1 staining was absent from capsular bags between 3 and 10 days PCS (Figure 4.4). These data in aggregate reveal that LECs rapidly initiate an inflammatory response after cataract surgery and/or lens wounding.

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Table 4.1: Inflammatory genes detected in this study differentially expressed between 24 hours and 0 hour post cataract surgery determined by RNA-seq.

Gene ID	Gene description	Fold change from 0 hour	P Value	24 hours Mean RPKM	0 hour Mean RPKM
CXCL1	chemokine (C-X-C motif) ligand 1	3866	1.81E-55	58.5	0
S100a9	S100 calcium binding protein A9	1505	2.86E-29	41.9	0
G-CSF/CSF3	colony stimulating factor 3 (granulocyte)	1119	7.57E-31	140.1	0.1
COX-2/Ptgs2	prostaglandin-endoperoxide synthase 2	248	2.01E-38	49.7	0.2
CCL2	chemokine (C-C motif) ligand 2	92	5.45E-95	330.8	3.3
LCN2	lipocalin 2	60	7.90E-40	5738.6	92.7
HMOX1	heme oxygenase 1	26.62	4.57E-28	207.11	7.22

RPKM- Reads Per Kilobase Million

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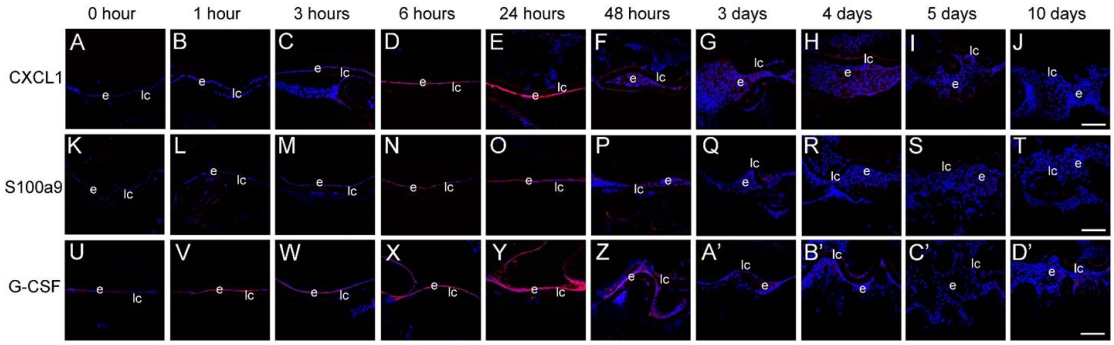

Figure 4.3: PCS expression time course in LECs for the three most differentially expressed genes in this study. (A-J) CXCL1 protein expression (red) in LECs after lens fiber removal. CXCL1 protein was not detected in LECs at 0 hour (A) and 1 hour (B) PCS. CXCL1 expression is first detected at 3 hours PCS (C), and this becomes robust by 6 hours PCS (D). The highest CXCL1 expression was detected at 24 hours PCS (E) followed by dramatically downregulation by 48 hours PCS (F). Weak CXCL1 expression persisted at 3 days PCS (G) and 4 days PCS (H), while nearly no CXCL1 staining was detected at 5 days PCS (I) and 10 days PCS (J) PCS. (K-T) S100a9 protein expression (red) in LECs PCS. There are little to no S100a9 expression in LECs at 0 hour (K), 1 hour (L), and 3 hours (M) PCS. S100a9 protein levels upregulated by 6 hours PCS (N), peaked around 24 hours PCS (O), then downregulated by 48 hours PCS (P). Low-level S100a9 expression is associated with capsular bags at 3 days (Q), 4 days (R), and 5 days (S) PCS, but largely disappears by 10 days PCS (T). (U-D') G-CSF protein expression (red) in LECs PCS. Weak G-CSF staining was observed in LECs at 0 hour PCS (U), and this staining upregulated gradually between 1 hour (V), 3 hours (W), and 6 hours (X) PCS. The highest level of G-CSF staining was seen at 24 hours PCS (Y), while this was attenuated at 48 hours PCS (Z). G-CSF levels are nearly undetectable at 3 days (A'), 4 days (B'), 5 days (C') and 10 days (D') PCS. For all panels, Blue= DNA as visualized by Draq5 staining; Scale bars: 100 μm; e, remnant lens epithelial cells/lens cells; lc, lens capsule.

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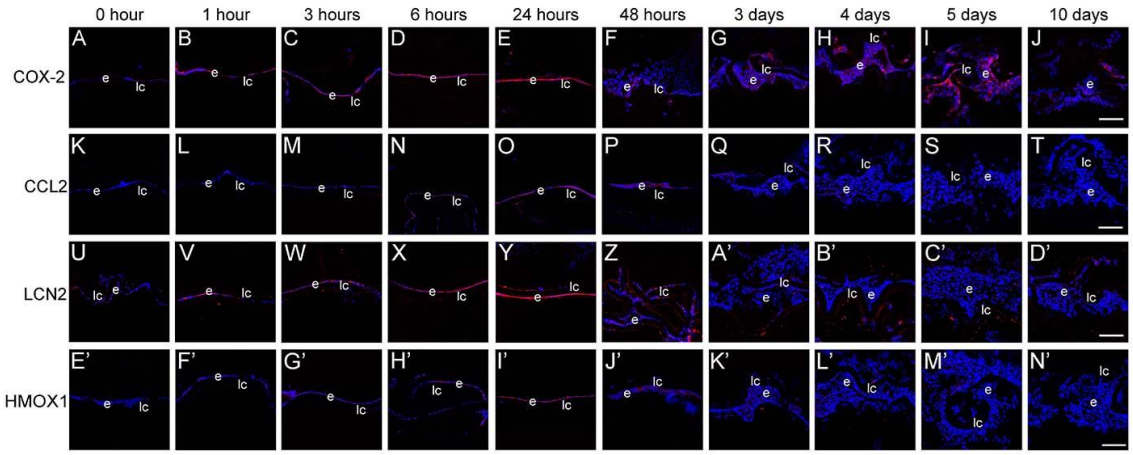

Figure 4.4: PCS expression time course for representative members of four different important inflammatory pathways in LECs. (A-J) COX-2 protein expression (red) in LECs PCS. COX-2 protein was not detected in LECs at 0 hour PCS (A), but it upregulated gradually between 1 hour (B), 3 hours (C), 6 hours (D) PCS with a peak at 24 hours PCS (E). COX2 decreases by 48 hours (F), remains low at 3 days PCS (G) but is upregulated at 4 days (H) and 5 days (I) PCS. COX2 levels are again low by 10 days (J) PCS. (K-T) CCL2 protein expression (red) in LECs after cataract surgery. Low COX-2 expression is observed in LECs at 0 hour (K), 1 hour (L), and 3 hours (M) PCS. CCL2 expression begins to upregulate by 6 hours (N) PCS, reaching a peak by 24 hours PCS (O) followed by a moderate decrease by 48 hours (P) PCS. By 3 days PCS (Q), CCL2 levels downregulate sharply and remain low through 4 days (R), 5 days (S), and 10 days (T) PCS. (U-D'). LCN2 protein expression (red) in LECs after cataract surgery. Minimal LCN2 expression was observed in LECs at 0 hour PCS (U), while it upregulated gradually between 1 hour (V), 3 hours (W), and 6 hours (X) PCS. After peaking at 24 hours PCS (Y), it is downregulated moderately by 48 hours PCS (Z), and while it was essentially undetectable by 3 days PCS (A'). LCN2 levels remained low through 4 days (B'), 5 days (C') and 10 days (D') PCS. (E'-N') HMOX1 protein expression (red) in LECs PCS. HMOX1 immunostaining was not detected in LECs at 0 hour (E'), 1 hour (F'), and 3 hours (G') PCS. HMOX1 is first detected at 6 hours PCS (H') and staining peaks at 24 hours PCS (I'). HMOX1 levels are decreased at 48 hours PCS (J') and while HMOX1 expression is not detected at 3 days (K'), 4 days (L'), 5 days (M'), and 10 days (N') PCS. For all panels, Blue= DNA as visualized by Draq5 staining; Scale bars: 100 μm; e, remnant lens epithelial cells/lens cells; lc, lens capsule.

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4.2.3 Inflammatory cells are associated with the lens capsular bag PCS

As many of the genes induced in lens capsular bags at 24 hours PCS are known chemokines that can attract neutrophils to injury sites, we then determined the timing of leukocyte infiltration into the mouse eye PCS. Immunostaining of lens capsular bags PCS with CD11b (ITGAM, integrin alpha M, 9 fold upregulated in lens capsular bags at 24 hours PCS by RNAseq), a widely accepted cell surface leukocyte marker with known roles in inflammation (Rosetti and Mayadas 2016), revealed no leukocyte infiltration into the eye prior to 12 hours PCS (Figure 4.5), while the first CD11b positive cells were detected associated with lens capsular bags at 18 hours PCS. The abundance of CD11b positive cells increases from 18 hours to 3 days PCS, remains appreciable at 4 and 5 days PCS, then falls to low levels by 10 days PCS. Similar results were obtained by immunostaining capsular bags with LY-6G, a GPI-linked protein that is a recognized marker of granulocytes and peripheral neutrophils (P. Y. Lee et al. 2013). Similar to CD11b, the first LY-6G positive cells did not arrive in the lens capsular bag until 18 hours PCS, although fewer cells stained overall (Figure 4.6) as would be expected since LY-6G is found on a more restricted set of leukocytes.

Since CD11b immunostaining is unable to distinguish between neutrophils and macrophages, we then immunostained capsular bags PCS with F4/80, an antibody that detects EMR1, a glycoprotein that is a very abundant and specific marker for mouse macrophages (McKnight et al. 1996). This experiment revealed that no F4/80 positive macrophages are associated with lens capsular bags for the first 24 hours PCS (Figure 4.7) as expected since the EMR1 gene is not appreciably expressed in capsular bags right after surgery, and is not differentially expressed in 24 hours PCS capsular bags by RNAseq (not shown). While occasional F4/80 positive cells were detected at 48 hours PCS, the first appreciable numbers of F4/80 positive cells were associated with

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lens capsular bags at 3 days PCS, while they become more abundant at 4 and 5 days PCS. F4/80 positive cells are still associated with lens capsular bags at 10 days PCS

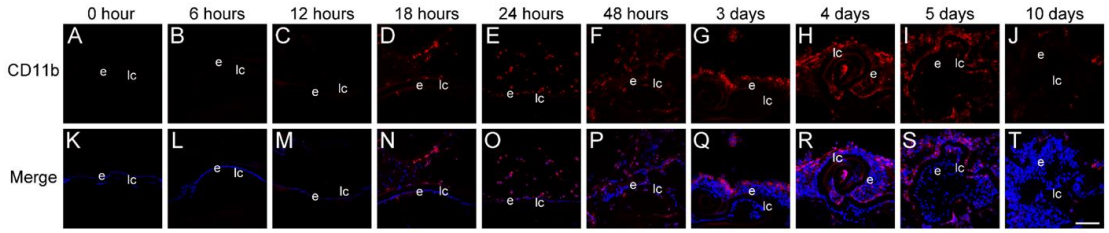

Figure 4.5: Neutrophil infiltration into the lens capsular bag PCS identified by CD11b immunostaining. (A-J) CD11b (red) staining alone; (K-T) CD11b expression (red) is merged with DNA detected by Draq5 (blue). No CD11b positive neutrophils are seen at 0 hour (A, K), 6 hours (B, L) and 12 hours (C, M) PCS. The first CD11b positive neutrophils are observed at 18 hours PCS (D, N), then increase by 24 hours PCS (E, O) and remain abundant through 48 hours (F, P), 3 days PCS (G, Q), 4 days PCS (H, R) and 5 days PCS (I, S). However, the number of CD11b positive cells sharply decrease by 10 days PCS (J, T). Scale bars: 100 μm. e, remnant lens epithelial cells/lens cells; lc, lens capsule.

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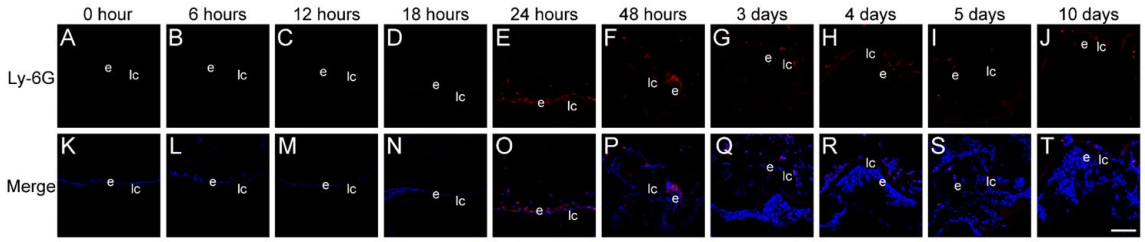

Figure 4.6: Neutrophil infiltration into the area surrounding lens capsular bags PCS identified by Ly-6G immunostaining. (A-J) Ly-6G (red) immunostaining of lens capsular bags PCS. (K-T) Merge of Ly-6G (red) and nuclear staining with the DNA stain, Draq5 (blue). No Ly-6G positive cells are seen at 0 hour (A, K), 6 hours (B, L), 12 hours (C, M), and 18 hours PCS (D, N). Ly-6G expressing cells are first seen near lens capsular bags at 24 hours PCS (E, O), and persist through 3 days (G, Q), 4 days (H, R), 5 days (I, S), and 10 days (J, T) PCS although they appear to become more sparse at later times PCS. Scale bars: 100 μm. e, remnant lens epithelial cells/lens cells; lc, lens capsule.

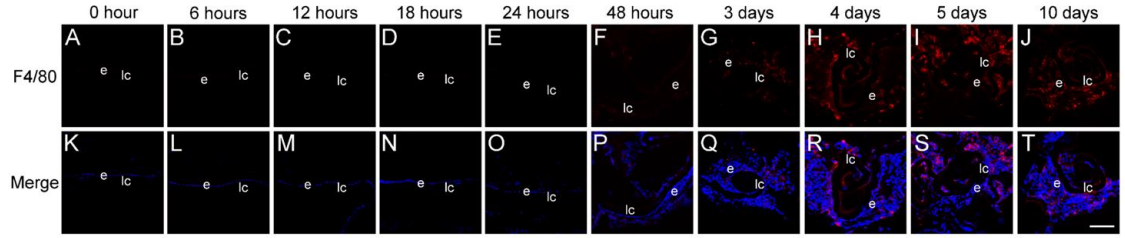

Figure 4.7: Macrophages infiltration into lens capsular bags following cataract surgery identified by F4/80 immunostaining. (A-J) F4-80 expression alone (red). (K-T) Merge between F4-80 immunodetection (red) and nuclear staining as detected by Draq5 labeling of DNA (Blue). No F4/80 staining (Red) is seen at 0 hour (A, K), 6 hours (B, L), 12 hours (C, M), 18 hours (D, N), and 24 hours (E, O) PCS. The first F4/80 positive cells are detected at 48 hours PCS (F, P) and robust numbers of F4/80 positive cells are first seen at 3 days PCS (G, Q), and increase dramatically at 4 days PCS (H, R). Robust numbers of F4/80 positive cells are maintained at 5 days (I, S) and 10 days (J, T) PCS. Scale bars: 100 μm; e, remnant lens epithelial cells/lens cells; lc, lens capsule.

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4.2.4 Pro-inflammatory cytokines co-localize with the epithelial marker, β1-integrin, in lens epithelial cells at 24 hours PCS, and in αSMA positive lens cells at 48 hours PCS, while these molecules generally were not found at high levels in infiltrating leukocytes

As most of the cytokines tested have been reported to be expressed by leukocytes, we then performed co-localization studies to confirm whether these genes were activating in LECs PCS, or whether the upregulation of these genes was simply reflecting leukocyte infiltration into the eye. Thus, we co-localized the cytokines of interest with β1-integrin, which is known to be abundant in LECs, particularly PCS (Mamuya et al. 2014), where it plays key roles in regulating the communication of lens cells with the capsule (Simirskii, Wang, and Duncan 2007; Y. Wang et al. 2017). This analysis revealed that all seven immune regulators studied, CXCL1, S100a9, G-CSF, COX-2, CCL2, LCN2, HMOX1 showed a near-complete overlap with β1-integrin positive LECs at 24 hours PCS (Figure 4.8). Further, double immunolabelling of 24 hours PCS lens capsular bags for the cytokines of interest and the leukocyte marker CD11b revealed that CXCL1, G-CSF, COX-2, CCL2, and LCN2 did not co-localize with CD11b positive cells (Figure 4.9). In contrast, the alarmin S100a9 was found in both LECs and a subset of CD11b positive cells (Figure 4.9) which would be expected as S100a9 has been reported to make up 40% of the cytoplasmic protein of circulating neutrophils(Kerkhoff et al. 1999).

In this cataract surgery model, the first induction of the fibrotic marker α-smooth muscle actin (αSMA) protein is seen in remnant LECs at 48 hours PCS (Mamuya et al. 2014). Co-immunostaining of the tested cytokines (purple) with CD11b (red) and αSMA (green) revealed that these pro-inflammatory markers were generally expressed in αSMA positive lens cells, not CD11b positive leukocytes at

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this stage (Figure 4.10). The exception was S100a9 which was observed in both αSMA positive lens cells as well as CD11b positive leukocytes (Figure 4.10, arrows).

While six of the seven inflammatory modulators showed a peak of expression in LECs at 24 hours PCS, followed by a rapid downregulation, COX2 showed a biphasic response, with the first upregulation seen at 24 hours followed by a rapid fall at 48 hours PCS (Figure 4.11)), while the second increase started at 4 days PCS with a peak in COX2 levels in 5 days PCS capsular bags. As this second wave of COX2 immunostaining matches the timing of macrophage infiltration into the capsular bag, and COX2 has been reported to be abundant in macrophages (Byun et al. 2014), we performed co-immunostaining of F4/80 (red) with COX2 (Green) (Figure 4.11). As expected, the second wave of COX2 expression corresponds with the influx of F4/80 positive macrophages at 4 days PCS, however, while some overlap between COX2 and F4/80 positive cells are seen, the majority of COX2 staining in capsular bags between 4 and 10 days PCS did not co-localize with F4/80.

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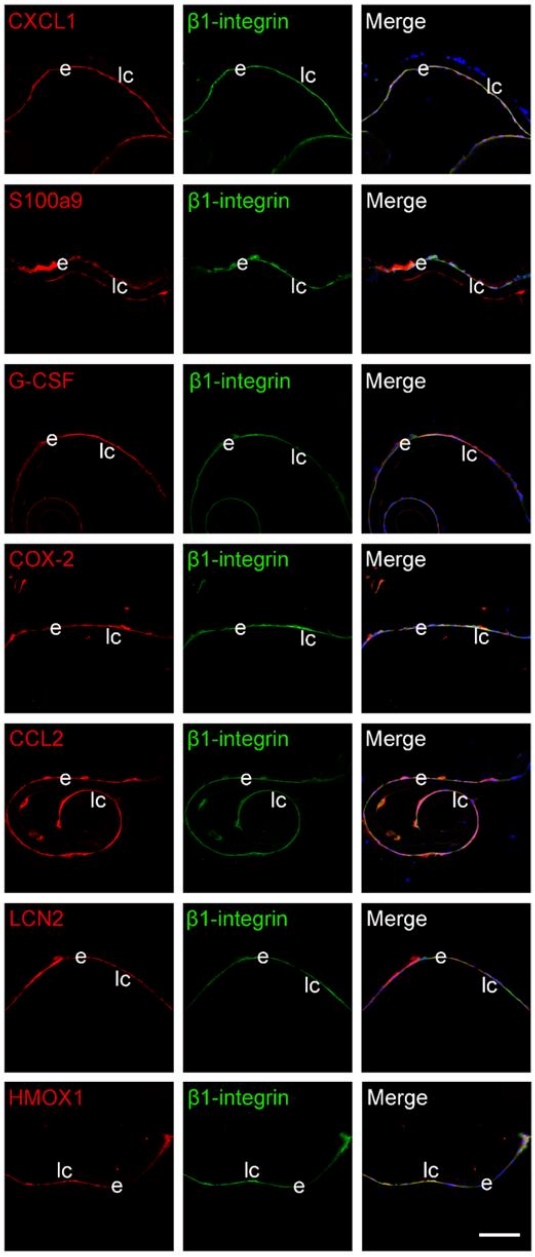

Figure 4.8: Pro-inflammatory genes (red) are expressed in LECs as assessed by their co-localization with β1-integrin (green) at 24 hours PCS. Merge co-localization of the proinflammatory molecule of interest (red), β1integrin which is used as a lens epithelial marker (green), and cell nuclei as assessed by DNA staining using Draq5 (blue). Scale bars: 100 μm. e, remnant lens epithelial cells/lens cells; lc, lens capsule.

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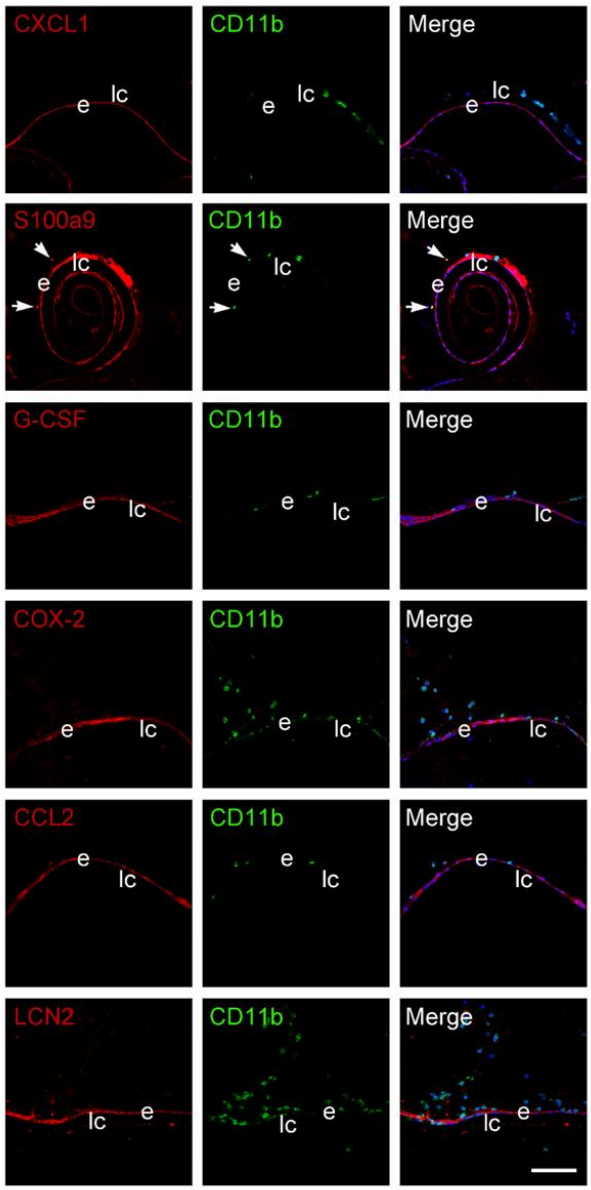

Figure 4.9: Pro-inflammatory gene expression (red) is generally not found in CD11b positive neutrophils associated with lens capsular bags at 24 hours PCS. Pro-inflammatory markers showed obvious positive staining (red) in LECs attached to the lens capsule, while, for most genes studied, these proteins were not detected in the CD11b positive cells (green) associated with the remnant LECs. However, S100a9 was detected in both the lens epithelial cells and CD11b positive neutrophils (arrows). Scale bars: 100 μm. e, remnant lens epithelial cells/lens cells; lc, lens capsule.

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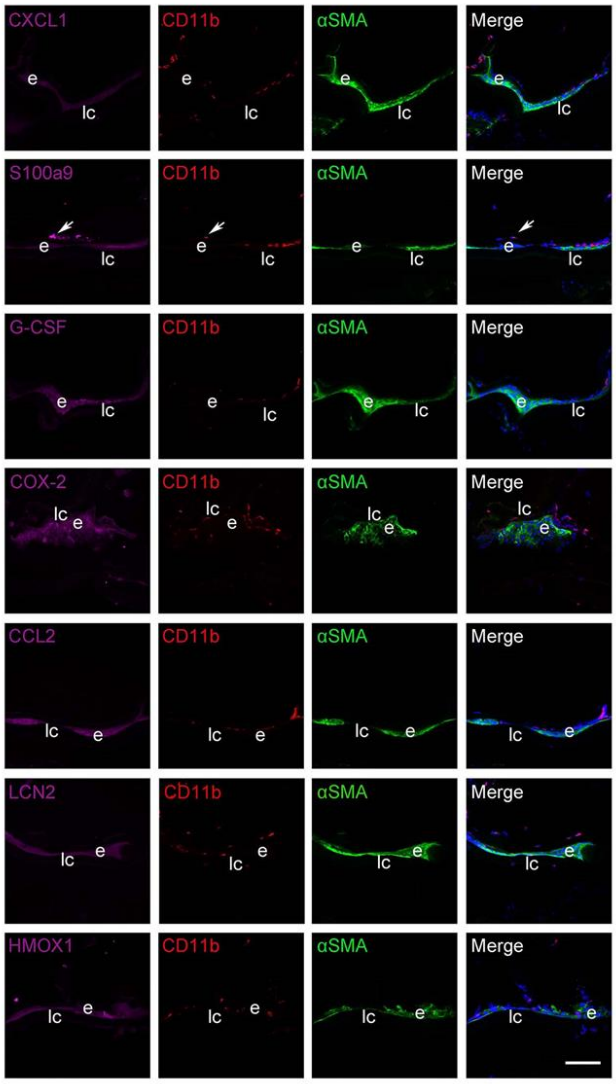

Figure 4.10: The residual pro-inflammatory gene expression detected in lens capsular bag associated cells at 48 hours PCS co-localizes with the fibrotic marker, αSMA. Triple immunostaining of inflammatory cytokines (purple) with CD11b (red) and αSMA (green) in capsular bags isolated at 48 hours PCS. Most inflammatory cytokines positive cells (purple) colocalized with αSMA (green), but not CD11b positive cells (red), although some CD11b positive cells were also S100a9 positive (Arrows). Merge cytokine- purple, CD11b- red; αSMA-green; Nuclei stained with the DNA dye DAPI- blue. Scale bars: 100 μm. e, remnant lens epithelial cells/lens cells; lc, lens capsule.

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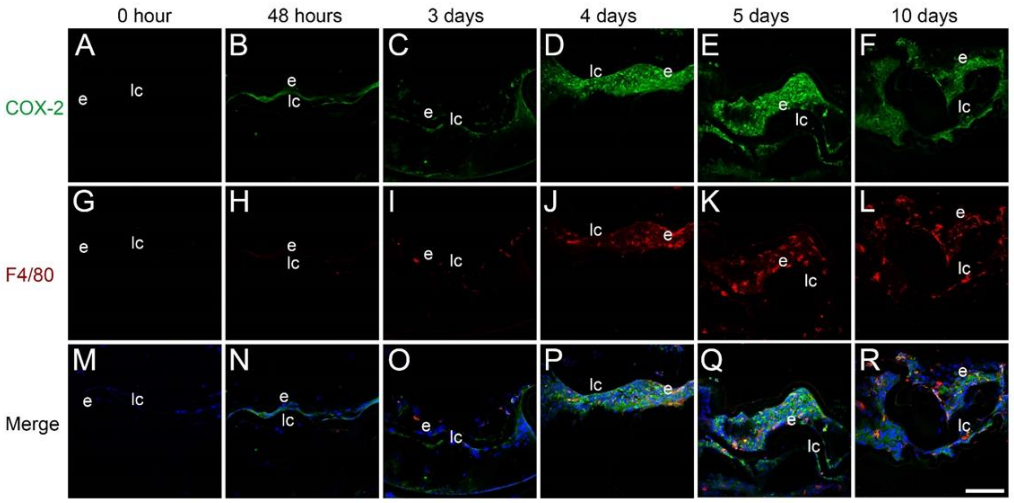

Figure 4.11: The late upregulation of COX2 protein levels PCS observed in lens capsular bags only partially co-localizes with F4/80 positive macrophages. (A-F) COX2 protein localization (green) alone. (G-L) F4-80 expression alone (Red). (M-R) Merge between F4-80 immunodetection (Red), COX2 immunostaining (Green), and nuclear staining as detected by Draq5 labeling of DNA (Blue). No F4/80 nor COX2 immunostaining is seen at 0 hours (A, G, M) At 48 hours PCS, only the occasional F4-80 positive cell is detected and these do not stain strongly for COX2 (B, H, N). At three days PCS, COX2 levels are low in all cells associated with capsular bags, although F4/80 positive cell numbers are increasing (C, I, O). At four days PCS, COX2 levels increase in most capsular bag cells and some co-localization of COX2 staining in F4/80 positive cells is seen (D, J, P), a pattern that is similar at five days PCS (E, K, Q). By 10 days PCS, the numbers of F4/80 positive cells appear to decline along with the intensity of COX2 immunostaining (F, L, R). Scale bars: 100 μm. e, remnant lens epithelial cells/lens cells; lc, lens capsule.

4.2.5 Macrophage influx and upregulation of SMAD3 phosphorylation (pSMAD3) during fibrosis post cataract surgery

Canonical (i.e. SMAD mediated) TGFβ signaling is recognized to be a major mediator of fibrotic PCO (Ian Michael Wormstone and Eldred 2016). However, TGFβ is produced in an inactive form and must be activated by tightly controlled mechanisms to elicit signaling (Robertson and Rifkin 2016). As macrophages have

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been implicated in the activation of TGFβ driving some fibrotic diseases (Brancato and Albina 2011; Wynn and Barron 2010), we compared the timing of macrophage influx into the lens capsular bag PCS with the onset of robust SMAD3 phosphorylation in remnant lens cells PCS (Figure 4.12). As we previously reported (Mamuya et al. 2014), pSMAD3 is undetectable by immunostaining in lens capsular bags prior to 24 hours PCS, while the first pSMAD3 positive nuclei are first detected in capsular bags at 48 hours PCS, although the staining is relatively weak (Figure 4.11, Figure 4.12). Robust upregulation of pSMAD3 staining in lens cells occurs between 48 hours and three days PCS which corresponds to the initial major influx of F4/80 positive macrophages into the area surrounding the capsular bag. The levels of pSMAD3 remain easily detectable in lens cells from 4-10 days PCS and these cells are in close proximity to F4/80 positive macrophages (Figure 4.12).

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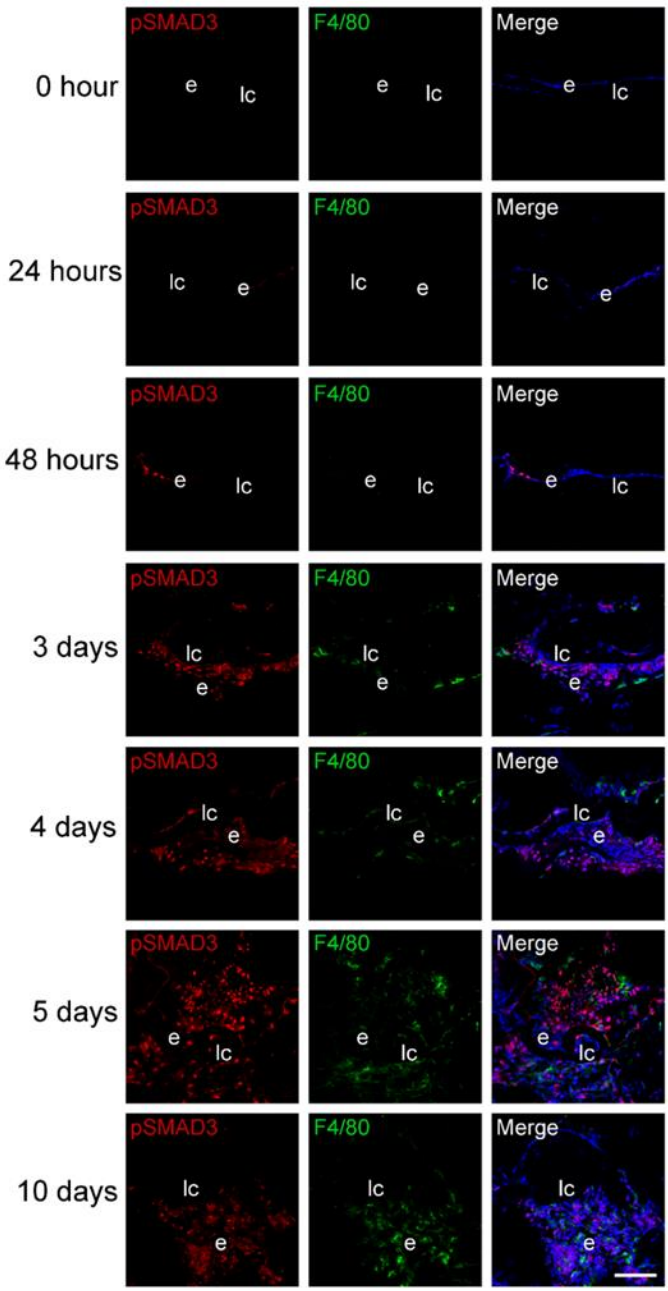

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Figure 4.12: Upregulation of pSMAD3 in LECs PCS correlates with the timing of F4/80 positive macrophage infiltration into lens capsular bags PCS. Neither pSMAD3 staining (red) nor F4/80 positive cells (green) are detected in lens capsular bags analyzed either immediately PCS or 24 hours later. The first pSMAD3 nuclei (red) are detected at 48 hours PCS, while both staining intensity and the number of pSMAD3 positive nuclei gradually increases through 3 and 4 days PCS, peaking at 5 days PCS. Occasional F4/80 positive macrophages (see figure 6) are detected at 48 hours PCS, but their numbers increase sharply by 3 days PCS, and these cells remain abundant in the capsular bag through 10 days PCS. Merge pSMAD3- red; F4/80- green; Nuclei stained with the DNA dye Draq5- blue. Scale bars: 100 μm. e, remnant lens epithelial cells/lens cells; lc, lens capsule.

4.3 Discussion

The epithelial to mesenchymal transition (EMT) of lens epithelial cells (LECs) to myofibroblasts is recognized to produce the fibrotic tissue seen in anterior subcapsular cataract as well as the fibrotic sequelae of cataract surgery including Soemmering's ring and the various forms of visual axis opacification (VAO) including anterior capsular contraction/phimosis, posterior capsular opacification (PCO) and VAO due to growth of myofibroblasts along the anterior hyaloid membrane (Shirai et al. 2018; Ian Michael Wormstone and Eldred 2016). There is robust experimental evidence supporting the hypothesis that canonical TGFβ signaling is both sufficient and necessary to induce LEC EMT (de Iongh et al. 2005; Shizuya Saika et al. 2004; Boswell et al. 2017) while the main signal transducer of the canonical TGFβ pathway (pSMAD2/3) is detected in both anterior subcapsular cataracts (Ishida et al. 2005; Frank J Lovicu et al. 2002) and fibrotic lens capsular bags, even years after surgery (S. Saika et al. 2002). However, TGFβ is produced in a latent form and must be activated to elicit signaling (Mamuya and Duncan 2012; Chang 2016), and we have previously shown that there is a 48 hour lag between lens injury and the ability to detect

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pSMAD2/3 in LECs in a mouse cataract surgery model (Mamuya et al. 2014). This work sought to elucidate the early response of LECs to cataract surgery that sets up the conditions necessary for the onset of TGFβ signaling and LEC EMT.

4.3.1 Lens epithelial cells rapidly change their phenotype in response to surgical lens fiber cell removal

LECs are polarized epithelial cells with basal attachments on the lens capsule and apical interactions with the apical tips of lens fiber cells (Zampighi, Eskandari, and Kreman 2000). These cells normally express many of the classical markers of an epithelium while also expressing genes more specific for lens function (Hoang et al. 2014). Comparison of the LEC transcriptome at the time of surgery with LECs remaining in the eye for 24 hours post cataract surgery (PCS) revealed that many genes known to be important for the lens phenotype exhibit altered expression. As expected for an EMT response, many regulators of lens cell fate and structure are downregulated including Sipa1l3, Foxe3, Tdrd7, Maf 51, Pitx3, Hsf4, FoxE3, and Pax6. However, at least five genes known to be important for lens development or physiology are upregulated PCS including Vim, Wfs1, Epha2, Ftl1, and Gcnt2). It is notable though that some of these upregulated genes are regulators or markers of mesenchymal cell fate or fibrosis (Z. Wang et al. 2018; Chao et al. 2017; St Laurent et al. 2017) in other systems, suggesting that their increased expression PCS also reflects the onset of LEC EMT. Finally, we detect the upregulation of transcripts encoding many myofibroblast markers in LECs at 24 hours PCS including α-smooth muscle actin, tenascin C, TGFβi, fibronectin, transgelin, lysyl oxidase, collagen type I, and α5 integrin. As we are unable to detect the pSMAD2/3 indicative of TGFβ signaling in LECs at this time point (Figure 4.12), this implies that the initial fibrotic response of

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LECs PCS is independent of TGFβ signaling although it is possible that some TGFβ signaling is active, but it is below the threshold of our pSMAD2/3 detection assay.

4.3.2 Lens epithelial cells remaining behind PCS rapidly induce the expression of genes important for the innate immune response

The uninjured lens epithelium expresses few genes with known roles in the innate immune response. However, RNAseq coupled with immunofluorescence revealed that a large number of genes involved in innate immunity, including those involved in numerous cytokine pathways, the prostaglandin synthesis pathway, and interleukins, were highly induced in LECs by 24 hours PCS. Many of the most upregulated genes encode either chemoattractants which induce neutrophil/macrophage/monocyte migration from the circulation to injury sites or modulate innate immune responses as would be expected after wounding of any epithelium.

Notably, though, it appears that the details of the initial inflammatory cascade initiated by lens epithelial cells may be unique to the lens. While RNAseq experiments testing the early stages of abrasive wound healing in mouse skin are qualitatively consistent with our results in the lens as mRNAs for genes involved in the cytokine response are elevated by 12 hours post wounding, remain quite high at 24 hours post wounding, and generally fall by 36 hours post wounding, none of the six genes that we highlighted for study in LECs (the top three most upregulated plus three others of biological interest) were included in the top 100 changed genes in abrasive skin wounding in mice (St Laurent et al. 2017). Further, the responses appear quite different quantitatively as well. For instance, while CXCL1 (the most elevated gene in LECs PCS) is also elevated after abrasive skin wounding, the response is much more

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mutated than in LECs while COX2, whose mRNA is elevated 248 fold in LECs PCS is not altered in skin post abrasive wounding at any time tested (St Laurent et al. 2017). The diversity of transcriptional responses to wounding are further highlighted by a recent paper demonstrating that human oral mucosa and skin have very different responses to incisional wounding, largely because the naïve oral mucosa already expresses many genes usually associated with inflammation, including S100A8/A9 (which are among the top upregulated genes in the injured lens epithelium, while CXCL1 and CCL2 (other top upregulated genes in injured mouse lens epithelium) do not upregulate after mucosal injury but are upregulated 48 hours and 5 days after incisional wounding of human skin (Iglesias-Bartolome et al. 2018).

Notably, human LECs have been previously reported to synthesize interleukins, prostaglandins, and G-CSF in culture (Nishi, Nishi, and Imanishi 1992; Dawes, Duncan, and Wormstone 2013), while the time course of inflammatory cell arrival in the mouse eye PCS is similar to the timing of the onset of "flare plus cells" in humans PCS (Findl et al. 2003). This suggests that the mouse cataract surgery model used in this study may accurately reflect the ocular inflammatory response subsequent to human cataract surgery. However, this requires confirmation as different species can induce different inflammatory responses to the same insult (Butler, Unger, and Grierson 1988; Laurell et al. 1997). Further, as most human cataract surgeries are performed on the elderly, while the results presented here were obtained on young adult mice, it will be important to test how age affects the post-surgical inflammatory response in the mouse model as it has been previously reported that LECs from elderly people produce a different profile of interleukins than those from younger individuals when cultured under serum-free conditions in an in vitro

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organ culture PCO model (Dawes, Duncan, and Wormstone 2013). Finally, little is known about the inflammatory cell types infiltrating the human eye PCS and neither the timing or identity of the major cytokines upregulated by human LECs PCS are known.

4.3.3 The possible significance of post-surgical inflammation

We found that the upregulation of the innate immune response in LECs likely occurs rapidly PCS as the levels for all of the pro-inflammatory proteins tested were elevated by 6 hours PCS, preceding the arrival of neutrophils into the eye PCS by at least 12 hours, and the arrival of macrophages by two-three days. Notably, we find that inflammatory mediators upregulate at least a day prior to TGFβ signaling PCS, while it is known that eyes with active inflammation (such as in uveitis) are more prone to aggressive fibrosis PCS(Abbouda et al. 2016; Mohammadpour, Jafarinasab, and Javadi 2007). Thus, the inflammatory response seen in LECs post wounding may be an initiator of PCO. Several prior studies have attempted to determine whether aggressive prevention of post-surgical inflammation can ameliorate PCO, however, the results are equivocal (Brookshire et al. 2015; Chandler et al. 2007; Zaczek, Laurell, and Zetterström 2004; Lois et al. 2005; Nibourg et al. 2015; Lewis 2013). However, in each case, only a subset of the pro-inflammatory pathways active PCS have been targeted, so these studies do not definitively rule out the therapeutic potential of shutting down PCS inflammation in PCO prevention.

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4.3.4 Conclusions

The past several decades have seen numerous advances in cataract surgery techniques and intraocular lens implants which have yielded huge decreases in the number of people suffering from blindness or visual disability due to cataract (Olson 2018; C. M. Lee and Afshari 2017). Despite these advances, post-surgical inflammation and ocular fibrosis derived from epithelial to mesenchymal transition of residual lens epithelial cells are still significant barriers preventing ideal visual outcomes (D. Spalton 2011; Aptel et al. 2017; Sundelin et al. 2014; Wielders, Schouten, and Nuijts 2018). Overall, this study provides new insights into the pathophysiology of cataract s side effects and implies that the LECs remaining behind following cataract surgery are signaling centers promoting PCS inflammation.

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Chapter 5

αVβ8 INTEGRIN- A POTENTIAL DRUGGABLE TARGET TO PREVENT PCO

All the contents included in this chapter are described in a manuscript which is under preparation -Mahbubul H. Shihan, Yan Wang, Dean Sheppard, Thomas D. Arnold, Amha Atakilit, Nicole M. Rossi, Adam P. Faranda and Melinda K. Duncan (2020) 'αVβ8 integrin- a potential druggable target to prevent posterior capsular opacification (PCO)'.

A provisional patent has been filed on December 5th, 2019 titled 'Prevention of Posterior Capsular Opacification with integrin αVβ8 blocking antibody', Application number- US 62/944, 151 and the inventors are- Dean Sheppard, Melinda K. Duncan, Amha Atakilit & Mahbubul H. Shihan.

5.1 Introduction

Cataracts, a major cause of blindness worldwide (Liu et al. 2017; C. M. Lee and Afshari 2017), are effectively treated by surgical removal of opaque lens fiber cells followed by implantation of an artificial intraocular lens (IOL) (Liu et al. 2017). However, months to years later, a significant proportion of patients experience an apparent recurrence of their cataract as Posterior Capsular Opacification (PCO) (I. Michael Wormstone, Wang, and Liu 2009). PCO occurs when the remnant lens epithelial cells (LCs) left behind post cataract surgery (PCS) migrate into the optical axis and transition into a mixture of myofibroblasts and aberrant lens fiber cells (I. Michael Wormstone, Wang, and Liu 2009). Approximately 25% of adults and

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veterinary patients and almost 100% of pediatric patients develop clinically significant PCO in a few months to years PCS (Shihan, Novo, and Duncan 2019). PCO is currently treated by Nd: YAG laser therapy (Beale et al. 2006; Burq and Taqui 2008; Billotte and Berdeaux 2004)(Shihan, Novo, and Duncan 2019). However, Nd: YAG laser therapy can have severe side effects, notably retinal detachment and macular edema while this therapy is often unsuitable/inconvenient for pediatric and veterinary patients suggesting that prevention may be a better option (Billotte and Berdeaux 2004; Beale et al. 2006; Burq and Taqui 2008)(Shihan, Novo, and Duncan 2019). The only FDA approved preventative approach for PCO utilizes specially designed prosthetic intraocular lenses which delay, but do not prevent, the onset of PCO (Shihan, Novo, and Duncan 2019).

Transforming growth factor β (TGFβ) signaling is known to mediate the epithelial to mesenchymal transition (EMT) of LCs to myofibroblasts (de Iongh et al. 2005). While TGFβ concentration in the aqueous humor is high before surgery, most of the TGFβ remains in the inactive form (Maier et al. 2006; Jampel et al. 1990). By using a mouse cataract surgery model, previously we have shown that TGFβ signaling activation is not detected until 48 hr PCS, and the robust activation is seen at 3 days PCS (Jiang et al. 2018). However, the mechanism by which TGFβ signaling is activated PCS is not well understood.

Integrins, heterodimeric extracellular matrix (ECM) receptors consisting of one α- and one β -subunit are involved in cell/ECM attachment, cell migration, and the transmission of tractional forces (Walker and Menko 2009). Integrins also cross talk with diverse growth factor signaling pathways (Sieg et al. 2000) including the TGFβ pathway (Henderson and Sheppard 2013) that is known to regulate PCO (de Iongh et

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al. 2005). Thus integrins have been proposed as therapeutic targets for PCO (Walker and Menko 2009). Previously we showed that αV integrin is critical for canonical TGFβ pathway mediated fibrotic PCO (Mamuya et al. 2014). Notably, αV integrin forms functionally distinct heterodimers with a variety of β integrins while four of the five possible β integrins (Sheppard 2004) were upregulated (β1, β5, β6, and β8) PCS (Mamuya et al. 2014) and are reported to participate in TGFβ activation in other systems (Munger et al. 1999; Tatler et al. 2011; Robertson and Rifkin 2016; Arnold et al. 2019; N. I. Reed et al. 2015; Mu et al. 2002a). Since each heterodimer has a different ligand binding profile/function and is inhibited by different compounds (Raab-Westphal, Marshall, and Goodman 2017), the identification of the β integrin that functions with αV integrin is critical to both the development of anti PCO therapies and the investigation of the operant signaling mechanisms. Thus, in this study, we aim to identify the β subunit that heterodimerizes with αV integrin subunit in TGFβ signaling mediated fibrotic PCO. We further characterize the effect of an integrin blocking antibody PCS to present possible effective therapeutics in preventing PCO development.

5.2 Results

5.2.1 Robust expression of β8 integrin by LCs is detected PCS

As β5 and β6 integrin are the most upregulated β integrins by LCs (Mamuya et al. 2014) and αvβ5 and αvβ6 integrins are widely studied concerning fibrosis (Munger et al. 1999; Tatler et al. 2011; Robertson and Rifkin 2016; Fontana et al. 2005), we first characterized the role of β5 and β6 integrin in the lens development and PCO. Our study reveals that both β5 and β6 integrin adult null lenses show normal lens

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morphology as WT (Figure 5.1) and undergo a robust fibrotic response similar as WT at 5 d PCS (measured by myofibroblasts marker α smooth muscle actin- αSMA) (Figure 5.2) (β5 null, P = 0.850; β6 null, P = 0.213) suggesting neither β5 integrin nor β6 integrin is critical for PCO development.

Next, we turned our focus to αvβ8 integrin as this heterodimer has gained attention recently due to its role in TGFβ activation using matrix metalloproteinase 14 (MMP14) cofactor (Robertson and Rifkin 2016; Mu et al. 2002a). At 0 h PCS, remnant LCs express little β8 integrin protein. By 48 hr PCS, β8 integrin protein upregulation around αSMA positive remnant LCs becomes significant (***P < 0.001) and the expression reaches a robust level at 3 d PCS (Figure 5.3A) (***P < 0.001), this time point correlates the robust activation of TGFβ signaling PCS (Jiang et al. 2018). The expression of β8 integrin protein is sustained until 5 d PCS (***P < 0.001). We further detected the expression of αV integrin, β8 integrin, and αSMA in a human PCO sample (Figure 5.3B) suggesting that αVβ8 integrin may be a potential candidate in PCO development. Thus, we decided to study the functional role of β8 integrin in PCO. To study this, we generated mice conditionally lacking a functional β8 integrin gene from the lens (β8ITGcKO) by mating mice carrying a floxed β8 integrin allele (Proctor et al. 2005) to mice harboring the lens-specific MLR10 CRE transgene (Figure 5.3C) whose activity is first detected in the lens beginning around embryonic day 10.5 (the lens vesicle stage) (H. Zhao et al. 2004). The complete deletion of the floxed region of the β8 integrin gene was confirmed by PCR analysis of genomic DNA isolated from adult lenses and tails (Figure 5.3D). Morphological analysis reveals that adult β8 integrin conditional knockout (β8ITGcKO) mouse lenses are transparent and show normal lens morphology as WT (Figure 5.3E)

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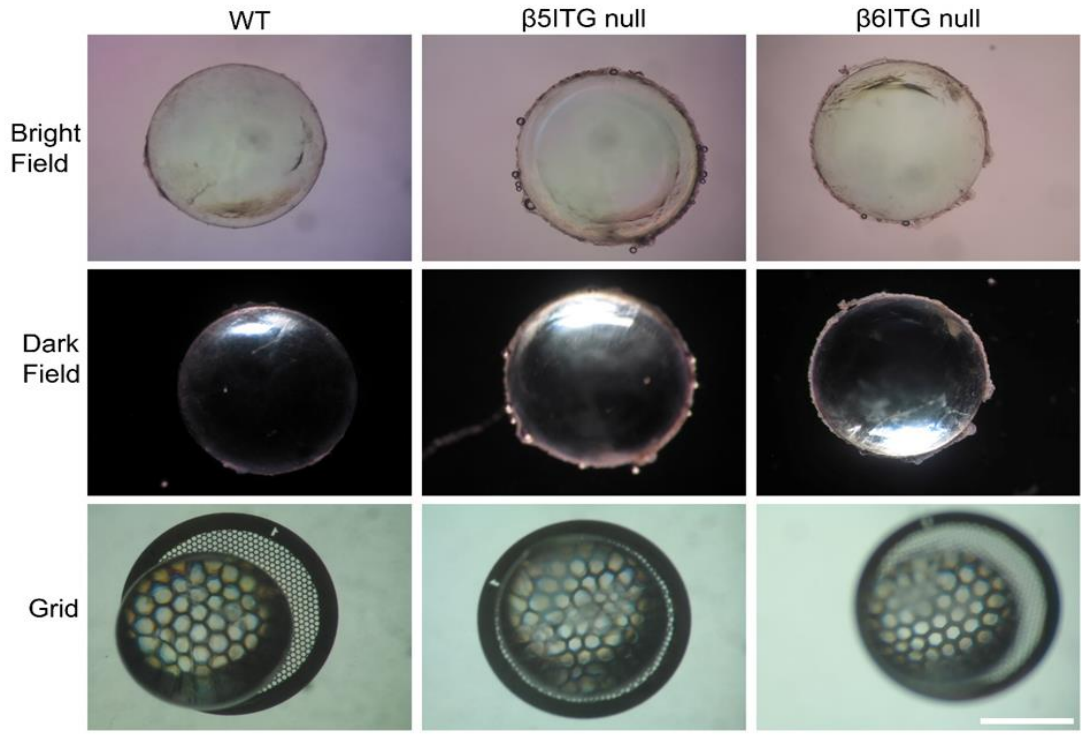

Figure 5.1: A bright field, a dark field and a 200- mesh electron microscopy grid analysis of 12 weeks old WT, β5ITG null and β6ITG null lenses reveal that β5ITG null and β6ITG null lenses are transparent and have refractive properties similar to WT. WT- wildtype, β5ITG- β5 integrin, β6ITG- β6 integrin, scale bar- 1 mm.

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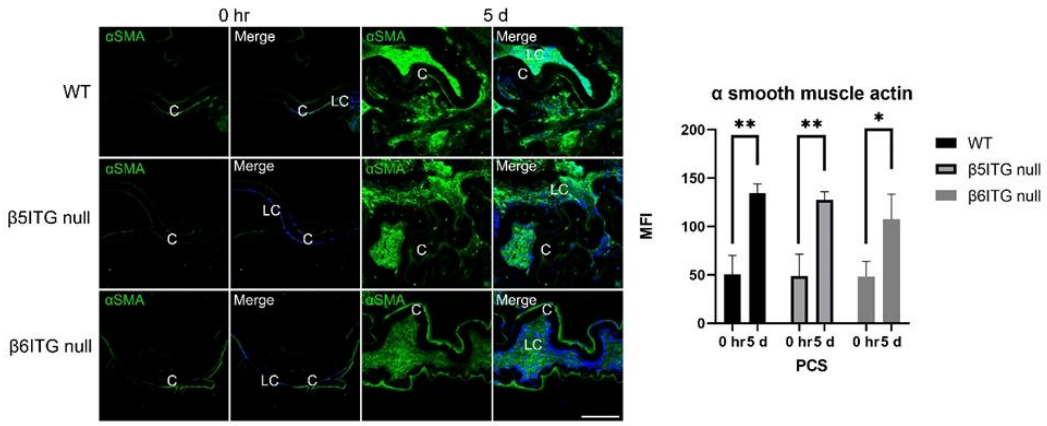

Figure 5.2: At 0 h PCS, all three mice strains (WT, β5ITG null and β6ITG null) express little levels of αSMA protein which becomes quite robust at 5 d PCS (WT, **P = 0.003; β5ITG null, **P = 0.005; β6ITG null, *P = 0.028). However, the difference of αSMA upregulation at 5 d PCS is not statistically significant in β5ITG null (P = 0.850) and β6ITG null (P = 0.213) compared to WT. C- lens capsule, LC- lens cells, h- hour, d- day, MFI- mean fluorescence intensity, PCS- post cataract surgery, WT- wildtype, β5ITG- β5 integrin, β6ITG- β6 integrin, scale bar- 72 µm. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between two PCS time points.

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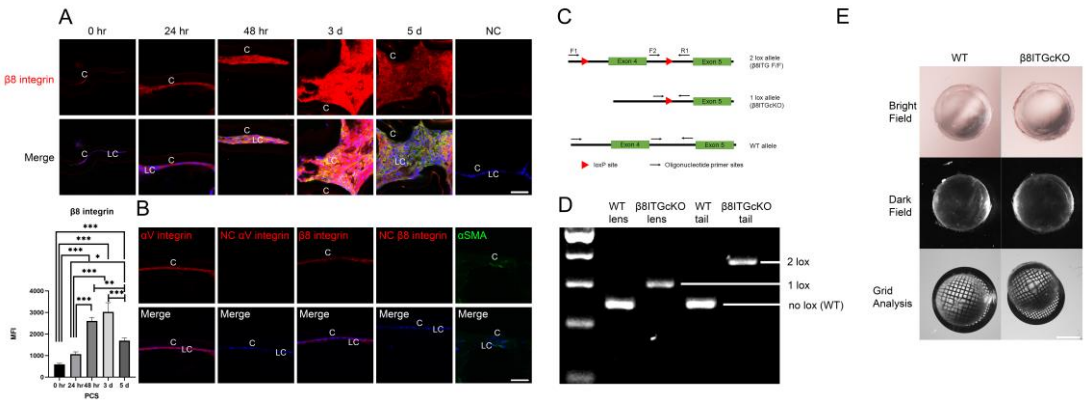

Figure 5:3: β8 integrin's role in the lens development and the dynamics of its protein deposition around remnant LCs PCS. (A) Dynamics of β8 integrin protein deposition around s LCs PCS reveals that β8 integrin (red) protein upregulation around αSMA (green) positive remnant LCs reaches a robust level at 3 d PCS (***P < 0.001). Scale bar- 36 µm. All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between two PCS time points of WT. (B) A human PCO sample showing the expression of αV integrin (red), β8 integrin (red), and myofibroblast marker αSMA (green). Scale bar- 36 µm (C) Diagram of β8 integrin gene locus showing the position of the loxP sites (D) PCR results from DNA obtained from 8 weeks old WT and β8ITGcKO lenses and tails demonstrating successful deletion of the floxed gene fragment in β8ITGcKO lenses (1 lox- recombined DNA, 2 lox- unrecombined DNA). (E) A bright field, a dark field, and a 200- mesh electron microscopy grid analysis of 12 weeks old WT and β8ITGcKO lenses reveal that β8ITGcKO lenses are transparent and have refractive properties similar to WT. Scale bar- 10 mm. C- lens capsule, LC- lens cells, hr- hour, d- day, MFI- mean fluorescence intensity, PCS- post cataract surgery, WT- wildtype, β8ITGcKO- β8 integrin conditional knockout, NC- negative control, scale bar- 72 µm. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between two PCS time points.

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5.2.2 Lenses lacking the β8 integrin gene show attenuated fibrotic response and proliferation while demonstrating the epithelial characteristics and fiber cell regeneration PCS

To test whether there is any change in the fibrotic response of lenses lacking the β8 integrin gene (β8ITGcKO), the expression of the common fibrotic markers αSMA, tenascin C and fibronectin was determined PCS (Figure 5.4A). As expected, little to no αSMA, tenascin C, and fibronectin protein is detected in remnant lens cells (LCs) associated with either WT or β8ITGcKO capsular bags at 0 h PCS. By 48 hr PCS, both WT and β8ITGcKO LCs upregulate all three proteins, however, β8ITGcKO LCs show attenuated upregulation of tenascin C (**P = 0.005) and fibronectin (*P = 0.022) protein compared to WT. WT LCs further upregulate all three fibrotic proteins (αSMA, ***P < 0.001; tenascin C, **P = 0.002; fibronectin, **P = 0.003) at 5 d PCS. In contrast, β8ITGcKO LCs fail to upregulate these proteins further and the attenuated fibrotic response is significant compared to WT LCs at 5 d PCS (αSMA, **P = 0.001; tenascin C, ***P < 0.001; fibronectin, **P = 0.005) suggesting that deletion of β8 integrin from the lens inhibits fibrotic response PCS.

Since we have detected that the cells formed in β8ITGcKO capsular bags have lost their fibrotic phenotype at 5 d PCS, next we attempted to determine the type of the cells formed in β8ITGcKO capsular bags PCS. To do so, we followed the expression of a classic epithelial cell marker, E cadherin, to determine if some of the myofibroblasts formed at 48 hr PCS change their phenotype to an epithelial characteristic in capsular bags of β8ITGcKO. As expected, both WT and β8ITGcKO LCs express appreciable amounts of E cadherin at 0 h PCS (Figure 5.4A). However, by 48 hr PCS, E cadherin protein levels are significantly downregulated in WT LCs (***P < 0.001), and this downregulation is sustained through 5 d PCS (***P < 0.001).

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In contrast, E cadherin levels remain unchanged in β8ITGcKO both at 48 hr (P = 0.651) and 5 d PCS (P = 0.390) and E cadherin levels are significantly higher in β8ITGcKO capsular bags compared to WT at 5 d PCS (**P = 0.005) suggesting that LCs lacking β8 integrin gene can preserve their epithelial characteristics PCS.

We further stained our PCS samples with aquaporin 0, a fiber cell preferred membrane protein, since some remnant LCs are known to differentiate into structurally aberrant lens fiber cells which contribute to the development of "pearl-like" PCO when present in the visual axis, and Soemmering's ring when restricted to the ocular periphery (I. Michael Wormstone, Wang, and Liu 2009). Remnant LCs from both WT and β8ITGcKO mice express little aquaporin 0 (Figure 5.4A). By 48 hr PCS, some remnant LCs express aquaporin 0 in both WT and β8ITGcKO eyes, and the expression aquaporin 0 becomes more robust by 5 d PCS (aquaporin 0, WT ***P < 0.001; β8ITGcKO **P = 0.003) suggesting that some of the lens cells may differentiate into lens fiber cells in β8ITGcKO capsular bags similar to WT.

Besides the finding that β8ITGcKO capsular bags contain a mixture of more epithelial and fiber cells and less fibrotic cells at 5 d PCS, the overall size of the capsular plaque appeared smaller qualitatively in β8ITGcKO at 5 d PCS suggesting that a proliferation defect of LCs could be the reason of fewer cells formation thus smaller size of capsular plaque in β8ITGcKO. Thus, a proliferation marker, Ki 67 which is present at all stages of the cell cycle except G0 (Scholzen and Gerdes 2000) is used to study this. At 0 h PCS, remnant LCs exhibit little to no cell proliferation (Figure 5.4B). In contrast, a significant upregulation of Ki 67 staining in LCs between 0 h and 48 hr PCS in both WT (**P = 0.001) and β8ITGcKO (***P < 0.001) is detected. However, the WT LCs show more bright signals of Ki 67 compared to

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β8ITGcKO LCs at 48 hr PCS (**P = 0.004) suggesting that β8ITGcKO LC proliferate less compared to WT LC at 48 hr PCS. This phenomenon correlates with significantly fewer associated cell nuclei in β8cKO capsular bags than WT at 5 d PCS determined by ImageJ (***P < 0.001) (Figure 5.4C). Overall, these data suggest that LCs lacking the β8 integrin gene show attenuated fibrosis, proliferation defects, elevated epithelial phenotype, and unhindered fiber cell differentiation in response to the lens fiber cell removal.

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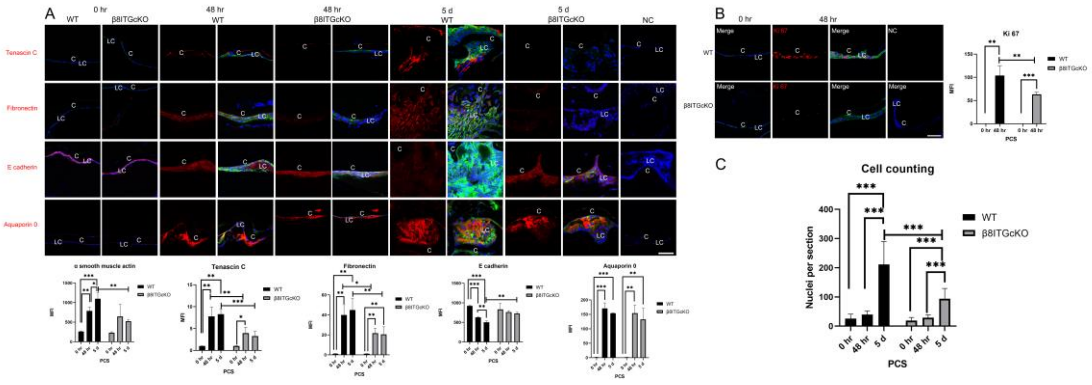

Figure 5.4: The response of LCs lacking the β8 integrin gene to lens fiber cell removal. (A) The deletion of the β8 integrin gene from the lens cells (LCs) leads to the attenuated expression of myofibroblast marker αSMA (**P = 0.001) at 5 d PCS and fibrotic proteins tenascin c (**P = 0.003; **P = 0.002) and fibronectin (**P = 0.022; **P = 0.005) at 48 hr and 5 d PCS compared to WT LCs. In contrast, the epithelial cell protein E cadherin levels remain unaltered in β8ITGcKO LCs while significant downregulation is seen in WT LCs at 5 d PCS (**P = 0.005) compared to β8ITGcKO. Fiber cell regeneration measured by aquaporin 0 is unhindered in β8ITGcKO capsular bags compared to WT PCS. (B) Appreciable numbers of bright Ki 67 positive LCs are detected in WT at 48 hr PCS while the bright signal is significantly less in β8ITGcKO LCs (**P = 0.004). (C) ImageJ reveals that the average number of nuclei associated with capsular bags is significantly less in β8ITGcKO compared to WT at 5 d PCS (***P < 0.001). Scale bar- 35 µm, C- lens capsule, LC- remnant lens cells, MFI- mean fluorescence intensity, PCS- post cataract surgery, tenascin C, fibronectin, E cadherin, aquaporin 0 and Ki 67 (red), αSMA (green), DNA detected by Draq5 (blue). All experiments had N = 3 (N=6 for figure C). Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI/nuclei per section between WT and β8ITGcKO at a PCS or between two PCS time points.

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5.2.3 RNAseq analysis reveals that genes associated with fibrosis and inflammation are differentially expressed in β8ITGcKO LCs PCS

To elucidate the mechanisms by which β8 integrin mediates the fibrotic response PCS, RNAseq was used as a global and unbiased approach to identify all genes whose expression levels change in WT LCs at 24 hr PCS (a common PCS time point when the robust upregulation of both fibrotic and inflammatory genes have been detected at the mRNA levels (Jiang et al. 2018)), and which of those genes require β8 integrin for their differential expression (GSE145492). This analysis revealed that 2312 genes are expressed at significantly different levels in WT LCs at 24 h PCS compared to 0 h PCS (1273 genes upregulated, 1039 genes downregulated) based on criteria we set previously (Jiang et al. 2018) and mentioned in the method section. These differentially expressed genes (DEGs) included many fibrotic genes that are known to upregulate either in LCs or other systems undergoing epithelial-mesenchymal transition (EMT) (Table 5.1). Further, consistent with our recent report (Jiang et al. 2018), this list of upregulated genes includes genes known to encode inflammatory cytokines (Table 5.2). Finally, the expression of many genes important for lens structure and function downregulate in LCs by 24 h PCS as well as would be expected in LCs undergoing EMT (Table 5.3).

Comparison of RNA expression profiles between WT and β8ITGcKO LCs at 24 h PCS revealed that the expression levels of 828 genes that meet the criteria for likely biological significance (Manthey et al. 2014) were significantly different. Of these, 97 were genes that normally upregulate in WT LCs by 24 h PCS but do not in β8ITGcKO lenses (Supplemental Table 1). Further, consistent with the muted fibrotic response by β8ITGcKO LCs PCS, the mRNA levels of several genes associated with fibrotic disease exhibit attenuated upregulation in β8ITGcKO LCs at 24 h PCS while

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another notable subset represents the attenuated upregulated DEGs plays known roles in inflammatory responses (Table 5.4).

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Table 5.1: Genes upregulated in LCs at 24 hr PCS that are known to be involved in fibrosis either in PCO or other systems.

Gene ID	Gene description	Fold_ Change	FDR	WT_0_Hour_Avg_ FPKM	WT_24_Hour_Avg_ FPKM
Tnc	tenascin C	175.38	3.92E-4	1.01	176.76
Grem1	gremlin 1, DAN family BMP antagonist	170.52	3.92E-4	0.97	165.86
Ecm1	extracellular matrix protein 1	79.11	3.92E-4	2.00	158.17
Tgfbi	transforming growth factor, beta induced	47.81	3.92E-4	5.80	277.16
Arg1	arginase, liver	46.89	1.93E-3	0.59	27.82
Fn1	fibronectin 1	34.21	3.92E-4	4.71	161.08
Fbln2	fibulin 2	28.91	3.92E-4	1.89	54.50
Spp1	secreted phosphoprotein 1	28.19	3.92E-4	1.02	28.89
Itga7	integrin alpha 7	23.71	3.92E-4	3.82	90.67
Tagln2	transgelin 2	10.54	3.92E-4	13.16	138.74
Nes	nestin	9.71	3.92E-4	22.12	214.74
Acta2	actin, alpha 2, smooth muscle, aorta	9.71	3.92E-4	83.37	809.26
Emp1	epithelial membrane protein 1	9.58	3.92E-4	4.66	44.63
Wisp2	WNT1 inducible signaling pathway protein 2	9.33	3.92E-4	1.32	12.35
Lox	lysyl oxidase	9.31	3.92E-4	0.57	5.32
Itga5	integrin alpha 5 (fibronectin receptor alpha)	9.06	3.92E-4	7.28	65.93
Emp3	epithelial membrane protein 3	8.01	3.92E-4	6.19	49.55
Thbs1	thrombospondin 1	6.12	3.92E-4	7.56	46.22

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Gene ID	Gene description	Fold_ Change	FDR	WT_0_Hour_Avg_ FPKM	WT_24_Hour_Avg_ FPKM
Runx1	runt related transcription factor 1	6.12	3.92E-4	4.21	25.72
Col1a1	collagen, type I, alpha 1	5.28	3.92E-4	2.06	10.89
E2f1	E2F transcription factor 1	3.57	3.92E-4	1.66	5.94
Ltbp1	latent transforming growth factor beta binding protein 1	3.51	3.92E-4	31.57	110.70
Tgfb1	transforming growth factor, beta 1	2.70	3.92E-4	21.88	59.05
Aebp1	AE binding protein 1	3.33	3.92E-4	32.16	107.15
Mmp14	matrix metallopeptidase 14 (membrane-inserted)	2.67	3.92E-4	7.80	20.81
Itgb1	integrin beta 1 (fibronectin receptor beta)	2.58	3.92E-4	69.11	178.41
Junb	jun B proto-oncogene	2.24	3.92E-4	35.58	79.86
Itgav	integrin alpha V	1.89	3.92E-4	49.77	93.90

Table 5.2: Genes known to be involved in inflammation are upregulated by LCs at 24 hr PCS.

Gene ID	Gene description	Fold_ Change	FDR	WT_0_Hour_Avg_ FPKM	WT_24_Hour_Avg_ FPKM
S100a9	S100 calcium binding protein A9 (calgranulin B)	∞	3.92E-4	0.00	36.69
Cxcl3	chemokine (C-X-C motif) ligand 3	∞	3.92E-4	0.00	33.77

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Gene ID	Gene description	Fold_ Change	FDR	WT_0_Hour_Avg_ FPKM	WT_24_Hour_Avg_ FPKM
S100a8	S100 calcium binding protein A8 (calgranulin A)	∞	3.92E-4	0.00	20.44
Csf3	colony stimulating factor 3 (granulocyte)	199.24	2.76E-2	0.24	47.06
Cxcl5	chemokine (C-X-C motif) ligand 5	140.46	3.92E-4	0.31	44.06
Lcn2	lipocalin 2	123.32	3.92E-4	28.66	3533.70
Ccl6	chemokine (C-C motif) ligand 6	44.75	3.92E-4	0.57	25.32
Ptgs2	prostaglandin-endoperoxide synthase 2	29.33	3.92E-4	0.77	22.58
Ptx3	pentraxin related gene	28.99	1.05E-3	1.95	56.58
Cxcl2	chemokine (C-X-C motif) ligand 2	11.78	3.92E-4	4.36	51.38
Ier3	immediate early response 3	9.42	3.92E-4	4.76	44.90
Ctsc	cathepsin C	5.02	3.92E-4	8.48	42.60
Csf1	colony stimulating factor 1 (macrophage)	4.98	3.92E-4	6.45	32.15
Cxcl1	chemokine (C-X-C motif) ligand 1	4.96	3.92E-4	7.18	35.62
S100a6	S100 calcium binding protein A6 (calcyclin)	4.05	3.92E-4	324.59	1314.14
Ptges2	prostaglandin E synthase 2	3.77	3.92E-4	2.51	9.47

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Table 5.3: Genes that are preferentially expressed in the lens or important for the lens cells homeostasis downregulate in LCs by 24 hr PCS.

Gene ID	Gene description	Fold_ Change	FDR	WT_0_Hour_Avg_ FPKM	WT_24_Hour_Avg_ FPKM
Crygd	crystallin, gamma D	-352.63	1.15E-2	236.24	0.67
Crygb	crystallin, gamma B	-280.30	3.92E-4	460.69	1.64
Crygc	crystallin, gamma C	-75.95	3.92E-4	597.43	7.87
Lenep	lens epithelial protein	-21.99	3.92E-4	125.29	5.70
Mip	major intrinsic protein of lens fiber	-8.58	3.92E-4	440.29	51.29
Bfsp1	beaded filament structural protein 1	-8.55	3.92E-4	471.63	55.17
Lim2	lens intrinsic membrane protein 2	-7.69	3.92E-4	213.55	27.76
Fgf1	fibroblast growth factor 1	-6.76	3.92E-4	14.89	2.20
Cryba4	crystallin, beta A4	-6.70	3.92E-4	2153.80	321.65
Crybb1	crystallin, beta B1	-5.61	3.92E-4	1660.04	295.71
Crygn	crystallin, gamma N	-5.53	3.92E-4	194.88	35.23
Cryba1	crystallin, beta A1	-5.43	3.92E-4	7251.02	1335.36
Lctl	lactase-like	-4.27	3.92E-4	50.70	11.87
Gas6	growth arrest specific 6	-4.04	3.92E-4	111.42	27.56
Cryba2	crystallin, beta A2	-3.97	3.92E-4	7397.48	1862.57
Gja3	gap junction protein, alpha 3	-3.96	3.92E-4	203.81	51.49
Dkk3	dickkopf WNT signaling pathway inhibitor 3	-3.33	3.92E-4	1032.30	309.94
Cryab	crystallin, alpha B	-2.92	7.12E-3	19582.00	6705.13
Tdrd7	tudor domain containing 7	-2.84	3.92E-4	96.90	34.07

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Gene ID	Gene description	Fold_ Change	FDR	WT_0_Hour_Avg_ FPKM	WT_24_Hour_Avg_ FPKM
Id3	inhibitor of DNA binding 3	-2.66	3.92E-4	77.94	29.29
Foxe3	forkhead box E3	-2.34	3.92E-4	126.31	53.87
Col4a4	collagen, type IV, alpha 4	-2.31	1.15E-2	194.46	84.08
Col4a3	collagen, type IV, alpha 3	-2.29	1.93E-3	211.67	92.42
Pitx3	paired-like homeodomain transcription factor 3	-2.20	3.92E-4	56.28	25.63
Prox1	prospero homeobox 1	-2.15	3.92E-4	160.02	74.56

Table 5.4: Genes known to be involved in fibrosis and inflammation are less upregulated in remnant LCs of β8ITGcKO at 24 hr PCS.

Gene ID	Gene description	Fold_ Change	FDR	WT_24_Hour_Avg_ FPKM	β8ITGcKO_24_Hour_Avg_ FPKM
Csf3	colony stimulating factor 3 (granulocyte)	-4.86	1.01E-3	50.87	10.47
Pttg1	pituitary tumor-transforming gene 1	-4.78	1.01E-3	11.91	2.49
Mylk2	myosin, light polypeptide kinase 2, skeletal muscle	-4.15	1.01E-3	3.49	0.84
Anxa8	annexin A8	-3.87	1.01E-3	22.77	5.88
Cxcl5	chemokine (C-X-C motif) ligand 5	-3.83	1.01E-3	47.41	12.39

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Gene ID	Gene description	Fold_ Change	FDR	WT_24_Hour_Avg_ FPKM	β8ITGcKO_24_Hour_Avg_ FPKM
S100a8	S100 calcium binding protein A8	-3.34	1.78E-2	21.86	6.54
Ptx3	pentraxin related gene	-3.03	1.01E-3	60.89	20.12
Grem1	gremlin 1, DAN family BMP antagonist	-2.94	1.01E-3	178.14	60.60
Nes	nestin	-2.86	1.01E-3	231.45	80.99
Itga5	integrin alpha 5 (fibronectin receptor alpha)	-2.76	1.01E-3	70.95	25.72
Snai1	snail family zinc finger 1	-2.69	3.07E-2	3.46	1.29
Lox	lysyl oxidase	-2.54	1.01E-3	5.74	2.26
S100a9	S100 calcium binding protein A9 (calgranulin B)	-2.51	3.33E-3	39.28	15.67
Ptgs2	prostaglandin-endoperoxide synthase 2	-2.50	1.01E-3	24.39	9.75
Mmp3	matrix metallopeptidase 3	-2.45	1.01E-3	15.58	6.35
Mmp19	matrix metallopeptidase 19	-2.32	4.24E-2	5.40	2.33
Thbs1	thrombospondin 1	-2.23	1.01E-3	49.51	22.17
Acta2	actin, alpha 2, smooth muscle, aorta	-2.18	1.01E-3	869.12	399.19
Tnc	tenascin C	-1.47	9.77E-2	189.85	128.98
Fn1	fibronectin 1	-1.44	1.19E-1	172.91	119.86

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Gene ID	Gene description	Fold_ Change	FDR	WT_24_Hour_Avg_ FPKM	β8ITGcKO_24_Hour_Avg_ FPKM
Itgb1	integrin beta 1 (fibronectin receptor beta)	-1.31	1.76E-1	191.15	146.02
Itgav	integrin alpha V	-1.24	3.30E-1	100.72	81.24

FDR- False Discovery Rate, Avg- Average, FPKM- Fragments Per Kilobase Million, ∞ indicates that fold change did not give a numerical value as FPKM of a specific gene appears 0 at WT 0 hour PCS

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5.2.4 Defects in TGFβ signaling are detected in β8ITGcKO LCs while the addition of active TGFβ1 to the β8ITGcKO capsular bags rescues the defects

It is well established that TGFβ signaling mediates fibrotic PCO (de Iongh et al. 2005). Previously, we have shown that αV integrin is critical in this process (Mamuya et al. 2014). αVβ8 integrin has been attributed in the activation of TGFβ signaling in other systems (Mu et al. 2002), while genes reported either to regulate (gremlin-1, thrombospondin-1, fibronectin), or be regulated by, TGFβ signaling (αSMA, tenascin C), are differentially expressed in β8ITGcKO LCs (Table 5.4, Figure 5.5A). Taken all together, we next determined the extent of canonical TGFβ pathway activation in WT and β8ITGcKO LCs PCS by following pSMAD3 levels (a downstream of TGFβ signaling). Activation of SMAD3 is seen in WT LCs at 48 hr PCS (*P = 0.013) while this is absent in β8ITGcKO (P = 0.213) (Figure 5.5B). WT LCs exhibit enhanced activation of canonical TGFβ signaling at 5 d PCS (***P < 0.001), pSMAD2/3 is barely detected in β8ITGcKO LCs (P = 0.019) which is significantly different from WT (***P < 0.001) suggesting that the activation of TGFβ signaling depends on the upregulation of the β8 integrin PCS.

As active TGFβ induces lens cells to convert to myofibroblasts(de Iongh et al. 2005) and we have identified TGFβ signaling activation defects in β8ITGcKO LCs, we then determined whether exogenous active TGFβ could rescue the TGFβ signaling and fibrotic defects in β8ITGcKO. We found that active TGFβ1 treated β8ITGcKO capsular bags show robust activation of pSMAD3 (***P < 0.001) and robust expression of the fibrotic markers αSMA (*P = 0.011), tenascin C (**P = 0.007), fibronectin (*P = 0.012) and collagen I (**P = 0.003) at 5 d PCS (Figure 5.5C) which further confirms the critical role of β8 integrin in TGFβ signaling activation PCS.

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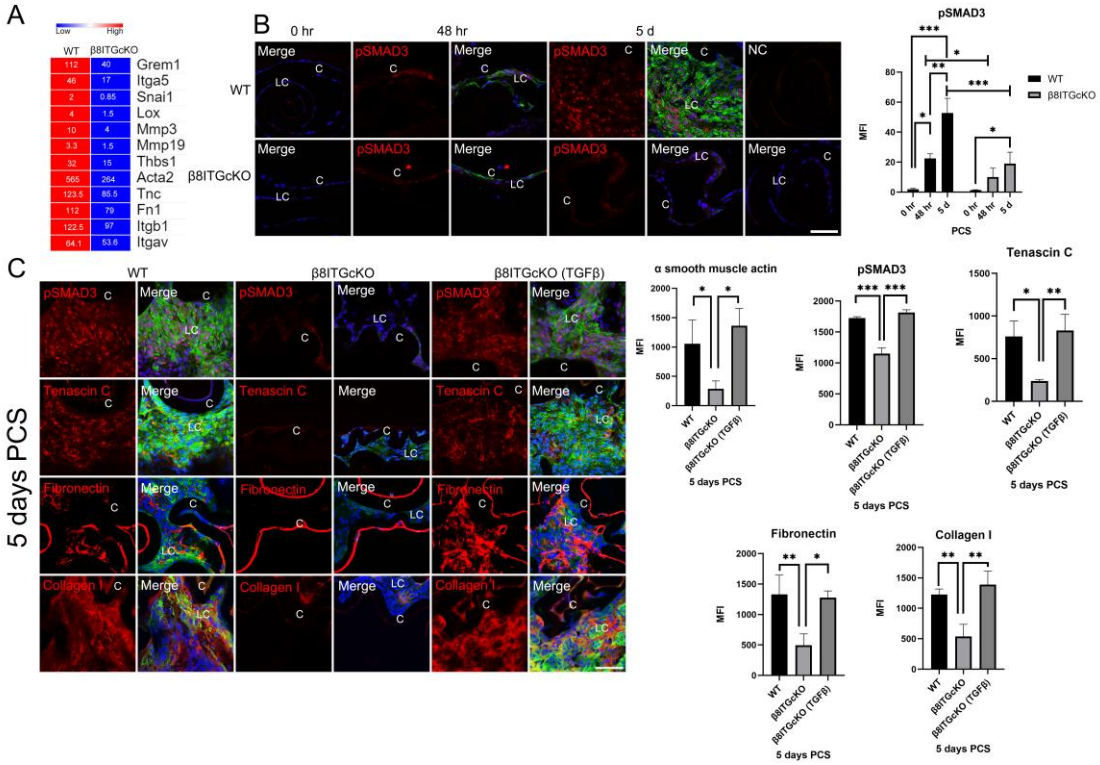

Figure 5.5: The effects of an αVβ8 integrin blocking antibody on LCs' response to TGFβ signaling activation and fibrosis PCS. (A) Differentially expressed genes related to TGFβ signaling between WT and β8ITGcKO at 24 hr PCS. Values in FPKM. (B) Administration of an αVβ8 integrin blocking antibody (αVβ8-IBA) to WT inhibits the fibrotic response by LCs compared to vehicle treated WT LCs detected by αSMA (**P = 0.002), tenascin C (**P = 0.003), fibronectin (***P < 0.001), collagen I (*P = 0.019) and SMAD3 activation (*P = 0.017) at 3 d PCS, the same response is detected in β8ITGcKO LCs. (C) Like β8ITGcKO, inhibition of fibrotic responses measured by αSMA (***P < 0.001), tenascin C (**P = 0.008), fibronectin (*P = 0.025), collagen I (*P = 0.016) and the inhibition of SMAD3 activation (**P = 0.008) are maintained till 5 d PCS compared to vehicle treated WT mice. Scale bar- 35 μm, C- lens capsule, LC- remnant lens cells, MFI- mean fluorescence intensity, PCS- post cataract surgery, αVβ8-IBA- αVβ8 integrin blocking antibody, pSMAD3, tenascin C, fibronectin and collagen I (red), αSMA (green), DNA detected by Draq5/DAPI (blue). All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between WT and/or β8ITGcKO and/or β8ITGcKO (αVβ8-IBA (Integrin Blocking Agent)) at a PCS.

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5.2.5 Blocking the interaction of LAP with αVβ8 integrin in WT LCs phenocopies the attenuated fibrotic response and TGFβ signaling PCS detected in β8ITGcKO

TGFβ is secreted from cells bound to its latency associated peptide (LAP) and latent TGFβ binding proteins (LTBPs) (Robertson and Rifkin 2016). Once the latent TGFβ complex is tethered to ECM by binding to matrix fibers, notably fibronectin (Robertson and Rifkin 2016; Shihan et al. 2020), the interaction of LAP with integrins is proposed to be essential for the subsequent release of TGFβ from the latent complex(Robertson and Rifkin 2016). Thus, we next tested an αVβ8 integrin blocking antibody ADWA-11(αVβ8-IBA (Integrin Blocking Agent)) that is shown to antagonize the LAP adhesion to the αVβ8 integrin receptor and thus blocks TGFβ activation(Sheppard, Atakilit, and Henderson 2020). We have found that the addition of αVβ8-IBA inhibits the fibrotic response of LCs detected by αSMA (**P = 0.002), tenascin C (**P = 0.003), fibronectin (***P < 0.001) and collagen I (*P = 0.019) concomitant with inhibition of TGFβ signaling activation (*P = 0.017) at 3 d PCS (the time point when the robust activation of TGFβ signaling is detected PCS (Jiang et al. 2018)) compared to WT (vehicle) (Figure 5.6A). We have detected the same response in β8ITGcKO LCs (Figure 5.6A) suggesting that αVβ8-IBA can block the activation of TGFβ signaling and subsequent fibrotic response in LCs PCS.

Next, we tested if αVβ8-IBA provides a sustained anti-fibrotic effects PCS. Thus, αVβ8-IBA was administered to WT mice following cataract surgery and the samples were harvested at 5 d PCS (the time point when the sustained fibrotic response and TGFβ signaling is detected PCS (Shihan et al. 2020)). Our analysis shows that upon addition of αVβ8-IBA to WT, the inhibition of fibrotic responses measured by αSMA (***P < 0.001), tenascin C (**P = 0.008), fibronectin (*P = 0.025), collagen I (*P = 0.016) and pSMAD3 (downstream signaling mediator of

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TGFβ signaling) (**P = 0.008) is maintained until 5 d PCS compared to WT and this mimics the attenuated fibrotic response detected in β8ITGcKO LCs (Figure 5.6B). This supports that αVβ8-IBA is capable of maintaining the anti-fibrotic effects at later time PCS besides its ability to block the activation of fibrosis at early time PCS.

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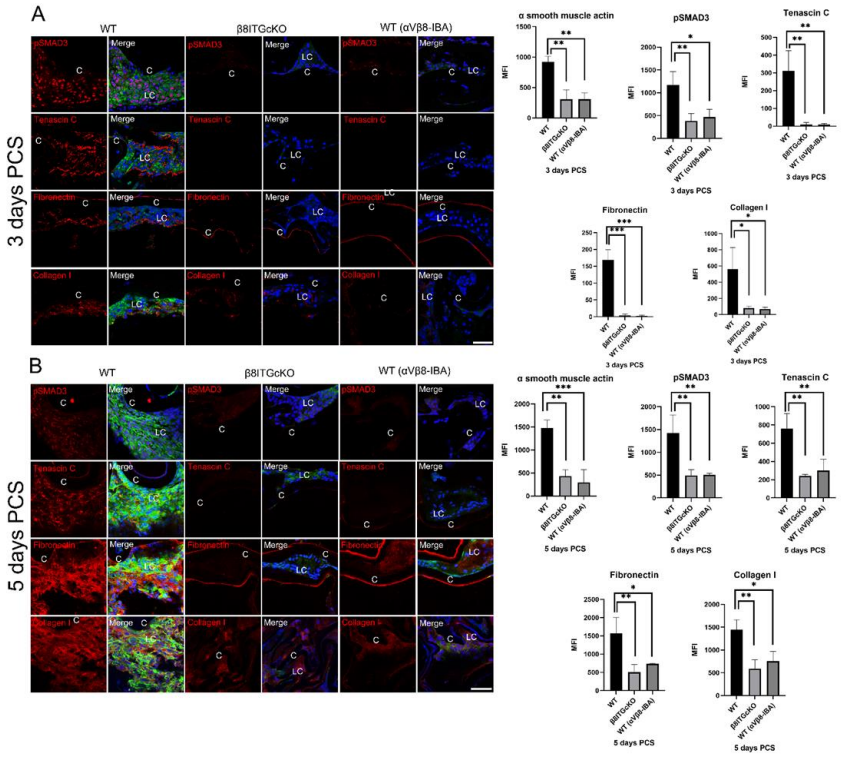

Figure 5.6: The effects of an αVβ8 integrin blocking antibody on LCs' response to TGFβ signaling activation and fibrosis PCS. (A) Administration of an αVβ8 integrin blocking antibody (αVβ8-IBA) to WT inhibits the fibrotic response by LCs compared to vehicle treated WT LCs detected by αSMA (**P = 0.002), tenascin C (**P = 0.003), fibronectin (***P < 0.001), collagen I (*P = 0.019) and SMAD3 activation (*P = 0.017) at 3 d PCS, the same response is detected in β8ITGcKO LCs. (B) Like β8ITGcKO, inhibition of fibrotic responses measured by αSMA (***P < 0.001), tenascin C (**P = 0.008), fibronectin (*P = 0.025), collagen I (*P = 0.016) and the inhibition of SMAD3 activation (**P = 0.008) are maintained till 5 d PCS compared to vehicle treated WT mice. Scale bar- 35 µm, C- lens capsule, LC- remnant lens cells, MFI- mean fluorescence intensity, PCS- post cataract surgery, αVβ8-IBA- αVβ8 integrin blocking antibody, pSMAD3, tenascin C, fibronectin and collagen I (red), αSMA (green), DNA detected by Draq5/DAPI (blue). All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between WT and/or β8ITGcKO and/or β8ITGcKO (αVβ8-IBA) at a PCS.

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5.2.6 The production of αVβ8 integrin by LCs is required for the upregulation of gremlin-1 expression PCS

To obtain further mechanistic insight into the function of β8 integrin PCS, we investigated the list of genes differentially expressed at the mRNA level in β8 integrin LCs at 24 hr PCS for those with the potential to mechanistically regulate TGFβ signaling. Gremlin-1, a secreted BMP antagonist (Brazil et al. 2015) and a profibrotic factor that can drive TGFβ signaling in lens cells (Shihan et al. 2020) is upregulated 170 fold in WT LCs at 24 h PCS and this upregulation was attenuated 3 fold in β8ITGcKO LCs (Table 5.1 & 5.4). Gremlin-1 protein levels were low in either WT or β8ITGcKO LCs immediately following surgery (Figure 5.7A). By 3 d PCS, significant upregulation of gremlin-1 is measured in WT capsular bags (***P < 0.001). In contrast, both β8ITGcKO (**P = 0.002) and WT (αVβ8-IBA) (***P < 0.001) LCs show attenuated expression of gremlin-1 at 3 d PCS compared to WT. We have observed the similar pattern at 5 d PCS where gremlin-1 protein levels are significantly less both in β8ITGcKO (*P = 0.017) and WT (αVβ8-IBA) (*P = 0.022) LCs compared to WT (Figure 5.7B) suggesting that αVβ8 integrin expression by LCs is critical for the upregulation of gremlin-1 PCS.

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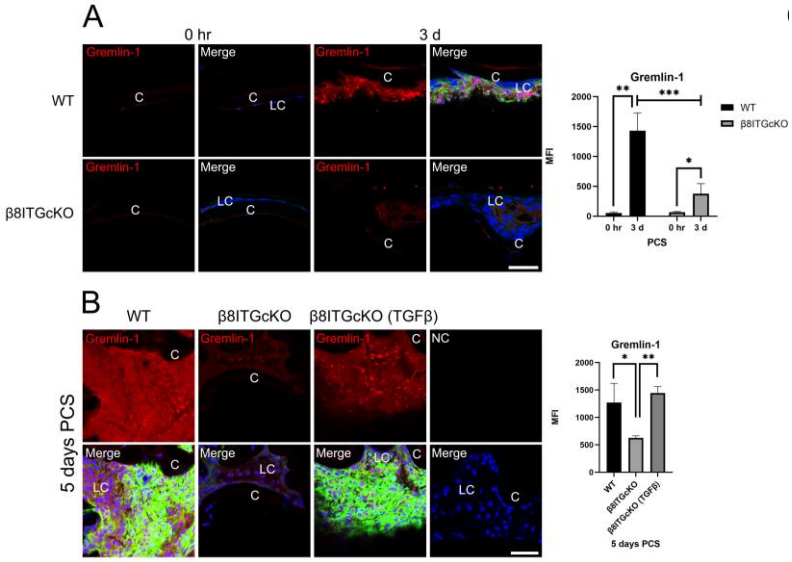

Figure 5.7: Crosstalk between αVβ8 integrin and gremlin-1 PCS. (A) WT LCs show the significant upregulation of gremlin-1 protein at 3 d PCS (***P < 0.001) while β8ITGcKO LCs fails to do so compared to WT (**P = 0.002). The addition of an αVβ8 integrin blocking antibody to WT further inhibits the upregulation of gremlin-1 expression compared to WT (vehicle treated) (***P < 0.001). (B) β8ITGcKO LCs show attenuated gremlin-1 protein levels compared to WT (vehicle) (*P = 0.017) and β8ITGcKO (TGFβ) (**P = 0.004) LCs at 5 d PCS. Like β8ITGcKO, the treatment of WT LCs with an αVβ8 integrin blocking antibody shows inhibition of gremlin-1 levels compared to WT (vehicle) LCs (*P = 0.022). Scale bar- 35 µm, C- lens capsule, LC- remnant lens cells, MFI- mean fluorescence intensity, PCS- post cataract surgery, αVβ8-IBA- αVβ8 integrin blocking antibody, gremlin-1 (red), αSMA (green), DNA detected by Draq5 (blue). All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between WT and/or β8ITGcKO and/or β8ITGcKO (TGFβ) and/or WT (αVβ8-IBA) at a PCS or between two PCS time points.

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5.2.7 Upregulation of integrins and integrin signaling by LCs depends on αVβ8 integrin mediated TGFβ signaling activation

The cross-talk between integrins and TGFβ signaling is well documented (Mamuya and Duncan 2012). Notably, LCs elevate the protein expression of several integrins attributed in fibrotic diseases such as α5β1-integrin and several αV class integrins PCS (Walker and Menko 2009; Mamuya et al. 2014; Shihan et al. 2020) while the addition of active TGFβ1 to a mouse model with attenuated integrin expression PCS rescues the integrin expression and signaling defect (Shihan et al. 2020). Consistent with the previous finding, we found that the addition of active TGFβ1 to β8ITGcKO capsular bags can rescue the attenuated integrin expression and signaling (pFAK) detected in β8ITGcKO LCs (α5 integrin **P = 0.002; αV integrin **P = 0.010; β1 integrin **P = 0.004; pFAK ***P < 0.001) (Figure 5.8B). The attenuated levels of integrins and its downstream signaling molecule pFAK are also detected in WT (αVβ8-IBA) LCs compared to WT (α5 integrin ***P < 0.001; αV integrin **P = 0.006; β1 integrin **P = 0.008; pFAK ***P < 0.001) as WT LCs express robust levels of integrins and pFAK at 5 d PCS (Figure 5.8B). We have recorded similar findings at 3 d PCS (Figure 5.8A). The expression of all three integrins and the pFAK levels are upregulated in WT LCs at 3 d PCS from 0 h PCS (α5 integrin ***P < 0.001; αV integrin ***P < 0.001; β1 integrin ***P < 0.001; pFAK **P = 0.006). In contrast, both β8ITGcKO (α5 integrin ***P < 0.001; αV integrin ***P < 0.001; β1 integrin ***P < 0.001; pFAK *P = 0.015) and WT (αVβ8-IBA) (α5 integrin ***P < 0.001; αV integrin ***P < 0.001; β1 integrin ***P < 0.001; pFAK *P = 0.029) LCs fail to upregulate them at 3 d PCS compared to WT LCs. Altogether, our finding suggests that a circle of regulation exists between integrins and TGFβ signaling PCS.

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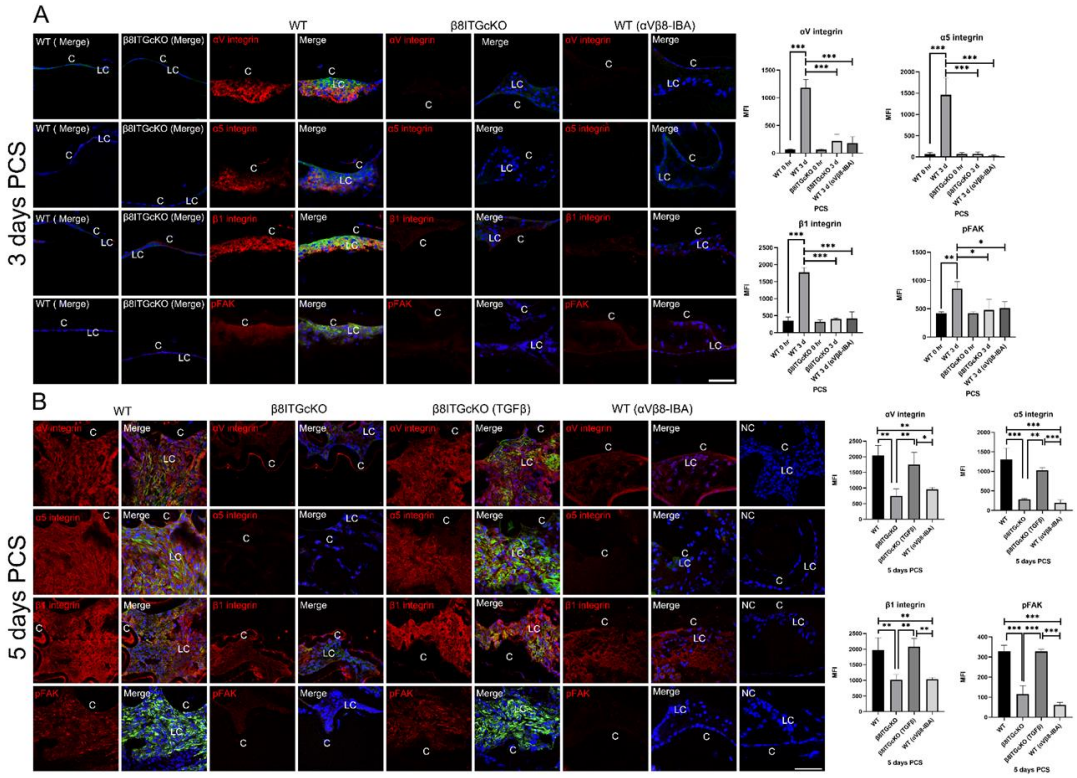

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Figure 5.8: The dynamics of integrins and integrin signaling regulation in relationship to TGFβ signaling activation PCS. (A) WT LCs upregulate all three integrins (αV integrin, α5 integrin, and β1 integrin) and pFAK levels at 3 d PCS while β8ITGcKO LCs fail to do so compared to WT (αV integrin, ***P < 0.001; α5 integrin, ***P < 0.001; β1 integrin, ***P < 0.001; pFAK, *P = 0.015). Like β8ITGcKO, WT LCs treated with αVβ8 integrin blocking antibody fail to upregulate integrins and pFAK levels compared to WT LCs (vehicle) (αV integrin, ***P < 0.001; α5 integrin, ***P < 0.001; β1 integrin, ***P < 0.001; pFAK, *P = 0.029). (B) Compared to WT LCs, both β8ITGcKO (vehicle) (αV integrin, **P = 0.002; α5 integrin, **P < 0.001; β1 integrin, **P = 0.007; pFAK, ***P < 0.001) and WT LCs (αVβ8-IBA) (αV integrin, **P = 0.006; α5 integrin, ***P < 0.001; β1 integrin, **P = 0.008; pFAK, ***P < 0.001) show attenuated expression of all three integrins and pFAK levels at 5 d PCS. The addition of active TGFβ1 to β8ITGcKO capsular bags rescues the attenuated integrins and pFAK levels detected in vehicle treated β8ITGcKO capsular bags (αV integrin, **P = 0.010; α5 integrin, **P = 0.002; β1 integrin, **P = 0.004; pFAK, ***P < 0.001). Scale bar- 35 μm, C- lens capsule, LC- remnant lens cells, MFI- mean fluorescence intensity, PCS- post cataract surgery, αVβ8-IBA- αVβ8 integrin blocking antibody, gremlin-1 (red), αSMA (green), DNA detected by Draq5/DAPI (blue). All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between WT and/or β8ITGcKO and/or β8ITGcKO (TGFβ1) and/or WT (αVβ8-IBA) at a PCS or between two PCS time points.

5.2.8 Fibrosis regression is observed in WT upon the addition of αVβ8 integrin blocking antibody at 5 days PCS

Finally, we determined if the αVβ8 integrin blocking antibody (αVβ8-IBA) can promote fibrosis regression PCS in addition to the prevention of PCO. To study this, three groups (each group consists of three mice at least) of wildtype (WT) mice and one group of β8ITGcKO were subjected to cataract surgery and let them develop fibrosis until 5 days PCS. At 5 days PCS, two WT mice group received one dose of (αVβ8-IBA) while another group of WT and β8ITGcKO mice group received vehicle and samples were harvested at 10 days PCS. To study further the dose-dependent

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relationship of αVβ8-IBA and fibrosis regression, one group of WT mice who previously received a single dose of αVβ8-IBA at 5 days PCS, received a second dose of αVβ8-IBA at 7.5 days PCS. The analysis revealed that the single dose of αVβ8-IBA significantly attenuated the expression of collagen I (WT vs WT (αVβ8-IBA -1 dose) *P = 0.012; WT vs β8ITGcKO ***P < 0.001), fibronectin (WT vs WT (αVβ8-IBA -1 dose) *P = 0.015; WT vs β8ITGcKO **P = 0.002), tenascin C (WT vs WT (αVβ8-IBA -1 dose) **P = 0.002; WT vs β8ITGcKO **P = 0.001) and the activation of pSMAD3 (downstream of TGFβ signaling) (WT vs WT (αVβ8-IBA -1 dose) ** P < 0.007; WT vs β8ITGcKO ** P = 0.009) at 10 days PCS similar as β8ITGcKO while the single dose of αVβ8-IBA was not enough for the regression of αSMA protein (WT vs WT (αVβ8-IBA -1 dose) P = 0.067) while two doses of αVβ8-IBA were able to attenuate the expression of αSMA protein at 10 days PCS (WT vs WT (αVβ8-IBA -2 doses) *P = 0.013). Overall this suggests that αVβ8-IBA may be used in the treatment of fibrotic PCO in addition to the prevention of PCO development.

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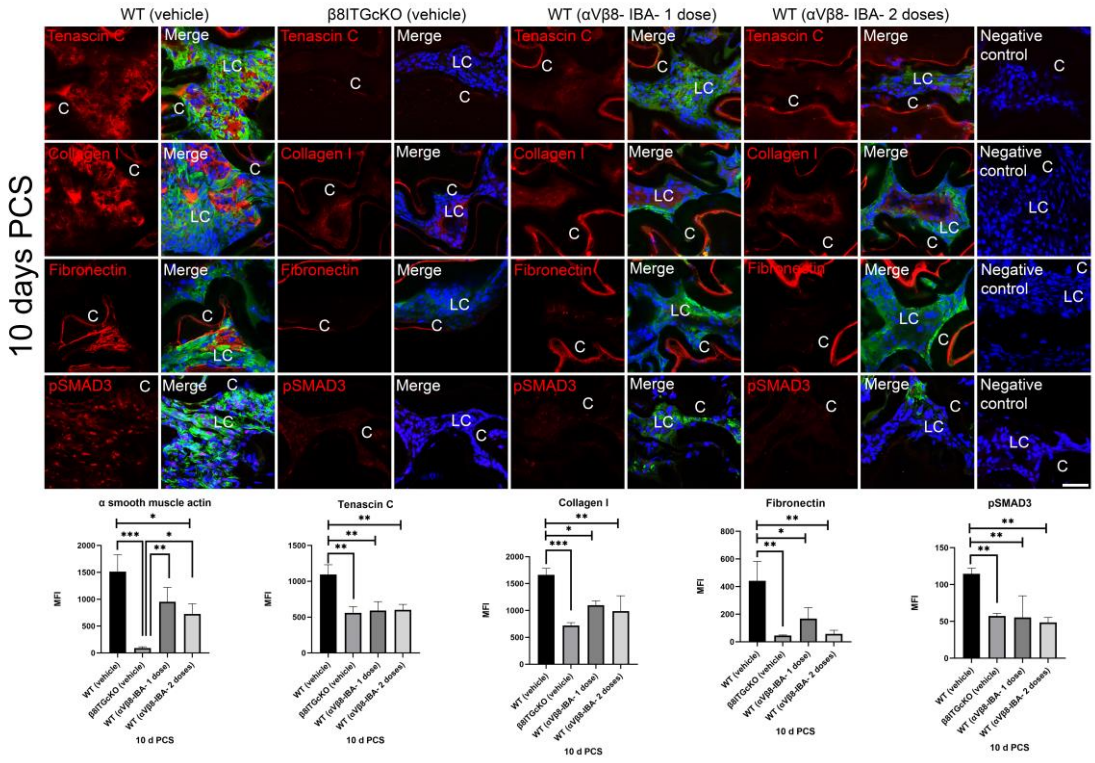

Figure 5.9: The effects of the αVβ8 integrin blocking antibody in fibrosis regression PCS. Administration of an αVβ8 integrin blocking antibody (αVβ8-IBA) at 5 days PCS significantly attenuated the expression of collagen I (WT vs WT (αVβ8-IBA -1 dose) *P = 0.012; WT vs β8ITGcKO ***P < 0.001), fibronectin (WT vs WT (αVβ8-IBA -1 dose) *P = 0.015; WT vs β8ITGcKO **P = 0.002), tenascin C (WT vs WT (αVβ8-IBA -1 dose) **P = 0.002; WT vs β8ITGcKO **P = 0.001) and the activation of pSMAD3 (downstream of TGFβ signaling) (WT vs WT (αVβ8-IBA -1 dose) ** P < 0.007; WT vs β8ITGcKO ** P = 0.009) at 10 days PCS similar as β8ITGcKO while the single dose of αVβ8-IBA was not enough for the regression of αSMA protein (WT vs WT (αVβ8-IBA -1 dose) P = 0.067) while two doses of αVβ8-IBA were able to attenuate the expression of αSMA protein at 10 days PCS (WT vs WT (αVβ8-IBA -2 doses) *P = 0.013). Scale bar- 35 μm, C- lens capsule, LC- remnant lens cells, MFI- mean fluorescence intensity, PCS- post cataract surgery, αVβ8-IBA- αVβ8 integrin blocking antibody, pSMAD3, tenascin C, fibronectin and collagen I (red), αSMA (green), DNA detected by Draq5/DAPI (blue). All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between WT and/or β8ITGcKO and/or β8ITGcKO (αVβ8-IBA) at 10 days PCS.

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5.3 Discussion

The rate of posterior capsular opacification (PCO) was high among adults (about 41% at 4 years post cataract surgery (PCS)) and young children (96% at 2 years PCS) 30 years ago (Moisseiev et al. 1989). Due to the innovations of modern cataract surgery and intraocular lenses of improved materials and shape, PCO rates have decreased (Awasthi, Guo, and Wagner 2009). However, despite these preventive strategies, PCO rates are still high among adults (28% at 5 years PCS) and children (40% at 2 years PCS) (Liu et al. 2017).YAG laser capsulotomy, the only approved treatment of PCO has some limitations either such as the subsequent side effects and limited availability and expertise in developing and underdeveloped countries (Shihan, Novo, and Duncan 2019). Altogether, it suggests that the additional ways are needed to prevent PCO. Notably, the majority of the clinicians treating PCO patients think that therapeutic measures to prevent PCO development would improve the outcome of cataract surgery (Shihan, Novo, and Duncan 2019).

The study of integrins, transmembrane cell surface receptors concerning tissue fibrosis have gained a lot of attention among cell biologists and translational researchers for several reasons (1) fibrosis mediated organ damage and failure are one of the major cause of natural death worldwide while there is no effective way to prevent or treat tissue fibrosis (Urban, Manenti, and Vaglio 2015); (2) integrins have been proposed as important activators of Transforming growth factor-beta (TGFβ) signaling, well-established signaling cascade of tissue fibrosis and PCO (de Iongh et al. 2005; Robertson and Rifkin 2016; Sheppard 2005); (3) integrins have been proposed as therapeutic targets in relationship to organ fibrosis and ocular diseases and several integrins blocking agents are under of the clinical trials (Walker and Menko 2009; Gonzalez-Salinas et al. 2018; Schnittert et al. 2018). Among all the

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integrins, αV integrins class are particularly significant as blocking this class of integrins has shown to ameliorate tissue fibrosis in several organs (Henderson and Sheppard 2013). Previously, we have found that the deletion of αV integrin from the lens protects the lens cells from undergoing epithelial mesenchymal transition (EMT) and we have also identified that TGFβ signaling is critical in this process (Mamuya et al. 2014). However, the missing piece is that which β subunit participates with αV integrin in this process since more than one β subunits reported to heterodimer with αV integrin are upregulated by LCs PCS (Mamuya et al. 2014).

To solve this puzzle, we have characterized the role of three β subunits (β5, β6, and β8) due to their potential role to participate in TGFβ activation hence tissue fibrosis in other organs (Munger et al. 1999; Tatler et al. 2011; Mu et al. 2002a). Although αVβ1 integrin is reported to play roles in tissue fibrosis (N. I. Reed et al. 2015), our study did not characterize the role of β1 integrin in relationship to PCO as we have previously shown that the β1 integrin is essential for the lens development and homeostasis while adult β1 integrin null mice are severely microphthalmic/anophthalmic (Pathania et al. 2016; Scheiblin et al. 2014; Wang et al. 2017), an eye not suitable for cataract surgery model.

The characterization of β5 and β6 null lenses subjected to cataract surgery reveal that β5 and β6 integrins are not critical for fibrotic PCO. This was surprising to us. However, we dug into literature and found that (1) the developmental and anti-inflammatory roles of αVβ5 and αVβ6 integrin have been proposed before (Sheppard 2004; Mohazab et al. 2013; Koivisto et al. 2018; Chauss et al. 2015) (2) αVβ5 and αVβ6 integrin's association in relationship to fibrosis is tissue and insults specific (X. Huang et al. 2000; Henderson and Sheppard 2013; B. Wang et al. 2007). Thus we

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turned our attention to αVβ8 integrin as this integrin has gained a lot of attention lately due to its ability to participate in tissue fibrosis and inflammation and its unique mechanism to activate TGFβ signaling by collaborating with a cofactor, membrane-type matrix metalloproteinase 1 (MT1-MMP/MMP14) (Arnold et al. 2019; Mu et al. 2002a; Lakhe-Reddy et al. 2014; Fenton et al. 2017; Melton et al. 2010; Greenhalgh et al. 2019). Thus, we comprehensively characterized the role of β8 integrin in epithelial-mesenchymal transition PCS. We took advantage of a global approach, RNA sequencing, and three different experimental designs were employed to characterize the role of αVβ8 integrin in PCO. Firstly, we generated mice lacking the integrin β8 gene from the lens (β8ITFcKO) since β8 integrin is essential for vascular morphogenesis and deletion of β8 integrin is embryonic lethal (Proctor et al. 2005; Zhu et al. 2002). The characterization of β8ITGcKO remnant lens epithelial cells (LCs) by RNA seq, Immunofluorescence/ImageJ quantification reveals that the deletion of β8 integrin from the lens attenuates fibrosis development and TGFβ signaling PCS. Not only have we detected that the TGFβ signaling and fibrotic proteins regulated PSC by this signaling (αSMA and tenascin C) are significantly less expressed in β8ITGcKO LCs, but also some of the regulators of TGFβ signaling and fibrosis (fibronectin and collagen I) are significantly attenuated at the protein levels and lost their fibrillar structure, a prerequisite to regulating fibrotic matrix assembly and latent TGFβ complex tethering (Shihan et al. 2020; Barker and Engler 2017), both mechanisms are critical for the subsequent activation of TGFβ signaling (Robertson and Rifkin 2016; Shihan et al. 2020). Besides, we show that gremlin-1, an agonist of TGFβ signaling (Shihan et al. 2020) and a potential mediator of PCO (Shihan et al. 2020; Ma et al. 2019) is under the regulatory control of β8 integrin PCS which is

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significant in the light of β8 integrin's ability to sustain fibrotic PCO since gremlin-1 has been proposed to play its fibrotic role at later times PCS (Shihan et al. 2020). We have also detected proliferation defects and a retention of epithelial cell phenotype in β8ITGcKO capsular bags which is further consistent with the previous findings that suggest that TGFβ overexpressing transgenic mice show the downregulation of E-cadherin and other epithelial cell markers in LCs (de Iongh et al. 2005; F. J. Lovicu et al. 2004).

The feedforward mechanism between αV integrins and TGFβ signaling is well defined in multiple systems (Mamuya and Duncan 2012). Consistent with this, we found that lenses lacking β8 integrin fail to upregulate the expression of αV, α5, and β1 integrins, and pFAK levels PCS. This finding adds further significance concerning the role of β8 integrin in PCO since all three integrins have been implicated in tissue fibrosis either by activating the TGFβ signaling or fibronectin matrix assembly (Shihan et al. 2020; Henderson and Sheppard 2013; Singh, Carraher, and Schwarzbauer 2010), hence targeting αVβ8 would suffice to prevent tissue fibrosis mediated by other integrins.

Since we have detected that canonical TGFβ signaling is critical for β8 integrin mediated fibrotic PCO, our next approach was to perform rescue experiments with two TGFβ signaling agonists – active TGFβ1 and gremlin-1- by adding them to the capsular bags of β8ITGcKO mice. By doing so, we were able to generate a robust fibrotic response, TGFβ signaling activation, and integrin upregulation PCS. This further confirms that the activation of TGFβ signaling is the core mechanism by β8 integrin mediated fibrotic PCO. Finally, we have used a novel αVβ8 integrin blocking antibody (ADWA-11) in our in vivo mouse cataract surgery model. ADWA-11 has

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been shown to prevent the adhesion of LAP to αVβ8 integrin, a critical step to release active TGFβ from its latent complex (Sheppard, Atakilit, and Henderson 2020). and we were able to phenocopy the attenuated fibrotic response, TGFβ signaling, and inhibited integrin expression by LCs detected in β8ITGcKO LCs. This finding is particularly significant as currently there is no pharmacological agent available to prevent the PCO development where our preclinical study offers a possible therapeutic target and its promising inhibitor in PCO prevention. Since this blocking antibody seems to both prevent the initiation of PCO, and we observed little fibrotic tissue at later stages, this suggests that blocking the initial wound healing response could be effective in preventing fibrotic PCO long term. This provides further added value in the light of PCO prevention as myofibroblasts can survive for years before triggering PCO which results in clinically significant vision impairment (Shirai et al. 2004). In addition to the role of αVβ8 integrin blocking antibody (ADWA-11) in blocking the initial wound healing response, we further observed that it may play in role in fibrosis regression PCS suggesting that ADWA-11 could be used to treat fibrotic PCO in patients in addition to its role as preventing fibrotic PCO.

In summary, our study provides a novel therapeutic target to prevent PCO and the effectiveness of a blocking agent to target it (Figure 5.10). As MT1-MMP has been implicated as a cofactor of αvβ8 integrin-mediated TGFβ activation (Mu et al. 2002), and as anti-MMPs inhibitors can prevent TGFβ regulates anterior subcapsular cataracts and other fibrotic like conditions (Fields 2019; Dwivedi et al. 2006), we further propose to study the role of MT1-MMP in lens EMT as dual blocking of αVβ8 integrin and MT1-MMP may further improve this PCO preventive approach.

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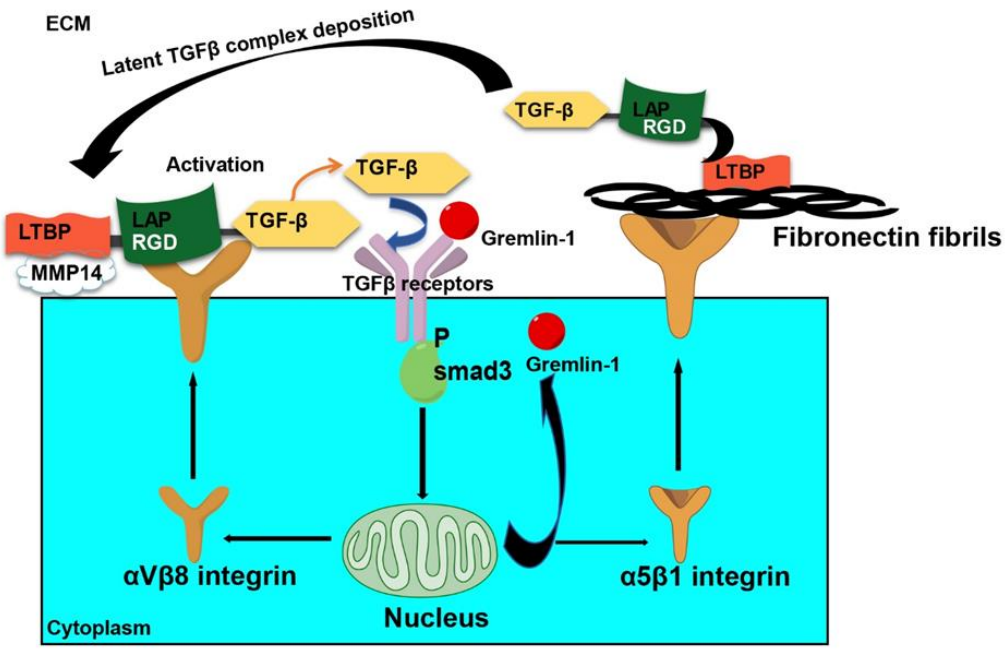

Figure 5.10: Following cataract surgery, the secreted latent TGFβ complex is tethered on to the extracellular matrix (ECM) by fibronectin fibrils leading to the binding of latency- associated peptide (LAP) of latent TGFβ complex to the αVβ8 integrin. This binding may make LAP more accessible to proteases, such as membrane-type matrix metalloproteinase 1 (MT1-MMP), that cleave LAP and release the active growth factor for its availability to binding to its receptors. Activation of TGFβ signaling leads to the expression of fibrotic proteins such as fibronectin, integrins, and profibrotic factor gremlin-1, all of which in turn may contribute to maintaining the TGFβ signaling mediated fibrotic response PCS.

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Chapter 6

FIBRONECTIN HAS MULTIFUNCTIONAL ROLES IN PCO

All the contents included in this chapter are described in a manuscript entitled Mahbubul H. Shihan, Mallika Kanwar, Yan Wang, Erin E. Jackson, Adam P. Faranda and Melinda K. Duncan (2020) 'Fibronectin has multifunctional roles in posterior capsular opacification (PCO)' Matrix Biology Volume 90, August 2020, Pages 79-108.

This paper is voted as Carson's Best Graduate Student Publication Award for 2020 by the Department of Biological Sciences, The University of Delaware.

6.1 Introduction

Fibronectin, a structurally complex extracellular matrix (ECM) protein, is essential for diverse physiological processes such as blood coagulation, opsonization, and embryogenesis (Blumenstock et al. 1986; Eriksen, Espersen, and Clemmensen 1984; Mezzenga and Mitsi 2019; Czop 1986; Astrof, Crowley, and Hynes 2007; Elizabeth L. George, Baldwin, and Hynes 1997; E.L. George et al. 1993). Plasma fibronectin is a compact, soluble protein produced by the liver that is present at high levels in body fluids (To and Midwood 2011). Tissue fibronectin, which is produced locally in tissues, is an alternatively spliced isoform of fibronectin possessing a more open conformation that allows it to assemble readily into insoluble fibrils (Schwarzbauer 1991; Mezzenga and Mitsi 2019; Paul et al. 1986; To and Midwood 2011). Fibronectin is known to concentrate at sites of wound healing and tissue repair

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(Lenselink 2015; Yamada and Clark 1988; CLARK 1983; Clark 1990), while chronic fibronectin deposition is a feature of numerous fibrotic diseases (Mezzenga and Mitsi 2019; Lenselink 2015; Walraven and Hinz 2018; Stoppacciaro et al. 2008; Altrock et al. 2015; Mamuya et al. 2014).

Plasma fibronectin complexes with fibrin immediately after cutaneous wounding to form the early provisional ECM necessary for primary wound closure (Barker and Engler 2017). Later, fibronectin is produced locally at the wound site as part of the late provisional matrix, which is then remodeled to facilitate the assembly of secondary scars rich in collagen I (Zhang et al. 2014). Fibronectin fibrils also serve as an extracellular depot for numerous growth factors, suggesting that fibronectin could play multifunctional roles in the wound healing response and fibrotic diseases (Fontana et al. 2005; Zollinger and Smith 2017). The importance of fibronectin in wound healing and fibrotic diseases has been confirmed in vivo using mice lacking the EDA exon which is often included in tissue fibronectin (Muro et al. 2003; Stenzel et al. 2011; Iwasaki et al. 2016; Moriya et al. 2012). Many of these in vivo studies suggest that fibronectin deposition drives fibrosis in their system. However, these studies only explore the function of one form of fibronectin produced by wounded tissue and do not typically explore other fibronectin functions such as its tethering of latent transforming growth factor-beta (TGFβ) to the ECM, which is crucial for subsequent activation of TGFβ, suggesting the need for the comprehensive in vivo study of the role of fibronectin in wound healing (Vega and Schwarzbauer 2016; Vogel et al. 1990; Kumra and Reinhardt 2016; Lenselink 2015).

Cataracts, a major cause of blindness worldwide (Khairallah et al. 2015; Liu et al. 2017; C. M. Lee and Afshari 2017), are effectively treated by surgical removal of

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opaque lens fiber cells followed by implantation of an artificial intraocular lens (IOL) (Liu et al. 2017). However, months to years later, a significant proportion of patients experience an apparent recurrence of their cataract as Posterior Capsular Opacification (PCO)(I. Michael Wormstone, Wang, and Liu 2009; Awasthi, Guo, and Wagner 2009; Vasavada and Praveen 2014; Julia M. Marcantonio and Vrensen 1999). PCO occurs when lens epithelial cells (LCs) left behind post cataract surgery (PCS) migrate into the optical axis and transition into a mixture of myofibroblasts embedded in a fibrotic ECM, and aberrant lens fiber cells (I. Michael Wormstone, Wang, and Liu 2009).

Transforming growth factor β (TGFβ) signaling is a major inducer of the epithelial to mesenchymal transition of LCs to myofibroblasts expressing numerous "fibrotic" markers, including fibronectin (Dawes et al. 2008; Gyorfi, Matei, and Distler 2018). However, the function of fibronectin in the pathogenesis of fibrotic PCO is unclear. In a mouse cataract surgery model, fibronectin mRNA levels upregulate in remnant LCs by 24 hours PCS, and fibronectin fibrils are first detected around LCs expressing fibrotic markers such as αSMA by 48 hours PCS (Mamuya et al. 2014), coincident with the onset of detectable TGFβ signaling. In vitro studies suggest that fibronectin is a negative regulator of posterior capsular wrinkling in PCO (Dawes et al. 2008) although disruption of fibronectin assembly attenuates LC conversion to myofibroblasts in culture (Tiwari et al. 2016). Most recently, it was reported that exposure of cultured chicken LCs to plasma fibronectin (as would occur after cataract surgery) led to the activation of the latent TGFβ being produced endogenously by cultured cells, indicating that fibronectin plays an important mechanistic role in PCO pathogenesis (VanSlyke, Boswell, and Musil 2018).

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However, the function of the cellular fibronectin produced autonomously by remnant LCs in vivo PCS is not well understood.

Here, we deleted the fibronectin gene from the lenses of adult mice and evaluated how this deletion affects the response of LCs to a lens fiber cell removal operation that models cataract surgery. This study reveals, for the first time, the multifunctional roles that cellular fibronectin plays in PCO pathogenesis and adds to our understanding of how fibronectin can contribute to the pathophysiology of fibrotic disease.

6.2 Results

6.2.1 Deletion of the fibronectin gene from the lens does not affect the later stages of lens development, while fibronectin protein expression increases during PCO progression

Fibronectin deposition around remnant lens epithelial cells (LCs) has long been a known feature of PCO and thus is often used as a "readout" for the progression of PCO in experimental models (Shirai et al. 2004; Frank J Lovicu et al. 2002; de Iongh et al. 2005). We previously reported that fibronectin mRNA levels upregulate in a mouse model of cataract surgery by 24 hours after fiber cell removal (post cataract surgery (PCS)), while cell associated fibronectin protein deposition can be detected around the remnant LCs by 48 hours PCS (Mamuya et al. 2014; Jiang et al. 2018). Consistent with this, here we found that fibronectin protein (red) is not readily detected around the remnant LCs at either the time of surgery (0 hr PCS) or 24 hours later (24 hr PCS) by immunofluorescence (IF) confocal imaging (Figure 6.1A), although some fibronectin is associated with the external surface of the lens capsule as previously reported (Mamuya et al. 2014). Cell-associated fibronectin (highlighted

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with arrow) is first detectable around the α smooth muscle actin (αSMA) positive remnant LCs (green) at 48 hours PCS, and this deposition greatly increases by 5 days PCS (Figure 6.1A). In addition to that, IF reveals all the classic features of fibrotic tissue in our cataract surgery model at 5 days PCS such as the absence of a normal cuboidal monolayer of epithelial cells, presence of multilayered spindle-shaped cells and capsular wrinkling as previously described (Hales, Chamberlain, and McAvoy 1995).

In order to test the function of fibronectin in PCO, we generated mice conditionally lacking a functional fibronectin gene from the lens (FNcKO) by mating mice carrying a floxed fibronectin allele (Sakai et al. 2001) to mice harboring the lens-specific MLR10 CRE transgene (Figure 6.1B, left) whose activity is first detected in the lens beginning around embryonic day 10.5 (the lens vesicle stage) (H. Zhao et al. 2004). The complete deletion of the floxed region of the fibronectin gene was confirmed by PCR analysis of genomic DNA isolated from adult lenses (Figure 6.1B, right). FNcKO lenses are transparent under dark field imaging (Figure 6.1C- A, B) and have refractive properties similar to wildtype (WT) lenses ((Figure 6.1C- C, D), while hematoxylin and eosin (H&E) staining demonstrated that both WT (Figure 6.1C- E, G) and FNcKO lenses (Figure 6.1C- F, H) exhibit similar morphology. This overall study suggests that fibronectin does not play a crucial role in regulating the structural properties of the adult lens.

To gain further insight into the role of fibronectin in adult lenses, RNA sequencing (RNAseq) was done on adult WT and FNcKO lenses, and the results submitted to the Gene Expression Omnibus (GEO) under accession number GSE119878. A total of 195 genes exhibited a statistically significant False Discovery

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Rate (FDR) ≤0.05; Fold change (FC) between adult WT and FNcKO null adult lenses of > 2 or < -2. However, only 121 of these genes met the criteria we have developed to identify biologically significant differentially expressed genes in the lens (FDR ≤0.05; FC > 2 or < -2; an absolute difference in group means > 2; and an expression level at least 2 Fragments Per Kilobase Million (FPKM) for at least one condition) (Manthey, Terrell, Lachke, et al. 2014). Notably, the FN1 (fibronectin1) gene which was deleted in this experiment did not make the list of "significant" differentially expressed genes because it is only expressed at very low levels (0.3 FPKM) in the unoperated adult lens. Analysis of these data for differentially expressed cellular components and pathways using iPathway guide (Advaita Corporation) revealed that the most significant gene ontology (GO) term calculated for the differentially expressed genes was "proteinaceous extracellular matrix" ($p < 5.4 \times 108$; data not shown), which included the upregulated genes Col1a2, Col9a1, Col9a2, and Col18a1, and the downregulated genes Col6a2 and Col6a3 (Supplemental Table A3), although in all cases the expression levels are low, and/or the fold changes modest.

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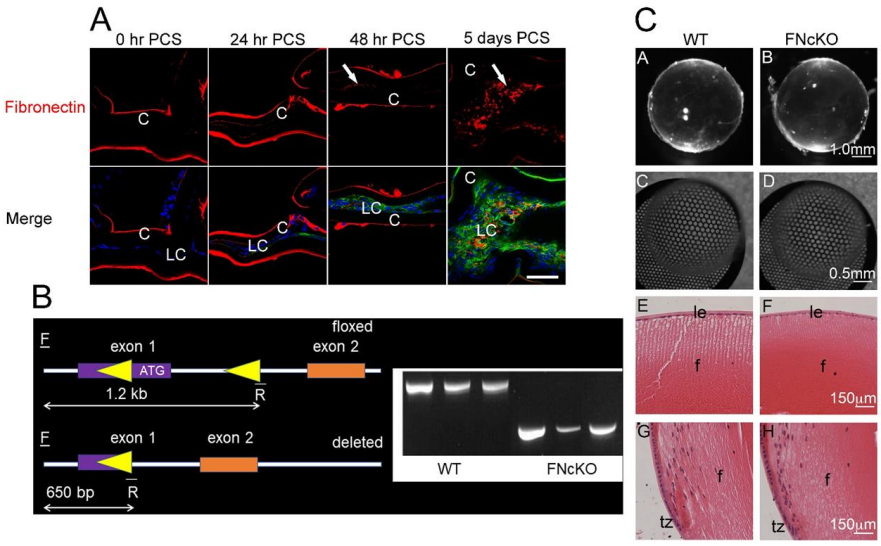

Figure 6.1: Fibronectin protein is not required for lens transparency, but deposits around remnant LCs PCS. (A) Dynamics of fibronectin protein deposition around remnant LCs PCS. At 0 hour PCS, little to no fibronectin is associated with remnant LCs, although the outer surface of the lens capsule is fibronectin positive (red). Fibronectin starts to deposit around αSMA positive remnant LCs by 48 hours PCS (arrow), and this deposition is more marked at 5 days PCS as PCO progresses. Fibronectin (red), αSMA (green), and DNA detected by Draq5 (blue). Scale bar- 35 µm, C- lens capsule, LC- remnant lens cells. (B) Deletion of the fibronectin gene from the developing lens. Diagram of fibronectin gene locus showing the position of the loxP sites (left) and PCR results from DNA obtained from 9 week old control (wildtype-WT) and FNcKO lenses demonstrating successful deletion of the floxed fibronectin gene fragment in FNcKO lenses (right). (C) FNcKO lenses are morphologically similar to WT lenses. A dark field image showing that 9 week old WT (A) and FNcKO lenses (B) are both transparent; 200-mesh electron microscopy grid analysis of 12 week old WT (C) and FNcKO lenses (D) showing that fibronectin null lenses have refractive properties similar to WT; Hematoxylin and eosin (H&E) staining showing the anterior epithelium of 9 week old WT lens (E) and FNcKO lens (F); H&E staining showing the transition zone of a 9 week old WT lens (G) and FNcKO lens (H) showing that FNcKO lenses are structurally normal although FNcKO fibers may stain more intensely with Eosin than WT. Abbreviations: le -lens epithelium, f - lens fiber cells, tz - transition zone. Scale bar Panels A, B - 1.0mm; Panels C, D - 0.5 mm; Panels E, F, G, H – 150 µm.

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6.2.2 Fibronectin is essential for prolonged cell proliferation and fibrotic responses post cataract surgery (PCS), with fibronectin null lenses retaining epithelial characteristics, and undergoing unhindered fiber cell regeneration PCS

To test whether there is any change in the fibrotic response of lenses lacking the fibronectin gene (FNcKO), the expression of the common fibrotic marker α smooth muscle actin (αSMA) was determined at both early and late times PCS (Figure 6.2A). As expected, little to no αSMA protein was detected in cells associated with either WT or FNcKO capsular bags at 0 hour PCS. By 48 hours PCS, both WT and FNcKO remnant lens cells exhibited detectable, but low, levels of αSMA staining which was significantly elevated by 3 days PCS (WT, ***P < 0.001; FNcKO, ***P < 0.001). This fibrotic response is sustained until 5 days PCS in WT LCs (0 hour vs 4 days PCS, *P = 0.011; 0 hour vs 5 days PCS, *P = 0.013). In contrast, lens capsular bags from FNcKO mice have significantly fewer associated αSMA positive cells compared to WT by the fourth day PCS (***P < 0.001), and this reduction persists at 5 days PCS (***P < 0.001). Overall, FNcKO capsular bags exhibit a significant reduction in αSMA staining between 3 and 4 days PCS (***P < 0.001) which is also true at 5 days PCS (***P < 0.001) (Figure 6.2A). The significant reduction in αSMA protein levels in FNcKO LCs compared to WT at 5 days PCS was confirmed by flow cytometry of LCs isolated from dissected lens capsular bags (**P = 0.005) (Figure 6.2A and Supplemental Figure B1). Not only do FNcKO capsular bags have fewer αSMA positive cells than WT controls at 5 days PCS, but the overall size of the capsular plaque appeared smaller.

Next, we determined whether the qualitative reduction in plaque size observed in FNcKO eyes at 5 days PCS reflected differences in cell number by quantitating the

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number of cell nuclei associated with capsular bags at different times PCS and comparing that with the number of cells in the cell cycle as measured by staining for the proliferation marker, Ki67 (Figure 6.2A) which is present at all stages of the cell cycle except G0 (Scholzen and Gerdes 2000). At 0 hour PCS, remnant LCs exhibit little to no cell proliferation. However, a sharp increase in the Average Number of Nuclei (ANN)/section is seen at 48 hrs. PCS which becomes statistically significant at 3 days PCS for both WT (***P < 0.001) and FNcKO (***P < 0.001) (Figure 6.2A) capsular bags. This finding correlates with a significant upregulation of Ki67 staining in LCs between 0 hour PCS and 48 hours PCS in both WT and FNcKO LCs (***P < 0.001 for both) which is sustained at 3 days PCS (***P < 0.001 for both). However, while the average number of nuclei detected per section remains steady in WT eyes at 4 days PCS (P=0.717), it is significantly decreased in FNcKO capsular bags at (**P = 0.003) leading FNcKO capsular bags to have significantly fewer associated cell nuclei at 4 days PCS compared to WT (***P < 0.001). This phenomenon correlates with the significant attenuation of LC proliferation between 3 and 4 days PCS (**P = 0.007) that appears more pronounced in FNcKO LCs (***P < 0.001). At 5 days PCS, WT capsular bags have significantly more associated cell nuclei than 4 days PCS (***P < 0.001), while this was not seen in FNcKO capsular bags (P = 0.712), leading the FNcKO capsular bags to have significantly fewer associated cell nuclei than controls at 5 days PCS (***P < 0.001). However, quantification of the Ki67 staining did not reveal a statistically significant difference between WT and FNcKO capsular bags (4 days PCS, P = 0.308; 5 days PCS, P = 0.310) largely due to the small numbers of Ki67 positive cells associated with capsular bags after 3 days PCS leading to variability in the measurements. However, these data in aggregate suggest that fibronectin is

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essential for the long-term, but the not initial, fibrotic response of LCs to cataract surgery.

As fewer αSMA positive myofibroblastic cells were observed in FNcKO capsular bags compared to controls at later time PCS, we attempted to determine the fate of the αSMA expressing LCs that were detected in FNcKO capsular bags prior to 3 days PCS. First, we determined if these cells are lost by apoptosis as this has been seen in the lens under some pathological conditions such as TGFβ induced cataract (Maruno et al. 2002). However, staining with cleaved caspase 3, a marker of conventional apoptosis, did not reveal any apoptotic cell death in either WT or FNcKO LCs at any time PCS tested, while tissue samples known to exhibit apoptosis stained appropriately (data not shown) suggesting that the loss of αSMA positive cells from the FNcKO capsular bag after 3 days PCS was not caused by apoptotic cell death.

After cataract surgery, some remnant LCs are known to differentiate into structurally aberrant lens fiber cells which contribute to the development of "pearl-like" PCO when present in the visual axis, and Soemmering's ring when restricted to the ocular periphery (I. Michael Wormstone, Wang, and Liu 2009). Thus, it is possible that the myofibroblasts formed in FNcKO capsular bags may transdifferentiate into the lens fiber cells after 3 days PCS. Remnant LCs from both wildtype (WT) and FNcKO mice express little protein for either the transcription factor cMaf, which controls lens fiber cell differentiation (Cvekl and Zhang 2017) or aquaporin 0, a lens fiber cell preferred membrane protein (Sindhu Kumari et al. 2015), immediately PCS (Figure 6.2B and Supplemental Figure B2). By 48 hours PCS, some remnant LCs express cMaf and aquaporin 0 in both WT and FNcKO eyes, and the expression of these lens

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fiber cell markers become more robust by 5 days PCS (aquaporin 0, WT **P = 0.002; FNcKO *P = 0.032) suggesting that fiber cell differentiation is unhindered in FNcKO capsular bags (Figure 6.2B and Supplemental Figure B2).

Finally, we followed the expression of a classic epithelial cell marker, E cadherin (F. J. Lovicu et al. 2004), to determine if some of the myofibroblasts convert back to an epithelial phenotype upon the deletion of fibronectin PCS. As expected, both WT and FNcKO lens cells express appreciable amounts of E cadherin at 0 hour PCS (Figure 6.2B). However, by 48 hours PCS, E cadherin protein levels are significantly downregulated in both WT and FNcKO capsular bags (WT *P = 0.019; FNcKO ***P < 0.001). However, while this downregulation is sustained through 5 days PCS in WT lens cells (0 hour vs 5 days PCS **P = 0.004), E cadherin protein levels significantly upregulate in FNcKO capsular bags between 48 hours and 5 days PCS (**P = 0.003). This results in E cadherin levels being significantly higher in FNcKO LCs than WT controls at 5 days PCS (***P < 0.001) (Figure 6.2B). Overall, these data suggest that mesenchymal to epithelial transition (Wells, Yates, and Shepard 2008), perhaps associated with reductions in cell proliferation and increases in lens fiber cell differentiation, may lead to the observed lack of sustained fibrotic response in FNcKO capsular bags at 5 days PCS.

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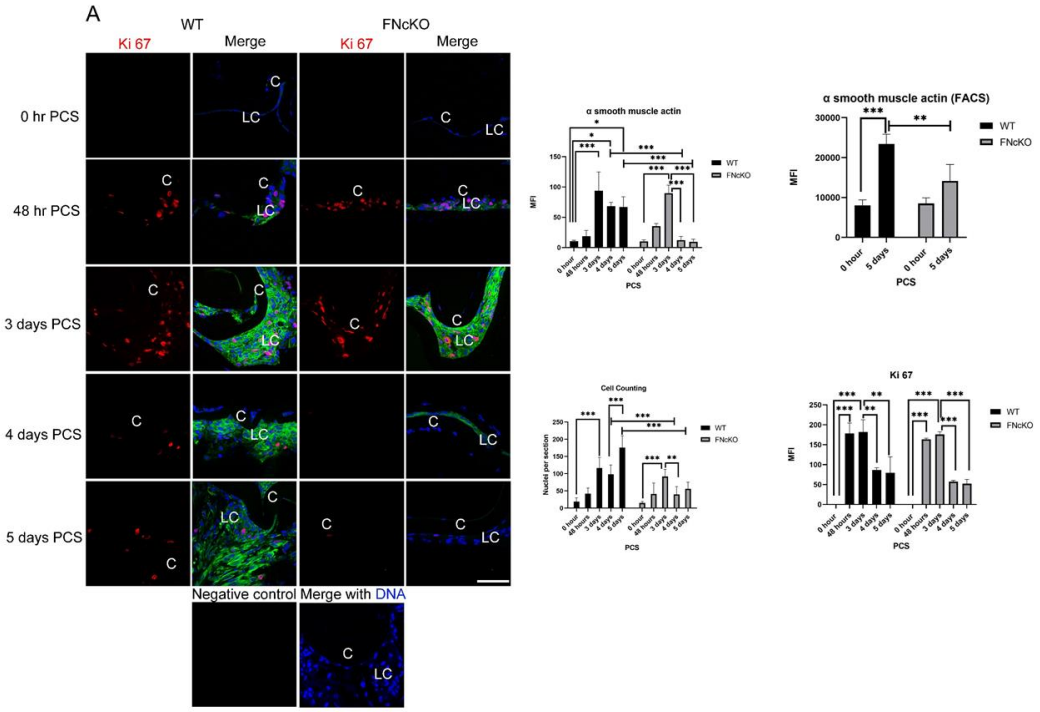

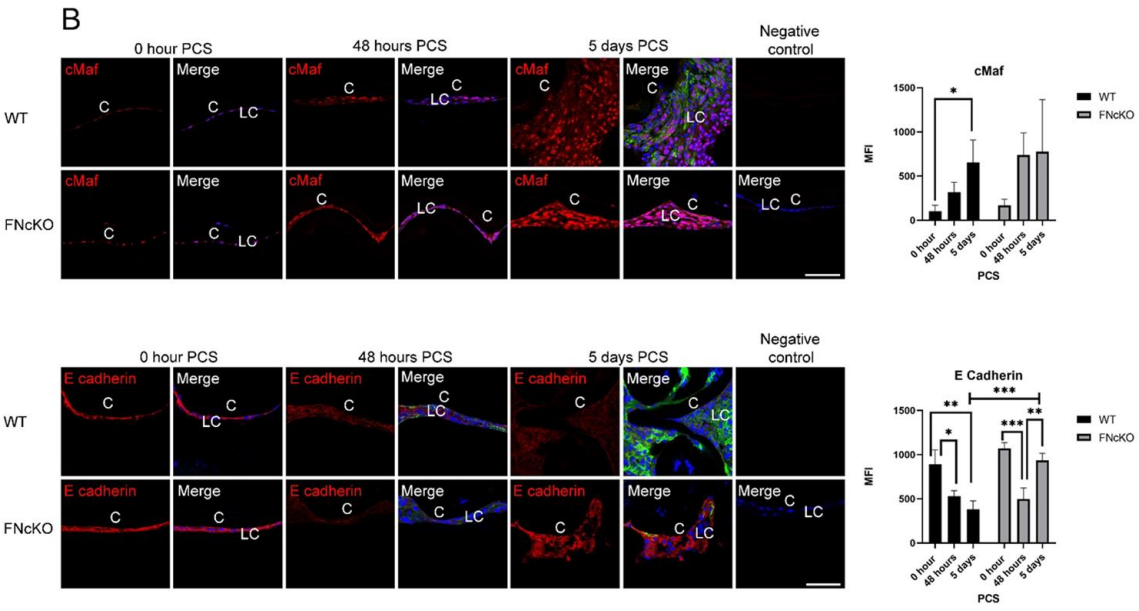

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Figure 6.2: The response of LCs lacking the fibronectin gene to lens fiber cell removal. (A) Neither WT nor FNcKO LCs express detectable levels of the proliferation marker Ki 67 or the common fibrotic marker α smooth muscle actin (αSMA) immediately PCS. By 48 hours PCS, appreciable numbers of Ki 67 positive LCs are detected in both WT and FNcKO capsular bags (WT ***P < 0.001; FNcKO ***P < 0.001), and this is sustained at 3 days PCS (WT ***P < 0.001; FNcKO ***P < 0.001). However, while the number of Ki 67 positive WT LCs was qualitatively attenuated by 4 days PCS, this effect is more prominent in FNcKOs which exhibit few to no Ki 67 positive LCs at either 4 or 5 days PCS [3 days vs 4 days PCS, WT **P = 0.007; FNcKO ***P < 0.001) (3 days vs 5 days PCS, WT **P = 0.004; FNcKO ***P < 0.001)] although mean fluorescence intensity (MFI) of Ki 67 staining determined by Image J is not statistically significant (T= 4 days PCS, P = 0.308; T= 5 days PCS, P = 0.310) between WT and FNcKO. A sharp increase in the average number of cell nuclei associated with capsular bags is seen at 48 hrs. which becomes statistically significant at 3 days PCS in both WT LCs (***P < 0.001) and FNcKO LCs (***P < 0.001). However, while the average number of nuclei associated with capsular bags is reduced in both WT (**P = 0.003) and FNcKO LCs beginning at 4 days PCS, this decrease is more pronounced in FNcKO LCs (***P < 0.001). At 5 days PCS, while WT capsular bags have an increase in the average number of nuclei detected compared to 4 days PCS (***P < 0.001), FNcKO capsular tissue fail to greatly expand the average number of nuclei between 4 and 5 days PCS (P = 0.712), leading 5 days PCS FNcKO capsular bags to have significant reductions in total nuclei count (***P < 0.001) compared to control. Similarly, both WT and FNcKO LCs begin expressing elevated amounts of αSMA protein by 48 hours PCS, and this becomes quite prominent by 3 days PCS (WT ***P < 0.001; FNcKO ***P < 0.001). However, while αSMA positive cells persist through 5 days PCS in WT capsular bags (*P = 0.013), few to no αSMA positive cells are detected in FNcKO capsular bags at either 4 days (***P < 0.001), or 5 days PCS (***P < 0.001). FACS analysis further supports the finding that FNcKO LCs express less αSMA protein at 5 days PCS (**P < 0.005) than controls. Ki 67 (red), αSMA (green), DNA detected by Draq5 (blue). Scale bars: 35 μm; LC, remnant lens cells; C, lens capsule. All experiments had and N = 3 except the cell counting analysis where N=6. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant changes between WT and FNcKO LCs at a time PCS or between two PCS time points. (B) Neither WT nor FNcKO LCs express detectable protein for the fiber cell marker cMaf immediately PCS, however, by 48 hours PCS, some of the remnant cells found in both WT and FNcKO capsular bags PCS are strongly cMaf positive which is maintained until 5 days PCS. Although the remnant cells of FNcKO qualitatively express more cMaf protein both at 48 hours and 5 days PCS compared to WT, this is not statistically significant (T= 48 hours PCS, P = 0.269; 5 days PCS P = 0.851). cMaf (red),

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DNA detected by Draq5 (blue). In contrast to cMaf, both WT and FNcKO lens cells express appreciable levels of E cadherin, an epithelial cell marker at 0 hour PCS. However, by 48 hours PCS, E cadherin protein levels are downregulated in both WT and FNcKO capsular bags (WT, *P = 0.019, FNcKO, ***P < 0.001) and this downregulation continues at 5 days PCS in WT lens cells (**P = 0.004). In contrast to WT, E cadherin protein levels upregulate in FNcKO capsular bags between 48 hours and 5 days PCS (***P < 0.001). All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between WT and FNcKO at a PCS or between two PCS time points.

6.2.3 RNAseq analysis revealed that WT LCs exhibit elevated mRNA levels for genes known to play roles in fibrosis and inflammation, and reduced expression of lens markers, at 48 hours PCS, while only a small subset of these expression differences is altered in FNcKO LCs

In order to elucidate the mechanisms by which fibronectin mediates the prolonged fibrotic response PCS, RNAseq was used as a global and unbiased approach to identify all genes whose expression levels change in WT lens epithelial cells (LCs) by 48 hrs. post cataract surgery- PCS (the time point when canonical TGFβ signaling is first detectable in LCs PCS) (Jiang et al. 2018), and which of those genes require fibronectin for their differential expression PCS (data deposited into the Gene Expression Omnibus (GEO) under accession number GSE119879). This analysis revealed that 2507 genes are expressed at significantly different levels in WT LCs at 48 hours PCS compared to 0 hours PCS (1569 genes upregulated, 938 genes downregulated) based on criteria that we have previously found to filter for biologically significant gene expression changes in lens cells (False Discovery Rate (FDR) ≤ 0.05; Fold Change (FC) in mRNA levels greater than 2; an absolute difference between group means > 2 RPKM (Reads Per Kilobase Million); expressed higher than 2 RPKM either immediately PCS or 48 hours later) (Manthey, Terrell, Lachke, et al. 2014). As expected, these differentially expressed genes (DEGs)

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included many fibrotic genes that are known to upregulate in LCs undergoing EMT (Table 6.1) as well as other genes known to be involved in fibrosis in other systems, but unreported or poorly described in PCO (Table 6.2). Further, consistent with our recent report describing gene expression changes observed in LCs at 24 hours PCS (Jiang et al. 2018), the most enriched biological pathway in WT LCs at 48 hours PCS identified by iPathway guide corresponds to cytokine-cytokine receptor interactions which included numerous known inflammatory proteins (Table 6.3), many of which are also upregulated at 24 hours PCS (Jiang et al. 2018). Finally, the expression of many genes important for lens structure and function downregulate in LCs by 48 hours PCS as well as would be expected in LCs undergoing EMT (Table 6.4).

Comparison of RNA expression profiles between WT and FNcKO LCs at 48 hours PCS revealed that the expression levels of 89 genes that meet the criteria for likely biological significance (False Discovery Rate (FDR) corrected p-value < 0.05, Fold Change (FC) ≥ 2, Reads Per Kilobase Million (RPKM) ≥ 2) were significantly different. Fifteen DEGs overlapped with the list of genes that were differentially expressed between unoperated WT and FNcKO lenses, leaving 74 DEGs differentially expressed in FNcKO lens cells at 48 hours PCS (Supplemental Table A3). Of these, 4 were genes that normally downregulate in WT LCs by 48 hours PCS but do not in FNcKO lenses, while 59 were genes that normally upregulate in remnant LCs whose upregulation was attenuated in FNcKO LCs (Supplemental Table A4, A5). Further, consistent with the muted fibrotic response that LCs from FNcKO lenses undergo PCS, the mRNA levels of several genes associated with fibrotic disease exhibit attenuated upregulation in FNcKO LCs at 48 hours PCS (Table 6.5), while another

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notable subset of attenuated DEGs plays known roles in inflammatory responses (Table 6.6) PCS.

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Table 6.1: Known markers of LC EMT upregulated in remnant LCs at 48 hours PCS.

Gene ID	**Gene description**	**Fold change (FC)**	**False discovery rate (FDR)**	**WT RPKM 0 hour**	**WT RPKM 48 hours**
Tnc	Tenascin C	116	7.8E-44	1	156
Col1a1	Collagen, type I, alpha 1	83	2.1E-42	0.82	79
MMP9	Matrix metallopeptidase 9	70	7.4E-14	0.47	40
Fn1	Fibronectin 1	53	1.3E-44	2	135
Tgfβi	TGFβ induced protein	42	5.7E-52	7	359
Itga5	Integrin alpha 5	7	5.1E-24	6	50
Acta2	Alpha smooth muscle actin	4	3.3E-08	74	380
Tgfβr2	Transforming growth factor, beta receptor II	3	3.4E-07	3	10
Tgfβ1	Transforming growth factor, beta 1	3	6.1E-08	32	99
Mylk	Myosin light chain kinase	3	0.001	1.75	7
Grem1	Gremlin-1	380	1.6E-40	1.4	642

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Table 6.2: Genes upregulated in LCs at 48 hours PCS that are known to be involved in fibrosis in other systems, but are unreported, or only poorly described, in PCO.

Gene ID	**Gene description**	**Fold change (FC) from WT 0 hour to WT 48 hours PCS in LCs**	**False discovery rate (FDR)**	**WT Mean RPKM 0 hour**	**WT Mean RPKM 48 hours**
Arg1	Arginase	411	8.2E-81	0.38	185
Spp1	Osteopontin	126	9.2E-76	3	461
ECM1	extracellular matrix protein 1	85	8.9E-68	4	425
Lox	Lysyl oxidase	44	5.0E-78	0.47	24
Thbs1	Thrombospondin 1	23	9.4E-22	3	85
Tagln	Transgelin	17	5.2E-44	6	119
Postn	Periostin	5	3.8E-10	3	19
Osmr	Oncostatin M Receptor	5	9.4E-18	5.5	30
Ltbp1	Latent transforming growth factor beta binding protein 1	3	1.894E-07	30	98

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Table 6.3: Genes known to be involved in inflammation are upregulated by LCs at 48 hours PCS.

Gene ID	**Gene description**	**Fold change (FC)**	**False discovery rate (FDR)**	**WT Mean RPKM 0 hour**	**WT Mean RPKM 48 hours**
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b	1587	8.0E-21	0	8
Cxcl1	Chemokine (C-X-C motif) ligand 1	1288	2.6E-19	0	20
S100a9	S100 calcium binding protein A9	643	1.8E-23	0.06	68
Cxcl3	Chemokine (C-X-C motif) ligand 3	213	6.7E-19	0.2	55
Igfbp3	Insulin-like growth factor binding protein 3	151	1.0E-102	0.45	80
Slfn4	Schlafen 4	110	1.7E-32	0.1	17
Ccl7	Chemokine (C-C motif) ligand 7	108	9.3E-44	0.4	56
S100a8	S100 calcium binding protein A8	102	4.8E-16	0.4	47
Lcn2	Lipocalin 2	60	1.9E-53	98	6715
Hmox1	Heme oxygenase 1	7	3.3E-08	15	123

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Table 6.4: Genes that are preferentially expressed in the lens that downregulate in LCs by 48 hours PCS.

Gene ID	Gene description	Fold change (FC)	False discovery rate (FDR)	WT Mean RPKM 0 hour	WT Mean RPKM 48 hours
Crygd	Crystallin, gamma D	-71	1.1E-06	306	5
Crygb	Crystallin, gamma B	-32	5.3E-05	650	24
Dnase2b	Deoxyribonuclease II beta	-24	1.6E-06	2.4	0.11
Crygc	Crystallin, gamma C	-20	1.5E-05	368	21
Cryba4	Crystallin, beta A4	-8	0.0002	2242	342
Bfsp1	Beaded filament structural protein 1	-7	8.3E-06	202	33
Mip	Major intrinsic protein of lens fiber	-7	7.9E-10	1264	215
Cryba1	Crystallin, beta A1	-6	3.9E-06	18405	3522
Lenep	Lens epithelial protein	-6	0.004	191	38
Crybb1	Crystallin, beta B1	-6	2.2E-05	1735	348
Crygs	Crystallin, gamma S	-5	6.7E-06	9090	2003
Cryba2	Crystallin, beta A2	-5	1.0E-05	5950	1415
Lim2	Lens intrinsic membrane protein 2	-5	1.3E-05	409	100
Crybb2	Crystallin, beta B2	-4	0.0004	31276	10027

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Table 6.5: Genes known to be involved in fibrosis are less upregulated in remnant LCs of FNcKOs at 48 hours PCS

Gene ID	**Gene description**	**Fold change (FC) from WT to FNcKO at 48 hours PCS in LCs (attenuated upregulation)**	**False discovery rate (FDR)**	**WT Mean RPKM At 48 hours**	**FNcKO Mean RPKM At 48 hours**
Grem1	Gremlin 1	-7	0.0001	642	87
Col1a1	Collagen, type I, alpha 1	-6.5	1.5E-10	79	11
Mylk	Myosin, light polypeptide kinase	-5.6	7.4E-06	7	1
Postn	Periostin	-4.6	2.7E-09	19	4
Lox	Lysyl oxidase	-4.5	2.1E-09	24	5

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Table 6.6: Genes known to be involved in inflammation are less upregulated by FNcKO LCs at 48 hours PCS.

Gene ID	**Gene description**	**Fold change (FC) from WT to FNcKO at 48 hours PCS in LCs (attenuated upregulation)**	**False discovery rate (FDR)**	**WT Mean RPKM At 48 hours**	**FNcKO Mean RPKM At 48 hours**
Serpina3f	Serine (or cysteine) peptidase inhibitor, clade A, member 3F	-157	1.3E-39	20	0.11
Serpina3m	Serine (or cysteine) peptidase inhibitor, clade A, member 3M	-94	4.1E-42	33	0.32
Serpina3c	Serine (or cysteine) peptidase inhibitor, clade A, member 3C	-60	2.9E-20	13	0.2
Serpina3h	Serine (or cysteine) peptidase inhibitor, clade A, member 3H	-14	1.4E-18	21	1.5
Lbp	Lipopolysaccharide binding protein	-11	2.4E-15	16	1.4
Slfn4	Schlafen 4	-7	1.1E-07	17	2.3
Crlf1	Cytokine receptor-like factor 1	-3	0.008	26	8
Slfn5	Schlafen 5	-3	0.01	5	1.5

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6.2.4 Fibronectin is required for the expression and assembly of a subset of fibrotic ECM molecules produced by lens cells undergoing EMT post cataract surgery

EMT of LCs produces myofibroblasts that synthesize a "fibrotic" extracellular matrix (ECM) which provides a scaffold for cell attachment, stiffens the tissue, and contributes to the light scatter caused by fibrotic PCO (I. Michael Wormstone, Wang, and Liu 2009). In other tissues/cell types, fibronectin is the initial scaffold that allows for fibrotic extracellular matrix (ECM) assembly (Yamada and Clark 1988; Barker and Engler 2017); however, its role in the formation of the fibrotic matrix associated with PCO has not been described. Notably, intact adult FNcKO lenses exhibit a 2 fold increase in the expression of the Col1a1 and Col1a2 genes (Supplemental Table A3) which encode the “pro-fibrotic” matrix molecule, collagen I (Hosper et al. 2013). However, at 48 hours PCS, Col1a1 levels upregulate over 80 fold in WT LCs compared to time 0 (Table 6.1), while 48 hour PCS FNcKO LCs express significantly lower levels of Col1a1 mRNA compared to WT (Table 6.5). Similarly, the mRNA encoding lysyl oxidase (Lox), an enzyme required for collagen I cross-linking (Trackman 2016), upregulates over 40 fold in WT LCs by 48 hours PCS (Table 6.2), while this response is also attenuated in FNcKO LCs (Table 6.5). Consistent with the RNAseq data, we have found that while FNcKO LCs produce collagen I and Lox proteins at 48 hours PCS, their levels are significantly attenuated compared to WT LCs (collagen I **$P = 0.009$; Lox ***$P < 0.001$). This result is more dramatic at 5 days PCS as FNcKO LCs are not associated with collagen I fibrils at this time (WT vs FNcKO ***$P < 0.001$), while the levels of Lox protein are still attenuated (WT vs FNcKO ***$P < 0.001$) (Figure 6.3). Overall, this suggests that cellular fibronectin produced by LCs PCS is required not just as a scaffold for collagen I assembly, but

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also triggers signal transduction cascades that regulate genes required for collagen I and Lox production during EMT.

In contrast, while RNAseq analysis reveals that the mRNA levels for tenascin C, which encodes another common fibrotic ECM protein (Mamuya et al. 2014; Jones and Jones 2000) upregulate 116 fold at 48 hours PCS in WT capsular bags, this gene still upregulates to a similar level in FNcKO capsular bags, suggesting that tenascin C gene expression is not under the control of fibronectin-induced signaling. However, as tenascin C also has been reported to be dependent on fibronectin for its incorporation into ECM (Singh, Carraher, and Schwarzbauer 2010) and colocalizes with cell-associated fibronectin PCS (Supplemental Figure- B3), we investigated the fate of tenascin C fibril formation PCS in the absence of fibronectin. While tenascin C mRNA levels increase in FNcKO LCs similar to wildtype at 48 hours PCS, tenascin C protein fibril deposition around FNcKO LCs is significantly reduced at both 48 hours (***$P < 0.001$) and 5 days PCS (***$P < 0.001$) compared to WT LCs (Figure 6.3) suggesting that fibronectin regulates tenascin C fibril formation but not tenascin C gene expression PCS.

Interestingly, thrombospondin-1 and extracellular matrix protein 1 are two other fibrotic extracellular matrix proteins (Murphy-Ullrich and Suto 2018; H. Chen, Jia, and Li 2016) whose mRNAs upregulate similarly in WT and FNcKO LCs PCS. While the matrix deposition of both is also proposed to be under fibronectin regulation (Tan and Lawler 2009; Sercu et al. 2008), both are still deposited around FNcKO LCs at 5 days PCS in a pattern similar to that seen in WT LCs (Supplemental Figure B4) although quantitation shows that significantly less thrombospondin is deposited around FNcKO LCs at 5 days PCS (**$P = 0.003$) while ECM1 deposition is

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unaffected in FNcKO lenses (P = 0.925). This suggests that fibronectin is essential for the deposition of only a subset of fibrotic ECM molecules PCS.

In aggregate, these data suggest that fibronectin is both critical for the assembly of the fibrotic ECM during PCO, as well as the activation of signal transduction cascades that elevate the expression of some fibrotic ECM genes in LCs PCS.

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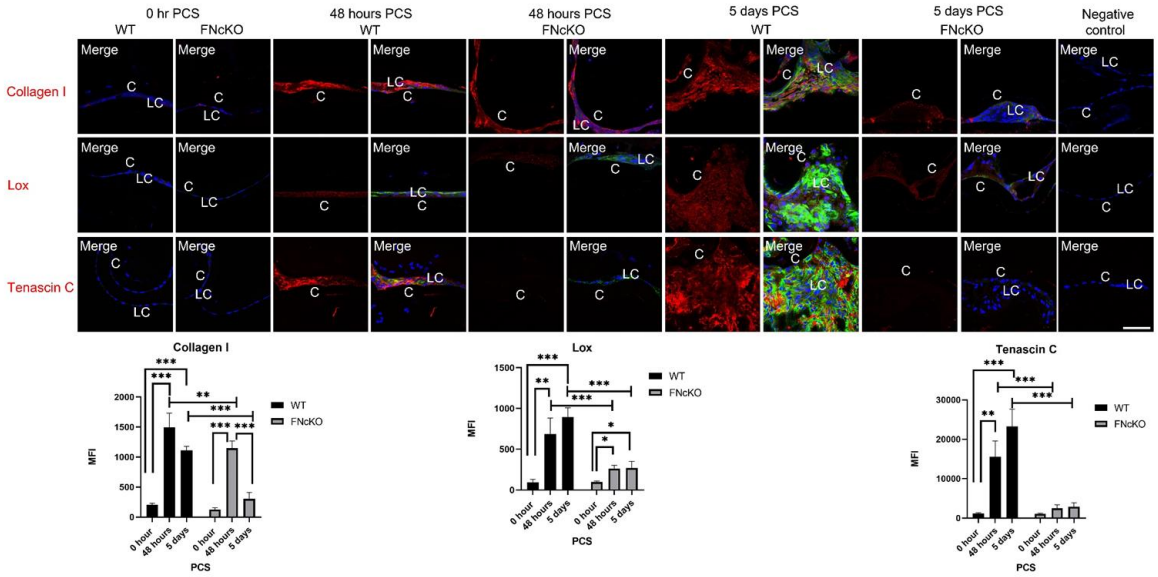

Figure 6.3: The production and assembly of fibrotic ECM PCS require fibronectin expression by LCs. At 0 hours PCS, only low levels of fibrotic ECM proteins (collagen I and tenascin C) and the enzymatic ECM crosslinker Lysyl oxidase (Lox) are detected in both WT and FNcKO LCs. Both WT and FNcKO LCs significantly upregulate collagen I protein expression by 48 hours PCS (WT ***P < 0.001; FNcKO ***P < 0.001) although FNcKO LCs exhibit less association with collagen I fibrils compared to WT (**P < 0.009). By 5 days PCS, WT LCs expressing αSMA are associated with a robust matrix of collagen I (***P < 0.001) while this is absent in the area surrounding FNcKO lens cells (WT vs FNcKO ***P < 0.001). Similarly, Lox protein upregulates in both WT and FNcKO LCs at 48 hours PCS (WT **P = 0.004; FNcKO *P = 0.020) while this signal is much less pronounced in FNcKO LCs compared to WT (***P < 0.001). There is a significant increase in tenascin C fibrils surrounding WT LCs both at 48 hours (**P = 0.005) and 5 days (***P < 0.001) PCS compared to 0 hour PCS. However, FNcKO lens cells are associated with significantly less tenascin C fibrils both at 48 hours PCS (***P < 0.001) and 5 days PCS (***P < 0.001) compared to WT. Collagen I, Tenascin C, and Lox (red), αSMA (green), are merged with DNA detected by Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule. All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between WT and FNcKO at a PCS or between two PCS time points.

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6.2.5 Deletion of fibronectin from the lens alters integrin expression and downstream signaling PCS

Notably, LCs elevate the protein expression of the fibronectin receptors α5β1-integrin and several αV class integrins PCS (Walker and Menko 2009; Mamuya et al. 2014; de Iongh et al. 2005). At 48 hours PCS, WT LCs upregulate the protein expression of α5 integrin (**P = 0.005), αV integrin (P = 0.070) and β1- integrin (P < 0.001) , and this upregulation remains robust at 5 days PCS (α5 integrin **P = 0.008; αV integrin **P = 0.001; β1 integrin ***P < 0.001) (Figure 6.4). However, compared to WT LCs, α5 and β1- integrin protein levels fail to upregulate in FNcKO LCs at either 48 hours (α5- integrin ***P < 0.001; β1-integrin, ***P < 0.001) or 5 days PCS (α5- integrin, ***P < 0.001; β1-integrin, ***P < 0.001). In contrast, FNcKO LCs still upregulate the expression of the αV-integrin subunit by 48 hours PCS (**P = 0.004) at levels similar to WT (P = 0.168). However, αV integrin levels are significantly attenuated in FNcKO LCs at 5 days PCS (***P < 0.001) compared to WT LCs (Figure 6.4). These data are suggesting that fibronectin expression in LCs is necessary for the upregulation of its integrin receptors PCS.

Since integrin expression fails to upregulate in FNcKO capsular bags PCS and the assembly of ECM around LCs is altered PCS (see Figure 6.3), next we sought to determine the levels of phosphorylated focal adhesion kinase (pFAK) which is the activated form of an important signaling molecule that transmits integrin signals (Kokkinos, Brown, and de Iongh 2007). Although pFAK levels upregulate to a similar extent in WT and FNcKO LCs at 48 hours PCS (P = .576), WT αSMA expressing LCs sustain elevated pFAK levels at 5 days PCS (*P = 0.022), while pFAK levels are significantly lower in FNcKO LCs compared to controls (*P = 0.013) at this time

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(Figure 6.4). These data show that fibronectin expression is required for LCs to sustain FAK activation post cataract surgery.

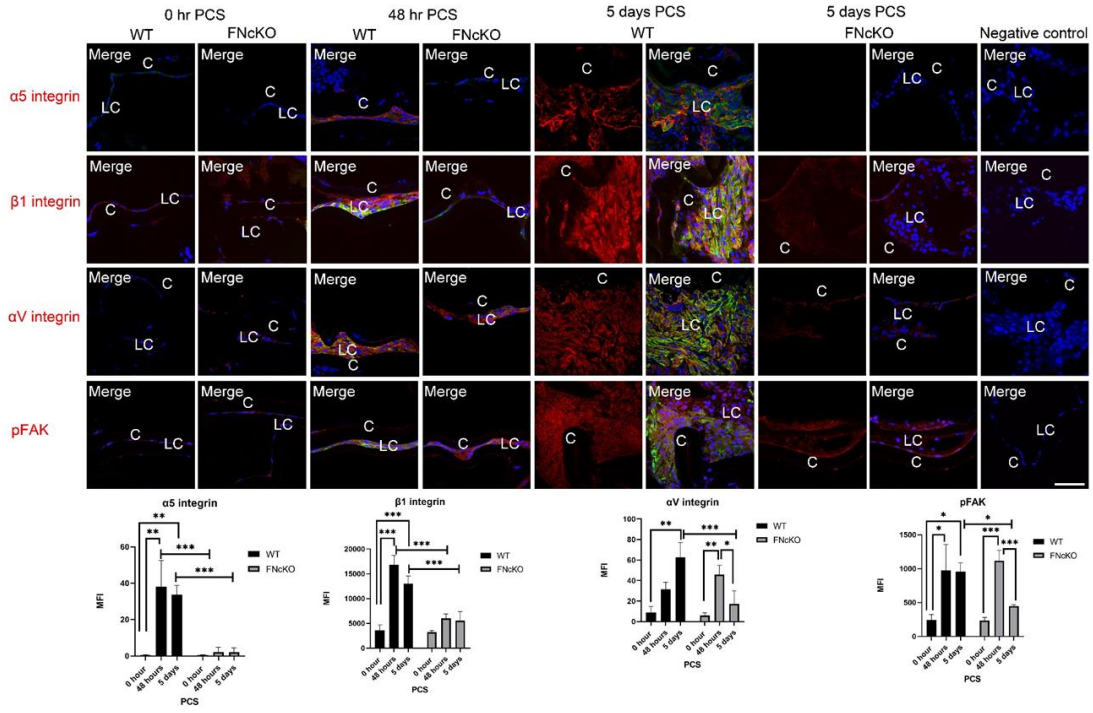

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Figure 6.4: Fibronectin expression by LCs is necessary for the upregulation of some integrin subunits and integrin signaling PCS. At 0 hour PCS, both WT and FNcKO LCs exhibit low protein expression for all three integrins (α5- integrin, β1- integrin, and αV-integrin) while pFAK levels are also low. However, by 48 hours PCS, WT LCs significantly upregulate the protein levels of α5 integrin (**P = 0.005) and β1 integrin (***P <0.001) while the upregulation of αV integrin did not reach 95% confidence of upregulation. (P = 0.070). However, the expression of all three proteins becomes quite robust by 5 days PCS (α5 integrin **P = 0.008; αV integrin **P = 0.001; β1 integrin ***P < 0.001). Concomitant with the detected elevation in integrin expression, pFAK levels are significantly elevated in WT LCs by 5 days PCS (*P = 0.022). However, FNcKO LCs fail to upregulate α5- and β1-integrin protein levels at both 48 hours and five days PCS compared to WT leading FNcKO LCs to have significantly less integrin staining than control at 48 hours(α5- integrin ***P < 0.001; β1-integrin, ***P < 0.001) and 5 days PCS (α5- integrin ***P < 0.001; β1-integrin ***P < 0.001) compared to WT. In contrast, FNcKO LCs also initially upregulate αV-integrin (**P = 0.004) and pFAK levels (**P < 0.004) at 48 hours PCS, at levels not significantly different from WT 48 hours (αV-integrin, P = 0.168; pFAK P = 0.576). Notably, this is not sustained as αV- integrin and pFAK upregulation is attenuated at 5 days PCS (αV- integrin ***P < 0.001; pFAK *P = 0.013) compared to WT. α5-integrin, β1- integrin, αV integrin, and pFAK (red) are merged with αSMA (green) and DNA detected by Draq5 (blue). Scale bars: 35 μm; LC, remnant lens epithelial cells/lens cells; C, lens capsule. All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate a statistically significant difference in MFI between WT and FNcKO at a time PCS or between two PCS time points.

6.2.6 Late PCS elevations in TGFβ signaling are attenuated in FNcKO LCs

As it is established that transforming growth factor beta (TGFβ) signaling is critical for sustained fibrotic PCO (de Iongh et al. 2005), and fibronectin plays a role in the regulation of the latent TGFβ complex in other systems (Robertson and Rifkin 2016; Griggs et al. 2017), we next determined the extent of canonical TGFβ pathway activation in WT and FNcKO LCs PCS by following pSMAD2/3 levels. Activation of TGFβ signaling is seen both in WT and FNcKO lens cells at 48 hours PCS (WT *P = 0.037; FNcKO *P = 0.014) and this was not significantly different between WT and

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FNcKO LCs (P = 0.216) (Figure 6.5) which supports the idea that fibronectin is not a major driver of the early fibrotic response PCS (Figure 6.2A). However, while WT LCs exhibit enhanced activation of canonical TGFβ signaling at 5 days PCS (***P < 0.001), pSMAD2/3 is barely detected in FNcKO LCs at 5 days PCS which is significantly different from WT (***P < 0.001) suggesting that the upregulation of fibronectin by LCs is required for sustained TGFβ signaling PCS (Figure 6.5).

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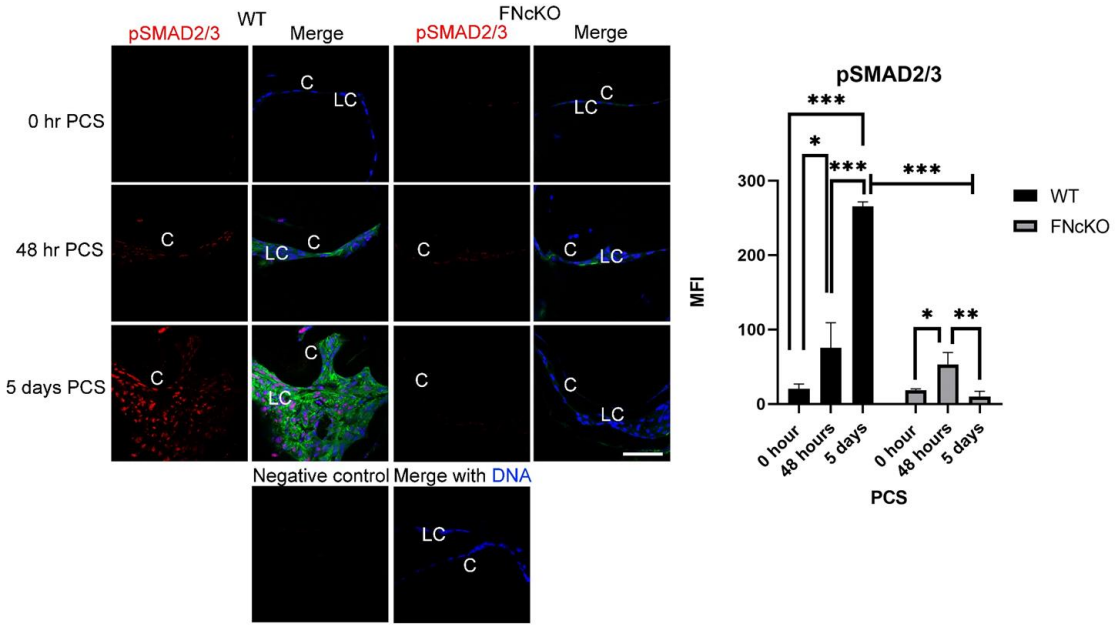

Figure 6.5: TGFβ signaling is attenuated in FNcKO lens cells at later times PCS. At 0 hour PCS, pSMAD2/3 is not detected in either WT or FNcKO LCs. However, at 48 hours PCS, pSMAD2/3 is first detected in WT LCs (*P = 0.037) which becomes robust at 5 days PCS (***P < 0.001). FNcKO LCs also upregulate pSMAD2/3 levels at 48 hours PCS (*P = 0.014) at levels quantitatively similar levels to WT (P = 0.216), while these levels do not continue to upregulate 5 days PCS and are significantly reduced (***P < 0.001) compared to WT. pSMAD2/3 (downstream effector of canonical TGFβ signaling) (red), αSMA (green), and DNA detected by Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule. All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate a statistically significant difference in MFI between WT and FNcKO at a time PCS or between two PCS time points

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6.2.7 Extracellular matrix deposition of the latent TGFβ complex around LCs PCS is dependent on fibronectin

Latent TGFβ is secreted from cells bound to latent TGFβ binding proteins (LTBPs) and is incorporated into the extracellular matrix prior to the activation needed for TGFβ to initiate signaling transduction upon injury (Robertson and Rifkin 2016; Hayashi and Sakai 2012). In other systems, fibronectin binds LTBPs directly or indirectly to tether the latent TGFβ to the ECM (Todorovic and Rifkin 2012; Rifkin, Rifkin, and Zilberberg 2018). Out of four LTBPs, LTBP1-3 are all abundantly expressed in adult LCs at the mRNA level (30, 83, and 70 RPKM, respectively). Notably, the mRNA for LTBP1, which uniquely binds to cell-associated fibronectin (Rifkin, Rifkin, and Zilberberg 2018), upregulates 3 fold in WT LCs by 48 hours PCS. (Table 6.2). Consistent with this, immunolocalization found that significant LTPB1 protein was associated with fibronectin deposits surrounding αSMA positive LCs at 5 days PCS (Figure 6.6A, B). In contrast, this LTBP1 deposition was significantly reduced around FNcKO LCs at 5 days PCS (Figure 6.6B) compared to WT (**P = 0.006) suggesting that fibronectin influences TGFβ signaling in LCs PCS, at least in part, at the level of matrix deposition of the latent TGFβ complex.

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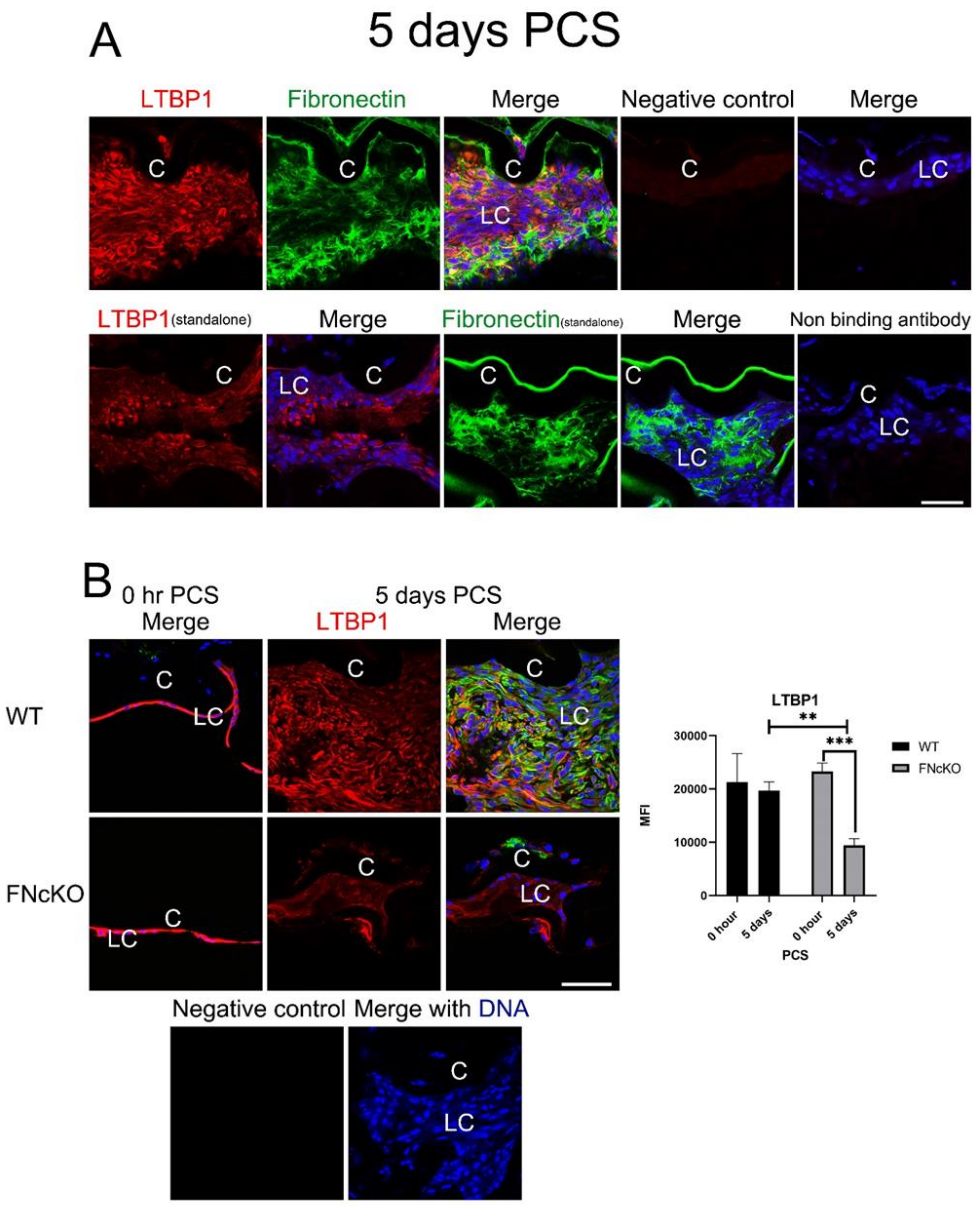

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Figure 6.6: LCs are associated with latent TGFβ binding protein at 5 days PCS, and this is highly attenuated in FNcKO LCs. (A) At 5 days PCS, WT LCs are associated with robust levels of cell-associated fibronectin and LTBP1. Fibronectin (green) and LTBP1 (red) merged with DNA detected by Draq5 (blue). (B) At 0 hours PCS, appreciable levels of LTBP1 protein are detected in both WT and FNcKO LCs whereas αSMA protein levels are low. However, by 5 days PCS, WT LCs maintain the robust levels of ECM-associated LTBP1 whereas extracellular deposition of LTBP1 around FNcKO LCs is greatly attenuated (**P = 0.006) compared to WT and is even reduced compared to 0 hours PCS (***P < 0.001). αSMA (green) and LTBP1 (red) merged with DNA detected by Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule. All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate a statistically significant difference in MFI between WT and FNcKO at a time PCS or between two PCS time points.

6.2.8 The attenuation of canonical BMP signaling in LCs PCS requires fibronectin

Canonical Bone Morphogenetic Protein (BMP) signaling is required for normal lens development (Faber et al. 2002; J. Huang et al. 2015; Boswell and Musil 2015), while it has been proposed that BMP signaling can counterbalance TGFβ signaling in fibrotic diseases (Brazil et al. 2015). Intact adult lenses have easily detectable levels of pSMAD1/5/8 (Supplemental Figure B5) in the lens epithelium, and this is not affected by the deletion of the fibronectin gene (Figure 6.7). After surgery, remnant LCs from WT mice significantly downregulate pSMAD1/5/8 signaling by 24 hours PCS (*P = 0.013), while both qualitatively and quantitatively, more FNcKO than WT LCs retain pSMAD1/5/8 at this time (P = 0.390). pSMAD1/5/8 levels continue to fall in WT LCs through 48 hours PCS (**P = 0.006) and by 5 days PCS, pSMAD1/5/8 staining is largely absent from WT LCs (0 hour vs 5 days PCS; (**P = 0.004). In contrast, pSMAD1/5/8 staining remains prominent in FNcKO LCs at all times PCS investigated, and is significantly elevated at 5 days PCS

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compared to WT (**P = 0.003) (Figure 6.7) suggesting that fibronectin is necessary for the sustained suppression of canonical BMP signaling in LCs PCS.

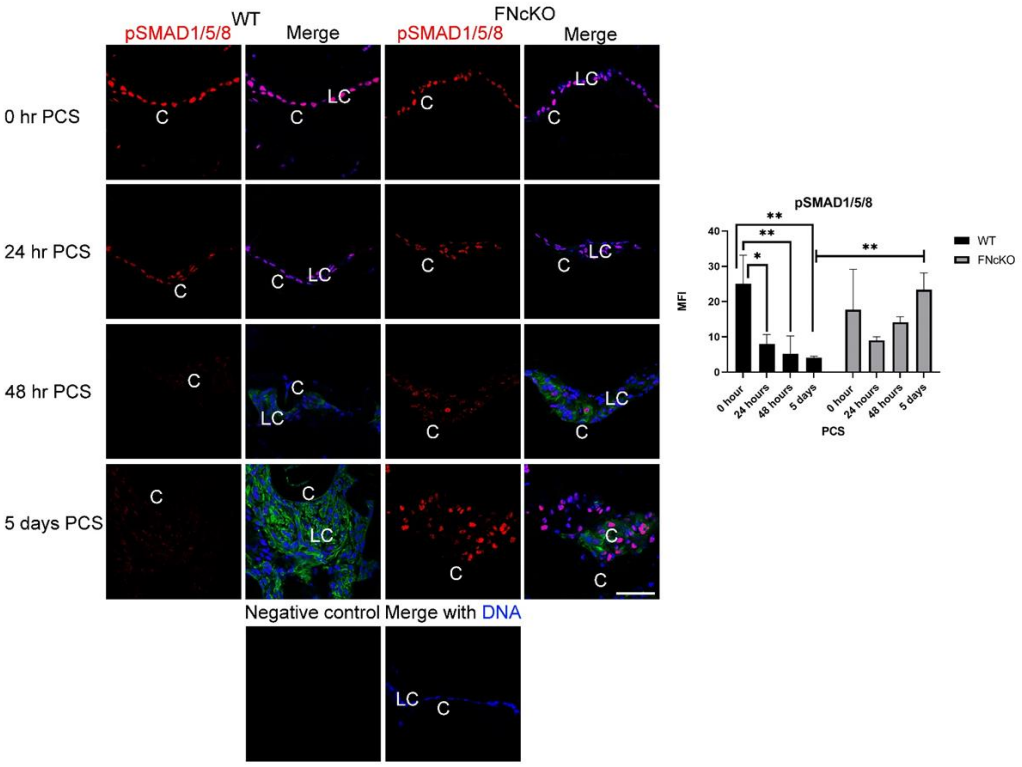

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Figure 6.7: The dynamics of BMP signaling in PCS LCs upon the deletion of the fibronectin gene. Immediately following lens fiber cell removal, both WT and FNcKO remnant lens cells stain robustly for pSMAD1/5/8, while this signaling begins to decrease at 24 hours PCS in WT LCs (P = 0.013) although this does not occur in FNcKO LCs (P = 0. 390). pSMAD1/5/8 levels continue to downregulate in WT LCs at 48 hours PCS (**P = 0.006), and this reduction of pSMAD1/5/8 levels persists through 5 days PCS in WT LCs expressing αSMA (**P = 0.004). In contrast, pSMAD1/5/8 levels never significantly downregulate in FNcKO LCs, so they have elevated levels of pSMAD1/5/8 at 5 days PCS (**P = .003) compared to WT LCs and do not express elevated levels of αSMA. pSMAD1/5/8 (downstream of BMP signaling) (red), αSMA (green), and DNA detected by Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule. All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate a statistically significant change in MFI between WT and FNcKO at a PCS or between two PCS time points.

6.2.9 Fibronectin production by LCs is required for the upregulation of gremlin-1 expression PCS

In order to obtain further mechanistic insight into the function of fibronectin PCS, we investigated the list of genes differentially expressed at the mRNA level in FNcKO LCs at 48 hours PCS for those with the potential to mechanistically regulate BMP and TGFβ signaling. Gremlin-1, a secreted BMP antagonist (Brazil et al. 2015) and profibrotic factor (Staloch et al. 2015; McDowell et al. 2015; G. Li et al. 2013), is upregulated 379 fold in WT LCs at 48 hours PCS and this upregulation was attenuated 7 fold in FNcKO LCs (Table 6.5). As gremlin-1 has been reported to regulate TGFβ signaling in different fibrotic conditions (Ma et al. 2019; Staloch et al. 2015; McDowell et al. 2015; G. Li et al. 2013; Church et al. 2017), we sought to determine the expression dynamics of gremlin-1 at the protein level PCS. As expected, based on the RNAseq data, no gremlin-1 protein was detected in either WT or FNcKO LCs immediately following surgery. Consistent with the upregulation of gremlin-1 detected at the mRNA level, WT LCs begin to express detectable levels of gremlin-1 protein by

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24 hours PCS (***P < 0.001), and this upregulation is also seen in FNcKO LCs (*P = 0.015). WT LCs continue to upregulate gremlin-1 protein levels through 48 hours PCS (***P < 0.001) and these levels remain quite high through 5 days PCS (***P < 0.001). In contrast, while gremlin-1 protein levels also continue to elevate in FNcKO LCs at 48 hours PCS (***P < 0.001), gremlin-1 levels are significantly lower than seen in WT 48 hours PCS (**P = 0.004) consistent with the RNAseq results (Table 6.5), resulting in FNcKO LCs exhibiting greatly reduced gremlin 1 staining compared to WT at 5 days PCS (***P < 0.001) (Figure 6.8A).

Since gremlin-1 can function as both an antagonist of BMP signaling (Brazil et al. 2015; Ma et al. 2019) and an agonist of canonical TGFβ signaling (McDowell et al. 2015; G. Li et al. 2013; Staloch et al. 2015), we next investigated if exogenous administration of gremlin-1 can rescue the defects in the fibrotic response and alterations in BMP and TGFβ signaling observed in FNcKO LCs PCS. Notably, exogenous gremlin-1 restored the ability of FNcKO LCs to upregulate the fibrotic marker αSMA (*P = 0.015) and deposit the fibrotic ECM proteins tenascin C (**P = 0.002) and collagen I (*P = 0.017) at 5 days PCS (Figure 6.8B) suggesting that the attenuation of gremlin-1 expression in FNcKO LCs plays a major role in the FNcKO phenotype. Thus, the effect of exogenous gremlin-1 on TGFβ and BMP signaling in FNcKO LCs was then determined. Consistent with the restoration of αSMA upregulation and collagen I/tenascin C distribution, FNcKO LCs treated with gremlin-1 exhibited robust pSMAD2/3 immunostaining at 5 days PCS compared to untreated FNcKO LCs (*P = 0.013) suggesting that gremlin-1 is working via its effects on the TGFβ pathway. Surprisingly though, in light of literature defining gremlin-1 as a BMP antagonist (G. Li et al. 2013; McDowell et al. 2015), gremlin-1 treated FNcKO LCs

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still exhibited sustained BMP signaling at 5 days PCS (P = 0.440), suggesting that gremlin-1 was largely acting via its effects on the TGFβ pathway (Figure 6.8B).

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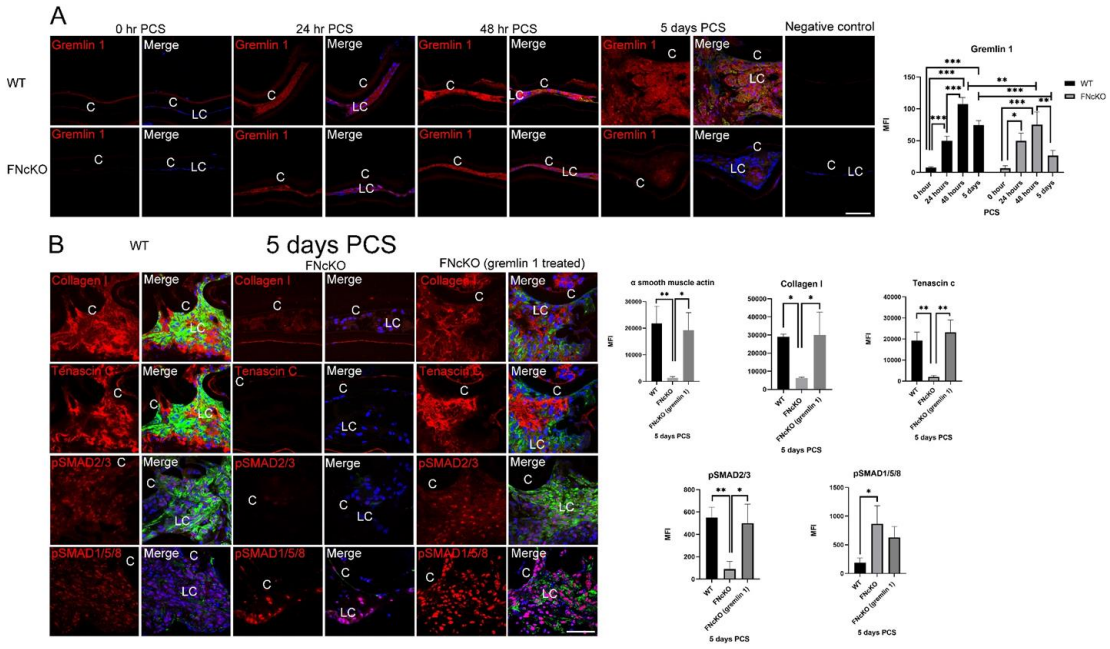

Figure 6.8: Exogenous gremlin-1 treatment of FNcKO capsular bags rescues the defect in TGFβ signaling and fibrotic marker expression PCS. (A) At 0 hour PCS, little expression of the gremlin-1 protein is detected in both WT and FNcKO LCs. However, by 48 hours PCS, gremlin-1 protein expression is elevated in both WT (***P < 0.001) and FNcKO LCs (***P < 0.001) although the expression is significantly less in FNcKO LCs (**P = .004) compared to WT. In contrast, gremlin-1 levels are greatly attenuated in FNcKO LCs by 5 days PCS (***P <.001) compared to WT whereas WT LCs maintain the robust expression of gremlin-1(***P < 0.001). Gremlin-1 (red) is merged with αSMA (green) and DNA detected by Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule. All experiments had N = 3. Values are expressed as mean + SEM. Asterisks (*) indicate statistically significant MFI between WT and FNcKO at a PCS or between two PCS time points. (B) Administration of exogenous gremlin-1 to FNcKO capsular bags elevates the levels of the fibrotic proteins αSMA (*P = 0.015), tenascin C (**P = 0.002), and collagen I (*P = 0.017) concomitant with elevated levels of pSMAD2/3 levels (*P = 0.013) at 5 days PCS. In contrast, exogenous gremlin-1 treatment did not reduce pSMAD1/5/8 levels in FNcKO capsular bags (P = 0.440). Collagen I, Tenascin C, pSMAD2/3 (downstream of TGFβ signaling), pSMAD1/5/8 (downstream of BMP signaling) (red), αSMA (green) and DNA detected by Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule. All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between WT and/or FNcKO and/or FNcKO (gremlin-1) at 5 days PCS.

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6.2.10 Fibronectin mediates sustained fibrotic PCO via TGFβ dependent pathway

Exogenous treatment of FNcKO lens capsular bags with gremlin-1 can rescue many aspects of the FNcKO phenotype including the defect in canonical TGFβ signaling as measured by pSMAD2/3 levels. As active TGFβ induces lens cells to convert to myofibroblasts (de Iongh et al. 2005), we then determined whether exogenous active TGFβ could also rescue the FNcKO phenotype. We found that active TGFβ1 treated FNcKO capsular bags show robust activation of pSMAD2/3 at 5 days PCS (***P < 0.001) as well as robust expression of the fibrotic markers αSMA (**P = 0.001) and collagen I (*P = 0.034), and the profibrotic factor gremlin-1(**P = 0.004) 5 days PCS (Figure 6.9A). Interestingly, like gremlin-1, active TGFβ1 treated FNcKO LCs still retain elevated pSMAD1/5/8 levels at 5 days PCS (P = 0.286) (Figure 6.9A) suggesting that TGFβ signaling may not inhibit BMP signaling in LCs.

Exogenous active TGFβ1 treatment was also able to induce the expression α5 –integrin (***P < 0.001), β1- integrin (**P = 0.001) and αV -integrin (*P = 0.018) in FNcKO LCs by 5 days PCS, consistent with previously described feedforward mechanisms between integrins and TGFβ signaling (Margadant and Sonnenberg 2010). These upregulated integrins are likely engaging with their ligands as pFAK levels are also increased in active TGFβ1 treated FNcKO LCs at 5 days PCS (*P = 0.025) compared to untreated capsular bags (Figure 6.9B); In contrast, active TGFβ1 treatment did not rescue the defect in tenascin C deposition observed in FNcKO LCs (P = 0.979) (Figure 6.9A) while exogenous gremlin-1 treatment did (Figure 6.8B) suggesting that gremlin-1 and TGFβ1 are not fully redundant. This is supported by the observation that gremlin-1 is more potent in rescuing the defect in periostin deposition observed in FNcKO capsular bags than TGFβ1 (Supplemental Figure B6). Notably,

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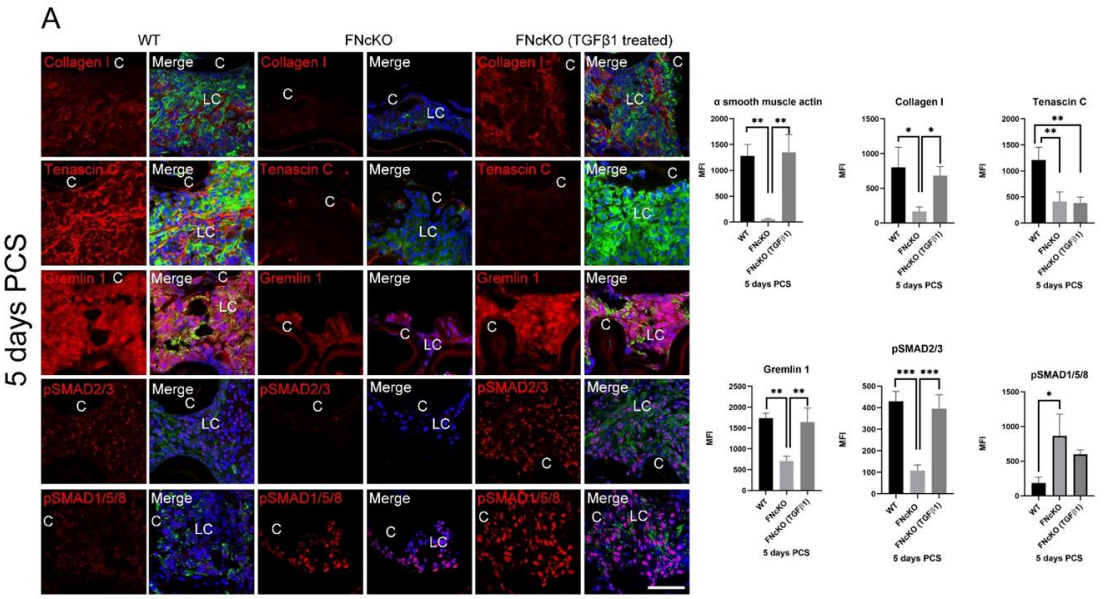
the precocious elevation of either the fibrotic response or activation of TGFβ signaling was not detected at 24 hours PCS after treatment of WT capsular bags with either exogenous active TGFβ1 or gremlin-1 PCS (Supplemental Figure B7).



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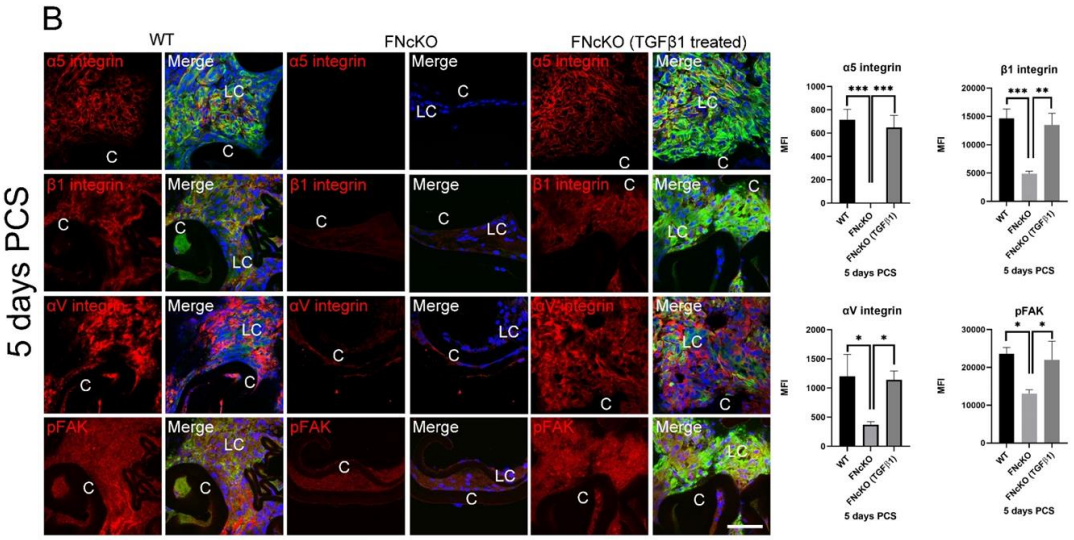

Figure 6.9: Treatment of FNcKO LCs with exogenous active TGFβ1 restores the fibrotic response. (A) Active TGFβ1 treated FNcKO capsular bags exhibit robust pSMAD2/3 levels (a measure of active TGFβ signaling) (***P < 0.001), as well as robust expression of the fibrotic markers αSMA (**P = 0.001) and collagen I (**P = 0.034) along with the profibrotic factor gremlin- (**P = 0.004) at 5 days PCS. In contrast, tenascin C deposition is not increased in FNcKO capsular bags after TGFβ1 treatment (P = 0.979) and the robust pSMAD1/5/8 levels indicative of active BMP signaling are also not affected at 5 days PCS (P = 0.286). (B) Treatment of FNcKO capsular bags with exogenous active TGFβ1 induces the upregulation of α5- integrin(***P < 0.001), β1- integrin (**P = 0.001), and αV- integrin expression (*P = 0.018), as well as pFAK levels (*P = 0.025), in FNcKO LCs at 5 days PCS. Collagen I, tenascin C, gremlin-1, pSMAD2/3 (downstream of TGFβ signaling), pSMAD1/5/8 (downstream of BMP signaling), α5 -integrin, β1 -integrin, αV- integrin, and pFAK (red) merged with αSMA (green) and DNA detected either by Draq5 or DAPI (blue). Scale bars: 35 μm; C, lens capsule; LC, remnant lens epithelial cells. All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate a statistically significant difference in MFI between WT and/or FNcKO and/or FNcKO (TGFβ1) at 5 days PCS.

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6.2.11 Fibronectin fibrils are detected in FNcKO capsular bags upon addition of active TGFβ1 and gremlin-1 PCS

In our study, exogenous addition of TGFβ1 and gremlin-1 rescue the sustained fibrotic response in FNcKO capsular bags PCS (Figure 6.8B and 6.9) including the deposition of (at least some) fibrotic ECM proteins and fibronectin binding integrins. Notably, a recent study has suggested that plasma fibronectin, which is abundant in aqueous humor (Vesaluoma et al. 1998) plays a critical role in sustained fibrotic PCO by regulating TGFβ and integrin signaling (VanSlyke, Boswell, and Musil 2018) as a small proportion of plasma fibronectin molecules are in the open conformation necessary for RGD presentation to integrins. Thus, we immunostained 5 day PCS WT or FNcKO eyes which have been treated with either vehicle, active TGFβ1, or gremlin-1 for fibronectin deposition. As expected, large numbers of fibronectin fibrils were detected around WT LCs, while this was not seen in FNcKO LCs (Figure 6.10) consistent with the proposal that cell autonomous fibronectin production is needed for fibronectin deposition PCS. However, treatment of FNcKO mice with exogenous TGFβ1 or gremlin-1 restored the deposition of fibronectin fibrils around LCs at 5 days PCS (Figure 6.10). As these LCs do not have the ability to produce their own fibronectin, this suggests that these fibrils are produced from fibronectin present in the aqueous humor. Overall, this result suggests that active TGFβ1 and gremlin-1 rescue the sustained fibrotic response of FNcKO LCs PCS by acting as agonists of the TGFβ signaling pathway which may allow for the upregulation of the integrins necessary for the assembly of plasma fibronectin into a matrix that allows for the assembly of fibrotic ECM PCS.

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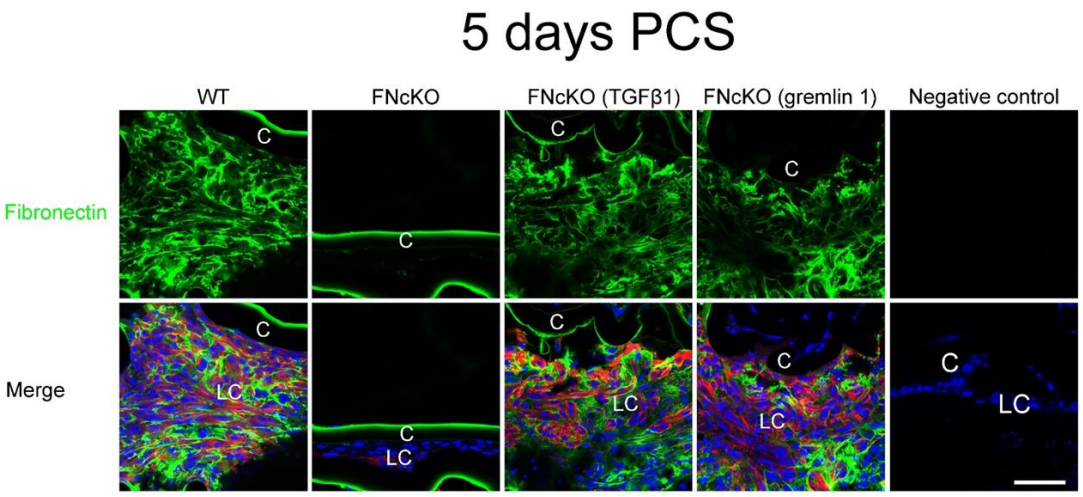

Figure 6.10: Fibronectin fibrils are detected in FNcKO capsular bags upon treatment with either active TGFβ1 or gremlin-1 at 5 days PCS. Active TGFβ1 and gremlin-1 treated FNcKO capsular bags are positive for fibronectin fibrils similar to WT capsular bags (vehicle-treated) at 5 days PCS. As expected, vehicle treated FNcKO capsular bags are not positive for fibronectin fibrils. Fibronectin (green), αSMA (red), and DNA detected by Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule.

6.3 Discussion

Cellular fibronectin, a multifunctional protein that regulates cellular behavior at diverse levels (To and Midwood 2011; D. Chen et al. 2015; Moriya et al. 2012; Fontana et al. 2005; Clark 1990; Zollinger and Smith 2017), has been long associated with lens development and fibrotic PCO pathogenesis (Parmigiani and McAvoy 1991; J. Huang et al. 2011; Boyd et al. 1992). However, its functions in the adult lens and PCO pathogenesis have been elusive. Here we deleted the fibronectin gene from the lens and used these conditional knockouts animals to characterize the role of fibronectin in adult lens homeostasis and the response of lens cells (LCs) to lens fiber

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cell removal which models cataract surgery. This work provides insight into the multifunctional roles of cellular fibronectin in the pathophysiology of fibrotic PCO as well as the multitude of other fibrotic conditions that feature fibronectin rich extracellular matrices (ECM).

6.3.1 Fibronectin is dispensable in the adult lens

Fibronectin is produced by the embryonic lens (Parmigiani and McAvoy 1991; Duncan et al. 2000), and its deposition in the ECM underlying the lens placode is required for the placode invagination (J. Huang et al. 2011). However, the role of fibronectin in the later stages of lens development was not known. Here we generated mice conditionally lacking a functional fibronectin gene from the lens (FNcKO) using MLR10 CRE, which has the potential to delete the FN1 gene from the lens as early as the lens vesicle stage although characterization of FNcKO animals indicated that FN1 deletion from LCs was not complete until after birth. These observations suggest that fibronectin plays little to no role in the uninjured adult lens as adult FNcKO lenses are transparent and structurally normal. RNAseq revealed that the 121 genes differentially expressed in FNcKO lenses were enriched in those encoding ECM proteins suggesting that FNcKO lenses may be compensating for fibronectin loss, although the expression of all of these genes, even after upregulation, was still quite low. This is consistent with the observation that uninjured adult lenses express levels of fibronectin mRNA (FPKM 0.3) that may be too low to be biologically significant to lens function (Manthey, Terrell, Lachke, et al. 2014). Notably, none of the DEGs included genes known to be important for lens homeostasis, although 27 of the 121 FNcKO lens DEGs exhibit lens enriched expression as defined by iSyte analysis of the P56 lens

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(not shown), so would be bioinformatically predicted to regulate lens biology (Kakrana et al. 2018).

6.3.2 Fibronectin is essential for the pathogenesis of fibrotic PCO

Fibronectin mRNA and protein levels upregulate sharply in lens epithelial cells (LCs) during the progression of anterior subcapsular cataract (Frank J Lovicu et al. 2002) and after lens fiber cell removal modeling cataract surgery (Mamuya et al. 2014). Fibronectin has been used as a fibrotic marker in PCO for years (Shirai et al. 2004; de Iongh et al. 2005; Frank J Lovicu et al. 2002; Das et al. 2019; Wernecke et al. 2018) and was implicated in PCO pathogenesis (I. Michael Wormstone et al. 2002; J. M. Marcantonio and Reddan 2004; Boyd et al. 1992). However, exogenous fibronectin, such as that present in blood/aqueous humor, has been proposed as both a positive and negative regulator of growth factors involved in PCO pathology (Dawes et al. 2008; Tiwari et al. 2016; VanSlyke, Boswell, and Musil 2018). Due to both conflicting literature on fibronectin function in PCO, and the dearth of studies on cellular fibronectin in this condition, we took advantage of the mouse cataract surgery model (Desai et al. 2010) to comprehensively characterize the role of endogenous tissue fibronectin in fibrotic PCO.

Here, we show that cellular fibronectin protein is robustly produced by LCs starting around 48 hours PCS, although fibronectin does not seem to be required for the early fibrotic response of LCs PCS as this was qualitatively and quantitatively normal in FNcKO LCs at this time, and fewer than 100 genes were differentially expressed between WT and FNcKO LCs at 48 hours PCS. The modest role that fibronectin produced by LCs plays in the initial response of LCs to cataract surgery is not surprising as its basal expression in the adult lens is very low, and its mRNA

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expression does not elevate in lens cells until 24 hours PCS (Mamuya et al. 2014). However, fibronectin deposition around LCs does become more robust at later times PCS, and this study found that it is critical for the maintenance of fibrotic PCO at 5 days PCS as little evidence of LC fibrosis was apparent in FNcKO mice at this time.

However, FNcKO LCs did initiate the fibrotic response PCS as both RNAseq and immunofluorescence revealed the upregulation of numerous fibrotic markers at 48 hours PCS, and this was maintained through 3 days PCS, although these cells diminish in number by 4 days PCS, and were largely absent by 5 days PCS. Our study found no evidence that these cells expressing fibrotic markers are lost via traditional apoptosis, although they may still be lost through one of the several known nonapoptotic cell death pathways (Tait, Ichim, and Green 2014). However, this study provides some evidence that LC derived FNcKO myofibroblasts may be returning to a lens epithelial cell phenotype and/or differentiating into lens fiber cells in the absence of cellular fibronectin. A definitive understanding of the fate of FNcKO myofibroblasts at later times PCS will require future cell lineage tracing experiments.

6.3.3 Fibronectin influences the pathogenesis of fibrosis via multiple mechanisms

Due to the critical role of autocrine fibronectin production in the maintenance of fibrotic PCO, we attempt to address the underlying molecular mechanisms by integrating RNAseq analysis of FNcKO lenses PCS with previous reports on fibronectin function in other systems.

6.3.3.1 Fibronectin and fibrotic matrix production and assembly

Tissue fibronectin is produced locally in tissues, where it assembles into insoluble fibrils, often in response to injury (To and Midwood 2011; Rousselle,

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Montmasson, and Garnier 2019). Later, fibronectin is remodeled to facilitate the assembly of secondary scars rich in collagen I and other fibrotic ECM proteins (Zhang et al. 2014; Zollinger and Smith 2017; Kii and Ito 2017; Karamanos et al. 2019). Numerous prior cell culture studies suggest that fibronectin is a master regulator of ECM assembly because of its ability to regulate a wide range of ECM molecules (Kumra and Reinhardt 2016). However, these findings had not been corroborated in vivo (Schwarzbauer and DeSimone 2011; Kumra and Reinhardt 2016; Lenselink 2015). Here we fill this knowledge gap by discovering that fibronectin is required for lens cells to upregulate both the mRNA expression and matrix assembly of some major fibrotic ECM components PCS including collagen I, tenascin C and Periostin (Figure 6.11-1). Interestingly, contrary to some reports (Sercu et al. 2008; Sottile and Hocking 2002), we found that fibronectin production by LCs was not required for the matrix deposition of the extracellular matrix proteins (ECM1) and thrombospondin-1 during the progression of LC fibrosis although thrombospondin-1 deposition was attenuated. This suggests either that the small amounts of exogenous fibronectin from aqueous humor that may deposit around FNcKO LCs is sufficient for ECM1 and thrombospondin-1 assembly or that LCs produce other mediators of their assembly (Sercu et al. 2008; S. Chen and Birk 2013). Overall, this study suggests that cellular fibronectin plays a previously unappreciated dual role in matrix formation in fibrotic disease in vivo as it is required for both the expression of fibrotic ECM genes and the assembly of their protein products.

6.3.3.2 Fibronectin and TGFβ superfamily signaling

It is well-established that TGFβ signaling mediates fibrotic PCO (de Iongh et al. 2005) while plating dissociated embryonic lens cells on plasma fibronectin can

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activate TGFβ signaling (VanSlyke, Boswell, and Musil 2018). Fibronectin is also crucial for the incorporation of the latent TGFβ complex into the ECM in other in vitro cell models (Fontana et al. 2005; Dallas et al. 2005). However, the relationship between the production of endogenous cellular fibronectin and the induction of TGFβ signaling in fibrotic conditions like PCO had not been explored in vivo.

In this study, we show that cellular fibronectin's role in driving TGFβ signaling is a major reason that endogenous expression of fibronectin by remnant LCs PCS is critical for sustained fibrotic PCO. In vitro studies have previously revealed that fibronectin interactions with latent TGFβ binding protein 1 (LTBP1) are critical to tether latent TGFβ to the ECM; a process necessary for its activation. In vivo, we found the LTBP1 normally associates with the fibrotic ECM that assembles around LCs PCS, while this does not occur around FNcKO LCs PCS, suggesting that the tethering of latent TGFβ (and its subsequent activation) cannot occur in the absence of cellular fibronectin expression by LCs (Figure 6.11-2).

However, the RNAseq analysis of FNcKO LCs at 48 hours PCS revealed that fibronectin is playing multifunctional roles in the regulation of the TGFβ pathway PCS. The expression of gremlin-1, a known activator of TGFβ signaling and antagonist of BMP signaling that has been implicated in the pathogenesis of fibrotic diseases including PCO (Church et al. 2017; Staloch et al. 2015; McDowell et al. 2015; G. Li et al. 2013; Rodrigues-Diez et al. 2012; Brazil et al. 2015; G. Li et al. 2013; Ma et al. 2019), is highly upregulated in LCs by 48 hours PCS, while its mRNA and protein levels are markedly attenuated in FNcKO LCs. Notably, the addition of exogenous gremlin-1 can also rescue the defects in TGFβ signaling, and fibrotic ECM production, observed in FNcKO LCs PCS, and its effects on tenascin C and periostin

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expression are qualitatively and quantitatively more potent than TGFβ1 (Figure 6.8B and supplemental figure B6). However, further study of the role of periostin in the assembly of tenascin C and other fibrotic ECM matrix components is required to understand these relationships better. Overall, this suggests that fibronectin could be playing multifunctional roles in regulating TGFβ pathway activation PCS which include both the regulation of the gene expression of a TGFβ pathway agonist (Figure 6.11-3) and the activation of latent TGFβ.

Notably, gremlin-1 is also well known to be an antagonist of BMP signaling (Brazil et al. 2015) which was particularly interesting as BMP signaling plays a critical role in lens development (Faber et al. 2002; Boswell and Musil 2015) while BMP signaling can play anti-fibrotic roles in epithelia (Brazil et al. 2015) as it can counterbalance TGFβ signaling (Brazil et al. 2015). A prior in vitro study on primary LCs suggested that the BMP signaling agonist BMP-7 can suppress TGFβ mediated epithelial mesenchymal transition (EMT) (Shu, Wojciechowski, and Lovicu 2017) and we show in this study that BMP signaling rapidly decreases in LCs PCS, a process that is attenuated in FNcKO LCs (Figure 6.11-4). To further understand how fibronectin regulates BMP signaling PCS, we tested whether the rescue of the fibrotic phenotype of FNcKO LCs by either gremlin-1 or TGFβ included the downregulation of BMP signaling and found that BMP signaling remained high in both cases. This was surprising as it shows that it was possible for both BMP and TGFβ signaling to be high in the same cell even though the fibrotic phenotype is qualitatively rescued suggesting that 1) BMP signaling is not sufficient to protect LCs from the fibrotic transformation in the presence of gremlin-1 or TGFβ-induced Smad2/3 activation and

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2) fibronectin's effect on BMP signaling PCS is not mediated by its effects on TGFβ pathway activation.

The rescue experiments performed by adding active TGFβ1 and gremlin-1 to FNcKO capsular bags revealed that plasma fibronectin can participate in sustained fibrotic PCO when TGFβ signaling is ectopically activated as fibronectin fibrils assembled around FNcKO LCs treated with either active TGFβ1 or gremlin-1. Based on previous studies, it is likely that this fibronectin is coming from the aqueous humor (an important source of plasma fibronectin (Vesaluoma et al. 1998)) as FNcKO LCs are unable to produce cell derived fibronectin and we did not observe any elevations in fibronectin expression by any other ocular structures besides LCs in active TGFβ1 or gremlin-1 treated eyes (data not shown). Overall, this study suggests that TGFβ1 and gremlin-1, by acting as agonists of the TGFβ signaling pathway, may allow for the integrin upregulation necessary for the assembly of plasma fibronectin into a matrix that allows for the assembly of fibrotic ECM PCS. This finding is consistent with a recent study that identified a critical role for plasma fibronectin in `sustained PCO (VanSlyke, Boswell, and Musil 2018).

6.3.3.3 Fibronectin and integrin signaling

Integrins have been proposed as therapeutic targets for PCO due to their roles in cell/ECM attachment, cell migration, and transmission of tractional forces (Tiwari et al. 2016; Kim et al. 2002; Walker and Menko 2009; Qin et al. 2017). As fibronectin is a well-known ligand for several integrin receptors that are upregulated by LCs PCS (Mamuya et al. 2014; de Iongh et al. 2005; Walker and Menko 2009), we investigated the effect of autocrine fibronectin on integrin pathways. Notably, our data revealed that cellular fibronectin is not just important for downstream integrin signaling PCS

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but is also necessary for the enhanced protein expression of several integrin receptors by LCs PCS. Notably, active TGFβ1 can rescue both the defects in integrin expression and downstream integrin signaling seen in FNcKO LCs PCS. As integrins can mediate the activation of latent TGFβ (Mamuya and Duncan 2012; Mamuya et al. 2014) whereas TGFβ signaling can upregulate their expression, this finding further supports a model by which the diverse functions of fibronectin, including its interaction with integrins, drives the epithelial mesenchymal transition of LCs in PCO, and potentially the pathogenesis of other disorders of EMT such as cancer and some fibrotic conditions (Figure 6.11-5).

6.3.4 Implications for the role of fibronectin in wound healing and fibrotic diseases

This comprehensive study shows that fibronectin production by LCs is required for the persistence of myofibroblasts PCS and we have laid out several possible mechanisms by which fibronectin mediates this response (Figure 6.11). This provides the first insight into why myofibroblasts, which are lost after initial wound healing responses in normal healing (Xue and Jackson 2013), are maintained at such extended times after surgery to cause fibrotic PCO in humans, an intractable complication PCS. Overall our study will provide important insights towards improving the outcome of cataract surgery (Shihan, Novo, and Duncan 2019).

Further, the destruction of tissue architecture by fibrosis has been estimated to cause at least one-third of natural deaths worldwide (Rockey, Bell, and Hill 2015). While numerous studies have identified important pathways driving fibrosis (Wynn and Ramalingam 2012; Ghosh, Quaggin, and Vaughan 2013; Y. Wang et al. 2018), much less is known about the mechanisms by which activated

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fibroblasts/myofibroblasts inappropriately persist after the initial injury/stress is removed (Xue and Jackson 2013; Leavitt et al. 2016). Notably, fibronectin has been extensively studied due to its important roles in the wound healing response and fibrosis (Lenselink 2015). However, most of the studies done on fibronectin are cell culture based and thus are difficult to correlate to wound healing in vivo (Kumra and Reinhardt 2016; Lenselink 2015; Schwarzbauer and DeSimone 2011). The few in vivo studies on the function of cellular fibronectin (Muro et al. 2003; Stenzel et al. 2011; Iwasaki et al. 2016; Moriya et al. 2012) mostly address only a single aspect of fibronectin's role in wound healing as most tissues consist of many cell types with complex interactions. This study has taken advantage of the lens's relative simplicity, as the cells left behind after cataract surgery consist of a monolayer of epithelial cells that undergo epithelial to mesenchymal transition (EMT) to form myofibroblasts that behave similarly to the myofibroblasts responsible for other fibrotic diseases (Shirai et al. 2018). This cellular simplicity has allowed for the dissection of the complex regulatory roles that cellular fibronectin plays in fibrosis (Figure 6.11). This work provides a new understanding of PCO pathogenesis and identifies new targets for the treatment/prevention of both fibrotic PCO (Shihan, Novo, and Duncan 2019) and numerous other fibrotic conditions resulting in death and disability (Walraven and

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Hinz 2018; Allinovi et al. 2018; Iozzo and Gubbiotti 2018; Hewlett, Kropski, and Blackwell 2018).

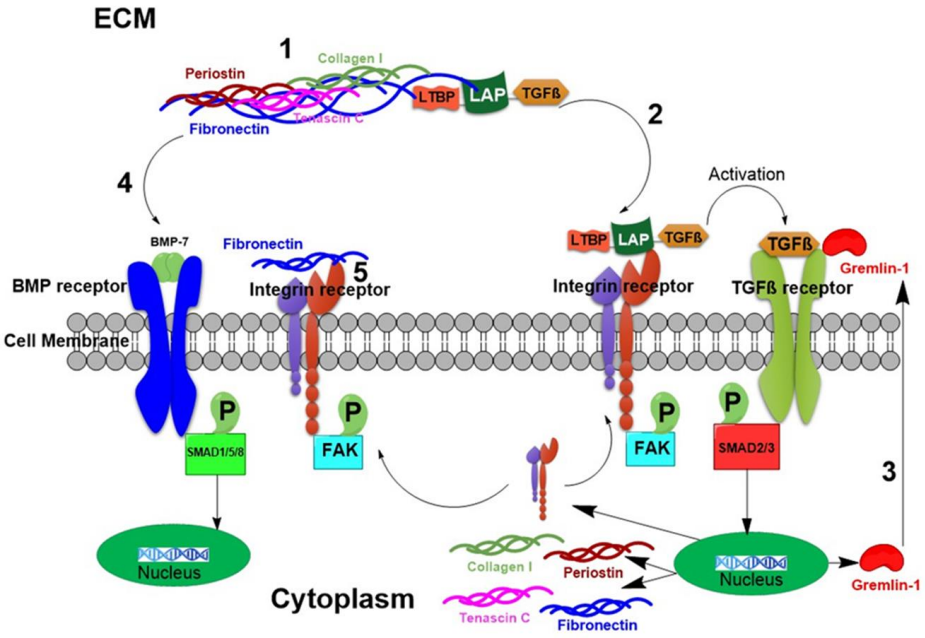

Figure 6.11: Multifunctional roles of fibronectin in PCO pathogenesis. This diagram depicts the multifunctional roles of cellular fibronectin in fibrotic PCO and potentially other fibrotic like conditions. (1) Regulation of fibrotic extracellular matrix protein assembly; (2) Extracellular deposition of latent TGFβ complex needed for its subsequent activation; (3) Regulation of the expression of the TGFβ signaling agonist, gremlin-1; (4) Regulation of BMP signaling; (5) Modulation of integrin signaling.

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Chapter 7

FUTURE DIRECTIONS

In total, there are four individual projects covered in this dissertation. The interview-based study (Chapter 3) provides a clear understanding that cataract surgeons agree that additional anti PCO preventive measures would improve the outcomes of cataract surgery. Chapter 4 reveals for the first time that proinflammatory cytokines are expressed by the lens epithelial cells post cataract surgery (PCS), setting up the novel hypothesis that this may set up a milieu to activate transforming growth factor-beta (TGFβ) signaling, a major mediator of fibrotic PCO. Chapter 5 has identified the critical role of αVβ8 integrin in TGFβ activation PCS and has shown the effectiveness of an antibody blocking agent to block the activity of αVβ8 integrin mediated TGFβ activation PCS. Chapter 6 revealed the critical role of fibronectin in regulating the latent TGFβ complex PCS and its implication in sustaining the fibrotic PCO. All contributed to a deep understanding of the molecular mechanisms of fibrotic PCO. However, there are still some questions that need to be addressed in the future.

7.1 Assessing Long Term Risks of Cataract Surgery using Longitudinal Medicare Data

Cataract surgery is the standard of care for cataract and has greatly reduced the world-wide burden of blindness. Despite this, cataract surgery can have negative sequelae including infection/endophthalmitis, exacerbation or onset of uveitis, post-surgical glaucoma, posterior capsular opacification, and retinal detachment, while some small epidemiological studies also suggest that cataract surgery may increase the

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risk of subsequent age-related macular degeneration, the most prevalent cause of blindness in America's elderly population. This is further confirmed by our study covered in chapter 3. However, while many studies have been performed to assess the benefits of cataract surgery related to fall prevention or mortality, few to no population-wide studies have been conducted to determine the risks of cataract surgery to ocular health, particularly at times later than 1 year following surgery. Further, our interview study (chapter 4) report that a significant number of patients develop diffent kinds of post cataract surgical complications following cataract surgery, however, the exact statistics about these patients is not well documented. Thus, this study will assess the rates of cataract surgery in the US population older than 65, and both the short term and long term side effects of cataract surgery by exploring 10 years of billing records obtained from the US Medicare system. The critical knowledge gap filled by this study will both allow for more fully informed decision making for patients diagnosed with mild cataracts or seeking "clear" cataract surgery for the treatment of presbyopia, and will identify areas where more research is needed to improve the long term outcomes of cataract surgery.

7.2 Identifying the transcriptional regulatory networks driving proinflammatory cytokine expression by the lens epithelial cells (LCs)

In chapter 4, I found that following cataract surgery, lens epithelial cells express pro-inflammatory cytokines as early as 3 hours post cataract surgery (PCS) while the infiltration of neutrophils in the capsular bags is first detected at 18 hours PCS, and macrophages at 48 hours PCS. However, the mechanisms by which these pro-inflammatory cytokines PCS are expressed not well understood. The first genes upregulated by a cellular stimulus are the primary response or Immediate Early Genes

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(IEGs) (Fowler, Sen, and Roy 2011; Bahrami and Drabløs 2016). Many IEGs encode transcription factors that drive cell proliferation, cell migration, and reprogram cell differentiation (Fowler, Sen, and Roy 2011; 2011). Among them, early growth response 1 (Egr1) protein and mRNA levels upregulate sharply (over 100 fold) in remnant lens cells (LCs) by 3 hours post cataract surgery (PCS) in our mouse cataract surgery model, which is the earliest biological effect ever detected in LCs in response to ocular trauma. Notably, Cxcl1 is a known DIRECT target of Egr1 (Brookshire et al. 2015), and many other genes upregulated (Kobayashi 2008) PCS is involved in homing of neutrophils to injury sites. Thus, further study is required to understand the role of Egr1 and other transcription factors (cJun, FosB, etc. (Kim, Ho Han, and Kwon 2003; Cervantes-Madrid, Nagi, and Asting Gustafsson 2017)) involved in the regulation of proinflammatory cytokines PCS. A graduate student Samuel Novo in Melinda K. Duncan lab has been working on this project.

7.3 Elucidating the role of MT1-MMP in the activation of TGFβ signaling PCS

MT1-MMP is a membrane-bound matrix metalloprotease that is often upregulated in fibrotic diseases (García-Alvarez et al. 2006). MT1-MMP can be critical for the pathogenesis of fibrosis as it is the αVβ8-integrin co-factor necessary for latent TGFβ activation (Mu et al. 2002) while I have found that αVβ8-integrin is critical in TGFβ activation PCS. In the lens, MT1-MMP levels are upregulated by TGFβ in an established human LC cell line (Eldred et al. 2012). In our mouse cataract surgery model, MT1-MMP mRNA levels upregulate 3 fold by 24 hours PCS (prior to robust TGFβ signaling), and over 6 fold by 48 hours PCS while MT1-MMP protein is robustly upregulated in remnant LCs by 24-48 hours PCS (unpublished). Thus understanding the role of MT1-MMP in relationship to TGFβ signaling activation PCS

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will further strengthen my study performed on the role of αVβ8 integrin in PCO (chapter 5).

7.4 Understanding the role of α5 integrin in PCO

In chapter 6, I have found that fibronectin fibril formation is critical in PCO pathogenesis as fibronectin fibrils can mediate latent TGFβ complex deposition on the extracellular matrix (ECM) and fibrotic ECM assembly PCS. It has been proposed that fibronectin fibril formation is dependent on its interaction with its major receptor α5β1 integrin (Singh, Carraher, and Schwarzbauer 2010). Notably, α5β1-integrin upregulates sharply in LCs by 48 hours PCS and is robustly expressed at 5 days PCS in our mouse cataract surgery model suggesting that α5β1-integrin/fibronectin interactions may be critical in PCO pathogenesis. Thus the future study is required to understand the relationship of α5β1-integrin in PCO pathogenesis.

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Appendix A

SUPPLEMENTAL TABLES

Table A1: Genes that normally upregulate in remnant LCs of WT, whose upregulation is attenuated in β8ITGcKO LCs at 24 hr PCS.

Table A2: Genes differentially expressed (DEGs) between adult WT and FNcKO unoperated lens which fall under the ontology (GO) term "proteinaceous extracellular matrix."

Table A3: Genes that are differentially expressed in the lens of FNcKO at 48 hours PCS.

Table A4: Genes that normally downregulate in WT LCs by 48 hours PCS but exhibit attenuated downregulation in FNcKO LCs.

Table A5: Genes that normally upregulate in remnant LCs of WT, whose upregulation is attenuated in FNcKO LCs.

Table A6: The expression of genes that encode different integrins at 48 hours PCS between WT and FNcKO LCs.

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Table A1: Genes that normally upregulate in remnant LCs of WT, whose upregulation is attenuated in β8ITGcKO LCs at 24 hr PCS.

Gene ID	WT_0 hr vs 24 hr _FC	WT_0 hr vs 24 hr_ FDR	24 hr_ WT vs β8ITGcKO_FC	24 hr_ WT vs β8ITGcKO_FDR	WT 24 hr_ Avg_ FPKM	β8ITGcKO 24 hr_ Avg _FPKM
Acod1	∞	3.92E-4	-2.42	9.36E-3	4.27	1.76
Acta2	9.71	3.92E-4	-2.18	1.01E-3	869.12	399.19
Akap2	2.27	6.93E-3	-2.41	1.01E-3	55.04	22.82
Ankrd1	14.76	3.92E-4	-4.90	1.01E-3	33.03	6.74
Anxa8	58.46	2.01E-2	-3.87	1.01E-3	22.77	5.88
Apbb1ip	11.03	3.92E-4	-2.31	1.04E-2	4.16	1.80
Apol9a	12.57	1.05E-3	-2.40	2.73E-2	4.93	2.06
Arc	2.43	3.92E-4	-2.60	1.01E-3	19.05	7.31
Asb5	4.05	1.74E-2	-5.12	2.04E-2	4.32	0.84
Blnk	6.42	3.92E-4	-2.43	1.01E-3	9.69	3.99
Calml3	2.52	3.86E-2	-15.66	6.59E-3	4.00	0.26
Car13	29.47	1.13E-2	-2.23	3.51E-2	3.80	1.71
Cbr2	5.10	3.92E-4	-2.48	4.74E-3	16.90	6.83
Cd33	22.05	3.92E-4	-3.13	1.01E-3	4.05	1.29
Cdk15	7.65	3.92E-4	-2.37	3.07E-2	4.81	2.03
Clmp	6.55	3.92E-4	-2.52	1.01E-3	6.62	2.63
Cmss1	2.72	8.97E-3	-2.02	2.62E-2	24.80	12.29
Col6a1	2.36	3.92E-4	-2.15	1.01E-3	22.96	10.67

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Gene ID	WT_0 hr vs 24 hr _FC	WT_0 hr vs 24 hr_ FDR	24 hr_ WT vs β8ITGcKO_FC	24 hr_ WT vs β8ITGcKO_FDR	WT 24 hr_ Avg_ FPKM	β8ITGcKO 24 hr_ Avg _FPKM
Crabp2	19.91	3.92E-4	-3.49	1.01E-3	21.17	6.06
Csf3	199.24	2.76E-2	-4.86	1.01E-3	50.87	10.47
Cth	2.43	5.83E-3	-2.92	4.04E-3	6.35	2.17
Cxcl2	11.78	3.92E-4	-3.09	1.01E-3	55.40	17.94
Cxcl5	140.46	3.92E-4	-3.83	1.01E-3	47.41	12.39

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Gene ID	WT_0 hr vs 24 hr _FC	WT_0 hr vs 24 hr_ FDR	24 hr_ WT vs β8ITGcKO_FC	24 hr_ WT vs β8ITGcKO_FDR	WT 24 hr_ Avg_ FPKM	β8ITGcKO 24 hr _Avg _FPKM
Defb1	18.52	2.71E-2	-10.79	1.68E-2	7.07	0.66
Dsg1b	2.61	3.92E-4	-5.32	1.01E-3	3.72	0.70
Dyrk3	3.41	2.47E-3	-2.09	2.23E-2	4.56	2.19
Ercc1	4.16	7.36E-4	-2.53	1.40E-2	27.49	10.88
Errfi1	2.45	3.92E-4	-2.11	1.01E-3	41.04	19.45
F3	8.71	3.92E-4	-2.24	1.01E-3	31.38	14.02
Fam25c	10.70	1.82E-2	-6.11	9.36E-3	22.71	3.71
Fgl2	10.34	3.92E-4	-3.52	1.01E-3	8.06	2.29
Gch1	2.12	9.94E-3	-2.97	1.01E-3	5.39	1.81
Grem1	170.52	3.92E-4	-2.94	1.01E-3	178.14	60.60
Gsta1	∞	3.92E-4	-3.21	4.52E-2	6.32	1.97
Gsta2	∞	3.92E-4	-3.14	1.50E-2	9.19	2.92
Hdc	14.13	1.64E-3	-4.48	5.37E-3	2.66	0.59
Hp	∞	3.92E-4	-3.27	1.01E-3	7.44	2.27
Ifit1	6.44	3.92E-4	-2.93	1.01E-3	18.61	6.35
Ifit3	5.59	3.92E-4	-3.18	1.01E-3	31.67	9.97
Ifit3b	6.57	3.92E-4	-2.60	4.74E-3	14.44	5.54
Il6ra	9.89	3.92E-4	-2.41	3.33E-3	4.18	1.73
Irak4	4.93	3.92E-4	-2.00	4.84E-2	5.46	2.72
Itga5	9.06	3.92E-4	-2.76	1.01E-3	70.95	25.72

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Gene ID	WT_0 hr vs 24 hr _FC	WT_0 hr vs 24 hr_ FDR	24 hr_ WT vs β8ITGcKO_FC	24 hr_ WT vs β8ITGcKO_FDR	WT 24 hr_ Avg_ FPKM	β8ITGcKO 24 hr _Avg _FPKM
Krt15	4.33	3.92E-4	-8.20	1.01E-3	29.50	3.60
Krt5	2.26	1.51E-2	-11.25	1.01E-3	5.66	0.50

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Table A1: Genes that normally upregulate in remnant LCs of WT, whose upregulation is attenuated in β8ITGcKO LCs at 24 hr PCS.

Gene ID	WT_0 hr vs 24 hr _FC	WT_0 hr vs 24 hr_ FDR	24 hr_ WT vs β8ITGcKO_FC	24 hr_ WT vs β8ITGcKO_FDR	WT 24 hr_ Avg _FPKM	β8ITGcKO 24 hr_ Avg _FPKM
Krt6a	5.83	3.92E-4	-10.69	5.37E-3	3.60	0.34
Lbp	4.53	3.92E-4	-3.07	1.01E-3	4.58	1.49
Lgals3	3.60	3.92E-4	-4.14	1.01E-3	205.36	49.63
Lmcd1	15.38	2.04E-2	-4.13	8.35E-3	3.47	0.84
Lox	9.31	3.92E-4	-2.54	1.01E-3	5.74	2.26
Ly6a	9.13	3.92E-4	-19.01	2.62E-3	13.92	0.73
Map3k6	6.36	3.92E-4	-2.12	1.01E-3	8.41	3.97
Mmp19	4.93	9.15E-3	-2.32	4.24E-2	5.40	2.33
Mmp3	∞	3.92E-4	-2.45	1.01E-3	15.58	6.35
Mt2	2.96	3.92E-4	-2.35	1.01E-3	205.55	87.37
Nes	9.71	3.92E-4	-2.86	1.01E-3	231.45	80.99
Noct	2.71	3.92E-4	-2.16	1.01E-3	42.35	19.64
Notum	3.23	3.92E-4	-2.15	1.01E-3	10.41	4.83
Nov	4.12	3.92E-4	-3.35	1.01E-3	4.18	1.25
Nppb	30.40	4.46E-3	-2.55	2.15E-2	13.67	5.37
Oas2	12.38	3.92E-4	-2.78	5.37E-3	3.40	1.22
Ocel1	3.15	9.94E-3	-2.21	4.61E-2	4.21	1.91
Pak1	3.72	3.92E-4	-2.06	1.01E-3	10.99	5.34

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Gene ID	WT_0 hr vs 24 hr _FC	WT_0 hr vs 24 hr_ FDR	24 hr_ WT vs β8ITGcKO_FC	24 hr_ WT vs β8ITGcKO_FDR	WT 24 hr_ Avg _FPKM	β8ITGcKO 24 hr_ Avg _FPKM
Phf11d	4.79	3.92E-4	-2.80	1.01E-3	5.19	1.85
Pla2g2e	∞	3.92E-4	-10.44	4.94E-2	2.64	0.25
Prrx2	2.45	4.25E-2	-2.76	2.70E-2	4.54	1.65
Ptgs2	29.33	3.92E-4	-2.50	1.01E-3	24.39	9.75
Ptx3	28.99	1.05E-3	-3.03	1.01E-3	60.89	20.12
Pxdc1	2.93	7.36E-4	-2.02	1.96E-2	8.78	4.34
Rhox8	4.28	2.99E-3	-3.19	7.15E-3	5.99	1.88
Rnf125	20.31	3.92E-4	-2.43	1.01E-3	18.73	7.71
Rsad2	24.10	3.92E-4	-4.66	1.01E-3	8.24	1.77
S100a8	∞	3.92E-4	-3.34	1.78E-2	21.86	6.54
S100a9	∞	3.92E-4	-2.51	3.33E-3	39.28	15.67
Serpina3h	3.35	2.74E-3	-21.48	4.74E-3	4.40	0.20
Serpinb6b	3.43	3.92E-4	-4.48	1.01E-3	37.74	8.42
Serpine1	36.42	3.92E-4	-2.32	1.01E-3	432.22	186.00
Sfn	2.05	2.99E-3	-11.68	1.01E-3	14.60	1.25
Slco2a1	7.02	3.92E-4	-2.23	1.01E-3	21.12	9.47
Slfn1	79.05	2.64E-2	-2.26	7.15E-3	7.49	3.32
Slfn4	∞	3.92E-4	-3.52	1.01E-3	37.08	10.53
Slpi	∞	3.92E-4	-3.51	2.81E-2	4.82	1.37
Snai1	5.46	3.72E-3	-2.69	3.07E-2	3.46	1.29
Sprr1a	24.75	3.92E-4	-4.39	1.01E-3	82.49	18.77

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Gene ID	WT_0 hr vs 24 hr _FC	WT_0 hr vs 24 hr_ FDR	24 hr_ WT vs β8ITGcKO_FC	24 hr_ WT vs β8ITGcKO_FDR	WT 24 hr_ Avg _FPKM	β8ITGcKO 24 hr_ Avg _FPKM
Sprr2b	∞	3.92E-4	∞	1.01E-3	2.67	0.00
Stac	2.74	3.92E-4	-2.88	1.01E-3	9.28	3.22
Stat5a	2.80	3.92E-4	-2.38	1.01E-3	8.65	3.64
Syt17	2.37	2.20E-3	-3.31	1.01E-3	9.84	2.98
Tgm1	11.21	3.92E-4	-4.83	1.01E-3	12.74	2.64
Thbs1	6.12	3.92E-4	-2.23	1.01E-3	49.51	22.17
Tm4sf1	2.74	3.92E-4	-2.31	1.01E-3	106.45	46.00
Tpd52l1	2.08	5.15E-3	-2.06	5.99E-3	15.26	7.40
Trim30c	∞	3.92E-4	-5.24	3.42E-2	2.89	0.55
Tuba1c	3.97	3.92E-4	-2.27	1.01E-3	34.21	15.08
Vaultrc5	11.64	3.92E-4	-3.05	1.01E-3	123.45	40.41
Xaf1	6.28	3.92E-4	-2.23	5.37E-3	16.33	7.33

FC- Fold Change, WT- Wild Type, β8ITGcKO - β8 integrin conditional knockout, FDR- False Discovery Rate, Avg- Average, Hr- hour, FPKM- Fragments Per Kilobase Million, ∞ indicates that fold-change did not give a numerical value as FPKM of a specific gene appears 0 at WT 0 hour PCS.

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Table A2: Genes differentially expressed (DEGs) between adult WT and FNcKO unoperated lens which fall under the ontology (GO) term "proteinaceous extracellular matrix."

Gene ID	**Gene description**	**Fold change (FC)**	**P Value**	**WT Mean FPKM Adult lens**	**FNcKO Mean FPKM Adult lens**
Col1a2	Collagen, type I, alpha 2	2	5.00E-05	1.5	3.2
Col9a1	Collagen, type IX, alpha 1	2	5.00E-05	3.5	7.8
Col9a2	Collagen, type IX, alpha 2	2	5.00E-05	1	2.2
Col18a1	Collagen, type XVIII, alpha 1	2	5.00E-05	3.5	7.8
Col6a2	Collagen, type VI, alpha 2	-2	5.00E-05	2	0.96
Col6a3	Collagen, type VI, alpha 3	-3	5.00E-05	16.6	2

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Table A3: Genes that are differentially expressed in the lens of FNcKO at 48 hours PCS.

Gene ID	FC_WT_48_Hour_vs_FN_48_Hour	FDR_WT_48_Hour_vs_FN_48_Hour	WT_48_Hour_Avg_RPKM	FN_48_Hour_Avg_RPKM	FC_WT_0_Hour_vs_WT_48_Hour	FDR_WT_0_Hour_vs_WT_48_Hour
Abcc3	-3.79	7.1E-03	2.79	0.69	9.27	2.8E-09
Adamts16	-9.91	2.6E-04	1.55	0.14	8.34	4.8E-05
C2	-13.30	2.0E-10	8.13	0.56	5.94	1.4E-09
Cda	-292.61	4.6E-07	5.26	0.00	14.63	1.0E-05
Cdh17	-42.53	1.2E-03	0.67	0.01	160.89	1.9E-06
Clmp	-4.82	1.6E-03	9.39	1.81	21.37	1.5E-11
Cnn1	-16.68	1.3E-03	1.11	0.06	40.89	2.8E-07
Col14a1	-5.95	3.2E-03	1.00	0.16	5.60	4.8E-05
Col1a1	-6.50	1.6E-10	79.38	11.45	83.45	2.1E-42
Col5a2	-3.28	3.5E-04	3.81	1.10	23.85	3.9E-25
Crabp2	-8.21	3.3E-09	64.87	7.34	81.37	9.0E-26
Crlf1	-2.94	8.1E-03	25.53	8.30	111.55	8.4E-43
Dct	-16.14	5.8E-09	56.17	3.20	2.36	1.1E-02
Dgkk	-183.25	1.4E-05	0.39	0.00	6.99	9.8E-04
Enpep	-2.33	4.1E-02	10.23	4.15	5.25	6.6E-10
F13a1	-2.53	3.8E-02	12.73	4.87	3.36	3.2E-06
Fstl4	-25.93	1.7E-05	2.83	0.09	2.98	3.7E-02
Gatm	-3.85	4.1E-05	12.31	3.02	4.81	2.6E-07
Gm10639	-96.03	8.3E-04	1.56	0.00	14.71	1.6E-04
Grb10	-3.17	1.3E-04	14.34	4.26	3.80	1.0E-06
Grem1	-6.95	1.0E-04	641.93	87.32	379.66	1.7E-40
Grem2	-4.63	1.2E-02	1.81	0.36	4.63	4.7E-04
Gsta2	-8.31	1.4E-02	3.24	0.34	51.11	1.9E-08
Gsta3	-8.38	1.6E-07	23.13	2.56	11.02	4.9E-11

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Gene ID	FC_WT_48_Hour_vs_FN_48_Hour	FDR_WT_48_Hour_vs_FN_48_Hour	WT_48_Hour_Avg_RPKM	FN_48_Hour_Avg_RPKM	FC_WT_0_Hour_vs_WT_48_Hour	FDR_WT_0_Hour_vs_WT_48_Hour
H19	-6.20	2.0E-02	6.71	1.02	46.70	5.1E-09
Hpse	-7.83	3.5E-04	5.47	0.64	7.39	1.1E-06
Hs3st3a1	-4.11	1.7E-02	1.77	0.40	2.73	7.7E-03
Lbp	-10.61	2.5E-15	15.67	1.40	5.25	9.9E-13
Lox	-4.58	2.1E-09	24.39	5.09	44.07	5.1E-78
Lrrc32	-4.03	4.3E-02	2.88	0.67	7.86	5.8E-06
Matn3	-6.60	2.0E-02	1.87	0.27	7.65	4.2E-06
Mchr1	-11.01	3.8E-04	1.88	0.15	14.18	1.6E-06
Mgll	-2.89	3.6E-05	30.00	9.77	2.54	1.2E-04
Mitf	-4.61	1.8E-04	5.81	1.16	3.34	7.5E-05
Mlana	-17.03	2.1E-09	43.60	2.37	6.47	5.7E-05
Mlph	-4.22	1.9E-04	9.44	2.12	2.57	1.9E-03
Mylk	-5.63	7.4E-06	6.83	1.14	3.39	1.1E-03
Nkd2	-2.41	3.6E-03	16.52	6.47	3.20	9.8E-08
Oas2	-4.40	2.4E-03	4.53	0.95	6.10	7.8E-07
Oas3	-4.44	4.3E-02	2.30	0.47	2.64	3.7E-02
Papss2	-3.49	8.3E-06	21.10	5.71	3.38	2.7E-08
Postn	-4.60	2.7E-09	19.33	3.91	5.47	3.9E-10
Ptger3	-17.25	1.1E-02	1.83	0.09	38.76	1.2E-06
Pyhin1	-3.75	2.9E-02	3.39	0.85	10.79	2.0E-07

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Gene ID	FC_WT_48_Hour_vs_FN_48_Hour	FDR_WT_48_Hour_vs_FN_48_Hour	WT_48_Hour_Avg_RPKM	FN_48_Hour_Avg_RPKM	FC_WT_0_Hour_vs_WT_48_Hour	FDR_WT_0_Hour_vs_WT_48_Hour
Rab27a	-3.09	3.4E-03	6.63	2.03	3.21	4.6E-04
Rhox8	-6.62	5.2E-04	12.44	1.76	2.82	9.5E-04
Serpina3c	-60.02	3.0E-20	13.37	0.20	25.26	1.6E-21
Serpina3f	-157	1.4E-39	20.32	0.11	35.98	4.7E-31
Serpina3g	-13.6	2.7E-05	2.25	0.15	34.12	1.2E-10
Serpina3h	-13.6	1.4E-18	21.15	1.47	8.40	1.3E-19
Serpina3i	-7.81	7.9E-03	3.49	0.42	6.97	2.0E-06
Serpina3k	-67.03	1.7E-02	0.58	0.00	5.56	2.3E-02
Serpina3m	-94.46	4.2E-42	32.75	0.32	36.45	2.4E-44

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Gene ID	FC_WT_48_Hour_vs_FN_48_Hour	FDR_WT_48_Hour_vs_FN_48_Hour	WT_48_Hour_Avg_RPKM	FN_48_Hour_Avg_RPKM	FC_WT_0_Hour_vs_WT_48_Hour	FDR_WT_0_Hour_vs_WT_48_Hour
Serpinf1	-3.01	4.8E-02	41.51	12.77	6.98	1.3E-10
Sfn	-9.43	4.1E-09	9.88	0.99	5.09	7.6E-08
Siglec1	-5.63	5.8E-03	3.12	0.51	2.58	2.0E-02
Slc16a10	-4.29	2.2E-03	4.62	1.00	2.49	7.5E-03
Slc24a5	-5.57	4.8E-04	8.92	1.47	4.23	1.5E-04
Slfn4	-7.12	1.1E-07	17.26	2.27	110.10	1.8E-32
Slfn5	-2.88	1.8E-02	4.80	1.57	4.64	7.5E-06
Slpi	-5.22	1.6E-02	18.72	3.30	294.73	8.3E-15
Sytl2	-8.32	6.5E-05	4.38	0.48	6.27	6.7E-06
Tagln	-2.41	1.4E-04	119.08	46.98	16.62	5.3E-44
Tbx20	-9.77	6.9E-08	8.15	0.76	7.51	6.0E-10
Tgm1	-3.71	6.8E-03	13.32	3.37	17.49	2.2E-15
Them7	-9.99	1.7E-02	1.83	0.16	5.66	3.5E-03
Tnfrsf19	-3.22	1.4E-02	7.59	2.19	2.61	1.4E-03
Tspan10	-11.08	1.7E-04	5.20	0.43	2.61	1.2E-02
Tyr	-11.84	6.9E-06	11.23	0.86	5.07	2.6E-06

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Gene ID	FC_WT_48_Hour_vs_FN_48_Hour	FDR_WT_48_Hour_vs_FN_48_Hour	WT_48_Hour_Avg_RPKM	FN_48_Hour_Avg_RPKM	FC_WT_0_Hour_vs_WT_48_Hour	FDR_WT_0_Hour_vs_WT_48_Hour
Ucp2	-2.45	1.1E-03	86.73	33.41	5.28	3.6E-19
Vat1l	-3.02	3.3E-03	41.47	12.74	2.90	5.1E-06
Vill	-3.70	9.4E-03	5.08	1.27	3.32	6.2E-04
Wnt16	-21.95	6.6E-07	15.33	0.63	50.65	5.1E-14
Wnt2	-8.97	2.6E-02	1.60	0.16	234.61	7.5E-10

FC- Fold Change, WT- Wild Type, FN- Fibronectin conditional knockout, FDR- False Discovery Rate, Avg- Average

Table A4: Genes that normally downregulate in WT LCs by 48 hours PCS but exhibit attenuated downregulation in FNcKO LCs.

Gene ID	FC_WT_48_Hour_vs_FN_48_Hour	FDR_WT_48_Hour_vs_FN_48_Hour	WT_48_Hour_Avg_RPKM	FN_48_Hour_Avg_RPKM	FC_WT_0_Hour_vs_WT_48_Hour	FDR_WT_0_Hour_vs_WT_48_Hour
Gpx3	2.91	1.8E-04	175.70	481.36	-2.80	1.2E-06
Hfe	2.65	9.8E-03	7.62	18.85	-2.31	1.3E-03
Jam2	2.30	9.6E-04	7.68	16.60	-4.45	1.4E-16
Slc22a8	3.32	1.7E-02	2.28	7.08	-12.07	1.8E-22

FC- Fold Change, WT- Wild Type, FN- Fibronectin conditional knockout, FDR- False Discovery Rate, Avg- Average

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Table A5: Genes that normally upregulate in remnant LCs of WT, whose upregulation is attenuated in FNcKO LCs

Gene ID	FC_WT_48_Hour_vs_FN_48_Hour	FDR_WT_48_Hour_vs_FN_48_Hour	WT_48_Hour_Avg_RPKM	FN_48_Hour_Avg_RPKM	FC_WT_0_Hour_vs_WT_48_Hour	FDR_WT_0_Hour_vs_WT_48_Hour
Abcc3	-3.79	7.1E-03	2.79	0.69	9.27	2.8E-09
C2	-13.30	2.0E-10	8.13	0.56	5.94	1.4E-09
Cda	-292.61	4.6E-07	5.26	0.00	14.63	1.0E-05
Clmp	-4.82	1.6E-03	9.39	1.81	21.37	1.5E-11
Col1a1	-6.50	1.6E-10	79.38	11.45	83.45	2.1E-42
Col5a2	-3.28	3.5E-04	3.81	1.10	23.85	3.9E-25
Crabp2	-8.21	3.3E-09	64.87	7.34	81.37	9.0E-26
Crlf1	-2.94	8.1E-03	25.53	8.30	111.55	8.4E-43
Dct	-16.14	5.8E-09	56.17	3.20	2.36	1.1E-02
Enpep	-2.33	4.1E-02	10.23	4.15	5.25	6.6E-10
F13a1	-2.53	3.8E-02	12.73	4.87	3.36	3.2E-06
Fstl4	-25.93	1.7E-05	2.83	0.09	2.98	3.7E-02
Gatm	-3.85	4.1E-05	12.31	3.02	4.81	2.6E-07
Grb10	-3.17	1.3E-04	14.34	4.26	3.80	1.0E-06
Grem1	-6.95	1.0E-04	641.93	87.32	379.66	1.7E-40
Gsta2	-8.31	1.4E-02	3.24	0.34	51.11	1.9E-08
Gsta3	-8.38	1.6E-07	23.13	2.56	11.02	4.9E-11
H19	-6.20	2.0E-02	6.71	1.02	46.70	5.1E-09
Hpse	-7.83	3.5E-04	5.47	0.64	7.39	1.1E-06
Lbp	-10.61	2.5E-15	15.67	1.40	5.25	9.9E-13
Lox	-4.58	2.1E-09	24.39	5.09	44.07	5.1E-78
Lrrc32	-4.03	4.3E-02	2.88	0.67	7.86	5.8E-06
Mgll	-2.89	3.6E-05	30.00	9.77	2.54	1.2E-04
Mitf	-4.61	1.8E-04	5.81	1.16	3.34	7.5E-05
Mlana	-17.03	2.1E-09	43.60	2.37	6.47	5.7E-05
Mlph	-4.22	1.9E-04	9.44	2.12	2.57	1.9E-03
Mylk	-5.63	7.4E-06	6.83	1.14	3.39	1.1E-03
Nkd2	-2.41	3.6E-03	16.52	6.47	3.20	9.8E-08
Oas2	-4.40	2.4E-03	4.53	0.95	6.10	7.8E-07

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Gene ID	FC_WT_48_Hour_vs_FN_48_Hour	FDR_WT_48_Hour_vs_FN_48_Hour	WT_48_Hour_Avg_RPKM	FN_48_Hour_Avg_RPKM	FC_WT_0_Hour_vs_WT_48_Hour	FDR_WT_0_Hour_vs_WT_48_Hour
Papss2	-3.49	8.3E-06	21.10	5.71	3.38	2.7E-08
Postn	-4.60	2.7E-09	19.33	3.91	5.47	3.9E-10
Pyhin1	-3.75	2.9E-02	3.39	0.85	10.79	2.0E-07
Rab27a	-3.09	3.4E-03	6.63	2.03	3.21	4.6E-04
Rhox8	-6.62	5.2E-04	12.44	1.76	2.82	9.5E-04
Serpina3c	-60.02	3.0E-20	13.37	0.20	25.26	1.6E-21
Serpina3f	-157.38	1.4E-39	20.32	0.11	35.98	4.7E-31
Serpina3g	-13.67	2.7E-05	2.25	0.15	34.12	1.2E-10
Serpina3h	-13.68	1.4E-18	21.15	1.47	8.40	1.3E-19
Serpina3i	-7.81	7.9E-03	3.49	0.42	6.97	2.0E-06
Serpina3m	-94.46	4.2E-42	32.75	0.32	36.45	2.4E-44
Serpinf1	-3.01	4.8E-02	41.51	12.77	6.98	1.3E-10
Sfn	-9.43	4.1E-09	9.88	0.99	5.09	7.6E-08
Siglec1	-5.63	5.8E-03	3.12	0.51	2.58	2.0E-02
Slc16a10	-4.29	2.2E-03	4.62	1.00	2.49	7.5E-03
Slc24a5	-5.57	4.8E-04	8.92	1.47	4.23	1.5E-04
Slfn4	-7.12	1.1E-07	17.26	2.27	110.10	1.8E-32
Slfn5	-2.88	1.8E-02	4.80	1.57	4.64	7.5E-06
Slpi	-5.22	1.6E-02	18.72	3.30	294.73	8.3E-15
Sytl2	-8.32	6.5E-05	4.38	0.48	6.27	6.7E-06
Tagln	-2.41	1.4E-04	119.08	46.98	16.62	5.3E-44
Tbx20	-9.77	6.9E-08	8.15	0.76	7.51	6.0E-10
Tgm1	-3.71	6.8E-03	13.32	3.37	17.49	2.2E-15
Tnfrsf19	-3.22	1.4E-02	7.59	2.19	2.61	1.4E-03

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Gene ID	FC_WT_48_Hour_vs_FN_48_Hour	FDR_WT_48_Hour_vs_FN_48_Hour	WT_48_Hour_Avg_RPKM	FN_48_Hour_Avg_RPKM	FC_WT_0_Hour_vs_WT_48_Hour	FDR_WT_0_Hour_vs_WT_48_Hour
Tyr	-11.84	6.9E-06	11.23	0.86	5.07	2.6E-06
Ucp2	-2.45	1.1E-03	86.73	33.41	5.28	3.6E-19
Vat1l	-3.02	3.3E-03	41.47	12.74	2.90	5.1E-06
Vill	-3.70	9.4E-03	5.08	1.27	3.32	6.2E-04
Wnt16	-21.95	6.6E-07	15.33	0.63	50.65	5.1E-14

FC- Fold Change, WT- Wild Type, FN- Fibronectin conditional knockout, FDR- False Discovery Rate, Avg- Average

Table A6: The expression of genes that encode different integrins at 48 hours PCS between WT and FNcKO LCs.

Gene ID	Gene description	Fold change (FC) from WT 0 hour to WT 48 hours PCS in LCs	False discovery rate (FDR) WT 0 hr vs. WT 48 hours PCS in LCs	Fold change (FC) from WT to FNcKO at 48 hours PCS in LCs	False discovery rate (FDR) WT 48 hours vs. FNcKO 48 hours PCS in LCs	WT Mean RPKM At 0 hour PCS	WT Mean RPKM At 48 hours PCS	FNcKO Mean RPKM At 48 hours PCS
Itgav	Integrin alpha V	1.26	0.248	1.13	1	45.50	66	71.5
Itga5	Integrin alpha 5	7.22	5.19E-24	1.05	1	6	49.5	50.44
Itgb1	Integrin beta 1	2.54	4.13E-05	-1.08	1	110	325	284.53

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Appendix B

SUPPLEMENTAL FIGURES

Figure B1: Representative figures of flow cytometric analysis of post cataract surgical lens cell suspensions. Single-cell suspensions obtained after enzymatic digestion of post cataract surgical samples were permeabilized and stained with antibodies against αSMA (right side of the gate marked as alpha SMA) or isotype control antibody (left side of the gate marked as negative) as described in methods. The gate marked as alpha SMA in histograms reveals events that stained with αSMA. This experiment had N=3 from each of post cataract surgical time point. A- Isotopic control; B- WT 0 hour PCS; C- FNcKO 0 hour PCS; D- WT 5 days PCS; E- FNcKO 5 days PCS.

Figure B2: Aquaporin 0 expression is undetectable at 0 hour PCS in both WT and FNcKO capsular bags while by 5 days PCS, significant levels of Aquaporin 0 is detected (WT **$P < 0.002$, FNcKO *$P < 0.032$). In addition to that, comparable levels of Aquaporin 0 expression are detected by Immunofluorescence and ImageJ (T= 48 hours, $P = 0.5495$; T= 5 days, $P = 0.9013$) both at 48 hours and 5 days PCS in WT and FNcKO capsular bags. Aquaporin 0 (red) is merged with DNA detected by Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule. All experiments had N = 3. Values are expressed as mean $\pm$ SEM. Asterisks (*)

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indicate a statistically significant change in MFI between WT and FNcKO at a PCS or between two PCS time points.

Figure B3: Colocalization of collagen I (A) and tenascin C (B) with cell associated fibronectin post cataract surgery (PCS) is detected at 5 days in WT lens cells (yellow). Fibronectin (green), collagen I and tenascin C (red) are merged with DNA detected by Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule.

Figure B4: FNcKO lens cells are positive for thrombospondin 1(THBS1) and extracellular matrix protein 1 (ECM1) staining at 5 days PCS although attenuated levels of THBS1 protein are detected in FNcKO LCs compared to WT LCs (**P = 0.003). αSMA (green), ECM1, and THBS1 (red) merged with DNA staining Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule. All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate a statistically significant change in MFI between WT and FNcKO at a PCS or between two PCS time points.

Figure B5: Adult lens epithelial cells stain robustly for pSMAD1/5/8, a downstream mediator of BMP signaling. pSMAD1/5/8 (red) merged with DNA staining Draq5 (blue). Scale bars: 35 μm. C, capsule; LC, remnant lens epithelial cells/lens cells; Tz, transition zone.

Figure B6: Gremlin-1 is more potent than active TGFβ1 in inducing periostin expression in FNcKO capsular bags at 5 days PCS. FNcKO LCs do not upregulate periostin protein levels at 5 days PCS (P = 0.919], while WT LCs do (**P = 0.005). Treatment of FNcKO capsular bags with active TGFβ1

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rescues the defect in periostin expression to levels qualitatively similar to WT [WT vs FNcKO (TGFβ1) P = 0.249], while gremlin-1 treated FNcKO lens cells exhibit qualitatively and quantitatively higher levels of periostin protein expression at 5 days PCS [WT vs FNcKO (gremlin-1) **P = 0.010], [FNcKO (TGFβ1) vs FNcKO (gremlin-1) ***P < 0.001]. Periostin (red), αSMA (green) and DNA detected by Draq5 (blue). Scale bars: 35 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule. All experiments had N = 3. Values are expressed as mean ± SEM. Asterisks (*) indicate statistically significant MFI between WT and/or FNcKO and/or FNcKO (TGFβ1) and/or FNcKO (gremlin-1) at 5 days PCS.

Figure B7: Precocious elevation of fibrotic response and activation of TGFβ signaling is not detected at 24 hours PCS either the addition of exogenous active TGFβ1 or gremlin 1 to WT capsular bags following cataract surgery. αSMA (green), pSMAD2/3 (red) merged with DNA staining Draq5 (blue). The positive αSMA staining reflects the endogenous expression of this protein that is known to exist in unoperated lens epithelial cells that is seen here due to the use of confocal settings that allow viewing of this basal expression Scale bars: 17 μm. LC, remnant lens epithelial cells/lens cells; C, lens capsule.

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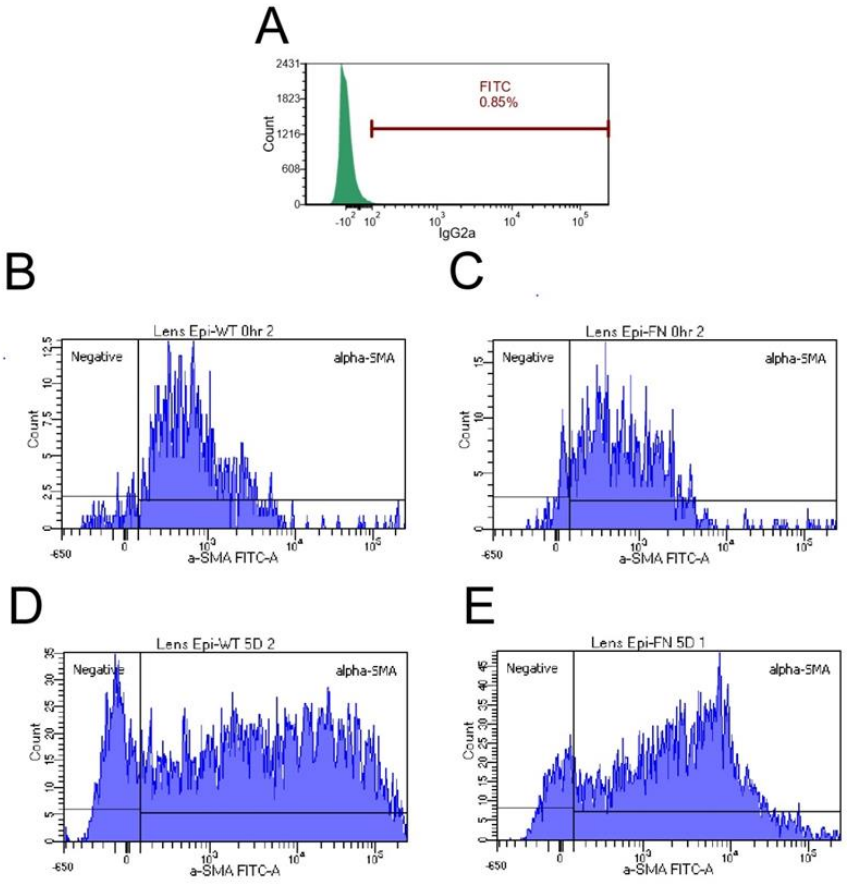

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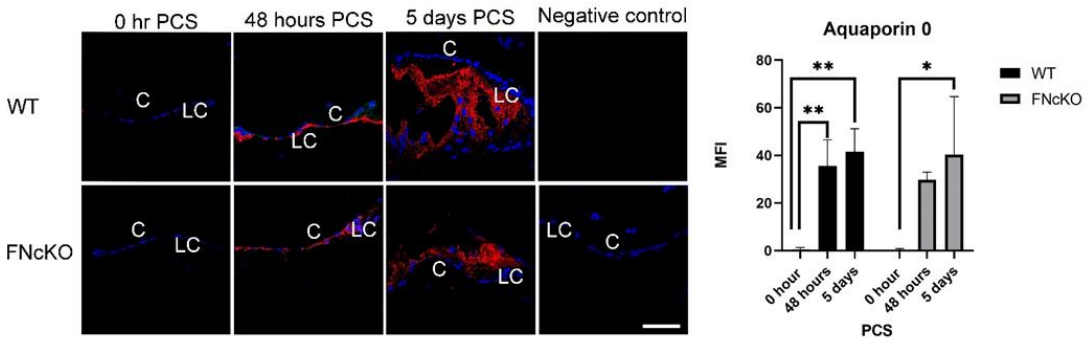

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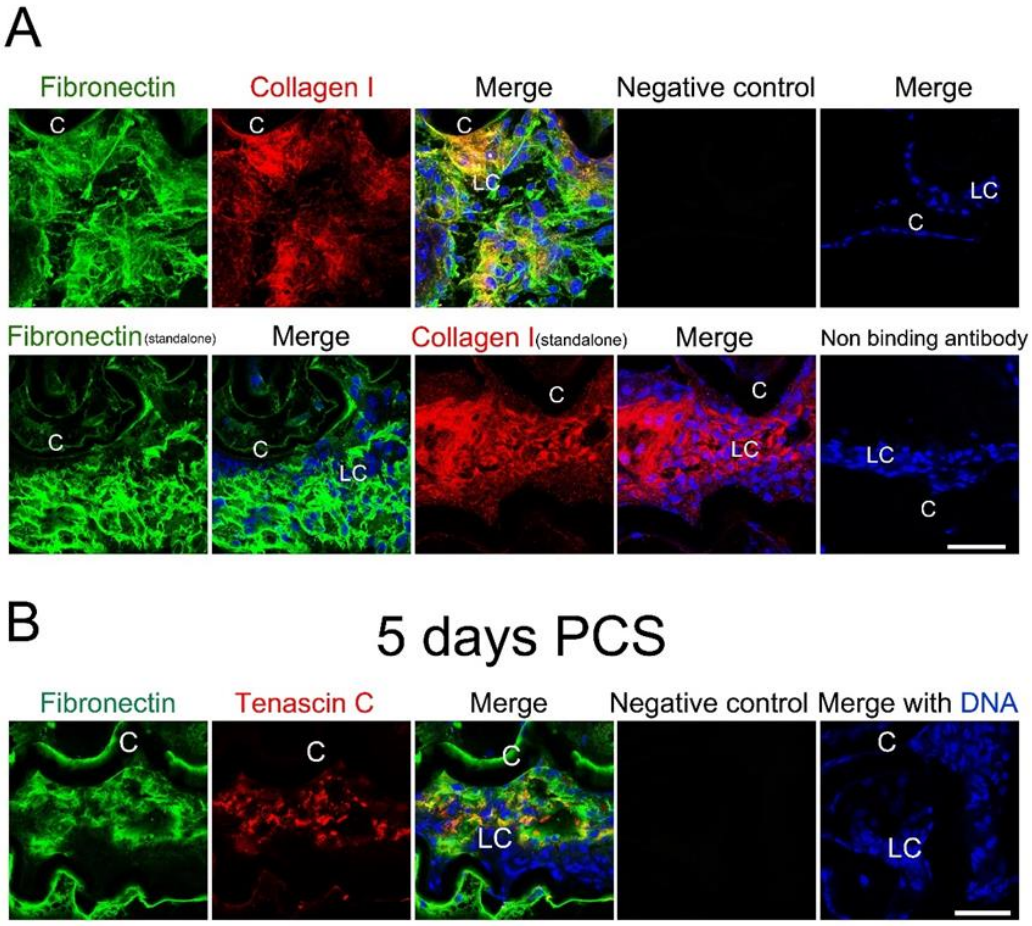

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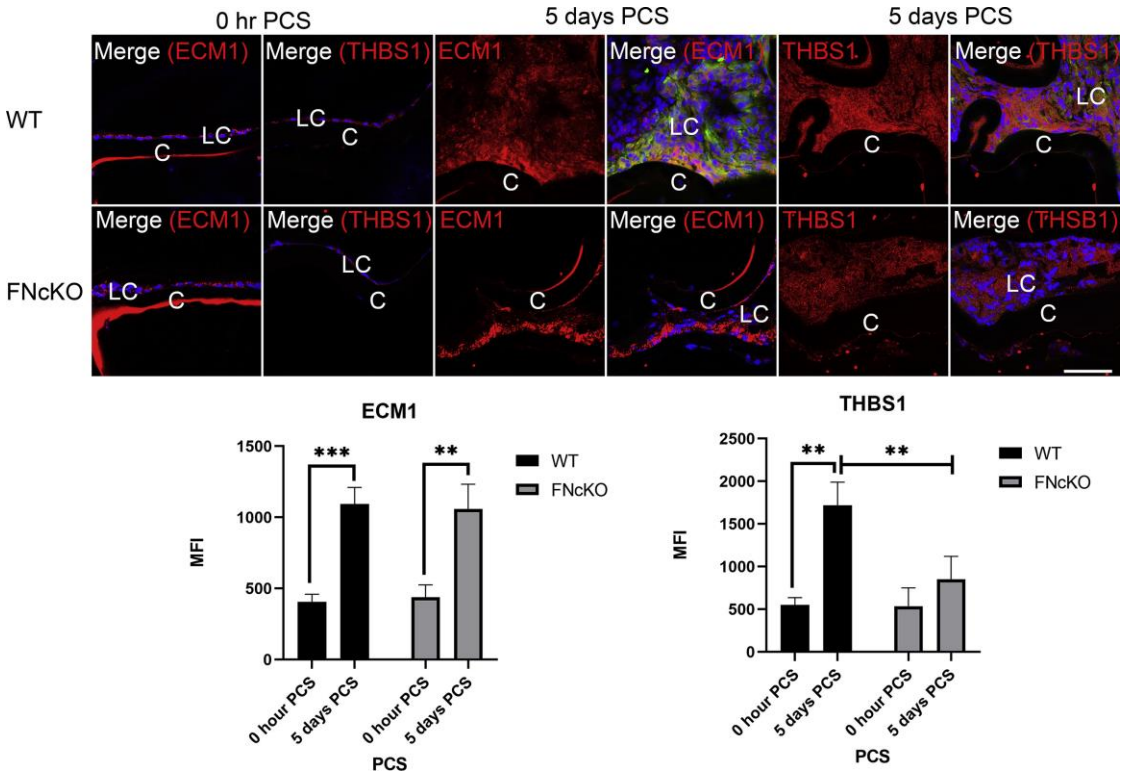

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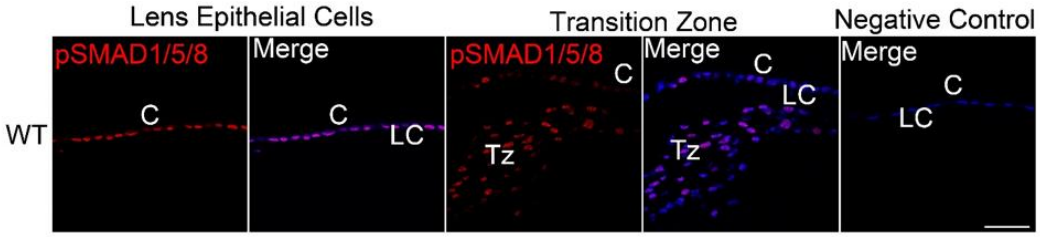

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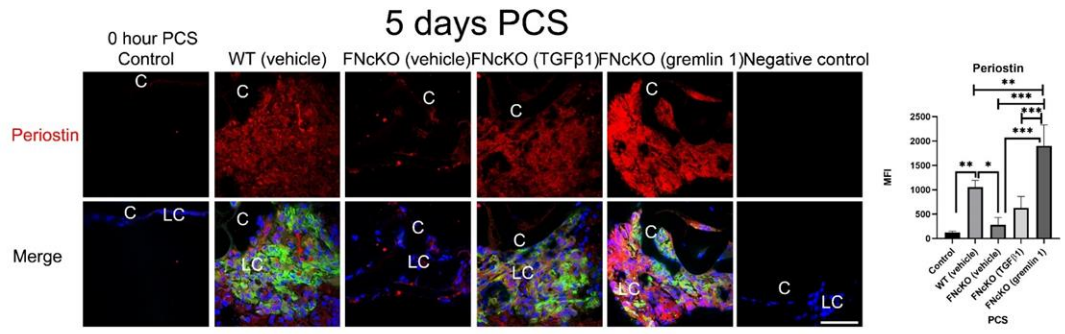

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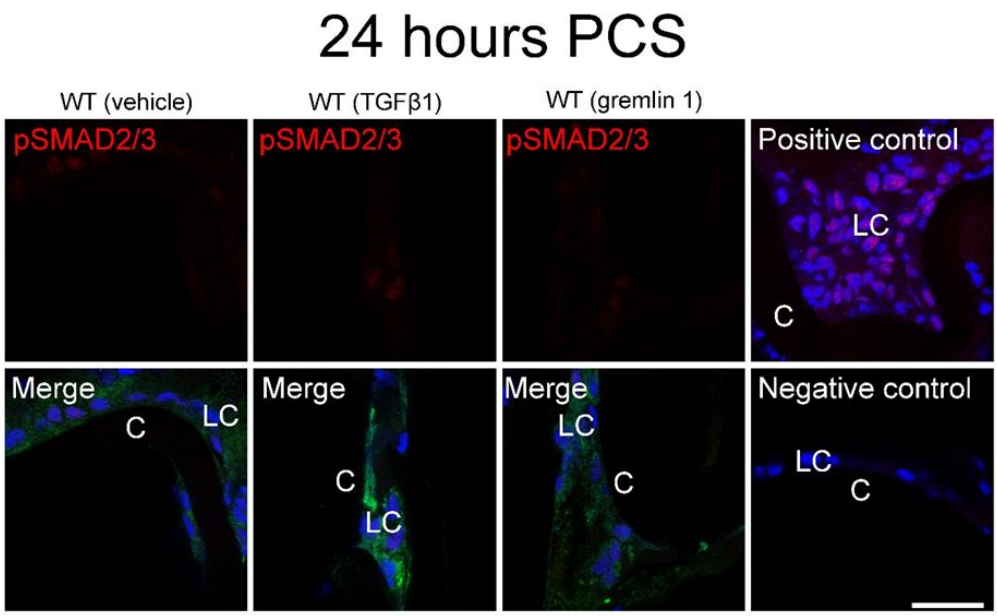

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Appendix C

INSTITUTIONAL APPROVALS

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RESEARCH OFFICE

210 Hullihen Hall
University of Delaware
Newark, Delaware 19716-1551
Ph: 302/831-2136
Fax: 302/831-2828

DATE: April 24, 2018

TO: Melinda Duncan, Ph.D.
FROM: University of Delaware IRB

STUDY TITLE: [1223629-1] market research on PCO therapeutics

SUBMISSION TYPE: New Project

ACTION: DETERMINATION OF NOT HUMAN SUBJECTS RESEARCH
DECISION DATE: April 24, 2018

Thank you for your submission of New Project materials for this research study. The University of Delaware IRB has determined this project does not meet the definition of human subject research under the purview of the IRB according to federal regulations.

We will put a copy of this correspondence on file in our office.

If you have any questions, please contact Maria Palazuelos at (302) 831-8619 or mariapj@udel.edu. Please include your study title and reference number in all correspondence with this office.

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RESEARCH OFFICE
210 Hullihen Ha
University of Delaware
Newark, Delaware 19716-1551
Ph: 302/831-2136
Fax: 302/831-2828

UNIVERSITY OF DELAWARE

DATE:	September 10, 2012
TO:	Melinda Duncan, Ph.D.
FROM:	University of Delaware IRB
STUDY TITLE:	[375348-1] Use of human cadaver eyes in research
SUBMISSION TYPE:	New Project
ACTION:	DETERMINATION OF NOT HUMAN SUBJECTS RESEARCH
DECISION DATE:	September 10, 2012

Thank you for your submission of New Project materials for this research study. The University of Delaware IRB has determined this project does not meet the definition of human subject research under the purview of the IRB according to federal regulations.

We will put a copy of this correspondence on file in our office.

If you have any questions, please contact Jody-Lynn Berg at (302) 831-1119 or jlberg@udel.edu. Please include your study title and reference number in all correspondence with this office.

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University of Delaware
Institutional Animal Care and Use Committee
Annual Review

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IACUC

Title of Protocol: Investigation of developmentally important genes in mouse and chicken	
AUP Number: 1039-2019-1	← **(4 digits only)**
Principal Investigator: Melinda Duncan	
Common Name: mouse, rat, chicken **Genus Species:** Mus musculus, Rattus rattus, Gallus gallus	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***

Category	Description
☐ B	Breeding or holding where NO research is conducted
☐ C	Procedure involving momentary or no pain or distress
☒ D	Procedure where pain or distress is alleviated by appropriate means (analgesics tranquilizers, euthanasia etc.)
☐ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

Official Use Only

IACUC Approval Signature: [signature]

Date of Approval: 12.1.19

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Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist listed on this AUP. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with **consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.**
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*
12. I understand that by signing, I agree to these assurances. _____ Signature of Principal Investigator _____ Date

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SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Melinda K. Duncan	
2. Yan Wang	
3. Samuel Novo	
4. Mahbubul Shihan	
5. Joseph StevensonClick here to enter text.	
6. Imane Assakhi	
7. Nicole Rossi	
8. Hira Peracha	
9. Ananya Garg	
10. Click here to enter text. Adam Faranda	
11. Click here to enter text.	
12. Click here to enter text.	
13. Click here to enter text.	
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IACUC approval of animal protocols must be renewed on an annual basis.

1. Previous Approval Date: 12/1/2018

Is Funding Source the same as on original, approved AUP?

X Yes **No**

If no, please state Funding Source and Award Number: Click here to enter text.

2. Record of Animal Use:

Common Name	Genus Species	Total Number Previously Approved	Number Used To Date
1. Mouse	Mus musculus	15,000	3003
2. Rat	Rattus rattus	100	0
3. Chicken	Gallus gallus	60	0
4. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Protocol Status: *(Please indicate by check mark the status of project.)*

Request for Protocol Continuance:

☒ A. Active: Project ongoing

☐ B. Currently inactive: Project was initiated but is presently inactive

☐ C. Inactive: Project never initiated but anticipated starting date is:

Click here to enter text.

Request for Protocol Termination:

☐ D. Inactive: Project never initiated

☐ E. Completed: No further activities with animals will be done.

4. Project Personnel: Have there been any personnel changes since the last IACUC approval?

☒ Yes ☐ No all amendments have been previously filed

If Yes, fill out the Amendment to Add/Delete Personnel form to "Add" Personnel.

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Project Personnel Deletions:

Name	Effective Date
1. Click here to enter text.	Click here to enter text.
2. Click here to enter text.	Click here to enter text.
3. Click here to enter text.	Click here to enter text.
4. Click here to enter text.	Click here to enter text.
5. Click here to enter text.	Click here to enter text.

5. **Progress Report:** If the status of this project is 3.A or 3.B, please provide a brief update on the progress made in achieving the aims of the protocol.

We have submitted our work on analyzing the injury responses of fibronectin lens knock out mice for publication, have learned numerous new things about the Pax6 mutant mice via RNAseq and have completed our microCT analysis of their eyes (data analysis in progress). We are finishing up the Bin3 and crystallin mutant analysis projects and how to have these papers submitted this year. We are finishing up our analysis of beta8 integrin null mice and have preliminary results showing that this is a good therapy target for posterior capsular opacification. We are still in the progress of making/analyzing several new strains (Runx1, mmp14, alpha5 integrin etc).

6. **Problems or Adverse Effects:** If the status of this project is 3.A or 3.B, please describe any unanticipated adverse events, morbidity, or mortality, the cause if known, and how these problems were resolved. If there were none, this should be indicated.

none

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Appendix D

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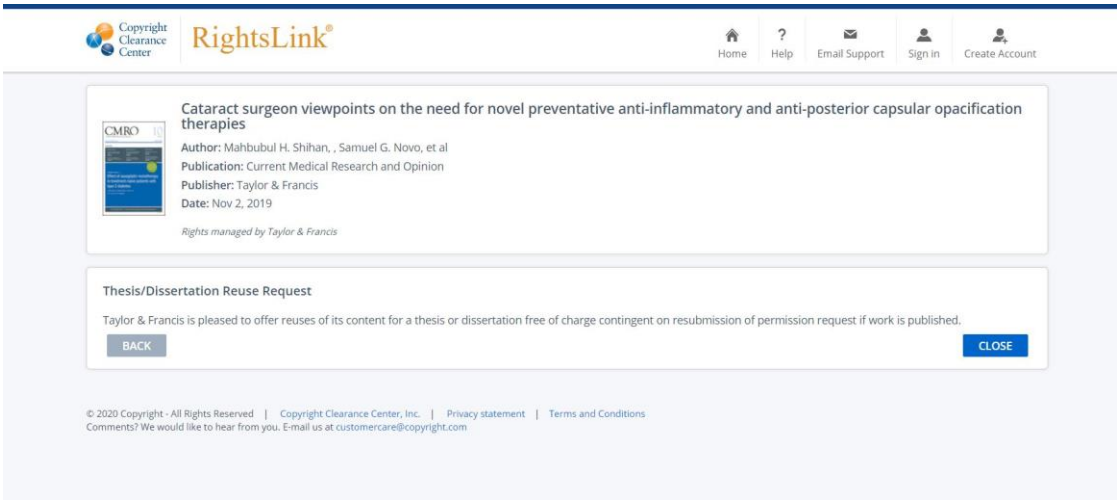

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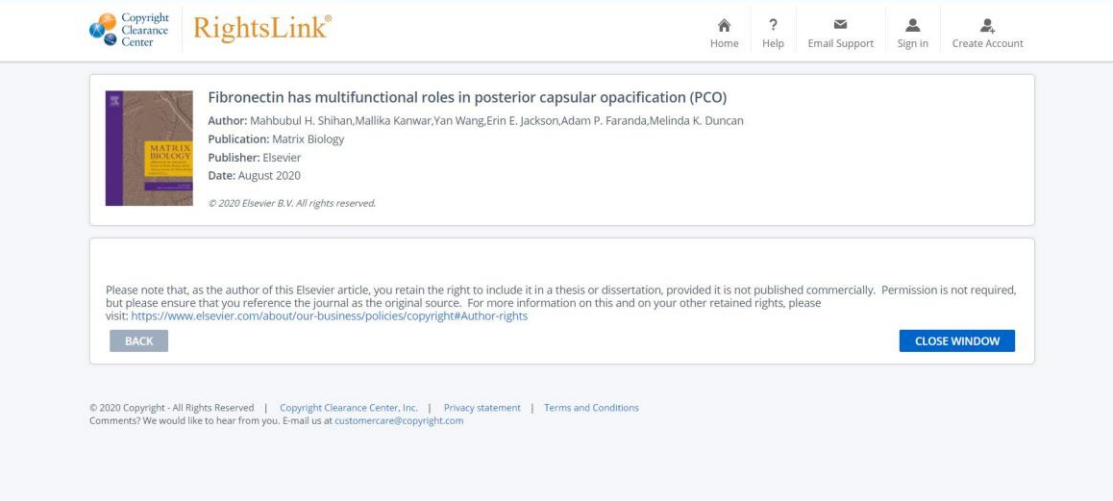

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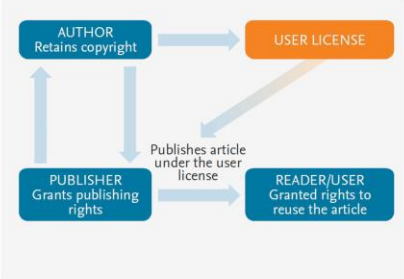

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