

**REGULATION OF LENS PHENOTYPE VIA TRANSLATIONAL
REPRESSION OF RETINAL MARKERS BY LACTASE LIKE & AN
INVESTIGATION OF POSTERIOR CAPSULAR OPACIFICATION**

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the Honors Bachelor of Science in Biological Sciences with Distinction

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REPRESSION OF RETINAL MARKERS BY LACTASE LIKE**

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ABSTRACT

The mammalian eye consists of many structures that work together to allow for proper vision. Importantly, the lens, a transparent structure which sits behind the cornea and iris, must develop properly and remain clear, otherwise it can cloud and form a cataract. A major pathway responsible for lens development is fibroblast growth factor (FGF) signaling. During this process, lens epithelial cells differentiate into fiber cells. Some fibroblast growth factors require extra proteins, klothos, to assist in binding their receptors. While alpha and beta family klothos are not expressed in the lens, gamma family klotho (LCTL) is expressed highly in adult lens and LCTL null mice develop mild lens opacities. However, the mechanisms underlying its function in the lens are unknown. Previous RNA sequencing of LCTL null lenses revealed that they upregulate the expression of retinal genes and neural markers, leading to my hypothesis that LCTL plays a role in repressing retinal genes in lens cells. However, this result could also simply be due to contamination of the dissected lenses used in the experiment with retinal tissue. Preliminary protein data is discordant with the upregulation of retinal gene expression observed via RNA sequencing. It is possible that this results from translational control of the upregulated messages, or contamination of the prior experiment with retinal tissue. Future testing of LCTL lenses by RT-qPCR, and investigation of the role of LCTL in lens injury responses is required to determine if LCTL plays alternative roles lens development and maintenance.

Chapter 1

INTRODUCTION: THE LENS AND CATARACT

1.1 Lens Structure and Development

The mammalian eye, the organ that receives and sends sensory information allowing us to visualize our environment, is composed of a complex of collaborative structures. The cornea and the lens refract incoming light, in the form of photons, to the back of the eye where the retina resides (Bassnett et al., 2011; Kellogg Eye Center). The retina then sends the incoming light as a neural signal to the brain, where it is processed as an image with colors, shapes, and dimension (Bassnett et al., 2011; Kellogg Eye Center). The ocular lens is a transparent biconvex structure that sits behind the cornea. It is composed of three important structures: fiber cells, the epithelial layer, and the capsular membrane (Bassnett et al., 2011) (Danysh & Duncan, 2009).

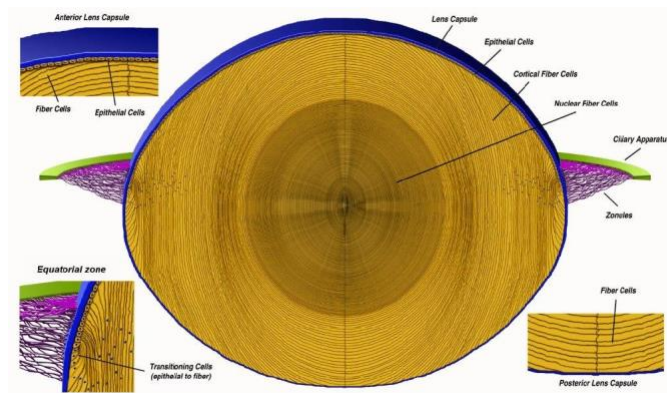


Figure 1: Components of the vertebrate lens (Danysh, BP., Duncan, MK., 2009).

Fiber cells the most abundant lens cells. They differentiate and elongate in response to Fibroblast Growth Factor Signaling (Robinson, 2006) from lens precursor cells (Cvekl & Duncan, 2007). If the lens precursor cells retain their original morphology, they progress into epithelial cells and establish the anterior epithelial lining of the lens (Cvekl & Duncan 2007). A capsular bag surrounds the entirety of the lens (Danysh & Duncan 2009). It is composed of laminin and collagen IV which form networks to protect and shape the lens (Danysh & Duncan, 2009).

The major structural features of the lens first begin as a sheet of surface ectoderm (Robinson, 2006). Following the introduction of many growth factors and inductive agents, the presumptive lens ectoderm comes in contact with the embryonic optic vesicle (Robinson, 2006). The lens ectoderm thickens to form the lens placode, which invaginates forming the optic cup (Lovicu et al., 2011;Robinson, 2006). The cells in the posterior lens placode elongate toward the anterior side of the developing lens, forming the epithelium (Lovicu et al., 2011). These epithelial cells generally retain the ability to proliferate. However, once differentiation progresses, they subsequently lose the ability to proliferate (Robinson, 2006;Lovicu et al., 2011). Secondary fiber cells develop later in differentiation and add layers to the lens. The differentiated primary and secondary fiber cells withdrawal from the cell cycle, which causes their elongation and inability to proliferate (Lovicu et al., 2011). Notably, Fibroblast Growth Factors (FGFs) and Prox1 are important for fiber cell differentiation and lens development (Lovicu et al., 2011;Audette et al., 2016). Paracrine FGF signaling requires a co-factor, heparan sulfate glycosaminoglycans (HSGAGS), in order to stabilize the FGF receptor (Frontiers, 2016). Once FGF is

bound, the complex dimerizes and activates tyrosine kinase domains and eventually leads to an intracellular signaling pathway (Frontiers 2016).

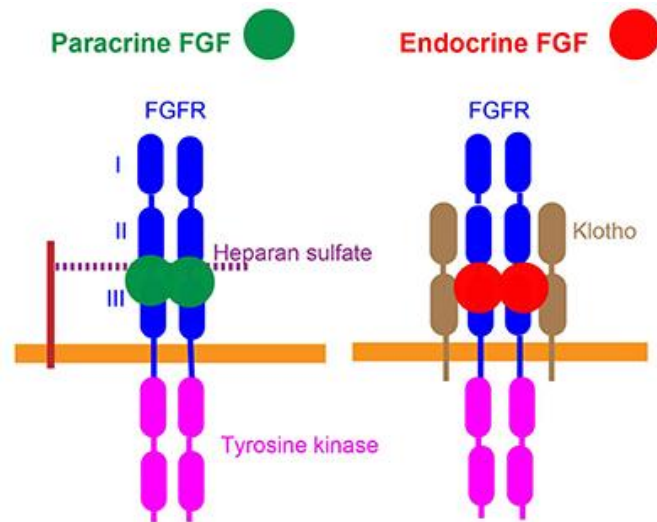


Figure 2: Independent and dependent klotho FGF signaling pathways (Itoh, N., Nakayama, Y., Konishi, M., 2016).

Fibroblast Growth Factors 15/19 are members of the endocrine family of FGFs (Fon Tracer et al., 2010). FGF15 is the mouse ortholog of the human FGF19 (Robinson, 2006). FGF15/19 have a low binding affinity for HSGAGS (Fon Tracer et al., 2010). Therefore, FGFRs recruit a klotho protein, through proposed interactions with lipid rafts on the plasma membrane (Dalton et al., 2017), to assist in binding FGFs to their receptors (Fon Tracer et al., 2010). Klotho proteins are single pass transmembrane proteins (Fon Tracer et al., 2010;Frontiers, 2016). Alpha, beta, and gamma klotho proteins have been identified. The gamma klotho, also referred to as Lactase-Like (LCTL), is the least studied of the three genes (Fon Tracer et al., 2010).

The transcription factor Prox1 is also responsible for lens development and fiber cell differentiation by promoting cell cycle exit (Audette et al., 2016). Prox1 expression increases during lens fiber differentiation. Eyes without Prox1 initially develop normally, except posterior lens cells do not differentiate into fiber cells (Wigle et al., 1999). Crystallins, structural protein elements in lens fiber cells, fail to localize to the fiber cells following Prox1 deletion (Audette et al., 2016). Prox1 conditional knockout lenses also resulted in a decrease expression of specific FGFs and the gamma klotho co-receptor, LCTL (Audette et al., 2016). FGFR-mediated signal transduction, such as mitogen-activated protein kinase (MAPK) or PI3K/ATK pathways that stimulate lens fiber differentiation (Wang et al., 2009) was also decreased following the Prox1 knockout and decreased FGFR expression (Audette et al., 2016). Western blot analysis revealed attenuation of Prox1 and FGF response during lens fiber differentiation with the addition of a MAPK inhibitor (Audette et al., 2016). However, no response was shown for PI3k/ATK inhibition. These results suggest that the expression of Prox1 and onset of FGF signaling during lens fiber cell differentiation are regulated by FGFRs and a MAPK-mediated signaling pathway (Audette et al., 2016).

Following Prox1 deletion, the expression of many genes downregulates in the lens, such as LCTL. Since LCTL is required for the binding of FGF15/19 to FGF receptors, Prox1 deletion would ultimately halt the binding response of FGF15/19 to their endocrine receptors (Audette et al., 2016). It is possible that Prox1 expression leads to the upregulation of LCTL during development in order to sensitize fiber cells to FGF15/19 (Audette et al., 2016). In epithelial cells, the lack of Prox1 expression causes lower levels of LCTL, and therefore cells would be less likely to respond to

FGF15/19 (Audette et al., 2016). If the FGFs cannot bind to their receptors due to the interruption of LCTL expression, then the FGF signaling pathways will not progress and the cells will not differentiate into fiber cells. Based on these findings, LCTL may be vital for differentiation during lens development.

1.2 Vision and cataract

In order for the eye to receive incoming light signals properly, the lens must remain clear. Since lens function requires that the tissue remain transparent, any deformities or defects could cloud the lens and impair vision. The clouding of the ocular lens is classified as a cataract. Cataracts are more commonly seen in older adults, although pediatric cataracts are possible as well. By the age of 65, more than 90% of people have some type of cataract or lens clouding (Kellogg Eye Center). Since cataract is a very common and burdensome disorder, it is important that we understand the science behind its progression in order to develop better treatments for those suffering.

Cataracts can occur due to injury or age which affects the tissues that comprise the ocular lens. As you age, the crystallin proteins, which help maintain the shape and stability of the lens, break down and clump together, leading to blurred vision (Moreau & King, 2012). Since lens fiber cells develop *in utero*, the mature cells lack the tools required for degradation and synthesis of aggregated crystallins in the lens interior (Moreau & King, 2012). A similar process happens when injury occurs to the eye or lens. As the healing response occurs, the regenerating tissue is misorganized, and the lens loses transparency.

Cataract can be treated by surgery. During cataract surgery, the clouded lens is removed and replaced with an artificial intraocular lens (Chong et al., 2009). Although

mostly effective, surgery can result in side effects such as inflammation and Posterior Capsular Opacification (PCO) (Chong et al., 2009). PCO, also termed as secondary cataracts, occurs when residual cells that remain on the lens capsule after initial surgery migrate and proliferate to the posterior lens capsule, causing the visual axis to cloud again (Chong et al., 2009; Wormstone 2002). PCO can be treated with Nd-YAG laser capsulotomy in attempt to restore some vision (Chong et al., 2009). However, this treatment can lead to further complications such as retinal detachment or cystoid macular edema (Sabbagh, Review of Ophthalmology). Inflammation also occurs after cataract surgery which can lead to delayed recovery, glaucoma, ocular hypertension, and other problematic side effects. Although both inflammation and PCO can be treated, it would be beneficial for ophthalmologists and patients to prevent these events from occurring, in order to avoid painful or risky procedures, and to improve the final visual outcome of cataract treatment (Shihan et al., 2019).

PCO causes lens clouding because the proliferating remnant epithelial cells migrate to the posterior lens capsule following surgery, while undergoing epithelial to mesenchymal transition (EMT) to form opaque scar tissue in the visual axis (Chong et al., 2009).

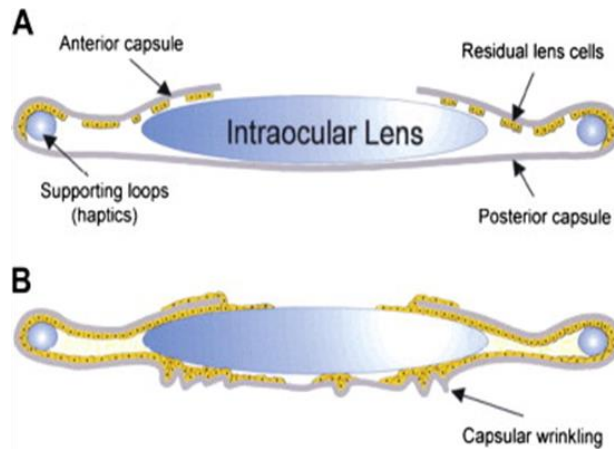


Figure 3: Posterior Capsular Opacification post-cataract surgery. Epithelial lens that remain on the anterior epithelium migrate to the posterior capsule and proliferate causing lens clouding (Wormstone, 2009).

Notably, EMT allows polarized epithelial cells to assume a mesenchymal cell phenotype (Kalluri & Weinberg, 2009). Mesenchymal cells are multipotent stromal cells that have roles in the fibrotic response, such as that of PCO. It is apparent that activation of TGF β receptors leading to Smad2/3 phosphorylation is an important player in fibrotic PCO as this regulates the transcription of fibrotic genes leading to scar tissue formation (Chong et al., 2009; Yang et al., 2003).

1.3 The Importance of LCTL

Lactase-like, also known as Klotho-related protein (KLPH), was first discovered through the NEIBank project that aimed to sequence the transcriptome of eyes tissues (Audette et al., 2016; Wistow et al., 2008; Wistow, 2006). LCTL was found as a novel protein that was highly expressed in humans lens tissue. Additional research showed LCTL's potential contributions to lens fiber cells differentiation via

FGF signaling (Audette et al., 2016). LCTL conditional knockout lenses in mice showed signs of early onset cataract in old age (Fan et al., 2018), which is more evidence for its implications in fibrosis and lens cell differentiation. In order to further investigate the function of this protein in the lens, RNA sequencing was performed on LCTL knockouts and wild-type lens cells (Fan et al., 2018). Interestingly, a few retinal transcripts appeared to be upregulated in the LCTL KO in comparison to low levels in the wild-type lenses (Fan et al., 2018). Researchers presumed that this difference was due to contamination during dissection. However, repeated RNA sequencing of LCTL KOs revealed similar levels of retinal and neural transcripts while similarly treated control lenses did not. Because we know of potential roles that LCTL plays in fiber cell differentiation in the lens, and the upregulation of retinal markers accompanying the knockout, I believe that LCTL has another function that is yet to be discovered. I hypothesize that LCTL regulates lens homeostasis by repressing markers of the retinal phenotype.

1.4 α V β 8-Integrin's Influence on Posterior Capsular Opacification

During my undergraduate studies and senior thesis project, I also made contributions to the work of Ph.D. student Mahbubul Shihan. His project investigated how specific transmembrane proteins contribute to inflammation and fibrosis after cataract surgery. Integrins, are heterodimeric integral membrane proteins consisting of an alpha and beta subunit. Integrins of the α v family have important function in lens cells (Mamuya, Duncan, 2012). α v integrins are upregulated at the protein level in lenses post-cataract surgery and helps regulate the epithelial mesenchymal transition (EMT) that is responsible for fibrotic events such as PCO (Mamuya, Duncan, 2012; Mamuya et al., 2014).

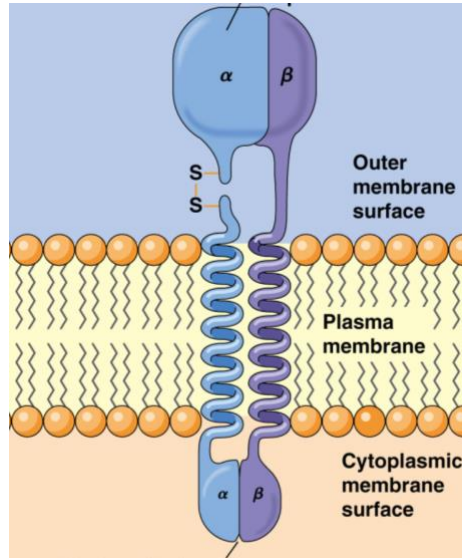


Figure 4: Alpha and beta subunit structure of a heterodimeric integrin within a plasma membrane (Staveley via Pearson Education 2012).

Following lens fiber cell removal, such as one would do during cataract surgery, subunits $\beta 1$, $\beta 5$, $\beta 6$, and $\beta 8$ were upregulated (Mamuya et al., 2014). In addition, lenses that lack αv integrins do not show robust proliferation after fiber cell removal, show low levels of α -SMA expression (a fibrotic marker), and decreased mesenchymal molecules (Mamuya et al. 2014). These results indicate that αV integrin is important for EMT during fibrotic PCO development (Mamuya et al. 2014) although the identity of the functional receptor was unknown.

Mahbubul Shihan found that lenses lacking either the $\beta 5$ or $\beta 6$ integrin subunit had normal responses to cataract surgery, so he began investigating the role of a lesser known beta subunit of the αV integrin, $\beta 8$ integrin, by making mice conditionally lacking the gene from the lens. He found that WT lens cells showed robust expression of α -SMA at 5 days post-cataract surgery (PCS) while the $\beta 8$ cKO showed practically no expression of this fibrotic marker.

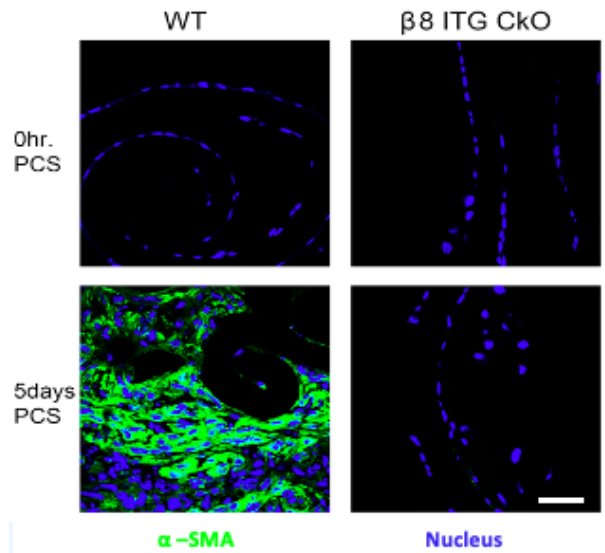


Figure 5: Deletion of the $\beta 8$ subunit downregulates expression of α -SMA 5 days PCS (Image from Mahbubul Shihan).

This and more evidence suggests that αV integrin and its $\beta 8$ integrin binding partner are essential for events that take place post-cataract surgery. Knowing that $\alpha V\beta 8$ integrin regulates PCO and EMT, while other $\alpha V\beta$ heterodimers regulate inflammation following injury in other systems (Breuss et al., 1995), I wanted to test the hypothesis that $\alpha V\beta 8$ integrin plays a role in post-cataract surgery inflammation. It would be beneficial to determine if $\alpha V\beta 8$ integrin regulates this process because untreated post-operative inflammation can lead to pain, photophobia, elevated intraocular pressure, glaucoma, and many other serious complications (Bodh et al., 2011).

Chapter 2 METHODOLOGY

2.1 Mouse Culture

The LCTL genomic region of about 5.5kb was deleted through homologous recombination in mouse embryonic stem cells (Fan et al., 2018). An artificial chromosome was generated in 129/SV mice and used as a targeting vector for recombination. The DNA targeting vector was electroporated into R1 mouse embryonic stem cells. The knockout of LCTL gene was confirmed and 129/SV origin mice were crossed with C57BL6/J mice (Fan et al., 2018).

The $\beta 8$ integrin gene was targeted by establishing loxP sites at exon 4 in embryonic stem cells. Conditional knockout mice were generated by mating mice with the $\beta 8$ integrin allele where exon 4 was flanked by loxP site with mice that express Cre recombinase in the lens (Shihan et al., manuscript in preparation). The finished knockout mice were mostly on the C57BL/6J genetic background, although they do likely carry 129/SV alleles (Proctor et al., 2005).

All studies were performed under the Animal Use Protocol #1039 approved by the University of Delaware IACUC in 2019.

2.2 Lens Fiber Cell Surgery Protocol

Mice, aged 3 to 4 months, were anesthetized with a ketamine/xylazine solution. Mouse pupils were dilated with dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride ophthalmic solution. The eye was flushed with a balanced salt solution (BSS). A 2-3 mm central corneal incision was made ending at the anterior lens capsule. The lens fiber cells were separated from the lens capsule by hydrodissection with BSS. The corneal incision was closed with a 10-0 nylon suture,

and BSS was inserted into the anterior chamber to maintain its original shape. Mice were allowed to recover and ointment was applied to the eye to aid in healing processes. For analysis, mice were sacrificed by carbon-dioxide inhalation 0 to 48 hours post-surgery. Operated eyes were removed for immunofluorescence microscopy. At least three independent animals were analyzed for each experiment. All surgeries were performed by Yan Wang.

2.3 Immunofluorescence

2.3.1 LCTL

Whole mouse eyes were dissected from non-operated 3 month old adult mice and LCTL knockout mice. Lenses were frozen at -80° Fahrenheit and sectioned using a Leica CM3050 cryostat. Slides were fixed in a 1:1 acetone methanol solution for 15 minutes in 4° Fahrenheit. Slides were incubated in a blocking buffer solution using 2% BSA, 5% NGS, and a drop of Triton X100 in PBS for 1 hour. Primary antibodies (LCTL, RHO, TYRP1, TRPM1, GNAT1, PMEL) were applied in a 1:100 ratio with the blocking buffer and left to sit overnight in -4°. Slides were washed in PBS 3x for 5 minutes. A secondary antibody (anti-rabbit Alexafluor 568) was applied in a 1:200 dilution and draq5, nuclear stain, at a 1:1000 dilution in blocking buffer for 1 hour. Slides were washed 3x for 5 minutes and then mounted for microscopy analysis.

2.3.2 Beta 8 Integrin

Whole lenses dissected from non-operated 3 month old adult WT mice and null mice. Lenses were frozen at -80° Fahrenheit and sectioned using Leica CM3050 cryostat. Slides were fixed in a 4% paraformaldehyde solution for 15 minutes in 4° Fahrenheit. Then soaked in PBS for 5 minutes Slides were cured in a blocking buffer

solution using 2% BSA, 5% NGS, and a drop of Triton X100 in PBS for 1 hour.

Primary antibodies were applied in a 1:100 ratio with the blocking buffer and left to sit overnight in -4°. Slides were washed in PBS 3x for 5 minutes. Secondary anti-rabbit 568/anti-rat 488 was applied in a 1:200 ratio and draq5, nucleus stain, at 1:1000 ratio in blocking buffer for 1 hour. Slides were washed 3x for 5 minutes and then mounted for microscopy analysis.

2.4 Confocal Microscopy

Fluorescently labeled slides were visualized using a Zeiss 780 LSM confocal microscope (Carl Zeiss, Inc., Gottingen, Germany), and comparisons between 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.

Chapter 3 RESULTS AND DISCUSSION: THE ROLE OF LCTL

3.1 Confirming LCTL Knockout in Lens

Before investigating the effects of removing LCTL from the lens, I needed to produce LCTL null lenses in order to properly compare them to wildtype (WT) mouse lenses. Mutant and WT mouse lenses were dissected and the tissues were sectioned and mounted on slides according to the methodology above. I stained my knockout lenses and WT lenses with LCTL as the primary antibody in order to determine if LCTL was knocked out of the lenses.

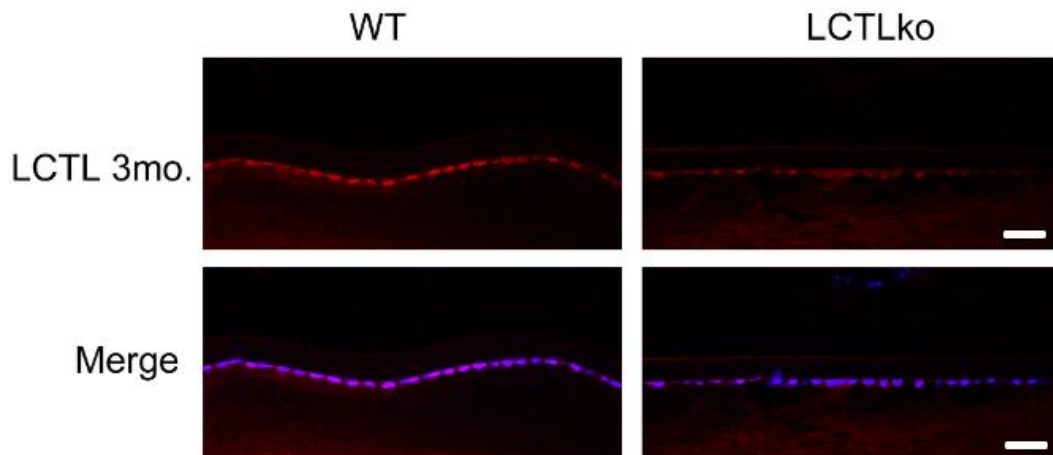


Figure 6: Expression of LCTL in 3 month old WT and LCTLko mouse lenses. LCTLcKO mice showed decreased expression of LCTL as compared to robust expression in WT lenses. Nucleus stain blue with DRAQ5.

Immunofluorescence staining revealed the downregulation of LCTL expression in the knockout lenses, which gave some confirmation that LCTL was successfully removed from the lens. However, the presence of residual staining suggests that the antibody

may not be strictly specific for LCTL. The loss of LCTL expression should be reanalyzed in the future by other methods such as western blotting and qPCR.

3.1.1 Confirming Markers in Retina

In order to test the efficacy of the secondary antibodies before experimentation, a retinal staining was performed in order to validate the antibodies were functional. All retinal markers (GNAT1 and TYRP1 shown) were stained for in WT 3 month old mouse retinas. Results revealed that markers are expressed in the retina in the expected cell type indicating that the antibodies used were for the specific gene of interest and were functional in immunolocalization studies.

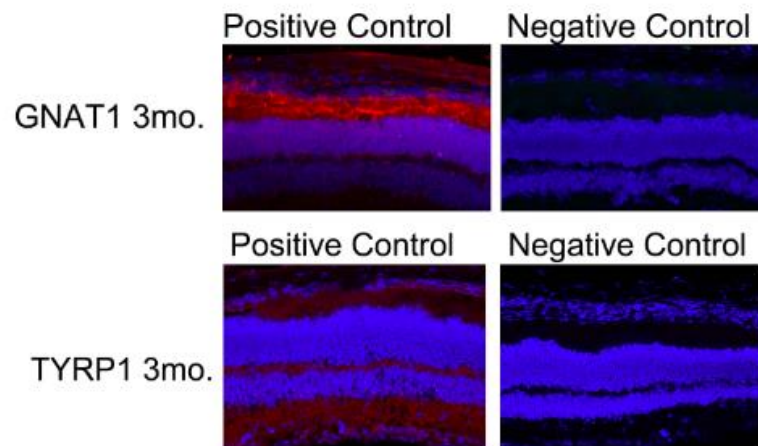


Figure 7: Expression of GNAT1 and TYRP1 in retina of WT adult mice under positive control and negative control conditions. Positive control condition included the application of primary and secondary antibodies while negative control lacked secondary antibodies.

3.2 RNA Sequencing Analysis

RNA sequencing data from LCTL null mice revealed the upregulation of several genes pertaining to the retinal pigmented epithelium or retinal function (Fan & Wistow, 2018). The initial response was to label these findings as an artifact of surgery since the retina and the lens are found quite close in proximity within the eye. However, as previously discussed, gamma klotho LCTL has a proposed role in lens fiber cell differentiation via the FGF signaling pathway (Audette et al., 2016).

(a)

Gene ID	P Value	RPKM - WT	RPKM – LCTL null
PMEL	5.00E-05	4.36019	115.652
TYRP1	5.00E-05	5.5513	74.9398
TRPM1	5.00E-05	8.50632	45.0414
DCT	5.00E-05	15.2708	244.148
RHO	5.00E-05	78.2528	26.6319
GNAT1	5.00E-05	41.4914	14.09

(b)

Gene ID	Description
PMEL	Transmembrane glycoprotein, synthesizes melanosomes
TYRP1	Enzyme found in melanocytes, help with production of melanin
TRPM1	Helps produce melanin through transportation of cations into cells
DCT	Plays role in melanogenic pathway
RHO	Produces rhodopsin, activated in photoreceptors of retina, transforms photons into neural signals.
GNAT1	Found in rods of retina, produces alpha subunit of transducin, transforms light signals into neural signals

Table 1: (a) Highest reads per kilobase million were the 6 chosen genes: PMEL, TYRP1, TRPM1, DCT, RHO, and GNAT1. (Data courtesy of Fan et al., Wistow, Dong, 2018 via NISC) (b) Descriptions and functions of the 6 most upregulated retinal markers in LCTL null lenses (vis NCBI Gene Database).

In order to test the hypothesis that LCTL maintains lens homeostasis by repressing retinal markers, the RNA sequencing data was examined for highly upregulated genes pertaining to the retinal phenotype. The table above (1a) lists the chosen retinal markers for experimentation. The descriptions of each gene is listed (1b). PMEL, TYRP1, TRPM1, and DCT generally manage melanin production. Melanin is a pigment located in the iris, choroid, and retinal pigmented epithelium (RPE) of the eye (Lapierre-Landry, Carroll, & Skala, 2018). It's main roles are to transport nutrients via blood vessels and to protect the retina from excess light and light-generated reactive oxygen species (Lapierre-Landry, Carroll, & Skala, 2018). RHO and GNAT1 are located in the photoreceptors of the retina. They assist in absorbing incoming light and converting the stimuli to a neural signal (Berg, Tymoczko, & Stryer, 2002). Although RHO and GNAT1 reads are higher in WT mice, their functions are important for maintain retinal function, hence why they were chosen.

3.3 Staining WT and LCTL null adult lenses

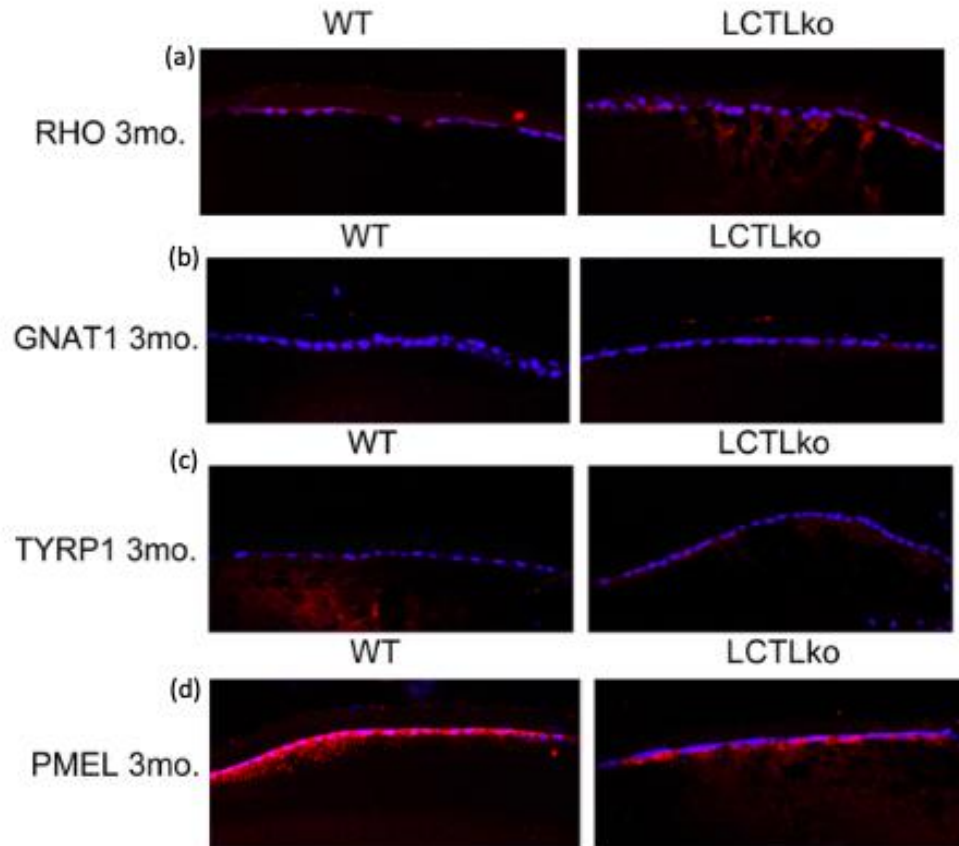


Figure 8: Expression of retinal markers in unoperated 3 mo. old WT and LCTL null mouse lenses. Expression levels for (a) RHO did not differ in the epithelial lining between WT and LCTL null lenses, but appears to express in lens fiber cell in LCTL null lenses. Protein expression for (b) GNAT1, and (c) TYRP1 did not differ between WT and LCTL null lenses. High expression of (d) PMEL was noted in WT mice and downregulates in the LCTL null mice.

Immunofluorescence staining revealed an unexpected result. I anticipated retinal markers to upregulate in the lenses without LCTL. However, the results show qualitatively equivalent protein expression of retinal markers between WT and LCTL null lenses. An exception to these results is PMEL staining, which revealed robust expression in the WT and downregulates in the LCTL null lenses. According to the RNA sequencing data, I anticipated that loss of LCTL would cause retinal proteins to be expressed in the lens. LCTL has implications in fiber cell differentiation and lens development via FGF signaling. Null lenses showed the upregulation of genetic material important for the retina. Therefore, I hypothesized that LCTL develops and maintains the lens phenotype by repressing retinal genes. However, my results are discordant with the preliminary RNA sequencing data. One explanation for these results is that a translational control mechanism is causing the retinal markers to not express on the protein level, even if they upregulate at the RNA level. Translational regulation is a fairly common cellular process. Examples of translational regulation within the eye include that of crystallin synthesis during lens development (Beebe, Piatigorsky, 1981). Alternatively, the upregulation of retinal markers was an artifact of isolating the tissues in the original analysis although it is typically easy to cleanly dissect lenses from retina.

Chapter 4

RESULTS AND DISCUSSION: THE ROLE OF β 8ITG

Previous research on post-cataract surgery (PCS) complications, such as PCO and inflammation, revealed the significance of α V integrin proteins (Mamuya et al., 2014; Mamuya, Duncan, 2012). α V integrins are upregulated PCS and during the EMT of PCO (Mamuya, Duncan, 2012). Subunits β 1, β 5, β 6, and β 8 of the α V integrin family were also upregulated PCS and during the fibrotic events of PCO. Some beta subunits are also known to regulate inflammation following surgery. It would be beneficial to determine is the lesser studied β 8 subunit does as well. Controlling inflammation from occurring could prevent dangerous post-operative inflammatory side effects. RNA sequencing was performed on WT mouse lenses PCS. RNA sequencing data was analyzed to determine which genes were upregulated in WT mice PCS. Table (a) lists the reads per kilobase of transcript, per million mapped reads (RPKM) which allows viewing of the more upregulated or downregulated genes at 24 hours after surgery. Table (b) displays the descriptions of highly upregulated genes from the RNA sequencing data. CXCL1 is a chemokine, a secreted growth factor that interacts through G-protein coupled receptors. CXCL1 has known roles in inflammation and as a white blood cell (WBC) neutrophil attractant, important for the wound healing response (Cao et al., 2014). Heterodimeric calcium binding protein S100A9 are released during the inflammatory response (Wang et al., 2018). As part of controlling inflammation, they stimulate leukocytes, another type of WBC and cytokines (Wang et al., 2018).

(a)

Gene ID	Fold Change from 0 Hour	P Value	24 Hours Mean RPKM	0 Hour Mean RPKM
CXCL1	3866	1.81E-55	58.5	0
S100a9	1505	1.86E-29	41.9	0
GCSF/CSF3	1119	7.57E-31	140.1	0.1
COX2	248	2.10E-38	49.7	0.2

(b)

Gene ID	Description
CXCL1	Recruits and activates neutrophils to tissues during immune response
S100a9	Facilitates neutrophil chemotaxis and adhesion, regulates leukocytes in inflammatory response
GCSF/CSF3	Stimulates production of granulocytes (WBC) for release into bloodstream
COX2	Enzyme induced by inflammatory cytokines at the site of inflammation, involved in fibrotic response

Table 2: (a) Most upregulated inflammatory mediators: CXCL1, S100a9, CSF3, and COX2 24 hours PCS in WT mice (Data courtesy of Mahbubul H. Shihan). (b) Descriptions and functions of the most upregulated markers in B8 null lenses (Sawant, 2016; Human Gene Database; Takai, 2013).

G-CSF, also known as CSF-3, is a granulocyte colony-stimulating factor which produces and controls neutrophil production (Boneberg, Hartung 2002). This cytokine is important for reducing inflammation after onset (Boneberg, Hartung 2002).

Lastly, cyclooxygenase- (COX-)2 was chosen as a well-known anti-inflammatory agent. COX2 is an enzyme which catalyzes the formation of prostaglandins (Fitzpatrick, 2004). Prostaglandins are lipids produced at the sight of injury and help maintain the inflammatory response (Ferrer et al., 2019).

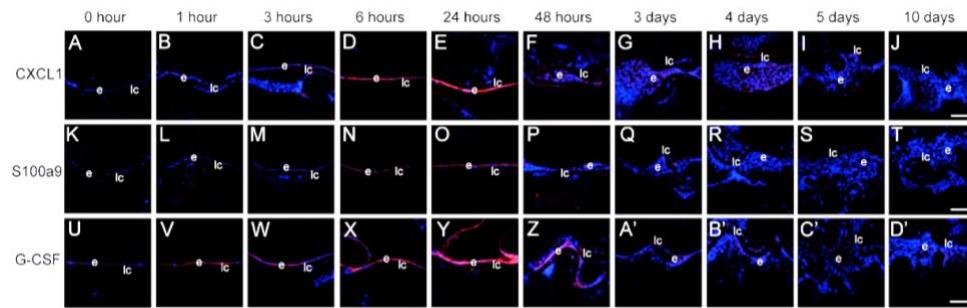


Figure 9: Preliminary data showed expression of inflammatory cytokines is robustly upregulated 24 hours PCS in WT mice (Jiang et al., 2018).

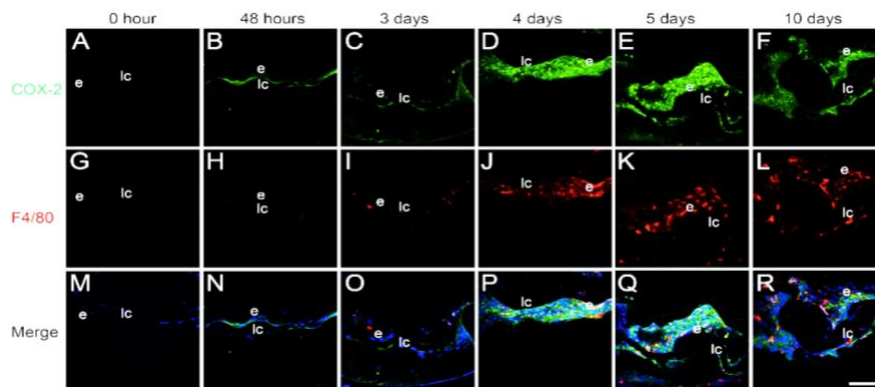


Figure 10: Time scale (0 hours to 10 days) of COX-2 and F4/80 macrophage expression in WT mice (Image courtesy of Mahbulul Shihan, 2018).

Before experimentation, initial staining of inflammatory markers was performed in WT mouse lenses from 0 hours to 10 days PCS. The immunofluorescence revealed that CXCL1, S100A9, and G-CSF were most robustly expressed on the protein level at 24 hours and COX2 was expressed the most around 5 days PCS. These time frames became the targets for staining in β 8ITG-cKO mice. WT and β 8 null lenses were stained with each of the most upregulated inflammatory cytokines 0 hours and 24 hours after lens fiber cell removal. CD11B, an integrin family member, was also applied to the lenses. CD11B has been shown to mediate

neutrophil and macrophage infiltration during inflammation (Coxon et al., 1996). Therefore, it makes a good marker for inflammation PCS and it was used for additional confirmation of inflammatory events during immunofluorescence staining.

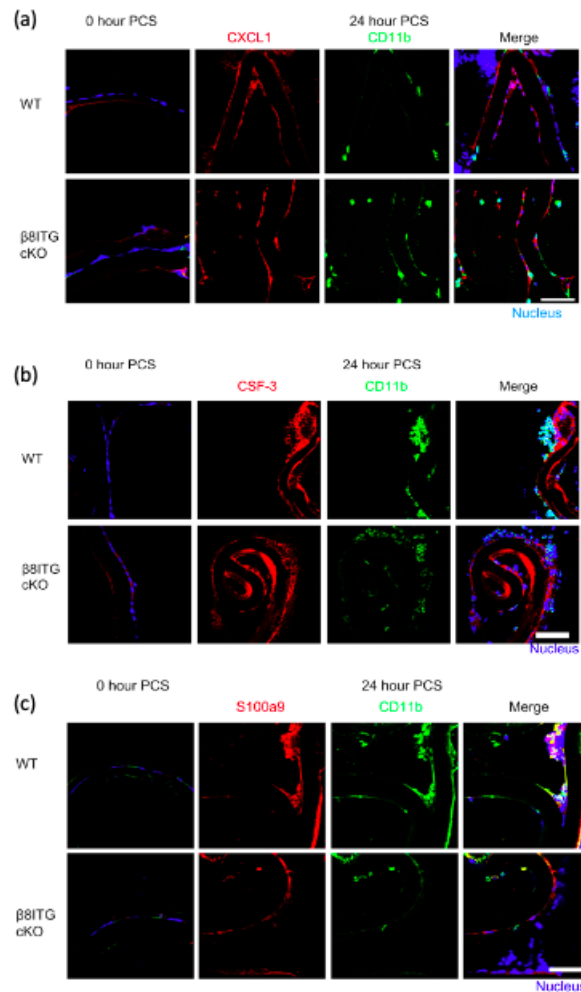


Figure 11: **(a)** CXCL1 is upregulated 24 hours PCS coincident with neutrophil infiltration in both WT and $\beta 8ITG$ -cKO mice. **(b)** CSF-3 is upregulated 24 hours PCS coincident with neutrophil infiltration in both WT and $\beta 8ITG$ -cKO mice. **(c)** S100a9 is upregulated 24 hours PCS coincident with neutrophil infiltration in both WT and $\beta 8ITG$ -cKO mice. Scale bar 70 μm .

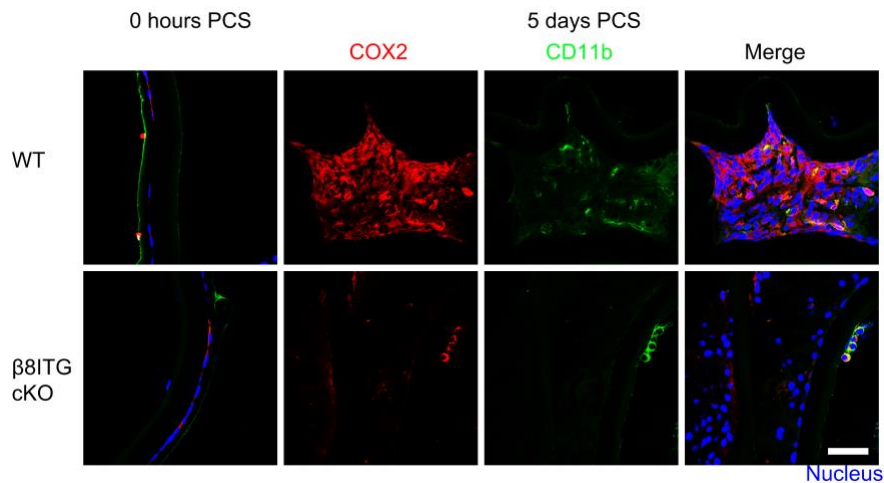


Figure 12: COX-2 is expressed 5 days PCS in both WT and β 8ITG-cKO mice. COX2 expression decreases slightly following removal of β 8-integrin. CD11B reveals similar patterns of neutrophil infiltration at 5 days in WT and β 8ITG-cKO mice. Scale bar 70 μ m.

Markers CXCL1, S100A9, and CSF3 all upregulate in WT lenses 24 hours PCS (Jiang et al., 2018). I was expecting to see decreased expression of the inflammatory markers in α V β 8 null lenses PCS if it does in fact regulate the inflammatory response after injury. However, expression levels appear to be unchanged between WT and α V β 8 null lenses. Another gene upregulated in WT mice PCS was COX2 (RNA sequencing data). Data from graduate student Mahbubul Shihan revealed the upregulation of COX2 5 days PCS in WT mouse lenses. Similarly, I stained for COX2 5 days PCS in WT and α V β 8 null lenses. COX2 expression appears to decrease in the null lenses, however the significance is unknown. This project was further explored by Mahbubul H. Shihan.

Chapter 5

FUTURE DIRECTIONS

For the LCTL project, I only stained for four out of six chosen retinal markers. I planned to review all of the upregulated retinal genes, but due to lack of time and after negative results from the first four, I did not think it would be worthwhile to stain for the others. Although, I do think it would be interesting if DCT or TRPM1 had unique protein expression, such as PMEL, that differed from the other results. Regardless, I believe we did not observe increased expression levels in the LCTL null lenses because of some unknown mechanism that prohibits retinal gene mRNAs from translating into proteins in the lens. This would explain the high levels of genetic expression but no protein expression.

LCTL has implications in wound healing and fibrosis events PCS. The EMT event occurring during PCO is similar to that of fiber cell differentiation during lens development. It was my plan to test the same six markers in WT and LCTL null mice PCS. I assumed that LCTL would play a role in maintaining the lens after surgery when epithelial cells are migrating and proliferating. However, I want to see if the lack of LCTL disrupts the normal lens response PCS and if retinal marker expression is different than what I already observed in WT and LCTL null lenses.

It would be beneficial to perform RT-qPCR in order to quantify the expression levels witnessed for each marker. This way, the original RNA sequencing data could be validated as more than just an artifact of surgery. However, preliminary RNA seq and staining data suggests that some other factor is blocking retinal protein expression in lens, but further analysis is required to determine the mechanism involved.

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