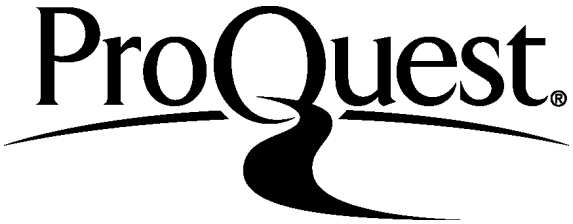
THE ROLE OF β1-INTEGRIN AND ITS LIGANDS IN LENS DEVELOPMENT AND DISEASE

by

Mallika Pathania

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 2015



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DEDICATION

This dissertation is dedicated to my parents, Usha and Rajendra S. Pathania, who instilled in me the virtues of perseverance, commitment and determination and relentlesly encouraged me to strive for excellence.

This dissertation is also dedicated to my husband, Manu Kanwar, for his love, encouragement, patience and support in what ever path I took.

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Most importantly, I would like to thank my family, my brother Bhanu and sister in law Shruti for the immense love and care with which they looked after my son, it made my life here easier to know how well taken care of he was. I thank my Parents in law, Vinod and Ramesh Chand Kanwar, my brother in law Munish Kanwar and sister in law Ruhi Kanwar for all their encouragement and support. A special thanks to my neice Riya Kanwar, for being such a great support with my son.

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ABSTRACT

This study has examined the fundamental cell biological processes that β1 integrins and their ligands control in the lens. I have demonstrated that β1 integrins play a vital role in the determination of cell fate during early lens development by modulating the activation of BMP and FGF signaling pathways. These observations highlight the temporal complexity in β1 integrin function during development.

Further, the β1 integrin ligand, laminin- subunit lamα1, is essential for the formation of the lens capsule including the deposition of collagen IV into the capsule and thus lens morphology/structure. Further, lamα1 is essential for the organization of the corneal epithelium including deposition of TGFβi underneath the corneal epithelium. These data suggest that the lama1a69/a69 (lamα1) mutant phenotype is due to a combination of both a structural and signaling function of the lens capsule and early corneal epithelial BM (basement membrane) during early eye development (Pathania, Semina et al. 2014).

Lastly I have shown that another β1 integrin ligand cFN (cellular fibronectin), similar to β1 integrins, may have a temporal complexity of function as well. While it appears to be essential for lens morphology in the early stages of lens development, its function is dispensable in a mature lens. However cFN is required for lens epithelial cells to undergo EMT during the late wound healing response following lens injury or cataract surgery. Notably, I have shown that cFN is required for TGFβ induced EMT signaling following lens injury or surgery.

Chapter 1

INTRODUCTION

The Lens

The ocular lens is a transparent structure responsible for focusing light onto the retina. The lens is situated behind the iris and lies between the aqueous and vitreous humor (Bloemendal 1977) (Figure1.1).

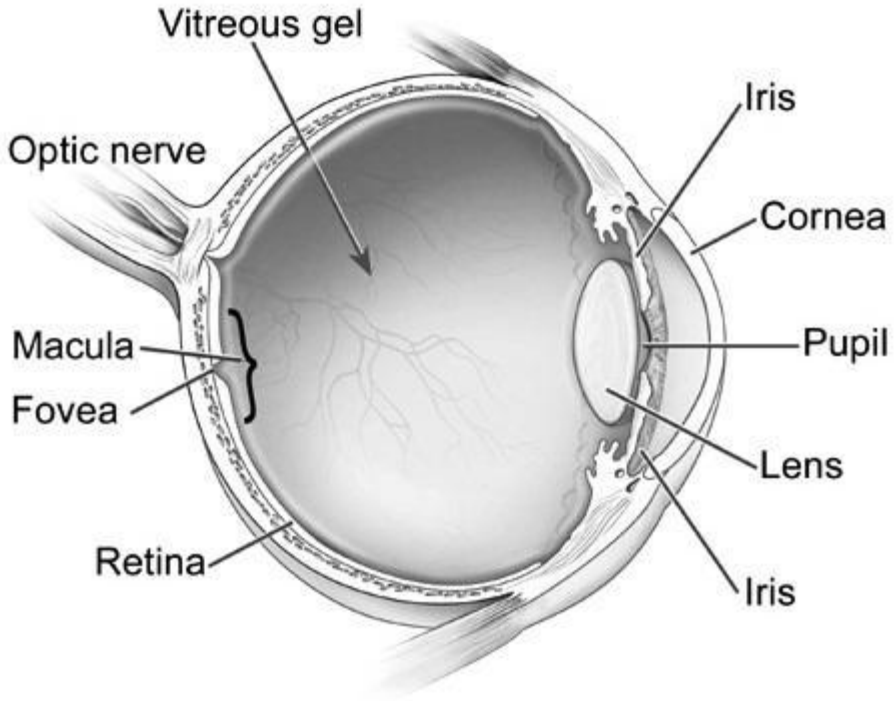


Figure 1.1: Eye Anatomy. Image adapted from NEI Catalog number NEA09

It is composed of two morphologically distinct polarized cell types, epithelial cells and fiber cells, whose basal surfaces interact with a thickened basement membrane called the lens capsule. The lens epithelial cells form a monolayer on the anterior surface of the lens and serve as progenitors for new lens fibers while also regulating homeostasis of the lens (Bassnett, Wilmarth et al. 2009). The posterior portion of the lens consists of concentric layers of elongated fiber cells, which serve to maintain transparency of the lens (Piatigorsky 1981; Bassnett, Wilmarth et al. 2009) (Figure1.2).

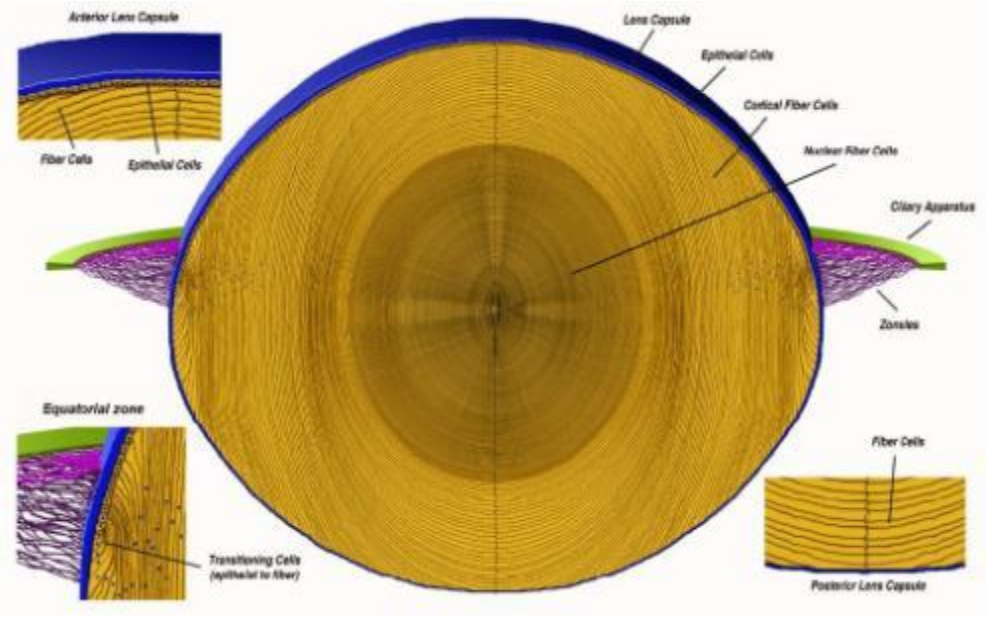


Figure 1.2: Lens anatomy showing that it is an epithelial tissue. Image adapted from Danysh and Duncan, 2009 (Danysh and Duncan 2009).

The aqueous humor and vitreous humor surrounding the lens are rich in growth and regulatory factors (Yamamoto 1976; Lovicu and McAvoy 2005; Bassnett, Wilmarth et al. 2009). The vitreous humor promotes fiber cell differentiation, whereas the aqueous environment promotes epithelial maintenance and growth. However, the mechanism(s) which establish the border between the differentiating lens fibers and the lens epithelium at the lens equator are not well understood (Lovicu and Robinson 2004; Lovicu, McAvoy et al. 2011).

Lens Development and Differentiation

Eye development and lens formation begins shortly after gastrulation coincident with neural tube closure as a portion of the head ectoderm becomes competent to become lens and corneal epithelium (Chow and Lang 2001; Donner,

Lachke et al. 2006; Gunhaga 2011; Jin, Fisher et al. 2012). By E8.5 of mouse development, the diencephalon gives rise to outpocketings which will form the optic vesicles (OV), and a portion of the head ectoderm acquires a lens forming bias to form a pre-placodal (PPR) region (Donner, Lachke et al. 2006). Around E9, the developing optic vesicle comes to lie in close apposition to the PPR (Figure 1.3A) and inductive signals from the OV function to convert the lens forming bias into specification. Around E9.5, lens development can morphologically be first visualized (Chow and Lang 2001; Gunhaga 2011) as thickening of the surface ectodermal cells overlying the developing optic vesicle (OV) to form the lens placode (LP) (Figure 1.3B).

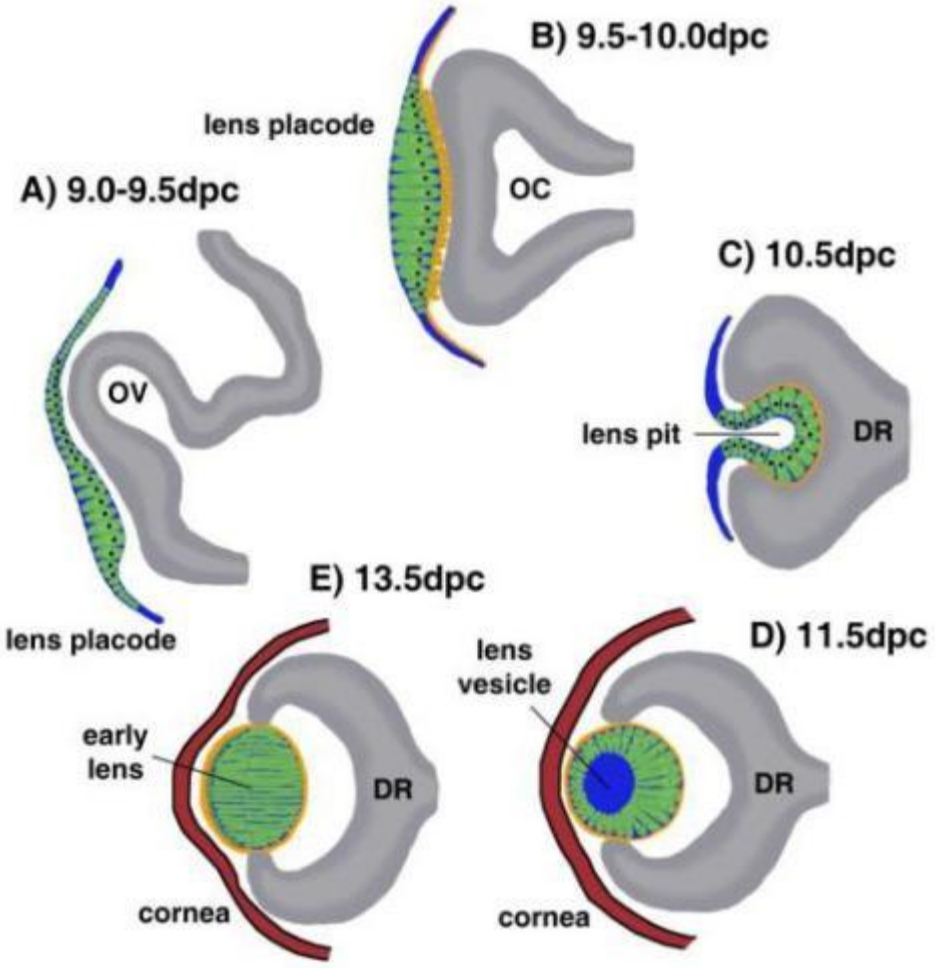


Figure 1.3: Stages of lens development. OV-optic vesicle, OC-optic cup, DR-developing retina. Image adopted from Lovicu FJ, McAvoy JW, 2005 (Lovicu and McAvoy 2005)

Coordinated invagination of the lens placode and optic vesicle (Figure 1.3C) leads to formation of the lens pit and optic cup (developing retina (DR). Subsequently, the lens pit pinches off from the overlying surface ectoderm, and forms the lens vesicle (Figure 1.3D). The lens vesicle consists of precursors of the cells that will contribute to the adult lens segregated from the remainder of the eye by the

surrounding basement membrane which will thicken to become the lens capsule (Chow and Lang 2001; Lovicu and Robinson 2004; Lovicu, McAvoy et al. 2011). In the lens vesicle, the apical surfaces of lens cells face the lumen while their basal surfaces are attached to the lens capsule and thus face outward. While shortly after the lens vesicle closes, all cells appear developmentally equivalent (Lovicu and Robinson 2004), shortly thereafter, the more posteriorly situated vesicle cells facing the developing retina (DR) exit the cell cycle, elongate (Figure 1.3D), and become terminally post mitotic (Lovicu and Robinson 2004). In contrast, the anterior cells facing the developing cornea remain as a monolayer of epithelial cells and form the lens epithelium. The apical ends of primary fiber cells eventually contact the apical surface of the anterior lens epithelium, filling the lumen of the lens vesicle (Figure 1.3E) (Lovicu and Robinson 2004; Lovicu, McAvoy et al. 2011). This establishes the distinctive polarity of the lens, which is maintained throughout life.

The lens then continues to increase in size as the lens epithelial cells proliferate and increase in number (McAvoy, Chamberlain et al. 1999; Lovicu and Robinson 2004). As the anterior epithelium of the lens (AEL) proliferates, adjacent epithelial cells move closer to the lens equator into a region called the transitional zone (Shi, De Maria et al. 2014). Within the transitional zone, epithelial cells withdraw from the cell cycle, differentiate and elongate into secondary fiber cells (Lovicu and Robinson 2004). Most molecules known to control fiber cell differentiation are either negatively acting signals such as Jagged/Notch/Rbpj (Jia, Lin et al. 2007; Rowan, Conley et al. 2008) or Pax6 (Duncan, Kozmik et al. 2000), or positively acting transcription factors such as cMaf (Ring, Cordes et al. 2000; Yoshida, Kim et al. 2001; Yoshida and Yasuda 2002), Prox1 (Wigle, Chowdhury et al. 1999), and Sox1 (Nishiguchi, Wood et

al. 1998; Donner, Ko et al. 2007) or receptor/ligand pairs like FGF/FGFr (Zhao, Yang et al. 2008), and BMP/BMPr (Belecky-Adams, Adler et al. 2002; Faber, Robinson et al. 2002; Boswell, Lein et al. 2008; Boswell, Overbeek et al. 2008; Boswell and Musil 2015) (Figure 1.4).

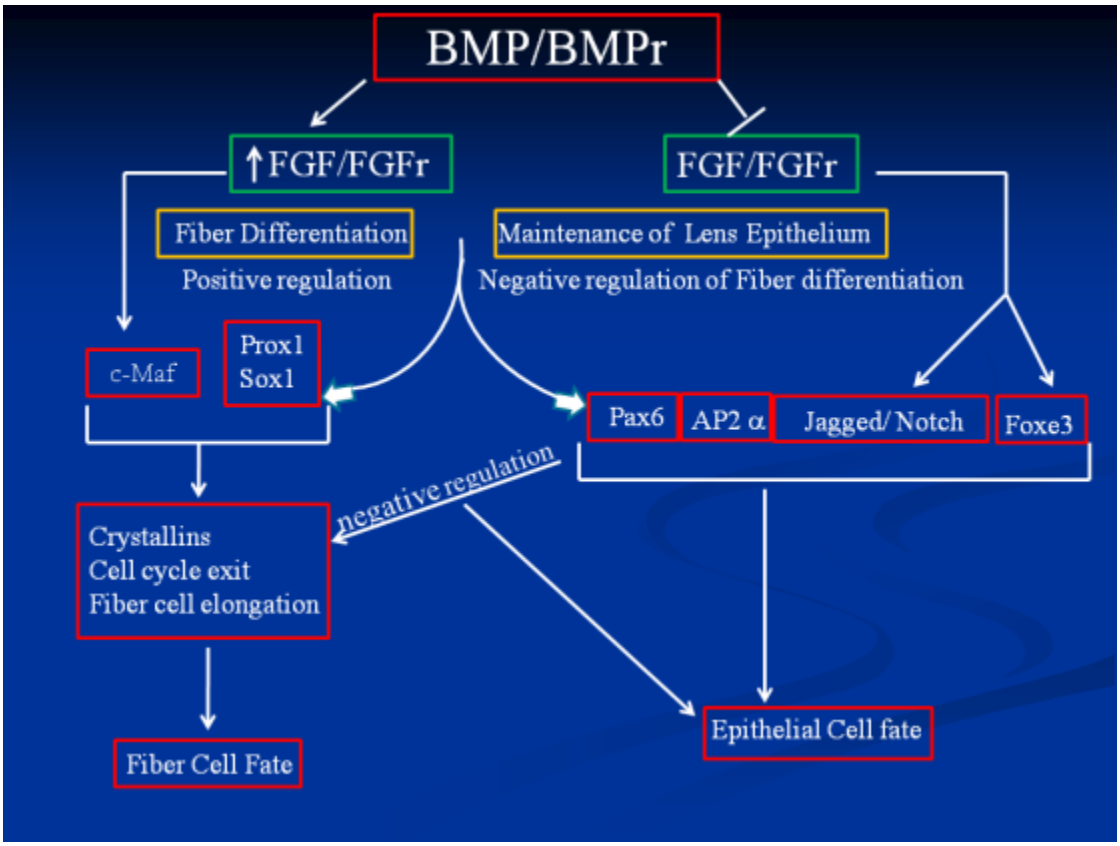


Figure 1.4: FGF as well other positive and negative regulators of fiber differentiation.

Particularly, fibroblast growth factor (FGF/FGFr) signaling is known to be a crucial player in differentiation of the posterior lens vesicle into the primary lens fibers (Zhao, Yang et al. 2008).

Fibroblast Growth Factor Signaling

Growth factors play key roles in influencing cell fate and behavior during development (Slack, Darlington et al. 1987; Grunz, McKeehan et al. 1988; Dorey and Amaya 2010). Various studies have shown that lens epithelial cell proliferation, migration and fiber cell differentiation occur in distinct spatial patterns that are related to the positions of these cells within the eye. If the lens of a five-day chick embryo is inverted within the eye so that its epithelium which normally faces the cornea (and is normally bathed in aqueous humor) was made to face the neural retina and be exposed to the vitreous compartment, the epithelial cells differentiate into lens fibers. While the cells which previously had begun to differentiate into lens fibers arrest elongation, leading to a complete reversal of lens polarity (Coulombre and Coulombre 1963; Coulombre and Coulombre 1969; Coulombre and Coulombre 1971). Similar results were obtained with inversion experiments on mouse lenses (Yamamoto 1976). Further work indicated that the elongation of epithelial cells into lens fibers depends on the presence of a diffusible factor(s) that are secreted by the retina (Muthukkaruppan 1965; Yamamoto 1976; Beebe, Feagans et al. 1980; McAvoy 1980; Campbell and McAvoy 1984; Beebe, Silver et al. 1987). Thus, it became evident that the polarity of the lens is regulated by its surrounding ocular environment. Experiments conducted using rat lens epithelial explants led to the identification of a retina derived diffusible lens 'fiber differentiation factor' (Campbell and McAvoy 1986) as a member of the FGF family (Chamberlain and McAvoy 1989). Since then, numerous studies have

provided compelling evidence that members of the FGF family play key roles in mammalian lens biology, particularly in relation to their ability to induce lens fiber differentiation (Zhao, Yang et al. 2008) .

FGF prototypes, FGF-1 and FGF-2 (de Iongh and McAvoy 1992; de Iongh and McAvoy 1993; Lovicu, de Iongh et al. 1997), and high-affinity FGF receptors (de Iongh, Lovicu et al. 1996; de Iongh, Lovicu et al. 1997) are expressed throughout the eye, in particular in the lens. However FGF concentrations and bioavailability appears to differ throughout the eye (de Iongh and McAvoy 1992; de Iongh and McAvoy 1993; Schulz, Chamberlain et al. 1993; de Iongh, Lovicu et al. 1997; Lovicu, de Iongh et al. 1997; Wu, Tholozan et al. 2014). These studies led to the proposal that the distinct polarity of the lens in the eye may be determined by an FGF gradient (Chamberlain and McAvoy 1997; Zhao, Yang et al. 2008; Wu, Tholozan et al. 2014) (Figure 1.5).

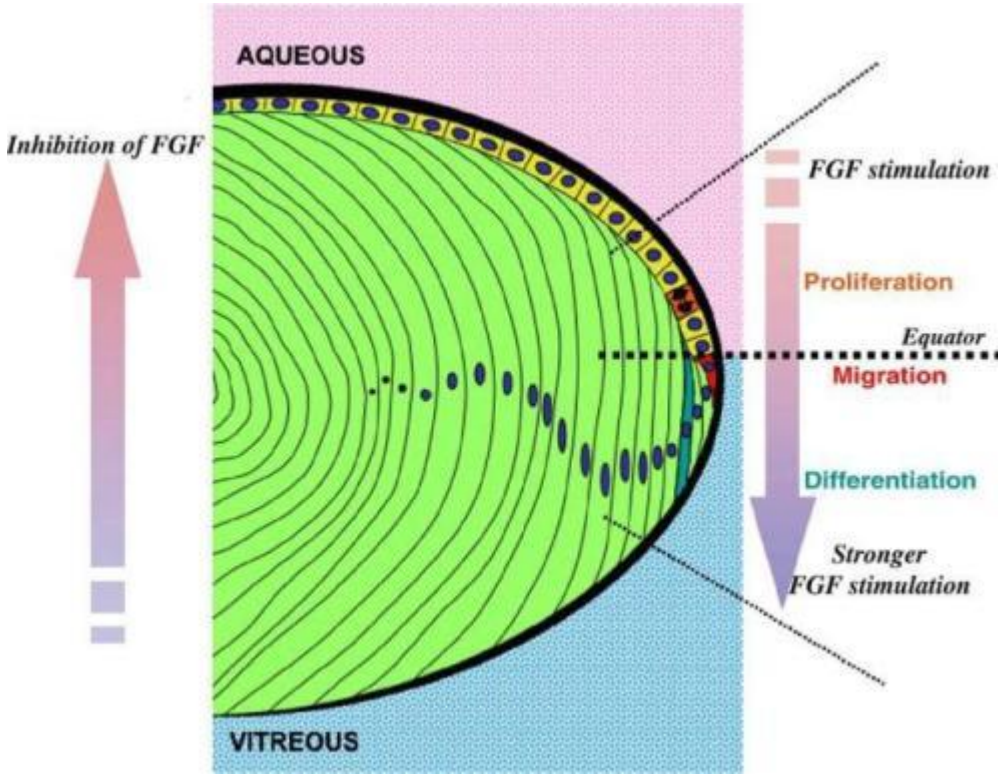


Figure 1.5: FGF gradient in the eye. Image adopted from Lovicu FJ, McAvoy JW. 2005 (Lovicu and McAvoy 2005).

While there is compelling evidence that FGF signaling is necessary for fiber cell differentiation (Govindarajan and Overbeek 2001; Lovicu and McAvoy 2005; Zhao, Yang et al. 2008), little work has been done to explore the factors necessary to prevent anterior LECs from responding to FGF, thereby restricting fiber cell differentiation only to the transition zone of the lens. It has been demonstrated that the developing lens epithelium expresses all four FGF receptors (de Iongh, Lovicu et al. 1997). Studies in lens epithelial explants have shown that low concentrations of FGF induce lens cell proliferation, whereas higher doses are required to induce epithelial cell migration and fiber cell differentiation (Chamberlain and McAvoy 1989)

demonstrating that the lens epithelium is competent to respond to FGF stimulation. However, the ciliary body, which is the source of aqueous production as well as an anatomical boundary between the anterior and posterior chambers, does not begin to form until days after the establishment of lens polarity and the transitional zone (15.5 dpc in mouse) (Cvekl and Tamm 2004; Davis-Silberman and Ashery-Padan 2008). Therefore, during establishment of lens polarity and the formation of transitional zone, this proposed FGF gradient is unlikely to be sharp, and the ocular environment surrounding the LECs may not be distinct enough from that around the lens fibers, to be solely responsible for the exquisite regulation of lens polarity and position of the transition zone observed in normal lens development. This has led us to the idea that there must be other factors that regulate or fine tune FGF stimulation to ensure the establishment and maintenance of the proliferating AEL and transitional zone during lens development (De Arcangelis and Georges-Labouesse 1999; Samuelsson, Belvindrah et al. 2007; Simirskii, Wang et al. 2007). **In chapter 3, I will demonstrate that integrins play a role in this process.**

Integrins in Lens

Integrins are a large family of heterodimeric, transmembrane; cell adhesion molecules that were first identified as cell– extracellular matrix (ECM) adhesion molecules (Wederell and de Iongh 2006; Walker and Menko 2009). While integrins act as linkers between the ECM and the cytoskeleton, they also act as principal transducers of signals between ECM and cells (Wederell and de Iongh 2006). Signaling via integrins is bidirectional, 'Outside-in signaling' results from engagement of integrins with their ligands leading to the induction of multiple and distinct signaling cascades that are crucial for cell proliferation, differentiation, morphogenesis

and survival. 'Inside-out signaling' occurs when a myriad of signals within the cell cause changes in integrin activation state that alters the way cells interact with their matrix environment (Hynes 2002; Wederell and de Iongh 2006).

In mammals, eighteen α and eight β subunits have been described that associate to form 24 distinct receptors comprised of αβ containing heterodimers that each bind to a specific ligand or set of ligands (Menko and Philip 1995; Hynes 2002; Walker and Menko 2009). The β1-integrin subunit is able to form functional receptors with the largest diversity of known α-integrins leading to the ability of cells to detect the composition of diverse ECM environments (Menko and Philip 1995). Therefore, it is not surprising that β1 integrins are expressed in embryonic lens cells at all stages of lens morphogenesis and development (Menko and Philip 1995; Bassnett, Missey et al. 1999; Duncan, Kozmik et al. 2000).(Figure 1.6).

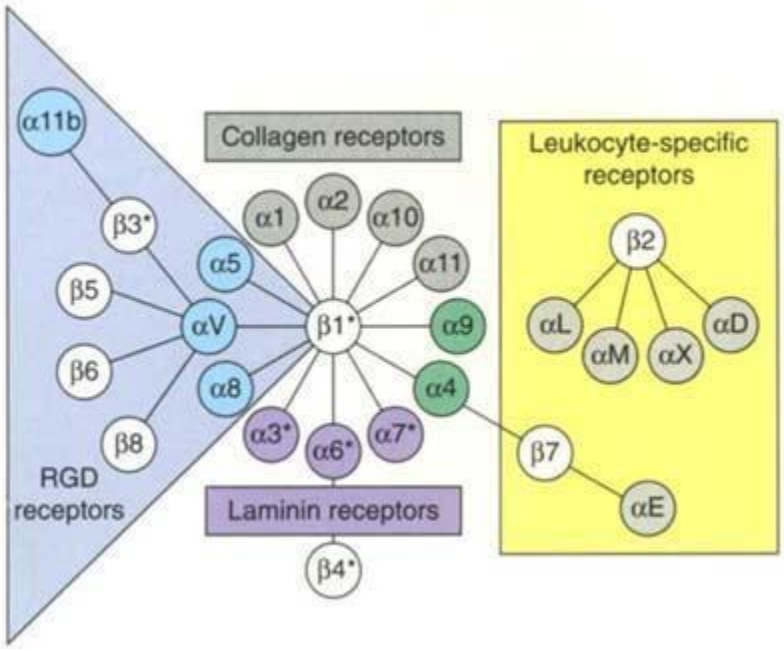


Figure 1.6: β1 integrins binding partners in mammals. Image adopted from Hynes RO, 2002 (Hynes 2002)

Role of β1 Integrins in Normal Lens Biology

β1 integrins are detected at basal surfaces of lens epithelial cells and at apical tips and lateral membranes of differentiating fiber cells (Menko and Philip 1995; Simirskii, Wang et al. 2007; Scheiblin, Gao et al. 2013). In the lens, β1 integrin interacts with α2, α3, αV,α5,α6 integrin subunits to form cell surface receptors for components of the lens capsule (Menko, Philp et al. 1998).

Various studies focused on the functions of integrin subunits have indicated a possible role of β1 integrins in lens development (Wederell and de Iongh 2006; Samuelsson, Belvindrah et al. 2007; Walker and Menko 2009). Loss of β1 integrins after lens morphogenesis is completed results in loss of epithelial cells by apoptosis and abnormal differentiation (Simirskii, Wang et al. 2007). The β1-integrin binding

partners α3 and α6 (Barbour, Saika et al. 2004; Wederell, Brown et al. 2005) integrins are also required for maintenance of anterior lens epithelium since α3/α6 double nulls show breaches in the anterior lens epithelium resulting in the extrusion of lens fibers (De Arcangelis, Mark et al. 1999; Wederell and de Iongh 2006). Further, knockdown of α6 integrin blocks fiber cell differentiation in chick lens explants (Walker, Zhang et al. 2002), while conditional deletion of integrin linked kinase (ILK), one possible downstream effecter of β1 integrins signaling in the lens, during lens formation results in non apoptotic death of lens epithelial cells (Cammas, Wolfe et al. 2012). While all these studies show that β1 integrins play a role in maintaining normal lens biology, the mechanism(s) by which β1 integrins exert their influence during early lens morphogenesis is not understood**. In chapter 3, I will demonstrate the role of β1 integrins during the cell fate decisions that occur as the lens vesicle transitions into the lens.**

The Lens Capsule

The lens capsule, also called the lens basement membrane, is a specialized extracellular matrix that serves as a semi-permeable barrier and mechanical scaffold for lens cells (Cammarata, Cantu-Crouch et al. 1986; Bassnett, Missey et al. 1999; Danysh and Duncan 2009; Danysh, Patel et al. 2010). The lens capsule is one of the thickest basement membranes in the body and is mainly composed of collagen IV, laminin, fibronectin, entactin, and heparan sulfate proteoglycans (Kelley, Sado et al. 2002; Wederell and de Iongh 2006; Walker and Menko 2009). (Figure 1.7).

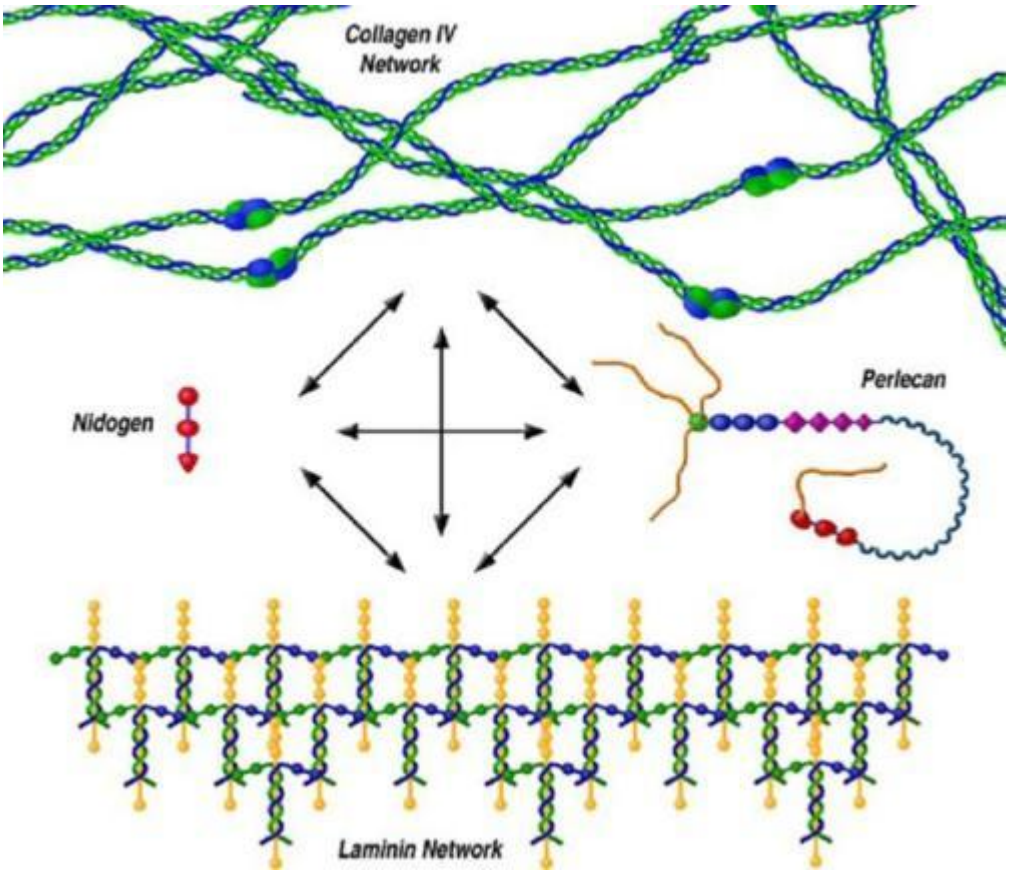


Figure 1.7: The lens capsule majorly consists of collagen IV and laminin networks. These two networks are bridged by other molecules such as entactin and perlecan. Image adapted from Danysh and Duncan, 2009 (Danysh and Duncan 2009).

Lens capsule proteins are involved in lens development as early as the lens determination stage when the optic vesicle interacts with the head ectoderm destined to become the lens (Huang, Rajagopal et al. 2011). It has been shown that from the time lens vesicle pinches off from the head ectoderm, it is surrounded by a basement membrane. This basement membrane rapidly increases in both area and thickness during embryogenesis and continues to grow into adulthood, albeit at a slower rate. It is continually produced and remodeled anteriorly by the lens epithelial cells and

posteriorly by newly differentiated fiber cells (Johnson and Beebe 1984; Haddad and Bennett 1988; Yurchenco, Amenta et al. 2004; Yurchenco 2011). Throughout their differentiation from epithelial to fiber cells, the basal ends of lens cells remain in contact with the capsule, releasing only when they reach the lens suture (Bassnett, Missey et al. 1999). The capsule components modulate various aspects of cell behavior such as adhesion, growth, and survival, (Cammarata and Spiro 1982; Olivero and Furcht 1993; Futter, Crowston et al. 2005; Tholozan, Gribbon et al. 2007; Tholozan and Quinlan 2007), making lens epithelial cells resistant to various forms of apoptosis (Cammarata and Spiro 1982; Futter, Crowston et al. 2005). Transgenic mice with altered lens capsule proteins exhibit abnormal lens fiber cell biology and develop cataracts suggesting the importance of lens capsule composition in lens homeostasis (Gilmour, Lyon et al. 1998; Dong, Chen et al. 2002; Yan, Clark et al. 2002; Rossi, Morita et al. 2003; Elamaa, Sormunen et al. 2005; Firtina, Danysh et al. 2009). Like all basement membranes, the lens capsule serves as an extracellular depot for growth factors and proteases (Tholozan, Gribbon et al. 2007; Wu, Tholozan et al. 2014) while also directly binding to cellular receptors such as integrins (Simirskii, Wang et al. 2007; Walker and Menko 2009) to provide signals which control the phenotype of the attached cells. The capsule also serves as a selectively permeable barrier between the lens and the ocular environment (Danysh, Patel et al. 2010), protecting the lens from infection while also conferring immune privilege (Piatigorsky 1981; Danysh and Duncan 2009). Finally, the lens capsule is important for lens structural integrity and serves as the attachment site between the lens and the zonules, which suspend the lens in the correct location within the eye (Hiraoka, Inoue et al. 2010; Shi, Tu et al. 2013) and transmit the forces necessary for accommodation in primates (Charman 2008).

Consistent with these functions, mutations in genes encoding either lens capsule components (Dong, Chen et al. 2002; Firtina, Danysh et al. 2009) or proteins necessary for lens capsule assembly (Takeda, Kondo et al. 2003; Chang, Winder et al. 2009; Qu, Hertzler et al. 2011) lead to diverse lens dysplasias (Rossi, Morita et al. 2003; Lee and Gross 2007).

Laminin

Laminin is a vital component of the extracellular matrix as it is both a structural component of the ECM and can activate cell signaling cascades that control cell migration, proliferation, cell survival and cellular phenotype via interactions with cell surface receptors, notably- integrins. There are five known α, four known β and three known γ-laminin subunits (Zinkevich, Bosenko et al. 2006; Sztal, Berger et al. 2011). These various subunits combine intracellularly and form sixteen different heterotrimeric laminin isoforms (Figure 1.8)

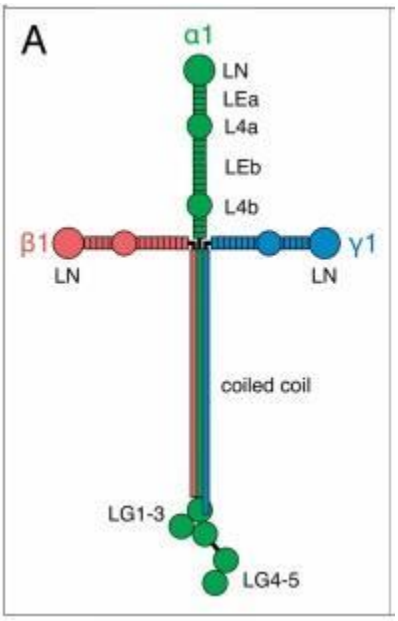


Figure 1.8: Schematic of laminin 111 heterotrimer. Image adapted from Erhard Hohenester and Peter D. Yurchenco, 2013 (Hohenester and Yurchenco 2013).

with different tissue distributions (Aumailley, Bruckner-Tuderman et al. 2005; Hamill, Kligys et al. 2009). During development, laminin 111 is the first network forming component to be expressed and is largely limited to epithelial basement membranes (Hamill, Kligys et al. 2009). The laminin 111 heterotrimer consists of an α1, a β1, and a γ1 subunit (Bystrom, Virtanen et al. 2006). Mice deficient in any component of the laminin 111 heterotrimer die at the postimplantation stage. The laminin α1 gene shows a tissue restricted expression pattern and is detected in nervous and urogenital systems, presomatic mesoderm, brain blood vessels and the developing and mature lens. Besides being involved in formation of laminin 111, *lama1* subunit also participates in one additional trimer, laminin 3 (Bystrom, Virtanen et al. 2006; Zinkevich, Bosenko et al. 2006; Sztal, Berger et al. 2011).

Mutations in different laminin subunits profoundly affect tissue morphogenesis. *lama1* mutant zebrafish have a shortened body axis and anterior segment dysgenesis with severely abnormal lenses (Semina, Bosenko et al. 2006). Ffurther, mutations in either *lamb1* or *lamc1* in zebrafish cause defects in the notochord and body axis and also lead to ocular abnormalities (Zinkevich, Bosenko et al. 2006).

Laminin in Lens

The lens capsule contains laminin α1, α5, β1, β2 and γ1 (Zinkevich, Bosenko et al. 2006; Sztal, Berger et al. 2011), and laminin binding to lens cells occurs principally via the α6β1, α3β1 and α6β4 integrin receptors on the cell surface (Bystrom, Virtanen et al. 2006). Mutations in human LAMB2 results in Pierson's syndrome, which is characterized by severe kidney disease associated with multiple ocular abnormalities including lens malformations and cataracts (Bredrup, Matejas et al. 2008). Notably, deletion of either the *lama1*, *lamb1* and *lamc1* genes result in post-implantation lethality in mice, apparently because laminin 111, the heterotrimer composed of laminin α1, β1, γ1, is critical for the initial assembly of epithelial basement membranes (Miner, Li et al. 2004). Further, mutations have been identified in the zebrafish *lama1* (bashful; bal), *lamb1* (grumpy; gup) and *lamc1* (sleepy; sly) genes, all of which result in profound body axis and brain defects (Stemple, Solnica-Krezel et al. 1996; Parsons, Pollard et al. 2002; Semina, Bosenko et al. 2006)

Zebrafish mutations in the *lamb1* and *lamc1* genes also result in retinal lamination defects, as well as severe lens defects by three days post fertilization including the ectopic position of the lens within the retina, loss of lens capsule integrity and inappropriate localization of the zebrafish lens marker ZL-1. By five

days post fertilization, the lens has fragmented and is largely lost from the eye (Lee and Gross 2007). Mutations and morpholino driven knockdown of the *lama1* gene result in similar lens degeneration/loss although the phenotype appears more severe with the first defects apparent by 30 hpf while the lens is absent by 72 hpf leading to the conclusion that fiber cell morphogenesis was disrupted. While these studies indicate that the laminin-1 heterotrimer is critical for eye and lens development and function, none of the prior studies on these laminin mutants characterized these lens defects further. **In chapter 4, I will re-evaluate the lens phenotype of the zebrafish *lama1* mutant, *lama1a69*, and show that the loss of the lens occurs upon its extrusion through the developing cornea, suggesting roles for laminin 1 in the structural integrity of the eye.**

Fibronectin

The homodimeric glycoprotein fibronectin (FN) is encoded by an 8kb mRNA to yield a largely soluble form secreted by hepatocytes into plasma (plasma FN), or through alternate splicing of the transcript, yielding a mixture of isoforms (cellular FN) in a tissue dependent, temporally regulated and cell specific manner (Singh, Carraher et al. 2010; To and Midwood 2011) (Figure 1.9).

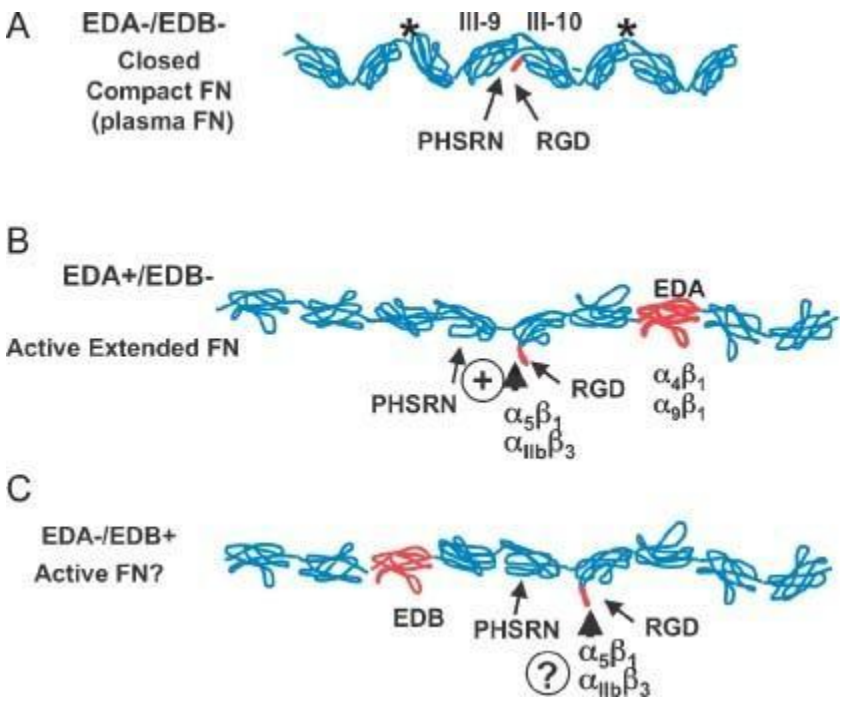


Figure 1.9: The closed or plasma form of FN (A) undergoes alternative splicing and contains neither EDA nor EDB domains and exists in a compact conformation that conceals the RGD sequence. Cellular fibronectin EDA+ FN (B) or EDB+ FN exists in an extended form with an accessible RGD sequence. Image adapted from White.et.al, 2011 (White and Muro 2011).

Cellular FN is an essential component of the extracellular matrix (ECM), serving as a scaffold for the assembly of other important ECM proteins such as collagens, fibrillin, fibulin and tensacin-C (Singh, Carraher et al. 2010), and increased expression of certain FN isoforms is observed during physiological and pathological tissue remodeling such as development and tissue injury (To and Midwood 2011). Thus, it is not surprising that complete deletion of fibronectin is embryonic lethal in mice (Huang, Rajagopal et al. 2011). FN matrix assembly and turnover both require association of FN with integrins, in particular $\alpha5\beta1$, as does binding of FN to other

ECM proteins like heparan sulfate proteoglycans (Ruoslahti 1981; Ruoslahti, Engvall et al. 1981; Ruoslahti, Jalanko et al. 1981; Pankov and Yamada 2002; To and Midwood 2011). The ability of FN to interact simultaneously with cells as well as other ligands in the ECM is largely conferred by its modular and multidomain structure. The three module types found in each of the FN subunits include 12 type I, 2 type II and 15-17 type III (Singh, Carraher et al. 2010). Some of the major functions of FN include cell adhesion, migration, differentiation and growth (To and Midwood 2011). It is also involved in several key physiological processes including wound healing, thrombosis, and embryogenesis (Smith, Symes et al. 1990).

Fibronectin in Lens

Fibronectin is produced in the lens throughout its development (Hayes, Hartsock et al. 2012) and is detectable in the embryonic rat and chick lens capsule (Parmigiani and McAvoy 1984), adult bovine lens capsule (Cammarata, Cantu-Crouch et al. 1986), and in the posterior aspects of the adult mouse lens capsule (Duncan, Kozmik et al. 2000). E16 rat lens epithelial cells are able to migrate on a FN substratum, while they lose this ability by E19, suggesting a developmental switch in its use (Parmigiani and McAvoy 1991), although adult rabbit lens epithelial cells are able to attach and spread when placed on FN-coated slides. Both FN and its binding partner $\alpha5\beta1$-integrin are involved in zebrafish lens fiber cell morphogenesis (Hayes, Hartsock et al. 2012), and functional perturbation of FN in chick embryos using injected RGD peptides suggests that cell–ECM interactions, possibly mediated by FN, are required for normal lens morphogenesis in vivo. Although both FN (George, Georges-Labouesse et al. 1993) and Itga5 (Yang, Rayburn et al. 1993) mouse knockouts are lethal, a recent study using a tamoxifen-inducible Cre to inactivate FN

at early stages of mouse development demonstrated that FN is required for lens placode thickening and invagination (Huang, Rajagopal et al. 2011).**In chapter 5, I will demonstrate that conditional deletion of FN using the Cre expressing transgenic mouse line , Le-Cre, which is expressed at the lens placode stage (Ashery-Padan, Marquardt et al. 2000; Yoshimoto, Saigou et al. 2005), leads to profoundly abnormal lenses at birth**.

Although FN is produced by the lens throughout its development, and mutations in FN as well as its receptor $\alpha5\beta1$,result in development of cataract in zebrafish, high levels of both these proteins are also associated with the myofibroblast-like epithelial cells found in patients with anterior subcapsular cataracts (Yoshino, Kurosaka et al. 2001; Hayes, Hartsock et al. 2012). . Further FN expression is up-regulated during the wound healing response that occurs after cataract surgery (Boyd, Peiffer et al. 1992; Wormstone, Tamiya et al. 2002; Marcantonio and Reddan 2004; Mamuya, Wang et al. 2014), a process called epithelial to mesenchymal transition (EMT), although its function in this process is not fully known. **In chapter 5, I will demonstrate the mechanisms by which FN may be involved in the EMT that occurs during development of Posterior Capsular Opacification (PCO), a complication of cataract surgery.**

Cataracts and Posterior Capsular Opacification

Cataract is a clouding of the ocular lens, which is the most common cause of blindness in the world (Asbell, Dualan et al. 2005). 43% of the worlds' blind population is suffering from cataract (WHO). Several factors are known to cause cataractsincluding trauma to the lens (Call, Grogg et al. 2004), oxidative stress (Thiagarajanand Manikandan 2013), metabolic dysfunction, loss of ion/water

balance(Donaldson,Chee et al. 2009), ultraviolet radiation (UVR) exposure (McCarty and Taylor 2002,Varma, Hegde et al. 2008), genetic defects such as mutation of lenticular proteins(Andley, Hamilton et al. 2008, Wang, Wang et al. 2011) as well as other non-geneticdefects that can occur during lens development (Firtina, Danysh et al. 2009, Yi, Yun etal. 2011) and simple ageing (Hejtmancik and Kantorow 2004).

Cataracts are usually treated by removal of the lens fibers, as well as most lens epithelial cells, while the lens capsule is retained to hold an artificial lens implant (Ashwin, Shah et al. 2009) (Figure 1.10).

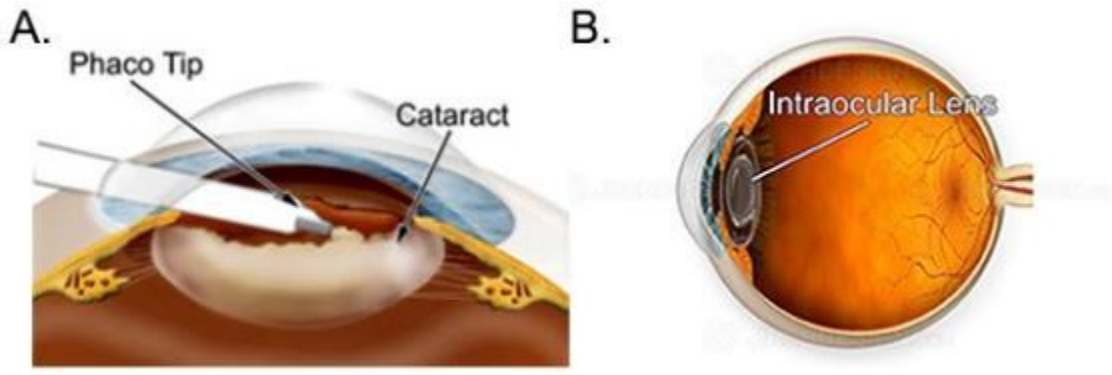


Figure 1.10: (**A**) Phacoemulsification procedure used during extracapsular lens extraction to remove a cataractous lens. (**B**) Completion of surgery showing the inserted IOL implant sitting at the position of the natural lens and surrounded by an intact elastic lens capsule. Images adopted from http://www.ocuclinic.com and http://www.jirehdesign.com

However, some lens epithelial cells (LECs) remain attached to the capsule and sense cataract surgery as an injury. This leads to residual LEC proliferation and migration resulting in Posterior Capsule Opacification (PCO) (Wormstone, Wang et al. 2009). (Figure 1.11).

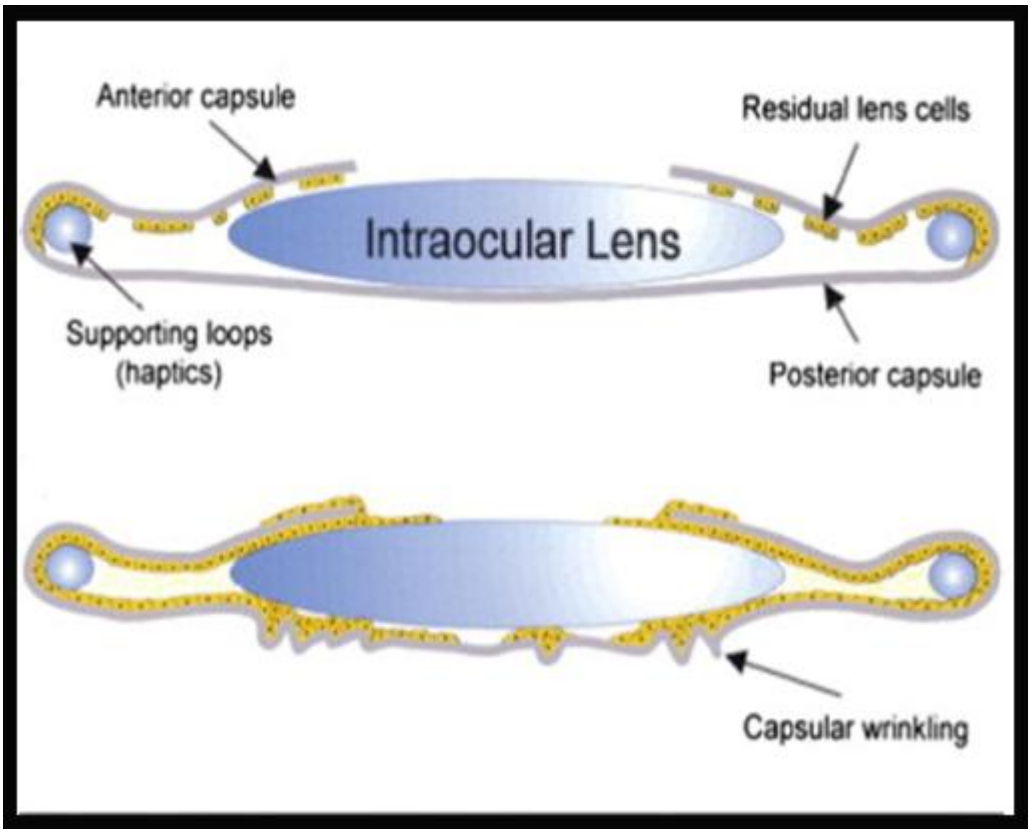


Figure 1.11: Cell proliferation and migration post cataract surgery. Image adapted from Wormstone et.al, 2009 (Wormstone, Wang et al. 2009).

PCO is the most common complication of cataract surgery, developing in about 30% of patients within a year, and 100% of patients within 7 years (Apple, Escobar-Gomez et al. 2011). Clinically, two morphological distinct types of PCO are the fibrosis-type and the pearl-type (Ashwin, Shah et al. 2009; Apple, Escobar-Gomez et al. 2011). The fibrosis-type is a result of proliferation and migration of LECs that undergo epithelial-mesenchymal transition (EMT) to transform into migratory myofibroblasts like cells. These migratory cells move onto the posterior capsule and, being contractile, cause folds and wrinkles on the posterior capsule, which was cell-free post-cataract surgery

(de Iongh, Wederell et al. 2005; Wederell and de Iongh 2006; Wormstone, Wang et al. 2009). Pearl-type PCO arises from LECs which attempt to undergo lens fiber regeneration but instead turn into abnormal lenticular fibers referred to as Elschnig pearls and Soemmering rings (Kappelhof, Vrensen et al. 1987; Awasthi, Guo et al. 2009; Wormstone, Wang et al. 2009). Both these phenomena lead to scattering of light, resulting in the reappearance of visual disturbances in patients post-cataract surgery (Wormstone, Wang et al. 2009).

The mechanisms underlying development of PCO are not well understood, thus there is no ideal prevention or cure for PCO. Different techniques such as improved IOLs that entrap migrating lens cells at the periphery, the use of therapeutic agents, or combination therapies have been used to reduce PCO prevalence, however, none of these strategies completely prevent the problem (Nagamoto and Eguchi 1997; Peng, Visessook et al. 2000; Beck, zur Linden et al. 2001). The most common treatment for PCO in developed countries, is an outpatient procedure using a Nd-YAG (neodymium-yttrium-aluminum-garnet) laser to disrupt the opacified posterior lens capsule, restoring clarity of the central visual axis to reestablish vision. However, side effects such as IOL subluxation, acute onset of macular hole, cystoid macular edema, retinal detachment, glaucoma and increases in intraocular pressure are seen commonly in patients undergoing Nd-YAG laser capsulotomy (Steinert, Puliafito et al. 1991; Asbell, Dualan et al. 2005; Sakimoto and Saito 2008; Waseem and Khan 2010) . Moreover, the procedure is expensive, and in most developing countries with irregular access to ophthalmic care, PCO is a significant cause of blindness. Further, PCO associated wrinkling/contraction of the posterior capsule can limit the movements of the postoperative capsule, hence limiting the movements of

advanced accommodating IOLs (McDonald 2007). Therefore, PCO not only results in vision loss after cataract surgery, but also impedes advances in implant engineering by limiting the possible use of current and advanced accommodating IOLs (Bertelmann and Kojetinsky 2001). Therefore, understanding the molecular mechanisms that mediate lens epithelial cell responses to cataract surgery is necessary to aid in developing pharmacological inhibitors of PCO, therefore enhancing effectiveness of cataract surgery and improving visual outcomes for these patients (Dewey 2006; Awasthi, Guo et al. 2009).

Epithelial to Mesenchymal Transition (EMT)

EMT is defined as the 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. This results in cells developing a fibroblastic morphology by rearranging their actin cytoskeleton, becoming migratory by forming filopodia and lamellopodia, interacting with stromal extracellular matrices (ECM) due to changes in cell surface matrix receptors such as integrins, and direct synthesis of stromal ECM to become contractile myofibroblasts (Hay, McElvaney et al. 1995; Kalluri and Neilson 2003; Thiery, Acloque et al. 2009). Various players suggested to contribute to lens EMT leading to PCO include growth factor signaling, extracellular matrix components, matrix metalloproteinases, as well as integrins (Wormstone, Wang et al. 2009). However TGF-β signaling (Saika, Miyamoto et al. 2002; de Iongh, Wederell et al. 2005) is the most well established player in the regulation of lens EMT during PCO development. LECs undergoing EMT are characterized by a fibroblastic morphology with the expression of alpha smooth muscle actin (αSMA) as the most reliable molecular marker. TGF-β is known to be a

potent inducer of this transdifferentiation of lens epithelial cells to a myofibroblast cell phenotype (Hales, Schulz et al. 1994; de Iongh, Wederell et al. 2005). Despite this understanding, the mechanisms underlying how TGF-β mediates these events in lens are still unclear. TGF-β is secreted in a latent form bound to latent TGFβ binding proteins (LTBP). TGF-β/LTBP complexes interact with the ECM to form an extracellular inactive TGF-β depot (Mamuya and Duncan 2012). Notably, LTBP1, the most abundant LTBP in the lens (unpublished) requires cell associated fibronectin to both tether (Zilberberg, Todorovic et al. 2012) latent TGF-β to the ECM and participate in interactions necessary for TGF-β activation (Fontana, Chen et al. 2005). **Thus, in chapter 5, I will demonstrate that fibronectin may be crucial for TGF-β activation critical for the development of fibrotic PCO.**

Overall, this study aims to place β1 integrins into the regulatory network controlling the epithelial to fiber cell fate decision in early lens. This will expand our current understanding of how positional cues influence the molecular mechanisms driving lens differentiation specifically and cellular differentiation in general. My work on the β1 integrin ligand laminin demonstrates both a structural and signaling function of the lens capsule in lens development. Further, understanding the roles of the β1 integrin ligand fibronectin during development will contribute to a better understanding of processes involved in the EMT that occurs as a complication of cataract surgery.

Chapter 2

MATERIALS AND METHODS

Animals

Mouse husbandry and identification of controls and mutants

All mice experiments described in this study conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Delaware Institutional Animal Care and Use Committee under protocol 1039.

B6;129-Itgb1^{tm1Efu}/J mice in which exon 3 of the β1-integrin gene is flanked by LoxP sites (β1 F/F) were obtained from The Jackson Laboratory (Bar Harbor, Maine) (Raghavan, Bauer et al. 2000). FVB/N mice hemizygous for the Le-Cre transgene (LE mice) were obtained from Dr. Richard Lang's lab at the Cincinnati Children's Hospital with permission from Ruth Ashery-Padan, Tel Aviv University (Ashery-Padan, Marquardt et al. 2000). β1 F/F mice were mated with Le-Cre mice to obtain mice homozygous floxed around exon 3 of the β1-integrin gene and hemizygous for Le-Cre (β1LE) or homozygous floxed around exon 3 of the β1- integrin gene and carrying no Cre (Control). Mice homozygous floxed around exon3 of the β1- integrin gene and homozygous for MLR10- Cre (β1-MLR10) mice have been previously generated in the lab (Simirskii, Wang et al. 2007).

Lenses conditionally lacking FN were created using two different strategies. For studying fibronectin in the early lens, mice were created by mating FN1$^{fx/fx}$

animals (B6;129-Fn1, originally created in Dr. Reinhard Fasslers lab (Sakai, Johnson et al. 2001)obtained from Dr. David Beebe, with FVB/N mice hemizygous for the Le-Cre transgene (LE mice) obtained from Dr. Richard Lang's lab at the Cincinnati Children's Hospital with permission from Dr. Ruth Ashery-Padan, Tel Aviv University. Lenses from mice homozygous for the floxed allele and Le-Cre positive were used for all experiments and compared to lenses from mice homozygous for the floxed allele and lacking cre as controls.

To study the role of fibronectin in PCO, mice were created by mating $FN1^{fx/fx}$ animals, with MLR10-cre mice which express Cre recombinase in all lens cells from the lens vesicle stage onward (Zhao, Yang et al. 2004) obtained from Michael Robinson (Miami University, Oxford, Ohio. The mutant mice studied are homozygous for the floxed FN allele and are MLR10 positive.

Embryo staging was done by designating the day that the vaginal plug was observed in the dam as embryonic day (E) 0.5. Post natal mice were staged by designating the day of birth as P0. All mice were maintained and bred at the University of Delaware animal facility in specific pathogen free conditions under a 14/10 hour light/dark cycle.

Zebrafish husbandry and identification of controls and mutants

The *lama1*^{a69} zebrafish mutant was previously isolated in a forward genetic screen for ocular phenotypes and originally named *a69* (B.A. Link 2001) then renamed *bal*^{a69} when *a69* was found to be allelic to the *bashful*(*bal*) mutation by complementation (Stemple, Solnica-Krezel et al. 1996). The causative mutation for the phenotype was identified in the *lama1* gene (Semina, Bosenko et al. 2006) and the allele is now denoted *lama1*^{a69} according to the 2013 Zebrafish Nomenclature

Guidelines https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines . Control embryos were obtained as a product of the *lama1*^{a69} mating scheme. All zebrafish (*Danio rerio*) were raised and maintained on a 14-h light/10-h dark cycle at 28.5°C. Embryos were obtained by natural spawning and their developmental stage was determined by time and morphological criteria. All experiments were conducted in accordance with the guidelines set forth by the Animal Care and Use Committees at the Medical College of Wisconsin and the University of Delaware.

DNA Isolation for Genotyping

Tail genotyping was performed using the Gentra Puregene Mouse Tail Kit (Qiagen Sciences, Germantown, Maryland, 158267). Briefly, mouse tails were mixed with 600µl of cell lysis solution (10mM Tris-HCl pH 8.0, 25mM EDTA, 0.5% SDS) supplemented with five µl of Proteinase K (20mg/ml) and incubated at 55°C overnight in a shaking water bath.

The next day, 200µl of protein precipitation solution (ammonium acetate) was added onto the lysed tails and mixed in by vortexing at high speed for 10-20 seconds. The mixture was then centrifuged at 14,000 rpm for three minutes to precipitate the protein. The supernatant containing the DNA was poured into a microcentrifuge tube containing 600µl of 100% isopropanol without disturbing the protein pellet. The tubes were then mixed by inverting 40-50 times, and centrifuged at 14,000 rpm for one minute. The supernatant was carefully discarded and the DNA pellet was washed one time by adding 600µl of 70% ethanol and centrifuging at 14,000 rpm for two minutes. After the ethanol was carefully poured off, the tubes were drained on a clean absorbent towel and allowed to air dry for 15 minutes.

The DNA pellet was rehydrated with 50-100 μl of DNA hydration solution (10mM Tris-HCl pH 7.5, 1mM EDTA) for one hour at 65°C. Hydrated DNA was stored temporarily at room temperature.

Mice were genotyped for the presence of floxed β1-integrin allele, floxed FN allele, Le-Cre transgene and MLR10-Cre transgene as appropriate (Table 2.1)

Table 2.1: List of all primers used for PCR and RT PCR used in this study

Gene	Forward primer	Reverse primer
β1-integrin	5′-CGG CTC AAA GCA GAG TGT CAG TC-3′	5′-CCA CAA CTT TCC CAG TTA GCT CT-3′
Fibronectin	5′-GTA CTG TCC CAT ATA AGC CT CTG-3′	5′-CTG AGC ATC TTG AGT GGA TGG GA-3′
Le-Cre	5′- ATG CCC AAG AAG AAG AGC AAA GT -3′	5′- GAA ATC AGT GCG TTC GAA CGC AA -3′
MLR10-Cre	5′-CTG AGC ATC TTG AGT GGA TGG GA-3′	5′-CTG AGC ATC TTG AGT GGA TGG GA-3′
LTBP1	5′-GCT GCAGGG GAT CAA CGT CT -3′	5′- AGC TGA GGC CGT AGA CAC AT -3′
Fibronectin (RT PCR)	5′-CTG GAG TCA AGC CAG ACA CA -3′	5′-CGA GGT GAC AGA GAC CAC AA-3′
Fibronectin (EDA)	5′-TGT GAC AGG CTA CAG AGT GAC C-3′	5′-ATT GGT CCT GTC TTC TCT TTC G-3′
Fibronectin (EDB)	5′-CAT GCT GAT CAG AGT TCC TG-3′	5′-GGT GAG TAG CGC ACC AAG AG-3′
β2MG	5′-TAC GCC TGC AGA GTT AAG CAT-3′	5′-TCA AAT GAA TCT GAG CAT CA-3′

Morphological Analysis

For gross documentation of alterations in lens structure, mouse lenses were isolated under a dissecting microscope and photographed under dark field optics. Lens transparency was assessed by placing lenses in Medium 199, (Mediatech Inc, Manassas VA) at 37°C to prevent cold cataract formation and photographs taken under both bright-field and dark-field conditions using a Cannon digital camera A420 mounted on a Zeiss Stemi SV 11 Apo Stereo Microscope (Zeiss, Thornwood, NY). For optical analysis, fresh lenses were placed on a 200-mesh electron microscopy grid and photographed as described (Shiels, King et al. 2007). The ratio between wet and dry lens weight was assessed by weighing, fresh lenses followed by drying lenses in an aseptic 50°C oven for 96hrs, and then reweighing.

For histological analysis, eyes (postnatal mice) or heads (embryos) were isolated and immediately fixed in one ml of Pen-Fix (Richard Allan Scientific, Kalamazoo, Michigan) in a test tube for two hours, followed by two, ten minute washes, then transferred into one ml of 70% ethanol and kept in ethanol until paraffin embedding by the Histology Core Laboratory, College of Agriculture, University of Delaware. Using the embedded sample blocks, serial six-micrometer sections were cut were cut using a Leica microtome and mounted on slides. Selected slides were stained by hematoxylin and eosin (H&E) using a standard method established in the lab. Slides were visualized for cellular morphology on a Zeiss Axiophot microscope fitted with a digital Nikon camera. For the β1LE lenses, the expression pattern of crystallins in the lens was determined by incubating deparafinized sections with rabbit anti-bovine β-crystallin and rabbit anti-bovine γ-crystallin (gifts of Dr. Samuel Zigler, The Wilmer Eye Institute, The Johns Hopkins School of Medicine) followed by detection with an anti-rabbit Dako Envision horseradish peroxidase kit (Dako Laboratories,

Carpinteria, CA) using diaminobenzidine as a substrate. pErk and pAkt levels in the lens was detected on deparafinized sections using the Catalyzed Signal Amplification (CSA) System. (Dako Laboratories, Carpinteria, CA, K150011-2) with Biotinylated Link Antibody (Tris diluent); CSA II Rabbit Link (K150180-2). Briefly, antigen retrieval was performed on deparafinized sections in 10mM Sodium Citrate, pH 6 by double boiling in a rice cooker for half an hour. Sections were then cooled to room temperature, briefly rinsed in 1X TBST (Tris buffered saline with Triton X 100), followed by blocking endogenous peroxidase activity, using the peroxidase block provided in the kit. This was followed by washes in 1X TBST, followed by blocking in blocking solution provided with kit. Sections were then incubated in primary antibody (see Table 2.2) diluted 1:50 in blocking solution for two hours at room temperature. Sections were then incubated with anti-rabbit immunoglobulins-HRP amplification reagent provided in the kit and then incubated with amplification reagent. Following this, anti fluorescein-HRP was applied to the section, and finally developed with liquid DAB substrate chromogen solution.

Scanning Electron Microscopy

SEM was performed as described by (Scheiblin, Gao et al. 2014). Briefly, eyes were enucleated and transferred to a solution containing 0.08 M sodium cacodylate, 1.25% glutaraldehyde, and 1% paraformaldehyde (pH 7.3). After two and a half hours of fixation, lenses were isolated from the eyes and placed in fresh fixative for another 48 hours. After fixation, the lens capsule and outermost layer of fiber cells were peeled away from the lens. The remaining lens mass was dehydrated by incubation in a graded ethanol series and left in 100% ethanol overnight. The following day, the lens was incubated two times in fresh 100% ethanol for 2.5 hours and then dried in 1:2

hexamethyldisilazane (HMDS)/ethanol for one hour, 2:1 HMDS/ethanol for one hour, and two times in 100% HMDS (30 minutes each). The lenses were subsequently placed in a vacuum desiccator and kept there until ready to analyze. The lenses were mounted on stubs, sputter coated for two minutes with gold-palladium and visualized with a Field Emission Scanning Electron Microscope (FE-SEM) Hitachi S-4700 (Tokyo, Japan).

Surgical Removal of Lens Fiber Cells

The effect of cataract surgery on lens cells was modeled in living mice by surgical removal of lens fiber cells as previously described (Call, Grogg et al. 2004; Desai, Wang et al. 2010). Briefly, 3-month-old mice were anesthetized with ketamine/xylazine and their pupils were dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride ophthalmic solution (HenrySchein, Melville,NY). Using an ophthalmic knife, a 3mm central corneal incision was made extending into the lens capsule. A balanced salt solution was used to separate the lens capsule from the lens fiber cells and the entire lens fiber cell mass was removed by a sharp forceps, leaving behind an intact lens capsule. The corneal incision was closed with a single 10-0 nylon corneal suture and normal saline was injected to inflate the eye back to its normal shape. Erythromycin ophthalmic ointment was applied topically and the mice were allowed to awaken from anesthesia. For analysis, mice were sacrificed with carbon dioxide following by cervical dislocation at various time intervals after surgery ranging from 24-hours to 5-days. Time zero controls were obtained by re-anesthesizing previously operated mice and the extracapsular lens extraction procedure was performed in the contralateral eye from the first surgery just prior to sacrifice. This minimized the number of animals used for these experiments. Previous

work from the lab did not observe any changes in expression for the markers used in this study comparing time zero samples obtained from naïve mice and those whose other eye had previously undergone lens fiber cell removal (Mamuya, Wang et al. 2014). At least 5 -10 independent animals were used for each analysis described here.

Immunofluorescence

Tissue was excised and embedded fresh in Optimum Cutting Temperature media (OCT, Tissue Tek, Torrance California). Sixteen micrometer thick sections were prepared on a cryostat and mounted on ColorFrost plus slides (Fisher Scientific, Hampton, New Hampshire). Slides were immersion fixed in 1:1 acetone–methanol at −20 C for 20 minutes or 4% paraformaldehyde at room temperature for 20 minutes depending on the antibody (Table2.2). Sections fixed with acetone-methanol were air-dried, while paraformaldehyde fixed sections were washed twice in 1X PBS for ten minutes each then blocked for one hour at room temperature. This was followed by incubation with primary antibody diluted in appropriate blocking buffer (as described in Table 2.2). Following washes either in 1X PBS (phosphate buffered saline) or 1X TBS, primary antibodies were detected with the appropriate AlexaFluor 568 or 488 labeled secondary antibody (Invitrogen, Grand Island, NY) diluted 1:200 in blocking buffer containing a 1:2000 dilution of the nucleic acid stain Draq-5 (Biostatus Limited, Leicestershire, United Kingdom). Sections were washed again in 1X PBS or 1X TBS and then mounted in mounting media (10 milliliters of PBS with 100 milligrams of p-phenylenediamine to 90 milliliters of glycerol; final pH 8.0) (Johnson 1981; Reed, Oh et al. 2001)

Table 2.2: List of all antibodies and other fluorescent reagents used in this study.

	Company	Product #	Fixation	Blocking	Secondary	Dilution
DRAQ5	Biostatus Limited	DR50200	acetone:methanol or 4% PFA in 1xPBS	2% BSA or 5% goat serum		1:2000
αSMA	SigmaAldrich	F3777	acetone:methanol or 4% PFA in 1xPBS	2% BSA or 5% goat serum	FITC conjugated	1:250
E-cadherin	Cell Signaling	4065	4% PFA in 1xPBS	5% goat serum	anti-rabbit	1:100
Aquaporin 0	Millipore	ab3071	acetone:methanol	2% BSA	anti-rabbit	1:200
Pax6	Millipore	AB2237	4% PFA in 1xPBS	5% goat serum	anti-rabbit	1:200
c-Maf	Santa Cruz Biotechnology	sc7866	acetone:methanol	2% BSA	anti-rabbit	1:100
Prox1	Belecky-Adams et al., 1997;Duncan et al., 2002		acetone:methanol	2% BSA	anti-rabbit	1:500
pERK	Cell signaling	s10572	acetone:methanol	2% BSA	anti-goat	1:200
pAkt	Cell signaling	s48789	4% PFA in 1xPBS	5% goat serum	anti-rabbit	1:100
Collagen IV	Abcam	AB6586	acetone:methanol	2% BSA	anti-rabbit	1:200
Keratin 8	Developmental Hybridoma Bank	Troma-1	acetone:methanol	2% BSA	anti-rat	1:100
β1 integrin	Millipore	MAB1997	acetone:methanol	2% BSA	anti-rat	1:200
Laminin	Abcam	11575250	acetone:methanol	2% BSA	anti rabbit	1:200
p27kip[1]	Santa Cruz Biotechnology	sc528	paraffin sections	10% BSA	anti rabbit	1:50
P57kip[2]	Santa Cruz Biotechnology	sc1039	paraffin sections	10% BSA	anti rabbit	1:50
β crystallin	Gift from Dr. Samuel Zigler		paraffin sections	5% goat serum	anti rabbit	1:100
γ crystallin	Gift from Dr Samuel Zigler/ Santa Cruz Biotechnology	sc22415	paraffin sections/ 4% PFA fixation	5% goat serum	anti goat	1:100
Jagged 1	Santa Cruz Biotechnology	sc8303	4% PFA fixation	5% goat serum	anti rabbit	1:100
Foxe3	Santa Cruz Biotechnology	sc134536	4%PFA fixation	5% goat serum	anti rabbit	1:100
Hes1	Santa Cruz Biotechnology	sc-25392	4%PFA fixation	2% BSA and 5% goat serum	anti rabbit	1:100

Table 2.2: List of all antibodies and other fluorescent reagents used in this study

pSmad1/5/8	Santa Cruz Biotechnology	sc-12353-R	4%PFA fixation	1% BSA and 3% goat serum	anti rabbit	1:100
BIGH3/TGFβi	Santa Cruz Biotechnology	sc-28660	acetone:methanol	2% BSA	anti rabbit	1:50
ZL1	Zebrafish International Resource Centre	NA	acetone:methanol	2% BSA	anti mouse	1:500
Laminin	SigmaAldrich	L9393	acetone:methanol	2% BSA	anti rabbit	1:200
Fibronectin	Abcam	ab23790	acetone:methanol	2% BSA	anti rabbit	1:200 -IF 1;1000-WB
LTBP1	Abcam	ab78294	4%PFA fixation	2% BSA and 5% goat serum	anti rabbit	1:200
TGFβ2	Abcam	ab36495	4%PFA fixation	2% BSA and 5% goat serum	anti rabbit	1:200
Phalloidan	Life technologies	A12379	4%PFA fixation	2% BSA and 5% goat serum	Alexa fluor 488 conjugated	1:200
Cleaved caspase3	Cell Signaling	9661	4%PFA fixation	2% BSA and 5% goat serum	anti rabbit	1:100
pSmad3	Cell Signaling	52903	4%PFA fixation	2% BSA,5% goat serum, 10%horse serum	Anti rabbit	1:100

TUNEL Labeling

Tissue was excised, fixed for two hours at room temperature in 4% paraformaldehyde, and transferred to 70% ethanol prior to paraffin embedding. Six micron thick sections were prepared, and nuclear DNA fragmentation was detected by TUNEL staining using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Indianapolis, IN, catalog # 11684795910) following the manufacturer's directions. Slides were counterstained with 1:2000 Draq-5 in 1X PBS to visualize cell nuclei. Following two, five minute 1X PBS washes, slides were mounted in mounting media.

Proliferation Assays

The number of lens epithelial cells that are in S phase was determined using 5-ethynyl-2′-deoxyuridine (EdU) Click-it proliferation assays (Invitrogen, Grand Island, NY, USA). Briefly, pregnant mice were injected intraperitoneally with 8 μg/mouse of EdU dissolved in normal saline. Two hours later, the dam was sacrificed and embryos were removed. Fetal heads were embedded in OCT; and 16-μm frozen sections were obtained by cryostat and mounted on charged glass slides. If needed, slides were stored at −80°C, or immediately fixed by 1:1 ice cold acetone-methanol at -20 C. Sections were allowed to air dry, and the EdU Click-it reaction was carried out following the manufacturer's instructions. Sections were counterstained and mounted as above.

Confocal Image Collection and Analysis

Slides were stored at -20^{o} C until they were visualized with a Zeiss LSM 780 confocal microscope configured with an 405 nm, 458nm, 488nm, 514nm, 561 nm and

633 nm excitation lines) (Carl Zeiss Inc, Göttingen, Germany). All comparisons of staining intensity between specimens were done on sections stained simultaneously and the imaging for each antibody was performed using identical laser power and software settings to ensure validity of intensity comparisons. In some cases, brightness and/or contrast of images were adjusted in Adobe Photoshop for optimum viewing on diverse computer screens. However, in all cases, adjustments were applied equally to both experimental and control images to retain the validity of comparison.

RNA Preparation, RT PCR and qRT PCR

RNA was isolated using the SV Total RNA Isolation System (Promega #Z3105). Briefly, up to 30mg of lenses were homogenized in ice-cold 175ml SV RNA lysis buffer (with BME added) using a pestle. After the tissue was thoroughly homogenized, 350ul of SV RNA dilution buffer was added, and the tube was placed in a 70°C water bath for no longer than three minutes. The lysate was then centrifuged for 10 minutes at 14,000 rpm and the clear lysate was carefully pipetted to an RNase-free microcentrifuge tube containing 200 µl of 95% ethanol. The ethanol:lysate solution was mixed by inverting the tube, transferred to a spin column assembly provided in the kit and centrifuged at 14,000 rpm for 10 minutes. The nucleic acids attached to the spin column membrane were then washed once with 600 µl of the SV RNA wash solution. To remove DNA contamination, the spin column membrane was incubated with 50µl DNase solution at room temperature for 15 minutes. The DNase reaction was terminated by adding 200µl of SV DNase stop solution and the spin column assembly was centrifuged for one minute at 14,000 rpm. The RNA on the spin column membrane was then washed twice with 600µl and 250µl of SV RNA wash solutions by consecutive centrifugation. Finally, the isolated RNA was eluted from the spin column membrane into an RNase-free elution tube through centrifugation

for one minute at 14,000 rpm with 100µl nuclease free water. A Nanodrop ND-1000 Spectrophotometer was used to analyze RNA concentrations of RNA isolates. The machine was blanked with two µL of water, cleaned and two µL of sample used to take appropriate measurements. The output data from the Nanodrop 3.1.2 software includes concentration (ng/µL), OD260 (Optical Density) and the OD ratios 260/230 and 260/280. The RNA was then stored at -80°C.

cDNA was synthesized from these samples using the RT^2qPCR Primer Assay (SABiosciences) according to the manufacturer's instructions, using 5ng of RNA as starting template.

For RT-PCR, 8ul of cDNA was used and the PCR reaction was run on an Eppendorf MasterCycler Gradient PCR machine. The PCR protocol involved a single hold stage at 95°C for 5 minutes. Next 32-34 cycles were run involving the denaturation, annealing and extension phases. Denaturation was done at 94°C for 30 seconds, annealing at variable temperatures and times depending on the primer (see table 1), and extension at 72°C for 1 minute. The sample was then held at 4°C in the PCR machine until use or transfer to the 4°C refrigerator. PCR products were run on either 2% agarose or 5% acrylamide gels. Samples were imaged on a CareStream Gel Logic 212Pro UV imager.

Quantitative RT-PCR (qRT-PCR) was performed using an ABI Prism 7000 Sequence Detection System. Samples were prepared in a MicroAmp® Optical 96-Well Reaction Plate. Each well contained: one µL of cDNA, 12.5 µL of SYBR Green Master Mix (SABiosciences), one µL each of forward and reverse primers and H_2O to 25 µL. Statistical analyses were done using log (base 10) transformed data in a nested ANOVA. The mean and standard deviation (S.D.) were then calculated for the log

transformed data and subsequently back transformed, thus providing the mean fold change, a positive standard deviation, and a negative standard deviation.

Western Blotting

Lenses were isolated and immediately homogenized with 0.1 ml of ice-cold lysis buffer (50mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS) supplemented with 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). The insoluble material was removed by centrifugation at 12,000g for 30 minutes. Final protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's specifications. 40 μg of total protein were resolved by SDS-polyacrylamide gel and transferred onto supported nitrocellulose membranes (Bio-Rad, Hercules, CA). The protein blots were blocked with SuperBlock T20 Blocking Buffer (Thermo Scientific, Rockford, IL) overnight at 4°C and incubated with the primary antibody (Table 2.2) in the same blocking buffer for 2 hours. After incubation with secondary antibodies conjugated with horseradish peroxidase (Calbiochem, San Diego, CA) for 1 hour at room temperature, the signals were detected using an enhanced chemiluminescence detection kit. (Amersham Biosciences, Piscataway, NY).

Chapter 3

THE ROLE OF β1 INTEGRIN IN LENS DEVELOPMENT

Introduction

Integrins are heterdimeric transmembrane adhesion molecules, assembled from 8α and 18β subunits in the endoplasmic reticulum to form 24 distinct integrins, which then transit, to the cell surface to mediate their function (Tiwari, Askari et al. 2011). Integrins are major metazoan receptors for extracellular matrix (ECM) proteins mediating cell-ECM adhesion and, in some cases cell-cell adhesions. Integrins also mediate transmembrane connections to the cytoskeleton and activate many intracellular signaling pathways (Campbell and Humphries 2011). Genes for the β subunits and all but four of the α subunits have been knocked out resulting in distinct phenotypes, reflecting the importance of integrins. The phenotypes range from preimplantation lethality (β1), through major developmental defects (α4, α5, αv, β8), to perinatal lethality (α3, α6, α8, αv, β4, β8) and defects in leukocyte function (αL, αM, αE, β2, β7), inflammation (β6), hemostasis (αIIb, β3, α2), bone remodeling (β3), and angiogenesis (α1, β3) among others. Thus integrins play key roles in development, immune responses, leukocyte traffic, hemostasis, and cancer thus resulting in genetic and autoimmune diseases. They are the target of effective therapeutic drugs against thrombosis and inflammation, and integrins are receptors for many viruses and bacteria (Danen and Sonnenberg 2003).

The importance of integrins in development lies in their ability to detect rapidly altering protein scaffolds allowing prompt response to ever changing cellular

and environmental cues characteristic of development (van der Flier and Sonnenberg 2001). In this context, β1 integrin is of special interest since it is the most widely expressed and promiscuous subunit capable of forming functional receptors with 12 of the 18 α subunits, leading to the ability of cells to detect the composition of diverse ECM environments (van der Flier and Sonnenberg 2001). It is essential for development and its role has been studied in various systems during development (Bokel and Brown 2002).

What is obvious from some of these studies is that β1 integrin changes its function during the course of development influencing cell migration, survival, cell fate specification and growth factor signaling. However the molecular bases of these functions are not well described. The ocular lens is an ideal model to study the molecular mechanisms by which of β1 integrins influence development. The lens is not essential for life, is completely derived from the surface ectoderm, has relatively simple morphology consisting of only two cell types- the proliferating lens epithelium and terminally differentiated lens fibers, with differentiation limited only to the equatorial zone, and extensive knowledge exists about the stages of lens development. Further, β1 integrin is expressed at all stages of lens development and is one of the systems in which the temporal complexity of β1 integrin function has been observed (Simirskii, Duncan et al. 2013; Scheiblin, Gao et al. 2014). While these studies have corroborated and shed light on the possible mechanisms by which β1 integrins play a role in maintaining cell phenotypes and regulating homeostasis, our knowledge on how β1 integrins influence cell fate decisions is still incomplete. In this study we use the lens as a model for expanding our understanding of how β1 integrins influence cell fate decisions during development.

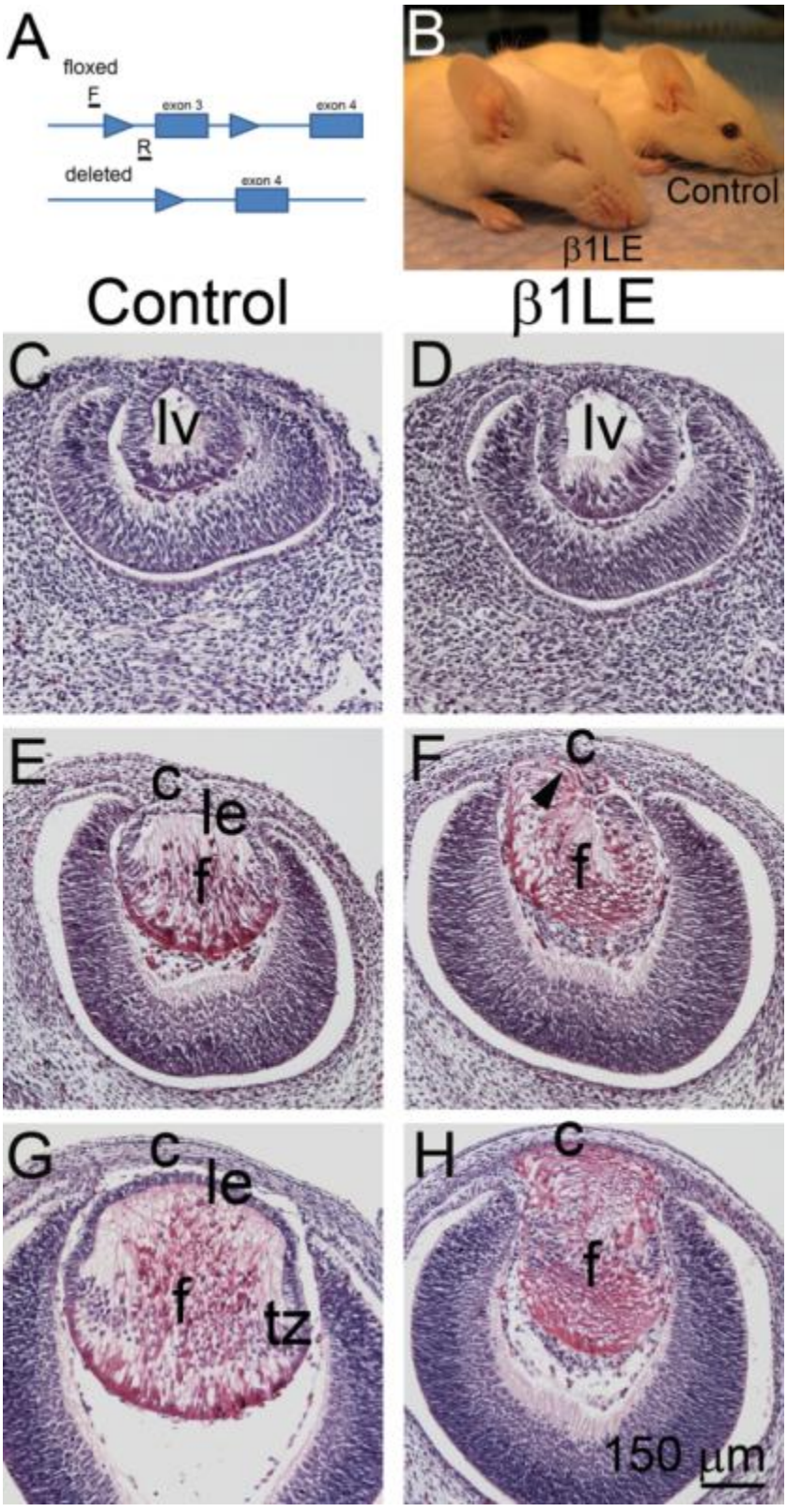
Results

Functional integrins are assembled as heterodimers in the endoplasmic reticulum from α- and β- subunits, then transit to the cell surface to mediate their function (Hynes 2002). The major β- subunit expressed in all lens cells at all stages of lens development is β1 integrin (Barbour, Saika et al. 2004; Simirskii, Wang et al. 2007; Wang, Stump et al. 2009; Scheiblin, Gao et al. 2014). Thus, the function of many integrins can be tested simultaneously by deletion of the β1-integrin gene. Prior work on integrin function in the lens *in vivo* demonstrated that β1 integrins are required for both for lens epithelial cell (LEC) phenotype and survival (Simirskii, Wang et al. 2007) as well as lens fiber cell structure at the later stages of lens development (Scheiblin, Gao et al. 2014). However, the previous studies did not address the role of β1 integrins during early lens development. Therefore we employed the mouse transgenic line, Le-Cre (Ashery-Padan, Marquardt et al. 2000; Yoshimoto, Saigou et al. 2005), to achieve specific deletion of β1 integrin during lens morphogenesis (β1LE; Figure 1A).

Loss of β1-integrin From the Lens Vesicle Reveals Temporal Complexity in β1-integrin Function During Lens Development.

The β1LE mice are microphthalmic as adults similar to the phenotype of adult β1-MLR10 mice (Figure 3.1B) (Simirskii, Wang et al. 2007). In contrast, mice either homozygous for the β1-integrin floxed allele (control) (Figure 3.1B) or heterozygous for both the floxed β1-integrin allele and LE-Cre (data not shown) had no apparent lens abnormalities. While Le-Cre has been reported to be active as early as E9.5 in the lens placode (Ashery-Padan, Marquardt et al. 2000; Yoshimoto, Saigou et al. 2005), both controls and β1LE mice show normal lens vesicle morphology at E11.5 (Figure 3.1C and D). At E12.5, control lenses show a normal lens epithelium anteriorly and

newly differentiated lens fibers posteriorly (Figure 3.1E). In contrast, E12.5 β1LE lenses show regions of lens epithelium loss, with some elongated eosinophilic cells extending beyond the normal anterior anatomical boundary of the lens and encroaching upon the developing cornea (Figure 3.1F, arrowhead). By E14.5, while control lenses show a hematoxylin stained anterior epithelium, a well established transition zone and an eosinophilic fiber cell mass posteriorly (Figure 3.1G), a hematoxylin stained anterior epithelium is completely absent in β1LE lenses and is replaced by eosinophilic elongated cells while no anterior chamber is evident (Figure 3.1).


A
floxed
F
exon 3
exon 4
R
deleted
exon 4
B
Control
β1LE
Control
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D
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E
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le
f
F
c
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tz
H
c
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150 μm

Figure 3.1: Mice homozygous for a floxed allele of β1-integrin and carrying one Le-Cre allele (β1LE) are microphthalmic as adults. (A) Diagram of a portion of the β1-integrin locus showing the location of the loxP sites (arrowheads) found in the floxed allele, the structure of the deleted allele, and the location of the PCR primers (F and R) used to genotype the mice. (B) Exterior appearance of β1LE and control (homozygous for the β1-integrin flox allele, not carrying cre) adult littermates. (C, E, G) Hematoxylin and eosin stained paraffin sections of the eye of control animals (C- E11.5; E- E12.5; G- E14.5), (D, F, H) Hematoxylin and eosin stained paraffin sections from the eyes of β1LE animals (D- E11.5; F- E12.5; H- E14.5). At E11.5, lenses from β1LE mice (D) look similar to controls (C). At E12.5, lenses from β1LE mice (F) show some loss of the anterior epithelium and the eosinophilic staining indicative of lens fiber cells (arrowhead) extends to the cornea as compared to controls (E). This loss of anterior epithelium is very pronounced at E14.5 with complete anterior epithelium loss (H) in β1LE mice as compared to controls (G). Abbreviations: lv - lens vesicle, le - lens epithelium; f – lens fiber cells; c – cornea; tz – transition zone. Scale bar - 150 μm.

While Le-Cre activity is first detected in the lens placode (Ashery-Padan, Marquardt et al. 2000), β1 integrin protein is known to have a long half-life in the lens and other tissues (Raghavan, Bauer et al. 2000; Li, Zhang et al. 2005; Simirskii, Wang et al. 2007; Scheiblin, Gao et al. 2014). Thus, the timing of β1 integrin protein loss in β1LE mice was determined by confocal immunofluorescence analysis (Figure 3.2A-F). At E9.5, β1-integrin protein (red) is detectable throughout the lens placode in controls (Figure 3.2A) while β1-integrin protein levels were lower, but still detectable in the lens placode of β1LE mice (Figure 3.2B). At E10.5, β1 integrin protein is detectable in all cells of the developing lens vesicle in controls (Figure 3.2C) while β1-integrin protein levels dropped below the limit of detection in the lens vesicle of β1LE mice at this age (Figure 3.2D). Consistent with this, E16.5 control lenses

express β1- integrin protein in all lens cells (Figure 3.2E), while β1-LE lenses lack detectable β1-integrin protein (Figure 3.2F).

Since the histological consequences of β1-integrin loss from the lens vesicle (see Figure 3.1) were different from that observed upon deletion later in development (Simirskii, Wang et al. 2007), we investigated whether the underlying molecular phenotype was distinct as well by investigating αSMA expression in β1LE lenses since the loss of β1-integrin from all lens cells later on (E11.5, after primary fiber cell elongation is complete) results in up-regulation of αSMA in the lens epithelium at E16.5 (Simirskii, Wang et al. 2007). Consistent with the histological appearance of β1LE lenses, immunolocalization studies between E11.5-16.5 showed no up-regulation of αSMA in the β1LE lenses (data not shown; Figure 3.2 F,H) as compared to the controls (data not shown, Figure 3.2E, G), suggesting a temporal complexity in the function of β1-integrins during lens development.

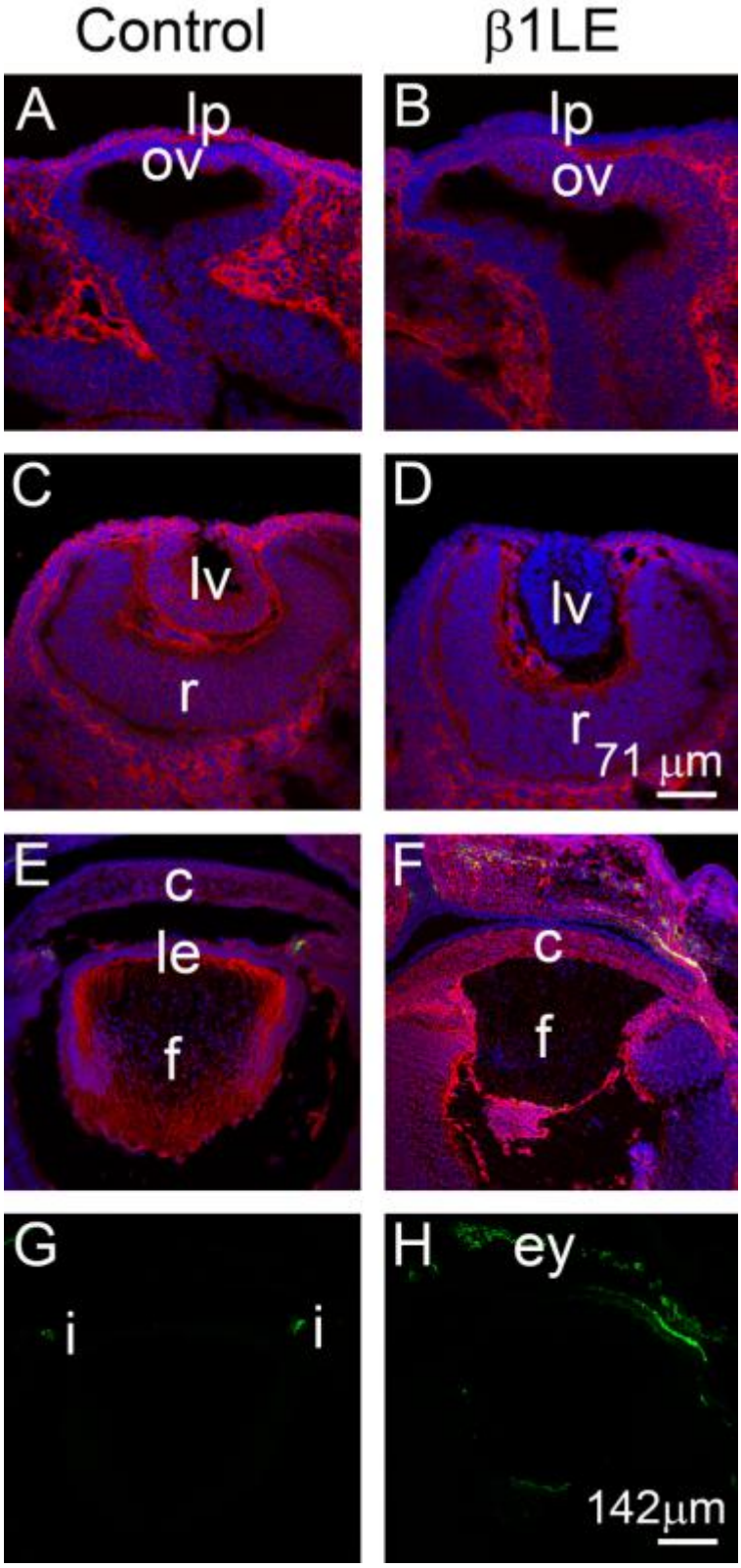
Control
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Figure 3.2: β1LE mice lose β1-integrin protein from the developing lens vesicle by E10.5. Immunofluorescent confocal microscopy showing β1-integrin protein expression in control (A, C, E) and β1LE (B, D, F) lenses at E9.5 (A, B), E10.5 (C, D) and E16.5 (E, F).At E9.5, β1-integrin protein expression is reduced in the β1LE lens placode (B), compared to control (A). By E10.5, β1-integrin protein is detected in all cells of the lens vesicle of control mice (C) whereas β1-integrin protein levels fall beyond the level of detection in β1LE lenses (D). At E16.5, β1-integrin is still detectable in all cells of control lenses (E) while, consistent with the result at E10.5, no β1-integrin was detected in β1LE lenses at this age (F). Co-staining of the E16.5 sections shown in panels E and F for αSMA did not reveal any αSMA signal (green) within the boundary of the lens in either in control (E, see panel G for αSMA channel only) or β1LE lenses (F, see panel H for αSMA channel only). Red - β1-integrin, Green - αSMA, Blue - DNA. Abbreviations: lp - lens placode, lv – lens vesicle, le – lens epithelium, c – cornea, f – lens fiber cells, ov – optic vesicle, r – retina, i – iris, ey - eyelids. Scale bars Panels A, B, C, D- 71μm; Panels E, F, G, H- 142 μm

β1LE Lenses Lose Their Anterior Lens Capsule.

The presence of lens cells outside of the normal anterior anatomical boundary of the lens suggested that the lens capsule, which completely surrounds the lens and sequesters it from other ocular tissues (Cotlier, Fox et al. 1968; Karkinen-Jaaskelainen, Saxen et al. 1975; Beyer, Vogler et al. 1984; Danysh and Duncan 2009), might be disrupted in β1LE mice. Confocal immunofluorescence using antibodies against the known lens capsule components- laminin (Figure 3.3A, B). collagen IV (data not shown) and perlecan (data not shown), show the expected intact lens capsule in control (Figure 3.3A) E12.5 lenses, while defects in the anterior lens capsule are seen in E12.5 β1LE lenses (Figure 3.3B. arrowheads). At higher magnification, all of the laminin associated with the lens is confined to the lens capsule in controls (Figure 3.3C), while β1LE lenses (Figure 3.3D) exhibit intracellular laminin immuno-

reactivity (arrowheads) suggestive of intracellular retention of newly synthesized laminin. Similar results were also obtained for collagen IV (not shown).

Cell adhesion to extracellular matrices via β1-integrins has long been proposed to protect cells from apoptosis/anoikis by signaling to cell survival pathways (Raghavan, Bauer et al. 2000). Therefore, the loss of lens capsule in β1LE lenses suggested that anoikis might be responsible for the loss of lens epithelium observed in these lenses. TUNEL assays revealed no TUNEL positive apoptotic cells in control lenses (Figure 3.3E, G) while only sporadic TUNEL signals were detected in β1LE lenses (Figure 3.3F, H, arrowheads) These data suggest that the absence of an anterior lens epithelium in β1LE lenses after E13.5 is unlikely to be primarily attributable to LEC apoptosis.

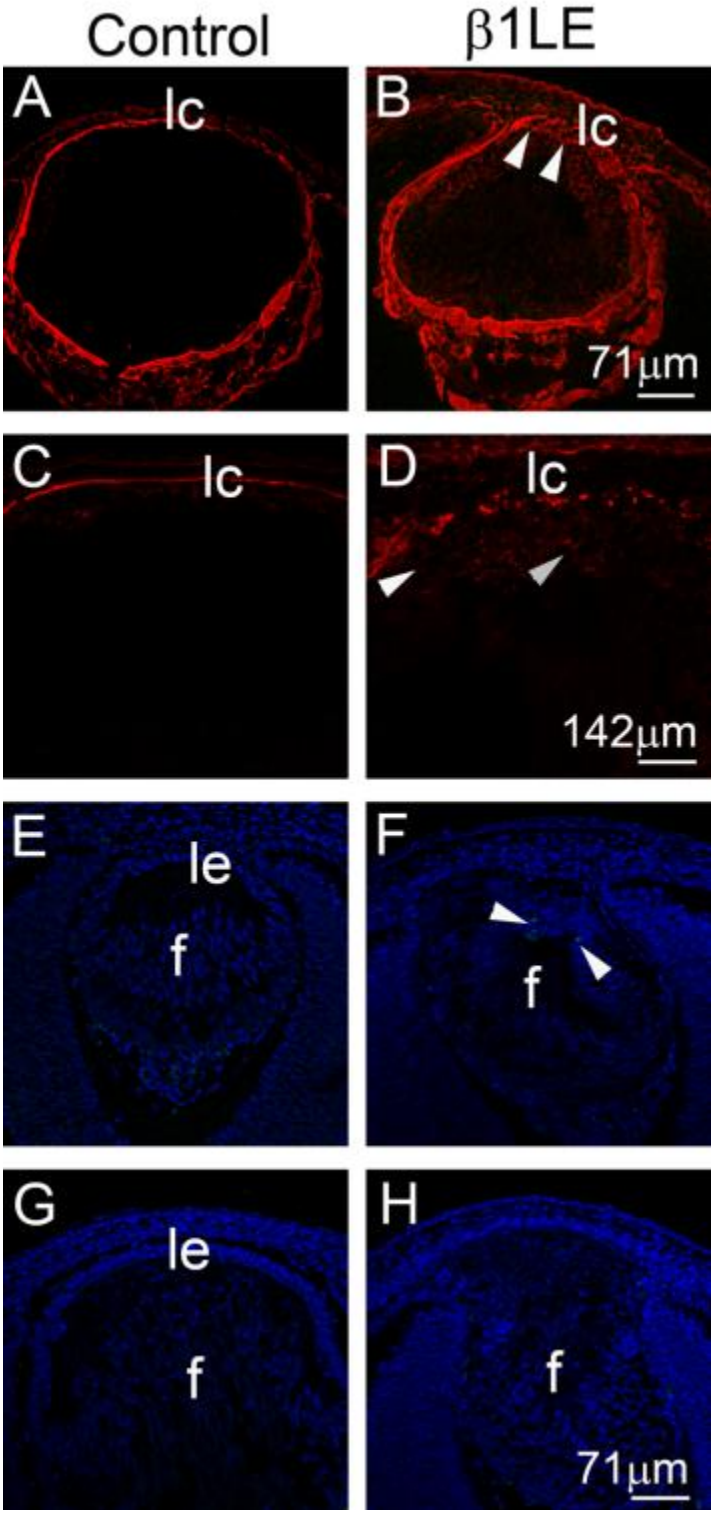
Control
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Figure 3.3: β1LE lenses show defects in anterior lens capsule starting at E12.5 without massive lens cell apoptosis. Confocal immunofluorescence showing the staining pattern of laminin in the lenses of control (A, C) and β1LE (B, D) mice at E12.5. Control lenses (A) exhibit continuous laminin staining around the lens at E12.5, while this pattern is interrupted, particularly on the anterior lens surface (arrowheads), in β1LE lenses (B). At higher magnification, E12.5 control lenses (C) only exhibit laminin staining associated with the lens capsule, while β1LE lenses (D) show intracellular laminin immuno-reactivity at this age (D-arrowheads).TUNEL assay for apoptosis (green), in controls (E– E12.5, G– E13.5) and β1LE lenses (F– E12.5, H– E13.5). Control lenses do not show anterior lens epithelium apoptosis as measured by TUNEL during normal lens development both at E12.5 (E) and E13.5 (G), β1LE lenses only show occasional TUNEL positive cells (F, arrowheads) but this is not consistently detected in every section (H, data not shown). Red (panels A, B, C, D)- Laminin; Green (panels E, F, G, H) - TUNEL; Blue - DNA. Abbreviations: f - lens fiber cells, lc - lens capsule, le - lens epithelium. Scale bars Panels A, B, E, F, G, H - 71μm, Panels C, D - 35μm.

β1LE Lens Epithelial Cells Show Less Proliferation.

Similar to the results seen in β1LE lenses (Figure 3.4E-H), conditional deletion of the β1-integrin gene from skin keratinocytes (Raghavan, Bauer et al. 2000), hair follicles (Brakebusch, Grose et al. 2000) or luminal mammary epithelial cells (Li, Zhang et al. 2005; Naylor, Li et al. 2005) does not lead to apoptosis *in vivo* even following cell detachment from the underlying basement membrane. These cells instead show reduction in proliferation rates. Therefore, we investigated if proliferation defects contribute to the loss of anterior epithelium in β1LE lenses. E12.5 control lenses (Figure 3.4A – arrowheads) shows large number of anterior epithelial cells actively synthesizing DNA as measured by EdU incorporation, while only a few EdU positive cells are seen in the anterior epithelium of E12.5 β1LE lenses (Figure 3.4B– arrowhead). By E14.5, control lenses (Figure 3.4C) show large number of

proliferating cells which are actively synthesizing DNA in the anterior epithelium, whereas we detected no cells actively synthesizing DNA in β1LE lenses (Figure 3.4D). In the normal lens, LECs up-regulate the cyclin-dependent kinase inhibitors $p27^{Kip1}$ (Figure 3.4E) and $p57^{Kip2}$ (Figure 3.4G) (Jia, Lin et al. 2007; Rowan, Conley et al. 2008; Saravanamuthu, Gao et al. 2009; Saravanamuthu, Le et al. 2012; Antosova, Smolikova et al. 2013), as they leave the cell cycle coincident with their differentiation into lens fibers. In contrast, abnormal up-regulation of both $p27^{Kip1}$ (Figure 3.4 F – arrowheads) and $p57^{Kip2}$ (Figure 3.4 H – arrowheads) was seen in the anterior lens epithelium of E12.5 β1LE mice.

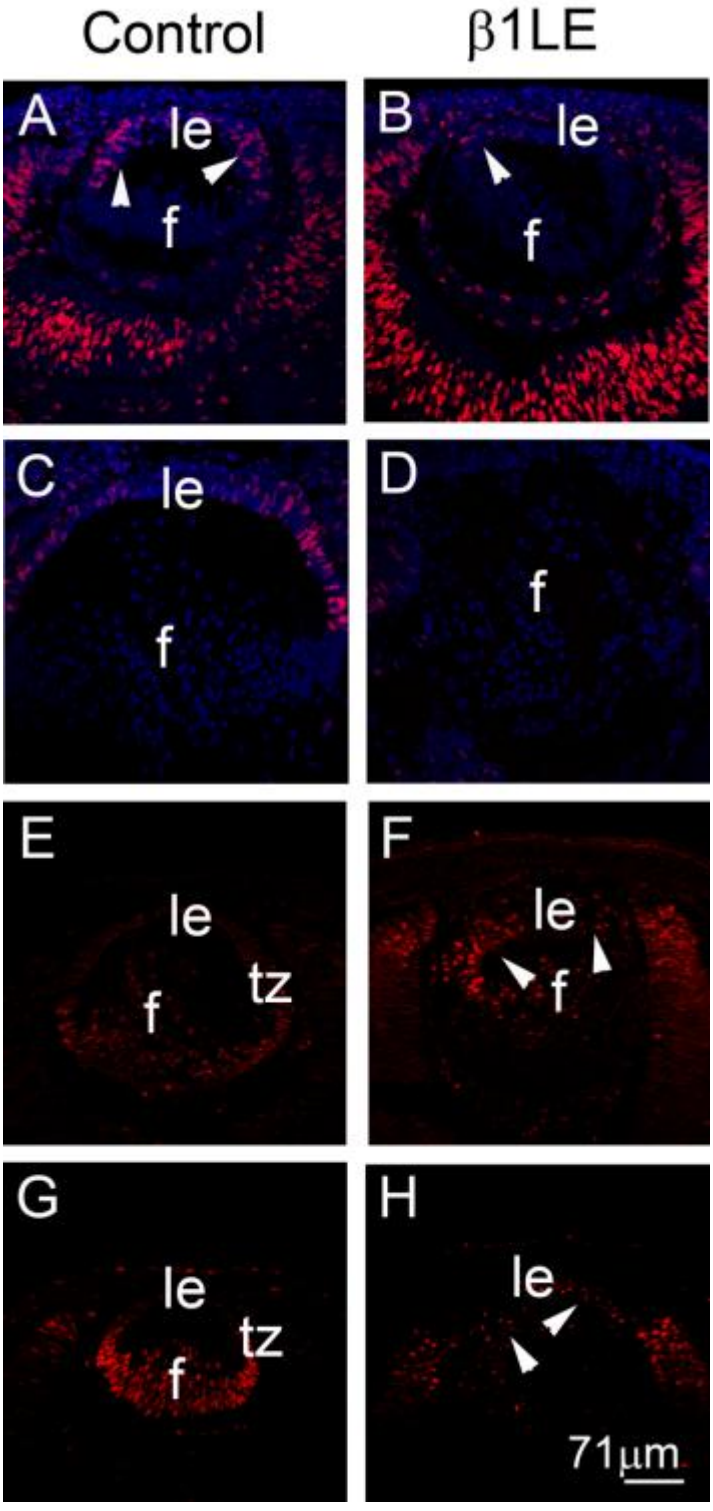
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Figure 3.4: β1LE lenses exhibit decreased LEC proliferation coincident with the up regulation of cell cycle exit markers. EdU cell proliferation assays (A-D) comparing control (A– E12.5; C– E13.5) with β1LE lenses (B– E12.5; D– E13.5). A decrease in the number of lens epithelial cells actively synthesizing DNA is seen starting at E12.5 in β1LE lenses (B) as compared to controls (A). By E13.5, β1LE lenses (D) show complete loss of cells actively synthesizing DNA as compared to controls (C) which maintain cell proliferation in the lens epithelium. Confocal immunofluorescence showing expression pattern of cell cycle exit markers in controls (E– $p27^{Kip1}$; G– $p57^{Kip2}$) versus β1LE lenses (F– $p27^{Kip1}$ and H– $p57^{Kip2}$). Control lenses showing little to no $p27^{Kip1}$ (E) as well as $p57^{Kip2}$ (G) in the anterior lens epithelium at E12.5, while β1LE lenses show large number of cells exiting the cell cycle as compared to controls, shown by both $p27^{Kip1}$ (F- arrowheads) staining and $p57^{Kip2}$ staining (H– arrowheads). Red (panels A, B, C, D)- Sites of active DNA synthesis, (panels E,F)- $p27^{Kip1}$, (panels G,H)- $p57^{Kip2}$; Blue- DNA. Abbreviations: f - lens fiber cells, le, lens epithelium, tz – transition zone. Scale bar - 71μm.

β1LE Lenses Lose E-cadherin Expression and Show Fiber Specific Marker Expression Throughout the Lens.

The loss of proliferation and up regulation of cell cycle exit markers in the anterior LECs of the β1LE mice, along with expanded domain of eosinophilic staining (diagnostic of cells expressing high concentrations of protein), suggested that the lens epithelium was differentiating inappropriately into lens fibers. To characterize this finding further, the expression pattern of the epithelium specific marker E-cadherin was analyzed. In control lenses (Figure 3.5A), E-cadherin staining is observed throughout the anterior LECs, while it is disappears coincident with fiber differentiation, as visualized by γ-crystallin immunoreactivity, at the transition zone (Figure 3.5A). In contrast, we observed a down regulation of E-cadherin starting at E12.5 in β1LE lenses (Figure 3.5B) and γ-crystallin immunoreactivity in the anterior portion of the lens. This was much more evident at E13.5, where β1LE lenses show

almost a complete loss of E-cadherin staining, while γ-crystallin immunoreactivity is detected at the most anterior aspect of the lens (arrowheads; Figure 3.5D) .

In order to support this evidence, we investigated whether other established fiber cells markers, β crystallin (data not shown) (Wigle, Chowdhury et al. 1999; Duncan, Xie et al. 2004), Aquaporin 0 (Figure 3.5E and 3.5F) (Bassnett, Missey et al. 1999; Bassnett, Wilmarth et al. 2009) and Jagged1 (Figure 3.5E-H) (Saravanamuthu, Gao et al. 2009; Saravanamuthu, Le et al. 2012) were expressed inappropriately in β1LE lenses. As expected, β–crystallin (data not shown) staining was confined to the lens fiber cells in controls at E12.5 and E13.5, while the anterior cells of β1LE lenses show β–crystallin (data not shown) staining, mirroring the domain of eosinophilic staining seen in Figure 3.5H. Aquaporin 0 is a water channel found exclusively in lens fiber cells (Bassnett, Missey et al. 1999; Bassnett, Wilmarth et al. 2009). As expected, Aquaporin 0 staining is confined only to the lens fiber cells both at E14.5 and E16.5 in controls (data not shown; Figure3.5E), while in the β1LE lenses, the most anterior lens cells express Aquaporin 0 (data not shown; Figure3.5F). Similarly, Jagged1, a membrane protein important for Notch signaling in lens, is confined to the developing lens fibers at E12.5 (Figure 3.5G), whereas this expression domain shifts anteriorly in β1LE lenses (Figure 3.5H – arrowheads). By E13.5, Jagged1 expression is restricted to the newly formed fiber cells in the transition zone of control lenses (Figure 3.5I), while β1LE lenses exhibit Jagged1 expression in all lens cells (Figure 3.5J – arrowheads)

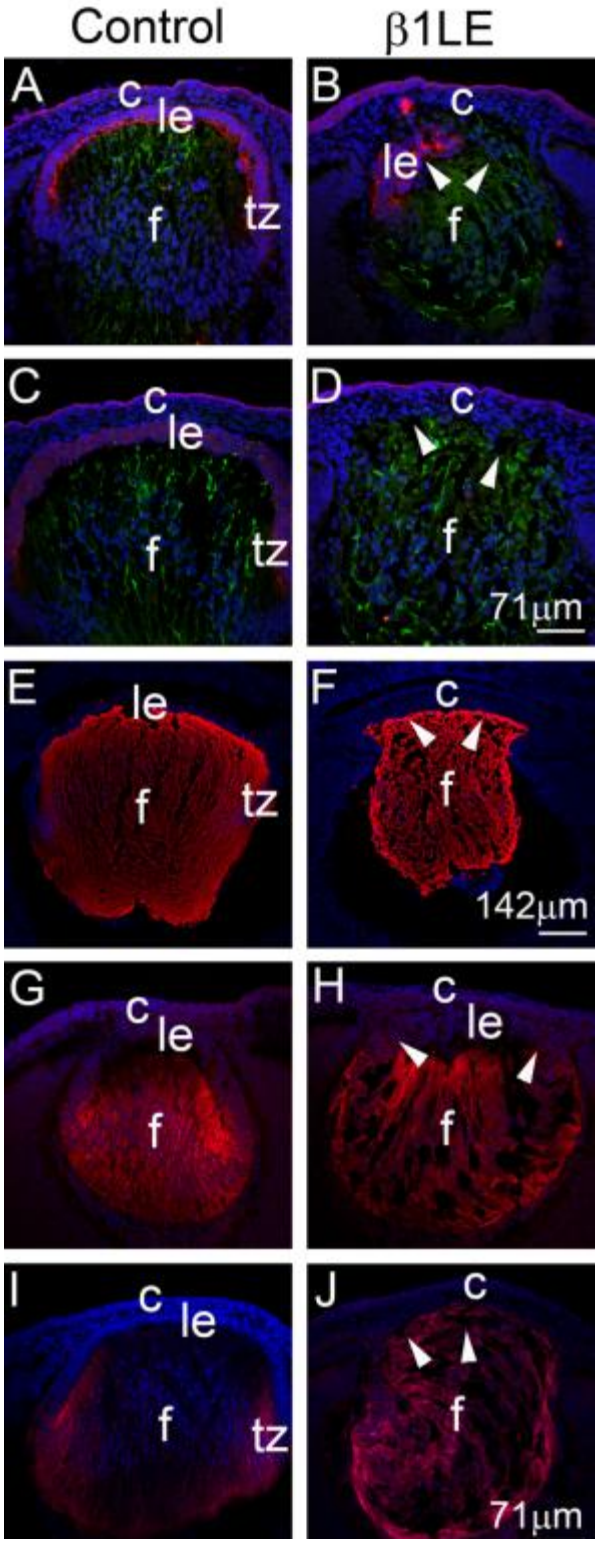
Control
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Figure 3.5: β1LE LECs down-regulate E-cadherin while exhibiting aberrant fiber cell marker staining Panels (A to D) show co-immunolocalization of E-cadherin (red) and γ crystallin (green) at E13.5 and E14.5. Control E13.5 (A) lenses show staining for E-cadherin (red) only in anterior LECs, with γ-crystallin (green) restricted only to fiber cells. In contrast, β1LE lenses at E13.5 (B-arrowheads) show γ-crystallin staining extending up-to cornea and only a few lens epithelial cells express E-cadherin. E14.5 control lenses (C) show uniform E-cadherin staining in the anterior LECs while γ-crystallin expression is confined to the lens fiber cells, whereas a complete loss of E-cadherin expression is seen in E14.5 β1LE lenses, with all lens cells positive for γ-crystallin (D - arrowheads). At E16.5, control lenses (E) show Aquaporin0 staining restricted to lens fiber cells, while β1LE lenses show Aquaporin0 (F) staining in almost all lens cells. Control lenses at E12.5 (G) show normal Jagged1levels up-regulating at the transition zone, while β1LE lenses show an anterior shift in Jagged1 expression (H – arrowheads). At E13.5, controls (I) show Jagged1 to be predominately expressed in the newly differentiated lens fibers, whereas in β1LE lenses, all lens cells are positive for Jagged1 (J–arrowheads). Red (panels A-D) – E-Cadherin, (panels E-F)– Aquaporin0, (panels G-J)-Jagged1; Green (panels A-D)- γ-crystallin, Blue - DNA. Abbreviations: le – lens epithelium, c- cornea, f - lens fiber cells, tz – transition zone. Scale bar Panels A,B,C,D,G,H,I,J = 71 μm; Panels E,F =142μm.

β1LE LECs Down Regulate the Expression of Transcription Factors Important for LEC Phenotype

In order to confirm that β1LE LECs were losing their lens epithelial identity, we looked at the expression of the transcription factors Foxe3 (Blixt, Mahlapuu et al. 2000), Pax6 (Duncan, Kozmik et al. 2000; Duncan, Xie et al. 2004; Donner, Ko et al. 2007),and Hes1 (Rowan, Conley et al. 2008) that are involved in maintaining lens epithelial cells. Foxe3 Pax6 and Hes1 are all expressed throughout the developing lens vesicle, however, once the lens forms, their levels fall in the lens fibers and they become more restricted to the LECs (Ashery-Padan, Marquardt et al. 2000; Blixt, Mahlapuu et al. 2000; Rowan, Conley et al. 2008). Consistent with their function in LECs, Foxe3 (Figure 3.6A), Pax6 (Figure 3.6C) and Hes1 (Figure 3.6E) staining was

seen in the LECs of E13.5 control lenses, while all three of these proteins (FoxE3, Figure 3.6B – arrowheads; Pax6, Figure 3.6D – arrowheads; Hes1, Figure 3.6F– arrowheads) are found at reduced levels in β1LE LECs.

In the normal lens, as LEC preferred negative regulators of fiber differentiation down regulate in the transition zone, the expression of positive regulators of lens fiber differentiation, such as such as Prox1 (Wigle, Chowdhury et al. 1999; Wigle and Oliver 1999) and cMaf (Kawauchi, Takahashi et al. 1999; Yoshida, Kim et al. 2001; Yoshida and Yasuda 2002) up regulate. Thus, control lenses exhibit cMaf (Figure 3.6G) and Prox1 (Figure 3.6I) staining in the lens fiber cells., In contrast, both cMaf (Figure 3.6H – arrowheads) and Prox1 (Figure 3.6J – arrowheads) are present in the cell nuclei found at anterior aspect of β1LE lenses, consistent with the proposition that these cells are undergoing inappropriate fiber cell differentiation.

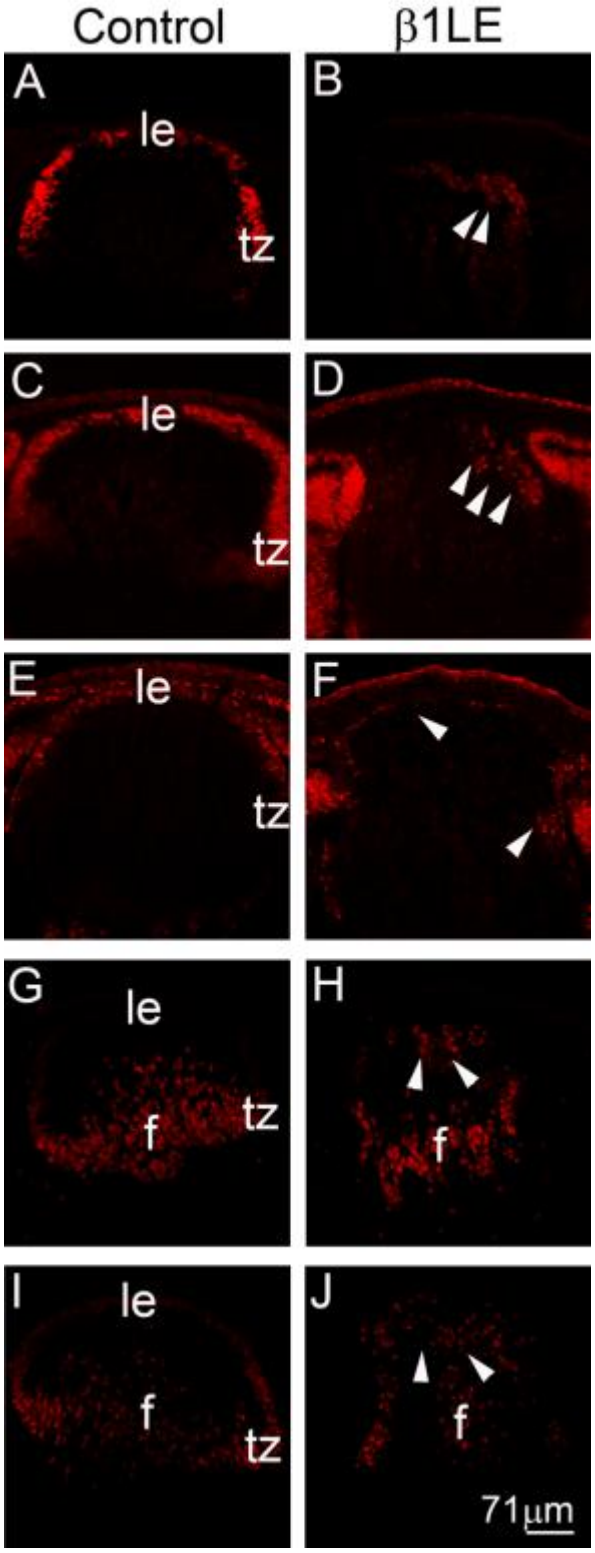
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Figure 3.6: β1LE lenses down regulate the expression of LEC preferred transcription factors and show ectopic fiber preferred transcription factor expression at E13.5. Immunolocalization of Foxe3 (A,B), Pax6 (C,D) and Hes1(E,F) show uniform expression in all lens epithelial cells of control lenses (A, C, E) while all of these factors down-regulate in the anterior epithelial cells of β1LE lenses (B, D, F; arrowheads).(G, H) Immunolocalization of cMaf and (I, J) immunolocalization of Prox1. Control lenses express cMaf (G) in the fiber cell compartment, while the anterior lens cells of β1LE lenses (H- arrowheads) ectopically express cMaf. In controls, Prox1 (I) is normally expressed in all lens cells, but its expression up regulates in the transition zone and this expression remains elevated in the fiber cells. In contrast, almost all lens cells of β1LE lenses (J- arrowheads) express high levels of Prox1 protein. Red –(panels A,B)-Fox3;(panels C, D)- Pax6; (panels E, F)- Hes1; (panels G-H)-cMaf,;(panels I-J)-Prox1; Blue -DNA. Abbreviations: le - lens epithelium; tz - transition zone, f - lens fiber cells. Scale bar - 71μm.

β1LE Lenses Up Regulate Downstream Effectors of Pathways Influencing Lens Fiber Differentiation.

Activation of FGF induced MAPK/ERK1/2 and PI3-AKT signaling is required for differentiation of lens epithelial cells to lens fibers (Le and Musil 2001; Lovicu and McAvoy 2001; Weber and Menko 2006; Weber and Menko 2006; Zhao, Yang et al. 2008; Wang, Stump et al. 2009) while lower levels pErk activity are essential for lens cell proliferation (Chandrasekher and Sailaja 2003; Iyengar, Patkunanathan et al. 2006). PI3-AKT signaling can be induced by many growth factors including FGF and is known to influence both LEC proliferation and lens fiber differentiation (Iyengar, Patkunanathan et al. 2006; Weber and Menko 2006). Thus, we tested whether β1-integrin deletion in the early lens influences the distribution and level of ERK1/2 and AKT phosphorylation at E12.5 (Figure 7A,D), using immuno-histochemistry.

In the normal embryonic lens, pERK1/2 is detectable by immuno-histochemistry only in cells undergoing fiber differentiation at the transition zone

(Figure 7A- arrowheads) (Madakashira, Kobrinski et al. 2012), whereas pERK 1/2 was detectable in a large number of anterior LECs of the β1LE lenses (Figure7B – arrowheads). During normal lens development pAKT is detectable by immuno-histochemistry (Figure 7C) in anterior LECs as well as in newly differentiating cells at the transition zone, whereas no staining is detectable in the already differentiated lens fibers (Li, Tao et al. 2014). In contrast, all the lens cells of β1LE lenses (Figure 7D- arrowheads) stain for pAKT.

FGF is frequently stated to be only factor essential and sufficient for initiating fiber cell differentiation. Although essential, recent work has shown that FGF is not sufficient to produce all changes characteristic of fiber cell differentiation (Lovicu, McAvoy et al. 2011). BMP signaling is essential for lens induction and is also required for both primary and secondary fiber differentiation (Belecky-Adams, Adler et al. 2002; Boswell, Lein et al. 2008; Boswell, Overbeek et al. 2008; Pandit, Jidigam et al. 2011). Further BMP and FGF crosstalk is shown to be essential for regulating proliferation and differentiation in the developing lens (Boswell, Lein et al. 2008; Jarrin, Pandit et al. 2012). Thus the changes in FGF signaling pattern upon β1 integrin deletion prompted us to investigate if BMP signaling was also affected by this deletion. Using immuno localization against BMP mediator pSmad1/5/8 (Beebe, Garcia et al. 2004) we found that BMP activity is restricted to the transition zone of the lens at E12.5 in controls (Figure 7E - arrowheads), while pSmad1/5/8 staining is observed extending into the anterior LECs in β1LE lenses (Figure 7F – arrowheads).

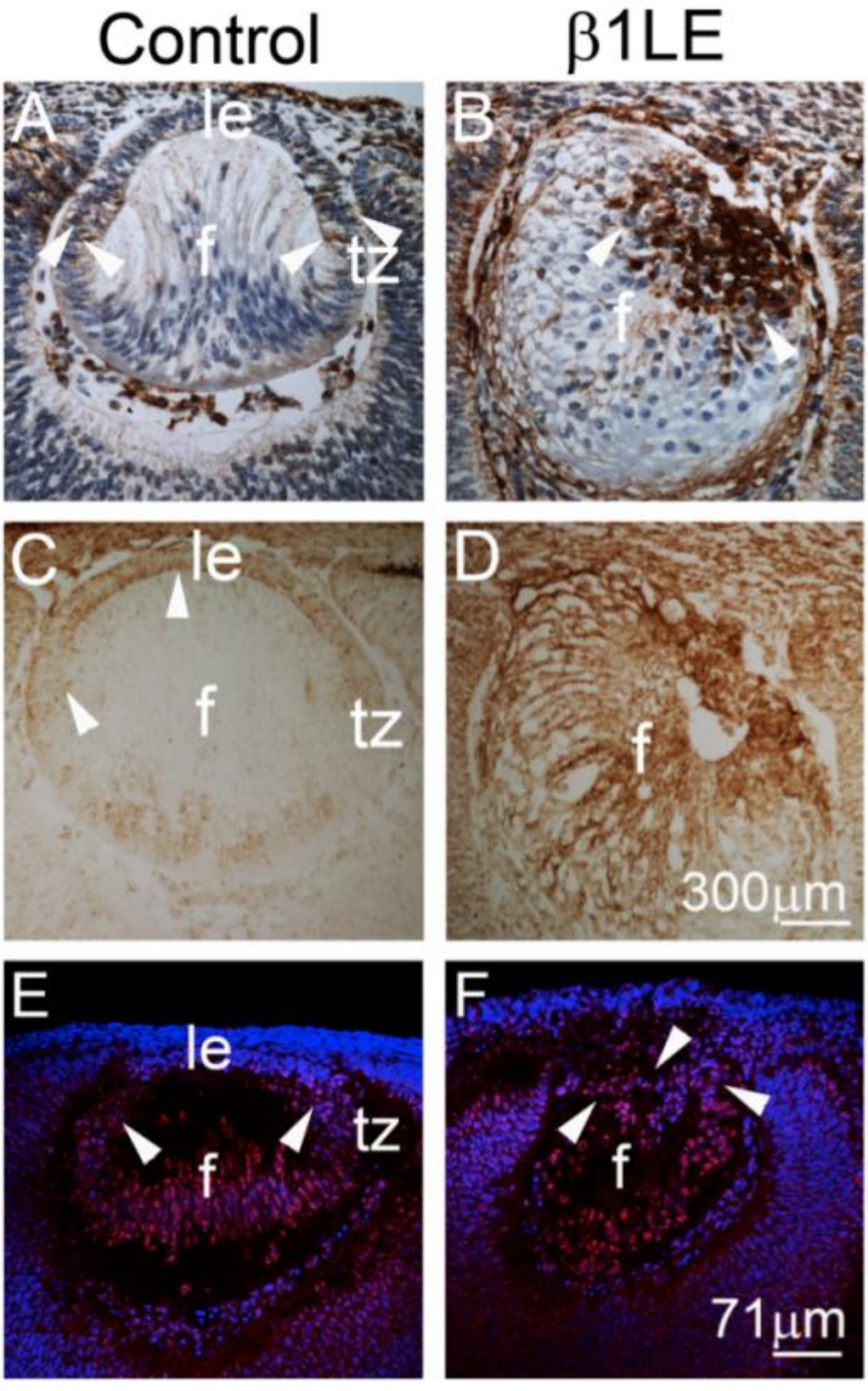
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Figure 3.7: Abnormal distribution of pERK1/2, Pakt, and pSMAD 1/5/8 in β1LE lenses at E12.5 Immunolocalization of pERK1/2 at E12.5 in control (A) and β1LE lenses (B) lenses. E12.5 control lenses (A) show normal distribution of pERK1/2 in the differentiating cells at the transition zone (arrowheads), while little to no signal is detectable by this method in the lens epithelium. In contrast, the β1LE lenses exhibit staining for pERK1/2 in the anterior lens cells (B– arrowheads). Immunohistochemical localization of pAKT at E12.5 in controls (C) and β1LE lenses (D). pAKT is normally distributed in the lens epithelium and newly differentiated lens fiber cells at the transition zone in controls (C– arrowheads) at E12.5, whereas in the β1LE lenses, it is up-regulated in almost all lens cells (D). Immunolocalization of pSmad1/5/8 at E12.5 in control (E) and β1LE lenses (F). E12.5 control lenses (E) show normal distribution of pSmad1 in the cells undergoing fiber differentiation at the tansition zone (arrowheads), while an up regulation of pSmad1 is seen in the anterior cells of β1LE lenses (F)Red –(panels E,F)- pSmad1; Blue – (panels E,F)- DNA Abbreviations; le - lens epithelium; f – lens fiber cells; tz – transition zone. Scale bar Panels : A, B, C, D =300μm; Panels: E, F = 71μm

Discussion

β1-integrins play diverse functions in the lens including mediation of lens cell -lens capsule interactions (Menko and Philip 1995; Danysh and Duncan 2009; Scheiblin, Gao et al. 2013) and lens development (Walker and Menko 1999; Walker, Zhang et al. 2002; Simirskii, Wang et al. 2007; Scheiblin, Gao et al. 2013). Further, β1-integrin expression up-regulates during epithelial-mesenchymal transition (EMT) of lens cells to myofibroblasts (Zuk and Hay 1994; de Iongh, Wederell et al. 2005), and blockade of β1-integrin function can prevent EMT. Despite this, the *in vivo* function of the β1-integrins expressed by LECs, as well their role in early lens development is not clearly delineated.

β1-integrins are Necessary for Initial Lens Capsule Assembly

Previously, we created mice lacking β1- integrins from the lens at E11.5 onwards (β1MLR10) (Simirskii, Wang et al. 2007). In these mice, early lens growth proceeds normally up to E15.5; however, later in development, the lens epithelial cells become spindle shaped, and begin expressing the mesenchymal marker, alpha smooth muscle actin (αSMA) and by birth undergo apoptosis, leading to microphthalmia in adulthood (Simirskii, Duncan et al. 2013). In contrast, in the present study, lenses that lose β1-integrin just a day earlier, at E10.5, show complete loss of a hematoxylin stained anterior epithelium, which is replaced by eosinophilic elongated cells by E14.5. Further, at the molecular level, E16.5 β1MLR10 mouse LECs down-regulated Pax6, and up-regulated αSMA and lens fiber cell markers with no changes in the lens capsule (Simirskii, Wang et al. 2007). Notably though, when β1-integrin protein is lost from the lens a day earlier in development (coincident with lens vesicle separation), the anterior lens capsule is abnormal by E13.5 and, the lens epithelium leaves the cell cycle. Coincidently, lens epithelial cells begin to elongate, and molecularly transition from an epithelial phenotype to a lens fiber cell one, in the absence of notable apoptosis. These data indicate that β1-integrins have multiple functions in the lens and these functions change as lens development proceeds.

The profound anterior lens capsular defects observed in β1LE lenses, along with the presence of laminin and collagen IV intracellular aggregates are likely directly related to integrin loss (Lohikangas, Gullberg et al. 2001; Li, Harrison et al. 2002; Li and Yurchenco 2006). Laminin is the first ECM component to be laid down during development, and β1-integrin dependant assembly of the laminin heterotrimer is required for its secretion and incorporation into the BM (Aumailley, Pesch et al. 2000). Collagen IV is ubiquitous in BMs including the lens capsule, integrating with

the laminin scaffold to provide stability and strength to the basement membrane (Aumailley, Pesch et al. 2000; Kelley, Sado et al. 2002; Danysh and Duncan 2009). Notably, both *lamininc1* mutant mice as well as *laminina1* mutant zebrafish which do not form the initial laminin111 network normally found in the epiblast, also do not form an organized Collagen IV network, instead, Collagen IV was detected in aggregates throughout the embryonic lens (Aumailley, Pesch et al. 2000; Pathania, Semina et al. 2014). This suggests that the lens, like the early embryo, requires β1-integrins for the appropriate secretion and assembly of the basement membrane.

β1-integrin Regulates Cell Fate Decisions Early in Lens Development

The transcription factors cMaf and Prox1 are expressed in the lens vesicle but as development progresses, their expression is up regulated in differentiating fiber cells (Cui, Tomarev et al. 2004). This expression is critical since null mutants for these proteins have defects in primary fiber cell elongation (Kawauchi, Takahashi et al. 1999; Wigle and Oliver 1999; Kralova, Czerny et al. 2002; Hu, Huang et al. 2012). In contrast, Pax6 and Foxe3 are predominately expressed in LECs and their over expression in the posterior lens vesicle disrupts fiber differentiation (Blixt, Mahlapuu et al. 2000; Yoshimoto, Saigou et al. 2005; Donner, Ko et al. 2007). The up regulation of Prox1 and cMaf coupled with down regulation of Pax6 and Foxe3 in the LECs upon β1- integrin removal from the lens indicated that β1 integrins might play a crucial role in lens cell fate determination by impinging upon fiber cell differentiation pathways in the lens. This was supported by our functional marker data where we saw down regulation of epithelium specific- E-cadherin in the LECs along with the inappropriate localization of fiber markers γ crystallin, aquaporin 0 and jagged 1 in the anterior cells of β1LE lenses. Further, during differentiation of LECs into fiber cells at the

equatorial zone of a normal developing lens a decrease in number of cells in S phase is coupled with an up regulation of inhibitors of cyclin dependant kinases (Cdks) $p27kip^1$ and $p57kip^2$ is seen (Lovicu and Robinson 2004). Both $p27kip^1$ and $p57kip^2$ are critical and redundant in controlling cell cycle exit and differentiation of LECs into fibers at the transition zone of the lens (Zhang, Wong et al. 1998). Consistent with these findings we see a loss of cells in S phase in the anterior β1 LE lenses, along with an up regulation of both $p27kip^1$ and $p57kip^2$ validating our observation that upon loss of β1 integrin from the lens vesicle, anterior LECs undergo inappropriate fiber differentiation.

β1-integrins Act as Negative Regulators of Growth Factor Signaling Required for Lens Fiber Cell Differentiation

There is compelling evidence implicating growth factors as key regulators of lens growth and development (Lang 1999; Walker, Zhang et al. 2002; Lovicu and McAvoy 2005). Both *in vitro* and *in vivo* studies have shown that lens cell can be stimulated to proliferate by many different growth factors including fibroblast growth factors (FGFs) (McAvoy, Chamberlain et al. 1999), bone morphogenetic proteins (BMP) (Boswell, Lein et al. 2008; Boswell, Overbeek et al. 2008) (platelet-derived growth factor (PDGF) (Reneker and Overbeek 1996), insulin and insulin-like growth factors (IGFs) (Chandrasekher and Sailaja 2003; Iyengar, Patkunanathan et al. 2006) epidermal growth factor (EGF) (Wang, Wormstone et al. 2005) and hepatocyte growth factor (HGF) (Choi, Park et al. 2004). Among these growth factors, both FGF and BMP are known not only to influence lens fiber differentiation (Boswell, Lein et al. 2008; Boswell, Overbeek et al. 2008; Lovicu, McAvoy et al. 2011; Jarrin, Pandit et al. 2012) but also interact during fiber differentiation (Boswell, Lein et al. 2008; Boswell,

Overbeek et al. 2008; Jarrin, Pandit et al. 2012), with both these factors being essential for but not sufficient to produce all changes associated with fiber differentiation (Boswell, Overbeek et al. 2008; Jarrin, Pandit et al. 2012). Further studies have shown that all lens cells are sensitive to FGF signals and that the level of FGF activity needs to be fine tuned in the two cellular compartments of the lens to ensure that the balance between proliferation and differentiation is maintained (Le and Musil 2001; Lovicu and McAvoy 2001; Iyengar, Wang et al. 2007; Carbe and Zhang 2011) While much of this regulation has been studied at the level of FGF bioavailability in ocular media and receptor expression, recent studies have identified other factors(Boros, Newitt et al. 2006; Jia, Lin et al. 2007; Boswell, Overbeek et al. 2008; Rowan, Conley et al. 2008; Saravanamuthu, Gao et al. 2009; Newitt, Boros et al. 2010; Jarrin, Pandit et al. 2012; Saravanamuthu, Le et al. 2012) particularly BMPs, which can regulate FGF signaling (Boswell, Overbeek et al. 2008; Jarrin, Pandit et al. 2012).

Phosphorylated Smad1/5/8 (pSmad1/5/8) staining is a readout of BMP signaling, as such, pSmad1,5,8 is found throughout the lens vesicle but becomes restricted to the equatorial zone once lens fiber differentiation begins (E12.5 in mouse)(Belecky-Adams, Adler et al. 2002; Rajagopal, Ishii et al. 2007. Similar to this pattern, the FGF mediator pErk is also restricted to the equatorial zone after onset of fiber differentiation {Zhao, 2008 #695; Zhao, Yang et al. 2008). Further BMP influences FGF signaling by selectively increasing FGF receptor1 (FGFR1) expression (Hayashi, Ishisaki et al. 2001; Hayashi, Ishisaki et al. 2003) and FGFR1 down regulation produces more profound defects than the down regulation of other FGFRs (FGFR2 and FGFR3) in the lens (Zhao, Yang et al. 2008). Further, β1 integrin is known to influence BMP signaling by localization of BMP receptors (BMPR) into

areas of cell membranes more conducive to their signal transduction (North, Pan et al. 2015).

Based on the simultaneous up regulation of FGF and BMP activity that we observe in the β1 LE LECs we propose that coordinated BMP and FGF signaling is required to maintain the balance between proliferating population of anterior LECs and differentiating population at the transition zone. BMP is constitutively active in the entire early lens (Boswell and Musil 2015) and lens epithelial cells are capable of responding to FGF signaling (Lovicu and McAvoy 2005; Lovicu, McAvoy et al. 2011). Therefore in the absence of a negative regulator BMP activity in the anterior lens would lead to up regulation of FGF, resulting in fiber differentiation similar to what is seen in β1 LE lenses. We propose a model (Figure 8) wherein β1 integrins are the key regulators of BMP activity and function by sequestering the BMP receptors away from lipid rafts in LECs which makes them insensitive to ligand.(North, Pan et al. 2015) This extra level of control allows for the precise positioning of the transition zone.

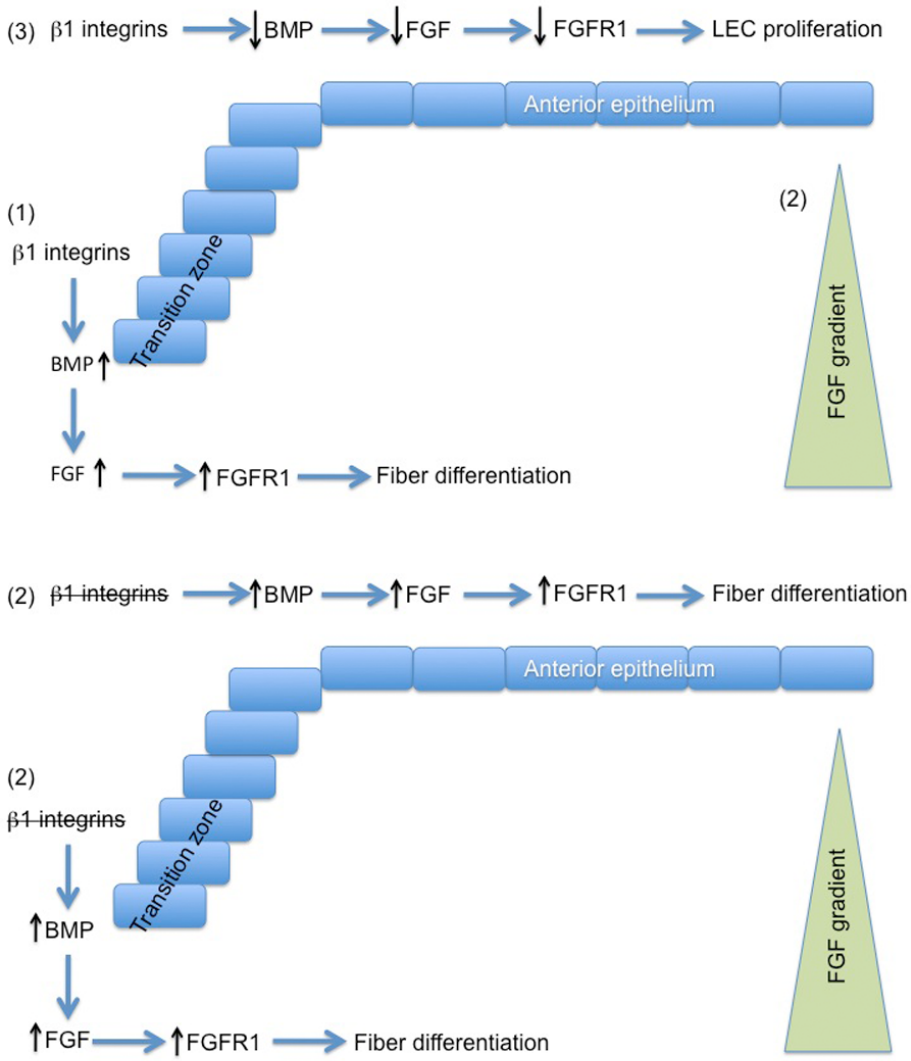


Figure 3.8: β1 integrin-BMP interaction modulates FGF activity to maintain to maintain the balance between proliferation and differentiation

Highest BMP and FGF activities are detected in cells undergoing fiber differentiation at the equatorial zone of the lens, (Figure 8A-1) against a backdrop of FGF antero-posterior gradient (Figure 8A-2).

BMP itself is regulated by β1 integrin at the level of partitioning and localization of the BMPR receptors into lipid rafts. β1 integrin prevents BMPR1b localization into lipid rafts, inhibiting its signaling. β1 integrins can alter their signaling in response to different cues from the ECM, supported by the observation that α1β1 (Collagen IV receptor) and α6Aβ1 (laminin receptor) are expressed at higher levels in cells undergoing fiber differentiation (Figure 8C) (De Arcangelis and Georges-Labouesse 1999; De Arcangelis and Georges-Labouesse 2000; Walker and Menko 2009). Thus, β1-integrins in the anterior epithelium limit differentiation by restricting BMPR1b entry into lipid rafts. Reduced BMP activity in turn limits FGFR1 expression in the anterior LECs (Figure 8A-3). At the equatorial zone a switch in β1-integrin activity results in enhanced localization of BMPR1b into lipid rafts, the resulting increase in BMP activity in turn up regulates FGFR1 expression (Figure 8-3). The result is increased FGF activity ultimately pushing these cells down the fiber differentiation pathway (Figure 8-3).

In our model the loss of β1 integrins creates results a situation similar to the transition zone (Figure 8B-2) in the anterior LECs (Figure 8B-1) wherein BMP is up-regulated in the lens epithelium, resulting in increased FGF activating up-regulating FGFR1 and ultimately resulting in inappropriate fiber differentiation of anterior LECs.

Thus, this investigation shows that β1-integrins impinge upon BMP-FGF crosstalk in the lens, likely modulating BMP signaling to maintain a balance between BMP and FGF induced LEC proliferation and differentiation.

Chapter 4

LAMININ IN THE LENS

Introduction

The lens capsule is secreted by the cells it surrounds and is a highly dynamic three dimensional network composed of laminin, collagen IV, fibronectin, entactin/nidogen, and heparan sulfate proteoglycans including perlecan (Cammarata, Cantu-Crouch et al. 1986; Bosman, Cleutjens et al. 1989; Danysh and Duncan 2009). Like all basement membranes, the lens capsule serves as an extracellular depot for growth factors and proteases (Tholozan, Gribbon et al. 2007) while also directly binding to cellular receptors such as integrins (Simirskii, Wang et al. 2007; Walker and Menko 2009) to provide signals which control the phenotype of the attached cells (Lovicu, de Iongh et al. 1997). The capsule also serves as a selectively permeable barrier between the lens and the ocular environment (Danysh, Patel et al. 2010), protecting the lens from infection while also conferring immune privilege (Piatigorsky 1981). Finally, the lens capsule is important for lens structural integrity and serves as the attachment site between the lens and the zonules, which suspend the lens in the correct location within the eye (Hiraoka, Inoue et al. 2010; Shi, Tu et al. 2013) and transmit the forces necessary for accommodation in primates (Charman 2008). Consistent with these functions, mutations in genes encoding either lens capsule components (Dong, Chen et al. 2002; Firtina, Danysh et al. 2009) or proteins necessary for lens capsule assembly (Takeda, Kondo et al. 2003; Chang, Winder et al.

2009; Qu, Hertzler et al. 2011) lead to diverse lens dysplasias (Rossi, Morita et al. 2003; Lee and Gross 2007).

Laminin is an extracellular matrix (ECM) component secreted as a heterotrimer of α, β and γ subunits. Currently, 16 different laminin heterotrimers have been identified; each comprised of a different combination of the five known α, four known β and three known γ subunits (Zinkevich, Bosenko et al. 2006; Sztal, Berger et al. 2011). The lens capsule has been reported to contain laminin α1, α5, β1, β2 and γ1 (Bystrom, Virtanen et al. 2006), and mutations in human LAMB2 results in Pierson's syndrome, which is characterized by severe kidney disease associated with multiple ocular abnormalities including lens malformations and cataracts (Bredrup, Matejas et al. 2008). Notably, deletion of the *lama1*, *lamb1* and *lamc1* genes result in post-implantation lethality in mice, apparently because laminin-111, the heterotrimer composed of laminin α1, β1, γ1, is critical for the initial assembly of epithelial basement membranes (Miner, Li et al. 2004). Further, mutations have been identified in the zebrafish *lama1* (bashful; bal), *lamb1* (grumpy; gup) and *lamc1* (sleepy; sly) genes, all of which result in profound body axis and brain defects (Stemple, Solnica-Krezel et al. 1996; Parsons, Pollard et al. 2002; Semina, Bosenko et al. 2006)

Zebrafish mutations in the *lamb1* and *lamc1* genes also result in retinal lamination defects, as well as severe lens defects by three days post fertilization including the ectopic position of the lens within the retina, loss of lens capsule integrity and inappropriate localization of the zebrafish lens marker ZL-1. By five days post fertilization, the lens has fragmented and is largely lost from the eye (Lee and Gross 2007). Mutations and morpholino driven knockdown of the *lama1* gene result in similar lens degeneration/loss although the phenotype appears more severe

with the first defects apparent by 30 hpf while the lens is absent by 72 hpf, leading to the conclusion that fiber cell morphogenesis was disrupted. While these studies make it apparent that the laminin-111 heterotrimer is critical for eye and lens development and function, none of the prior studies on these laminin mutants characterized these lens defects further. Here I re-evaluate the lens phenotype of the zebrafish *lama1* mutant, *lama1*^{a69}, and find that the loss of the lens occurs upon its extrusion through the developing cornea suggesting roles for laminin111 in the structural integrity of the eye. This work has been published (Pathania, Semina et al. 2014)

Results

Laminin Alpha 1 Mutation Leads to Loss of Laminin Immuno Reactivity in the Lens Capsule

The zebrafish lens forms when a region of the head ectoderm thickens at 18 hours post fertilization (hpf) to form a ball of cells that delaminates from the overlying cell sheet between 20 and 24 hpf, at which time the lens epithelium and fiber cells are already apparent (Greiling and Clark 2012). Laminin is found at all stages of this process as it is a component of the BM underlying the head ectoderm at 16 hpf and completely surrounds the newly delaminated lens at 24 hpf (Figure 4.1A,B)(Greiling and Clark 2012). In contrast, the *lama1*$^{a69/a69}$ zebrafish lens exhibits little to no immunoreactivity against pan-laminin antibody at 24 hpf (Figure 4.1C, D).

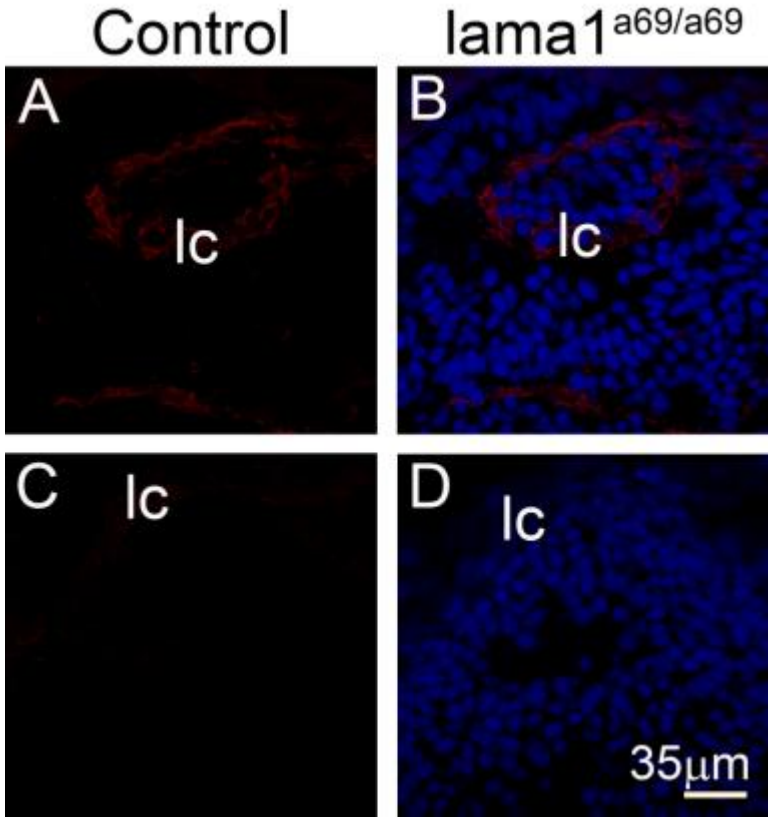


Figure 4.1: Laminin levels are downregulated in *lama1*$^{a69/a69}$mutants. Immunofluorescent confocal microscopy showing laminin protein expression at 24-hpf. Eye from a wild type zebrafish embryo (A,B) showing normal distribution of laminin in the lens capsule at this stage. Eye from a *lama1*$^{a69/a69}$ mutant embryo showing downregulation of laminin expression (B,D). Laminin - Red; DNA/Draq5 - Blue. Abbreviations: lc, lens capsule. Scale bar = 35µm. Modified from Pathania et al, 2014 (Pathania, Semina et al. 2014)

lama1$^{a69/a69}$ Mutant Zebrafish Lenses have Defects in Collagen IV Organization and Secretion

Like laminin, Collagen IV is another heterotrimeric molecule ubiquitous to BMs including the lens capsule (Kelley, Sado et al. 2002), integrating with the laminin scaffold to provide stability and strength to the basement membrane (Aumailley,

Pesch et al. 2000; Lee and Gross 2007; Danysh and Duncan 2009) Since the lens capsule was nearly absent from *lamc1* mutant zebrafish, we investigated whether collagen IV was correctly assembled around *lama1*$^{a69/a69}$ mutant lenses. At 60 hpf, the wildtype lens was completely surrounded by a well formed collagen IV matrix while little to no staining was detected outside of the capsule (Figure 4.2 A, B). In contrast, collagen IV was not found in this sharply demarcated distribution in *lama1*$^{a69/a69}$ mutants, instead, most of the staining was found within the lens, in a distribution consistent with the presence of collagen IV aggregates (Figure 4.2 C, D).

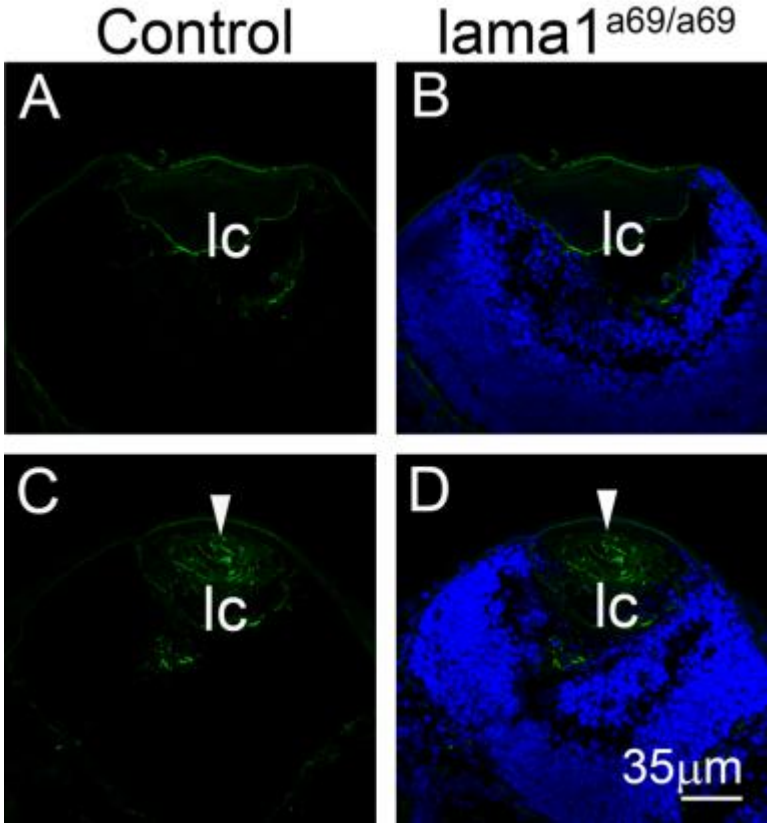


Figure 4.2: Collagen IV aggregates are seen in *lama1*$^{a69/a69}$ mutant lens fibers. Immunofluorescent confocal microscopy showing collagen IV protein expression at 60 hpf. Eye from a wild type zebrafish embryo (A,B) showing normal distribution of collagen IV in the lens capsule at this stage. Eye from a *lama1*$^{a69/a69}$ mutant embryo showing downregulation of collagen IV expression in the lens capsule, while Collagen IV retention is seen in the lens fibers (C,D-arrowheads). Collagen IV - Red; DNA/Draq5 - Blue. Abbreviations: lc, lens capsule. Scale bar = 35µm. Modified from Pathania et al, 2014 (Pathania, Semina et al. 2014)

Laminin Alpha 1 Mutation does not Disturb Fiber Cell Marker Expression but Leads to Lens Extrusion from the Eye

The lens expresses the laminin receptors α6β1, α6β4 and α3β1 integrin and mice lacking either both the *itga3* and *itga6*, or *itgb1* genes from the lens develop

profound lens abnormalities including loss of the lens epithelium and fiber cell defects (De Arcangelis, Mark et al. 1999; Samuelsson, Belvindrah et al. 2007; Simirskii, Wang et al. 2007). Further, lens cells grown in vitro are commonly cultured on laminin to allow for their survival in serum free culture (Musil 2012), while laminin/α6β1 integrin interactions are necessary for fiber cell differentiation in vitro (Walker and Menko 1999). Since defects in lens fiber cell differentiation have been proposed to cause the lens defects in *lama1*$^{a69/a69}$ mutants, I evaluated these lenses for the expression of lens fiber cell markers. Aquaporin 0 is the most abundant membrane protein found in vertebrate lens fiber cells (Bassnett, Wilmarth et al. 2009) that serves as both a water channel and cell adhesion molecule necessary for fiber cell physiology (Clemens, Nemeth-Cahalan et al. 2013). In the zebrafish, aquaporin 0 is encoded by two genes (*aqp0a* and *aqp0b*), and both initiate mRNA expression in the lens at 22 hpf, and this expression is maintained at high levels throughout development (Froger, Clemens et al. 2010). Consistent with this, an aquaporin 0 antibody expected to react similarly with both zebrafish isoforms robustly labels the lens fiber cell membranes but not the lens epithelium of 60 hpf wildtype zebrafish lens fiber cells (Figure 4.3 A, B). Importantly, *lama1*$^{a69/a69}$ mutant lenses also stain robustly for aquaporin 0, although the distribution is more disorganized reflecting the morphological defects seen in these lenses (Semina, Bosenko et al. 2006). (Figure 4.3 C, D). Notably though, clusters of aquaporin 0 positive cells were routinely detected adhered to the outer surface of the developing cornea suggesting that while fiber cell differentiation per se is not affected in this mutant, the lens is rupturing through the cornea (Figure 4.3 C,D-arrowheads). Similarly, staining lenses with the monoclonal antibody, ZL1, which recognizes a marker of zebrafish fiber cell differentiation which is first

expressed in the lens between 20 and 23 hpf (Greiling, Aose et al. 2010), showed that the lens fibers of *lama1*$^{a69/a69}$ mutants appropriately entered the lens fiber cell differentiation pathway although their structural organization is abnormal.

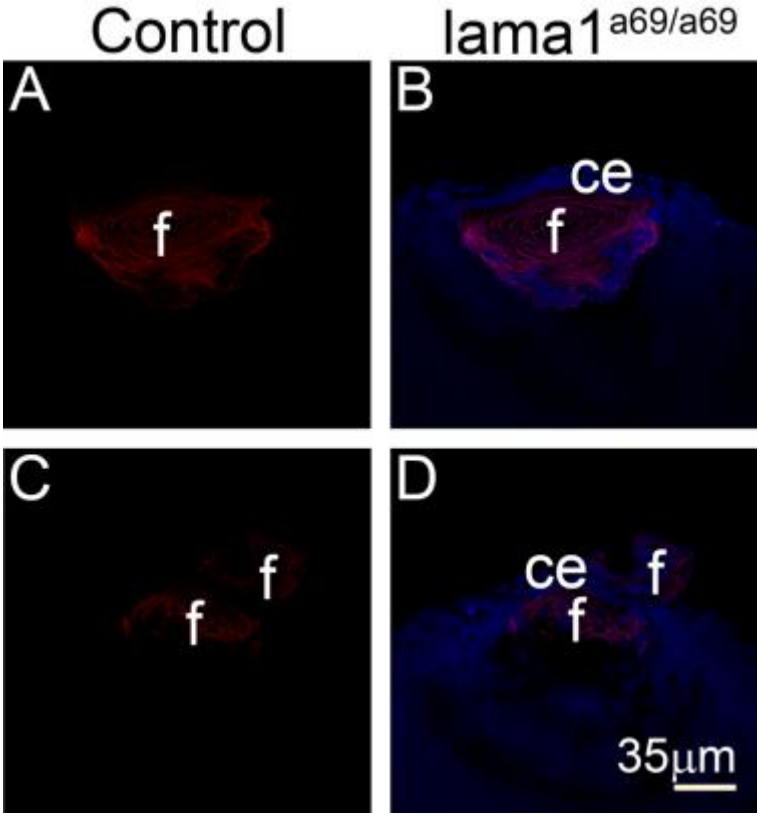


Figure 4.3: Lens fibers extrude from the eye in *lama1*$^{a69/a69}$ mutant zebrafish. (A, B) Immuno-histochemical confocal microscopy of aquaporin 0 in zebrafish wild type and lenses at 60 hpf shows that the expression of this lens fiber cell marker confined to the lens (A,B). In contrast, aquaporin 0 expression is detected both in the malformed lens and in material extruding out of the eye anteriorly in the mutants (C,D). Red – Aquaporin 0; Blue - Draq5. Abbreviations: f, lens fiber cells; ce, corneal epithelium. Scale bar = 35μm. Modified from Pathania et al, 2014 (Pathania, Semina et al. 2014)

Laminin Mutant Zebrafish have Defects in Corneal Integrity

The loss of the lens capsule and lens fragmentation seen in *lama1*$^{a69/a69}$ (Figure 1 and 2) as well as *lamb1* and *lamc1* mutants (Lee and Gross 2007) implies that Laminin 111 is important to form the lens capsule and is consistent with our prior understanding of the role of the lens capsule in the maintenance of lens structural integrity (Rossi, Morita et al. 2003; Danysh and Duncan 2009). However, we also routinely observed that a portion of the lens fiber mass extruded to the exterior of the cornea by 60 hpf, indicating that the structural integrity of the cornea was also compromised.

Immunolocalization using a pan-laminin antibody revealed that at 60 hpf, laminin was found both in the lens capsule as well as the basement membrane underlying the developing corneal epithelium (Figure 4.4 A, B). This staining was absent from the region surrounding the *lama1*$^{a69/a69}$ lens as expected, while some laminin immunoreactivity was still detected underlying the corneal epithelium, although it was discontinuous (Figure 4.4 C, D-arrowheads), suggesting that the corneal BM structure is compromised.

Transforming growth factor, beta-induced (TGFβi, BIGH3) is an extracellular matrix protein first named for the induction of its expression by transforming growth factor β (Skonier, Neubauer et al. 1992; Skonier, Bennett et al. 1994). In the cornea, it is found beneath the corneal epithelium associated with the BM where it serves as an adhesion matrix for the epithelial cells (Kim, Kim et al. 2000).. Since *lama1*$^{a69/a69}$ mutants have defects in the BM underlying the presumptive corneal epithelium and exhibited an extrusion of lens fiber cells anteriorly, we sought to determine whether TGFβi was appropriately found in the developing cornea. TGFβi was detected in a discrete line below the corneal epithelium (green) in wildtype eyes (Figure 4.4 E, F-

arrowheads) while the zebrafish lens marker Zl-1 was confined to the lens fiber cells (Figure 4.4 F) at 60 hpf. However, TGFβi was not detected in *lama1*$^{a69/a69}$ eyes (Figure 4.4 H), and cells staining with Zl-1 were found outside of the anatomical boundaries of the eye (Figure 4 G-arrowheads) compared to the wild type zebrafish embryos (Figure 4.4 E,F).

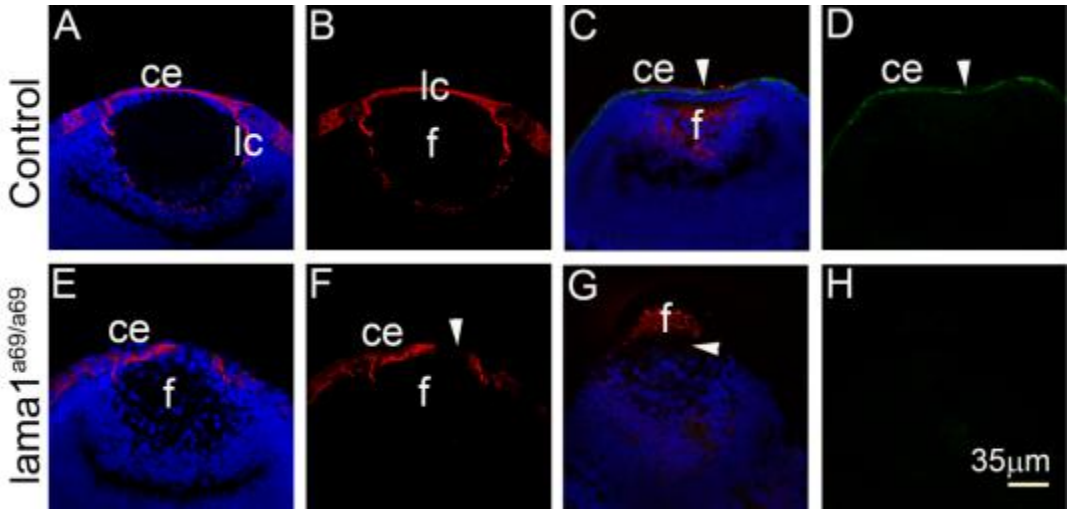


Figure 4.4: Laminin and BIGH3 expression downregulates in the developing cornea of *lama1*$^{a69/a69}$ mutant zebrafish. Immuno-histochemical confocal microscopy showing normal expression and distribution of Laminin at 60 hpf in wild type embryos (A, B). Zebrafish *lama1*$^{a69/a69}$ mutants show downregulation of laminin in lens capsule (E) and discontinuous laminin staining in the developing cornea (F-arrowheads). BIGH3 co-staining with lens fiber cell specific marker ZL1 shows normal distribution at 60 hpf, in wild type embryos (C, D). Zebrafish *lama1*$^{a69/a69}$ mutant embryos show downregulation of corneal BIGH3 (H) and ZL1 positive cells were detected anterior to the anatomical boundary of the eye (G-arrowheads). Laminin - Red; (A, B, E, F), ZL1- Red (C, D, G, H); BIGH3 – Green (C,D,G,H); Draq5 - Blue. Abbreviations: f, lens fiber cells; ce, corneal epithelium; lc, lens capsule. Scale bar = 35µm. Modified from Pathania et al, 2014 (Pathania, Semina et al. 2014)

Discussion

Basement membranes (BM) play diverse roles in vertebrates which include serving as a selectively permeable barrier between cells and the extracellular environment (Miner 2012), providing signals that allow cells to sense their extracellular environment and respond by changing/maintaining cellular phenotype/behavior (Yurchenco 2011), the maintenance of an extracellular depot of growth factors/matricryptins (Mott and Werb 2004) and the preservation of tissue

structural integrity (Tanner 2012; Breitkreutz, Koxholt et al. 2013). The lens capsule, an unusually thick BM (7-48 μm depending on age, genetic background, region measured, and species (Danysh, Czymmek et al. 2008) has been proposed to have all of these functions (de Iongh and McAvoy 1992; Danysh and Duncan 2009), although the contribution of different BM components to these diverse roles has not been comprehensively investigated.

Laminins are heterotrimeric molecules that are found in all BMs that appear to provide the primary scaffolding necessary to assemble other BM components such as collagen IV, nidogen/entactin and heparan sulfate proteoglycans into a fully functional ECM (Yurchenco and Cheng 1993; Adam, Gohring et al. 1997; Kadoya, Katsumata et al. 1997; Yurchenco, Quan et al. 1997; Yurchenco, Amenta et al. 2004). The human lens capsule has been reported to contain the Laminin α1, α5, β1, β2, and γ1 chains (Falk, Ferletta et al. 1999; Bystrom, Virtanen et al. 2006) while these were also found to be the most abundant laminin mRNAs expressed by the embryonic mouse lens by RNAseq (Manthey, Lachke et al. 2013), thus the lens capsule has the potential to contain the laminin111, laminin 121, laminin 511 and laminin 521 heterotrimers (Aumailley, Bruckner-Tuderman et al. 2005). No human diseases have been associated with mutations in *LAMA1*, *LAMA5*, and *LAMC1* (encodes laminin γ1) to date, although *lama1*, *lama5*, *lamb1* and *lamc1* null mice are embryonic lethal (Smyth, Vatansever et al. 1998; Nguyen, Miner et al. 2002; Miner, Li et al. 2004), while a hypomorphic allele of *lama1* results in retinal defects in mice (Edwards, Mammadova-Bach et al. 2010), point mutations in *LAMB1* result in lissencephaly-5 in humans (Radmanesh, Caglayan et al. 2013) and mutations of *LAMB2* result in Pierson syndrome (Matejas, Hinkes et al. 2010), which causes severe nephrosis and ocular

abnormalities including lens malformations and cataracts demonstrating the critical role that these laminins play in development.

In zebrafish, mutations in the *lama1*, *lamb1* and *lamc1* genes all result in a variety of severe defects in the notochord, body axis, muscle formation, and nervous system development. Notably, mutation or knockdown of any of these genes, also results in a variety of ocular phenotypes including defects in retinal lamination, corneal defects, and lens malformations/degeneration although the timing and severity of the phenotype varies between alleles (Gross, Perkins et al. 2005; Semina, Bosenko et al. 2006; Zinkevich, Bosenko et al. 2006). Previous studies of $lama1^{a69/a69}$ mutant embryos have shown that the lenses are profoundly abnormal with severe lens degeneration leading to the speculation that the lens epithelium and fiber cells did not differentiate normally (Semina, Bosenko et al. 2006). This study further clarifies the role of laminin in lens development, by a more detailed analysis of the morphological and molecular consequences of the $lama1^{a69/a69}$ mutation on the lens.

Defects in Laminin Networks Subsequently Lead to Defects in Collagen IV Assembly and Secretion

This loss of laminin from the lens capsule that we observed, likely occurs because the C56S mutation responsible for the $lama1^{a69}$ mutant phenotype is expected to disrupt one of the disulfide bridges necessary for laminin heterotrimer assembly (Zinkevich, Bosenko et al. 2006; Yurchenco 2011) while assembly of the laminin heterotrimer is required for its secretion and assembly into the BM (Aumailley, Pesch et al. 2000). This suggests that the Laminin 111 or Laminin 121 networks are the main laminin heterotrimers present in the zebrafish lens capsule at this age. This is consistent with the prior detection of laminin111 in the embryonic zebrafish lens

capsule (Lee and Gross 2007) and the known preference for laminin 111 in embryonic epithelial basement membranes (Virtanen, Gullberg et al. 2000; Ekblom, Lonai et al. 2003).

Notably, mice mutant for *lamc1*, which do not form the initial laminin111 network which is normally found in the epiblast, also do not form an organized collagen IV network, instead, collagen IV was detected in aggregates throughout the embryo (Smyth, Vatansever et al. 1999). This suggests that the lens, like the early embryo, requires a laminin111 scaffold for the appropriate assembly of the lens capsule. This loss of collagen IV organization is likely to contribute to the phenotype of these lenses as mutations in the *COL4A1* gene cause anterior segment defects (Van Agtmael, Schlotzer-Schrehardt et al. 2005; Gould, Marchant et al. 2007), while mutations in the *COL4A3* or *COL4A4* genes result in Alport Syndrome in humans, which is associated with anterior and posterior lenticonus, capsular ruptures and cataracts (Colville, Savige et al. 1997; Colville and Savige 1997; Olitsky, Waz et al. 1999; Takei, Furuya et al. 2001; Van Agtmael, Schlotzer-Schrehardt et al. 2005; Wilson, Trivedi et al. 2006).

Laminin Alpha 1 Mutation Leads to Defective Structural Organization of Lens Fibers

Currently, the role of laminin in regulating the differentiation of lens fiber cells is unclear. The observation that lens fiber cell marker expression in *lama1*$^{a69/a69}$ mutants is preserved despite the morphological abnormalities seen in these lenses is consistent with a prior report showing that Zl-1 expression is retained in *lamc1* mutant lenses (Lee and Gross 2007). The lens expresses the laminin receptors α6β1, α6β4 and α3β1 integrin and mice lacking either both the *itga3* and *itga6*, or *itgb1* genes

from the lens develop profound lens abnormalities including loss of the lens epithelium and fiber cell defects (De Arcangelis, Mark et al. 1999; Samuelsson, Belvindrah et al. 2007; Simirskii, Wang et al. 2007; Scheiblin, Gao et al. 2014). Further in vitro studies show that laminin is required for optimum differentiation (Walker and Menko 2009) and also survival of lens cells in serum free culture (Musil 2012), while laminin/α6β1 integrin interactions are necessary for fiber cell differentiation in vitro (Walker and Menko 1999). Experiments utilizing chick lens cultures and microdissected embryonic lenses show that the expression and cytoskeletal linkage of α6-integrin, a component of α6β1 and α6β4 integrin, the most abundant laminin receptors in the lens, changes during fiber cell differentiation and knockdown of α6 integrin expression in cultured LECs blocks their differentiation into fibers (Walker, Zhang et al. 2002). In contrast, β1-integrin is necessary for the maintenance of the mouse lens epithelium with its loss corresponding to the upregulation of some lens fiber cell markers and the EMT marker α-smooth muscle actin followed by epithelial cell apoptosis. While it has been proposed that β1-integrins are also important for lens fiber cell survival (Samuelsson, Belvindrah et al. 2007), conditional deletion of β1-integrin from lens fibers leads to defects in lens fiber cell structure, but not lens fiber cell survival or differentiation per se (Scheiblin, Gao et al. 2014). These data in aggregate lead to the proposition that laminin interactions with integrins expressed by lens cells are important for the proper morphological organization of lens fibers, with the caveat that both α6 and β1 integrin are also localized to the lateral membranes of lens fibers away from the laminin of the lens capsule and may be playing roles independent of their function as laminin receptors (Walker, Zhang et al. 2002; Scheiblin, Gao et al. 2014).

Laminin Alpha 1 Mutation Results in Loss of Structural Integrity of the Cornea

The laminin composition of the zebrafish corneal BM has not been reported, however, in humans, lamα3 and lamα5 are found to be the predominant laminin α chains in the BM underlying the adult corneal epithelium, while lamα1 was not detected (Bystrom, Virtanen et al. 2006). Thus, lamα1 may be necessary for the initial organization of the corneal BM but later in development, it is replaced by other laminin α proteins. This would be consistent with the observation that laminin111 is deposited early in the development of most epithelia, although in most cases it is replaced by other laminins later in development (Ekblom, Lonai et al. 2003).

Further, TGFβi interacts with several ECM components including laminin and this interaction is important for the maintaining integrity of the corneal epithelium by inhibiting cell migration and promoting cell-cell and cell-ECM adhesion (Kim, Park et al. 2002). In the cornea, it is found beneath the corneal epithelium associated with the BM where it serves as an adhesion matrix for the epithelial cells (Kim, Kim et al. 2000). Mutations in this gene result in a variety of human corneal dystrophies and its expression has been detected in the developing cornea of mice, rabbits and zebrafish (Munier, Korvatska et al. 1997; Hirate, Okamoto et al. 2003). Thus the discontinuous laminin staining in *lama1*$^{a69/a69}$ (figure 4.4) as well as loss of TGFβi (figure 4.4) in aggregate show that the structural integrity of the corneal epithelium is disrupted in *lama1*$^{a69/a69}$ mutants, suggesting that laminin 111 is playing both structural and signaling functions in the developing zebrafish eye.

Overall our data demonstrate that lamα1 is essential for the formation of the lens capsule including the deposition of collagen IV into the capsule and thus lens morphology/structure. Further, lamα1 is essential for the organization of the corneal

epithelium including deposition of TGFβi underneath the corneal epithelium. These data suggest that the *lama1*$^{a69/a69}$ mutant phenotype is due to a combination of both a structural and signaling function of the lens capsule and early corneal epithelial BM during early eye development.

Chapter 5

THE ROLE OF FIBRONECTIN IN THE LENS

Introduction

FN is either synthesized by hepatocytes as a soluble dimer and secreted directly into the circulation (plasma FN or pFN), or is produced locally by diverse cell types and secreted as insoluble fibrils which incorporate into their surrounding ECM or pericellular matrix (cellular FN, or cFN) (Pankov and Yamada 2002). The two principle FN isoforms differ from each other by the presence of the two, type III domains- called Extra Domains A (EDA) and B (EDB). As a result of alternative splicing, pFN lacks these alternatively spliced EDA and EDB sequences, while cFN contains variable combinations of these domains. The EDA and EDB cFN domains show a very high degree of homology among vertebrates, and can be independently spliced-in or out from the pre-mRNA. They are very abundant in embryonic stages and show a very tight spatial and temporal regulation, with both domains only expressed in embryonic tissue and during wound healing, while no expression is found in normal adult tissues (Peters and Hynes 1996; Pankov and Yamada 2002; To and Midwood 2011).

Fibronectin is essential for development as a null mutation of the FN gene is embryonic lethal (George, Georges-Labouesse et al. 1993). Replacement of the EDB exon by a neomycin- resistance cassette or by a longer cDNA sequence including the EDB and flanking exons also results in early embryonic lethality (Georges-Labouesse, George et al. 1996). Mutant mice bearing a homozygous mutation in the RGD site

(RGE) necessary for fibrillar fibronectin assembly mediated by integrins, die at embryonic day 10 (Takahashi, Leiss et al. 2007). Homozygous mutant mice constitutively lacking the EDA or EDB domains (individually), or constitutively expressing the EDA domain, are viable, do not appear to have any postnatal defect,s and reproduce normally (Fukuda, Yoshida et al. 2002; Astrof, Crowley et al. 2004; Astrof and Hynes 2009). In contrast, simultaneous deletion of both the EDA and EDB exons from the FN gene results in embryonic lethality due to multiple embryonic cardiovascular defects (Astrof, Crowley et al. 2007).

Both the EDA and EDB forms of cFN are up regulated in specific conditions such as tissue repair, tissue fibrosis, angiogenesis, and cell migration (To and Midwood 2011). While the role of EDB-cFN in these conditions remains unclear, *in vitro* studies have shown that EDA-cFN along with TGF-β1 is responsible for the differentiation of fibroblasts into the αSMA expressing myofibroblasts seen in fibrosis (Serini, Bochaton-Piallat et al. 1998) and TGF-β activation promotes the inclusion of EDA exon in the fibronectin transcript (White and Muro 2011). Thus cFN- TGF-β interactions appear to be important during pathogenesis of fibrosis. TGF-β exists in an ECM-bound complex containing TGF-β, latency-associated peptide (LAP), and latent TGF-β-binding protein (LTBP). LTBP requires a fibronectin substrate for binding and localizing latent TGF-β complexes to the ECM to form an inactive extracellular TGF-β depot (Murphy-Ullrich and Poczatek 2000).

In the lens, fibronectin is produced throughout its development (Parmigiani and McAvoy 1984) and its expression increases during PCO progression (Mamuya, Wang et al. 2014). Zebrafish with fibronectin mutations have developmentally abnormal lenses, while fibronectin deposition in the ECM underlying the lens placode

is required for placode thickening and invagination (Huang, Rajagopal et al. 2011). Further, fibronectin is up regulated during PCO development and it is a ligand for both the αVβ1 and α5β1-integrins (Walker and Menko 2009), which are also up regulated during PCO (Wormstone, Wang et al. 2009; Wormstone and Wride 2011; Mamuya, Wang et al. 2014). Conversely, fibronectin is proposed to be a negative regulator of matrix contraction in fibrotic PCO (Wormstone, Wang et al. 2009). Thus the function of fibronectin in the lens during its morphogenesis and its role in lens EMT/PCO are not clear, highlighting the need for further study of this molecule in the lens in vivo. This study determined for the first time the functional significance of fibronectin in both lens development and in the pathogenesis of PCO.

Results

Although fibronectin is reported to be a minor component of various basement membranes (Lovicu and Robinson 2004), it is known to be involved in diverse cellular processes such as proliferation, migration, wound repair and fibrosis (White and Muro 2011). Fibronectin is reported to be present in the lens capsule of various species, however no consensus exists about the spatiotemporal distribution as well as function of fibronectin in embryonic as well adult lenses (Parmigiani and McAvoy 1984; Danysh and Duncan 2009).

While investigating the role of fibronectin in lens placode formation, Dr. David Beebe's lab created conditional FN knockouts using the Le-Cre transgene (FN-LE). While these mice had normal lenses at E12.5, they developed severe morphological defects by birth. Since studying these lenses was beyond the scope of their study, they agreed to collaborate with Dr. Melinda Duncan's lab and $FN1^{flox/+}$ mice were transferred to our lab in the Fall 2011. We've established the FN-LE line in our lab, since the phenotype of these mice was much too severe to be used to study PCO, we also established another mouse line using MLR10-Cre (FN-MLR10)

Fibronectin is Expressed in the Embryonic Lens and During PCO Progression

As mentioned earlier, the cFN isoforms EDB-cFN and EDA-cFN are expressed primarily in embryonic tissues and during wound repair and fibrosis. Although FN is reported to be present in the lens capsule and is postulated to play roles in both lens development and PCO (Hayes, Hartsock et al. 2012), much less clarity exists on the isoform type, source, and temporal distribution of fibronectin in the lens (Lovicu and Robinson 2004).

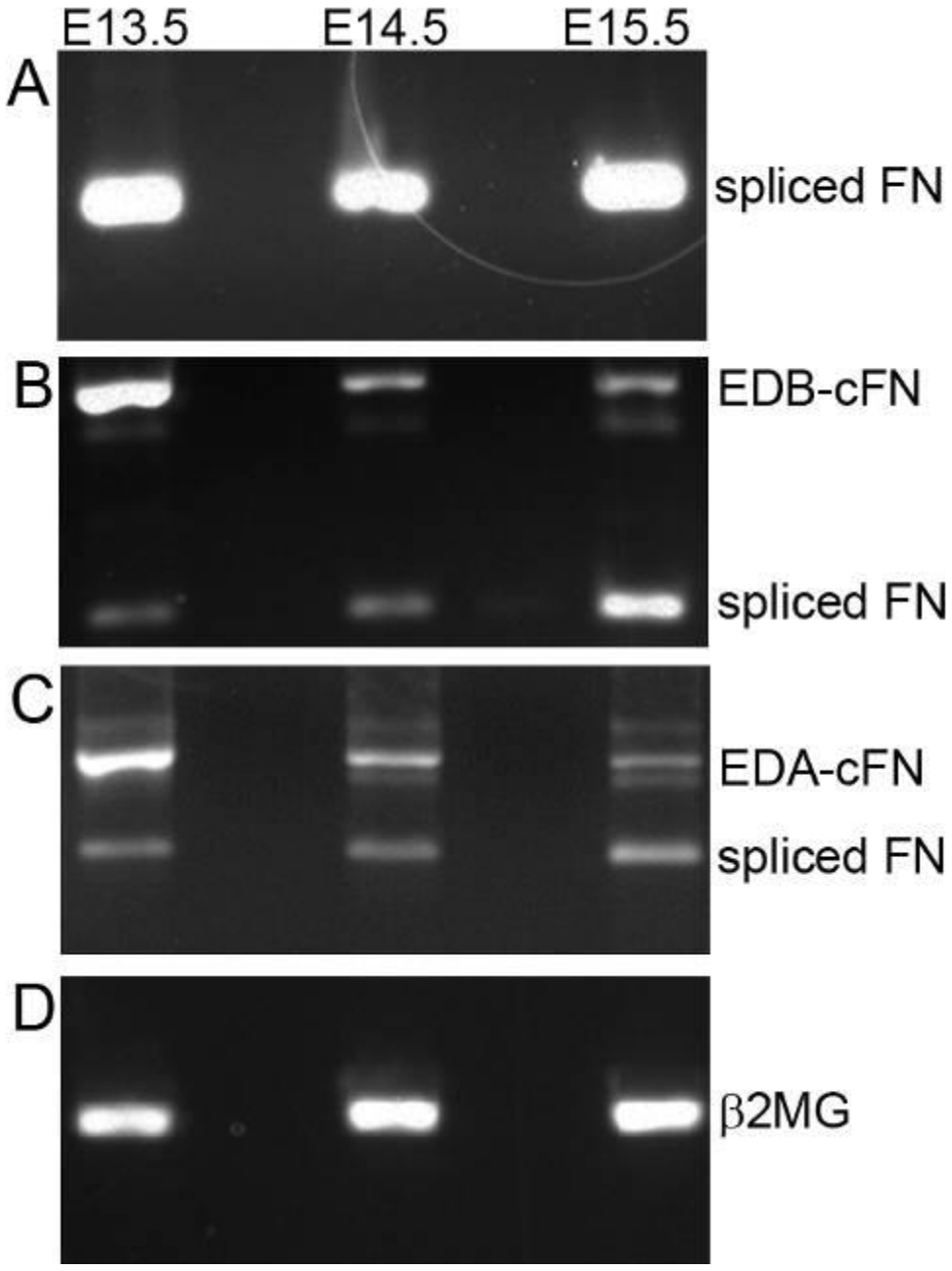

E13.5
E14.5
E15.5
A
spliced FN
B
EDB-cFN
spliced FN
C
EDA-cFN
spliced FN
D
β2MG

Figure 5.1: Cellular fibronectin is expressed in the embryonic mouse lens. (A,D) RT-PCR analysis of cellular fibronectin expression. (A) The spliced FN mRNA is detected in embryonic wild type lenses. β2-microglobulin was used as a control. (B) EDB-cFN is expressed in mouse embryonic lenses. (C) EDA-cFN expression is detected in mouse embryonic lenses. (D) β2-microglobulin is used as control

Using semi-quantitative RT PCR to analyze both the type and distribution of FN isoforms in the lens, I found that spliced fibronectin is expressed (single 100bp band seen here) during embryonic lens development (Figure 5.1 A). The EDB cFN isoform is also expressed in the lens during embryonic development (Figure 5.1B). Two amplification products are seen on the gel, with the higher band corresponding to the isoform that has the EDB exon and the lower band corresponding to spliced fibronectin. Similarly EDA-cFN is also expressed in the embryonic lens (Figure 5.1 C), with two bands seen on the gel. The higher band corresponds to the EDA-cFN containing the EDA exon while the lower band corresponds to spliced FN. β2-microglobulin (β2MG) was used as the internal control (Figure 5.1 D).

Semi-quantitative RT PCR using 9 weeks old control mouse lenses shows that only plasma FN (100 bp band) is expressed in the adult whole mouse lens (Figure 5.2 A) while no expression of either EDB- or EDA-cFN was detected in the lens at this age (data not shown).

Similar to the adult mouse lenses, no EDB and EDA-cFN message could be amplified from the lens epithelium either directly after (zero hours), or 24 hours after fiber cell removal, by 48 hours after fiber cell removal however, I was able to detect expression of both EDB-cFN (Figure 5.2 C) (upper band 650 bp) and EDA-cFN (Figure 5.2 D) obtained (upper band 550bp). β2MG was used as internal control

(Figure 5.2 E). This data is further supported by immunofluorescence data, showing cell associated fibronectin protein expression (arrowheads) in capsular bags at 48 hours post surgery (Figure 5.2 F (red channel-fibronectin), G- merge of αSMA (green) and fibronectin (red)).

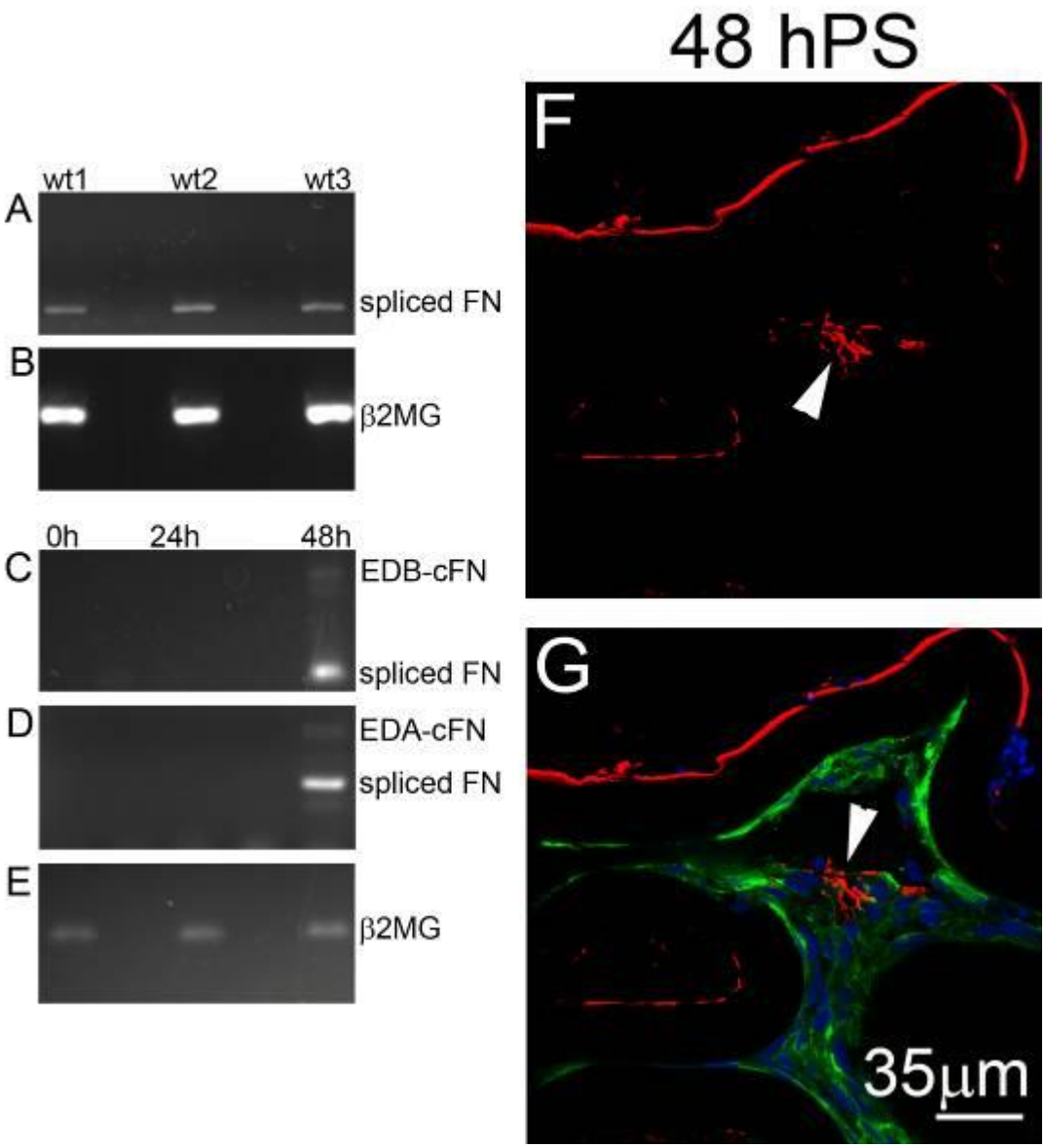


Figure 5.2: Cellular fibronectin expression up-regulates in response to surgery.(Panel A) RT-PCR analysis showing(A)Cellular fibronectin is not detected in adult (9 week) mouse lenses by RT-PCR analysis. (B) β2-microglobulin is used as control. (C) EDB-cFN expression is detected by RT-PCR in capsular bags 48 hours post surgery. (D) EDA-cFN is detected by RT-PCR of mouse lens cells 48 hours post capsular surgery. (E) β2-microglobulin is used a control.

(B) Confocal immunofluorescent analysis of FN in the capsular bags post surgery.(Panel B (A,B - arrowheads) Cellular fibronectin is detected in lens cells by immunolocalization 48 hours post cataract surgery.Red (panel B(A, B) – fibronectin, Green (panel b(B) – αSMA, Blue (panels B(A, B) - DNA. Scale bars – 35μm.

Fibronectin Plays an Essential Role Early in Lens Development.

In order to gain an understanding of the functional significance of fibronectin expression in the embryonic lens, I re established the FN-LE mice by mating $FN1^{flox/+}$ mice obtained from Dr. David Beebe with mice harboring the LeCre transgene, whose activity is first detected at E9 (lens placode stage) (Ashery-Padan, Marquardt et al. 2000). I then mentored Saleena Malik, an undergraduate student in Dr. Melinda Duncan's lab, as she characterized them as part of her senior thesis. PCR analysis of genomic DNA isolated from adult control and FN-LE lenses showed that the deletion of the floxed region of the fibronectin gene is nearly complete in the FN-LE lenses (Figure 5.3 A-B). Further, we observed that by 16 days post natal (dpn), FN-LE mice are micropthalmic as compared to controls. (Figure 5.3 C) and dark field images show that the 16dpn FN-LE lenses are severely abnormal as compared to controls (Figure 5.3 D).

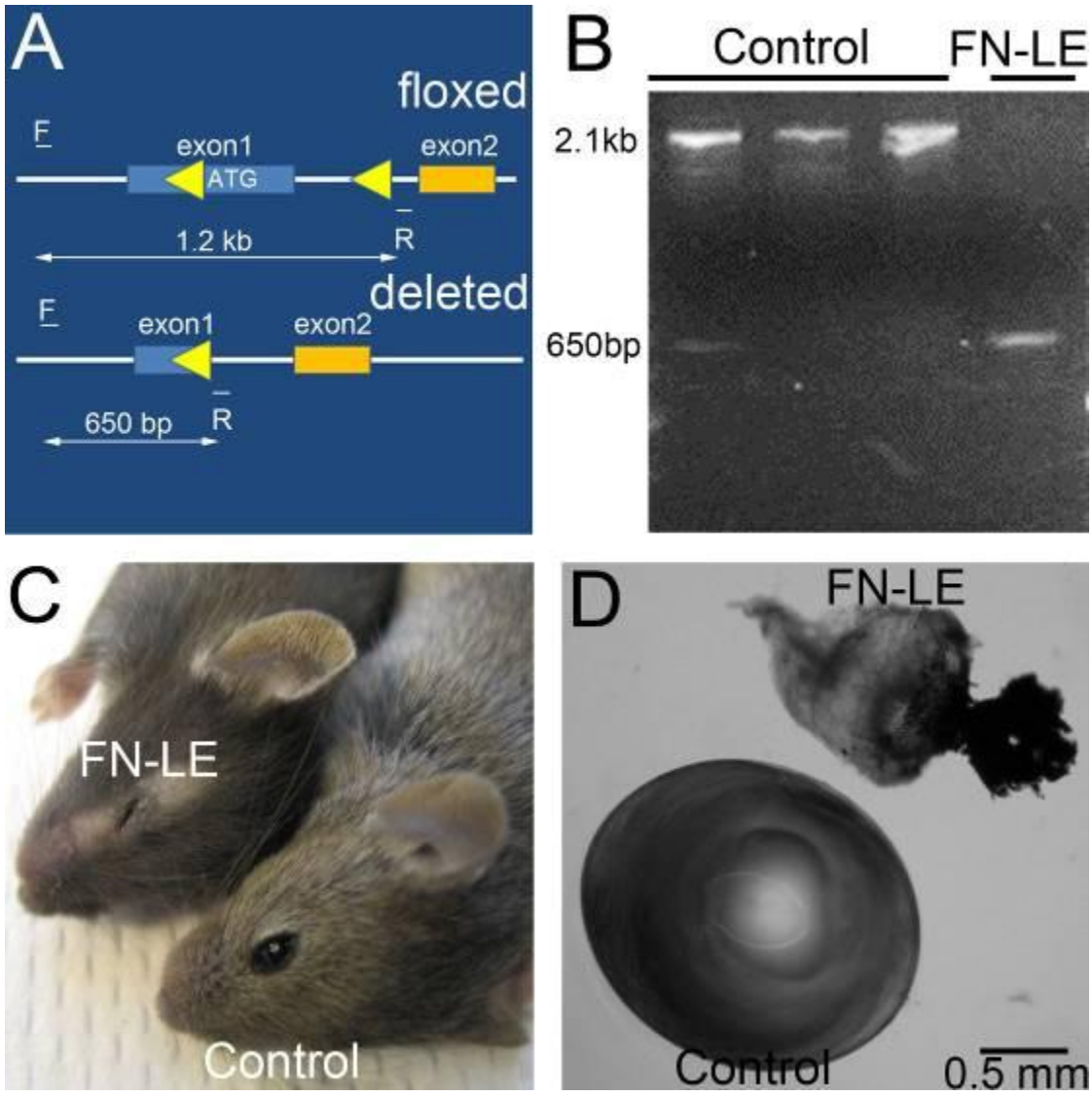


Figure 5.3: Analysis of fibronectin gene deletion and morphological analysis of FN-LE lenses. (A) Diagram of FN gene locus showing the position of loxP sites and the PCR primers used for analysis of lens specific deletion. (B) PCR results from DNA obtained from 9 weeks old control and FN-LE lenses demonstrating successful deletion of the FN gene fragment in the FN-LE lenses. (C) External ocular phenotype of control and FN-LE littermates at 16 dpn. (D) A dark field image showing 16dpn control and FN-LE lenses

Saleena Malik carried out a detailed morphological analysis of these lenses as well as conducted experiments to look at the ocular mechanism underling this phenotype. She successfully defended her undergraduate honors thesis on this project. Ramachandran Balasubramaniam, a MS student in Dr. Melinda Duncan's lab, is now carrying this work forward.

Loss of Fibronectin During Late Lens Development Does not Affect Lens Morphology.

In order to study the role of fibronectin in PCO, I created conditional knockout mice lacking fibronectin specifically in the lens using the MLR10-Cre transgenic mouse line (Figure 5.4 A). MLR10-Cre activity is first detected in the lens beginning at E10.5 (Zhao, Yang et al. 2004) and PCR analysis of genomic DNA isolated from adult lenses (9 weeks old) showed that the deletion of the floxed region of the fibronectin gene is nearly complete in the FNMLR10 lenses (Figure 5.4 B). Confocal immuno fluorescence analysis using polyclonal fibronectin antibody, revealed cell-associated fibronectin staining in the control lenses (Figure 5.4 C - arrowheads), while no staining of cellular fibronectin was seen in the FNMLR10 lenses (Figure 5.4 D). The lens capsule stains with equal intensity in both the control and FNMLR10 due to the presence of aqueous humor derived plasma fibronectin in the lens capsule (Vesaluoma, Mertaniemi et al. 1998; Chowdhury, Madden et al. 2010; Anshu, Price et al. 2011).

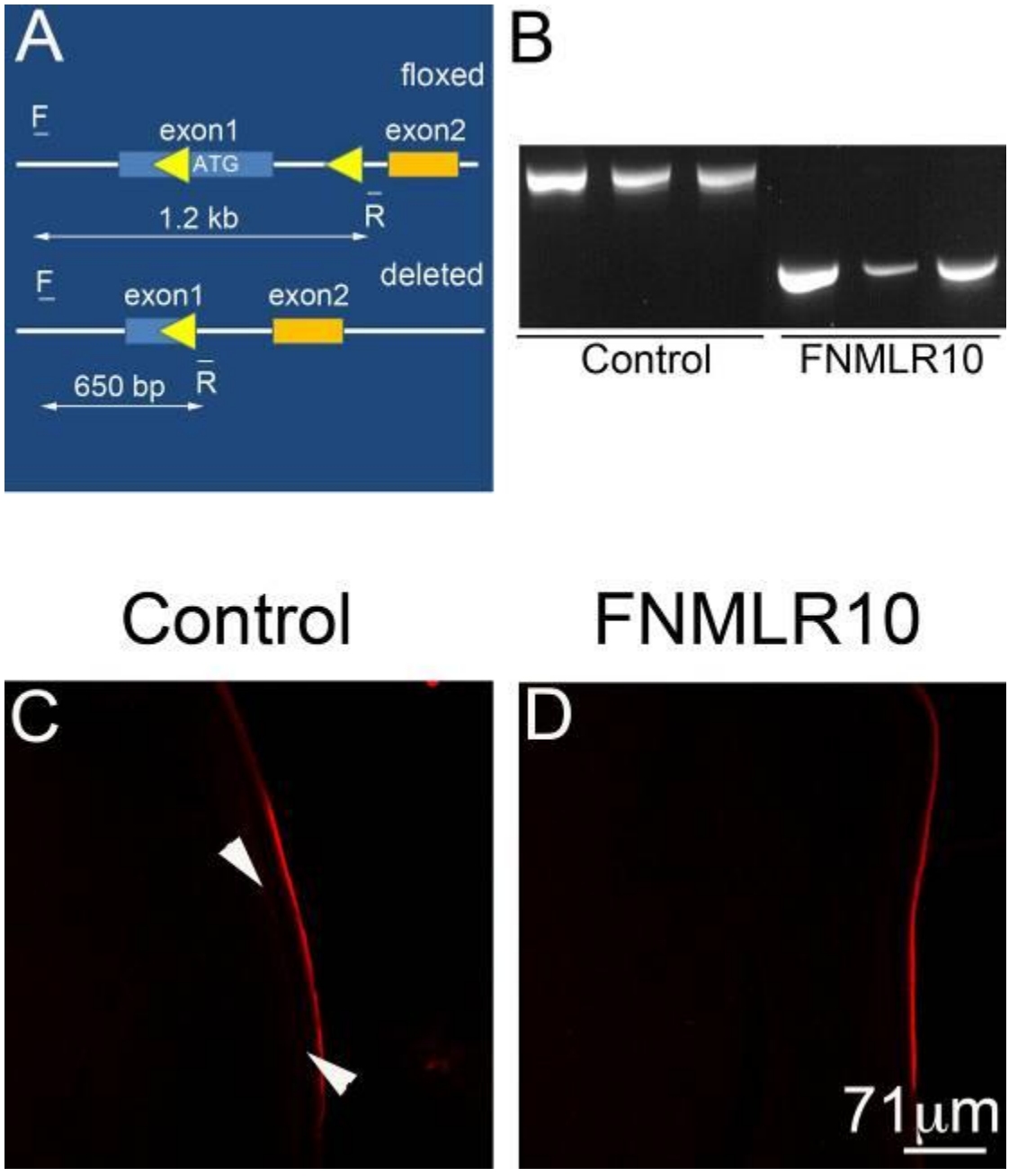

A
floxed
F
exon1
ATG
exon2
R
1.2 kb
deleted
F
exon1
exon2
650 bp
R
B
Control
FNMLR10
Control
FNMLR10
C
D
71μm

Figure 5.4: Analysis of fibronectin gene deletion in FN mlr10 lenses. (A) Diagram of FN gene locus showing the position of loxP sites and the PCR primers used for analysis of lens DNA. (B) PCR results from DNA obtained from 9 weeks old control and FN mlr10 lenses demonstrating successful deletion of the FN gene fragment in the FN mlr10 lenses. (C) By immuno localization 9 weeks old control lenses show presence of plasma fibronectin in the lens capsule while very low levels of cell associated fibronectin (arrowheads) staining is also detectable in these lenses. (D) FN mlr10 lenses show only presence of plasma fibronectin in the lens capsule with no cell associated FN staining found in these lenses.Red (panels C, D) – fibronectin, Blue (panels C, D) - DNA. Scale bars – 71μm.

FNMLR10 Lenses are Morphologically and Optically Indistinguishable from Controls.

Unexpectedly, an approach that was presumably deleting the fibronectin gene just a day later than would be expected in the FN-LE lenses, resulted a very different phenotype. Similar to controls (Figure 5.5 A), FNMLR10 lenses (Figure 5.5 B) were morphologically normal and appeared transparent under dark field imaging. There was also no difference either in the dry or wet weight of the FNMLR10 lenses as compared to controls (Table 5.1). Further, like controls (Figure 5.5 C), the FNMLR10 lenses refracted a hexagonal grid normally (Figure 5.5 D), suggesting that fibronectin is not important either for the transparency or refractive properties of the lens. Using H&E staining, both control (Figure 5.5 E, G) and FNMLR10 lenses (Figure 5.5 F, H) exhibit similar morphology and no obvious defects in lens fiber cell structure were observed in the FNMLR10 lenses (Figure 5.5 J) by scanning electron microscopy when compared to controls (Figure 5.5 I).

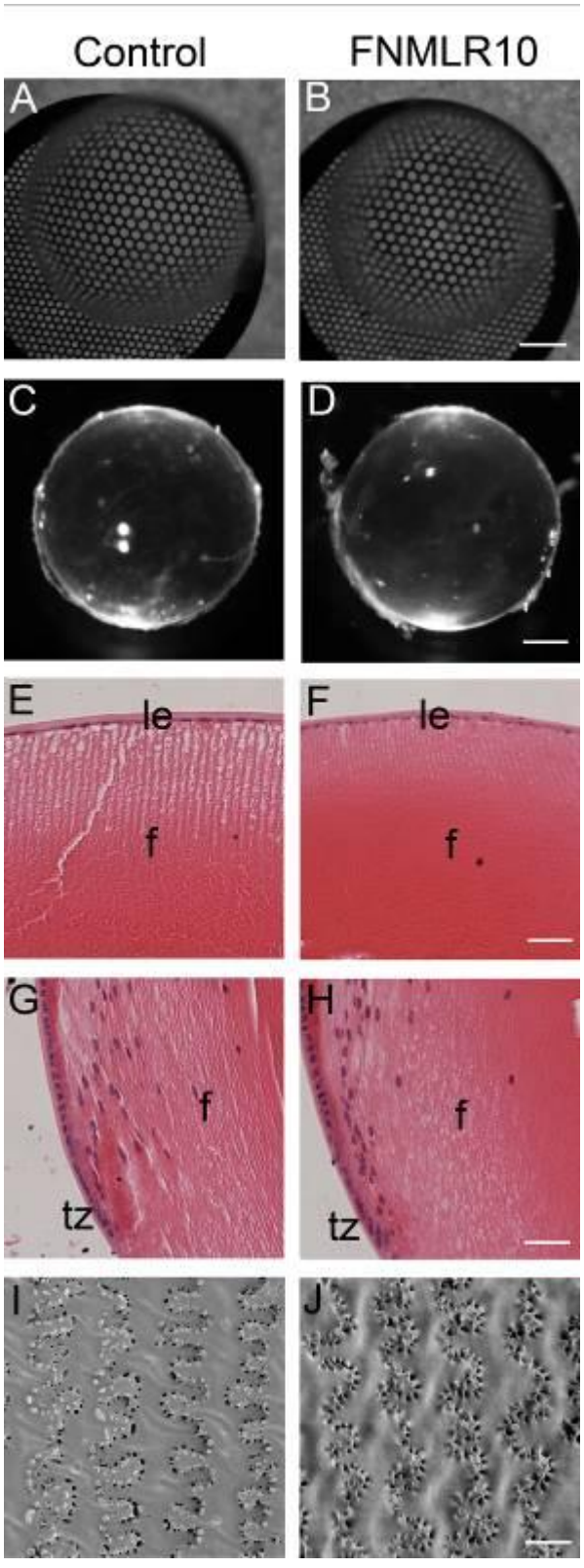

Control
FNMLR10
A
B
C
D
E
le
f
F
le
f
G
f
tz
H
f
tz
I
J

Figure 5.5: FN mlr10 lenses are morphologically similar to control lenses. A dark field image showing 9 weeks old control (A) and FN mlr10 lenses (B). 200-mesh electron microscopy grid analysis of 12 weeks old control (C) and old FN mlr10 lenses. (D) (E) Hematoxylin and eosin (H&E) staining showing anterior epithelium of 9 weeks old control lens. (F) H&E staining showing anterior epithelium of 9 weeks old FN mlr10 lenses. (G) H&E staining showing transition zone of 9 weeks old control lenses. (H) H&E staining showing transition zone of 9 weeks old FN mlr10 lenses. (I) SEM analysis of the fiber cell structure and organization of a 12 weeks old control lens.(H) SEM analysis of the fiber cell structure and organization of a 12 weeks old FN mlr10 lens. SEM analysis courtesy of Dr. David Scheiblin.Abbreviations: le -lens epithelium, f - lens fiber cells, tz - transition zone. Scale bar Panels A, B - 1.0mm; Panels C,D - 0.5mm; Panels E,F,G,H - 150μm; Panels I,J - 4μm.

FNMLR10 Lenses Lose αSMA Expression at Five Days Post Surgery, While Maintaining Fiber Cell Marker Expression

The absence of any obvious defects in the development, morphology or function of the FNMLR10 lenses meant that I could use the FNMLR10 mice as a model to study the role fibronectin plays in the cellular and molecular changes that occur post cataract surgery, when residual LECs undergo EMT leading to PCO. Following cataract surgery/lens injury, while some residual LECs undergo EMT (as part of wound healing response), whileothers begin to express lens fiber cell markers, presumably in an attempt to regenerate the injured lens (Wormstone and Wride 2011). As expected, immediately after surgical fiber cell removal, the residual LECs in both control and FNMLR10 mice (data not shown) exhibit no appreciable expression of αSMA, an EMT marker, or cMaf, a lens fiber cell marker. However, 48 hours later, the residual lens cells in both control (Figure 5.6 A, C) and FNMLR10 mice (Figure 5.6 B, D) are seen forming clusters that either express the EMT marker αSMA (Figure

5.6 A, B) or the fiber cell marker cMaf (Figure 5.6 C, D). It is of interest to note that expression of αSMA is seen both in the control (Figure 5.6 A) and the FNMLR10 lenses (Figure 5.6 A) at 48 hours post surgery (hPS). Further, no difference is observed in the expression of cMaf between the control (Figure 5.6 C) and FNMLR10 (Figure 5.6 D) lenses 48 hPS.

However by five days post surgery (dPS), while the cell clusters in control lenses continue to up regulate αSMA (Figure 5.6 E) and cMaf (Figure 5.6 G) protein expression, the FNMLR10 lenses do not exhibit any αSMA protein expression (Figure 5.6 F) expression while still continuing up regulation of cMaf expression. (Figure 5.6 H).

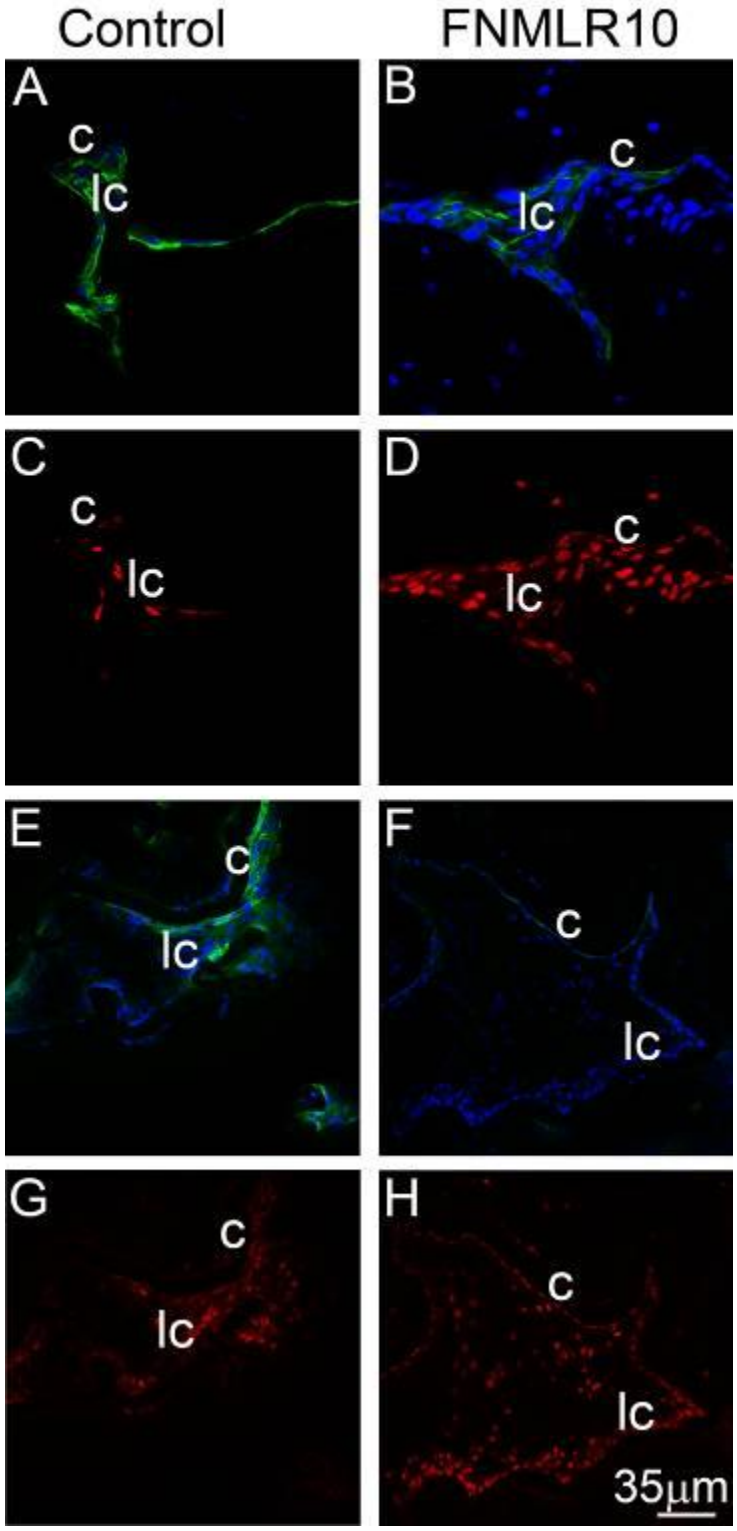
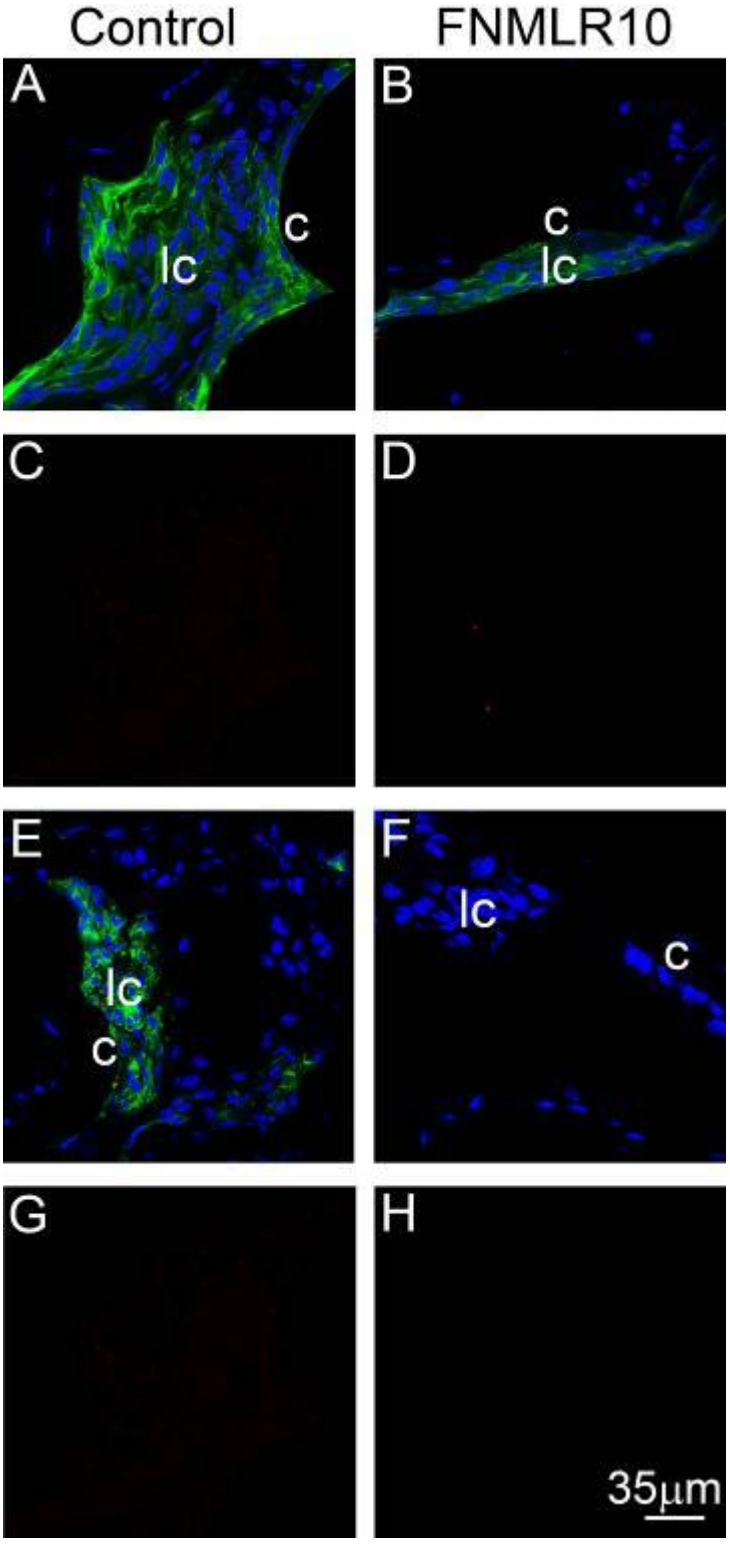

Control
FNMLR10
A
B
C
D
E
F
G
H
c
lc
35μm

Figure 5.6: FN mlr10 lenses show attenuation of αSMA expression. (A) Immunofluorescent confocal microscopy showing αSMA + cmaf protein expression in control (A, C, E ,G) and FN mlr10 (B, D, F, H)) capsular bags. At 48 hours post surgery (48 hPS) αSMA protein expression is similar between the control (A) and the FN mlr10 capsular bags (B). By five dPS, αSMA protein expression is maintained in controls (E) whereas αSMA protein levels fall beyond the level of detection in FN mlr10 lenses (F). These sections were co-stained with cmaf (see panels C,D,G,H for cmaf channel only). Both at 48 hPS (C,D) as well as five dPS (G,H) no major difference is seen in cmaf protein expression between controls (C-48 hPS, G- five dPS) and FN mlr10 (D-48 hPS, H-five dPS) capsular bags. Red - cmaf, Green - αSMA, Blue - DNA. Abbreviations: lc - lens cells, c – capsule, f – lens fiber. Scale bars – 35μm.

No Induction of Cell Death is Seen in FNMLR10 Lenses

The lack of αSMA expression but continued cMaf expression in FNMLR10 lenses by five dPS, raised the possibility that the cells expressing αSMA were undergoing apoptosis. During progression of PCO in the wildtype lenses no cell death is observed at any time point post surgery (Mamuya 2014). Consistent with this, control lenses did not exhibit apoptosis at either 48 hPS (Figure 5.7 A, C) or five dPS (Figure 5.7 E, G). Similar results were obtained for FNMLR10 lenses both at 48 hPS (Figure 5.7 B, D) and five dPS (Figure 5.7 F.H).


Control
FNMLR10
A
B
c
lc
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c
lc
c
G
H
35µm

Figure 5.7: Lens cell apoptosis is not detected in capsular bags after lens fiber cell removal. Sections were co-stained for cleaved caspase 3 and αSMA. Panels C, D, G, H show cleaved caspase 3 channel only. Control lenses at 48 hPS (A, C) as well as five dPS (E, G) do not exhibit any cleaved caspase3 staining. Similar results are seen in FN mlr10 lenses both at 48hPS (B,D) and five dPS (F,H). Red (panels C, D, G, H) – cleaved caspase3; Green- (panels A, B, E, F) - αSMA; Blue - DNA. Abbreviations: lc - lens cells, c - capsule. Scale bars – 35μm.

FNMLR10 Lenses Fail to Maintain Up Regulation of SMAD3 Phosphorylation by Five dPS.

TGF-β signaling plays a central role during LEC EMT leading to development of fibrotic PCO. and previous work in an *in vivo* lens injury model has shown that phosphorylation of SMAD3 is central to this process (Saika, Kono-Saika et al. 2004; de Iongh, Wederell et al. 2005). Consistent with these reports, SMAD3 phosphorylation was detected in both control (Figure 5.8 A, C) as well as FNMLR10 (Figure 5.8 B, D) LECs by 48 hPS. By five dPS SMAD3 phosphorylation levels are greatly elevated, especially in cells expressing αSMA in the controls (Figure 5.8 E, G). However, FN MLR10 LECs exhibit a down regulation of phosphorylated SMAD3 at five dPS (Figure 5.8 F, H).

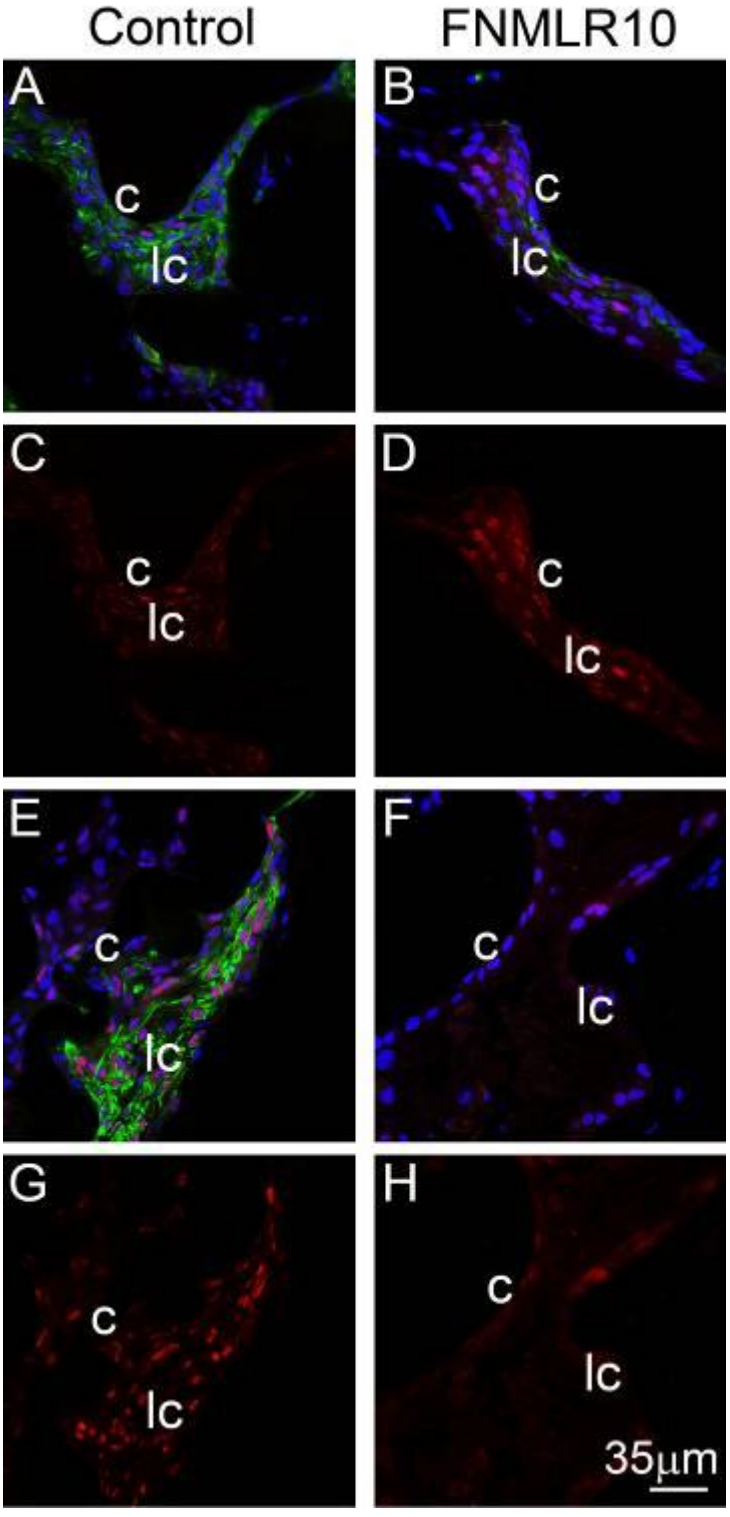
Control
FNMLR10
A
B
C
D
E
F
G
H
c
lc
35μm

Figure 5.8: FN mlr10 lenses show loss of sustained SMAD3 activation (pSMAD3). Immunofluorescent analysis of αSMA and pSMAD3 in controls and FN mlr10 lens cells from capsular bags collected at 48 hPS (A, B, C, D) and five dPS (E, F, G, H). (A) pSMAD3 + αSMAexpression in control lenses at 48 hPS. (B) pSMAD3+ αSMA expression in FN mlr10 lenses at 48 hPS. (C) pSMAD3 expression alone in controls at 48 hPS compared to pSMAD3 expression alone in FN mlr10 lenses (D), shows no difference in activation status of pSMAD3 up to 48 hPS. (E) pSMAD3 + αSMA expression in control lenses at five dPS. (F) pSMAD3 + αSMA expression in FN mlr10 lenses at five dPS. (G) pSMAD3 expression alone in control lenses at five dPS compared to(H) pSMAD3 expression in FN mlr10 lenses at five dPS shows failure to maintain sustained SMAD3 activation. Red (panels C, D, G, H) – pSMAD3; Green- (panels A, B, E, F) - αSMA; Blue - DNA. Abbreviations: lc - lens cells, c - capsule. Scale bars – 35μm.

Higher Levels of Intracellular LTBP1 are Observed in FNMLR10 Lenses.

Fibronectin interaction with LTBP 1,3 and 4, a family of ECM molecules is required for proper secretion, deposition and activation of the large latent complex containing latent Tgf β (LLC) (Zilberberg, Todorovic et al. 2012), it is however unclear whether any of these LTBPs are expressed in the LECs. RNA seq data on embryonic lenses from the Duncan lab (Manthey, Lachke et al. 2014) and data on adult lenses from ISYTE2.0 (Anand et.al, In preparation) suggested that LTBP1 is the most abundant LTBP expressed in the mouse lens. By using semi quantitative RT PCR, I was able to show the expression of LTBP1 in the epithelium of 9 week-old control mouse lenses (Figure 5.9 A, B). Further I used confocal immunofluorescence on whole mounts from 12 week-old unoperated lenses to determine if the loss of fibronectin might affect LTBP1 and hence LLC secretion from the lens. The FNMLR10 mice show higher levels of intracellular LTBP1 protein (red) (Figure 5.9 F) as compared to the controls (Figure 5.9 G). Surprisingly we detected higher F-actin

polymerisation (green) in FNMLR10 lenses (Figure 5.9 D) as compared to controls (Figure 5.9 E)

This raised the possibility that higher intracellular LTBP1 protein levels could be a result of higher LTBP1 expression in FNMLR10 lenses, and not necessarily due to improper secretion of the LLC. QRT PCR using RNA derived from the LECs of 9 week-old un-operated control and FNMLR10 mice showed no significant difference in the expression levels of LTBP1 between the control and FNMLR10 lenses, p value = 0.1204 (Figure 5.9-2) using ANOVA.

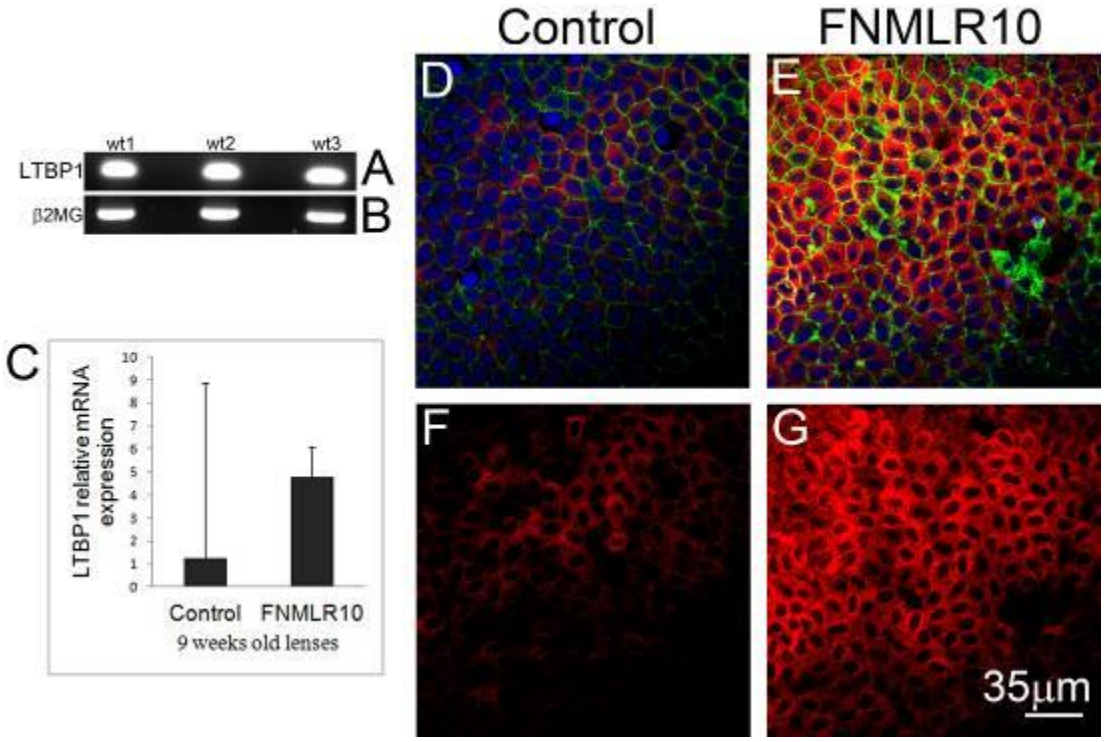

Figure 5.9: FN mlr10 lenses show intracellular accumulation of LTBP1. (A) RT-PCR analysis of 9 weeks old wild type lenses shows that LTBP1 is expressed in the normal mouse lens. (B) Co immuno-localization of F-actin and LTBP1 in whole mounts from 9 weeks old control (A) and FN mlr10 (B) lenses. Panels C, D show only LTBP1 channel demonstrating higher intracellular LTBP1 protein expression in FN mlr10 (D) lens epithelium as compared to controls (C). Red – LTBP1; Green – (panels A, B) F-actin; Blue - DNA. Scale bars – 35μm. (C) QRT-PCR quantification of LTBP1 mRNA expression levels in 9 weeks old control and FN mlr10 lens epithelial cells. mRNA expression was normalized to β2- microglobulin (β2M), n=4. No significant difference is seen in LTBP1 mRNA level between control and FN mlr10 lenses, p= .1204. Higher levels of intracellular TGFβ2 are observed in FN mlr10 lenses

Reduced SMAD3 phosphorylation post surgery (refer Figure 5. 8) as well as LTBP1 accumulation within the FNMLR10 lenses (refer Figure 5.9) indicated that

there might be intracellular retention of TGFβ as part of the LLC in these lenses, resulting in impaired TGFβ signaling. TGFβ2 is the major TGFb isoform expressed in the mouse eye (ISYTE 2.0, Anand et.al, In preparation and (Manthey, Lachke et al. 2013), and is more effective than other TGF-β isoforms in causing changes associated with human subcapsular cataract (Nishi, Nishi et al. 1999; Wormstone, Tamiya et al. 2002). Using confocal immunofluorescence on whole mounts from 12 week-old un-operated FNMLR10 lenses had higher levels of intracellular TGFβ2 protein (red) (Figure 5.10 B (A) as compared to the controls (Figure 5.10 B (B). Surprisingly, F-actin (green) labeling was also higher in FNMLR10 lenses (Figure 5.10 B (B) as compared to controls (Figure 5.10 B (A)

I needed to again examine the possibility that higher intracellular TGF-β2 protein levels could be a result of higher expression of TGF-β2 in FNMLR10 lenses, and not necessarily due to improper secretion of the LLC. QRT PCR using RNA derived from the LECs of 9 week-old un-operated control and FNMLR10 mice showed no significant difference in the expression levels of TGF-β2 between the control and FNMLR10 lenses, p-value = 0.0668 (Figure 5.10 A). Similarly, no significant difference was observed in TGFβ2 expression in post surgical samples at 0h, p-value = 0.828 (data not shown), 48 hours, p-value = 0.3361 and five days post surgery, p-value = 0.0667 (Figure 5.11).

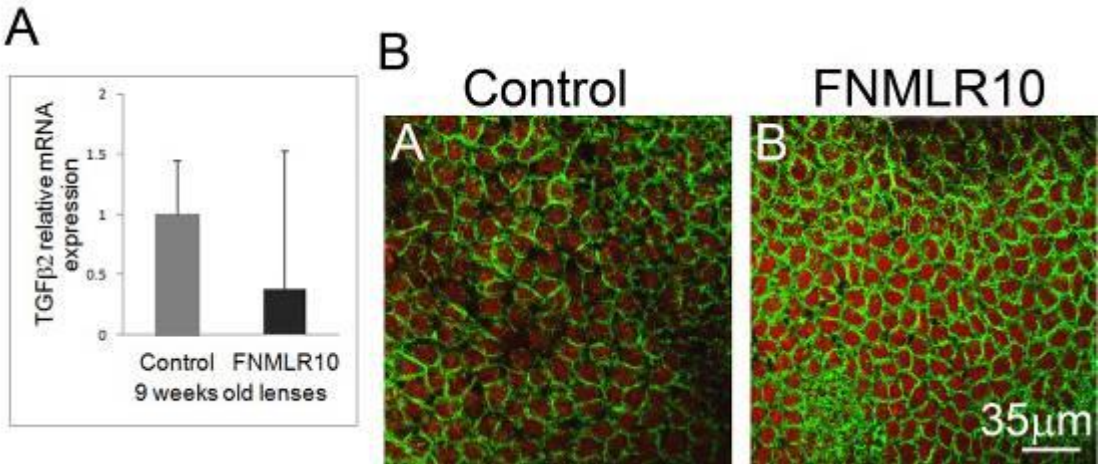

Figure 5.10: FN mlr10 lenses show intracellular accumulation of TGF-β2. (A) QRT-PCR quantification of TGF-β2 mRNA expression levels in 9 weeks old control and FN mlr10 lenses. mRNA expression was normalized to β2-microglobulin (β2M), n=6. (B) Co immunolocalization of F-actin and TGF-β2 in whole mounts from 9 weeks old control (A) and FN mlr10 (B) lenses, demonstrating higher intracellular TGF-β2 protein expression in FN mlr10 (B) lens epithelium as compared to controls (A). Red – TGF- β2; Green – F-actin; Blue - DNA. Scale bars – 35µm.

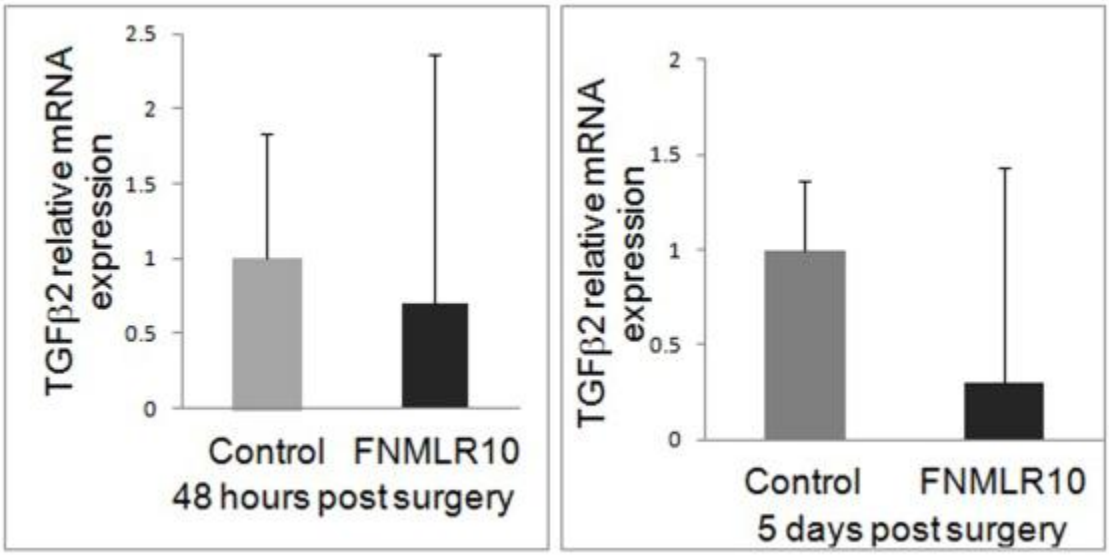

Figure 5.11: RT-PCR quantification of TGFβ2 mRNA levels in control residual lens cells from capsular bags at 0hrs, 48hrs and five days post-surgery normalized to β2M n=3.

Discussion

The ECM is known to provide structural support for organs and tissues, for cell layers in the form of basement membranes, and for individual cells as substrates for cell motility. The role of ECM has been well studied in cell adhesion and in signaling to cells through adhesion receptors such as integrins and, more recently, the idea has been developed that mechanical characteristics of the matrix (stiffness, deformability) also provide inputs into cell behavior. Thus signals from the ECM play a role in governing processes such as cell fate determination, differentiation, proliferation, survival, polarity and migration of cells. Further ECMs undergo dynamic rearrangements and exhibit compositional differences during development (Ruoslahti 1981; Sadaghiani, Crawford et al. 1994; Li and Yurchenco 2006), thus, studying ECM

components at various developmental stages is critical to understanding ECM functions at key points in development.

ECM proteins are also known to bind, sequester and modulate the distribution and presentation of growth factors thus regulating cell signaling, though this remains an understudied area (Murphy-Ullrich and Poczatek 2000; Wu, Tholozan et al. 2014).

Cellular Fibronectin is Expressed in the Developing Lens and During Lens EMT

FN is an abundant constituent of plasma and other body fluids and also part of the insoluble extracellular matrix (To and Midwood 2011). On the basis of its solubility, FN can be subdivided into two principle forms -soluble plasma FN (pFN) and less-soluble cellular (cFN) FN. Plasma FN is synthesized and secreted by hepatocytes and the alternatively spliced EDA and EDB domains are absent. cFNs contain variable proportions of these EDA and EDB domains, resulting from cell-type-specific and species-specific splicing patterns (To and Midwood 2011).

Fibronectin has been detected in the lens capsule of developing zebrafish, chicks, mice and rats, however it was not detected in rat embryonic day nineteen lens capsules (Parmigiani and McAvoy 1984; Duncan, Kozmik et al. 2000; Duncan, Xie et al. 2004; Lovicu and Robinson 2004). In the adult, fibronectin, believed to be plasma derived (Vesaluoma, Mertaniemi et al. 1998; Chowdhury, Madden et al. 2010; Anshu, Price et al. 2011), has been detected throughout the adult mouse posterior capsule (Duncan, Kozmik et al. 2000). but only on the outer surfaces of the anterior and equatorial capsules of adult rats (Sramek, Wallow et al. 1987), mice (Duncan, Kozmik et al. 2000), and humans (Kohno, Sorgente et al. 1987). Therefore, while fibronectin in the lens, as in other tissues, has been proposed to play a role in wound healing

responses, such as that seen during PCO (Wormstone, Wang et al. 2009; Wormstone and Wride 2011), no clarity existed about the expression pattern of its isoforms in the lens.

Therefore, an RT-PCR analysis was carried out of cFN expression in the lens and found that cFN is expressed in the embryonic lens, while cFN expression is undetectable in adult lenses. However, cFN mRNA is detectable in post surgery capsular bags by 48 hours after surgery (Figure 5.2 C, D). In direct contrast to these data, immuno localization studies showed the presence of fibronectin in the lens capsules of embryonic, adult and post surgery lens capsules (data not shown, Figure 5.2 F, G). A major fraction of ECM-FN is plasma derived and it has been seen that addition or injection of soluble FN into the culture medium of cells or into the plasma of mice resulted in the incorporation of FN into the extracellular matrix (Oh, Pierschbacher et al. 1981; Moretti, Chauhan et al. 2007) Therefore, using commercial polyclonal fibronectin antibodies, I was detecting pFN, likely derived from the aqueous humor (Vesaluoma, Mertaniemi et al. 1998; Chowdhury, Madden et al. 2010; Anshu, Price et al. 2011), in these lenses.

Fibronectin May Play an Essential Role During Early Stages of Lens Development but is Dispensable in the Fully Formed Lens.

The impact of ECM components on cellular functions critical for lens development has been well documented in various models (Kurkinen, Alitalo et al. 1979; Parmigiani and McAvoy 1991; Wederell and de Iongh 2006). Consistent with these findings, mutations in genes encoding either lens capsule components or proteins necessary for lens capsule assembly lead to diverse lens dysplasias (Parmigiani and McAvoy 1991; Rossi, Morita et al. 2003; Danysh and Duncan 2009; Firtina, Danysh

et al. 2009; Pathania, Semina et al. 2014). While cFN is well studied during development in other systems, its role in lens development remains an understudied area. Following an analysis of cFN expression during development, I found that although the gene is expressed even in later stages of lens development (E13.5 onwards), only lenses that lose cFN early during lens formation (FN-LE) exhibit a developmental phenotype. Thus, cFN is dispensable for maintaining transparency and morphology once lens formation is complete (FNMLR10).

During development, cells need the ability to respond to rapidly changing cues from neighboring cells and chemical gradients. Embryonic cells synthesize and secrete ECM beginning at the earliest stages of development. The ECM via its receptors mediates physical linkages with the cytoskeleton, providing a substrate for cells to migrate upon. Further ECM also acts as a repository for growth factors influencing growth factor signaling by restricting or promoting access of ligands to cognate cell-surface receptors, modulating the spatial distribution of a diffusible morphogen, or by sequestering factors for subsequent release (Brown 2011; Yurchenco 2011). It is important to note however that the composition and organization of the ECM components changes throughout development, influencing bidirectional flow of information, resulting in specific developmental events (Daley, Peters et al. 2008; Bonnans, Chou et al. 2014). Variations in temporal distribution of fibronectin isoforms and their influence on specific developmental processes have been reported in various systems (Peters and Hynes 1996; Fukuda, Yoshida et al. 2002; Astrof, Crowley et al. 2004; Astrof, Crowley et al. 2007; Astrof and Hynes 2009). Therefore, it is not surprising that loss of fibronectin early in lens development has a profound effect on normal lens biology, while later in lens development, fibronectin seems dispensable .

cFN Plays a Crucial Role in EMT but has no Effect on Lens Fiber Regeneration during PCO Development.

Posterior Capsule Opacification (PCO) is the most common complication of cataract surgery. In a significant number of cases, PCO leads to secondary loss of vision (Awasthi, Guo et al. 2009; Apple, Escobar-Gomez et al. 2011). Cataract surgery involves excision of the central anterior lens capsule followed removal of lens fibers; finally the surgeon polishes the lens capsule to remove LECs. This generates a capsular bag, which comprises the remainder of the anterior and the entire posterior capsule. The capsular bag partitions the aqueous and vitreous humors, and in majority of cases, houses an intraocular lens (IOL). Free passage of light occurs along the visual axis through the transparent IOL and thin acellular posterior capsule (Ashwin, Shah et al. 2009). However attached to the remaining anterior capsule are residual LEC which cannot be completely removed during surgery. These LECs sense cataract surgery as an injury resulting in concurrent LEC proliferation and migration onto the posterior lens capsule (Wormstone, Wang et al. 2009; Chan, Mahroo et al. 2010). Some of these cells undergo epithelial-mesenchymal transition (EMT) resulting in these cells overexpressing αSMA, depositing mesenchymal ECM proteins and contraction of the posterior capsule leading to light scatter and visual disability seen in fibrotic PCO. In contrast, other LECs that migrate onto the posterior capsule undergo fiber cell differentiation , in an attempt to regenerate the lens. Since they do not form the correct cellular organization, they induce light scattering instead of forming a transparent lens which leads to Pearl type PCO. Finally, many of the intraocular lens implants used in cataract surgery are designed to trap and prevent posterior migration of residual LECs at the lens equator, these cells often attempt lens fiber differentiation

forming an opacity outside of the visual axis known as Soemmering's ring (Awasthi, Guo et al. 2009; Wormstone, Wang et al. 2009).

PCO thus arises from two distinct cellular responses to cataract surgery, EMT and fiber cell regeneration. While elevation of fibronectin expression in LECs is often used as a marker of LEC EMT and its levels are known to increase during PCO progression, its functional role during lens EMT/ PCO was not established (Boyd, Peiffer et al. 1992; Wormstone, Wang et al. 2009; Wormstone and Wride 2011).

Therefore, I tested the response of FNMLR10 mice to lens fiber cell removal. αSMA, a hallmark marker for LECs undergoing EMT, was found to up regulate by 48 hPS in FNMLR10 lenses similar to controls. This response however was greatly attenuated at 5 dPS with no αSMA expression seen in the FNMLR10 lenses. Further, no appreciable differences were seen in the fiber cell marker expression between controls and FNMLR10 lenses.

These data suggest that cFN has no role in regulating the lens fiber cell regeneration response but plays an essential role in the maintenance of EMT response, during fibrotic PCO progression, while not being involved in the early regulation of the EMT post cataract surgery. This is consistent with reports that there exists a temporal distinction of fibronectin function wherein pFN functions during early wound-healing responses (Chandrasekhar, Norton et al. 1983; White and Muro 2011), whereas cFN is expressed following initiation of wound healing response, assembled locally into the ECM and functions during later wound-healing responses (To and Midwood 2011).

The Attenuation of the EMT Response 5 dPS Post Surgery in cFN Lenses is likely due to Impaired TGF-β Signaling

TGFβ signaling plays an important role in development of fibrotic PCO. Treatment of LECs with TGF-β in vitro can induce most cellular and molecular changes associated with fibrotic PCO, including formation of myofibroblast like cells, the expression of ECM proteins characteristic of EMT, LEC proliferation, and capsule wrinkling. Further, transgenic mice overexpressing an active form of TGF-β in lens fiber cells develop anterior subcapsular cataracts which share many features with fibrotic PCO (Sponer, Pieh et al. 2005; Symonds, Lovicu et al. 2006; Mamuya, Wang et al. 2014; Saika, Werner et al. 2014).

TGF-β mediates effects via SMAD2/3 dependent (canonical) signaling or through SMAD-independent (non canonical signaling). The classical Smad signaling pathway is considered to be the main player in the pathogenesis of PCO (Saika, Miyamoto et al. 2002; Saika, Kono-Saika et al. 2004; Dawes, Sleeman et al. 2009). pSmad3 and pSmad4 are present in cell nuclei of post-operative human lens epithelial cells and in injury-induced epithelial to mesenchymal transition (EMT) of murine lenses (Wormstone and Eldred ; Mamuya, Wang et al. 2014). Further Smad3/Smad4 knockout models also provide evidence that TGFβ Smad3/Smad4 signalling may regulate the transdifferentiation of LECs seen in PCO (Saika, Kono-Saika et al. 2004; Dawes, Sleeman et al. 2009). SMAD-3 activation (SMAD-3 phosphorylation) is not appreciably detected until 48 hours after surgery in our model (Mamuya, Wang et al. 2014), coincident with the upregulation of cFN protein expression (Figure 5.4) (Mamuya, Wang et al. 2014). Phosphorylated SMAD-3 levels then continue to increase through five days postsurgery in wildtype mice. Notably, SMAD-3 phosphorylation is detected in LECs of FNMLR10 lenses at levels comparable with

controls at 48 hPS, while much less phosphorylated SMAD3 is detected in FNMLR10 lenses by 5 dPS compared to controls. TGFβ is present in the aqueous humour of the eye and also synthesized by the lens cells (Lovicu and Robinson 2004; Eldred, Hodgkinson et al. 2012) and exists largely in a latent, inactive form regulated by ECM proteins and α2-macroglobulin, which has a high affinity for free active TGFβ (Meeting, Kinoshita et al. 1999). As the blood aqueous barrier is breached during cataract surgery, TGFβ activators such as plasmin proteases MMP-2 and -9, thrombospondin-1 and reactive oxygen species activate TGFβ resulting in downstream signaling (Wormstone, Wang et al. 2009). This initial TGFβ activation serves to up regulate αSMA and other EMT mediators in the early post injury period initiating the wound healing response. However, this is a transient phenomenon as repair of blood aqueous barrier and removal of fibrin clot starts within 24 hPS (Meeting, Kinoshita et al. 1999), highlighting the existence of another mechanism to sustain TGFβ activation during fibrosis. This initial TGFβ activation also increases EDA exon incorporation into FN mRNA, about 3 days after injury in other systems (Muro, Chauhan et al. 2003; Muro, Moretti et al. 2008; To and Midwood 2011). Similar to my findings: EDA-cFN deficient mice show reduced αSMA expression by myofibroblasts (Muro, Moretti et al. 2008; Kohan, Muro et al. 2011), ultimately preventing fibrosis since EDA-cFN in ECM facilitates proper incorporation of LLC into the ECM (Dallas, Sivakumar et al. 2005) and also plays a role in αV integrin mediated release of TGFβ1 from the LLC (Fontana, Chen et al. 2005). This sustained activation of TGFβ signaling, seen as a hallmark of fibrosis is thus cFN dependent.

cFN Regulates TGF-β via Interactions with LTBP1

Lens cells *in vivo* synthesize all three TGFβ isoforms as inactive precursors (Saika, Miyamoto et al. 2000; Mamuya, Wang et al. 2014) that contain a pre-region (Signal peptide) and pro-region (N terminal peptide – latency associated peptide - LAP). After proteolytic cleavage of signal peptide from pre-pro-TGF-βs, they dimerize. These dimers are cleaved by proteases (e.g. Furin) into C-terminal mature peptides and N-terminal LAP (Latency Associated Peptide). TGF-βs containing LAP form small latent complexes (SLP) that further covalently bind to latent TGF-β binding protein (LTBP) to form a large latent complex (LLC) (Kubiczkova, Sedlarikova et al. 2012). LTBP facilitates secretion of the LLC from the cell (Miyazono, Olofsson et al. 1991) and targets latent TGFβ to the ECM for storage (Taipale, Miyazono et al. 1994). Notably, LTBP1, the most abundant LTBP in the lens (unpublished), requires cFN to both tether latent TGFβ to the ECM and participate in the interactions necessary for TGFβ activation (Fontana, Chen et al. 2005). Thus, fibronectin is crucial for TGFβ activation critical for the development of fibrotic PCO via its interactions with the LTBP1. Further, fibronectin is known to provide the template for initial as well as continuous LTBP1 containing LLC incorporation into the ECM (Dallas, Sivakumar et al. 2005). Therefore, similar to my data, FN null fibroblasts fail to incorporate LTBP1 into the ECM, while no differences were observed in the LTBP1 expression of FN null fibroblasts as compared to controls (Dallas, Sivakumar et al. 2005). Interestingly similar to my data, no differences were observed between the expression of TGFβ in FN null fibroblasts as compared to controls, suggesting that the loss of TGFβ activation upon cFN deletion may be a result of loss of LTBP1 –cFN interactions (Fontana, Chen et al. 2005).

This work provides for the first time insight into the multiple roles of fibronectin in the lens. While it supports work done in other systems on the role of cFN in regulating TGF β signaling, in τηε context of PCO, these findings have the potential to identify novel targets to block PCO in order to improve outcome of cataract surgery.

Chapter 6

CONCLUSIONS AND FUTURE DIRECTIONS

Integrins have been implicated in maintenance of the normal structure and function of lens epithelium as well homeostasis of lens fiber cells (De Arcangelis and Georges-Labouesse 1999; Simirskii, Wang et al. 2007; Cammas, Wolfe et al. 2012; Simirskii, Duncan et al. 2013; Scheiblin, Gao et al. 2014). β1 integrins are detected at basal surfaces of lens epithelial cells and at apical tips and lateral membranes of differentiating fiber cells (Menko and Philip 1995). In the lens, β1 integrin interacts with α3,α6,αV,α5 and α2 subunits to form cell surface receptors for components of the lens capsule (Menko, Philp et al. 1998).

Various studies focused on the functions of integrin subunits have indicated a possible role of β1 integrins in lens development (Wederell and de Iongh 2006; Samuelsson, Belvindrah et al. 2007; Simirskii, Wang et al. 2007; Walker and Menko 2009). Loss of β1 integrins after lens morphogenesis is complete results in loss of epithelial cells by apoptosis and abnormal differentiation (Simirskii, Wang et al. 2007). The β1 integrin binding partners α3 and α6 (Black, Walker et al. 2002; Barbour, Saika et al. 2004; Wederell and de Iongh 2006) integrins are also required for maintenance of anterior lens epithelium since α3/α6 double nulls show breaches in the anterior lens epithelium resulting in the extrusion of lens fibers (De Arcangelis and Georges-Labouesse 1999; Wederell and de Iongh 2006). Further, knockdown of α6 integrin blocks fiber cell differentiation in chick lens explants (Walker, Zhang et al. 2002), while conditional deletion of integrin linked kinase (ILK), one possible

downstream effecter of β1 integrins signaling in the lens, during lens formation results in non apoptotic death of lens epithelial cells (Cammas, Wolfe et al. 2012). While all these studies show that β1 integrins play a role in maintaining normal lens biology, the mechanism(s) by which β1 integrins exert their influence during early lens morphogenesis is not understood understood. This aim of this study was to elucidate the role of β1 integrins during morphogenic movements and cell fate decisions that occur as the lens placode transition into the lens vesicle.

A fundamental and highly conserved process during morphogenesis is cellular sheet folding and invagination (Lecuit and Le Goff 2007; Lecuit and Lenne 2007). Invagination of cellular sheets begins when dynamic actin rearrangements occurring within a few cells lead to apical constriction of these cells (Hardin and Keller 1988). Apical constriction creates conical or wedge shaped cells with reduced apical surface area. This leads to local mechanical stress that forces the epithelial sheets to bend. Further biochemical remodeling or compaction of cells as a result of changes in size and number of cells on a rigid ECM changes its compliance (Odell, Oster et al. 1981; Hardin and Keller 1988; Ingber 2003; Ingber 2006). These two mechanical processes acting in concert lead to invagination. These mechanical signals are transferred across the cell surface via numerous molecules and sub cellular structures including the transmembrane adhesion molecules- integrins (Ingber 2003; Ingber 2006). Mechanical stress application to integrins alters cytoskeleton structure and activates signal transduction and gene expression in a stress dependant manner. This process is called mechanotransduction and leads to changes in the ability of the cells to respond to soluble cues and chemical gradients involved in cell fate determination that occurs during embryogenesis (Ingber 2006; Chauhan, Disanza et al. 2009; Chauhan, Lou et

al. 2011). Both invagination and cell fate specification occur during early lens morphogenesis, thus underscoring the potential importance of integrins in the early lens (Chow and Lang 2001; Donner, Lachke et al. 2006). Since β1 integrin is the most promiscuous of the integrin subunits, its deletion can help us simultaneously study the role of integrins in early lens morphogenesis (Walker and Menko 2009).

To characterize the role of β1 integrins in the early lens, I used a mouse model with the potential to conditionally delete β1 integrin from the lens at the lens placode stage (β1 LE mice) (Ashery-Padan, Marquardt et al. 2000). My data showed that loss of β1 integrin protein in the β1 LE mice does not occur until the lens pit stage (10.5 dpc) thus making this model unsuitable for the study of morphogenic movements occurring during early lens development.

Surprisingly though, my data showed that the loss of β1 integrins from the lens at 10.5 dpc resultsin the ectopic differentiation of anterior LECs into fiber cells. Thus β1 integrins fine tune the position of the border at the lens equator between the LEC and lens fibers proposed to be set up by FGF signaling gradient (Lovicu and McAvoy 2001). However I have shown that modulation of BMP via β1 integrins in the lens epithelium actively blocks induction of FGF dependent fiber differentiation pathways in the anterior lens epithelium, thus playing a fundamental role in the decision between lens epithelial and fiber cell fates in the early lens.

Overall my work has addressed for the first time the role-played by β1integrins in controlling the decision between epithelial and fiber cell fates in the early lens. This will expand our understanding of how positional cues via integrins can influence the molecular mechanisms driving lens differentiation specifically and cellular differentiation in general.

Future Perspectives: It would be interesting to note if the BMP- β1 integrin interaction in the LECs occurs at the level of modulating FGFR expression as is seen in some other systems (Hayashi, Ishisaki et al. 2001). The localization of these receptors is challenging presently since immunostaining techniques used in the lab have not yielded results. In situ hybridization may work as an alternative technique to look at the relative expression profile of the lens FGFRs in the LECs. Also localization BMPR1b inside lipid rafts, if observed in specific regions of the lens epithelium, may shed more light on the proposed modulation of BMP signaling by β1 integrins (North, Pan et al. 2015).

The lens capsule- lens cell interactions are critical for lens morphogenesis and function (Webster, Silver et al. 1984; Lee and Gross 2007). Fibronectin is an ECM protein produced by the lens throughout its development (Parmigiani and McAvoy 1991; Duncan, Kozmik et al. 2000) and its expression increases during PCO progression (Boyd, Peiffer et al. 1992; Wormstone, Tamiya et al. 2002). Zebrafish with fibronectin mutations have abnormal lenses (Hayes, Hartsock et al. 2012), while fibronectin deposition in the ECM underlying the lens placode is required for placode thickening and invagination (Huang, Rajagopal et al. 2011). Fibronectin is also involved in fibrotic processes since this gene is a direct target of TGFβ signaling (Hocevar, Brown et al. 1999; White and Muro 2011) and the EDA splice variant of fibronectin collaborates with TGFβ to drive myofibroblast development during fibrosis (To and Midwood 2011; White and Muro 2011). Further, fibronectin is a ligand for α5β1 integrin and αV integrins (Hynes 1992) which are upregulated during PCO (Menko and Philip 1995{Mamuya, 2014 #1331; Dawes, Eldred et al. 2008)}. Fibronectin may also act as a negative regulator of matrix contraction in fibrotic PCO

(Dawes, Eldred et al. 2008), highlighting the need for further study of this molecule in the lens.

RNA-seq data from the lab suggested that fibronectin is expressed high levels in E15.5 mouse lenses (Manthey, Lachke et al. 2014). Further, during investigations of fibronectin function in the lens placode (Huang, Rajagopal et al. 2011), fibronectin was deleted from the lens using Le Cre (FN-LE), fibronectin protein however was not lost until after the end of lens morphogenesis. While these lenses were normal at E12.5, they had a severe phenotype at birth. Since characterization of these animals was beyond the scope of their studies, we collaborated and set up the FN-LE colony in our lab to study fibronectin in the maturing lens. The phenotype of these lenses was characterized by the undergraduate that I mentored, Saleena Malik.

It is known that fibronectin, along with its canonical receptor α5β1 integrin as well as αV integrins, increase in expression during LEC EMT (de Iongh, Wederell et al. 2005; Dawes, Eldred et al. 2008; Mamuya, Wang et al. 2014). Also treatment of cultured LECs with an α5β1 function-blocking antibody inhibited cell migration (Yao, Tan et al. 2007) and peptides blocking integrin interactions with fibronectin may be useful to prevent PCO in rabbit models (Nishi, Nishi et al. 1997). However our knowledge of mechanisms by which fibronectin contributes to the development of PCO are still incomplete. Since the phenotype of FN-LE mice was too severe to use in the study of PCO we created mice which lose fibronectin later in development employing the MLR10- Cre mice.

The role of TGFβ signaling in inducing lens epithelial cells to undergo an EMT resulting in PCO (de Iongh, Wederell et al. 2005, Dawes, Sleeman et al. 2009, Wormstone, Wang et al. 2009) is well established. TGFβ signaling is carried out by a

diversity of complex pathways, which, in part, contribute to the challenge in understanding how these pathways are integrated to drive lens EMT (de Iongh, Wederell et al. 2005; Dawes, Sleeman et al. 2009; Wormstone, Wang et al. 2009; Wormstone and Wride 2011). Consistent with previous studies on fibrosis outside the lens (Dallas, Sivakumar et al. 2005; Fontana, Chen et al. 2005), my work has shown cFN has the capacity to regulate TGFβ signaling by modulating both the secretion of latent TGFβ into the ECM and also by being essential to αV integrin mediated TGFβ activation. TGFβ activation in turn promotes the incorporation of the EDA exon into fibronectin (To and Midwood 2011), thus establishing a positive feed forward loop that acts as a continuous driver of EMT, thereby promoting the changes associated with fibrotic PCO.

Overall this work has been able to establish not only role of fibronectin both in maturing lens as well as in PCO, but in the process has revealed a temporal complexity in its functon during development. Further the knowledge of how fibronectin impinges upon TGFβ activation and thus drives fibrotic PCO could in the future be useful in creating effective therapies for preventing PCO.

Future perspectives: The cell biological effect of fibronectin on developing lens needs to be fully addressed by looking at cell proliferation/apoptosis pathways and fiber cell differentiation pathways. If this approach is inconclusive, RNA-seq analysis of FN-LE lenses could be used to identify the molecular pathways influenced by lens specific deletion of fibronectin.

To further explore the temporal complexity of cFN function in the developing lens a charecterization of fibronectin protein loss when using the MLR10- Cre system would

be useful. We need to evaluate expression and localization of other TGFβ isoforms both in intact and post surgery FN mlr10 lenses, since TGFβ1 is also a contributor to lens EMT. As with TGFβ2 it would interesting to note whether the effect on TGFβ1 is also at the level of secretion of LLC or is it due to defective cFN dependent- αV integrin mediated TGFβ1 activation (Fontana, Chen et al. 2005).

The lens capsule-lens cell interactions are an understudied, but crucial regulator of lens biology. This work sheds light on the molecular mechanisms by which lens capsule components and their principal receptors integrins regulate lens differentiation specifically and cellular differentiation in general. Further we have gained insights into the role of lens capsule componenst in maintaining structural integrity of not only the lens but also the cornea. This work has also identified the molecular mechanisms by which extracellular matrix components regulate the transdifferentiation of lens epithelial cells to myofibroblasts during the pathogenesis of posterior capsular opacification, validating molecular targets to prevent PCO.

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Lens Extrusion from Laminin Alpha 1 Mutant Zebrafish

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Appendix B

IRB APPROVAL

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

Title of Protocol: The investigation of developmentally important genes in the mouse and chicken

AUP Number: 1039-2015-2 ← **(4 digits only)**

Principal Investigator: Melinda K. Duncan

Common Name: mouse, chicken, rat

Genus Species: *Mus musculus, Gallus gallus, Rattus rattus*

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: _____

Date of Approval: 12/1/2014

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.

Melinda Duncan — Signature of Principal Investigator

10/7/14 — Date

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. Dylan Audette	
3. Mallika Pathania	
4. Yichen Wang	
5. Ramachandran Balasubramanian	
6. Troy Rubenstein	
7. Priyha Mahesh	
8. YAN WANG. ~~Click here to enter text.~~	
9. Click here to enter text.	
10. Click here to enter text.	
11. Click here to enter text.	
12. Click here to enter text.	
13. Click here to enter text.	
14. Click here to enter text.	
15. Click here to enter text.	

IACUC approval of animal protocols must be renewed on an annual basis.

1. Previous Approval Date: 12.1.13

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

xYes ☐ 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	12,000	6455
2. Rat	Rattus rattus	30	none
3. Chicken (embryo)	Click here to enter text.	9000	600
4. Chicken (posthatch)		60	none
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

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

Project Personnel Deletions:

Name	Effective Date
1. all have been previously reported	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.

1. Abby L. Manthey, Salil A. Lachke, Paul G. FitzGerald, Robert W. Mason, David A. Scheiblin, John H. McDonald, and **Melinda K. Duncan** (2014) Loss of Sip1 leads to migration defects and retention of ectodermal markers during lens development *Mechanisms of Development,* **131,** 86-110.

2. Fahmy A. Mamuya, Yan Wang, Victoria H. Roop, David A. Scheiblin, Jocelyn C. Zajac and **Melinda K. Duncan** (2014) The Roles of α_V Integrin in Lens EMT and Posterior Capsular Opacification *Journal of Cellular and Molecular Medicine,* **18,** 656-670.

3. David A. Scheiblin, Junyuan Gao, Jeffrey L. Caplan, Vladimir N. Simirskii, Kirk J. Czymmek, Richard T. Mathias and **Melinda K. Duncan** (2014) Beta-1 integrin is important for the structural maintenance and homeostasis of differentiating fiber cells *International Journal of Biochemistry and Cell Biology,* **50,** 132-145.

4. Abby L. Manthey, Anne Terrell, Yan Wang, Jennifer R. Taube, Alisha R. Yallowitz, and **Melinda K. Duncan** (2014) The Zeb proteins, δEF1 and Sip1, may have distinct functions in lens cells following cataract surgery *Investigative Ophthalmology and Visual Sciences, In press.*

These papers describe progress towards our understanding of Sip1, and integrins in lens biology. We are preparing 6 other manuscripts for publication that describe the function of Bin3, integrins, crystallins and Prox1 in the lens.

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