THE ROLE OF ALPHA V INTEGRINS IN LENS EPITHELIAL MESENCHYMAL TRANSITION AND POSTERIOR CAPSULAR OPACIFICATION

by

Fahmy A. Mamuya

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

Winter 2014

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DEDICATION

This dissertation is dedicated to my lovely, brilliant daughter

Leyla Lynn Mamuya

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ABSTRACT

Posterior Capsular Opacification (PCO) is the major complication after cataract surgery. PCO occurs when residual lens cells (LCs) remaining following cataract surgery undergo a wound healing response producing a mixture of αSMA expressing myofibroblasts and lens fiber cells which impair vision. Prior investigations have proposed that integrins play a central role in PCO while my data indicated that αV integrin and its interacting β-subunits; β1, β5, β6, β8 along with α-smooth muscle actin (αSMA) are upregulated in residual lens epithelial cells in a mouse model of cataract surgery. To test the hypothesis that αV integrins are functionally important in PCO pathogenesis, I created mice lacking the αV integrin subunit in all lens cells using a conditional knockout approach. Adult lenses lacking αV integrins were analyzed for function and cellular organization by grid analysis, conventional light, confocal and scanning electron microscopy. At three months of age, fiber cells were surgically removed from αV integrin null and control lenses and the extent of EMT post-surgery was measured by QRT-PCR and immunofluorescent detection of EMT markers.

Lenses lacking αV integrin subunits are transparent and show no apparent morphological abnormalities when compared to control lenses. When challenged with surgery/injury, control lenses developed LC multilayering along with upregulation of αSMA, β1-integrin, fibronectin, vitronectin, tenascin-C and TGF-β induced protein within 48hrs. In contrast, LECs lacking αV integrins showed no increase in LC multilayering with little to no upregulation of αSMA, fibronectin, vitronectin, and TGF-

β induced protein 48hrs post-surgery. In addition, I saw a significant increase in the mRNA expression of αSMA, fibronectin, tenascin-C and TGF-β induced protein 24hrs post-surgery in control lenses with no significant increase in lenses lacking the αV integrin subunit.

This effect appears to result from the known roles of αV integrins in latent TGF-β activation since αV integrin null lenses do not exhibit detectable SMAD-3 phosphorylation after surgery, while this occurs robustly in control lenses, consistent with the known roles for TGF-β in fibrotic PCO as well as Anterior Subcapsular Cataracts (ASC). To look further into whether αV integrins play a role in the TGF-β associated fibrotic lens EMT, I utilized mice homozygous for a 12 nucleotide deletion in the βB2-crystallin gene (Crybb2$^{Phil/Phill}$), which have been previously demonstrated in our lab to undergo a juvenile lens epithelium EMT as early as four weeks postnatal, leading to ASC which is followed by the development of severe lens abnormalities. At 2 months age, Crybb2$^{Phil/Phil}$ demonstrates identical EMT characteristics as seen in wildtype mice at 48hrs post-surgery. Their lens epithelium becomes multilayered and upregulates αSMA, fibronectin, vitronectin, TGF-β induced protein and phosphorylated SMAD-3. Moreover, a SuperArray RT-PCR gene expression analysis on mRNA expression of TGF-β associated genes showed that, while all TGF-β ligands 1, 2 and 3 and the vast majority of the down-stream canonical TGF-β signaling associated players, including SMAD, 2, 3 or 4 were not upregulated in Crybb2$^{Phil/Phil}$ epithelium, TGF-β receptor II, TGF-βi and Follistatin which are genes known to upregulate in the aftermath of TGF-β signaling, were significantly upregulated. These findings suggested that the

Crybb2$^{Phil/Phil}$ epithelium was undergoing an extreme unregulation of TGF-β signaling which seem to be regulated mainly at the TGF-β activation level point. This was attested by an active TGF-β assay which confirmed that Crybb2$^{Phil/Phil}$ lenses had four times the amount of active TGF-β as compared to wildtype lenses. In addition, αV integrins were found to upregulate at both the mRNA and protein level in Crybb2$^{Phil/Phil}$ lens epithelium EMT similar to that seen in the lens post-surgery suggesting that βB2-crystallin may have a non-refractive function in maintaining lens epithelial integrity which will be a topic for future investigations.

Overall, while αV integrin only subtlety regulates the development and function of the lens, it is involved in regulating the phenotypic alterations in lens epithelial cells that occurs following lens injury/cataract surgery leading to PCO. This is the first study to examine the roles of αV integrins in lens development, refractive functions and the pathogenesis of PCO/ASC. These data suggest that therapeutics antagonizing αV integrin function could be used to prevent TGF-β fibrotic PCO following cataract surgery as well as ASC development.

Chapter 1

GENERAL INTRODUCTION

1.1 The Lens: Structure and Function

The mammalian lens, also known as the crystallin lens, is a transparent avascular tissue located between the aqueous and vitreous humors in the anterior half of the eye. The main function of the lens is to focus light onto the retina where further vision processing occurs. (Land 1988). It provides almost 33% of the eye's refractive power with the remaining 66% coming from the cornea (Hung 2001).

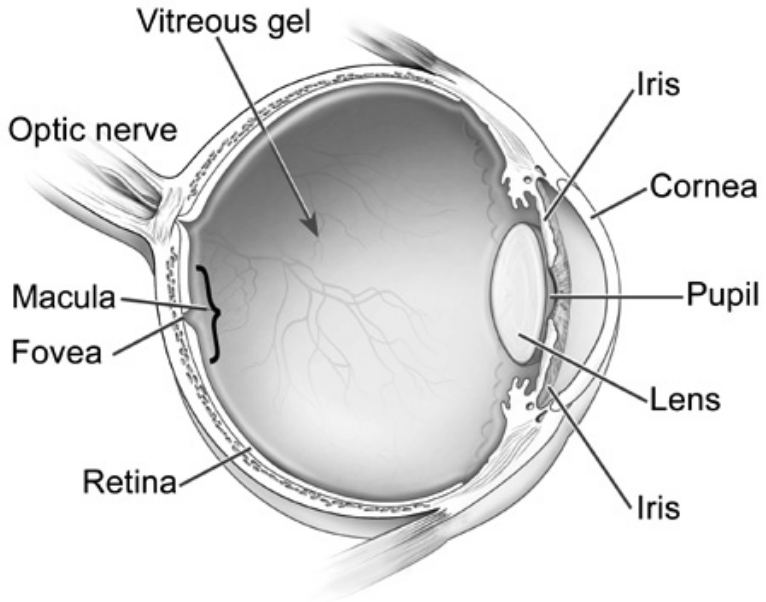


Figure 1.1: Eye Anatomy. Image adapted from NEI Catalog number NEA09

The entire lens is encapsulated by a basement membrane, also known as the lens capsule, which compartmentalizes lens cells from the rest of the eye (Danysh and Duncan 2009). The lens is composed of two types of cells, a monolayer of cuboidal lens epithelial cells (LECs) on the anterior face and concentric elongated layers of fiber cells that are formed continuously by the differentiation of the epithelium making up the vast majority of the lens. To further enhance transparency, fiber cells in the center of the lens degrade their nuclei and most of their organelles (Bassnett and Mataic 1997). Fiber cells contain high concentrations of water soluble crystallin proteins, with the lens' protein concentration twice that of other body tissues; about 30-35% w/w compared to an average of 15% w/w (Purves, Sadava et al. 2004, Hoehenwarter, Klose et al. 2006). This high concentration of crystallins provides the lens with a high refractive index while maintaining its transparency throughout life (deJong, Lubsen et al. 1994).

Most of the crystallins found in the mammalian lens belong two super families, α and β/γ crystallins (Andley 2007). They make up about 90% of the proteins found in the lens and are distinguished by their high intrinsic stability against stress and thermal stability, which is a hallmark sign of lifelong function (Donaldson, Kistler et al. 2001).

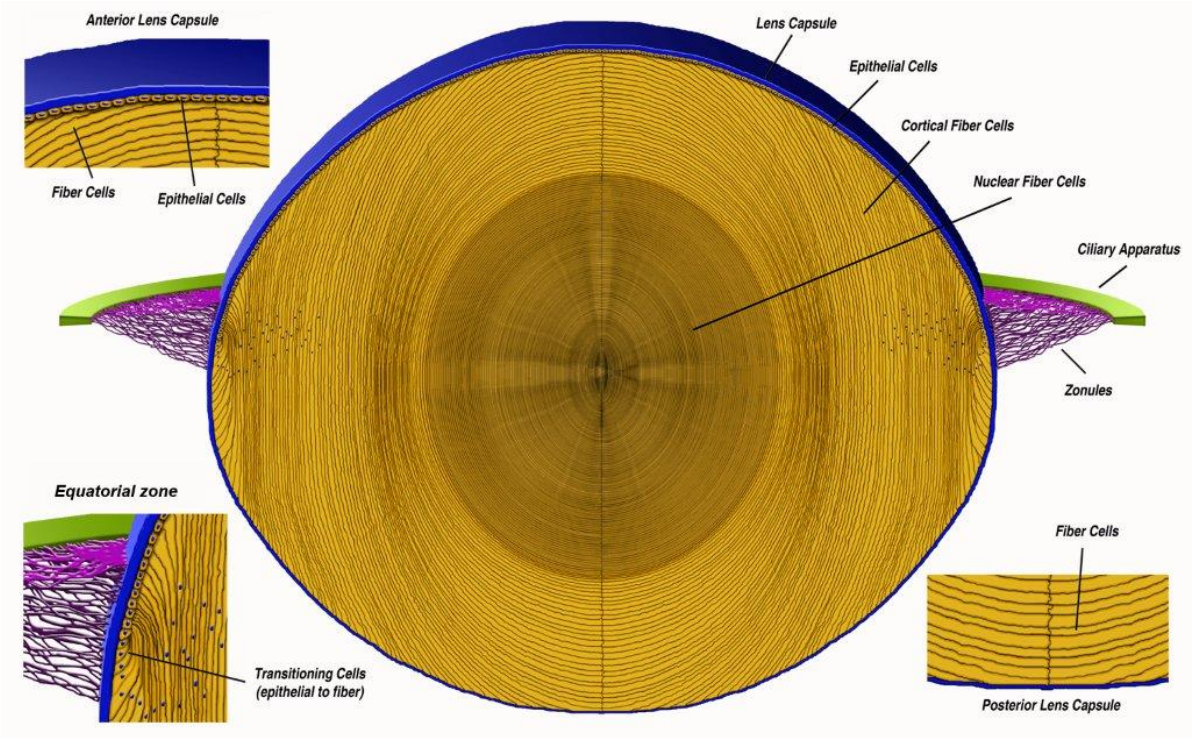


Figure 1.2: Lens anatomy showing it is an epithelial tissue. (Image adapted from Danysh and Duncan 2009)

1.2 Lenticular Crystallins

1.2.1 α-crystallins

α-crystallins are expressed in both lens epithelial and fiber cells. Structurally, they are often compared to small heat shock proteins and recent studies have come to an agreement that they function as chaperones in lens (Horwitz 2003, Bloemendal, de Jong et al. 2004). α-crystallin's chaperone activity (or that of its subunits) can suppress the aggregation of proteins denatured by oxidation, heat, and other stressors hence maintain lens transparency (Horwitz 1992). However, in order to maintain this chaperone activity, α-crystallins must remain dynamic throughout life, with its subunits

constantly disassociating and reassociating (Harding 2002). If this vital process is impaired, whether due to changes that are associated with aging or inherited genetic defect, it can impair lens transparency (Wistow, Slingsby et al. 1981, Oyster 1999). In addition to their chaperone activity, the α-crystallins also play central roles in conferring the lens' high refractive index due to their high concentration.

There are two α-crystallin genes, α-A, and α-B (Wistow, Slingsby et al. 1981). αA crystallin is found mainly in the lens, with trace amounts in other tissues, while αB is essentially considered to be a ubiquitous protein (Bhat and Nagineni 1989, Iwaki, Kume-Iwaki et al. 1990). Approximately two decades ago, several studies begin to show that α-crystallins are expressed in diverse tissues such as the spleen and the heart, suggesting important non-refractive functions for this protein (Srinivasan, Nagineni et al. 1992). Not long after, several investigations began highlighting α- crystallin activities' beyond the lens (Jaenicke and Slingsby 2001, Narberhaus 2002, Horwitz 2003). Ultimately, α-crystallins, which were for so many years thought to only play refractive roles in the lens, became recognized for their chaperone activity functions in lens (Horwitz 1992, Bloemendal, de Jong et al. 2004, Koteiche and McHaourab 2006). Concomitantly, there were also multiple reports of αB crystallin involvement in various non ocular activities, especially in neurological diseases such as Creutzfeldt-Jacob, Alzheimer's, Alexander's disease, Amyotrophic lateral sclerosis (ALS) and Parkinson's disease (van Rijk and Bloemendal 2000). Altogether, it is now well accepted that α-crystallins have important cellular functions outside of the lens.

1.2.2 β/γ-crystallins

The other members of the lenticular crystallins are the β and γ crystallin superfamily. They are characterized by the Greek key motif: four continuous anti-parallel β-strands (Slingsby and Clout 1999). They are distinguished by their βγ-crystallin fold which is a double domain structure containing a series of four highly stable "Greek key" motifs (Hutchinson and Thornton 1993). See (Figure 1.3). These Greek key motifs are super-secondary protein structural folds that are evolutionarily conserved from bacterial through vertebrate species that offers the crystallins structural compactness necessary for stability (Lapatto, Nalini et al. 1991, Wistow, Wyatt et al. 2005). Both β and γ crystallins share similar secondary structures, however, β-crystallins associate into hetero-and homotypic dimers and large oligomers in solution, while γ-crystallins remain as monomers (Sathish, Koteiche et al. 2004).

For the past two decades, there has been an evolution in the understanding β-crystallins' function in the mammalian lens (Slingsby, Wistow et al. 2013), some of which suggest non-refractive roles in lens epithelial cell differentiation, maintenance and survival (Boyle and Takemoto 2000, Xi, Bai et al. 2003, Wang, Garcia et al. 2004, Morozov and Wawrousek 2006). Recent studies are proposing more possible non-refractive roles for β-crystallins, particularly in the ocular stress response (Sakaguchi, Miyagi et al. 2003), lysosomal activities (Zigler, Zhang et al. 2011), anoikis, and even in extracellular signal-regulated kinase pathways (Ma, Sen et al. 2011).

More than one study found that β-crystallins upregulate dramatically in diverse retina damage conditions such as mechanical injury, light injury and even diabetic retinopathy (Sakaguchi, Miyagi et al. 2003, Vazquez-Chona, Song et al. 2004, Kumar, Haseeb et al. 2005). A very recent study done on rats showed that βA3/A1-crystallin, a crystallin belonging to the β-crystallin family, functions as a lysosomal protein in retinal pigmented epithelium (RPE), essential for degradation of phagocytosed material (Zigler, Zhang et al. 2011). Furthermore, βA3/A1-crystallin has also been shown to play a role in anoikis via the mediation of insulin-like growth factor-II, (PI3K)/AKT/mTOR and extracellular signal-regulated kinase pathways (Ma, Sen et al. 2011). However, despite speculation that the β-crystallins' might have non-refractive functions, the precise mechanisms of their functions in the lens beyond refraction, are not well understood.

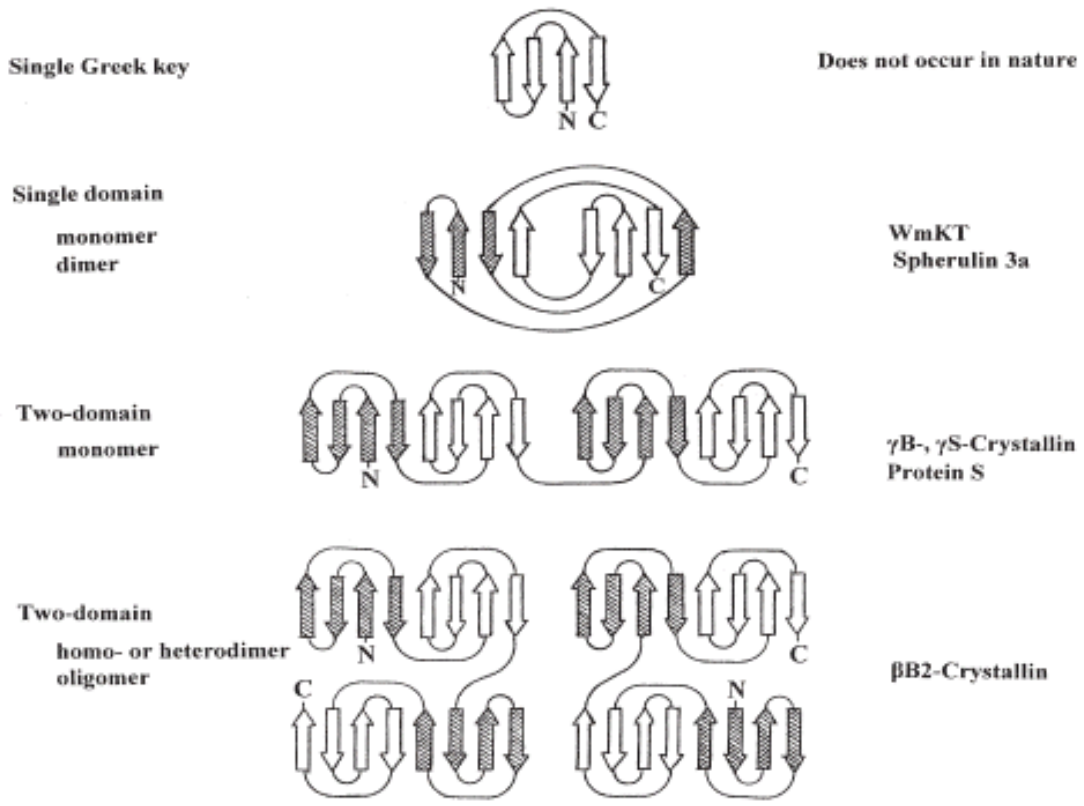


Figure 1.3: Schematic diagram of protein folds that involve Greek key domains (Jaenicke and Slingsby 2001).

1.2.3 βB2-crystallins

β-crystallins are primarily known for their refractive functions by providing the lens with a high protein concentration necessary for light refraction (Ueda, Duncan et al. 2002, Sathish, Koteiche et al. 2004). Six different β-crystallins exist in the lens and are classified as acidic or basic molecules resulting from dissimilarities between their N- and C-terminal regions. βB2-crystallin, a member of the basic group, can form large oligomeric complexes and it is the most abundant protein in adult human lens fiber cells (Slingsby and Clout 1999).

Despite the fact that β-crystallins are extremely resistant to protein modifications (Zhang, David et al. 2001), their N- and C-terminal extensions are vulnerable to post-translational modifications and oxidative damage; both of which can interfere with their correct folding, causing insolubility and protein aggregation that can impair lens transparency (Bloemendal, de Jong et al. 2004, Sathish, Koteiche et al. 2004). Further, studies have shown that proteolytic and oxidative damage to βB2-crystallins can disrupt folding patterns and specific protein-protein interactions, leading to the accumulation of light-scattering aggregates (Matsushima, David et al. 1997, Slingsby and Clout 1999).

Different studies have unanimously established that βB2-crystallin malfunctions whether due to genetic and non-genetic causes, lead to cataract development in both mice (Uga, Kador et al. 1980, Chambers and Russell 1991, Zhang, Li et al. 2008) and humans (Fujii, Kawaguchi et al. 2011, Wang, Wang et al. 2011). However, although this is widely accepted, the precise molecular mechanisms responsible for this pathology are not well known. Similar to other lenticular crystallins, non-refractive roles of ββ2-crystallin are also starting to emerge with multiple reports proposing βB2-crystallin's roles outside the eye. For example, studies on mice either lacking or mutant in the βB2-crystallin gene reported a reduced fertility due to disordered proliferation and apoptosis of germ cells in the testis (Duprey, Robinson et al. 2007, Xiang, Cui et al. 2012). Furthermore, βB2-crystallin upregulates in the axonal elongation of retinal ganglion cells during retinal regeneration (Bohm, Melkonyan et al. 2013), suggesting potential roles in neurodegenerative diseases (Liedtke, Schwamborn et al. 2007). Then again, the mechanisms behind these findings are not well understood.

1.3 **Cataract**

Cataract is a clouding of the ocular lens which is the most common cause of blindness in the world (Asbell, Dualan et al. 2005) with 43% of the 44.8 million blind suffering from cataract (WHO). There are several factors known to cause cataracts ranging from trauma to the lens (Call, Grogg et al. 2004), oxidative stress (Thiagarajan and 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-genetic defects that can occur during lens development (Firtina, Danysh et al. 2009, Yi, Yun et al. 2011) and simple aging (Hejtmancik and Kantorow 2004).

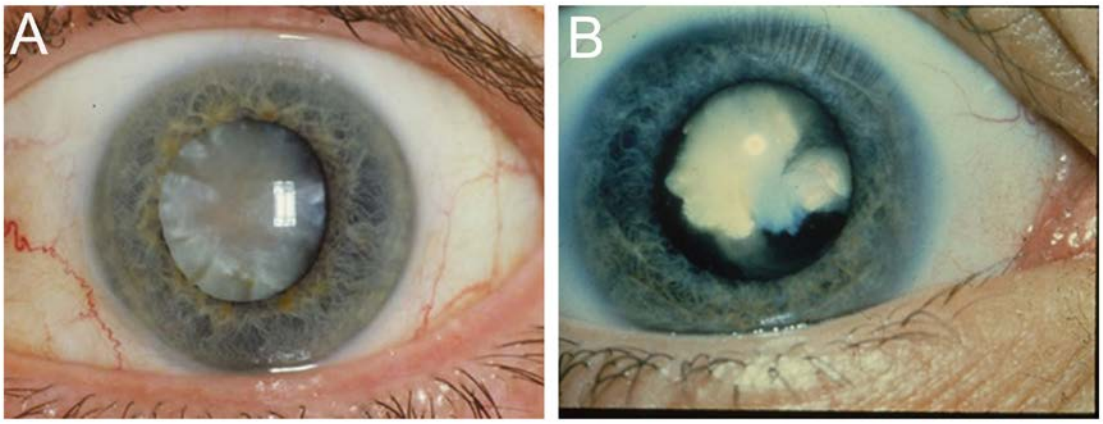


Figure 1.4: (**A**) Adult eye showing a hypermature age-related cortico-nuclear cataract with a brunescent (brown) nucleus. (**B**) Infant eye showing a white congenital cataract. Images adapted from http://www.nei.nih.gov/photo.

1.3.1 Congenital *vs.* age-related cataracts

Although treatable, a congenital cataract requires immediate treatment since infants who undergo late treatment may develop other visual impairments such as deprivational amblyopia which is a neurodevelopmental disorder of vision resulting from the brain not knowing how to perceive vision (Mansouri, Stacy et al. 2013). Congenital cataracts cause visual impairment and blindness during infancy with an estimated prevalence of up to 6 cases per 10,000 live births (Figure 1.4: B) (Santana and Waiswo 2011). Since the lens formation results from a series of inductive complex process, it is not surprisingly that up to fifty percent of all congenital cataracts are due to genetic causes (Santana and Waiswo 2011). Studies done on hereditary congenital cataract have led to the identification of several vital candidate genes that encode proteins such as crystallins, lens specific connexins, aquaporin-0, cytoskeletal structural proteins, and other developmental regulators that are essential for lens development (Yi, Yun et al. 2011). Since the transparency and high refractive index of the lens highly depend on the precise architecture of the fiber cells and the homeostasis of the lens proteins in terms of their concentration, stability, and organization, some of the findings from congenital cataract studies also aided in the identification of genetic modifiers that are responsible for age-related cataract (Figure 1.4: A). However, unlike congenital cataracts, age-related cataract also depends on environmental factors in addition to the identified genetic modifiers (Benedek 1997, Bloemendal, de Jong et al. 2004, Asbell, Dualan et al. 2005). Studies in age related cataract have shown that simple aging, Ultraviolet Radiation (UVR), injuries, lifestyles choices such as cigarette smoking and

disorders such as obesity, uncontrolled blood glucose levels, corticosteroid exposure, as well as alcohol consumption, can play a role in triggering age-related cataract onset (Hejtmancik and Kantorow 2004). Besides, inherited genetic abnormalities can still stand alone as risk factors for age-related cataract (Benedek 1997).

1.3.2 Lenticular crystallins and cataracts

As lens proteins, α-, β- and γ– crystallins undergo very little turnover, however, they do undergo a number of modification during aging such as truncation, oxidation, deamidation (Zhang, Smith et al. 2003), and disulfide bond formation (Hanson, Smith et al. 1998), all of which induce a high degree of crystallin proteolysis, fragmentation and aggregation leading to cataractogenesis (Bloemendal, de Jong et al. 2004). Since proper subunit interaction between crystallins is necessary to prevent the formation of light-scattering aggregates of crystallins (Delaye and Tardieu 1983), these modifications, whether induced by age-related changes or other mechanisms, may affect the short-range interactions among α-, β- and γ– crystallins. This increased homoaggregation of proteins can induce fluctuations in protein densities that are sufficient to cause increased light scattering which eventually leads to the development of cataracts (Delaye and Tardieu 1983, Takemoto and Sorensen 2008).

In addition, genetic and non genetic crystallin abnormalities play a major role in age-related cataract. For example, the loss of α-crystallin's chaperone activity due to simple aging plays a major role in human cataracts (Horwitz 1992, Rao, Huang et al. 1995). Notably, the lens has a refractive index gradient that increases as you move

towards the central part of the lens (the lens nucleus) (Donaldson, Kistler et al. 2001). This refractive index gradient is created continuously as the lens grows by tightly compacting fibers cell in the center of lens compared to those on the periphery. This results in the lens nucleus being the most water-insoluble part of the lens. Added to that, normally 50% of the lens proteins become water-insoluble at around the average age of 55yrs old (Wistow, Slingsby et al. 1981). Thus, this gradual insolubilization increases faster as we age, resulting in an extremely water-insoluble lens nucleus consequently leading to light-scattering properties and eventually cataracts (Benedek 1997, Hains and Truscott 2007, Harrington, Srivastava et al. 2007). Not surprisingly, mutations in either α-crystallin subunit, αA- and αB-crystallin alone, have been associated with a number of adult-onset cataracts (Brady, Garland et al. 1997, Andley, Hamilton et al. 2008, Hayes, Devlin et al. 2008, Xi, Bai et al. 2008).

βB2-crystallin is the major β-crystallin in the lens and is thought to play a major role in hetero-oligomer formation in the lens (Lampi, Ma et al. 1997). Since it is the least modified crystallin during aging (Zhang, David et al. 2001), βB2-crystallin is most soluble of the β-crystallins, remaining relatively more soluble during aging (Feng, Smith et al. 2000). In rats, βB2-crystallin is detected in the lens at birth but its expression increases dramatically after birth and peaks at about 2 months postnatally (Aarts, Lubsen et al. 1989), a similar expression pattern is seen in mice (Decker 2008). Since the vast majority of nuclear lens fibers cells are synthesized during embryonic stages, this explains why nuclear lens fibers contain less βB2-crystallin than the newly formed postnatal differentiated fiber cells at the periphery of the lens. Furthermore, this

phenomenon contributes to a more water-insoluble lens nucleus (Wistow, Slingsby et al. 1981). Since there is a tendency for other β-crystallins to precipitate when separated from βB2-crystallin, it is proposed that βB2 in maintains the solubility of other β-crystallins that are heavily modified during aging and is needed to maintain the solubility of beta-crystallin aggregates (Bateman and Slingsby 1992, Zhang, David et al. 2001).

Other investigations have proposed that βB2-crystallins' play a role in maintenance and calcium lens homeostasis by acting as a calcium buffer in the lens, actively protecting the lens from calcium-induced cataract, suggesting additional non refractive roles for the crystallin (Jobby and Sharma 2007). Not surprising, impairments of βB2-crystallins' structure are known to contribute to both age-related and inherited progressive cataract conditions (Chambers and Russell 1991, Graw, Klopp et al. 2001, Graw, Loster et al. 2001, Ueda, Duncan et al. 2002, Duprey, Robinson et al. 2007)..

1.3.3 Antioxidants and free radicals role in cataracts

The lens is constantly exposed to oxidative stress from reactive oxygen species (ROS) such as H_2O_2 and hypochlorous acid (HClO) and other xenobiotics; thus, it has to constantly protect its susceptible proteins from oxidation (Lou 2003). For example, the lens has the highest levels of reduced glutathione (GSH), a major antioxidant molecule, of all mammalian tissues (Giblin 2000). Studies have shown that GSH-deficient mice, which lack glutathione from the lens, undergo DNA strand fragmentation upon exposure to H_2O_2 (Kleiman and Spector 1993, Reddy, Lin et al.

1997). Unfortunately, the synthesis and recycling of GSH falls as we age, leading to a progressive loss of GSH and a rise in its oxidized form (GSSG) (Spector 1995). With the increasing levels of oxidative stress that occur during aging, lens proteins become thiolated by GSSG to form the mixed disulfides which may be further oxidized to form protein–protein disulphides (PSSPs) leading to an increase in the intramolecular disulfide bonding of lenticular crystallins during aging and eventually cataract formation (Takemoto 1996, Lou 2003).

On the other hand, both ultraviolet-A (UV-A) and UV-B radiation have been shown in many studies to be a major contributor in age-related cataract (Taylor, West et al. 1988, McCarty and Taylor 2002). UVR is a risk factor for retina damage in children as well as the cause of many other diseases, while exposure to intense ambient radiation can pose additional ocular hazards particularly to individuals who are over 40 years of age (Roberts 2011). This is due to the ability of UVR to catalyze the generation of ROS which can induce oxidative stress in the lens leading to disruption of lens fiber cells and finally cataracts (Wang, Lofgren et al. 2010).

Although it is well known that free radicals play a major role in age-related cataracts, the question to whether antioxidant supplementation will protect the lens against exogenous and endogenous ROS are still not conclusive (Varma, Hegde et al. 2008, Thiagarajan and Manikandan 2013). Thus, despite the current understanding of cataract pathogenesis, the complexity of the pathology, whether due to genetics or the current known and unknown risk factors, have led to a failure to prevent cataract despite numerous clinical trials over the past 30 years (Kinoshita, Fukushi et al. 1979). Thus,

the need for surgical procedures to treat cataract will continue to increase along with the aging population (Erie, Baratz et al. 2007).

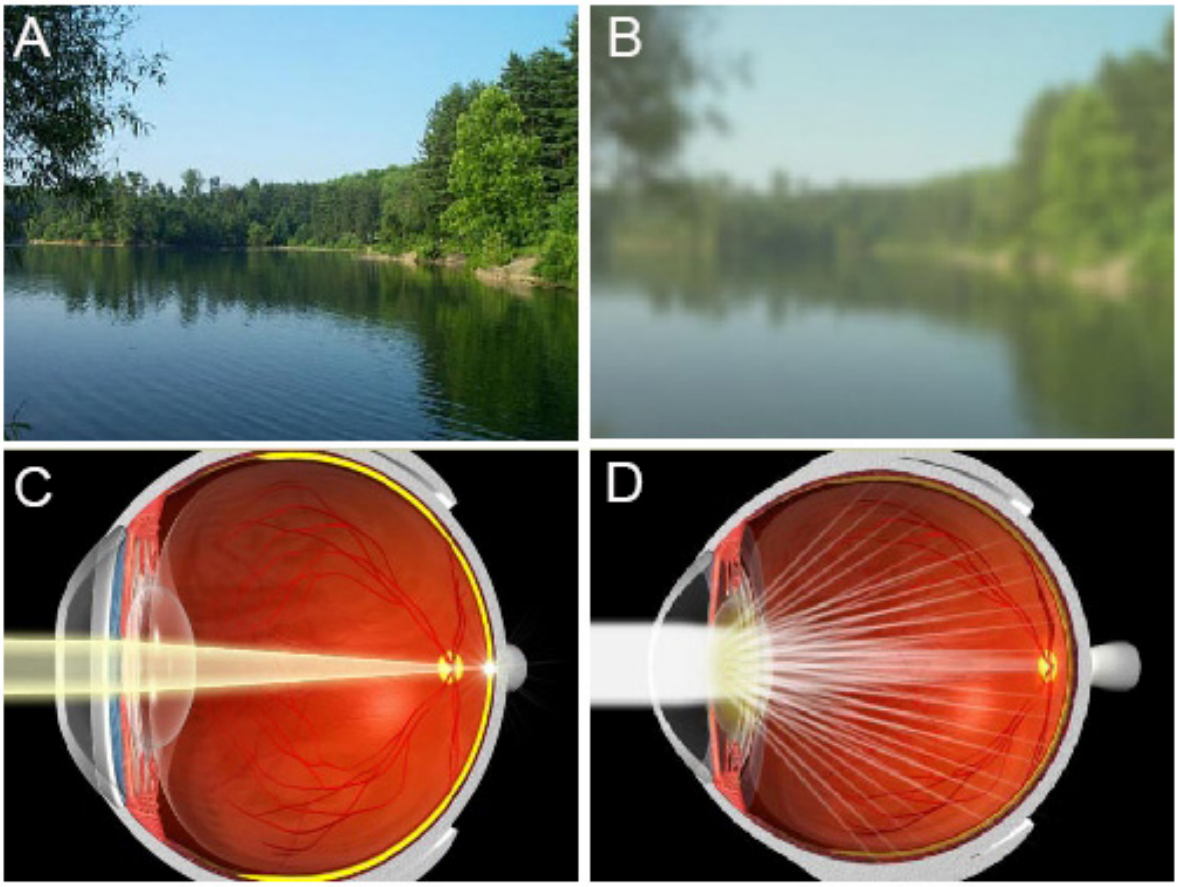


Figure 1.5: Light rays entering the eye are focused onto the retina to initiate clear vision (**A, C**). Cataract is associated with poor vision due to light scatter away from the focal point (**B**). Light rays entering the eye are scattered by the cloudy hardened lens (**D**). Image adapted from http://www.ynewyorkeyedoctor.com

1.4 Cataract Treatment

Cataracts are treated by an outpatient surgical procedure that involves extracapsular cataract extraction (ECCE) followed by implantation of an artificial intraocular lens (IOL) (Steinberg, Javitt et al. 1993, Laroche 2013). This procedure takes an average of 40 minutes to one hour, and is the most commonly performed outpatient surgery performed in the USA. Since its development in the late 1970s, ECCE with IOL implantation has successfully restored the vision of over 30 million cataract patients in the USA alone. It is projected that 30 million cataract surgeries per year will be done globally by the year 2020 (Foster 2001). However, this surgical volume means that even low complication rates have a significant societal impact, due to increased medical costs, aging population and other related visual disabilities expenses.

1.4.1 Extracapsular Lens Extraction

There are two main types of extracapsular lens extraction surgeries: Phacoemulsification and conventional extracapsular cataract extraction (ECCE) (Pershing and Kumar 2011). In phacoemulsification (Figure 1.6: A), which is the current standard of care, the surgeon begins the procedure by making a 2-3mm incision on the peripheral side of the cornea or sclera followed by a gentle tear of the lens capsule, a procedure referred to as capsulorhexis (Chercota 2005). Following the incision, the surgeon inserts a tip of a hand set piece device called a Phacoemulsifier, which vibrates at an ultrasonic frequency of around 40,000 Hz which is capable of breaking down the opacified lens into microscopic fragments (Hsu and Wu 2005). In addition, the

Phacoemulsifier device has a suction mechanism, which allows it to simultaneously emulsify and aspirate the resulting lens fragments through the incision while leaving behind an intact elastic lens capsule (Nishi 1988). Unlike phacoemulsification, ECCE method requires manual removal of the natural crystalline lens through a larger (10-12mm) insertion (Ohrloff 1990). Due to the large incision size, ECCE usually requires a cornea suture after completion and it now only used when phacoemulsification is a problem; for example in patients with a very hard cataract or in third world countries where Phacoemulsification is not feasible due to cost or unavailability of the equipment (Ohrloff 1990, Pershing and Kumar 2011). In both cataract treatments, refraction is restored by implantation of a foldable Intraocular Lens (IOL) (Figure 1.6: B) into the remaining capsular bag (Riaz, Mehta et al. 2006).

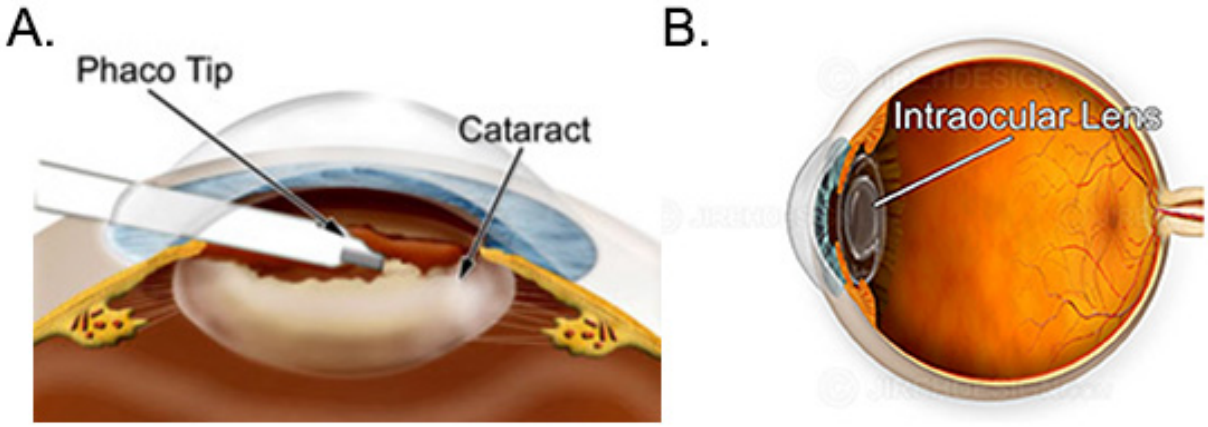


Figure 1.6: (**A**) Phacoemulsification procedure used during extracapsular lens extraction to remove a cataractous lens. (**B**) Completion of surgery showing the inserted IOL implant sitting at the position of the natural lens and surrounded by an intact elastic lens capsule. Images adopted from http://www.ocuclinic.com and http://www.jirehdesign.com.

1.4.2 Intraocular lenses

The technology involved in cataract treatment has evolved dramatically, especially the design of IOLs, which currently do not only restore vision at the time of surgery but also may reduce complications that may occur following surgery (Lichtinger and Rootman 2012). The Monofocal IOL is the most commonly used implant, with the IOL power chosen for optimum distance vision (Lindstrom 1993). Although this IOL is effective, it requires reading glasses for efficient near vision (Kirschfeld and Land 2011). To solve the problem, Multifocal IOLs have been designed however, the main problem with this IOL is the reduction in contrast sensitivity and formation of glare and

haloes due to the multiple concentric focusing powers within it (Haring, Dick et al. 2001, Chiam, Chan et al. 2006, Terwee, Weeber et al. 2008).

On the other hand, accommodating IOLs have also been designed and marketed (Findl and Leydolt 2007, Nishi, Mireskandari et al. 2009). For example, some of these IOLs have several unique features, including a hinge to allow its lens to move via the contraction of the ciliary muscle during accommodation, and hence restoring accommodating abilities (Strenk, Semmlow et al. 1999, Cumming, Colvard et al. 2006, Menapace, Findl et al. 2007). Although, in order to achieve these capabilities, the IOL must sit in a completely ideal position in the capsular bag and any slight differences in its position will impact its accommodating ability (Menapace, Findl et al. 2007).

Since most IOL designs are implanted within the lens basement membrane (lens capsule) to fix the IOL in the optical path; most of the lens capsule is left intact in the eye (Menapace, Findl et al. 2007). However, it is not possible to completely remove the lens epithelial cells (LECs) attached to the intact lens capsule, and these residual lens cells (LCs) undergo a wound healing response that includes cell proliferation, migration, and epithelial-mesenchymal transition (EMT) into migratory myofibroblasts, while others undergo lens fiber cell differentiation in an attempt to regenerate the lens (de Iongh, Wederell et al. 2005, Wormstone, Wang et al. 2009). If these LCs, which at this point of time are no longer phenotypically normal LECs, remain outside of the visual axis, a Soemmering's ring results, which can stabilize some of the current IOL designs within the eye (Werner, Tassignon et al. 2010). However, if they migrate along the lens

capsule into the visual axis, posterior capsular opacification (PCO) results, leading to impairment of vision (Awasthi, Guo et al. 2009).

1.5 The Problem: Posterior Capsular Opacification (PCO)

The most common negative outcome of cataract surgery is PCO or secondary cataract which is reported to occur following 3%-100% of cataract surgeries within months to 6 years post-surgery depending on numerous factors including patient age, surgical method, type of IOL implanted, and post-surgical follow up time (Apple, Escobar-Gomez et al. 2011). Substantial efforts have been made to prevent and treat PCO, leading to a reduction of its incidence shortly following cataract surgery (Dewey 2006). However, longer term, PCO, is still a major barrier to the long-term restoration of high acuity vision in cataract patients (Apple, Escobar-Gomez et al. 2011). In addition, PCO also stands as a barrier towards the successful use of the next generation accommodating IOLs. This is because PCO is generally associated with wrinkling/contraction of the posterior capsule as well as increases in cell aggregation on the peripheral capsule, which can substantially reposition the IOL or limit the movement of the postoperative capsule hence impairing the efficiency of accommodating IOLs (Kappelhof, Vrensen et al. 1987, McDonald, Croft et al. 2007, Wormstone, Wang et al. 2009).

Despite efforts to eradicate PCO, such as by designing improved IOLs that can trap migrating lens cells at the lens equator; majority of these attempts only reduce PCO but do not eradicate the problem (Bertelmann and Kojetinsky 2001). The current, most

successful means to alleviate PCO is by the quick outpatient procedure, Nd-YAG (neodymium-yttrium-aluminum-garnet) laser capsulotomy. This procedure uses a quick-pulsed Nd:YAG laser to apply a series of focal ablations in the posterior capsule, creating a small circular opening in the visual axis, hence reestablishing visual acuity (Aron-Rosa, Aron et al. 1980). In the USA, Nd-YAG laser capsulotomy is the seventh most common outpatient surgery performed on the elderly (www.cms.hhs.gov/Medicare). Unfortunately, Nd-YAG laser capsulotomy can cause damage to intraocular lenses (Newland, McDermott et al. 1999) or IOL dislocation (Framme, Hoerauf et al. 1998). Moreover, up to 2.5 % of patients who undergo Nd-YAG laser capsulotomy develop cystoid macular edema (Bath and Fankhauser 1986), up to 2.0% develop retinal detachment (Ranta, Tommila et al. 2000), and about 0.2% develop a new onset of glaucoma (Bath and Fankhauser 1986). Moreover, retinal detachment and cystoid macular edema often develops several months after the capsulotomy procedure, therefore requiring ongoing medical observation to detect and treat these serious complications (Steinert R.F., Puliafito C.A. et al. 1991, Steinberg, Javitt et al. 1993). Overall, Nd-YAG laser capsulotomy restores patient's vision it does not eradicate the problem (Gillies, Brian et al. 1998).

Thus, understanding the molecular mechanisms mediating lens epithelial cell responses to cataract surgery leading to PCO is important to understand the mechanism behind ASC, improve visual outcomes and retaining accommodation following cataract surgery or other surgical treatments that require lens extraction and replacement (Dewey 2006).

1.6 Epithelial Mesenchymal Transition

Epithelial-mesenchymal transition (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. Concomitantly, these cells develop a fibroblastic morphology by rearranging their actin cytoskeleton, become migratory by forming filopodia and lamellopodia, interact with stromal extracellular matrices (ECM) due to changes in cell surface matrix receptors such as integrins, begin direct synthesis of stromal ECM and become contractile myofibroblasts (Hay 1995, Kalluri and Neilson 2003, Thiery JP, Acloque H et al. 2009). However, due to its functional diversity and complexity, scientists have long disagreed on its clear definition and thus have now classified EMT into three different types: Type-1, Type-2 and Type-3 (Kalluri and Weinberg 2009, Zeisberg and Neilson 2009). Type-1 EMT occurs during the earliest stages of development, for example, during implantation and embryogenesis. Type-2 EMT is defined as that which occurs in more mature epithelial tissues. In contrast to Type-1 EMT, Type-2 EMT can be triggered by inflammation or wound-healing responses and may lead to fibrosis. Finally, Type-3 EMT is the loss of epithelial and the gain of mesenchymal characters associated with cancer progression and metastasis. Thus, lens EMT that occurs either post-surgery or after ocular injury is a Type-2 EMT, resulting from a wound healing response that involves myofibroblast transdifferentiation, increased proliferation, and migration of residual LECs (Marcantonio and Vrensen 1999, de Iongh, Wederell et al. 2005).

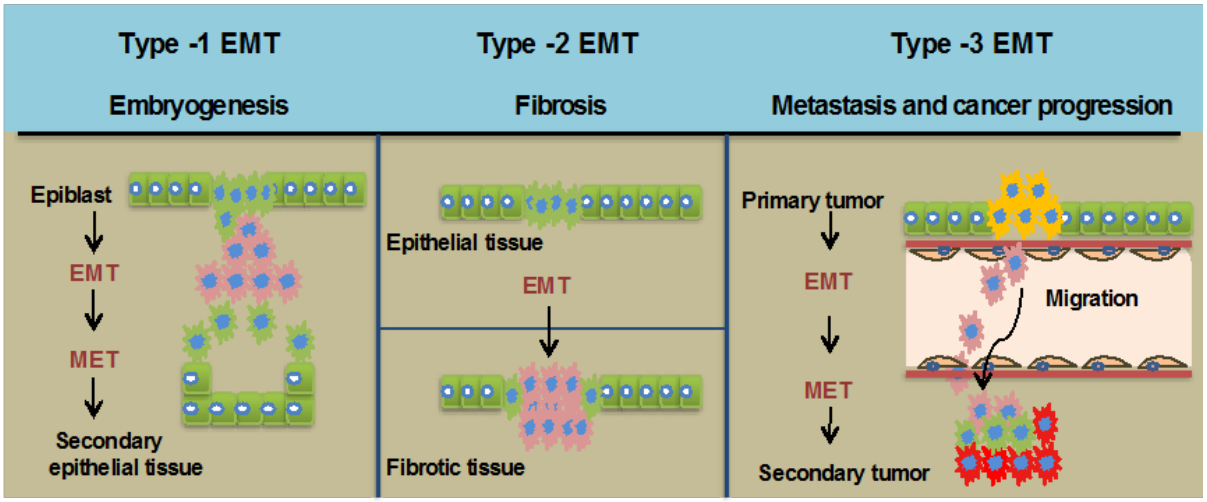


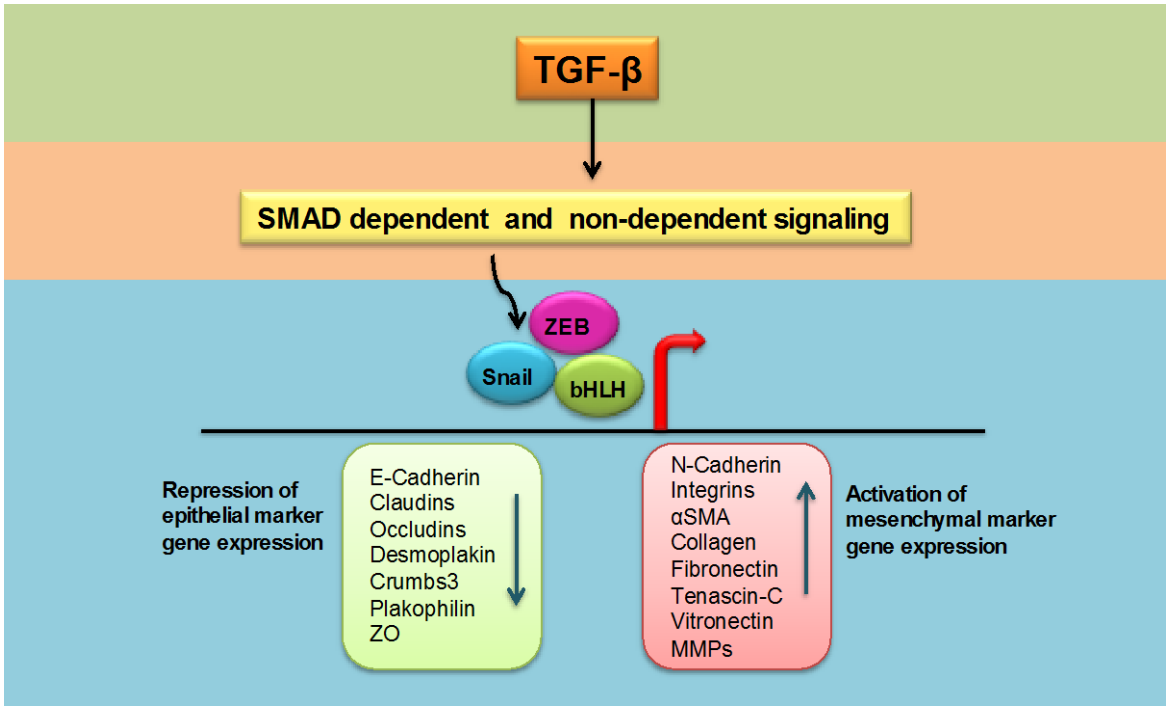
Figure 1.7: Epithelial mesenchymal transition (EMT) occurs when epithelial cells lose their epithelial cell characteristics and become mesenchymal. Mesenchymal cells can return to an epithelial phenotype, a process called mesenchymal-epithelial transition (MET). Type-1 EMT: During embryogenesis, the primitive epithelium (the epiblast) undergoes EMT forming primary mesenchyme that can migrate and undergo MET to form secondary epithelia that differentiate into new epithelial tissues. Type-2 EMT: In mature or adult tissues, epithelial cells can also undergo EMT following stress, inflammation or wounding but fail to undergo MET leading to fibroblast production and finally fibrosis. Type-3 EMT: Epithelial cancer cells can undergo EMT to acquire a more migratory mesenchymal phenotype that allows them to invade secondary epithelia and proliferate as secondary tumors. *Green (epithelial cells), Pink (mesenchymal), Yellow (primary tumor, Red (secondary tumor).* Adapted from (Mamuya and Duncan 2012)

1.7 Transforming Growth Factor-beta Induced EMT

Evidence suggests that Transforming Growth Factor-beta (TGF-β) can drive all three classes of EMT (Bhowmick, Ghiassi et al. 2001, Strutz, Zeisberg et al. 2002, Nawshad, LaGamba et al. 2005). Further, TGF-β also regulates a wide array of other cellular processes including cell division, differentiation, motility, apoptosis and tumor suppression (Taipale, J. Saharinen et al. 1998, Yue and Mulder 2001). There are three known isoforms of TGF-β in mammals, TGF-β1, TGF-β2 and TGF-β3 (Derynck, Lindquist et al. 1988). The expression and function of these three isoforms varies dramatically among tissues and can also vary from species to species (Massague, Cheifetz et al. 1992, Massagué 2008); this makes the mechanism by which TGF-β function is regulated and its signals transmitted within cells very complex. TGF-β is synthesized within the secretory pathway as a precursor molecule, which is cleaved to form the functional 25kDa TGF-β homodimer and the latency-associated peptide (LAP). While in the secretory pathway, LAP and TGF-β homodimer remain associated forming a non-covalent complex known as the small latent complex (SLC). The SLC remains in the secretory pathway until it is bound by Latent TGF-β-Binding Protein (LTBP) to form the Large Latent Complex (LLC). The LLC is secreted and bound either covalently or non-covalently to the ECM (Nunes, Gleizes et al. 1997, Chen, Sivakumar et al. 2007). In most cases, the LLC will remain in the ECM until it is further processed to release active TGF-β ligand (Annes, Munger et al. 2003). This process is referred to as TGF-β activation. Upon its release, the active TGF-β cytokine ignites TGF-β signaling by first binding to the type II TGF-β receptor, inducing a conformational

change in its serine/threonine kinase domain. The type II TGF-β receptor recruits the type I TGF-β receptor to form the active receptor complex. Once the receptor complex is formed, the type II receptor kinase phosphorylates multiple serine and threonine residues in the TTSGSGSG sequence of the cytoplasmic GS region of the type I receptor, leading to the activation of TGF-β induced signaling (Shi and Massagué 2003).

Figure 1.8: TGF-β signaling can instigate a TGF-β induced EMT via both



canonical (SMAD dependent) and non-canonical (SMAD independent pathway by repressing epithelial makers gene expression while activation mesenchymal marker gene expression. Adapted from (Mamuya and Duncan 2012)

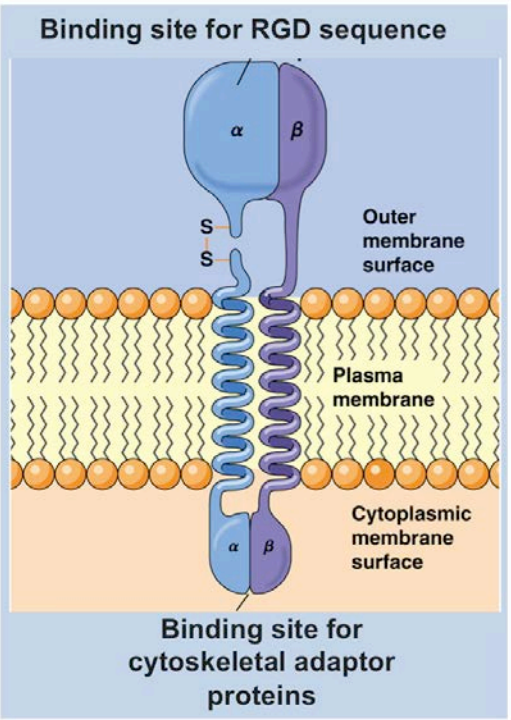
The best studied pathway mediating TGF-β function is the SMAD dependent (canonical) pathway which initiates when receptor-regulated SMAD2 and/or 3 (r-SMAD2/3) is recruited to the activated TGF-β receptor by the SMAD anchor for receptor activation (SARA) (Wu, Chen et al. 2000). The type I TGF-β receptor then phosphorylates r-SMAD2/3 inducing a conformational changes in the MH2 domain of r-SMAD2/3 and its subsequent dissociation from the receptor complex. Once phosphorylated and released, the phosphorylated r-SMAD2/3 attains a high affinity towards the co-SMAD (SMAD4) and binds to it (Souchelnytskyi, Rönnstrand et al. 2001). The complex formed by r-SMAD2/3 and co-SMAD4 translocates to the nucleus where, in the case of EMT, it represses epithelial gene transcription while transcriptionally activating the expression of mesenchymal genes as well as other transcription factors capable of regulating EMT, such as members of Snail, ZEB, and bHLH families (Peinado, Olmeda et al. 2007, Massagué 2008). This canonical pathway can also induces EMT via cross talk with other signaling pathways. For instance, SMAD-mediated TGF-β signaling can activate integrin linked kinase (ILK) which allows ILK to phosphorylate GSK-3β and Akt, leading to β-catenin nuclear translocation and activation of other transcription factors, resulting in EMT of renal tubular epithelial cells (Delcommenne, Tan et al. 1998, Li, Tan et al. 2009). TGF-β can also mediate EMT through its non-canonical (SMAD independent) pathways (Derynck and Zhang 2003). For instance, TGF-β induced activation of Erk/MAP kinase, Rho GTPase and the PI3 kinase/Akt pathways can result in all three EMT types (Zavadil, Bitzer et al. 2001, Xu, Lamouille et al. 2009).

Not surprisingly, more non-canonical pathways and additional levels of TGF-β regulation are still being discovered (Zhang 2009). Recently it was shown that TGF-β signaling can regulate the expression of microRNAs which play a crucial role in regulating EMT (Zavadil, Narasimhan et al. 2007). MicroRNAs belonging to the miR-200 and miR-205 family can prevent EMT by downregulating the EMT associated transcription factors ZEB1 and SIP1 (Gregory, Bert et al. 2008). Since miR-200 and miR-205 expression is reduced upon TGF-β stimulation, this suggests that TGF-β is a key regulator of the expression of microRNAs that block EMT (Pandit, Corcoran et al. 2010). It is likely that other microRNAs either synergize or antagonize TGF-β signaling during TGF-β induced EMT. Thus, investigating different pathways of how TGF-β signaling is regulated will help understanding the mechanism of lens EMT and eventual development of PCO therapeutics.

1.8 Integrins and Their Role in EMT

Integrins are heterodimeric extracellular matrix (ECM) receptors consisting of one α- and one β-integrin subunit, which play major roles in cell proliferation, adhesion and migration of numerous cell types (Hynes 1992, Hynes 2002). Interactions between cells and ECM are necessary to convey micro-environmental cues essential for regulating cell behavior and function (Slavkin 1982, Lukashev and Werb 1998). Integrins play this role by linking the cytoskeleton to the ECM components influencing a number of cell signaling cascades ranging from cell proliferation, differentiation, adhesion to cell migration. They are encoded by 18 α-subunit and 8 β-subunit genes that

can only function as paired α-β heterodimers. There are at least 24 different functional integrin heterodimers (Hynes 1992, Hynes 1996, Hynes 2002). These heterodimers are specific in function making integrins one of the most diverse types of cell receptors (Hynes 2002). Not surprisingly, with such a complex repertoire, integrins can recognize diverse components of the ECM including laminin, collagen, heparan sulfate proteoglycans, vitronectin, fibronectin, osteopontin, bone sialoprotein, thrombospondin, fibrinogen, von Willebrand factor and tenascin (Ruoslahti 1996, Desgrosellier and Cheresh 2010).



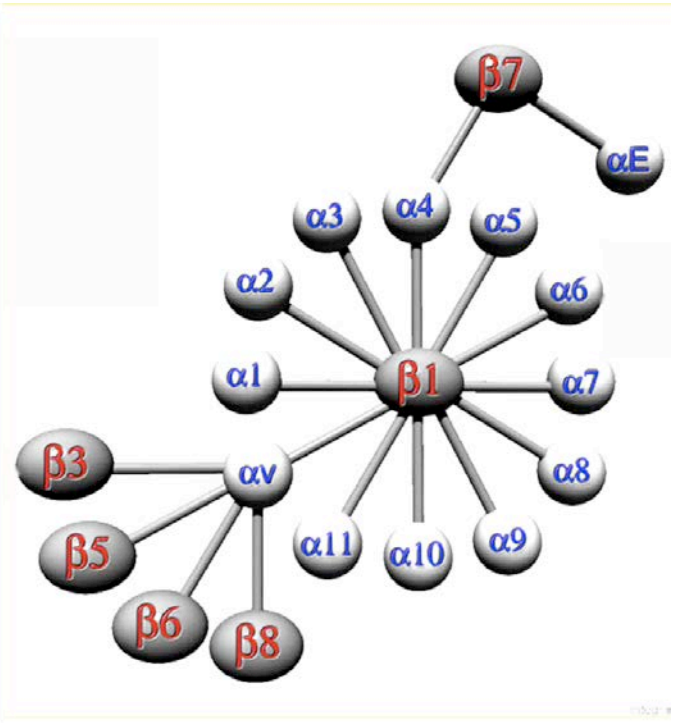


Figure 1.9: (**Right**) A schematic diagram showing a α- and β-integrin subunit interaction forming a non-covalently bound transmembrane receptor heterodimer. (**Left**) A schematic diagram showing α- and β-integrin subunit dimerization preferences (Images adapted from Pearson Education, Inc. copyright © 2009).

Intracellular cues resulting from different cell signaling pathways can activate integrin associated proteins affecting their affinity to extracellular ligands, a process known as inside-out signaling (Anthis and Campbell 2011). Changes in ECM composition, such as the increased expression of fibronectin, vimentin, vitronectin or collagen seen during EMT, can also activate integrins by increasing their ligand binding affinity state (Boudreau and Jones 1999, Imamichi and Menke 2007). In result, this can induce an outside–in integrin signaling, mediated via adaptor proteins such as ILK, paxillin, FAK and PINCH which bind to the cytoplasmic tail of integrins (Giancotti and Ruoslahti 1999). Thus, in the case of EMT, ECM microenvironment perturbations or intracellular cues can persuade integrins to dictate adhesion changes between cells and the ECM or cells to cells which in favorable conditions can induce the disassembly of tight and adherens junctions, dissolution of desmosomes, actin reorganization and loss of epithelial apical-basal polarity leading to EMT. In extremis, these changes can initiate focal adhesion complex formation leading to cell migration and invasion (Yilmaz and Christofori 2009). Further, integrins can also facilitate changes in cell-ECM contacts during EMT by the co-localizing proteases such as membrane type matrix metalloproteinase (MMPs) (Gonzalo, Moreno et al. 2010). This ability of integrins to

simultaneously bind ECM proteins and recruit MMPs is essential in the activation of latent TGF-β, a cytokine that not only executes EMT but also regulates the transcription of genes that encode numerous integrin subunits; this is discussed in the next section.

1.9 Activation of Transforming Growth Factor-beta by Alpha V Integrins

TGF-β is secreted in a latent form that must be activated extracellularly to efficiently trigger receptor-mediated TGF-β signaling. Diverse activation mechanisms have been demonstrated which allow for regulation of TGF-β function in different cellular/tissue contexts. (Miyazono and Heldin 1991). Among the three TGF-β isoforms, only the LAPs of TGF-β1 and TGF-β3 contain an integrin-binding motif, arginine–glycine–aspartic acid (RGD). Notably, many αV integrins including $\alpha_V\beta1$, $\alpha_V\beta3$, $\alpha_V\beta5$, $\alpha_V\beta6$ and $\alpha_V\beta8$ can interact with this RGD sequence resulting in activation of TGF-β1 and TGF-β3 (Munger, Huang et al. 1999, Annes, Rifkin et al. 2002, Mu, Cambier et al. 2002, Ludbrook, Barry et al. 2003, Annes, Chen et al. 2004). Not surprisingly, mice with a nonfunctional variant of the RGD sequence in their TGF-β1 LAP express normal levels of latent TGF-β1, but display features similar to that of TGF-β1 knockouts (Munger, Huang et al. 1999, Yang, Mu et al. 2007). Moreover, mice lacking $\alpha_V\beta6$ and $\alpha_V\beta8$, phenocopy TGF-β1 and TGF-β3 knockouts confirming the importance of αV integrins in TGF-β1 and TGF-β3 activation (Aluwihare, Mu et al. 2009). Furthermore, antibody-mediated blockade of αV integrin function, particularly $\alpha_V\beta6$, downregulates the TGF-β induced EMT and inflammation known to causes fibrosis, metastasis and cancer (Bates, Bellovin et al. 2005, Wang, Dolinski et al. 2007,

Horan, Wood et al. 2008, Koopman Van Aarsen, Leone et al. 2008). Two different models of how αV integrins can activate TGF-β are proposed, both of which have significant experimental support. It appears that the choice of TGF-β activation mechanism is cell type specific and influenced by whether the appropriate integrin is expressed and the specific cell physiological context. However, it is not known whether the use of a particular TGF-β activating mechanism influences the resulting TGF-β signaling.

1.9.1 Conformational change activation mechanism:

Upon binding to the LAP of TGF-β1 and TGF-β3, αV integrins can exert adhesion-mediated cell forces inducing conformational changes of the LAP, which results in structural deformation of the latent complex and liberation of active TGF-β (Munger, Huang et al. 1999). Drugs that block myofibroblast contraction such as ML-7, blebbistatin, cytochalasin D and the *α*-SMA contraction inhibitor SMA-FP, also inhibited activation of TGF-β1 by contraction-inducing drugs (Hinz, Gabbiani et al. 2002, Wipff, Rifkin et al. 2007). Analogously, studies that inhibited $\alpha_V\beta5$ integrin function by forming αvβ5 -Thy1 complexes also inhibit contraction-induced latent TGF-β1 activation (Zhou, Hagood et al. 2010). Since TGF-β signaling induces a contractile cytoskeleton along with *α*-SMA expression during EMT, this can result in further TGF-β activation (Kurosaka, Kato et al. 1995). While this direct activation does not require proteolysis of either the LAP or LTBP, it does require a mechanically stiff

ECM and contractile cytoskeleton, which provides sufficient forces to liberate TGF-β from its latent complex (Annes, Munger et al. 2003, Wipff and Hinz 2008).

1.9.2 Conformational change activation mechanism with proteolysis:

Not all integrin interactions with the LAP directly activate TGF-β, particularly in the absence of a stiff ECM. Moreover, active TGF-β must be physically proximal to a type II TGF-β receptor for activation of TGF-β signaling. Notably, several MMPs such MMP-9 and MMP-2 can proteolytically cleave the LAP and/or LTBP to liberate TGF-β from its ECM bound stores (Yu and Stamenkovic 2000, Dallas, Rosser et al. 2002). Further, αV integrins can interact with MMPs, such as MMP2 and MMP9, to tether them to the cell surface (Brooks, Stromblad et al. 1996, Rolli, Fransvea et al. 2003). Thus, in certain physiological contexts, αV integrins can simultaneously promote proximity of MMPs to the LAP and sequester the LLC close to the type II TGF-β receptor. Depending on the cell/tissue type, this mechanism can function without significant cell traction (Yu and Stamenkovic 2000, Wipff and Hinz 2008), although a contractile cytoskeleton and a mechanically resistant ECM can also participate (Wipff, Rifkin et al. 2007, Wipff and Hinz 2008).

1.10 **Overall**

Upon cataract surgery, lens epithelial cells (LECs) that left behind in the capsular bag will perceive the surgery as a wound, inducing a wound-healing response

which drives these cell into a migratory myofibroblast or lens fiber cell regeneration phenotype associated with, deposition of mesenchymal proteins and cell migration across the posterior capsule (Cobo, Ohsawa et al. 1984, Zhu 1990).

It is known that TGF-β plays a central role in the cell biology of PCO. However, as elaborated earlier on this chapter, TGF-β is a multifunctional growth factor with a wide range of opposing effects on cellular processes (Massague, Cheifetz et al. 1992). Thus the precise mechanism of how it regulates PCO is not well understood. Similarly, TGF-β signaling has also been associated with Anterior Subcapsular Cataract (ASC) (Hales, Chamberlain et al. 1995, Hales, Chamberlain et al. 1997). ASC is a condition that results from aberrant growth and differentiation of LECs to form fibrotic plaques that obscure vision (Pau, Novotny et al. 1985). Unlike PCO, ASC occurs following ocular trauma, from unrelated eye surgery, or can result from diseases such as atopic dermatitis (Novotny and Pau 1984, Sasaki, Kojima et al. 1998). However, in ASC, the lens epithelial cells undergo similar morphological and molecular changes to those occurring during PCO (Ishibashi, Hatae et al. 1994, Saika, Kawashima et al. 1998). Furthermore, in *vitro* and in *vivo* rodent cataract models used to study ASC are also useful for investigating the molecular and cellular basis of PCO and vise versa (de Iongh, Wederell et al. 2005, Martinez and de Iongh 2010).

There has been speculation that integrins play a role in PCO and several studies have reported integrin overexpression as LECs undergo EMT. Further these studies also showed that integrin antagonists can attenuate lens EMT (Zuk and Hay 1994, Sponer, Pieh et al. 2005, Walker and Menko 2009). However, the identity of the

particular integrins involved and their function still remains uncertain. αV integrins can activate TGF-β and trigger a TGF-β induced EMT, which in return can upregulate more αV integrin gene expression as well as creating a mesenchymal microenvironment ideal for further unregulated TGF-β activation resulting in pathological TGF-β induced EMT (Mamuya and Duncan 2012). Since therapeutics targeting αV integrins have been developed and are in clinical trials for other diseases (Nemeth, Nakada et al. 2007), further understanding of these unknown complex mechanisms it executes will be essential in developing therapeutics for lens EMT and other associated TGF-β-EMT pathologies.

Chapter 2

MATERIALS AND METHODS

2.1 Animals

All the experiments conducted 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. All mice were bred and maintained under pathogen free conditions at the University of Delaware animal facility under a 14/10-hour light/dark cycle.

2.1.1 αV integrin conditional knockout mice

C57BL/6 mice carrying an αV integrin allele with loxP sites flanking exon 4 ($\alpha V^{[+/flox]}$) (McCarty, Lacy-Hulbert et al. 2005) were obtained from Adam Lacy-Hulbert (Harvard School of Medicine, Boston, MA). FVB/N mice expressing *cre* recombinase in all lens cells from the lens vesicle stage onward (MLR10-*cre*) (Zhao et al., 2004) were obtained from Michael L. Robinson (Miami University, Oxford, OH) and backcrossed 10 generations to C57BL/6<har> mice to create a congenic line. Homozygous male FVB/N mice carrying a *cre* recombinase gene under the control of the adenovirus EIIA-promoter, *EIIa-cre/EIIa-cre* (TgN(EIIa-Cre)C5379Lmgd) (Lakso, Pichel et al. 1996), were obtained from The Jackson Laboratories, Bar Harbor, Maine. Heterozygous αV integrin flox mice ($\alpha V^{[flox/+]}$) were mated to *EIIa-cre/EIIa-cre* mice to generate mice carrying a germline αV integrin null allele $\alpha V^{[-/+]}$. These animals were mated to $\alpha V^{[flox/flox]}$ to generate $\alpha V^{[-/flox]}$ mice. $\alpha V^{[-/flox]}$ were mated to MLR10-*cre* mice

to generate mice lacking αV integrin in their entire lens αV $^{[-/flox]}$; MLR10-*cre* (αVMLR10). All control mice used in the α_V integrin study are αV $^{[flox/flox]}$ that lack a *cre*-recombinase expressing transgene. All embryos were staged based on E0.5 being the day that a vaginal plug was found in the dam.

2.1.2 Mutated βB2-crystallin mice

Mice homozygous for the mutated βB2-crystallin gene STOCK Crybb2^{Phil} (Phy/Phy) mice on a Swiss Webster derived background were obtained from the National Institutes of Health, Animal Genetics Resource, Veterinary Resources Program (Bethesda, MD). Swiss-Webster and C57Bl/6NHsd mice were acquired from Harlan-Sprague Dawley (Indianapolis, IN). The Crybb2^{Phil} allele was moved onto the C57Bl/6NHsd genetic background by over 10 generations of backcrossing to establish mice carrying βB2-crystallin mutation in a C57Bl/6NHsd genetic background Crybb2$^{Phil/Phil}$ (homozygous) and Crybb2$^{+/Phil}$ (heterozygous).

2.2 DNA Isolation

DNA was isolated from tail snips or whole lenses using PureGene Tissue and Mouse Tail kit (Gentra Systems, Minneapolis, MN). Briefly, 0.5cm length of mouse tail or one whole mouse lens was immersed in a microfuge tube containing 600µl of PureGene cell lysis solution cocktail containing Ethylenediaminetetraacetic Acid (EDTA), Tris[hydroxymethyl]aminomethane (Tris), Sodium Dodecyl Sulfate (SDS) and 5µl of 20mg/ml Proteinase K solution (Life Technologies, Grand Island, NY).

The microcentrifuge tube was inverted several times and incubated at 55°C overnight (or for two nights if isolating DNA from the lens) in a gentle shaking water bath. After incubation, 200µl of protein precipitation solution [ammonium acetate] was added to the cell lysate solution and vortexed at high speed for about 20 seconds followed by 6 minutes of centrifugation at 14,000 rpm. The supernatant containing the DNA was separated from the precipitated protein pellet and poured into a microcentrifuge tube containing 600µl of 100% isopropanol, mixed by inverting 25 times, and centrifuging at 14,000 rpm for 1 minute. The supernatant was carefully discarded and 600µl of 70% ethanol was added to the microfuge tube containing the DNA pellet, washed by inverting the tube several times followed by a 1 minute centrifugation at 14,000 rpm. The ethanol was carefully discarded without disturbing the pelleted DNA. The microcentrifuge tube containing the pelleted DNA was left open to air dry in room temperature for 2 hours or till completely dry. Once dry, the pelleted DNA was rehydrated with 100µl of pureGene DNA hydration solution or by nuclease free molecular grade water and incubated overnight at room temperature. Hydrated DNA was stored temporarily at 4°C or at -20°C for storage longer than a week.

2.3 PCR and Genotyping

Polymerase Chain Reaction (PCR) was performed using a Taq DNA Polymerase Kit (QIAGEN Inc., Valencia CA). Briefly, the isolated genomic DNA was quantified in ng/µl using the Nanodrop (ND-1000 spectrophotometer) (Thermo Fisher) and diluted

down to about a 100-150ng/µl final concentration. DNA was stored at 4°C or PCR was carried out as follow. A 25µl PCR mix cocktail containing 1µl of the 100-150ng/µl DNA, 1µl of each desired primer (Table 2.1), 10µl of *Taq* PCR Master Mix and 7µl of water was added to a 0.2ml PCR tubes and mixed gently by pipetting while kept on ice at all times. PCR was carried out with an Eppendorf Mastercycler® PCR Cycler – (model #5333 000.018). The cycling conditions for all genotyping primers used were 5 minutes at 95 °C, 30 seconds at 94 °C, 58 °C for 45 seconds, 72 °C for 90 seconds, repeat the cycle 33 times followed by 72 °C for 10 minutes and hold at 4°C. PCR product bands were analyzed by 2.5% agarose gel electrophoresis with ethidium bromide concentration of 0.5µg/ml ran at 150V for 1.25 hours and examined under UV transilluminator. For the βB2-crystallin mutants, the 120-nucleotide product of the Crybb2^{Phil} allele and 132-nucleotide product of the wildtype allele was resolved by electrophoresis on a 10% polyacrylamide gel ran at 150V for 2 hours or until the gel front reached the bottom of the gel.

2.4 RNA Isolation

RNA for reverse-transcription PCR was isolated from either two whole frozen lenses or least 5-pooled capsule bags collected at different times post-surgery using the SV Total RNA Isolation System (Promega, Madison, WI). Due to their smaller size, four or six whole lenses were used for 2-month or 4-month-old Crybb2$^{Phil/Phil}$ respectively. Briefly, fresh or frozen lenses stored at -80°C were immediately submerged in 175µl of RNA Lysis buffer and the mixture was

homogenized. 350µl of SV RNA Dilution Buffer was added to the lysate, mixed by inversion and followed by incubation in a 70°C water bath for exactly 3 minutes. The lysate was centrifuged at 13000 rpm for 10 minutes to pellet protein. The supernatant was transferred to a fresh microcentrifuge tube, and 200µl of 95% ethanol was added and mixed by flicking the tube.

The resulting mixture was transferred to a spin column assembly containing a silica glass membrane that can bind RNA attached to a collecting tube. The spin column was centrifuged at 13000rpm for 1 minute. 600µl of SV RNA Wash solution was added to the spin column and centrifuged for another 1 minute at 13000 rpm. The spin column was incubated at room temperature with a DNase solution containing: 40µl Yellow Core Buffer; 5ul 0.09M $MnCl_2$; and 5µl DNase I enzyme. After exactly 15 minutes of incubation, 200µl of SV DNase Stop Solution was added to the spin column, and centrifuged at 13000rpm for 1 minute. The spin column membrane was washed with 600µl SV RNA Wash solution, centrifuged for 1 minute, rewashed again with 250ul SV RNA Wash solution. Through every each step, liquid collected in the spin column collecting tube was discarded. Finally, RNA was eluted from the membrane with 30ul of nuclease free molecular grade water. Isolated RNA was stored at -80°C and the final concentrations were quantified before further use. The total RNA eluted from at least a pool of five capsular bags collected post-surgery, ranged between 450 to 900ng of total RNA.

2.5 cDNA Synthesis

cDNA was synthesized using a The RT² First Stand Kit (catalog #330401 , QIAGEN Inc., Valencia CA) under a RNase free environment. RNA was thawed on ice and quantified by ND-1000. The RT² First Stand Kit can work with initial total RNA ranging from 25ng to 5µg. To make sure that all the samples in one experimental set had similar RNA concentrations, all eluted RNA concentrations were diluted down to match the sample that had the lowest eluted RNA concentration. After matching the RNA concentrations between samples, 8µl of each sample and 2µl of 5× gDNA Elimination Buffer were added to a microcentrifuge tube and mixed gently with a pipettor followed by short centrifugation. The microcentrifuge tubes were incubated at 42ºC for 5 minutes. The reverse transcriptase cocktail solution was made as described in the RT² First Stand Kit manual and 10µl was added to each sample and incubated at 42ºC for exactly 5 minutes. The samples were incubated at 95ºC for 5 minutes to stop the reaction. 91µl of nuclease free molecular grade water to each 20µl of cDNA synthesis reaction mixture, mixed well and stored at -20°C.

2.6 RT^2 ProfilerTM PCR Array

Megan Fisher used the RT² Profiler™ PCR Array System (SA Bioscience) to investigate the expression of 84 genes involved in TGF-β signaling in the Crybb2$^{Phil/Phil}$ and wildtype 3-month-old lenses (Fisher 2009). Briefly, an experimental reaction mixture of 1275µl of Master Mix, 8µl of cDNA and 1194µl of nuclease free

molecular grade water was prepared. 25µl of this experimental reaction mixture was added to all 96 wells of the SuperArray plate. The real-time PCR reaction was performed using an ABI7300 Real-Time PCR system (Applied Biosystems, Foster City, CA). The following cycling conditions were used: 1 cycle of 10 minutes at 95°C; 40 cycles of 15 seconds at 95°C followed by 1 minute at 60°C. Results were analyzed using the data analysis program provided on the SA Bioscience website (http://www.SABiosciences.com/pcrarraydataanalysis.php). This program analyzes the data obtained from multiple plates and calculates the relative expression of genes in two sample groups. The program uses the $\Delta\Delta_{CT}$ method described to calculate the relative expression of 84 genes involved in TGF-β signaling.

2.7 **Primer Design**

Primers for Quantitative Real-Time PCR (QRT-PCR) were designed using the Primer3 program at (http://bioinfo.ut.ee/primer3/). Suitable target areas were determined using exon sequences found in the Ensembl Genome Database (http://www.ensembl.org) and analyzed by the San Diego Supercomputer Center Biology Work Bench database (workbench.sdsc.edu/). The optimal product size for each primer was set to 100-200 base pairs and the optimal primer size was set to 21 base pairs. Primer specificity was checked using NCBI's Primer BLAST function (http://blast.ncbi.nlm.nih.gov).

2.8 Quantitative Real-Time PCR

Real-time PCR was performed using a QuantiTect SYBR Green PCR Kit (QIAGEN Inc. Valencia CA) using an ABI Prism 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) using a standard cycle temperature set of 50 °C for 2 minutes, 95°C for 10 minutes, 95°C at 15 seconds for 45 cycles followed by 60°C for 1 minute. mRNA levels for each gene were normalized to the expression levels of the housekeeping gene β2-microglobulin (β2M) or hypoxanthine phospho-ribosyl-transferase-1 (HPRT-1). Table 2.1 shows a list of all primers used. Relative expression was calculated by $\Delta_{CT}^{experimental} - \Delta_{CT}^{wild\ type\ at\ 0hrs\ post\text{-}surgery}$. The fold difference was obtained by the following equation: $2^{\Delta\Delta CT}$.

Table 2.1. List of all primers used for both PCR and QRT –PCR used in this study

	Gene/cDNA detected	Forward primer	Reverse primer
1	αV integrin [null allele]	5'-GGTGACTCAATCGACCTTCAGC	5'-CAGAAATCAAGGACCAAACTGAG
2	αV integrin [floxed]	5'-TTCAGGACGGCACAAAGACCGTTG	5'-CACAAATCAAGGATGACCCTGAG
3	αV integrin [qrt-pcr]	5'-GATGCAGTGTGAGGAACTGGT	5'-GAGTGAACTGGTTCAGGATGG
4	αSMA	5'-GCACAGCTTCTCCTTGATGTC	5'-5'CCGAGATCTCACCGACCT
5	β2M	5'-TACGCCTGCAGAGTTAAGCAT	5'-TCAAATGAATCTGAGCATCA
6	β1 integrin	5'-TCCTTCAATTGCTCACCTTGT	5'-GCGCACTGCTGACTTAGGAAT
7	β5 integrin	5'-AGGATCTACGGACCTTTCTGC	5'-CATTTGCATTCTCCACAGTGA
8	β6 integrin	5'-GCAGAACGCTCTAAGGCCAA	5'- AAAGTGCTGGTGGAACCTCG
9	β8 integrin	5'-AAGCAAAGGCTGTCCAGTTG	5'-TCCACGGGGTATTTCTTCAG
10	Cre-recombinase	5'-ATGCTTCTGTCCGITTGCCG	5'-CTTGTTTTGCACGTTCACCG
11	Fibronectin	5'-CTGGAGTCAAGCCAGACACA	5'-CGAGGTGACAGAGACCACAA
12	Tenascin -C	5'-AAAGTAACCACAACCCGCCT	5'-AGGTGATCAGTGCTGTGGTG
13	TGF-β induced protein	5'-CCTCACCTCCATGTACCAGAA	5'-TGGAAATGACCTTGTCAATGAG
14	Vitronectin	5'-CAAAGCTCGCACTGACA	5'-CCCCTGAGGCCCTTTTTCATA
15	Col1α1	5'-AGGAGCTAGAGGCTCTGAAGG	5'-AGCAATACCAGGAGCACCATT
16	Crybb2	5'-AGGACAGACTCCCTCAGCTCT	5'-GGCACATCGTCGTCTACAATC
17	Crybb2$^{Phil/Phil}$	5'-CTACCGTGGGCTGCACCTGC	5'-GTGGAAGGCACCTCGCTGGTGC
18	FST	5'-TGGATAGCCTATGAGGGAAAG	5'-GACACAGCTCATCGCAGAGA
19	IGF-bp3	5'-CTAAGCGGGAGACAGAATACG	5'-GTCACAGTTTGGGATGTGGAC
20	TGF-bi	5'-CCTCACCTCCATGTACCAGAA	5'-TGGAAATGACCTTGTCAATGAG
21	TR2	5'-TGTGTGCCTGTAACATGGAAG	5'-GGTGGACACGGTAGCAGTAGA
22	Hprt1	5'-CAAGGGCATATCCAACAACA	5'-CAAACTTTGCTTTOCCTGGT

2.9 Morphology, Size and Optical Analysis

Three and six month-old mice were sacrificed with carbon dioxide followed by cervical dislocation. Eyes were removed and lenses were dissected. 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 previously described (Shiels, King et al. 2007). The ratio between wet and dry lens weight, fresh lenses were weighed and placed in an aseptic 50°C oven for 96hrs to dry, and then reweighed.

2.10 Scanning Electron Microscopy

Eyes were immersion fixed in 0.08M Sodium Cacodylate buffer pH 7.4 (Electron Microscopy, Hatfield, PA), 1.25% glutaraldehyde (Electron Microscopy, Hatfield, PA), and 1% paraformaldehyde (Electron Microscopy, Hatfield, PA) for five hours. The lens was excised and transferred to fresh fixative for an additional 48 hours. After fixation, lenses were washed in 1× phosphate buffered saline (PBS). The lens capsule was peeled and the superficial fiber cell layers were removed with fine forceps to expose the cortical fiber cells, some lenses were peeled further to expose the nuclear fiber cells. Peeled lenses were subjected to an ethanol dehydration series (25%, 50%, 75%, 100%) followed by overnight incubations in fresh 100% ethanol followed by an

additional two 2.5-hour 100% ethanol incubation. Finally, the dehydrated, peeled lenses were dried using hexamethyldisilazane (HMDS) (Electron Microscopy, Hatfield, PA) as previously described (Duncan et al., 2000), mounted on aluminum stubs and coated with gold/palladium for 2.5 minutes. Samples were viewed with a Field Emission Scanning Electron Microscope (FE-SEM) Hitachi S-4700 (Tokyo, Japan).

For hematoxylin and eosin (H&E) staining, mice were euthanized, the eye excised, immersed in fixed in Pen-Fix (Richard Allan Scientific, Kalamazoo Michigan) for four hours, then stored in 70% ethanol prior to paraffin embedding. Six-micrometer sections were cut and stained by H&E by standard methods to visualize cellular morphology.

2.11 **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 (Schein). Using an ophthalmic knife, a 3mm central corneal incision was made extending into the lens. 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. We 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. At least 5 -10 independent animals were used for each analysis described here.

2.12 **Immunofluorescence**

All immunofluorescence analyses were carried out as previously described (Reed, Oh et al. 2001). Briefly, to confirm αV integrin deletion, heads were removed from embryos, and eyes removed from postnatal mice, and embedded directly in OCT (Sakura). For analyzing post-surgery samples, whole eyes containing capsular bags collected at different times post surgery were carefully embedded in TissueTek OCT Compound in 10mm x 10mm x 5 mm TissueTek Crymold Biopsy molds and immediately stored at -80ºC. 16µm sections were collected on Fisher Colorfrost/Plus slides using a Leica CM3050 S Cryostat at -15ºC to -17ºC and stored at -80ºC. Immunostaining were carried out under different conditions depending on the primary antibody. Briefly, 1:1 acetone:methanol fixation at −20°C for 20 minutes or in 4%

paraformaldehyde fixation for 30 minutes at room temperature. Appropriate blocking sera were diluted in either 1×PBS or 1×Tris-buffered saline (TBS) and slides were blocked for one hour at room temperature. Primary antibodies were diluted in blocking serum (see Table 2.2 for antibodies and dilutions used) and applied onto the slides and incubated in a humid chamber at room temperature for another one hour or overnight at 4°C. Three, 10-minute washes were performed with either 1×PBS or 1×TBS at room temperature. For secondary antibody staining, slides were stained with 100ul cocktail containing 1:2000 dilutions of DRAQ5™ (Biostatus, Leicestershire, United Kingdom), 1: 250 dilution of αSMA (Sigma-Aldrich, St. Louis, Missouri USA) and 1:200 dilution of the appropriate AlexaFluor 568 labeled secondary antibodies (Invitrogen, Carlsbad, California). The slides were incubated for one hour at room temperature in a dark humid chamber followed by another three, 10-minute washes with either 1×PBS or 1×TBS in dark at room temperature. Excess solution surrounding the tissue was wiped with a Kimberly-Clark® Kimwipes and approximately 150 - 200ul of mounting media was pipetted over the tissue and carefully sealed with a coverslip (Fisher). The slides were stored at -20°C and analyzed not later than two weeks.

2.13 **Antibodies**

Table 2.2. List of all antibodies used in this study

Primary antibody name	1hr room temperature blocking conditions	Primary Antibody	Company and Location
αSMA Clone 1A4, F3777	1% BSA in PBS	1 hour @ RT 1:250 dilution	Sigma-Aldrich (St. Louis, MO)
αV integrin AB1930	5% Horse Serum + 5% Goat Serum in PBS	Overnight @ 4°C 1:400 dilution	Millipore (Billerica, MA)
β1 integrin clone MB1.2,1997	1% BSA in PBS	1 hour @ RT 1:250 dilution	Millipore (Billerica, MA)
β5 integrin AB 1925	1% BSA in PBS	1 hour @ RT 1:200 dilution	Chemicon (Temecula, CA)
β6 integrin (H-1100): sc-15329	5% Goat Serum in PBS	Overnight @ 4°C 1:100 dilution	Santa Cruz (Santa Cruz, CA)
β8 integrin Pro Sci Xw 7802	1% BSA in PBS	1 hour @ RT 1:200 dilution	Chemicon (Temecula, CA)
Phospho-SMAD 3 Clone EP823Y	10min wash in 5% BSA followed by 5% Goat Serum + 10% Horse Serum in PBS	Overnight @ 4°C 1:100 dilution	Epitomics (Burlingame, CA)
TGF-β -induced (H58) sc-28660	5% Goat Serum in PBS	1 hour @ RT 1:100 dilution	Santa Cruz (Santa Cruz, CA)
Fibronectin Ab23750	1% BSA in PBS	1 hour @ RT 1:400 dilution	abcam (Cambridge, MA)
Vitronectin Ab28023	10% Goat Serum in TBS	1 hour @ RT 1:500 dilution	abcam (Cambridge, MA)
Tenascin C AB19011	5% Goat Serum in PBS	Overnight @ 4°C 1:400 dilution	Millipore (Billerica, MA)
Prox 1 Duncan et al., 2002	1% BSA in PBS	1 hour @ RT 1:500 dilution	University of Delaware (Newark, DE)
cMaf sc7866	1% BSA in PBS	1 hour @ RT 1:100 dilution	Santa Cruz (Santa Cruz, CA)
Caspase -3 (Asp175)# 8120	5% Goat Serum in TBS	Overnight @ 4°C 1:50 dilution	Cell signaling (Danvers, MA)
βB2-crystallin takemlj@ksu.edu	10% Goat Serum in TBS	1 hour @ RT 1:250 dilution	Larry Takemoto, (Manhattan, KS)
Pax-6 PRB-278B-Covance	5% Goat Serum in PBS	1 hour @ RT 1:150 dilution	Santa Cruz (Santa Cruz, CA)

2.14 **Confocal Microscopy**

After staining, slides were washed, cover slipped, and stored at −20°C or directly imaged with a Zeiss LSM 780 Confocal Microscope (Carl Zeiss, Inc., Gottingen, Germany) equipped with a 405 nm diode laser, an Argon laser with 458/488/514 nm lines, DPSS 561 nm and HeNe 633 nm laser. 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, images were processed post-imaging to optimize brightness and contrast for viewing on diverse computer screens. In all cases, such manipulations were applied identically to experimental and control images. Some image comparisons were quantitatively analyzed using the open-source application Fiji controlled with a custom Java code (Schindelin, Arganda-Carreras et al. 2012).

2.15 **EdU Click-it Proliferation Assay**

The number of LECs in DNA synthesis or S-phase of the cell cycle at different times post-surgery was determined by 5-ethynyl-2´-deoxyuridine (EdU) click-it proliferation assays (Invitrogen, need location). EdU is a nucleoside analog of thymidine that is incorporated into DNA during DNA synthesis (Warren, Puskarczyk et al. 2009, Kotogany, Dudits et al. 2010). Briefly, mice were injected intraperitoneally

with 400μg of EdU for 25g of mouse body weight (16μg per 1g body weight) dissolved in normal saline. Two hours later, the animals were sacrificed, eyes were removed, ocular tissue was embedded in OCT. 16μm frozen sections were obtained by cryostat and mounted on glass slides. Specimen slides were stored at −80°C or right away fixed by 4% paraformaldehyde for 30 minutes at room temperature followed by methanol fixation at −20 °C for 10 minutes. Sections were allowed to air dry, then blocked with 3% BSA in 1×PBS, for 15 minutes. The EdU click-it reaction cocktail was prepared as instructed in the Click-it reaction kit protocol (Warren, Puskarczyk et al. 2009, Kotogany, Dudits et al. 2010). Excess blocking solution surrounding the tissue was wiped with a Kimberly-Clark® Kimwipes and the appropriate amount of the 100μl of the EdU click-it reaction cocktail was added to the slides and incubated for 30min at room temperature in a dark humid chamber. After the click-it reaction slides were washed with 1% BSA in 1x PBS for 15 minutes followed by a counterstain with 100μl cocktail of 1:2000 dilution of DRAQ5™ and 1: 250 of αSMA for one hour at room temperature in a dark humid chamber. Slides were washed, mounted media and coverslip was applied as described above.

2.16 **Active TGF-β Assay**

2.16.1 Lens extract preparation for TGF-β assay

Four, fresh 3-month-old lenses were placed in a 24 well plate. All 4 lenses were dissected and the lens capsule and fiber cells were carefully separated. 500μl of DMEM/

0.1% BSA (DMEM/BSA) was added and while pipetting up and down 15 times. The mixture was incubated at 37° C for 30min. The supernatant (lens extract) was carefully pipetted out and transferred to a microfuge tube. The remaining lens materials were discarded. The lens extract (which had few loose precipitates) was stored at -80° C until needed or on ice and continued with assay to the final stage.

2.16.2 Acid activation of lens extracts for TGF-β assay

For acid activation assay, 40µl of 1N HCl was added to 200ul lens extract and rocked for 10min at room temperature. Samples were neutralized by adding 40µl 1.2N NaOH containing 0.5 M HEPES. (After neutralizing, the tubes had very thick viscous precipitate). The acid treated tubes were centrifuge for 5 minutes at 4°C at full speed and 200µl of clear supernatant was transferred into new microfuge tube and continued with assay to the final stage.

2.16.3 TGF-β Assay with MLECs

Mink Lung Epithelial Cells (MLEC) stably transfected with Plasminogen Activator Inhibitor-1 promoter (PAI-1)/luciferase construct (Khan, Joyce et al. 2012), were cultured in minimum essential medium (MEM) (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum containing 250ug/ml Geneticin (Invitrogen Life Technologies, Carlsbad, CA). For assays, cells were trypsinized and approximately $4x10^4$ MLEC were plated in each of the desired number of wells in a 96-well plate and allowed to settle for 3 hours in a 37°C incubator.

After the cells were allowed to attach, the medium was removed and replaced with assay media (see below). To obtain a standard luciferase activity curve in response to bioactive TGF-β1, different concentrations (25, 50, 75 and 100pg/ml) of human recombinant TGF-β1 solution (Roche Diagnostics Corporation, Indianapolis, IN) in 200µl of DMEM/BSA containing 250µg/ml Geneticin was added to the wells containing attached MLEC in triplicate. For the active TGF-β in lens assay, 100µl of each prepared lens extract (discussed in section 2.3.1) was added to the well of a 96-well plate containing MLEC in duplicate. Similarly, 100µl of each acid activated sample (discussed in section 2.3.2) was added to the well of a 96-well plate containing MLEC in duplicate. The final volume in the plate was made up to 200µl with DMEM/BSA containing 250µg/ml Geneticin and cells were incubated for 20h at 37°C then harvested for luciferase assay.

Once the media was removed, the wells were washed with 200µl of 1× PBS twice. 30ul of 1x passive lysis buffer ((Promega, Madison, WI)) was added and the plate was placed on shaker for 5min. The plates were stored at –80°C overnight and thawed at 37°C for 5-10 minutes and placed on shaker for 5 minutes. 100ul of luciferase assay reagent (Promega, Madison, WI) was added per well by injector and the relative luciferase unit (RLU) was read on a 2030 Multilabel Reader (Perkin Elmer, Waltham MA). Corresponding TGF-β levels were calculated by subtracting the RLU of control from the RLU of study samples because MLEC itself has some endogenous TGF-β

activity. The TGF-β concentration was calculated with a standard curve using straight-line formula.

Chapter 3

THE ROLE OF ALPHA V INTEGRINS IN LENS EPITHELIAL TO MESENCHYMAL TRANSTIONS AND POSTERIOR CAPSULAR OPACIFICATION

3.1 Introduction

Posterior capsular opacification (PCO) is the most common negative outcome of cataract surgery triggered by residual lens (LCs) cells wound healing responses after cataract surgery (Apple, Escobar-Gomez et al. 2011). It is characterized by LCs proliferation, migration, and epithelial-mesenchymal transition (EMT) into migratory myofibroblasts, while others LCs undergo lens fiber cell differentiation in an attempt to regenerate the lens (de Iongh, Wederell et al. 2005, Wormstone, Wang et al. 2009). If these LCs, which at this point of time are no longer phenotypically normal LECs, remain outside of the visual axis, a Soemmering's ring results, which can stabilize some of the current IOL designs within the eye (Werner, Tassignon et al. 2010). However, if they migrate along the lens capsule into the visual axis, PCO results, leading to impairment of vision (Awasthi, Guo et al. 2009). In the last three decades, substantial efforts have been made to prevent and treat PCO, leading to a reduction of its incidence shortly following cataract surgery (Dewey 2006). This includes but not limited to, improved surgical techniques and improved IOLs that are capable of trapping residual lens epithelial cells to prevent their migration to the posterior capsule, However, in spite of such efforts, longer term, PCO is still a major barrier to the long-term restoration of high acuity vision in cataract patients (Apple, Escobar-Gomez et al. 2011).

Integrins are expressed by both lens epithelial and fiber cells and play major roles in lens development and epithelial cell survival (Simirskii, Wang et al. 2007, Walker and Menko 2009). There is also some descriptive evidence that integrins play a major role in lens EMT and thus PCO pathogenesis. This suggests that integrin antagonists can ameliorate PCO (Zuk and Hay 1994, Kim, Lee et al. 2002, Sponer, Pieh et al. 2005, Walker and Menko 2009). Not surprisingly, several integrin subunits, including β1-integrin, are highly overexpressed in LECs undergoing EMT, while blocking of β1-integrin function using anti-β1 integrin antibodies also blocked this EMT (Zuk and Hay 1994). Analogously, treatment of LECs with salmosin, a disintegrin derived from snake venom, significantly decreased PCO both in vivo and in vitro (Kim, Lee et al. 2002). Further, studies on human lens capsule cultures revealed that the residual LECs, which give rise to PCO, highly overexpressed αVβ6 integrin shortly after lens extraction (Sponer, Pieh et al. 2005). Thus, it is compelling to hypothesize that integrins play a role in the lens EMT that leads to PCO development, however, the identity of the particular integrins involved and the mechanistic function that integrins adapt to participate in PCO was not known prior to this dissertation.

With at least 24 different functional integrin heterodimers encoded by 18 α-subunits and 8 β-subunits (Hynes 2002, Desgrosellier and Cheresh 2010), a clear understanding of which integrins are expressed after cataract surgery and the possible mechanism involved during the process will open a door towards the elucidating molecular signaling pathways responsible in driving LECs EMT and towards PCO development. However, mechanistic studies into these observations are yet to be done.

Numerous studies suggest that TGF-β signaling is crucial for the pathogenesis of fibrotic PCO (Hales, Schulz et al. 1994, de Iongh, Wederell et al. 2005). However, less is known about how cataract surgery initiates TGF-β mediated EMT since high levels of latent TGF-β are constitutively present in the ocular fluids (Cousins, McCabe et al. 1991). Further, mesenchymal ECM ligands such as type I collagen, tenascin-C, vitronectin, and fibronectin are known to upregulate during PCO and their overexpression has been often used as markers for LECs undergoing EMT (Colitz, Malarkey et al. 2000, Latvala, Uusitalo et al. 2000, Wunderlich, Pech et al. 2000). Concomitantly, it is well established that αV-β integrins' interactions with mesenchymal ECM ligands, can activate integrin signaling leading to TGF-β induced EMT (Breuss, Gillett et al. 1993, Breuss, Gallo et al. 1995, Boudreau and Jones 1999, Munger, Huang et al. 1999, Guo and Giancotti 2004, Hazelbag, Kenter et al. 2007, Imamichi and Menke 2007, Honda, Yoshida et al. 2010, Mamuya and Duncan 2012).

In order to test the hypothesis that αV integrins are functionally important in PCO pathogenesis, I created mice lacking the αV integrin subunit in all lens cells using a conditional knockout strategy. The phenotype of these mice was characterized and their lenses were subjected to a mouse model of cataract surgery that is routinely performed in our lab. Following surgery, the residual lens epithelium was examined for markers of EMT and lens fiber regeneration known to occur during PCO onset. Furthermore, since αV integrins are proposed to modulate TGF-β leading to TGF-β induced EMT activation in other system, I also examined the expression pattern of known TGF-β signaling markers, especially those reported to occur during the PCO

development. These results will support the hypothesis that the loss of αV integrin function in lens attenuates the molecular pathways provoked by TGF-β during PCO pathogenesis.

3.2 Results

3.2.1 αV integrin and its interacting β-subunits are overexpressed 48hrs post-surgery in lens

It has been previously postulated that integrins play a major role in the development of lens EMT and thus PCO (Walker and Menko 2009). Although little experimental evidence for this idea has been reported, post-operative studies on human residual lens epithelial cells (LCs) suggested that integrins, especially those belonging to the αV family, are significantly upregulated during PCO (Sponer, Pieh et al. 2005, Walker and Menko 2009). Thus, in order to characterize the distribution pattern of integrin expression in LCs after a lens injury similar to cataract removal, I used a mouse surgical model of extracapsular cataract extraction (ECCE) that is routinely performed in our laboratory by Dr. Yang Wang. This mouse surgical model was first reported by Call and collaborators (Call, Grogg et al. 2004) and refined as described by Desai (Desai, Wang et al. 2010).

By immunofluorescence, I found that the αV integrin subunit is only modestly expressed in LCs at the time of surgery, but its levels upregulated dramatically by 48 hours post-surgery in cells that exhibit the multilayering and increased αSMA expression associated with PCO (Figure 3.1: A-C). In addition to the αV integrin subunit, four of αV integrin's interacting β subunits: β1, β5, β6 and β8 integrin subunits were also overexpressed by 48 hours post-surgery (Figure 3.1: 1D-O). In contrast, the levels of α5 and α6 integrin were only slightly upregulated, while α1, α2, α3, β2 and β3

integrin protein levels did not change or were undetectable over this time frame (data not shown).

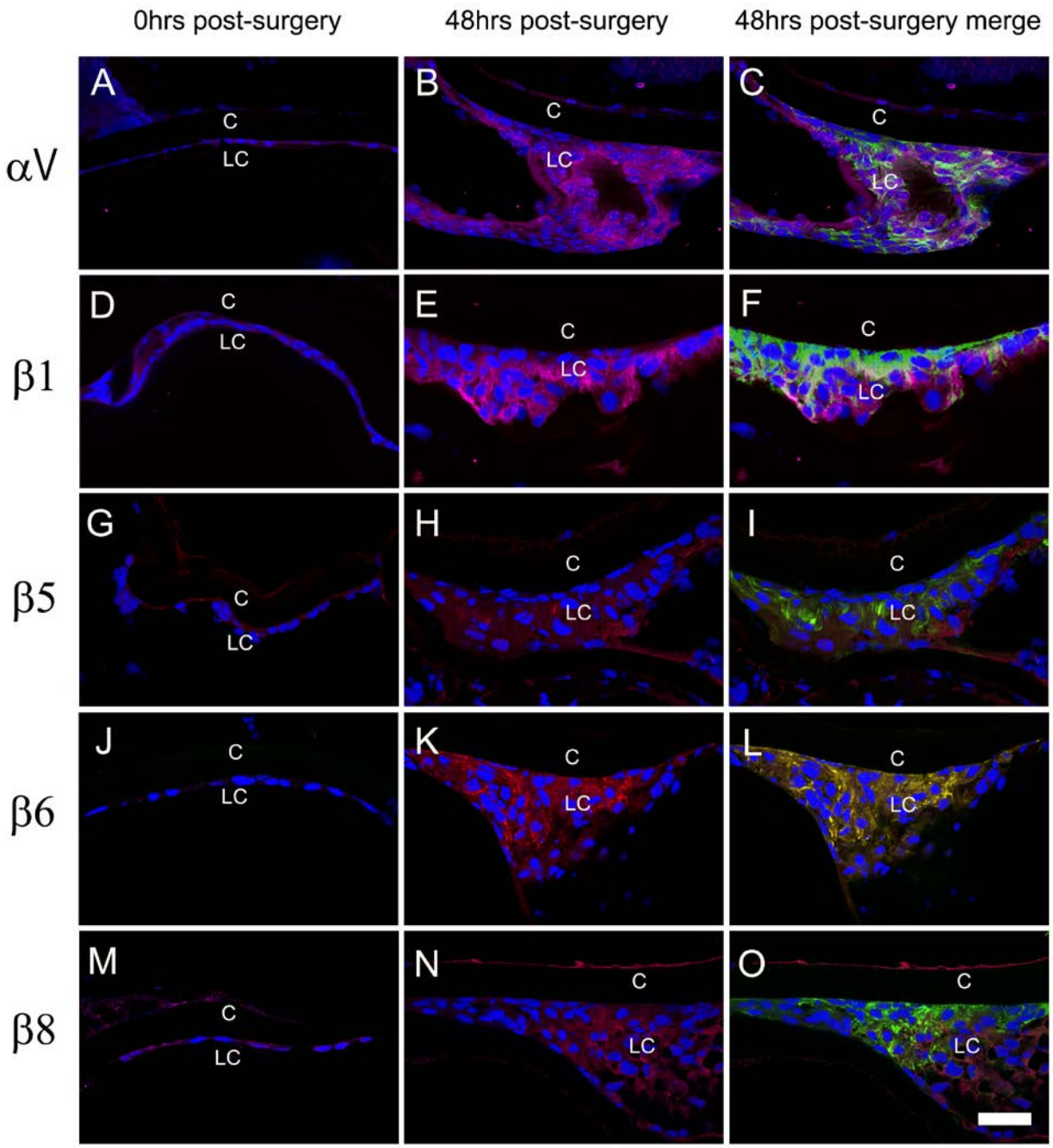
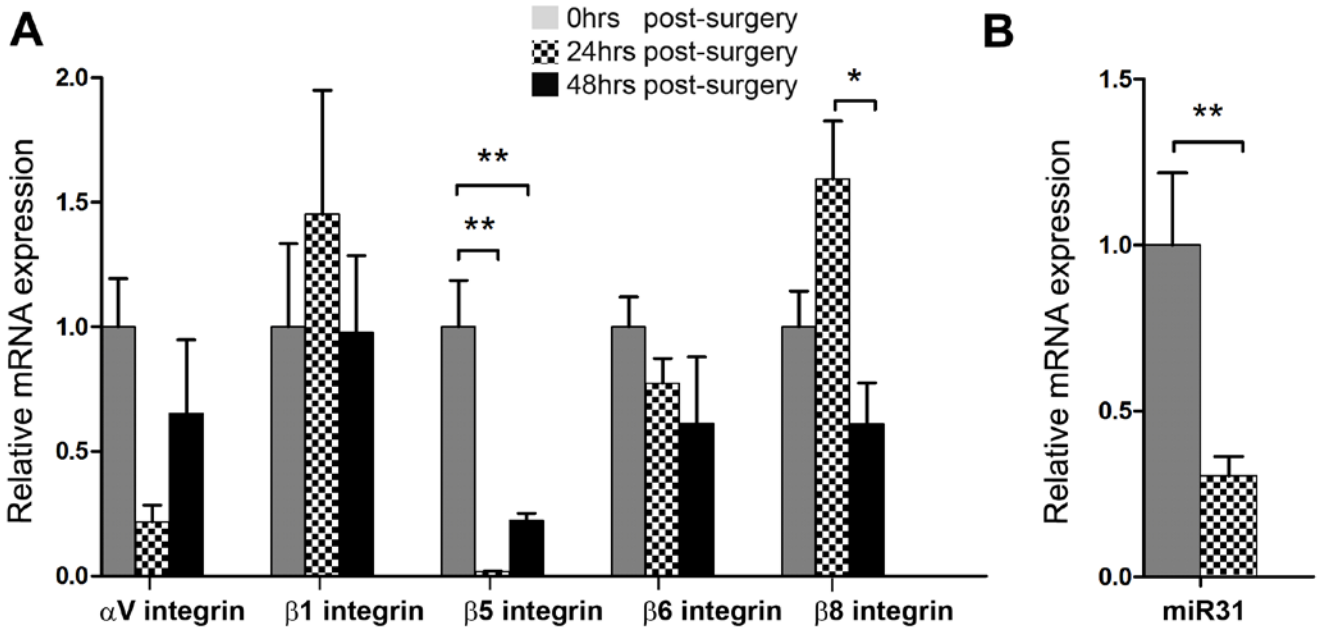


Figure 3.1: Immunofluorescent analysis showing that αSMA and αV-β integrin levels increase in wildtype residual lens cells (LC) at 48hrs post-surgery (**A**) αV integrin + αSMA expression, 0hrs post-surgery. (**B**) αV integrin expression at 48hrs post-surgery. (**C**) αV integrin +

αSMA expression, 48hrs post-surgery. (**D**) β1 integrin + αSMA expression, 0hrs post-surgery. (**E**) β1 integrin expression, 48hrs post-surgery. (**F**) β1 integrin + αSMA expression, 48hrs post-surgery. (**G**) β5 integrin + αSMA 0hrs post-surgery. (**H**) β5 integrin expression at 48hrs post-surgery. (**I**) β5 integrin + αSMA expression at 48hrs post-surgery. (**J**) β6 integrin + αSMA expression 0hrs post-surgery. (**K**) β6 integrin expression at 48hrs post-surgery. (**L**) β6 integrin + αSMA expression 48hrs post-surgery. (**M**) β8 integrin + αSMA expression 0hrs post-surgery. (**N**) β8 integrin expression at 48hrs post-surgery. (**O**) β8 integrin + αSMA expression, 48hrs post-surgery. Scale bar = 35μm, red=integrin; blue=nucleus; green = αSMA, LC = residual lens cells, C= lens capsule. Adapted from (Mamuya, Wang. et al. 2014)

It should be noted that the upregulation of αV integrin expression was only seen at the protein level, as the mRNA levels for αV integrin and its interacting β-integrins either did not change significantly or decreased significantly by 48hrs post-surgery (Figure 3.2: A). I believe this effect could be mediated by microRNAs since miR31, a microRNA known to negatively regulate integrin translation as well as mRNA stability (Valastyan, Reinhardt et al. 2009, Augoff, Das et al. 2011), did down regulate significantly by 24hrs post-surgery (Figure 3.2: B).

Figure 3.2: (**A**) RT-PCR quantification of integrin mRNA levels in wildtype



residual lens cells (LC) at 0hrs, 24hrs and 48hrs post-surgery normalized to β2-microglobulin n=4. αV integrin subunit mRNA expression appeared attenuated at 24hrs but this did not reach significance (P=0.083). β5 integrin subunit mRNA expression was significantly reduced at both 24hrs and 48hrs post-surgery (**P=0.001). No significant changes were observed in β1 or β6 integrin subunit mRNA expression post-surgery. β8 integrin subunit mRNA appeared to increase slightly at 24hrs although the difference was not significant. Its abundance did significantly fall at 48hrs post-surgery (*P=0.014). (**B**) Quantification of miR-31 mRNA levels in wildtype LCs at 24hrs post-surgery normalized to snoRNA202 levels (**P=0.0087, n=5). The miRNA data was courtesy of Joceyln Zajac. All fold changes post-surgery were calculated by setting values obtained at 0hrs post-surgery in each specific group to one. Values are expressed as mean ± S.E.M.

Asterisks (*) indicate statistically significant fold changes from 0hrs post-surgery. Adapted from (Mamuya, Wang. et al. 2014).

3.2.2 Deletion of αV integrin from the developing lens

In order to study the role of αV integrin in the lens during its development and in PCO, I created mice lacking αV integrin solely in the lens using MLR10-*cre* conditional knockout approach whose activity is first detected in the lens beginning around embryonic day 10.5 (the lens vesicle stage) (Zhao et al., 2004). PCR analysis of genomic DNA isolated from adult 3-month-old lenses showed that the deletion of the floxed region of the αV integrin gene is nearly complete (Figure 3.3: A-B) and immunofluorescence analysis revealed significant reduction of αV integrin protein beginning at about 12.5 dpc (data not shown) with the near total loss of the protein in adult αV $^{[-/flox]}$; MLR10-*cre* (αVMLR10) lenses when compared to αV $^{[flox/flox]}$ (wildtype) (Figure 3.3: C-D).

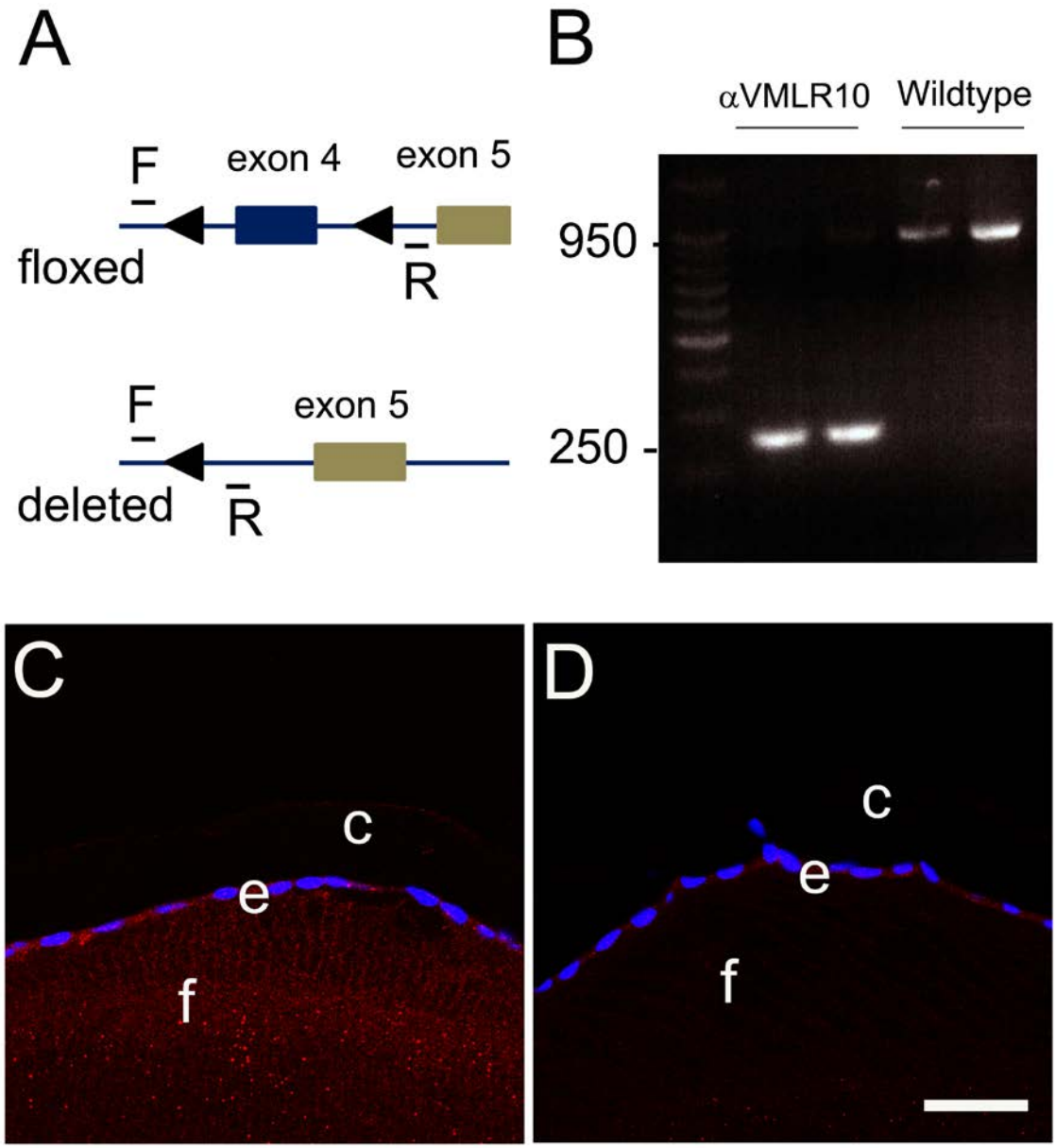


Figure 3.3: αV integrin gene deletion analysis. (**A**) Diagram of the αV integrin locus showing the position of the PCR primers and the loxP sites (McCarty, Lacy-Hulbert et al. 2005). (**B**) PCR results from DNA obtained from 3-month-old lenses demonstrating successful deletion of exon 4 in mice lacking αV integrin in all lens cells αV

[-/flox]; MLR10-cre (αVMLR10). (**C**) Immunofluorescence showing αV integrin protein expression in a 3-month-old wildtype lens. (**D**) Immunofluorescence showing αV integrin protein expression in a 3-month-old αVMLR10 lens Key: Scale = 35μm. Red = αV integrin, blue = nucleus, e = epithelial lens cells, f= lens fiber cells and c= lens capsule. Adapted from (Mamuya, Wang. et al. 2014)

3.2.3 αV integrin null lenses are morphologically and optically indistinguishable from wildtype

To my surprise, lenses lacking αV integrin were morphologically normal and did not show any abnormalities. They appeared transparent under dark field imaging (Figure 3.4: A-B) and refracted a hexagonal grid similar to wildtype lenses (Figure 3.4: B-C), suggesting that αV integrin is not important for the transparency or refractive properties of the lens. At the light level, I found both wildtype and αVMLR10 lenses exhibit similar morphology (Figure 3.4: E-F) and no obvious defects in lens fiber cell structure were observed by scanning electron microscopy (Figure 3.4: G-H).

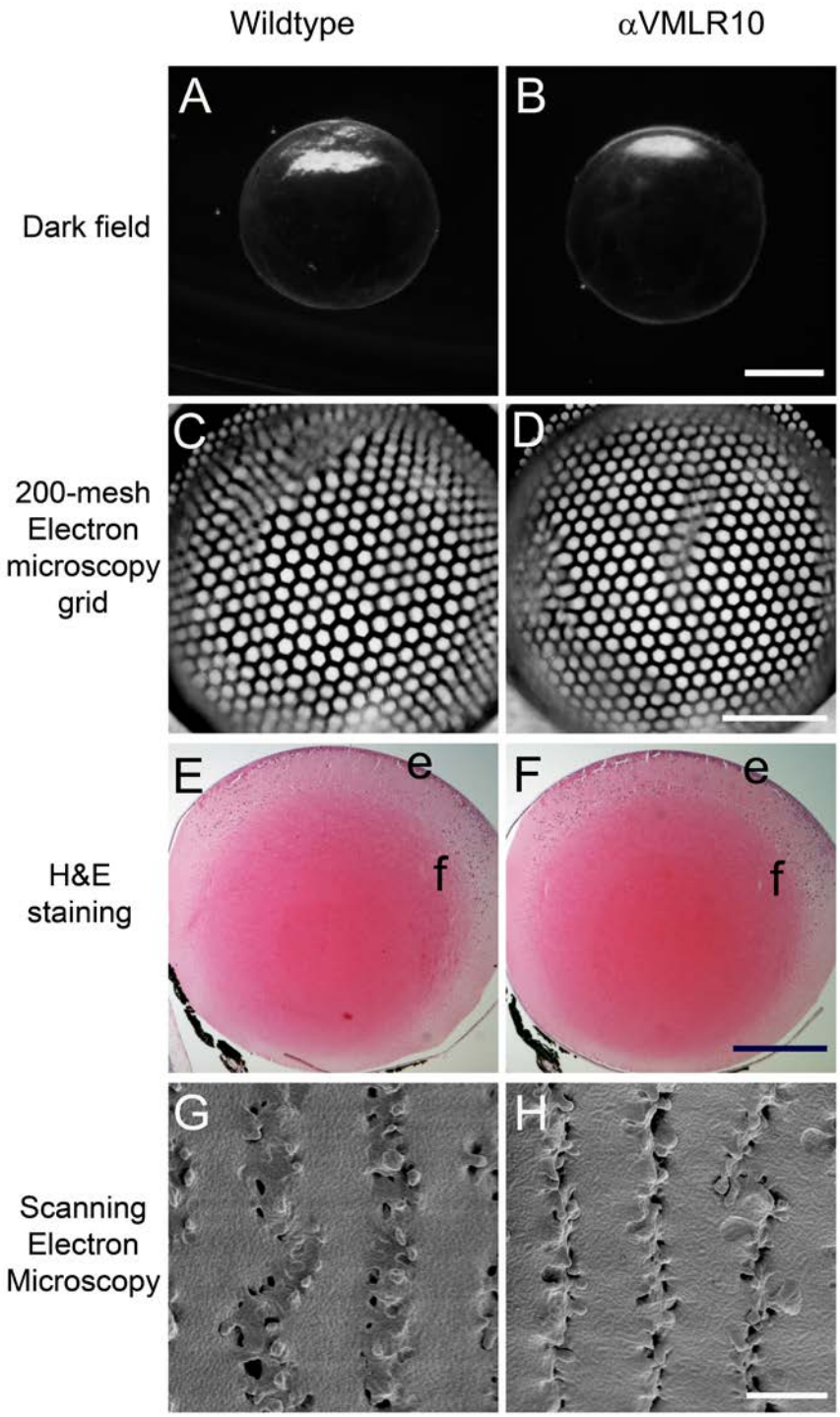


Figure 3.4: Morphological analysis of αV integrin null lenses. (**A**) A dark field

image showing a 3-month-old wildtype lens. (**B**) A dark field image showing a 3-month-old αVMLR10 lens. (**C**) A 200-mesh electron microscopy grid analysis of a 4-month-old wildtype lens. (**D**) A 200-mesh electron microscopy grid analysis of a 4-month-old αVMLR10 lens. (**E**) Hematoxylin and eosin (H&E) staining showing 4-month-old wildtype lens (**F**) H&E staining showing 4-month-old αVMLR10 lens. (**G**) SEM analysis of the fiber cell organization of a 4-month-old wildtype lens. (**H**) SEM analysis of the fiber cell organization of a 4-month-old αVMLR10 lens fiber cell organization. SEM analysis courtesy of David Scheiblin. Scale bar for (A, B) = 1.0mm, (**C, D**) = 0.5mm, (**E, F**) = 0.5mm and (**G, H**) = 4.0μm. e =lens epithelium, f = lens fiber cells. Adapted from (Mamuya, Wang. et al. 2014).

Unexpectedly, I found that αVMLR10 null lenses weighed significantly more than controls at three months of age. Although by six months, this difference was no longer statistically significant (Table 3.1). However, the basis for this observation is unclear since the ratio of wet lens to dry lens weight between αVMLR10 and wildtype is unchanged (Table 3.1) and no differences in cell proliferation were detected (data not shown).

Table 3.1. Lens wet and dry weight comparison between 4-month-old αV [-/flox]; MLR10-*cre* (αVMLR10) lenses and wildtype lenses. αVMLR10 lenses are significantly heavier than wildtype at three months of age (P = 0.02; n = 8). However, by six months of age, these lenses were no longer significantly different in size (P = 0.30, n = 6). In addition, the ratio of wet lens to dry lens between wildtype and αVMLR10 lenses was similar. All results are expressed on a per lens basis Table from (Mamuya, Wang. et al. 2014).

Lens age and Genotype	**Average wet lens weight (mg)**	**Average dry lens weight (mg)**	**Wet lens /dry lens ratio**
3 month old αVMLR10 lenses	7.78 ± 0.35*	3.54 ± 0.24	2.26
3 month old wildtype lenses	7.26 ± 0.42*	3.29 ± 0.18	2.21
6 month old αVMLR10 lenses	8.67 ± 0.30	4.12 ± 0.19	2.10
6 month old wildtype lenses	8.44 ± 0.49	3.91 ± 0.20	2.16

3.2.4 LCs from lenses lacking αV integrin do not elevate cell proliferation and αSMA expression 48hrs post-surgery

Since the absence of αV integrin from lens did not obviously affect normal lens development, morphology or function, I took this outcome as an advantage and utilized the αVMLR10 mice as a model to study the role of αV integrin in the cellular and molecular changes that occur in LCs after lens injury/during PCO. As expected, immediately after fiber cell removal, the LCs in both wildtype (Figure 3.5: A) and αVMLR10 mice (Figure 3.5: B) exhibit neither appreciable cell proliferation nor do they express the EMT marker, αSMA. However, 48 hours later, the LCs of wildtype lenses proliferate (Figure 3.5: C, E) and initiate robust αSMA expression (Figure 3.5:

G). In contrast, I found that the residual LCs remaining in αVMLR10 capsule after fiber cell removal exhibit very little cell proliferation (Figure 3.5: D, F) and do not appreciably upregulate αSMA expression by 48 hours after surgery (Figure 3.5: H). In addition, there was a question to whether the absence of residual lens epithelial cells in the αVMLR10 was due to an induction of cell death; however, apoptosis, as assayed by active caspase-3 levels, was not detected in either wildtype nor αVMLR10 lenses after lens fiber cell removal (data not shown).

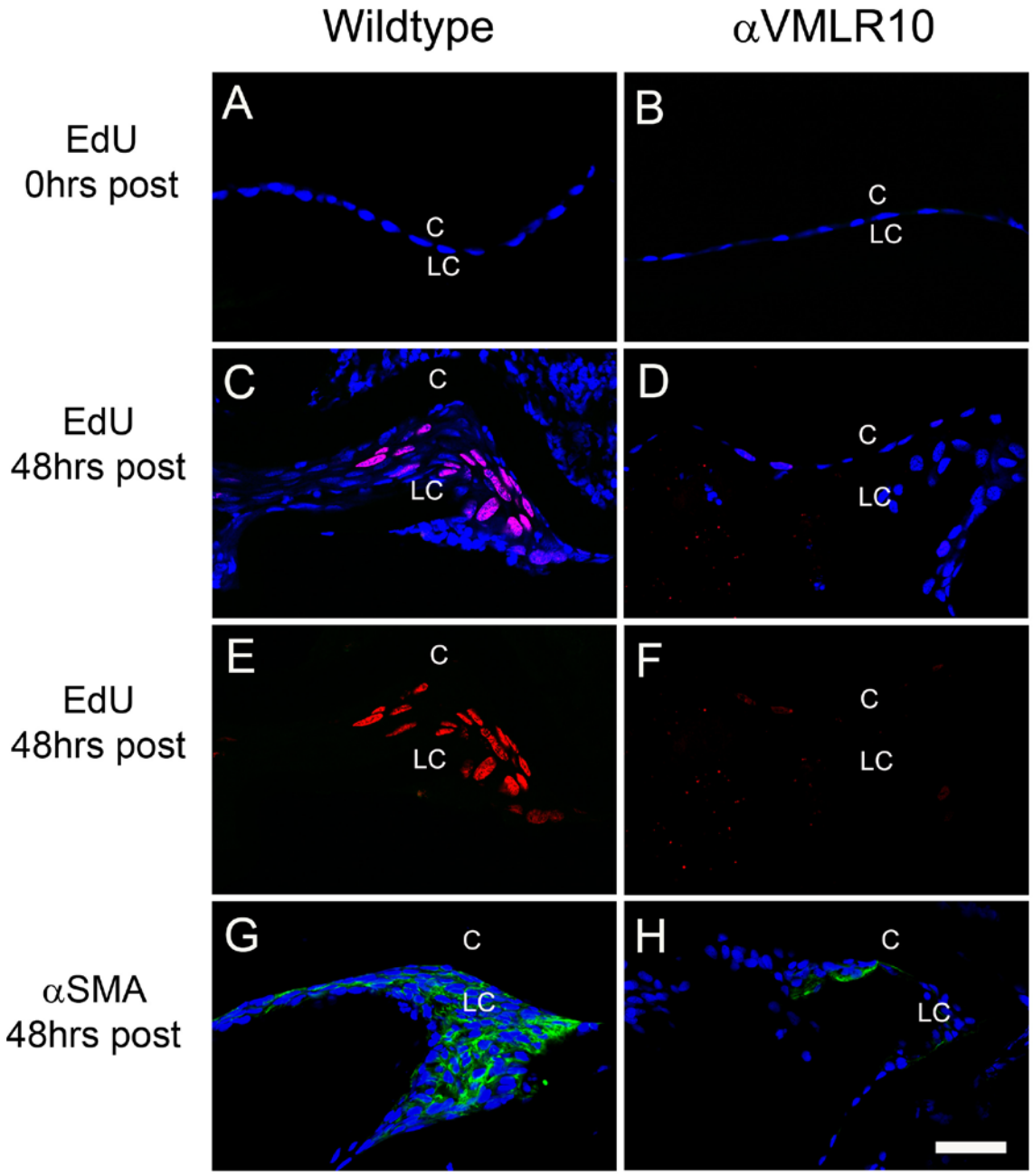


Figure 3.5: Immunohistochemistry analysis of αSMA expression and EdU (5-ethynyl-2´-deoxyuridine) click-it labeling of wildtype and αVMLR10 residual lens cells (LC) post-surgery. (**A**) EdU labeling + αSMA expression in wildtype LCs at 0hrs post-surgery. (**B**) EdU

+ αSMA expression in αVMLR10 LCs at 0hrs post-surgery. (**C**) EdU staining of proliferating wildtype LCs at 48hrs post-surgery. (**D**) EdU labeling of αVMLR10 LCs at 48hrs post-surgery. (**E**) EdU labeling alone in proliferating wildtype LCs at 48hrs post-surgery. (**F**) EdU labeling alone in αVMLR10 LCs at 48hrs post-surgery. (**G**) αSMA expression in wildtype LCs at 48hrs post-surgery. (**H**) αSMA expression on αVMLR10 LCs at 48hrs post-surgery. Scale bar (A, B, C, D, E, & F) = 70μm, (**G, H**) = 35μm. Red = EdU positive (Proliferating LCs), blue = nucleus, green = αSMA, LC = residual lens cells, C= lens capsule. Adapted from (Mamuya, Wang. et al. 2014).

3.2.5 αVMLR10 lenses do not upregulate EMT markers in LCs remaining in the eye after fiber cell removal

The expression levels of αSMA (Figure 3.6: A), fibronectin (Figure 3.6: B) and tenascin-C (Figure 3.6: C) upregulate significantly in wildtype LCs during lens EMT as previously reported in human PCO (Tanaka, Saika et al. 2002, Tanaka, Sumioka et al. 2010, Eldred, Dawes et al. 2011). Interestingly, I found that αSMA mRNA levels did not increase in αVMLR10 lenses 24hrs post-surgery (Figure 3.6: A) while the extent of fibronectin (Figure 3.6: B) and tenascin-C (Figure 3.6: C) upregulation was greatly attenuated in lenses lacking the α_V integrin gene. In contrast though, the mRNA levels of the αV integrin ligand vitronectin did not change significantly post-surgery. Compared to wildtype, vitronectin mRNA levels were initially lower in αVMLR10

lenses and increased slightly at 24hrs post-surgery, however, none of these changes were statistically significant (Figure 3.6: D).

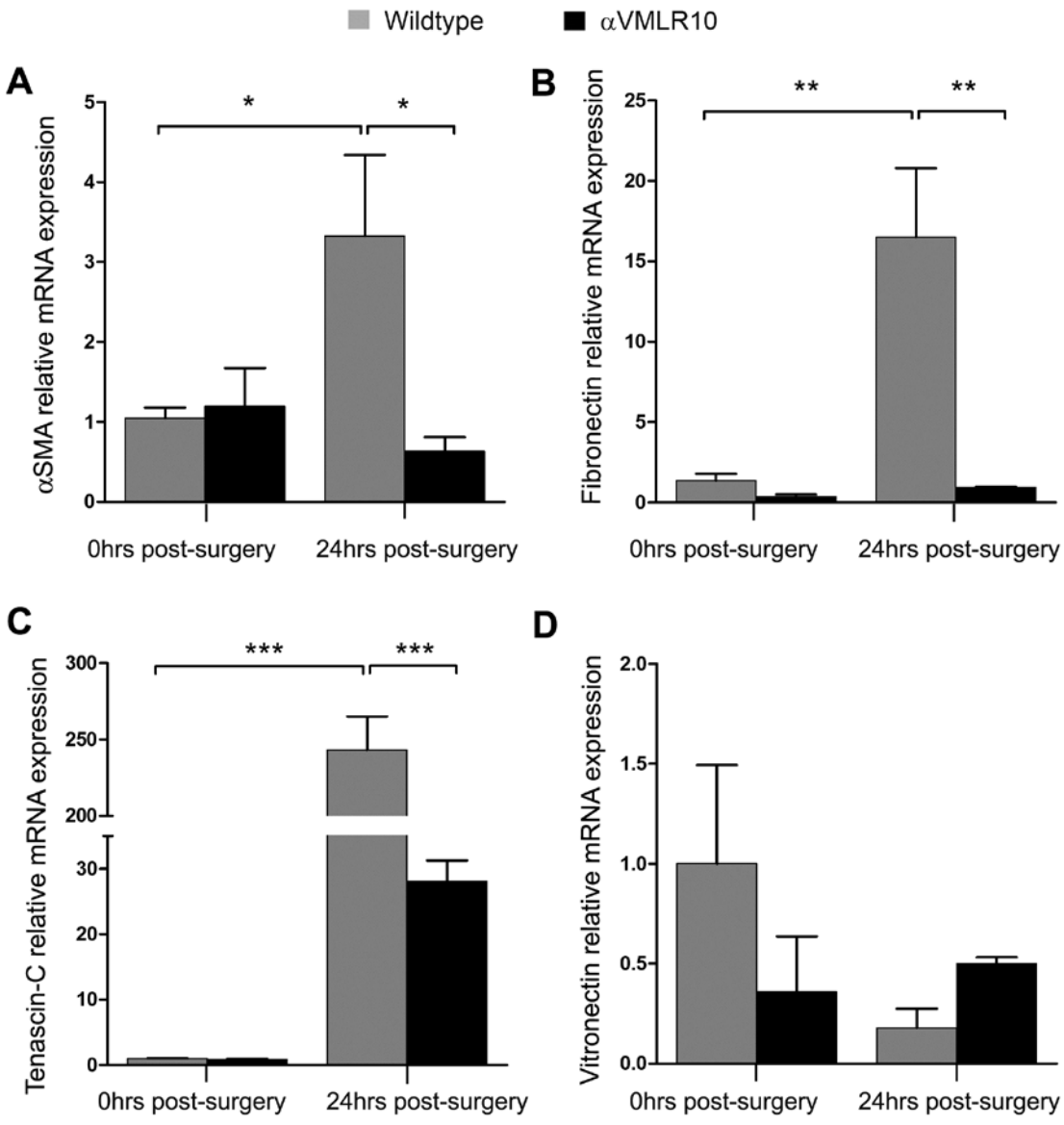


Figure 3.6: RT-PCR quantification of mRNA expression levels in wildtype and αVMLR10 residual lens cells on capsular bags collected at 0hrs and 24hrs post-surgery. For each gene, mRNA expression was normalized to β2M and fold-change were calculated based on the mean 0hrs post-surgery wildtype mRNA level equated to 1. (**A**) αSMA relative mRNA expression post-surgery, *P=0.02. There was no significant changes in αSMA mRNA levels between 0hrs

and 24hrs αVMLR10 post-surgery lenses, P=0.54 (**B**) Fibronectin relative mRNA expression post-surgery, **P = 0.001. There was no significant changes in fibronectin mRNA levels between 0hrs and 24hrs αVMLR10 post-surgery lenses, P=0.90 (**C**) Tenascin-C relative mRNA expression post-surgery ***P = 0.0001. There was no significant increase in tenascin-C mRNA expression in αVMLR10 24hrs post-surgery lenses, P=0.08 (**D**) No significant changes were observed in vitronectin relative mRNA expression in any group post-surgery when compared to wildtype 0hrs, P=0.21. The decrease in vitronectin mRNA expression in wildtype at 24hrs post-surgery was not significant, P = 0.79; neither was the slight increase in vitronectin mRNA expression in αVMLR10 at 24hrs post-surgery, P=0.41. All experiments had n=5. Values are expressed as mean ± S.E.M. Asterisks (*) indicate statistically significant fold changes from 0hrs post-surgery. Adapted from (Mamuya, Wang. et al. 2014).

By immunofluorescence, I found that fibronectin, tenascin-C and vitronectin proteins were nearly undetectable in both wildtype (Figure 3.7: A-C) and αVMLR10 (Figure 3.7: D-F) LCs immediately and even 24 hours following surgery (data not shown here). However, all were upregulated 48hrs post-surgery in wildtype (Figure 3.7: G-I) Further, this upregulation was observed highly in LCs that overexpressed αSMA (Figure 3.7: J-L). However, αVMLR10 LCs show mild to almost no upregulation of fibronectin, tenascin-C or vitronectin (Figure 3.7: M-O) and showed a much less robust αSMA elevation as compared to wildtype lenses 48 hours post-surgery (Figure 3.7: P-R).

Immunofluorescence analysis also revealed that fibronectin, which is normally present in the lens capsule of both wildtype (Figure 3.7: A) and αVMLR10 (Figure 3.7: D) lenses prior to surgery, is not obviously elevated around LCs at 24 hours post-surgery (not shown) but is deposited around LCs expressing αSMA by 48hrs post-surgery especially on the leading LCs in wildtype lenses (Figure 3.7: G). However, I did not observe such extensive fibronectin deposition in αVMLR10 LCs (Figure 3.7: M). Surprisingly though, unlike the QRT-PCR results, vitronectin protein levels were upregulated at 48 hours surgery in wildtype (Figure 3.7: I&L) but not in αVMLR10 lenses (Figure 3.7: O&R).

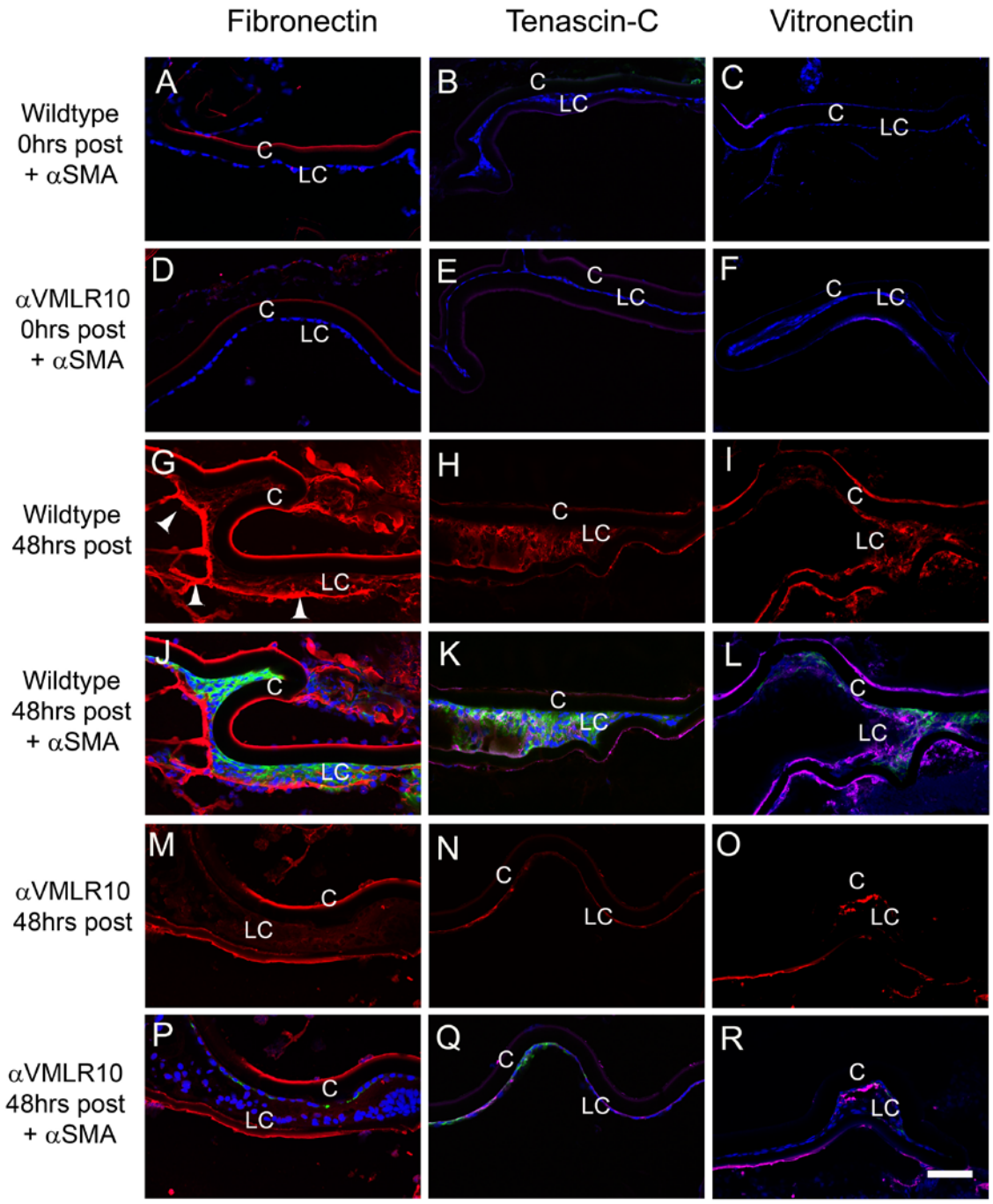


Figure 3.7: Immunofluorescent analysis of fibronectin, tenascin-C and vitronectin deposition in wildtype and αVMLR10 residual lens

cells (LC) post-surgery. (**A**) Fibronectin + αSMA expression in wildtype LCs at 0hrs post-surgery. (**B**) Tenascin-C + αSMA expression in wildtype LCs at 0hrs post-surgery. **(C**) Vitronectin + αSMA expression in wildtype LCs at 0hrs post-surgery. (**D**) Fibronectin + αSMA expression in αVMLR10 LCs at 0hrs post-surgery. (**E**) Tenascin-C + αSMA expression in αVMLR10 LCs at 0hrs post-surgery. (**F**) Vitronectin + αSMA expression in αVMLR10 LCs at 0hrs post-surgery. (**G**) Fibronectin expression in wildtype LCs 48hrs post-surgery with arrowheads showing fibronectin deposition on the leading LCs. (**H**) Tenascin-C expression alone in wildtype LCs at 48hrs post-surgery. (**I**) Vitronectin expression alone in wildtype LCs at 48hrs post-surgery. (**J**) Fibronectin + αSMA expression in wildtype LCs at 48hrs post-surgery. (**K**) Tenascin-C + αSMA expression in wildtype LCs at 48hrs post-surgery. (L Vitronectin + αSMA expression in wildtype LCs at 48hrs post-surgery. (**M**) Fibronectin expression in αVMLR10 LCs at 48hrs post-surgery. (**N**) Tenascin-C expression alone in αVMLR10 LCs at 48hrs post-surgery. (**O**) Vitronectin expression in αVMLR10 LCs at 48hrs post-surgery. (P) Fibronectin + αSMA expression in αVMLR10 LCs at 48hrs post-surgery. (**Q**) Tenascin-C + αSMA expression in αVMLR10 LCs at 48hrs post-surgery (**R**) vitronectin + αSMA expression in αVMLR10 LCs at 48hrs post-surgery. Scale bar = 60μm. Red = Fibronectin, tenascin-C or Vitronectin, blue = nucleus, green = αSMA. LC = residual lens cells, C= lens capsule. Adapted from (Mamuya, Wang. et al. 2014).

3.2.6 Lens fiber differentiation markers still upregulate post-surgery in the absence of αV integrin

After cataract surgery/lens injury, LCs do not exclusively undergo EMT. Instead, some LCs begin to express lens fiber cell markers, presumably in an attempt to regenerate the injured lens (Gwon 2006, Awasthi, Guo et al. 2009, Wormstone, Wang et al. 2009). At five days post-surgery in the mouse model used here, the residual wildtype LCs are found in cell clusters either expressing EMT markers such as αSMA (Figure 3.8: C, G) or lens fiber cell markers such as cMaf (Figure 3.8: A,C) or Prox1 (Figure 3.8: E,G). However, similar to the results at 48 hours post-surgery, αVMLR10 LCs do not express appreciable αSMA (Figure 3.8: D, H), although they do begin to express both cMaf (Figure 3.8: B, D) and Prox1 (Figure 3.8: F, H) similar to wildtype.

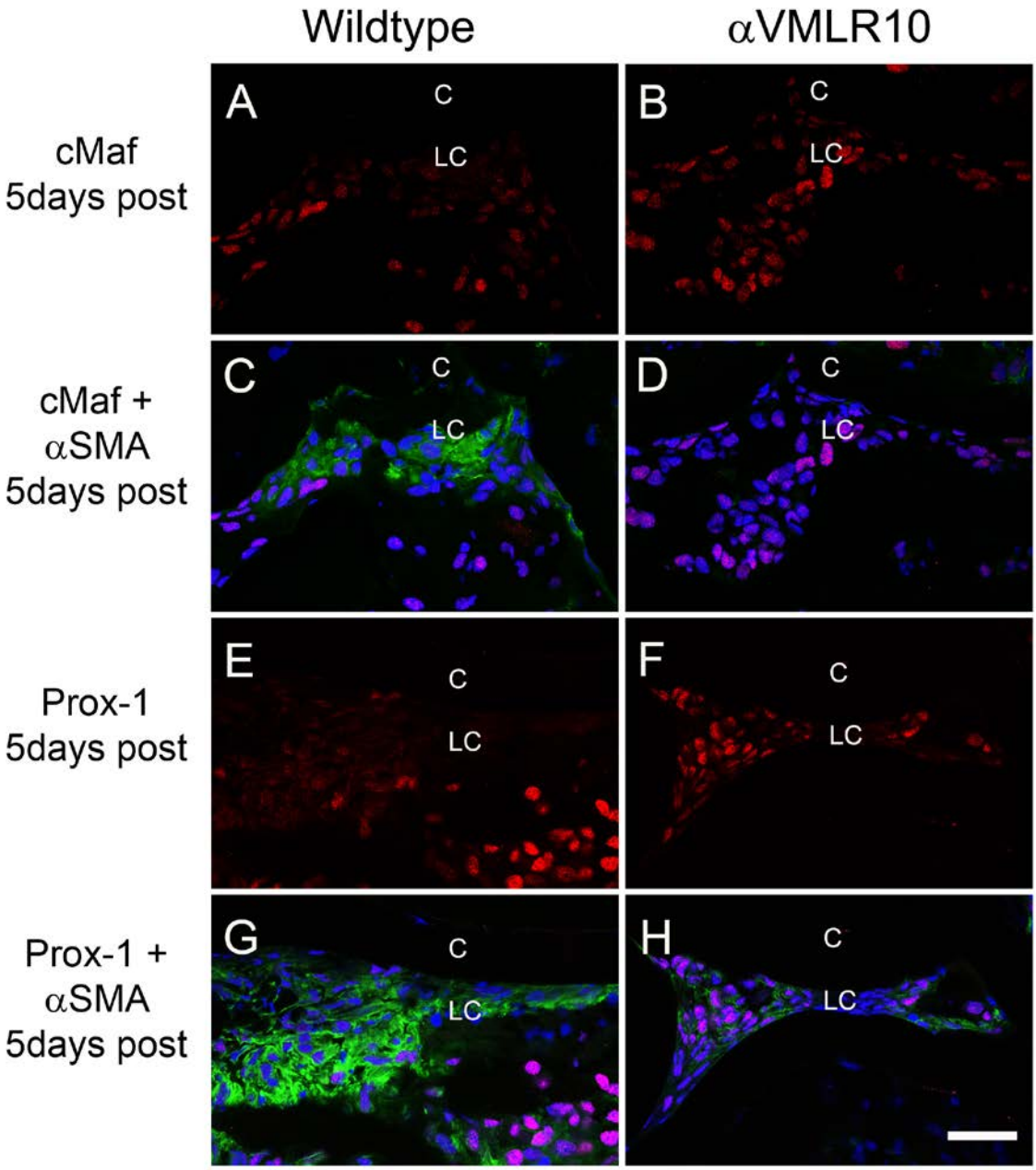


Figure 3.8: Immunohistochemistry of αSMA and the lens fiber differentiation markers Prox-1 and cMaf in wildtype and αVMLR10 residual lens cells (LC) from capsular bags collected at 5 days post-surgery. (**A**) cMaf expression alone in wildtype LCs at 5 days post-surgery. **(B)**

cMaf expression alone in αVMLR10 LCs at 5 days post-surgery. (**C**) cMaf + αSMA expression in wildtype LCs at 5days post-surgery. (**D**) cMaf + αSMA expression in αVMLR10 LCs at 5days post-surgery. (**E**) Prox-1 expression alone in wildtype LC at 5 days post-surgery. (**F**) Prox-1 expression alone in αVMLR10 LCs at 5 days post-surgery. (**G**) Prox-1 + αSMA expression in wildtype LCs at 5days post-surgery. (**H**) Prox-1 + αSMA expression in αVMLR10 LCs at 5 days post-surgery. Scale bar = 35μm. Red = Prox-1 and cMaf, blue = nucleus, green = αSMA, LC = residual lens cells, C= lens capsule. Adapted from (Mamuya, Wang. et al. 2014).

3.2.7 αVMLR10 lenses fail to upregulate SMAD-3 phosphorylation post-surgery.

Many studies have demonstrated that TGF-β signaling plays a central role in fibrotic PCO/LEC EMT and previous work in an *in vivo* lens injury model has shown that phosphorylation of SMAD-3 is central to this process (Saika, Kono-Saika et al. 2004, de Iongh, Wederell et al. 2005). Consistent with these reports, I detected SMAD-3 phosphorylation in wildtype LCs by 48hrs after surgery (Figure 3.9: A, C). I also noticed that these SMAD-3 phosphorylation levels are greatly elevated by five days post-surgery, especially in cells expressing αSMA (Figure 3.9: E, G). However, αVMLR10 LCs do not exhibit elevated levels of phosphorylated SMAD-3 at either 48 hours (Figure 3.9: B, D) or five days (Figure 3.9: F, H) after surgery.

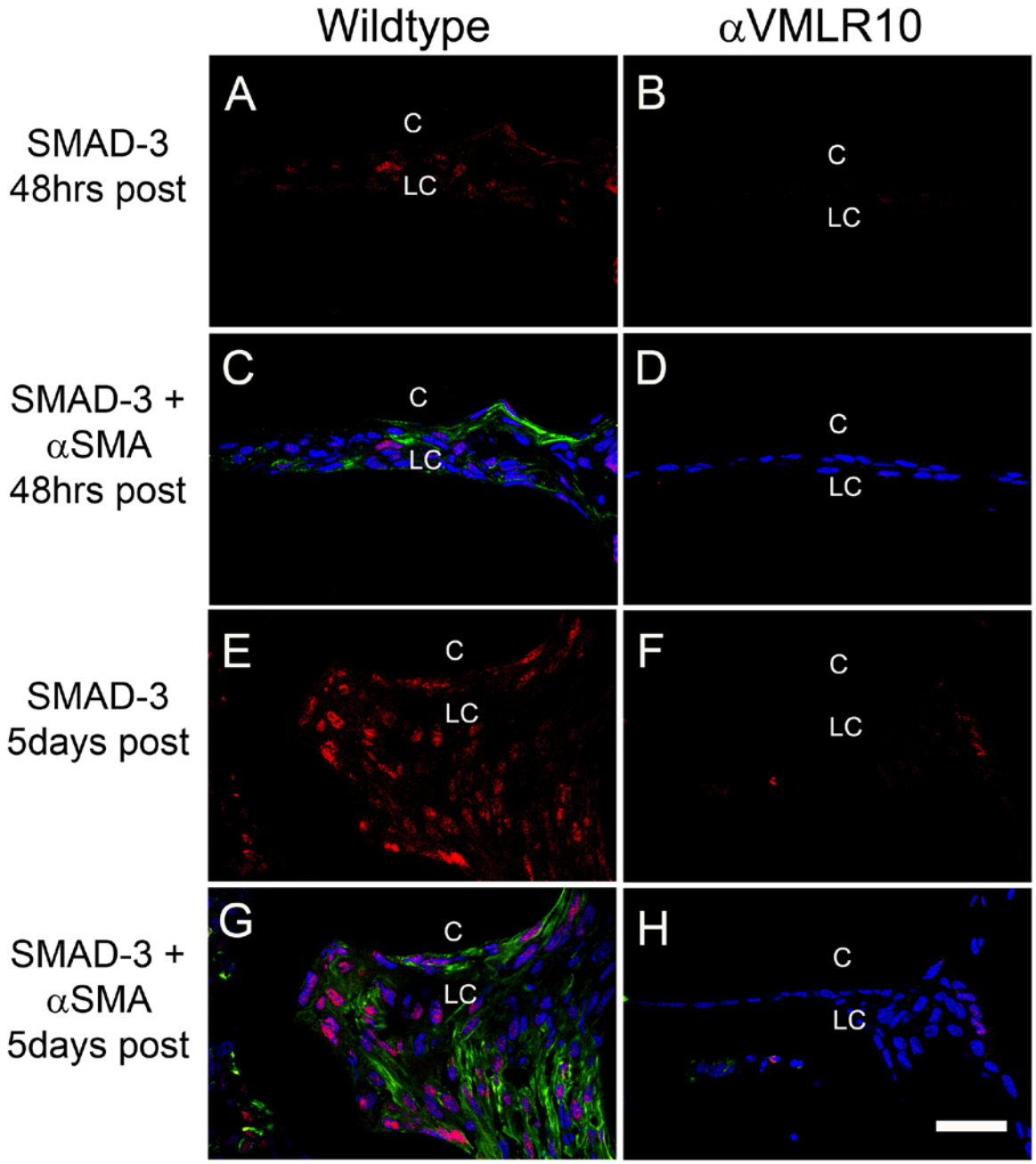


Figure 3.9: Immunofluorescent analysis of αSMA and phospho-SMAD-3 in wildtype and αVMLR10 residual lens cells (LC) from capsular bags collected at 48 hrs and 5 days post-surgery. (**A**) Phospho-

SMAD-3 expression alone in wildtype LCs at 48 hrs post-surgery. (**B**) Phospho-SMAD-3 expression alone in αVMLR10 LCs at 48 hrs post-surgery. (**C**) Phospho-SMAD-3 + αSMA expression in wildtype LCs at 48 hrs post-surgery. (**D**) Phospho-SMAD-3 + αSMA expression in αVMLR10 LCs at 48 hrs post-surgery. (**E**) Phospho-SMAD-3 expression alone in wildtype LCs at 5 days post-surgery. (**F**) Phospho-SMAD-3 expression alone in αVMLR10 LECs at 5 days post-surgery. (**G**) Phospho-SMAD-3 + αSMA expression in wildtype LCs at 5 days post-surgery. (**H**) Phospho-SMAD-3 + αSMA expression in αVMLR10 LCs at 5 days post-surgery. Scale bar = 35μm. Red = phospho-SMAD-3, blue = nucleus, green = αSMA, LC = residual lens cells, C= lens capsule.

3.2.8 TGF-β induced protein is not deposited in αVMLR10 ECM 48hrs post-surgery

TGF-β induced protein (TGF-βi) is an ECM molecule and α_V integrin ligand (Nam, Kim et al. 2003), whose expression and ECM deposition robustly upregulate in response to TGF-β signaling (Jeon, Kim et al. 2012). Consistent with this, I found that TGF-βi mRNA is robustly upregulated in the remnant LCs of wildtype mice by 24 hours after fiber cell removal (Figure 3.10: A), and obvious TGF-βi protein deposition is detectable around wildtype LCs by 48 hours after surgery (Figure 3.10: D, F). In contrast, despite a significant TGF-βi mRNA upregulation in αVMLR10 LCs, such upregulation was significantly lower (P=0.001) than that observed in wildtype (Figure

.10: A); moreover, TGF-βi protein deposition was not detected in αVMLR10 LCs at 48 hours after lens fiber cell removal (Figure 3.10: E, G).

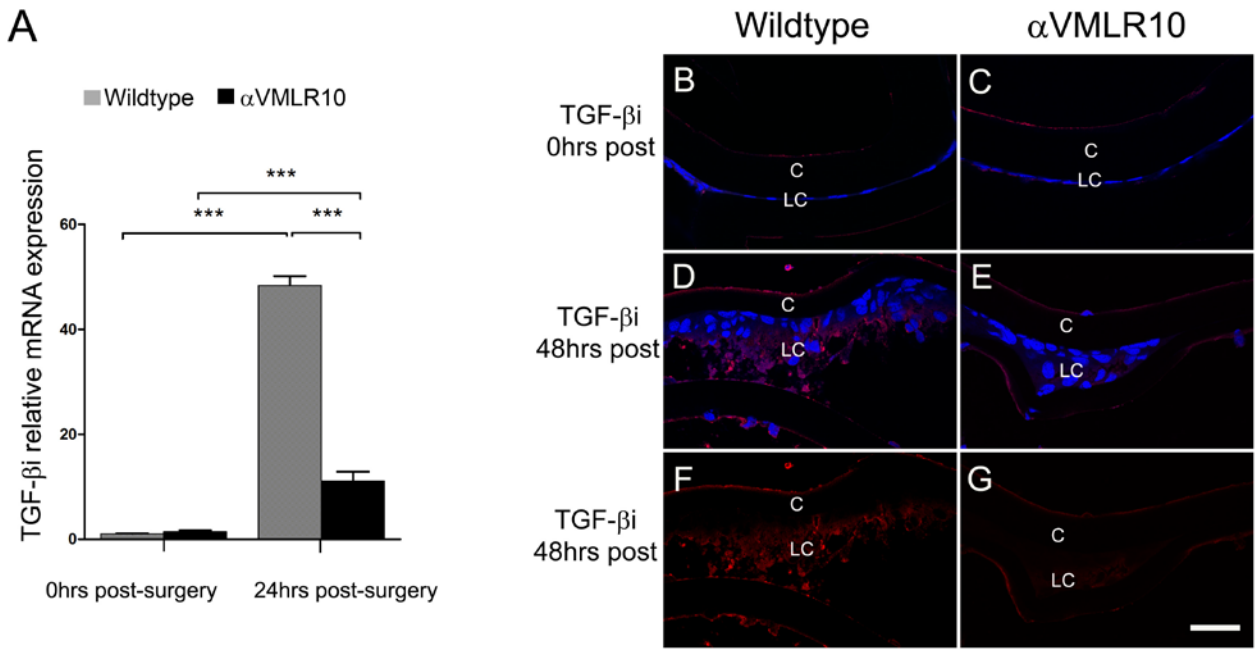


Figure 3.10: (**A**) RT-PCR quantitation of TGF-βi mRNA levels in wildtype and αVMLR10 mice residual lens epithelial cells (LCs) on capsular bags collected at 0hrs and 24hrs post-surgery showing almost no expression of TGF-βi mRNA at 0hrs post-surgery in both wildtype and αVMLR10 LCs but a significant increase in mRNA levels in both wildtype and αVMLR10 LCs ***P=0.0001. TGF-βi mRNA levels in wildtype LCs at 24hrs post-surgery was significantly higher than that of αVMLR10 LCs at 24hrs post-surgery (***P=0.001). mRNA expression was normalized to β2M and fold change differences were calculated based on 0hrs post-surgery wildtype mRNA expression. All experiments had n=5. Values are expressed as mean ± S.E.M. Asterisks (*) indicate statistically significant fold changes from 0hrs post-surgery. (**B**) Immunohistochemistry results showing TGF-βi protein expression 0hrs post-surgery in wildtype LCs and (**C**) αVMLR10 LCs. (**D**) TGF-βi expression in wildtype LCs at 48hrs post-surgery. (**E**)

TGF-βi expression in αVMLR10 LCs at 48hrs post-surgery. (**F**) TGF-βi expression alone in wildtype LCs at 48hrs post-surgery. (**G**) TGF-βi expression alone in αVMLR10 LCs at 48hrs post-surgery. Scale bar 35μm. Red = TGF-βi, blue = nucleus, LC = residual lens cells, C= lens capsule. Adapted from (Mamuya, Wang. et al. 2014).

3.3 Discussion

3.3.1 αV integrin is not necessary for the latter stages of lens development, morphology or the maintenance of lens optical quality

Integrins are heterodimeric transmembrane proteins best known as cellular receptors for diverse extracellular matrix proteins (Hynes 2002). In the normal lens, the expression of α_V integrin and its beta integrin partners have been previously reported, while mice lacking both α3 and α6 integrin (De Arcangelis, Mark et al. 1999) or β1 integrin from the lens (Simirskii, Wang et al. 2007) have profound lens epithelial defects; zebrafish lacking α_5 integrin have lens fiber cell(Hayes, Hartsock et al. 2012). After a careful analysis of α_V integrin protein expression during development, I found out that, despite the fact that the protein is detectable in the lens, particularly along the lateral membranes of both adult (Figure 3.3) and embryonic (not shown) lens fibers, lenses lacking αV integrins are transparent and morphologically normal when examined by both light and scanning electron microscopy.. However, αVMLR10 (αV $^{[-/flox]}$; MLR10-*cre*) lenses were significantly larger than wildtype lenses in early adulthood, although this difference disappeared by six months of age. Further investigation on this lens size difference implied that the larger size of αVMLR10 lenses was not the result of misregulation of water homeostasis as has been seen in some lens pathologies (Shiels, Bassnett S et al. 2001) since the dry/wet lens ratio is unchanged. Instead, it is likely that

α_V integrin plays a subtle role in regulating lens growth that was not revealed by proliferation analyses.

3.3.2 αV integrin proteins are upregulated in residual LCs by 48 hours after lens fiber cell removal

α_V integrin is the only α_- integrins subunit that is known to dimerize with more than two β–integrin subunits, making a class of six distinct heterodimeric proteins $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$ and $\alpha_V\beta_8$, which are known as promiscuous receptors for diverse ECM proteins associated with mesenchymal cells including fibronectin, vitronectin, tenascin C and TGF-βi (Kerr, Slee et al. 2002). α_V integrins commonly upregulate during EMT/tissue fibrosis and cancer and have been proposed to play diverse roles in this process in multiple cell types (Nemeth, Nakada et al. 2007). I found that the proteins levels of α_V integrin and four of five of its β-integrin partners (β1, β5, β6 and β8) are upregulated in LCs following fiber cell removal while β3 integrin was expressed at very low levels both before and after surgery. While few prior investigations of α_V integrin expression in the lens have been published, my findings are consistent with prior reports of elevated $\alpha_V\beta_5$ expression in an established human lens epithelial cell line treated with TGF-β, and elevated $\alpha_V\beta_6$ expression in post-operative human lens capsule and human primary LEC explants induced to undergo EMT in an in vitro PCO model (Sponer, Pieh et al. 2005, Dawes, Elliott et al. 2007).

However, while the protein levels of α_V integrins were robustly upregulated by 48 hours after lens fiber cell removal, the levels of α_V integrin mRNA and that of its β-

subunits were not, suggesting that this phenomenon is regulated at the level of either protein translation or protein stability.

miR-31 is a microRNA known for its ability to negatively regulate invasion-metastasis cascades in cancer progression by repressing the expression of proteins important for this process (Valastyan, Reinhardt et al. 2009, Sossey-Alaoui, Downs-Kelly et al. 2011). I found abundant miR-31 in the lens epithelium consistent with a prior report (Karali, Peluso et al. 2010). Adding to these findings, a recent study demonstrated that miR-31 can repress α_V integrin translation by directly binding to the 3'UTR of the α_V integrin mRNA (Augoff, Das et al. 2011). Since miR-31 levels decrease more than 50% by 24hrs post-surgery in wildtype mice (Figure 3.2: B), it is possible that the upregulation of α_V integrin protein levels by 48 hours after surgery is regulated via this downregulation of miR-31. This postulate can be tested in the future to gain insight into the earliest events occurring in LCs following lens fiber cell removal/cataract surgery.

3.3.3 αv integrin plays a crucial role in fibrotic type but not Pearl type PCO development

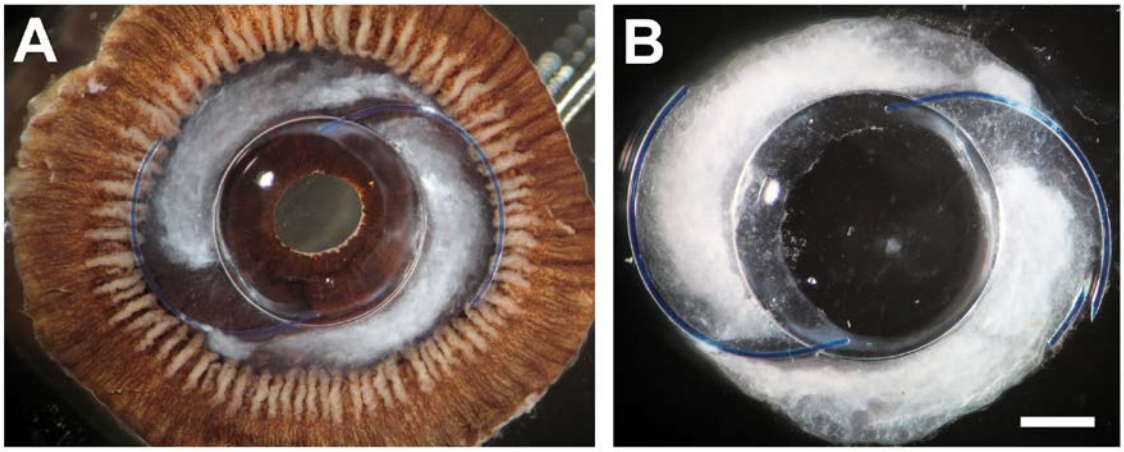
Clinically, two different types of PCO occur following cataract surgery, the 'fibrotic type' and the 'pearl type' (Figure 3.11). The fibrotic type is typically attributed to the migration of LCs into the optical path concomitant with their EMT resulting in these cells overexpressing αSMA, depositing mesenchymal ECM proteins and contraction of the posterior capsule leading to light scatter and visual disability

(Wormstone, Wang et al. 2009, Eldred, Dawes et al. 2011, van Bree, van der Meulen et al. 2011). In “pearl type" PCO, the LCs which migrate onto the posterior capsule enter the fiber cell differentiation pathway, presumably in an attempt to regenerate the lens. However, since they do not form the correct cellular organization for transparency, they instead induce light scattering (Dewey 2006). Finally, many designs of intraocular lens implants used in cataract surgery seek to trap residual lens epithelial cells at the lens equator, and these cells also often attempt to undergo lens fiber differentiation to form an opacity outside of the visual axis known as Soemmering’s ring (Kappelhof, Vrensen et al. 1987, Huang and Xie 2007) (Figure 3.11). Therefore, it is apparent that PCO arises from two distinct cellular responses to cataract surgery, with some LCs undergoing EMT, while others attempt fiber cell regeneration. (Marcantonio and Vrensen 1999, de Iongh, Wederell et al. 2005).

Since αV integrins are upregulated following fiber cell removal in a mouse model (Figure 3.1), I tested the response of αVMLR10 LCs to fiber cell removal. In wildtype mice, I noticed a robust increase in residual epithelial cell proliferation along with a significant increase in αSMA expression by 48 hours post-surgery. αSMA is often used as a hallmark marker for LCs undergoing EMT. In addition, wildtype LCs upregulate a number of TGF-β associated mesenchymal ECM proteins particularly fibronectin, tenascin-C, vitronectin and TGF-βi. Since all four ECM proteins have been reported to be ligands for αV integrins, these data suggest that a functional αV integrin/ ECM ligand network upregulates in LCs by 48 hours of surgery in this model. In contrast, αVMLR10 lenses, which lack αV integrins, do not exhibit robust LC

proliferation, do not upregulate αSMA expression and have greatly attenuated expression of the mesenchymal ECM molecules. These data suggest that αV integrins play an essential role in the early regulation of the EMT that occurs during the development of fibrotic PCO.

Figure 3.11: (A) IOL inside a human capsular bag attached to the zonular fibers as observed from the posterior side of the eye showing an extensive Soemmering's ring (B) The image of the IOL inside a human lens capsule after it was removed/detached from the zonular fibers.



At later times after surgery, wildtype LCs continue to proliferate, forming additional αSMA expressing myofibroblasts embedded in an ECM rich in fibronectin, tenascin-C and vitronectin (Figure 3.7). However, by five days post-surgery, not all cells express αSMA and islands of cells which instead express fiber cell markers become

obvious (Figure 3.8). Notably, αVMLR10 lenses still begin expressing fiber cell markers at this time although they still do not express appreciable levels of fibrotic markers. This suggests that αV integrin does not play a role in regulating the lens regenerative pathway which is activated post-surgery (Call, Grogg et al. 2004). This is consistent with my observation that αV integrin is also not involved in regulating normal lens fiber cell differentiation during development. Overall, these data point to a role for αV integrin in regulating pathways that are critical for the establishment of fibrotic, but not pearl type PCO.

3.3.4 The loss of αV integrin impairs TGF-β signaling post-surgery

Treatment of LCs with TGF-β in vitro can induce most cellular and molecular changes associated with fibrotic PCO, including myofibroblast formation, the expression of fibrotic ECM proteins and LC proliferation and capsule wrinkling (de Iongh, Wederell et al. 2005, Dawes, Sleeman et al. 2009, Wormstone, Wang et al. 2009) while transgenic mice overexpressing an active form of TGF-β in lens fiber cells develop anterior subcapsular cataracts which share many features with fibrotic PCO (Lovicu, Schulz et al. 2002).

TGF-β can mediate its biological effects via canonical (SMAD2/3 dependent) signaling or through non-canonical pathways (SMAD-independent signaling). My results show that SMAD-3 activation (SMAD-3 phosphorylation) which has been previously shown to be important for LEC EMT in a lens injury model (Saika, Okada et al. 2001, Saika, Miyamoto et al. 2002, Saika, Kono-Saika et al. 2004, Wederell and

de Iongh 2006, Dawes, Sleeman et al. 2009), is not appreciably detected until 48 hours after surgery in our model, coincident with the upregulation of αV integrin protein expression. Further, the expression of TGF-βi, a known direct transcriptional target of pSMAD-3 (Jeon, Kim et al. 2012), is also upregulated at the protein level around this time frame. The levels of SMAD-3 phosphorylation then continue to increase at later times post-surgery consistent with TGF-β activation increasing through five days post-surgery in wildtype mice. Notably, no appreciable SMAD-3 phosphorylation is detected in LCs lacking αV integrins (Figure 3.9). Further, while the mRNA levels of TGF-βi upregulated to a certain extent in αV integrin null lenses at 24 hours post-surgery, this upregulation was insufficient to deposit detectable levels of TGF-βi protein in the ECM by 48hrs post-surgery, suggesting that αV integrins are playing a fundamental role in regulating the TGF-β pathway post lens injury/cataract surgery.

3.3.5 αV integrins may be playing a role in activating TGF-β signaling during lens EMT

All three isoforms of TGF-β; TGF-β1, 2, and 3 are synthesized by lens cells *in vivo*, and the latent forms of these molecules are abundant in the aqueous and vitreous humor of the eye (Lee and Joo 1999). TGF-βs are activated by numerous cellular mechanisms, all of which result in liberation of the active TGF-β molecule from its latency associated peptide (LAP) /Latent TGF-β binding proteins (LTBPs). Notably, αV integrins can activate latent TGF-β by at least three distinct mechanisms (Wipff, Rifkin et al. 2007, Wipff and Hinz 2008, Mamuya and Duncan 2012). αV integrins can

bind to an RGD sequence present in the LAP of either TGF-β1 or TGF-β3, inducing these molecules to undergo a conformational change to liberate the active TGF-β molecule (Munger, Huang et al. 1999, Annes, Rifkin et al. 2002, Ludbrook, Barry et al. 2003, Annes, Chen et al. 2004). αV integrins can also interact with matrix metalloproteases (MMPs), particularly MMP2 and MMP9, which tethers them to the cell surface promoting proximity of MMPs to the LAP and sequesters the large latent complex (LLC) close to the type II TGF-β receptor (Brooks, Stromblad et al. 1996, Mu, Cambier et al. 2002, Rolli, Fransvea et al. 2003).

Alternatively, cross-talk between TGF-β and αV integrin signaling can occur downstream of initial receptor activation and regulate various cellular processes (Cary, Han et al. 1999). Signals propagated intracellularly by integrin associated adaptor proteins such as ILK, Src, PTKs and FAK can subsequently activate other downstream TGF-β induced EMT and cell proliferation players such as MAPK, Ras/Rho, small GTPases, PI3K and AKT (Dedhar 1999, Zhang 2009). Altogether, all of these pathways may override the normal brakes on TGF-β signaling levels resulting fibrotic PCO. This suggests that functional blocking of αV integrins may prevent fibrotic PCO pathogenesis and possibly other TGF-β associated lens fibrotic disorders such as ASC. This is discussed in chapter five.

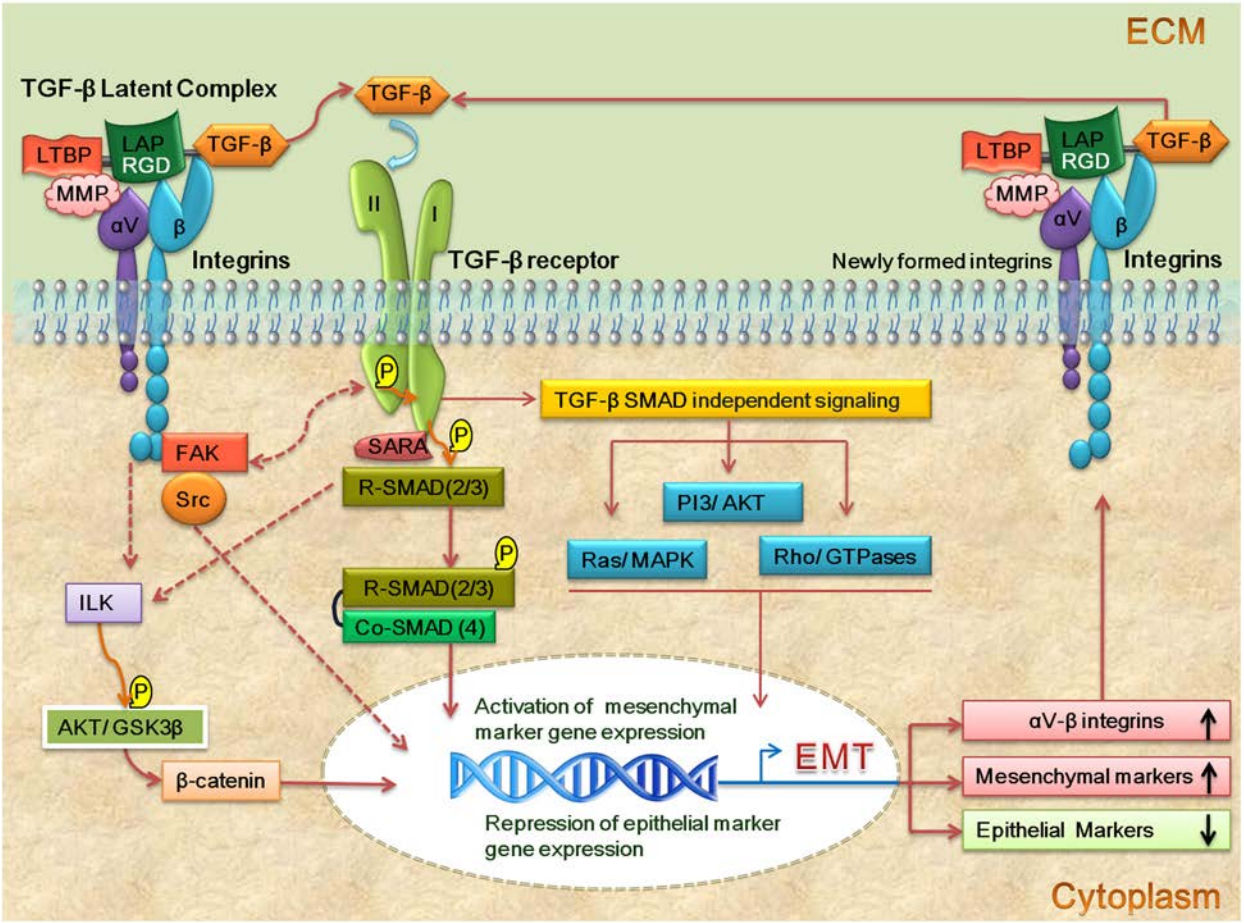


Figure 3.12: αV integrins recognize a RGD motif present in the LAP of TGF-β. This binding induces either adhesion-mediated cell forces and/or brings latent TGF-β into the proximity of MMPs, which consequently lead to the liberation/activation of the TGF-β homodimer from its latent complex (Yu and Stamenkovic 2000, Dallas, Rosser et al. 2002). Upon activation, the TGF-β homodimer will bind to the Type II TGF-β receptor initiating TGF-β-Smad signaling which upregulates the expression of αV integrins in addition to that of other EMT markers (Boudreau and Jones 1999, Wu, Chen et al. 2000, Imamichi and Menke 2007). These newly formed integrins can liberate more TGF-β from its latent complex;

sustaining and reinforcing TGF-β induced EMT progression. This cooperative feed forward loop between αV integrins and TGF-β can lead to the unregulated TGF-β signaling responsible for a number of TGF-β-associated disorders. Adapted from (Mamuya and Duncan 2012).

Chapter 4

CHARACTERIZATION OF EMT IN CRYBB2 ^{PHIL} MUTANT MICE

4.1 Introduction

The mammalian lens, also known as the crystallin lens, is a transparent tissue that focuses light onto the retina where further vision processing occurs. In order to perform this task, the crystallin lens must maintain a high refractive index and remain transparent throughout the lens' life (Hung 2001). This is achieved by the high concentration of water soluble crystallin protein found in the lens (300mg/ml) (Fagerholm, Philipson et al. 1981), which is about 30-35% w/w compared to an average of 15% (w/w) in other tissues (Donaldson, Kistler et al. 2001, Purves, Sadava et al. 2004, Hoehenwarter, Klose et al. 2006). In mammals, these proteins mostly belong to the two superfamilies of lenticular crystallins, α and β/γ crystallins, which comprise about 90% of the proteins found in the lens (Andley 2007). A detailed overview of lenticular crystallins is discussed in Chapter 1.

For the past two decades, there has been much progress made towards understanding crystallins' function in the mammalian lens (Slingsby, Wistow et al. 2013). Mutations in both αA and αB -crystallins can cause cataract and myopathy (Horwitz 2003). They are expressed in both lens epithelial and fiber cells, and were for many years thought to only play refractive functions in lens. It is now very well established that both αA and αB-crystallins possess chaperone-like functions and are members of the small heat-shock protein family (Horwitz 1992). Like any other

members of the small heat-shock proteins, they are also found outside the lens having an extensive tissue distribution (de Jong, Caspers et al. 1998). Currently, their non-refractive roles in lens and other tissues are well established (Boyle and Takemoto 2000, Xi, Bai et al. 2003, Wang, Garcia et al. 2004, Morozov and Wawrousek 2006).

However, the majority of lenticular crystallins expressed in the lens are members of the β and γ crystallin superfamily. Although both β and γ crystallin proteins have been found in other tissues beyond lens, very little is known about their functions beyond lens refractive roles (Ueda, Duncan et al. 2002, Sathish, Koteiche et al. 2004). Recent studies have proposed possible non refractive roles of β-crystallins, particularly in ocular stress response (Bohm, Melkonyan et al. 2013), as they are found to upregulate in response to diverse types of retinal damage such as mechanical injury, light injury and even diabetic retinopathy (Sakaguchi, Miyagi et al. 2003, Vazquez-Chona, Song et al. 2004, Kumar, Haseeb et al. 2005). βA3/A1-crystallin, an abundant β-crystallin protein in the lens fibers is also expressed outside of the lens and has been proposed to have non-refractive roles (Aarts, Lubsen et al. 1989, Parthasarathy, Ma et al. 2011). A spontaneous mutation in the Cryba1 gene, coding for *β*A3/A1-crystallin, was found to inhibit the normal denucleation of lens fibers (Sinha, Hose et al. 2005), while another study proposed that *β*A3/A1-crystallin plays diverse lysosomal functions in the RPE (Zigler, Zhang et al. 2011), suggesting essential roles of *β*A3/A1-crystallin in ocular homeostasis and function. *β*A3/A1-crystallin is also expressed by astrocytes in the neural retina where it is believed to play an essential role in the migration, proliferation and patterning of retinal astrocytes (Sinha, Klise et al. 2008, Zhang, Asnaghi et al.

2011). Furthermore, that loss of βA3/A1-crystallin induces IGF-II and increases cell survival by regulating the PI3K/AKT/mTOR and ERK pathways, thereby protecting astrocytes from anoikis-mediated cell death (Ma, Sen et al. 2011).

On the other hand, βB2-crystallin is the most abundant protein in the adult mammalian lens (Jobby and Sharma 2007). It is proposed to play a major role in maintaining the solubility of other β-crystallins (Bateman and Slingsby 1992, Zhang, David et al. 2001). Not surprisingly, βB2-crystallin mutant and knockout mice are known to develop cataract (Uga, Kador et al. 1980, Chambers and Russell 1991, Zhang, Li et al. 2008). In humans, impairments of βB2-crystallins' structure are known to contribute to both age-related and inherited progressive cataract conditions (Chambers and Russell 1991, Graw, Klopp et al. 2001, Graw, Loster et al. 2001, Ueda, Duncan et al. 2002, Duprey, Robinson et al. 2007). For example, Q155X mutation is a βB2-crystallin mutation in the known to cause human cataracts. It is characterized by the conversion of 475C → T, which creates a stop codon and prevents the translation of the final 51 amino acids of the normal protein (Bateman, von-Bischhoffshaunsen et al. 2007). The Q155X mutation has been found in several unrelated families, causing a variety of cataract phenotypes (Vanita, Sarhadi et al. 2001), with cataract formation beginning prenatally or during childhood, but usually becoming severe only during adulthood (Litt, Carrero-Valenzuela et al. 1997).

βB2-crystallin is expressed in lens epithelial and fiber cells, as well as other tissues outside the lens where like other crystallins; it is proposed to have functions beyond lens refractive (Magabo, Horwitz et al. 2000, Duprey, Robinson et al. 2007,

Ganguly, Favor et al. 2008). For example, mice lacking the βB2-crystallin gene experience reduced fertility due to disordered proliferation and apoptosis of their germ cells in the testis (Xiang, Cui et al. 2012). βB2-crystallin studies outside of lens provide substantial evidence of βB2-crystallin roles in axonal elongation in retinal ganglion cells during retinal regeneration (Bohm, Melkonyan et al. 2013), suggesting potential roles in neurodegenerative diseases (Liedtke, Schwamborn et al. 2007). Yet again, the mechanistic basis of these proposed non-refractive functions, are not well understood (Slingsby, Wistow et al. 2013).

To address the roles of βB2-crystallin, a spontaneous βB2-crystallin mutant mouse known as Crybb2$^{Phil/Phil}$ that was first discovered as an autosomal dominant cataract mutant have been investigated since the late 1970's. This mouse harbors a 12-nucleotide in-frame deletion in the fourth Greek key of the βB2-crystallin locus leading to four-amino acid-loss that results in inherited cataract (Chambers and Russell 1991). Morphological studies on this βB2-crystallin mutant noted a number of lens defects, including failure of lens fiber cells to denucleate during differentiation as well as an increase in intercellular space in the posterior fiber cells. Furthermore, Crybb2$^{Phil/Phil}$ is characterized by a significant decrease in lens size (Uga, Kador et al. 1980). It was later established that this mutation causes destabilization of βB2-crystallin along with precipitation of other crystallins in the lens, leading to a more severe phenotype than just cataracts (Chambers and Russell 1991).

Previous students in our lab, Megan Fisher and Corrine Decker, reevaluated the Crybb2$^{Phil/Phil}$ cataract phenotype. Unlike many known mutations associated with

cataract, they found that Crybb2$^{Phil/Phil}$ cataract is associated with induction of fibrotic lens EMT that is very similar to that observed in ASC and PCO (Figure 4.5). Since in *vitro* and in *vivo* model for ASC have been routinely used in studying PCO (de Iongh, Wederell et al. 2005, Martinez and de Iongh 2010), I utilized these mice to further understand the role of αV integrins in the TGF-β associated fibrotic lens EMT that leads to PCO. Furthermore, since βB2-crystallin has been proposed to engage functions that are beyond lens refraction, these findings may open a door towards the understanding of mechanism behind βB2-crystallin's non-refractive roles.

4.2 **Results**

4.2.1 **Crybb2$^{Phil/Phil}$ adult lenses develop cataracts, significant decrease in size and undergo a severe lens epithelium disorganization**

Similar to the previous morphological studies performed on Crybb2$^{Phil/Phil}$ lenses (Uga, Kador et al. 1980), we found that Crybb2$^{Phil/Phil}$ lenses are morphologically normal and transparent one week after birth and remain transparent until about 2 months of age. In order to reevaluate the morphological changes associated with this mutation, 4-month-old eyes from Crybb2$^{Phil/Phil}$, Crybb2$^{+/Phil}$ and wildtype were dissected; lenses isolated and visualized by dark field microscopy. Both Crybb2$^{Phil/Phil}$ and Crybb2$^{+/Phil}$ lenses were significantly clouded by opaque masses in the lens when compared to wildtype, while Crybb2$^{Phil/Phil}$ lens also were much smaller than either wildtype or Crybb2$^{+/Phil}$ (Figure 4.1. A-C). Notably, H&E staining showed defects in not just the lens fibers of the Crybb2$^{Phil/Phil}$ lens, but also severe abnormalities in the lens epithelium. Further, similar abnormalities were also found in small focal spots in Crybb2$^{+/Phil}$ lenses (Figure 4.1. D-F).

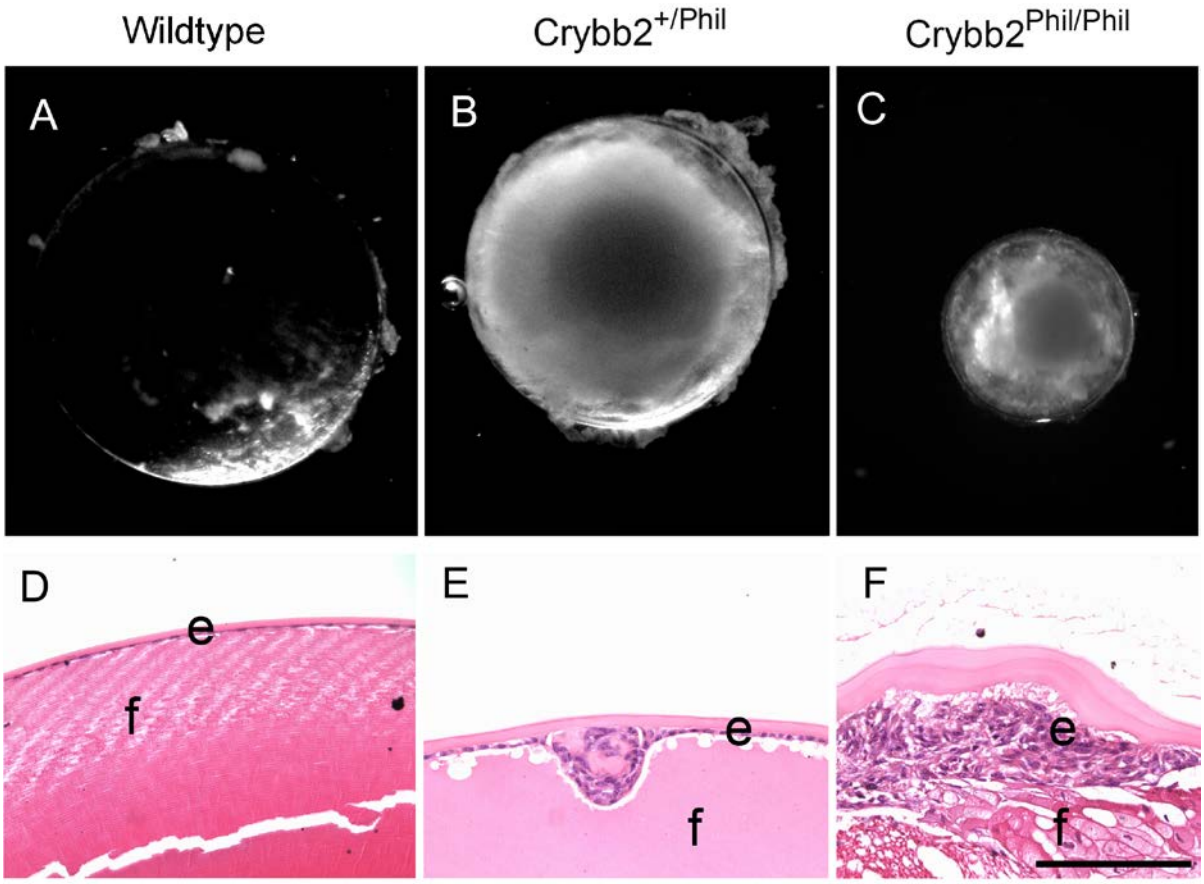


Figure 4.1: Gross morphology of 4-month-old adult lenses: (**A**) Dark field image showing a wildtype lens maintaining transparency through adulthood. (**B**) A heterozygous mutant lens (Crybb2+$^{/Phil}$) showing a total cortical cataract associated with a moderately smaller lens as compared to wildtype. (**C**) A Crybb2$^{Phil/Phil}$ lens displaying a total cataract and severe reduction in size compared to either wildtype or heterozygous mutant lens. H&E staining showing: (**D**) A normal wildtype adult lens showing a normal lens epithelium and organized mass of elongated fiber cells (**E**) A Crybb2$^{+/Phil}$ lens disrupted fiber cells organization and a focal abnormality of the lens epithelium. (**F**) Abnormal lens histology of Crybb2$^{Phil/Phil}$ showing epithelial fibrosis and disrupted fiber cell organization.

Scale A, B, C =1mm. D, E, F = 0.125mm, pink = cytoplasm blue = cell nucleus, e = lens epithelium, f = lens fibers. (Image adapted from Mamuya, F.A., et al, manuscript in preparation)

4.2.2 The Crybb2$^{Phil/Phil}$ lenses have abnormal βB2-crystallin expression since birth

QRT-PCR revealed that βB2-crystallin mRNA expression changed as the lens ages (Figure 4.2. A). At 1 week of age, βB2-crystallin mRNA was almost undetected in wildtype lenses but increased significantly at 3 weeks of age and peaked at 2 months postnatal. However, Crybb2$^{Phil/Phil}$ lenses had almost a 30 fold higher than normal βB2-crystallin mRNA expression at 1 week of age. Although, βB2-crystallin mRNA peaks at 2 months postnatally in wildtype mice, in Crybb2$^{Phil/Phil}$ mice, βB2-crystallin mRNA levels are almost 10 fold lower than seen in wildtype lenses at the same age. At 4 months of age, the levels of βB2-crystallin mRNA expression eventually fall dramatically in both wildtype and Crybb2$^{Phil/Phil}$ lenses.

A previous student in our lab, Ms. Corinne Decker, looked at βB2-crystallin protein expression levels at birth. Immunohistochemistry analysis showed that βB2-crystallin was expressed in the lens fibers of both wildtype and Crybb2$^{Phil/Phil}$ lenses. Interestingly, in Crybb2$^{Phil/Phil}$ lenses, the protein was overexpressed in lens fibers as well as in lens epithelial cells. (Figure 4.2. B-C). This overexpression of βB2-crystallin in Crybb2$^{Phil/Phil}$ lens epithelium is also seen at 2 months of age when most of the lens fiber cell βB2-crystallin is comparatively low (Figure 4.2. D-E).

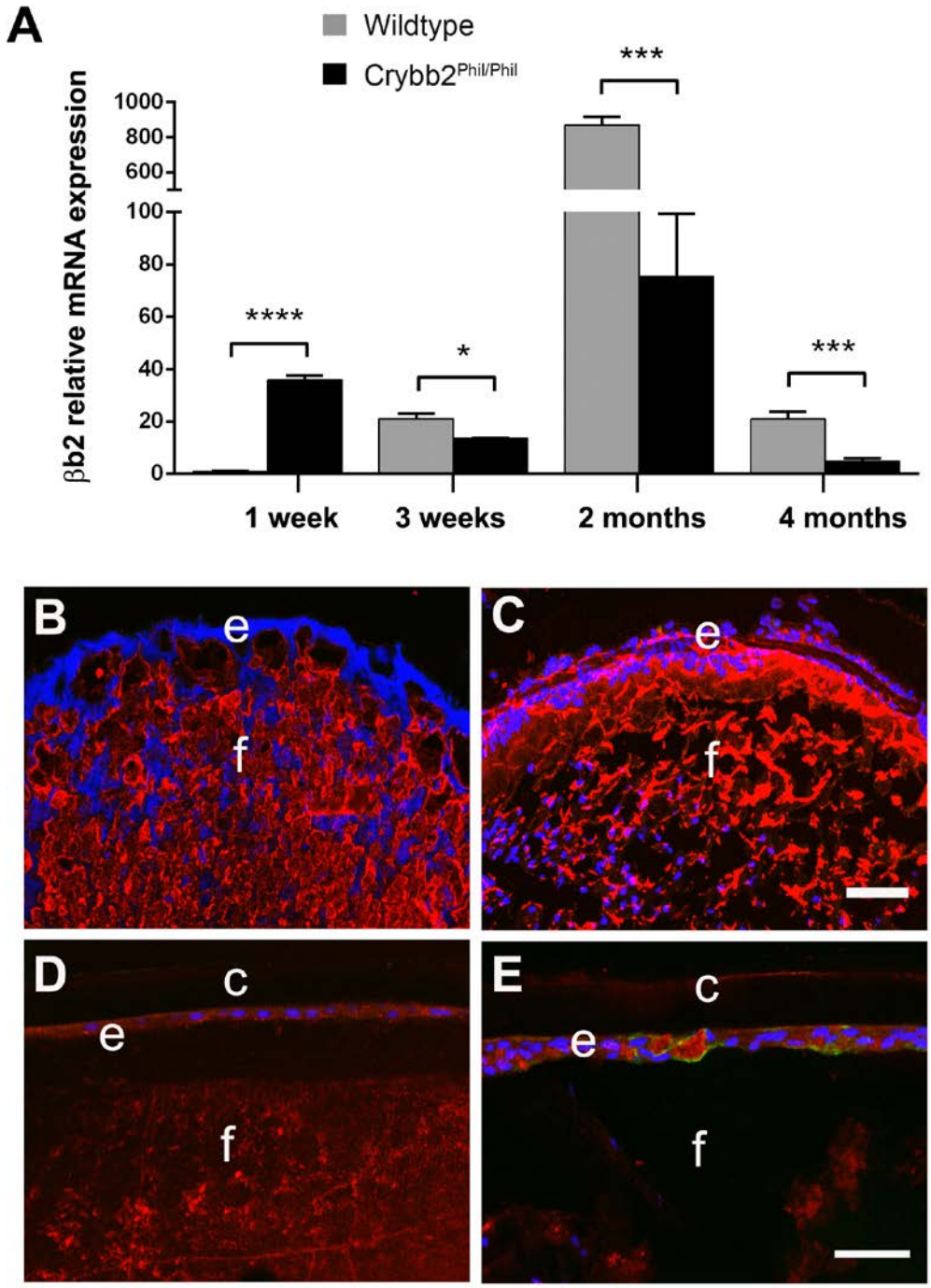


Figure 4.2: Relative expression of βB2-crystallin mRNA in lens epithelium over time. (**A**) At 1 week of age, βB2-crystallin mRNA is almost

undetected in wildtype lenses but significantly higher in Crybb2$^{Phil/Phil}$ lenses, with an almost 40 fold upregulation (****p=0.0001). At 3 weeks of age, βB2-crystallin mRNA expression in wildtype lenses dramatically increases, surpassing that of Crybb2$^{Phil/Phil}$ lenses (*p=0.02). The peak of βB2-crystallin mRNA expression in normal lenses is at around 2 months of age and is almost 10 fold higher in wildtype lenses compared to Crybb2$^{Phil/Phil}$ lenses (***p=0.0001). βB2-crystallin mRNA expression eventually begins to falls off at around 4 months of age. However, at this time, there was still a significant elevation of expression in wildtype lenses (***p=0.006). βB2-crystallin protein expression in wildtype and Crybb2$^{Phil/Phil}$ over time: (**B**) Wildtype newborn lens expresses βB2-crystallin only in the fiber cell mass. (**C**) Crybb2$^{Phil/Phil}$ newborn lens expression appears to be upregulated and more dispersed in the epithelial cell layer of the mutant lens compared to the wildtype lens. (**D**) 2-month-old wildtype lens showing normal βB2-crystallin expression in both lens epithelium and fiber cells (**E**) 2-month-old Crybb2$^{Phil/Phil}$ showing overexpression of βB2-crystallinin the lens epithelium will with remote expression in lens fiber cell. Scale bar: B-C= 65μm, D-E =38μm, red = βB2-crystallin, green = αSMA, blue = cell nucleus, e = lens epithelium, C = lens capsule, f = lens fibers. (Image adapted from Mamuya, F.A., et al, manuscript in preparation)

4.2.3 Collagen 1α1 and αSMA mRNA are overexpressed in Crybb2$^{Phil/Phil}$ lens epithelium over time

In order to confirm that the EMT in Crybb2$^{Phil/Phil}$ lenses is similar to that established in ASC, PCO and in our mouse cataract surgery model, which have all shown to upregulate αSMA and collagen-1α1gene expression, I performed a QRT-PCR analysis to investigate the expression of these two genes in the lens epithelium. I found out that αSMA mRNA expression was significantly upregulated in Crybb2$^{Phil/Phil}$ lenses as early as 2 months of age. This upregulation remained relatively high throughout 4 months of age as compared to wildtype lenses (Figure 4.3. A). Further, normal lens epithelial cells predominantly deposit type IV collagen, however as they transform to a more mesenchymal state, they start to deposit collagen type I (Leask and Abraham 2004). I found collagen-1 α1, whose mRNA expression was relatively low in both wildtype and Crybb2$^{Phil/Phil}$ lenses at 1 week of age, increased dramatically in 2-month-old Crybb2$^{Phil/Phil}$ lenses and remained significantly high throughout 4 months of age in Crybb2$^{Phil/Phil}$ as compared to wildtype (Figure 4.3. B).

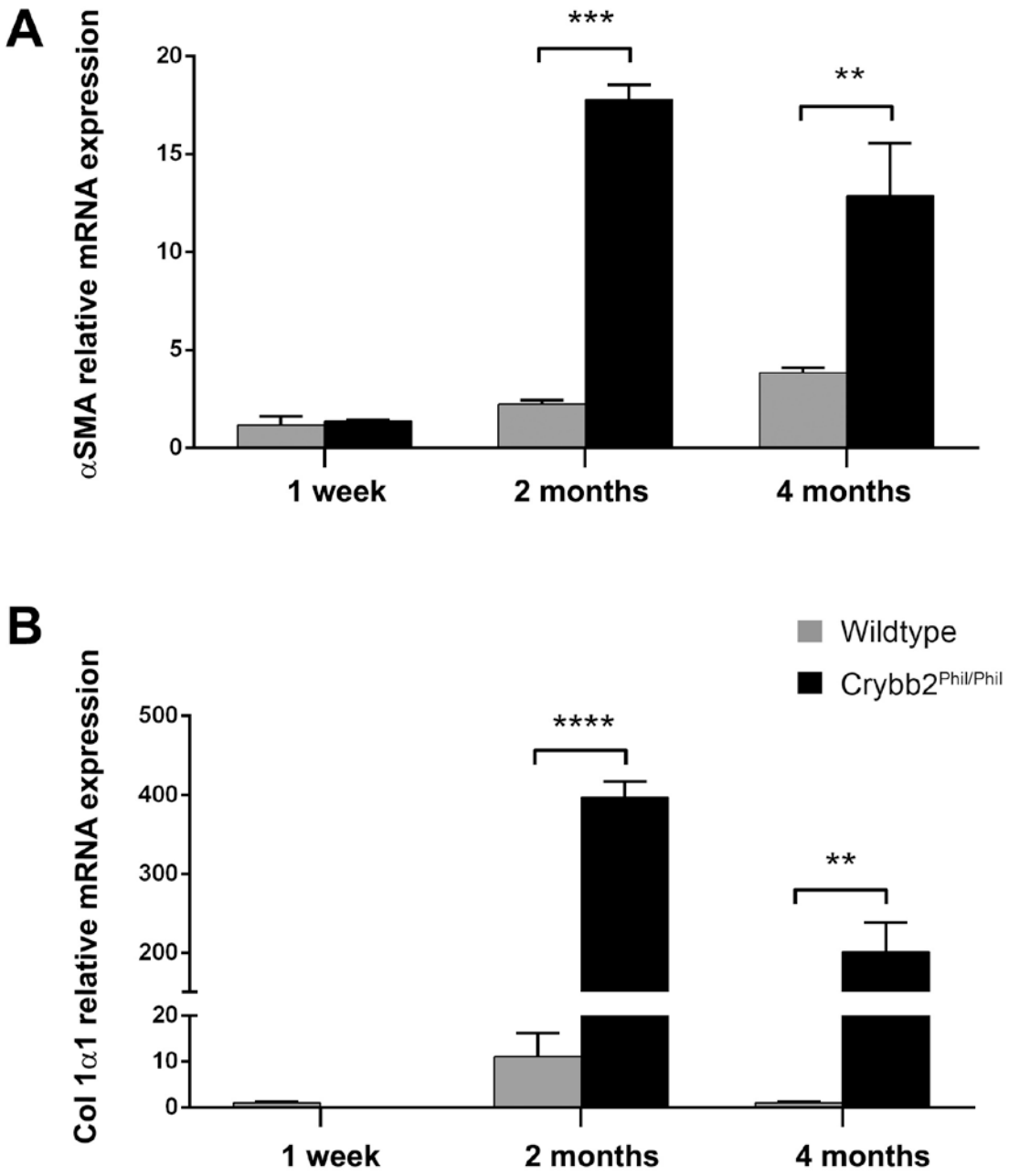


Figure 4.3: αSMA and Collagen-1α1 mRNA expression in wildtype and Crybb2$^{Phil/Phil}$ lens epithelium over time: (**A**) A graph showing a gradual increase in αSMA mRNA expression of in Crybb2$^{Phil/Phil}$ lens epithelium. At 1 week of age, αSMA mRNA levels in Crybb2$^{Phil/Phil}$ lens is approximately equal to that of

wildtype. At 2 months of age, αSMA mRNA levels in Crybb2$^{Phil/Phil}$ lenses are significantly higher than wildtype lenses (***p=0.0001), and was still significant higher at 4 months of age (**p=0.005). (**B**) A graph showing gradual increase in collagen 1α1 mRNA expression in Crybb2$^{Phil/Phil}$ lens epithelium. At 1-week of age, collagen 1α1 mRNA levels in Crybb2$^{Phil/Phil}$ lenses are approximately equal to that of wildtype. By 2 months of age, collagen 1α1 mRNA expression in Crybb2$^{Phil/Phil}$ lenses is significantly higher (almost 100-fold) when compared to wildtype (****p=0.0001), and continue to stay significantly high (almost 200-fold upregulated) at 4 months of age (**p=0.006). (Image adapted from Mamuya, F.A., et al, manuscript in preparation)

The αSMA upregulation in Crybb2$^{Phil/Phil}$ lenses was also confirmed by confocal imunohistochemistry analysis, which showed a completely disorganized Crybb2$^{Phil/Phil}$ lens epithelium overexpressing αSMA by 4 months of age. At this age, even the Crybb2$^{+/Phil}$ showed a slight increase in αSMA protein expression, although, this was only found in small fibrotic plaques (Figure 4.4. A-C). Confocal analysis on whole mount 4-month old lens epithelium stained by phalloidin revealed a loss of polymerized filamentous F-actin and severe disorganization in Crybb2$^{Phil/Phil}$ lens epithelium. This disorganization was mildly seen the Crybb2$^{+/Phil}$ lens epithelium as compared to the organized actin filaments seen in the same age wildtype lens epithelium (Figure 4.4. D-F). Since, LECs undergoing EMT are characterized by their fibroblastic morphology (Hales, Schulz et al. 1994, Zuk and Hay 1994), David Scheiblin examined the

morphological changes in Crybb2$^{Phil/Phil}$ lenses as they undergo EMT using SEM. We saw a progressive lens epithelial cell fibrosis, distortion and some form of multi-layering in the Crybb2$^{Phil/Phil}$ as the lens aged which eventually covered the entire epithelium at about 4 month of age (Figure 4.4. G –I).

4.2.4 The lens epithelial cell marker Pax-6 is upregulated in Crybb2$^{Phil/Phil}$ EMT.

Pax-6 is an evolutionarily conserved transcription factor known for its role in regulating eye and brain development in humans, mice, zebrafish, and *Drosophila* (Callaerts, Halder et al. 1997). It also controls transcriptional expression of genes encoding transcription factors responsible for lens development and maintenance of lens epithelial cell morphology (Chauhan, Reed et al. 2002). Notably, Pax-6 is known to downregulate as lens epithelial cells lose their epithelial properties during fiber cell differentiation (Madhavan, Haynes et al. 2006). We found that the protein is localized in the wildtype lens epithelium (Figure 4.5. A-C) but downregulated as epithelial cells undergo EMT in at least 3 days post surgery in our mouse model of cataract surgery (Figure 4.5. G-I). Immunohistochemistry staining on Pax-6 performed by Corrine Decker surprisingly showed that Pax-6 was upregulated in the Crybb2$^{Phil/Phil}$ epithelium as early as 4 weeks postnatal in cells overexpressing αSMA (Figure 4.5. D-F).

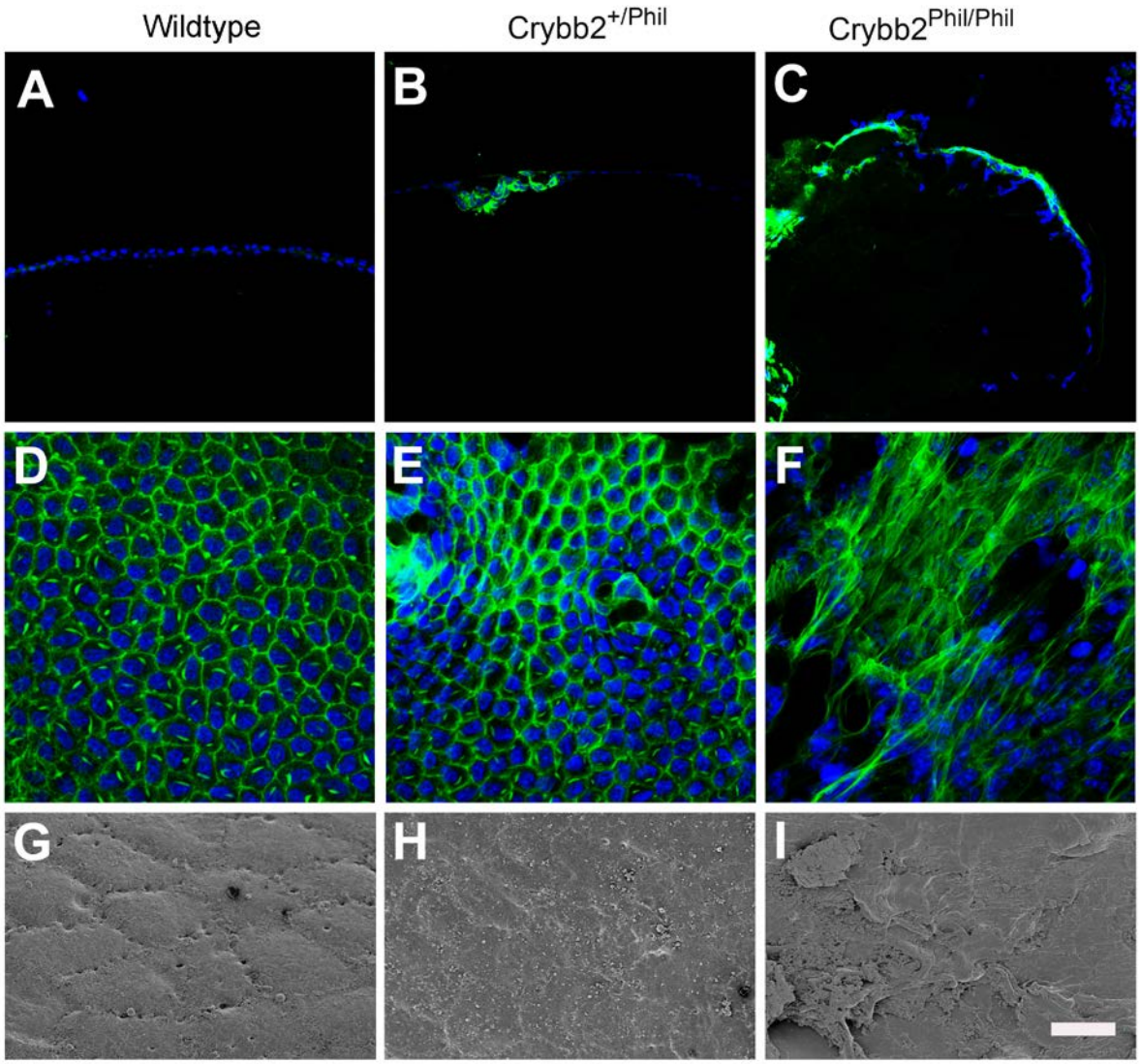


Figure 4.4: Expression of αSMA at 2 months postnatal: (**A**) Immunohistochemistry showing undetected levels of αSMA with a maintained epithelial cell organization in wildtype lens. (**B**) Crybb2$^{+/Phil}$ (heterozygote) epithelium shows small fibrotic plaque EMT with αSMA expression. (**C**) Uniformly malformed Crybb2$^{Phil/Phil}$ epithelium showing extreme αSMA expression. Scale bar: (A-C) 75µm,, Green = αSMA, blue = cell nuclei, e = lens epithelium, f = lens fibers. Phalloidin staining of a 2-month-old

whole mount lens epithelium: (**D**) Whole mount wildtype lens epithelium showing a normal cell shape with uniform F-actin organization. (**E**) Whole mount Crybb2$^{+/Phil}$ lens epithelium showing the appearance of fibrotic plaques, misshapen lens epithelium and a moderate level of F-actin disorganization. (**F**) Uniformly malformed Crybb2$^{Phil/Phil}$ epithelium showing the severity of epithelial fibrosis and F-actin disorganization. Scanning Electron Microscopy analysis showing: (**G**) Wildtype lens anterior epithelium. (**H**) Crybb2$^{+/Phil}$ lens anterior epithelium with a mild abnormality. (**I**) Crybb2$^{Phil/Phil}$ lens epithelium with progressive lens fibrosis and distortion. Scale bar: (D-F) = 30μm, (G-I) =6μm. Green = F-actin, blue = cell nuclei. (Image adapted from Mamuya, F.A., et al, manuscript in preparation)

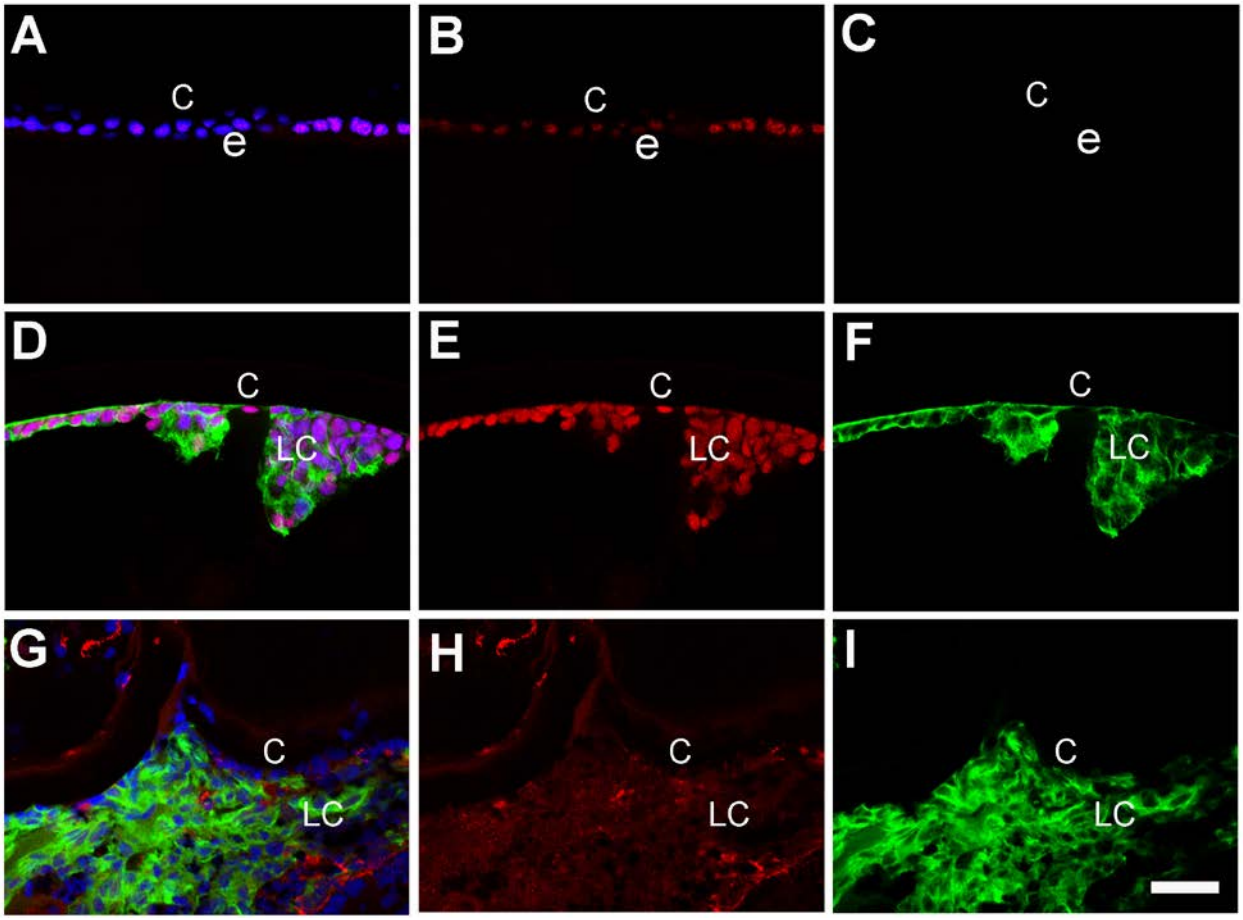


Figure 4.5: Pax-6 and αSMA expression in the 4-week old lens epithelium and in 3-days post-surgery (A model of lens injury response after cataract surgery): (**A**) Wildtype Pax-6 + αSMA +nucleus staining. (**B**) Wildtype Pax-6 alone. (**C**) Wildtype αSMA alone. (**D**) Crybb2$^{Phil/Phil}$ Pax-6 + αSMA +nucleus staining, Showing Crybb2$^{Phil/Phil}$ lens epithelial cells become multilayering as seen in wildtype lenses post-surgery. (**E**) Crybb2$^{Phil/Phil}$ Pax-6 alone, showing overexpression of Pax-6 (**F**) Crybb2$^{Phil/Phil}$ αSMA alone showing that Crybb2$^{Phil/Phil}$ lenses undergo EMT similar to that observed post-surgery (**G**) Wildtype lens 3-days post-lentectomy Pax-6 + αSMA +nucleus staining. (**H**) Wildtype lens 3-days post-lentectomy pax-6 alone (**I**) Wildtype lens 3-days post-lentectomy

αSMA alone. Scale = 38μm. Red = Pax-6, green = αSMA, blue = cell nucleus, LC= lens cells, C= lens capsule. (Image adapted from Mamuya, F.A., et al, manuscript in preparation)

4.2.5 Crybb2$^{Phil/Phil}$ lenses have a significantly elevated amount of active TGF-β compared to wildtype lenses

The overexpression of αSMA, collagen 1α1 and lens epithelial cell multilayering in Crybb2$^{Phil/Phil}$ lenses confirmed that Crybb2$^{Phil/Phil}$ lenses undergo an epithelial EMT similar as that seen in ASC as well as in wildtype lens post surgery. Since it is well established that TGF-β is the key player in lens EMT (de Iongh, Wederell et al. 2005), Megan Fisher, a previous student in our lab, used a TGF-β/BMP Signaling Pathway RT^{2}Profiler^{TM} PCR to examine the mRNA expression of TGF-β associated genes involved in this EMT pathway between 4-month-old Crybb2$^{Phil/Phil}$ lens EMT compared to the wildtype. Her results suggested that a number of TGF-β signaling associated genes were upregulated in 4-month-old Crybb2$^{Phil/Phil}$ lenses (Table 4.1).

To examine whether the Crybb2$^{Phil/Phil}$ lenses have more activated free TGF-β ligand than wildtype lenses, I redesigned a bioassay capable of measuring the level of active TGF-β signaling in a specific tissue (Khan, Joyce et al. 2012). I found a significantly elevated amount of active TGF-β in Crybb2$^{Phil/Phil}$ lens compared to wildtype lenses. On average, I found that a single 3-month-old wildtype lens contains about 100pg/ml of active TGF-β. In contrast, Crybb2$^{Phil/Phil}$ lens had, on average, almost 4 times the amount of active of TGF-β compared to the wildtype lens (Figure 4.6. A).

In addition, I found a dramatic increase in SMAD-3 phosphorylation in Crybb2$^{Phil/Phil}$ 3-month-old lenses when compared to wildtype (Figure 4.6. B-C).

Table 4.1. TGF-β/BMP Signaling Pathway RT^{2}Profiler^{TM} PCR to examine the mRNA expression of TGF-β associated genes involved in this EMT pathway between 4-month-old Crybb2$^{Phil/Phil}$ lens EMT compared to the wildtype. (Table adapted from Mamuya, F.A., et al, manuscript in preparation)

	Gene name	**Abbreviation**	**Fold change in Crybb2$^{Phil/Phil}$**	**pValue *vs.* Widltype**
1	Latent TGF-beta binding protein 4	Ltbp4	26.2	0.00003
2	Latent TGF-beta binding protein 2	Ltbp2	15.1	0.0006
3	Transforming growth factor, beta receptor 2	Tgfbr2	12.2	0.02
4	Plasminogen activator inhibitor-1	PAI-1	11.5	0.004
5	Latent TGF-beta binding protein 1	Ltbp1	2.7	0.02
6	Receptor-regulated SMAD3	Smad3	2.2	0.13
7	Receptor-regulated SMAD2	Smad2	1.1	0.80
8	Common-mediator SMAD4	Smad4	-3.3	0.02
9	Transforming growth factor beta-1	Tgfb1	-1.0	0.98
10	Transforming growth factor beta-2	Tgfb2	-1.4	0.20
11	Transforming growth factor beta-3	Tgfb3	-1.4	0.80
12	Transforming growth factor, beta receptor I	Tgfbr1	-1.3	0.39
13	Transforming growth factor, beta receptor 3	Tgfbr3	-6.2	0.06

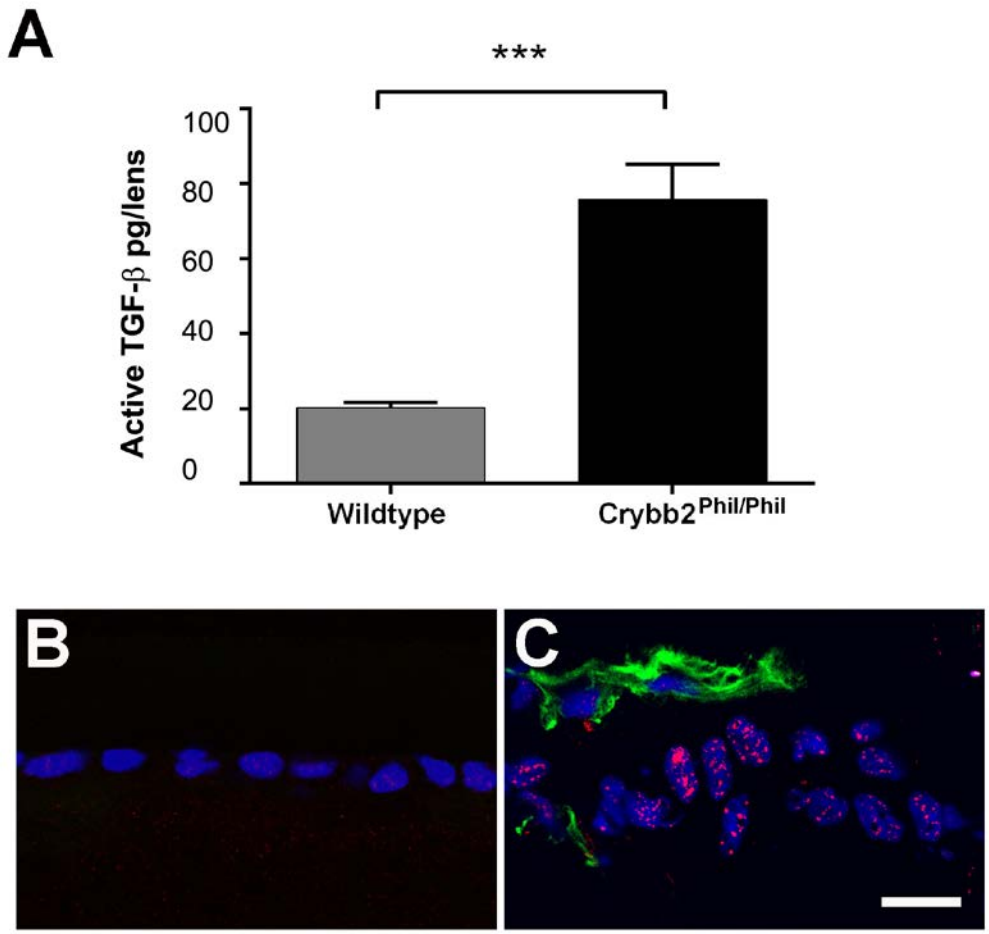
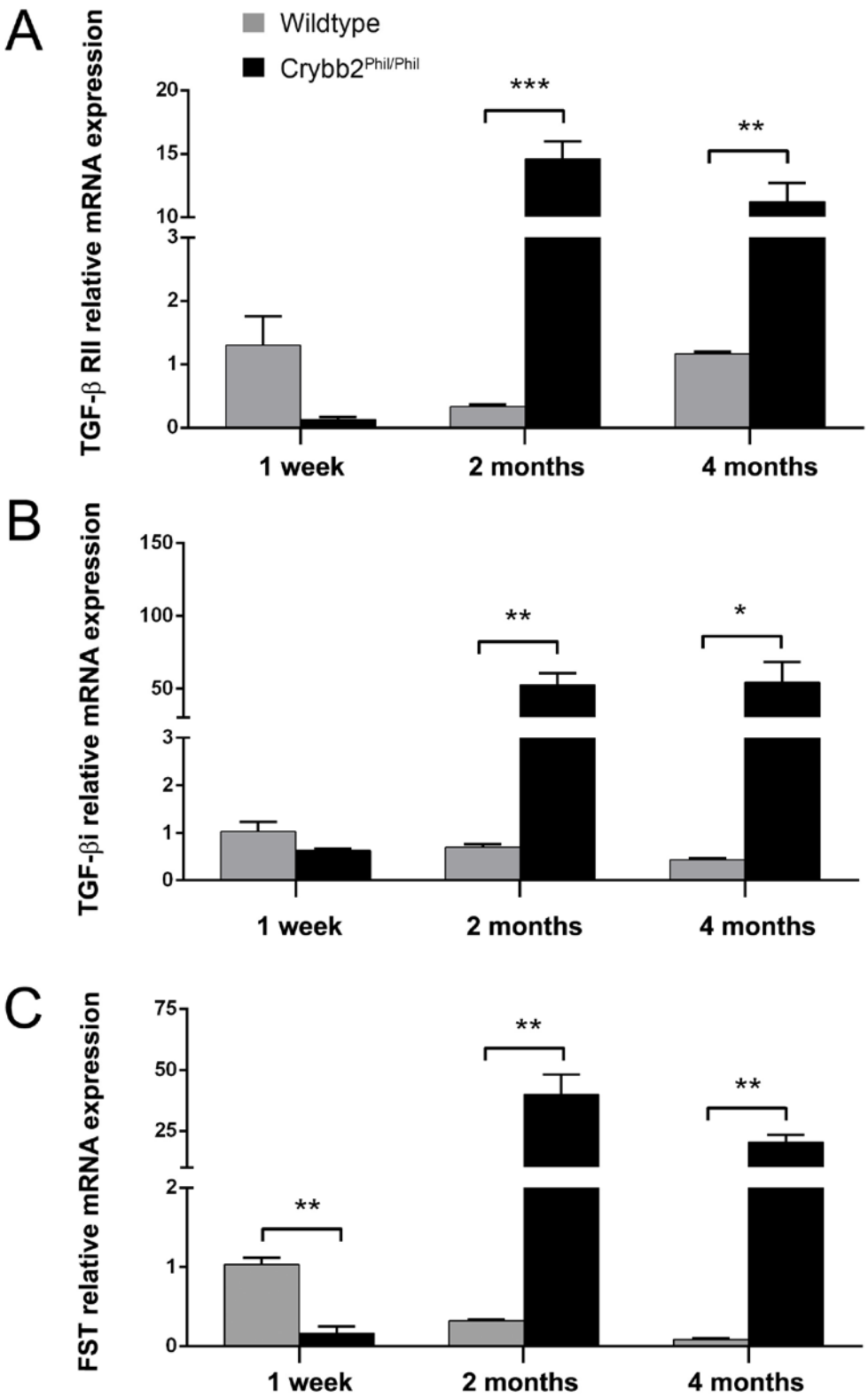


Figure 4.6: (**A**) Active TGF-β levels in 3-month-old wildtype and Crybb2$^{Phil/Phil}$ lens epithelium: On average, a single wildtype lens contains about 21 pg/lens of active TGF-β. Crybb$^{2Phil/Phil}$ lens had an average of 75 pg/lens of active TGF-β, almost 4 times higher than wildtype levels (***P=0.002). All assay experiments had n=4. Values are expressed as mean ± S.E.M. Asterisks (*). SMAD-3 activation in 3-month-old wildtype and Crybb2$^{Phil/Phil}$ epithelium: (**B**) Phosphorylated SMAD-3 protein expression wildtype epithelium (**C**) Phosphorylated SMAD-3 protein expression Crybb2$^{Phil/Phil}$ epithelium. Scale bar = 20μm. Red = phospho-SMAD-3, blue = nucleus, green = αSMA.

4.2.6 TGF-β induced EMT associated players are significantly upregulated in Crybb2$^{Phil/Phil}$ lenses

TGF-βi is an ECM molecule whose mRNA expression and ECM deposition strongly upregulate in response to TGF-β signaling (Jeon, Kim et al. 2012). Consistent with this, by QTR-PCR, I found that TGF-βi mRNA, which is found to be very low in both wildtype and Crybb2$^{Phil/Phil}$ lens epithelium immediately after birth, is robustly upregulated in Crybb2$^{Phil/Phil}$ lens epithelium compared to wildtype at 2-months of age, and remains high through 4 months of age (Figure 4.7. A). Similarly, QRT-PCR also showed that Follistatin, which has been recently reported to play a key role in regulating TGF-β induced SMAD-3/AKT/mTOR pathway (Winbanks, Weeks et al. 2012) was also significantly upregulated in Crybb2$^{Phil/Phil}$ lens epithelium compared to wildtype at 2 months of age throughout 4-month of age (Figure 4.7. B). This upregulation of TGF-βi and Follistatin mRNA was parallel with the overexpression of TGF-β receptor 2 (TGF-β RII), which was significantly higher in Crybb2$^{Phil/Phil}$ lens epithelium beginning 2 month of age and stayed high throughout 4 months of age (Figure 4.7. C).

Figur


A
Wildtype
Crybb2Phil/Phil
TGF-β RII relative mRNA expression
20
15
10
3
2
1
0
***
**
1 week
2 months
4 months
B
TGF-βi relative mRNA expression
150
100
50
3
2
1
0
**
*
1 week
2 months
4 months
C
FST relative mRNA expression
75
50
25
2
1
0
**
**
**
1 week
2 months
4 months

and

Crybb2Phil/Phil lens epithelium over time. (**A**) Crybb2$^{Phil/Phil}$ lenses show lower mRNA expression levels of TGF-βi receptor 2 (TGF-β RII) at 1-week of age. At 2 months of age TGF-β RII mRNA expression significantly increases Crybb2$^{Phil/Phil}$ lenses with very low expression in wildtype lenses (***p=0.003). At 4 months of age, there is almost no TGF-β RII mRNA expression in wildtype but high expression of TGF-β RII in Crybb2$^{Phil/Phil}$ lenses (**p=0.01). (**B**) Crybb2$^{Phil/Phil}$ lenses show lower mRNA expression levels of TGF-βi at 1 week of age. At 2 months of age, TGF-βi mRNA expression significantly increases Crybb2$^{Phil/Phil}$ lenses with very low expression in wildtype lenses (*p=0.01). At 4 months of age, there is a mild expression of TGF-βi mRNA in wildtype but high expression of TGF-βi in Crybb2$^{Phil/Phil}$ lenses (**p =0.002). (**C**) Crybb2$^{Phil/Phil}$ lenses show significantly lower mRNA expression levels of Follistatin (FST) at 1 week of age (**p=0.002). At 2 months of age, FST mRNA expression levels fall dramatically but significantly increase Crybb2$^{Phil/Phil}$ (**p=0.008). At 4 months of age, there is almost no FST mRNA expression in wildtype but high expression of TGF-β RII in Crybb2$^{Phil/Phil}$ lenses (**p=0.003). Values are expressed as mean ± S.E.M. Asterisks (*) indicate statistically significant fold changes from wildtype epithelium normalized to Hprt-1 mRNA level, n=4.

4.2.7 αV, β1 and β8 integrin subunits and their respective ligands are upregulated in Crybb2$^{Phil/Phil}$ EMT

Integrins have been reported to play a role in lens EMT leading to PCO (Zuk and Hay 1994, Kim, Lee et al. 2002, Sponer, Pieh et al. 2005, Walker and Menko 2009). In chapter three, I reported that αV integrin and its interacting β-subunits; β1, β5, β6, β8 are upregulated concomitant with αSMA and their ligands in lens epithelial cells remaining behind in a mouse fiber cell removal model of cataract surgery, suggesting the roles of αV integrin in activation the SMAD-3 associated TGF-β induced EMT (Leask and Abraham 2004). Similarly, I found αV integrin and its interacting β-subunits; β1, β5, β6, β8 are also upregulated concomitant with αSMA in 2-month-old Crybb2$^{Phil/Phil}$ lens epithelium as compared to wildtype (Figure 4.8) In addition, αV integrin ligands, TGF-βi, fibronectin and vitronectin, were also upregulated in 2-month-old Crybb2$^{Phil/Phil}$ lens epithelium as compared to wildtype (Figure 4.9). In addition, QRT-PCR analysis showed αV integrin and its interacting β-subunits, β1 and β8, were also up regulated significantly at the mRNA level during the Crybb2$^{Phil/Phil}$ lens epithelium EMT around the same age (Figure 4.10).

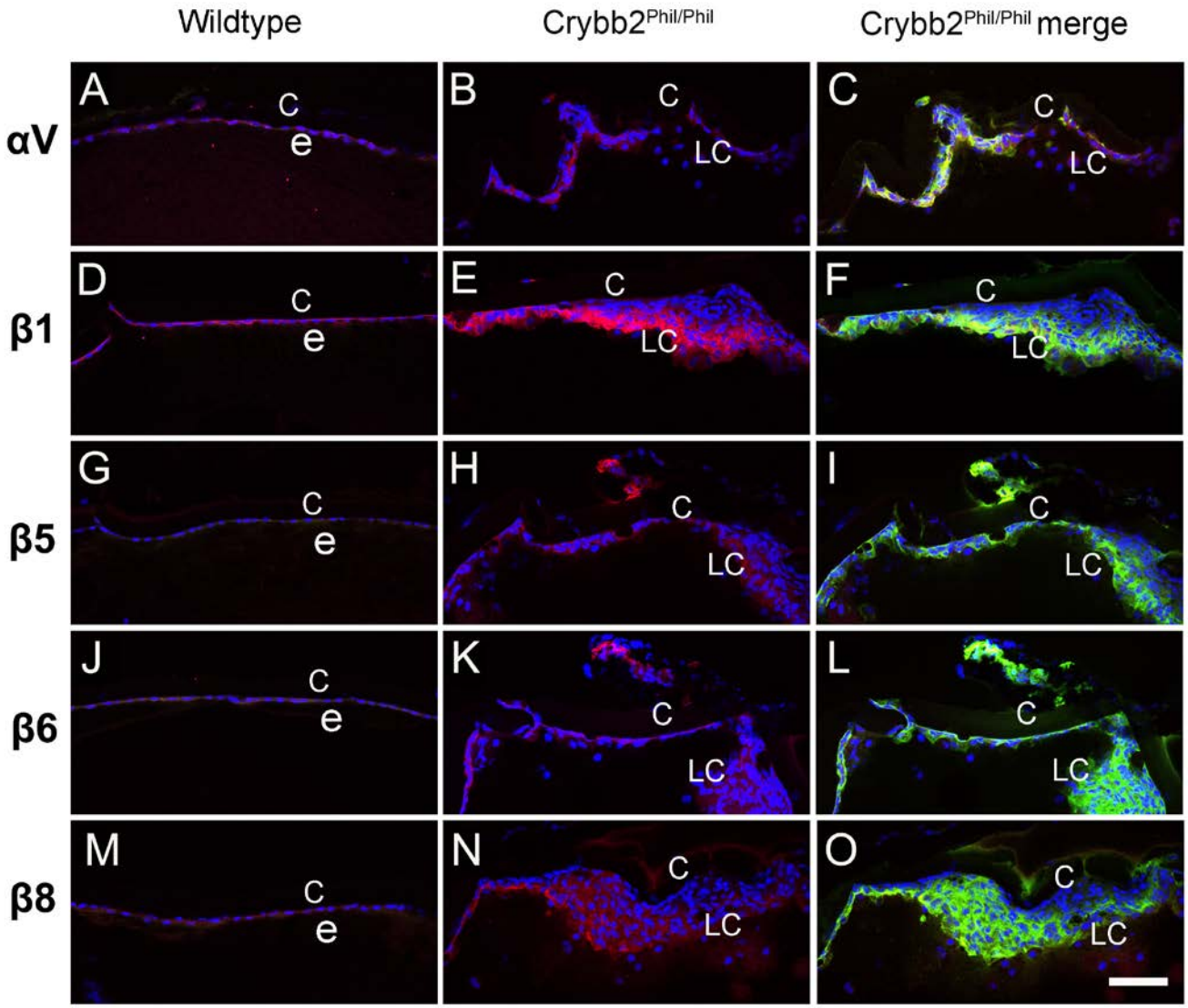


Figure 4.8: Immunofluorescent analysis showing that αSMA and αV-β integrin levels increase in wildtype residual lens cells (LC) at 48hrs post-surgery (**A**) αV integrin + αSMA expression, in wildtype epithelium. (**B**) αV integrin expression in Crybb2$^{Phil/Phil}$ epithelium. (**C**) αV integrin + αSMA expression, in Crybb2$^{Phil/Phil}$ epithelium. (**D**) β1 integrin + αSMA expression in wildtype epithelium. (**E**) β1 integrin expression, in Crybb2$^{Phil/Phil}$ epithelium. (**F**) β1 integrin + αSMA expression in Crybb2$^{Phil/Phil}$ epithelium. (**G**) β5 integrin +

αSMA wildtype epithelium (**H**) β5 integrin expression in Crybb2$^{Phil/Phil}$ epithelium. (**I**) β5 integrin + αSMA in Crybb2$^{Phil/Phil}$ epithelium. (**J**) β6 integrin + αSMA expression wildtype epithelium (**K**) β6 integrin expression in Crybb2$^{Phil/Phil}$ epithelium. (**L**) β6 integrin + αSMA expression in Crybb2$^{Phil/Phil}$ epithelium. (**M**) β8 integrin + αSMA expression wildtype epithelium. (**N**) β8 integrin expression in Crybb2$^{Phil/Phil}$ epithelium. (**O**) β8 integrin + αSMA expression in Crybb2$^{Phil/Phil}$ epithelium. Scale bar = 35μm, red=integrin; blue=nucleus; green = αSMA, LC = residual lens cells, C= lens capsule.

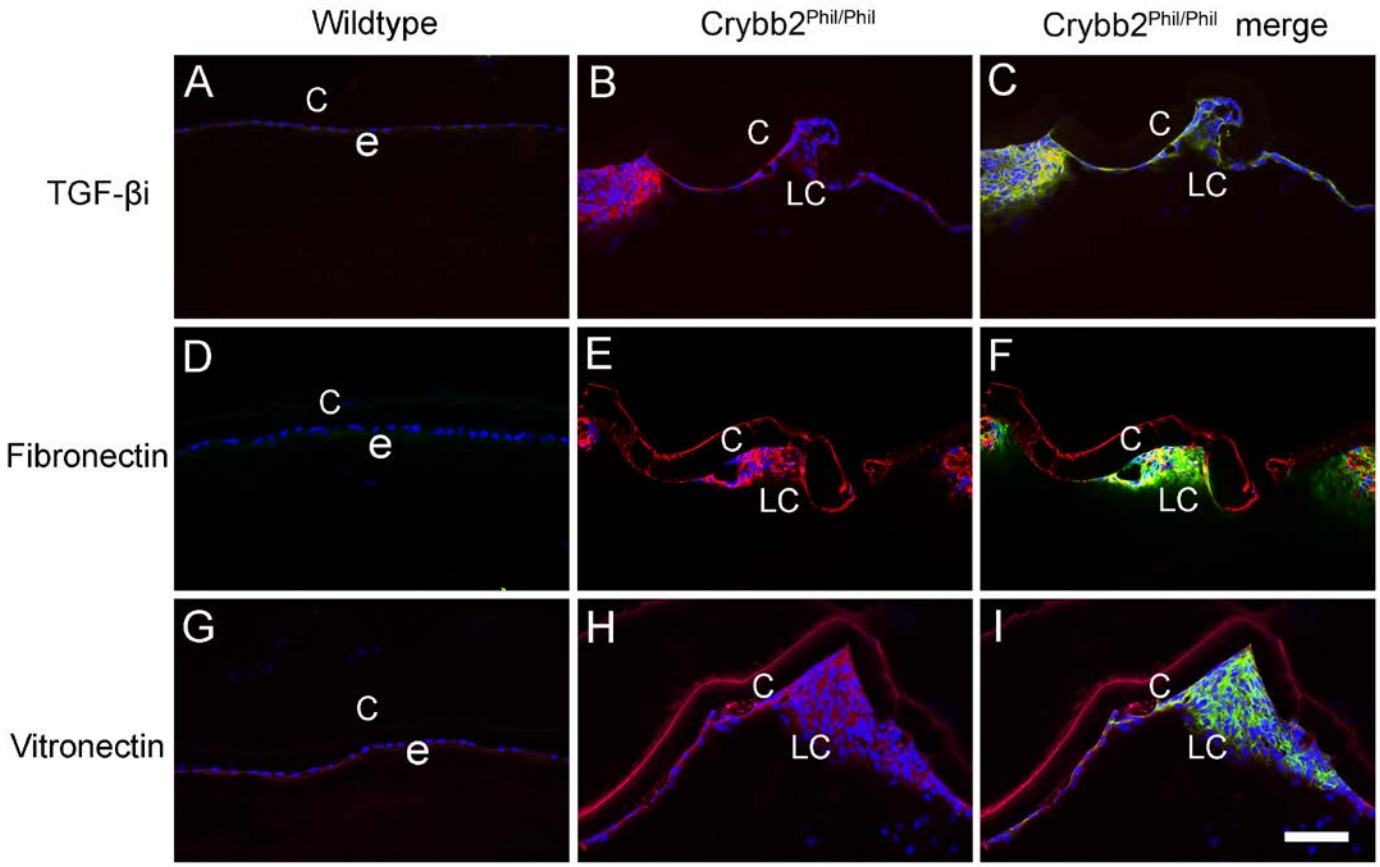


Figure 4.9 Immunohistochemistry analysis of αV integrin ligands, TGF-βi, fibronectin and vitronectin deposition in 2-month old wildtype and Crybb2$^{Phil/Phil}$ lens epithelium: (**A**) TGF-βi + αSMA expression in wildtype lens epithelium. (**B**) TGF-βi expression in Crybb2$^{Phil/Phil}$ lens epithelium. (**C**) TGF-βi + αSMA expression in Crybb2$^{Phil/Phil}$ lens epithelium. (**D**) Fibronectin + αSMA expression in wildtype lens epithelium (**E**) Fibronectin expression in Crybb2$^{Phil/Phil}$ lens epithelium. (**F**) Fibronectin + αSMA expression in Crybb2$^{Phil/Phil}$ lens epithelium. (**G**) Vitronectin + αSMA expression in wildtype lens epithelium. (**H**) Vitronectin expression in Crybb2$^{Phil/Phil}$ lens epithelium. (**I**) Vitronectin + αSMA expression in Crybb2$^{Phil/Phil}$ lens epithelium. Scale bar = 60μm. Red

= TGF-βi or fibronectin or vitronectin, blue = nucleus, green = αSMA. LC = residual lens epithelial cells, C= lens capsule.

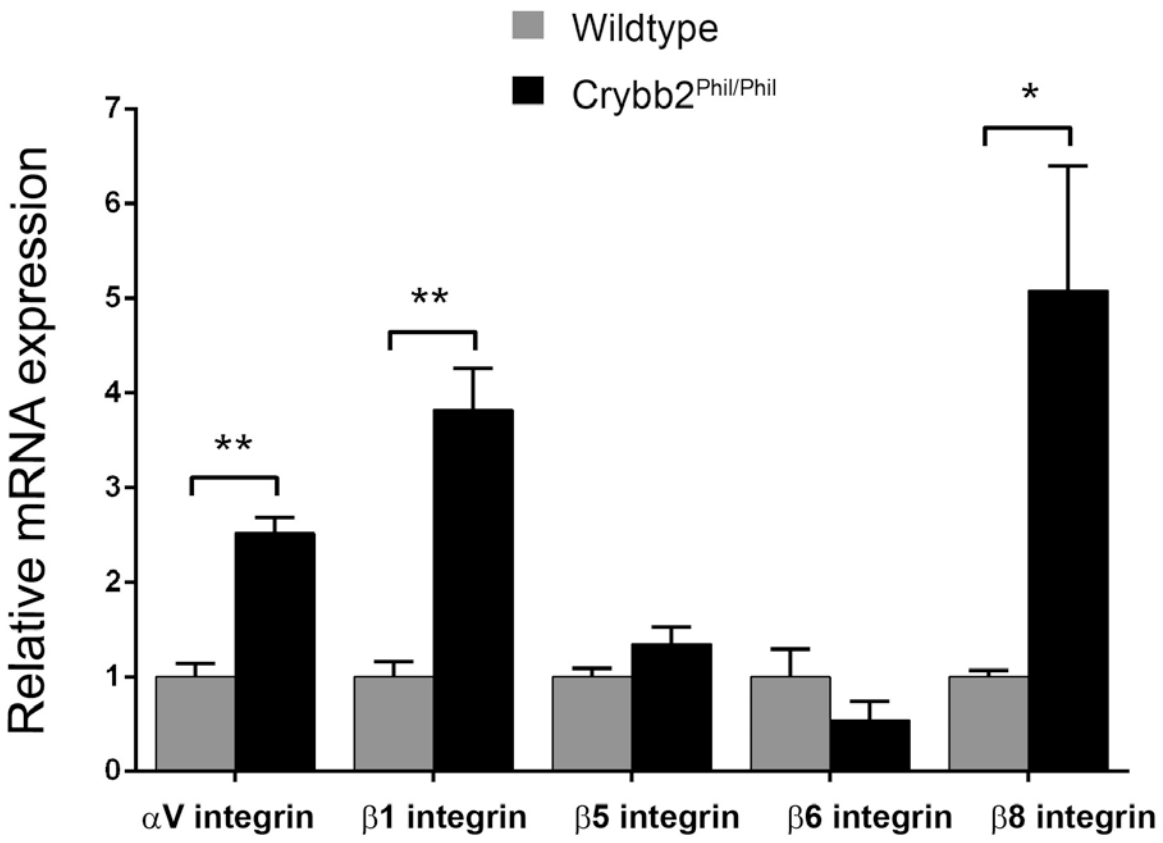


Figure 4.10: RT-PCR quantitation of integrin mRNA levels in 2-month old wildtype and Crybb2$^{Phil/Phil}$ lens epithelium normalized to Hprt-1, n=4. αV integrin subunit mRNA expression was significantly upregulated at 2-month of age in Crybb2$^{Phil/Phil}$ lens epithelium as compared to wildtype (**P=0.002). αV integrin β–interacting subunits were also significantly upregulated in Crybb2$^{Phil/Phil}$ lens epithelium as compared to wildtype, β1–integrin (**P=0.004) and β8–integrin (**P=0.035). No significant changes were observed in either β5 or β6 integrin subunit mRNA expression. β6-integrin subunit mRNA appeared to decrease slightly in Crybb2$^{Phil/Phil}$ lens epithelium although the difference was not significant. All fold changes in integrin subunit expression in Crybb2$^{Phil/Phil}$ lens

epithelium were calculated by setting values obtained in wildtype in each specific subunit group to one. Values are expressed as mean ± S.E.M. Asterisks (*) indicate statistically significant fold changes from wildtype.

4.2.8 IGFbp-3 is significantly upregulated in Crybb2$^{Phil/Phil}$ mutant lenses

The TGF-β/BMP Signaling Pathway RT^{2}Profiler^{TM} PCR performed by Ms. Megan Fisher, reveled IGFbp-3 mRNA was highly overexpressed in Crybb2$^{Phil/Phil}$ lenses. To further investigate, this I performed QRT-PCR on the expression of IGFbp-3 on Crybb2$^{Phil/Phil}$ lens epithelium at different time postnatal. I found out that IGFbp-3 mRNA expression, which is almost undetected in wildtype lenses, was not only significantly overexpressed in Crybb2$^{Phil/Phil}$ lenses but this overexpression increased as the Crybb2$^{Phil/Phil}$ lenses aged (Figure 4.11. A). In addition, immunohistochemistry performed by Corrine Decker showed that cleaved caspase-3 was expressed in 4-month-old Crybb2$^{Phil/Phil}$ lenses. Moreover, this caspase-3 expression appeared to be greater in cells lacking nuclear staining in the epithelium fibrotic plaque (Figure 4.11. B). No upregulation in cleaved caspase-3 was observed in either wildtype (Figure 4.11. C), or Crybb2$^{+/Phil}$ lenses (data not shown here). Neither of these observations have been reported in lens EMT that occurs after surgery nor in lens EMT associated with ASC.

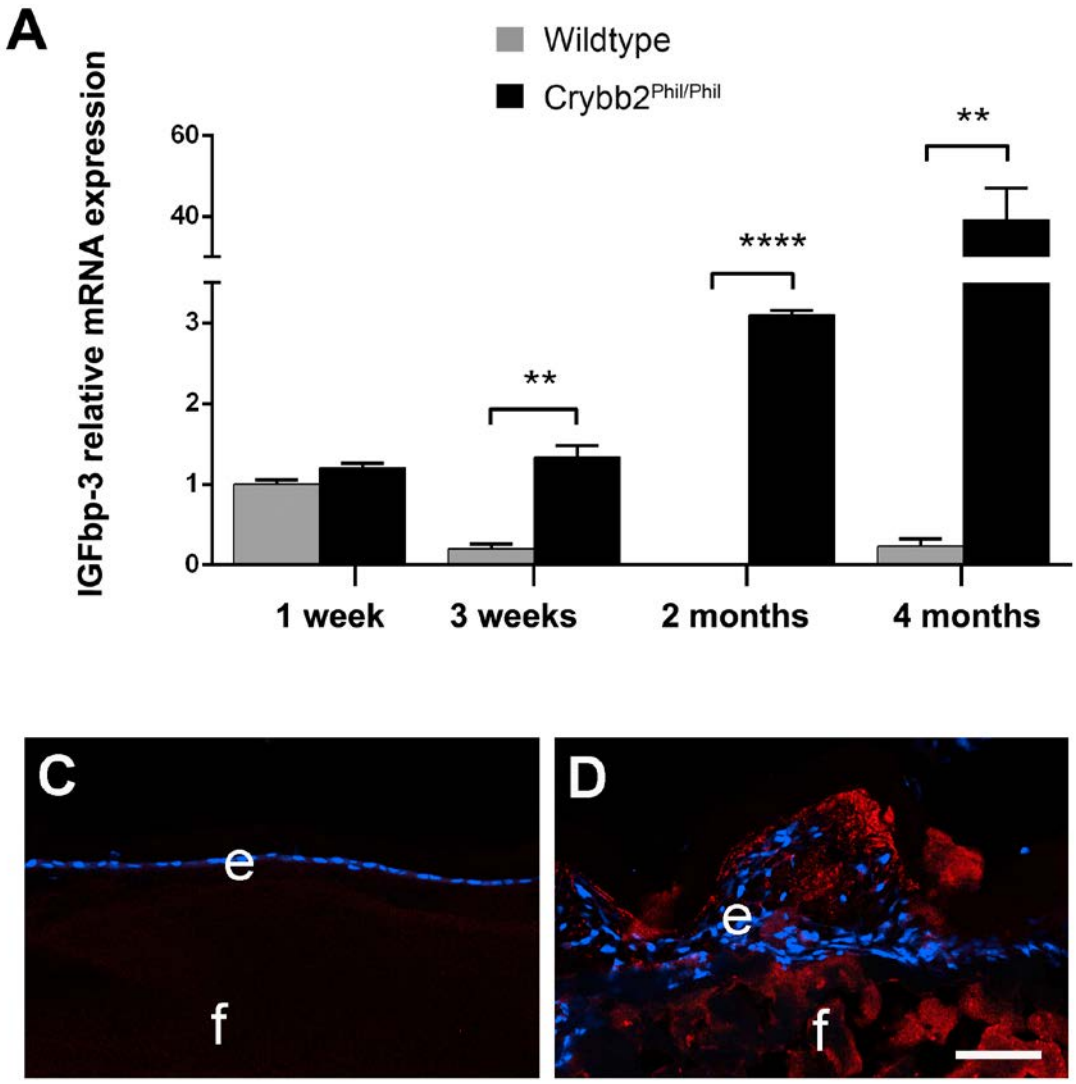


Figure 4.11: Relative expression of IGFbp-3 mRNA in lens epithelial cells over time. (**A**) Both wildtype and Crybb2$^{Phil/Phil}$ lenses have similar mRNA expression levels of IGFbp-3 at 1 week of age. At 3 weeks of age, IGFbp-3 mRNA expression significantly falls in wildtype lenses (**p=0.002). At 2 months of age, almost no IGFbp-3 mRNA expression in wildtype but high expression in Crybb2$^{Phil/Phil}$ lenses (****p=0.0001) which continued to increase throughout 4 months

of age (**p=0.007). Values are expressed as mean ± S.E.M. Asterisks (*) indicate statistically significant fold changes from wildtype epithelium normalized to Hprt-1, n=4. Cleaved caspase-3 expression: (**B**) Cleaved caspase-3 expression in wildtype lens epithelium. (**C**) Upregulated Cleaved caspase-3 expression in fibrotic plaques and fiber cells underling the epithelial layer in Crybb2$^{Phil/Phil}$ lens. Scale = 75µm, red = cleaved caspase-3, blue = cell nucleus, e = lens epithelium f = lens fibers.

4.3 **Discussion**

4.3.1 The βB2-crystallin mutation (Crybb2$^{Phil/Phil}$)

There was a drastic difference between the heterozygous and the Crybb2$^{Phil/Phil}$ lens' phenotype. Both the heterozygous and the Crybb2$^{Phil/Phil}$ develop cataracts suggesting the important role of βB2-crystallins in lens transparency. However, unlike lenses heterozygous for the mutation, which predominately exhibited cortical cataracts, the Crybb2$^{Phil/Phil}$ lenses were severely impacted, and underwent a significant reduction in lens size. Crybb2$^{Phil/Phil}$ lenses weigh about a 1/4 of heterozygous' lens weight and about a 1/6th of a wildtype lens. In addition, Crybb2$^{Phil/Phil}$ exhibited severe lens epithelial distortions. This implies that a functioning βB2-crystallin gene /protein is required to maintain lens epithelium morphology.

I also noticed that Crybb2$^{Phil/Phil}$ lenses had significant higher βB2-crystallin mRNA and protein expression 1-week after birth as compared to wildtype. Since βB2-crystallin is not highly expressed in lens until after birth (Uga, Kador et al. 1980, Chambers and Russell 1991, Zhang, Li et al. 2008), this expression pattern suggests that the mutant βB2-crystallin protein in Crybb2$^{Phil/Phil}$ lenses induces a positive feedback response which signals to make more βB2-crystallin so to compensate for the mutated form at birth. As a result, the upregulation of a mutated form of βB2-crystallin could disrupt the normal lenticular crystallin ratio, impairing the proper folding of other lens crystallin proteins, thus disrupting crystallins' organization leading to crystallin aggregation and hence an early onset cataract as early as five to six weeks after birth.

This upregulation of mutant βB2-crystallin may also explain why full βB2-crystallin knockouts which do not express βB2-crystallin at all do not develop cataracts until about 4-6 months of age (Zhang, Li et al. 2008).

Moreover, unlike wildtype lenses which only express βB2-crystallin protein levels in lens fibers cells at birth, Crybb2$^{Phil/Phil}$ lenses overexpressed βB2-crystallin protein levels in both lens fiber cells and lens epithelial cells (LECs). This overexpression of βB2-crystallin in LECs which normally do not express the protein at such an early age may interfere with the normal molecular and physiological pathways necessary in maintain lens epithelial integrity. This may explain why Crybb2$^{Phil/Phil}$ lenses develop severe lens epithelium abnormalities.

4.3.2 Crybb2$^{Phil/Phil}$ lens epithelial undergoes and EMT at around 1-2 months after birth.

Unlike many mutations that causes cataract, the Crybb2$^{Phil/Phil}$ lens epithelium developed severe epithelial EMT by 8 weeks postnatal (Figure 4.12). In conjunction with αSMA overexpression, this phenotype also involves an extreme upregulation of collagen 1α1 which remained elevated throughout the Crybb2$^{Phil/Phil}$ lenses' life. Both αSMA and collagen-1α1 upregulation have been reported in numerous lens EMT studies (Hales, Schulz et al. 1994, Zuk and Hay 1994, Gotoh, Perdue et al. 2007) and are considered as hallmark markers for lens EMT. αSMA expression also occurs in Crybb2$^{+/Phil}$ lenses, however, only as small focal fibrotic plaques, which again reemphasize the fact that the presence of a functional βB2-crystallin is important in

maintaining the lens epithelium integrity. In addition to overexpressing lens EMT markers, the Crybb2$^{Phil/Phil}$ lens epithelium also becomes uneven and multilayered as early as 4 weeks postnatal; phenotype that is similar to the phenotypes seen in PCO and ASC. This distortion in epithelial cell shape gets more extreme in the 16-week-old lens, with protruding plaques that drastically deform the normally smooth surface of the lens.

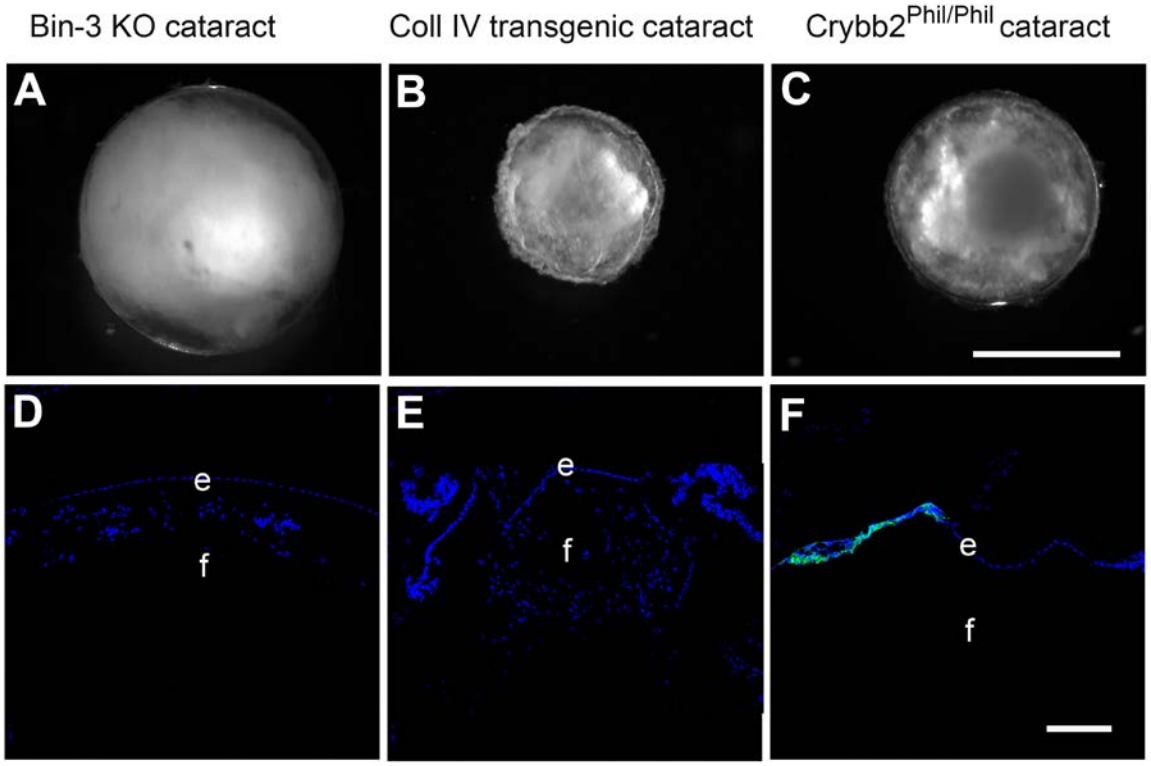


Figure 4.12: Comparison of Crybb2$^{Phil/Phil}$ cataract to other profound cataract mutation known in lens. (**A**) Dark field image showing Bin-3 knockout cataract (Ramalingam, Duhadaway et al. 2008). (**B**) Dark field image showing the collagen IV transgenic mouse cataract with significant reduction in size (Firtina, Danysh et al. 2009). (**C**) Dark field image showing Crybb2$^{Phil/Phil}$ cataract. (**D**) Immunohistochemistry showing no αSMA expression in Bin-3 knockout cataract (**E**) Immunohistochemistry showing no αSMA expression in collagen IV mutant cataract but a severe lens capsule wrinkling. (**F**) Immunohistochemistry showing αSMA overexpression in Crybb2$^{Phil/Phil}$ cataract with severe lens capsule wrinkling. Scale bar: (A-C) =1mm, (D-F) = 140μm.

4.3.3 Crybb2^{Phil} lens EMT results from unregulated Transforming growth factor-β activation.

TGF-β regulates a wide array of cellular processes including cell division, differentiation, motility, apoptosis and tumor suppression (Taipale, J. Saharinen et al. 1998, Yue and Mulder 2001). Notably, the TGF-β pathway is well known to play a role in lens EMT following cataract surgery leading to Posterior Capsular Opacification (PCO) (Saika, Okada et al. 2001, Leask and Abraham 2004, Mamuya and Duncan 2012) as well as in ASC (Lovicu, Ang et al. 2004). *In vitro* and *in vivo* lens studies have clearly shown the effects of TGF-β signaling in lens development, while inappropriate TGF-β signaling resulted in lens EMT and cataracts (de Iongh, Wederell et al. 2005). I saw a significant increase in SMAD-3 phosphorylation, TGF-β receptor II mRNA overexpression as well as TGF-βi and follistatin upregulation, genes known to upregulate aftermath of TGF-β signaling, suggesting that Crybb2$^{Phil/Phil}$ epithelium was undergoing extreme unregulated TGF-β signaling. This was confirmed when I looked at the amount of active TGF-β associated with Crybb2$^{Phil/Phil}$ lenses which were found to be about four times higher than the levels found in wildtype lenses. Crybb2$^{Phil/Phil}$ lenses had 75 pg/lens of active TGF-β per lens compared to 21 pg/lens in wildtype lenses. And this was without taking into account that Crybb2$^{Phil/Phil}$ lenses are almost 4 times smaller in size as compared to wildtype lenses. In addition, mRNA levels for none of the three TGF-β 1, 2 and 3 ligands mRNAs nor SMAD 2 or 3 were significantly upregulated in Crybb2$^{Phil/Phil}$ lenses, suggesting that this increase in TGF-β induced EMT is regulated mainly at the TGF-β activation level. However, the answer to why

mutation of βB2-crystallin leads to unregulated TGF-β activation and EMT will need further investigation.

4.3.4 Crybb2$^{Phil/Phil}$ EMT suggests a different pathway from previously known lens EMTs

Both *in vitro* and *in vivo* lens studies have clearly shown that inappropriate TGF-β signaling can result in lens EMT and cataracts (de Iongh, Wederell et al. 2005). The vast majority of these studies have shown that lens epithelial cells undergoing EMT, especially in PCO and ASC do share very similar characteristics (Leask and Abraham 2004, Lovicu, Ang et al. 2004). These include but not limited to LECs multilayering, upregulation of ECM mesenchymal proteins and a transformation to migratory myofibroblasts which do not undergo apoptosis (Lovicu, Steven et al. 2004, Mamuya, Wang et al. Submitted 2013).

When I examined the protein levels of Pax-6, a transcriptional factor that plays a major role in maintaining the epithelial phenotype of the lens (Callaerts, Halder et al. 1997, Chauhan, Reed et al. 2002), I found that Pax-6, which is expressed in normal lens epithelium, was downregulated 3-days post-surgery in wildtype mice. Other reports from PCO studies have reported similar findings (Mansfield, Cerra et al. 2004, Mansfield, Cerra et al. 2004). In contrast, Crybb2$^{Phil/Phil}$ EMT is associated with LECs undergoing apoptosis (Figure 4.11 C-D). Moreover, unlike PCO and ASC, Pax-6 is extremely upregulated during Crybb2$^{Phil/Phil}$ EMT. Altogether this may suggest that βB2-crystallin mutations instigate an alternate lens EMT pathway that, although dissimilar

to the EMTs seen in PCO and ASC, it is still mediated by unregulated TGF-β signaling. Thus, understanding how βB2-crystallin mutations lead to this extreme level of active TGF-β in the lens, can be essential in understanding the pathogenesis of PCO and ASC which till this day, are not well known. However, a mechanism on how βB2-crystallin mutations lead to this extreme level of active TGF-β in the lens is proposed in chapter 5.

4.3.5 αV integrins are upregulated in Crybb2$^{Phil/Phil}$ EMT and might be playing a role in sustained TGF-β induced EMT

Due to their function in activating latent TGF-β which triggers a TGF-β-induce-EMT known to play roles in inflammation, cancer and fibrosis, αV integrins have been implicated in many developmental and pathological processes (Munger, Huang et al. 1999, Ludbrook, Barry et al. 2003, Wipff, Rifkin et al. 2007, Yang, Mu et al. 2007, Wipff and Hinz 2008, Mamuya and Duncan 2012, Mamuya, Wang. et al. 2014). I have discussed these roles of αV integrins in lens EMT in my 3rd chapter which have also been investigated and proposed in several other lens studies and are known to play a significant role in both ASC and PCO (Zuk and Hay 1994, Kim, Lee et al. 2002, Sponer, Pieh et al. 2005, Walker and Menko 2009). Notably, αV integrins can activate TGF-β, provoking a TGF-β-induced-EMT, which in return can further stimulate αV integrin gene expression (Munger, Huang et al. 1999, Hazelbag, Kenter et al. 2007, Pandit, Corcoran et al. 2010). This phenomenon then creates a positive feed forward loop that drives TGF-β-induced-EMT (Mamuya and Duncan 2012, Mamuya, Wang. et al. 2014).

I found that both the residual lens cells left after mouse cataract surgery and the Crybb2$^{Phil/Phil}$ epithelium deposit fibronectin, tenascin-C and TGF-βi, while upregulating αV, β1, β5, β6 and β8 integrin subunit protein levels as they undergo EMT and become multilayered. Interestingly, unlike the residual lens cells remaining 48hrs after mouse cataract surgery, the Crybb2$^{Phil/Phil}$ lens epithelium also overexpressed αV, β1 and β8 integrin subunit mRNA levels. This may be due to the fact that, unlike the residual lens left 48hrs post cataract surgery, a time which there is only a small amount active TGF-β which is incapable of stimulating αV integrin mRNA expression, by 2 month of age, the Crybb2$^{Phil/Phil}$ lens epithelium already has elevated levels of active TGF-β. This explains why the Crybb2$^{Phil/Phil}$ lens epithelium overexpresses αV, β1 and β8 integrin subunit mRNA levels and the post-surgery model failed to do so. However, it is unclear whether a functional βB2-crystallin protein is essential in regulating integrin function in lens.

4.3.6 What is the role of IGFbp-3 in lens EMT?

The roles of IGFbp-3 in lens are yet to be explored. Nonetheless, there was a significant upregulation of IGFbp-3 in both the mRNA and protein (data not shown here) expression levels throughout the entire life of the Crybb2$^{Phil/Phil}$ lens epithelium. In some systems, IGFbp-3 has been shown to promote cell proliferation and metastasis (Xi, Nakajima et al. 2006). While in other cases, IGFbp-3 has been associated with growth inhibition, proliferation and motility suppression of both epithelial and other cell types, alleviating cancer growth and progression. (Huang and Huang 2005)

(Ullmannova and Popescu 2007, Fuchs, Goldberg et al. 2008). Interestingly, IGFbp-3 levels have also been reported to rise in response to TGF-β signaling, and recent studies have shown that IGFbp-3 clearly activates the canonical TGF-β -SMAD pathway inhibiting proliferation of numerous cell types (Fanayan, Firth et al. 2002, Kuemmerle, Murthy et al. 2004, Izumi, Kurosaka et al. 2006, Forbes, Souquet et al. 2010). However, similar to TGF-β, the precise mechanisms of IGFbp-3 cross-talk with other signaling pathways are not well understood (Baxter 2013). On the other hand, IGFbp-3 has been also shown to induce apoptosis in corneal epithelium as well as in other different cell types (Butt, Firth et al. 2000, Robertson, Ho et al. 2007). This was either directly or by potentiating other apoptotic members (Gill, Perks et al. 1997). Thus, IGFbp-3 involvement in all these biological processes raises a question, what is its function in lens EMT?

Since, Crybb2$^{Phil/Phil}$ lenses are significantly smaller that wildtype, and show an increase in caspase-3 activity, I first thought this might be due to IGF-bp3 growth inhibition and apoptotic roles. However, most if not all studies on IGF-bp3 induced apoptosis involves the roles of caspase-8 and -9 (Kim, Ingermann et al. 2004, Butt, Dickson et al. 2005, Galluzzi, Vitale et al. 2012). Moreover, Crybb2$^{Phil/Phil}$ lens epithelium is associated with increase in lens cell multilayering at the beginning, with apoptosis only occurring in late stages. This weakened the idea that IGFbp-3 might be inhibiting LEC proliferation during Crybb2$^{Phil/Phil}$ EMT. However, IGFbp-3 is expression in the Crybb2$^{Phil/Phil}$ lenses is influencing the Crybb2$^{Phil/Phil}$ phenotype raising a question on what functions does IGFbp-3 play the lens and whether if whether these

function are associated to non-refractive roles of βB2-crystallin.This is a topic of future investigation.

4.4 **Conclusion**

Zhang et al. created a systemic βB2-crystallin knockout mouse. Surprisingly, this mouse exhibited only mild lens morphological defects at birth. This can be explained by the fact that the majority βB2-crystallin expression does not begin until after birth. However, since these full knockouts showed a delayed and milder phenotype as compared to the Crybb2$^{Phil/Phil}$ mutants, suggests that early unregulated overexpression of the mutated βB2-crystallins protein, impairs lenticular crystallin's organization leading to an early onset cataract. On the other hand, the presence of TGF-β-induced-EMT in Crybb2$^{Phil/Phil}$ mutant lenses along with loss of polymerized filamentous F-actin, apoptosis and severe decrease in lens size, suggest that fully functioning β-crystallins proteins are necessary for maintenance of lens epithelial cell integrity. Further, the induction of EMT in heterozygous mutant lenses despite of possessing one fully functioning βB2-crystallin allele, strongly suggests that mutations in βB2-crystallin impair certain specific lens signaling tasks that are beyond refractive roles.

Chapter 5

DISCUSSION AND FUTURE PERSPECTIVES

5.1 Summary

In this study, I have answered three fundamental questions in regards to the functions of αV integrins in the lens. First, I demonstrated that αV integrins are necessary for neither lens development, nor the maintenance of lens morphology. Secondly, I demonstrated that αV integrins are required for lens epithelial cells to undergo EMT following a lens injury or cataract surgery. Further, I suggest that αV integrins are required for TGF-β induced EMT signaling following lens injury or surgery, perhaps via their known roles in the activation of latent TGF-β.

While enormous advancements have been made in understanding the mechanisms that drive PCO, the precisely mechanisms that drive PCO are not well known (Awasthi, Guo et al. 2009). Nonetheless, for almost two decades, considerable evidence has been presented confirming that TGF-β signals induce lens epithelial cells to undergo an EMT resulting in both ASC and PCO (de Iongh, Wederell et al. 2005, Dawes, Sleeman et al. 2009, Wormstone, Wang et al. 2009). Most importantly, αV integrins have the capacity to regulate TGF-β which in turn can upregulate the expression of more αv integrins creating a positive feed forward loop that stands as a continuous driver of EMT (Dawes, Elliott et al. 2007, Mamuya and Duncan 2012). TGF-β 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 (Wederell and de Iongh 2006, Dawes, Sleeman et al. 2009). However, taking in consideration the fact that integrins have already been implicated to play a role in lens EMT (Walker and Menko 2009), my findings along with others that I will briefly explain below, strongly suggest that αV integrins play an essential role in driving the lens EMT that leads to PCO, and, most likely, the EMT associated with ASC as well.

Several prior studies support the idea that αV integrin plays an essential role in PCO. Prior studies have shown that αV integrins' ECM ligands such as lumican, osteopontin and tenascin-C (Saika, Miyamoto et al. 2003, Saika, Shirai et al. 2007, Tanaka, Sumioka et al. 2010) play a major role in PCO development. Knockouts of all these three ECM ligands exhibited reduced or delayed EMT as shown by altered expression of α-SMA or reduced TGF-β1 expression and SMAD-2/3 activation. Likewise, studies on MMP9 and MMP2suggest that TGFβ-induced EMT depends on the metalloproteinase functions of MMP9 and MMP2 (Nathu, Dwivedi et al. 2009). This importance of MMPs in EMT was strengthened by findings that MMP inhibitors (Ilmostat) can prevent ASC formation and capsule contraction in both *in vitro* and *in vivo* models (Wong, Daniels et al. 2004, Dwivedi, Pino et al. 2006, Morarescu, West-Mays et al. 2010). Moreover, Ilmostat, a broad-spectrum matrix metalloproteinase inhibitor, was also found to significantly decrease LECs migration on the posterior capsule (Wong, Daniels et al. 2004). MMP9 and MMP2 are known to activate latent TGF-β, and intriguingly, αV integrins have been shown to recruit both MMP9 and MMP2, bringing them into closer proximity with latent TGF-β (Brooks, Stromblad et al. 1996, Rolli, Fransvea et al. 2003). However, my data also suggest that βB2-crystallin

mutations instigate an alternate lens EMT pathway different than that activated in PCO. A proposed mechanism of how a non-refractive function for βB2-crystallin may lead to an extreme level of TGF activation is discussed in the following section.

5.2 Crybb2$^{Phil/Phil}$ Lens EMT Might Result From PI3K/AKT/mTOR and ERK Cell Survival Pathways

In addition to βB2-crystallin, βA3/A1-crystallin, an abundant β-crystallin protein in the lens fibers, is also expressed outside of the lens and has been proposed to have non-refractive roles including the regulation of anoikis-mediated cell death (Aarts, Lubsen et al. 1989, Parthasarathy, Ma et al. 2011). It has been demonstrated that Bit1 (Bcl-2 inhibitor of transcription-1), a mitochondrial protein mediated by its upstream regulator protein kinase-D, induces caspase-independent apoptosis upon its release into the cytoplasm (Biliran, Jan et al. 2008). Studies have also shown that Bit1 localizes to the early secretory pathway in the endoplasmic reticulum (ER) where it negatively regulates the ERK-MAPK signaling pathway in the Golgi (Zhan, Zhao et al. 2004, Yi, Nguyen et al. 2010).

Ma and collaborators showed that βA3/A1-crystallin is required for trafficking of Bit1 to the Golgi, which is essential for anoikis-mediated cell death, while the loss of βA3/A1-crystallin induces IGF-II expression and increases cell survival by regulating the PI3K/AKT/mTOR and ERK pathways. It has also been found that this Bit1 -mediated cell death is reversed by integrin-mediated cell attachment to fibronectin (Jan, Matter et al. 2004), suggesting that the loss of βA3/A1-crystallin indirectly leads to the

activation of survival signaling pathways known to induce integrin-mediated cell attachment. My results show that showed that in additional to significant upregulation in IGFbp-3, cleaved caspase-3 is also upregulated in Crybb2$^{Phil/Phil}$ EMT. Since both situations are not observed in the EMT arising in the post-surgery model, it is possible that similar to βA3/A1-crystallin, βB2-crystallin is also involved in the IGF-II mediated activation of the PI3K/AKT/mTOR and ERK cell survival pathways. Altogether, this suggests that Crybb2$^{Phil/Phil}$ EMT might initiate via PI3K/AKT/mTOR and ERK cell survival pathways that are induced by mutations in βB2-crystallin protein.

5.3 Alpha V Integrin Antagonists as Potential PCO Therapeutics

Since my findings suggest that the absence of αV integrins abrogate the TGF-β induced EMT known to drive PCO after cataract surgery, I propose that blocking of αV integrins' function may interfere with the TGF-β induced EMT known to drive PCO. Thus, appropriate αV integrin antagonists, such as the RGD peptide derivatives discussed in the next section, may be efficient in the treatment or prevention of PCO, ASC and other TGF-β associated ocular fibrotic disorders.

αV integrin antagonists are currently in clinical trials to treat cancer and other TGF-β mediated diseases (Derynck, Akhurst et al. 2001, Derynck and Akhurst 2007, Mamuya and Duncan 2012). Notably, a liberated TGF-β ligand has a high affinity for its receptors, so, in most cases, once released/active, and as long as TGF-β receptors are within reach, it will initiate a TGF-β signaling cascade (Massague 1985). Since different cellular functions require distinct levels of TGF-β signaling (Wipff and Hinz 2008),

tight regulation of latent TGF-β activation is necessary to prevent diverse diseases including inflammation, autoimmune disorders, fibrosis, cancer and cataract (Derynck, Akhurst et al. 2001, Roberts and Wakefield 2003, Ulrike, Stefan et al. 2005, Ghannad, Nica et al. 2008, Irina, Nevins et al. 2009). Conversely, inadequate levels of active TGF-β due to mutations of either the TGF-β genes or those for TGF-β activators can lead to pathology. For instance, TGF-β1 deficient mice exhibit a multifocal, mixed inflammatory cell response and tissue necrosis, leading to organ failure and death 20 days after birth (Shull, Ormsby et al. 1992, Sterner-Kock, Thorey et al. 2002). In humans, inadequate TGF-β signaling can result in various disorders including brain hemorrhage and immune system-associated disorders (Cambier S, Stephanie Gline et al. 2005, Travis, Reizis et al. 2007). Notably, restoring normal TGF-β signaling and/or inhibiting its inappropriate expression in experimental animals reverses some TGF-β associated pathologies and stands as a promising therapeutic approach (See table 5.1).

TGF-β induced signaling is also known to destabilize E-cadherin mediated cell-cell adhesion during EMT (Vogelmann, Nguyen-Tat et al. 2005). An important early onset event in the EMT that occurs in TGF-β-induced EMT in lens is the induction of MMP9 and MMP2, which coincides with cell multilayering seen in PCO and ASC development (Nathu, Dwivedi et al. 2009). αV integrins can activate TGF-β by binding to its LAP and evidence of such activation has been linked to EMT progression. Therefore, blocking the undesirable activities of αV integrins without interfering with their beneficial functions could impede EMT progression during PCO, ASC, cancer, wound healing and fibrosis.

Integrin antagonists show clinical promise for the treatment of TGF-β induced EMT associated disorders such as inflammation, fibrosis and cancer (Wang, Chui et al. 2010). Most of the therapeutic approaches currently under investigation target integrin function using anti-integrin agents including both naturally occurring and engineered peptides that can mimic their RGD ligand, or antibodies that can act as integrin antagonists (Palmade F 1994, Chernousov and Carey 2003, Oharazawa H 2005). For example, clinical administration of a peptide antagonist of the αVβ3 receptor successfully inhibits pathological angiogenesis seen in cancer, proliferative retinopathy, rheumatoid arthritis, and psoriasis (D'Andrea, Del Gatto et al. 2006, Del Gatto, Zaccaro et al. 2006). Likewise, TGF-β-mediated enhancement of glioma cell migration via the upregulation of αVβ3 integrin expression is abrogated by echistatin, a Arg-Gly-Asp (RGD) containing snake venom which is a potent antagonist of αVβ3 integrin (Platten, Wick et al. 2000).

Table 5.1. Attempts to target αV integrin function as a therapeutic strategy to treat TGF-β associated disorders Adapted from (Mamuya and Duncan 2012).

Integrin	Disorder	Experimental findings
αVβ3 αVβ5	1. Atherosclerosis 2. Rheumatoid arthritis 3. Systemic sclerosis	1. Blockade of αVβ3 reduced neointima formation by reducing TGF-β activity (Coleman, Braden et al. 1999) 2. Integrin αVβ3 as a target for the treatment of rheumatoid arthritis and related rheumatic diseases(Wilder 2002). 3. Increased expression of integrin αVβ5 contributes to the establishment of autocrine TGF-β signaling in scleroderma fibroblasts.(Asano, Ihn et al. 2005)
αVβ6	1. Inflammation 2. Carcinoma 3. Fibrosis 4. Cataracts	1. αVβ6 protects against inflammatory periodontal disease through activation of TGF-β (Ghannad, Nica et al. 2008). 2. Blockade of integrin αVβ6 inhibits tumor progression in vivo by a TGF-β regulated mechanism (Koopman Van Aarsen, Leone et al. 2008). 3. Inhibitors of αVβ6 integrin or TGF-β down-regulate fibrosis following acute or ongoing pulmonary, biliary injury, renal injury (Wang, Dolinski et al. 2007, Horan, Wood et al. 2008). 4. αVβ6 was hypothesized to be the main activator of TGF-β1 in the lens capsule and represents a possible target for the prevention of posterior capsular opacification (Ulrike, Stefan et al. 2005).
αVβ8	1. Immune dysfunction 2. COPD 3. Brain Hemorrhage	1. αVβ8 -mediated TGF-β activation by dendritic cells is essential to prevent inflammatory bowel disease and autoimmunity (Travis, Reizis et al. 2007). 2. αVβ8 integrin-mediated TGF-β activation amplifies pathologic epithelial-mesenchymal in chronic obstructive pulmonary disease patients (Araya, Cambier et al. 2007). 3. αVβ8 acts as a central regulator of brain vessel homeostasis through its regulation of TGF-β activation (Cambier S, Stephanie Gline et al. 2005).

Several integrin targeted therapies are in clinical development for the treatment of cancer (Nemeth, Nakada et al. 2007). For instance, Cilengitide or EMD12197 (Merck

KGaA, Darmstadt, Germany), is a small cyclic RGD designed peptide that selectively and competitively antagonizes ligand binding to αVβ3 and αVβ5 (Nisato, Tille et al. 2003), which is being evaluated in a phase III clinical study for treatment of glioblastoma (Tabatabai, Weller et al. 2010). A number of monoclonal antibodies are also in clinical development. CNTO 95™ is a fully humanized monoclonal antibody targeting αV integrin which shows anti-tumor and anti-metastatic activity in animal models and is in a Phase I clinical trial for the treatment of solid tumors (Mullamitha, Ton et al. 2007, Chen, Manning et al. 2008). Likewise, Vitaxin™, also known as MEDI-522 or Abegrin, is also a humanized monoclonal antibody that can block the interaction of αVβ3 with various ligands such as osteopontin, latent TGF-β and vitronectin (Wilder 2002). Vitaxin™ is currently in clinical trials for the treatment of stage IV metastatic melanoma and androgen-independent prostate cancer (Tucker 2006). Notably, MedImmune Inc. ended advanced human testing of Vitaxin™ to treat rheumatoid arthritis and psoriasis in 2004 because it failed to show clinical benefits in initial studies (Rosenwald 2004). More recently, it was shown that pre-treament of osteoclasts with macrophage colony stimulating factor (M-CSF), which is known to activate αVβ3, enhanced Vitaxin's™ inhibitory effect. Furthermore, the PI3-kinase inhibitor wortmannin abolished M-CSF's effects on the action of Vitaxin™ suggesting that Vitaxin's™ inhibitory effects require an activated form of αVβ3 integrin and that PI3-kinase signaling is involved in the process (Gramoun, Shorey et al. 2007). On the other hand, numerous studies have shown that PI3K-Akt signaling is involved in TGF-β induced EMT and cell migration (Bakin, Tomlinson et al. 2000, Runyan, Schnaper et

al. 2004). This exemplifies how understanding the cross-talk between αV integrins and TGF-β signaling can enhance the therapeutic potential of not only Vitaxin^{TM}, but other integrin antagonists as well, to make better and more successful therapeutics.

5.4 **Conclusion**

Integrins and their signaling can regulate EMT by both perturbing cell adhesion and stimulating EMT associated gene expression. In addition, integrins containing the αV subunit can activate latent TGF-β to result in TGF-β induced EMT. Concomitantly, TGF-β signaling can activate integrins and also upregulate integrin expression. TGF-β signaling can also induce a contractile cytoskeleton and a stiff cellular microenvironment to further facilitate latent TGF-β activation by αV integrins. This altogether creates a feed-forward circle of cross regulation between αV integrins and TGF-β that can drive the EMT responsible for fibrotic PCO.

Whether the EMT seen in Crybb2$^{Phil/Phil}$ mutant lenses is induced in a similar fashion as that occurring post-surgery or via the PI3K/AKT/mTOR and ERK cell survival pathways, this requires further investigation. Nonetheless, in this study, I demonstrated that integrins belonging to the αV family are not essential for lens maturation or homeostasis but are upregulated at the protein level in the LCs remaining on the lens capsule in a mouse model of cataract surgery. Further, LCs lacking the αV integrin gene fail/delay entrance into the EMT that is known to cause fibrotic PCO. Since therapeutics targeting αV integrins have been developed and are in clinical trials

for other diseases (Nemeth, Nakada et al. 2007), my current findings strongly indicate that such drugs may also be used as therapeutics for preventing PCO and treating ASC.

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Appendix A

JCMM CONSENT

Appendix B

IRB APPROVAL

RESEARCH OFFICE

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

UNIVERSITY OF DELAWARE

DATE: September 10, 2012

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

STUDY TITLE: [375348-1] Use of human cadaver eyes in research

SUBMISSION TYPE: New Project

ACTION: DETERMINATION OF NOT HUMAN SUBJECTS RESEARCH
DECISION DATE: September 10, 2012

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

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

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

University of Delaware
Institutional Animal Care and Use Committee
Annual Review

(Please complete below using Arial, size 12 Font.)

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

AUP Number: 1039-2014-1 ← **(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
X ☐ 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: 12/1/2013

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 10/1/13 — 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	Melinda Duncan
2. Yan Wang	[illegible]
3. Yichen Wang	Yichen Wang
4. Dylan Audette	[illegible]
5. David Scheiblin	David Scheiblin
6. Mallika Pathania	OFF ON MATERNITY LEAVE W/SIGN W/SHE RETURNS TO CAMPUS
7. Saleena Malik	Saleen
8. Fahmy Mamuya	[illegible]
9.	
10.	
11.	
12.	
13.	
14.	
15.	

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

1. **Previous Approval Date:** 12/1/2012

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

   X Yes ☐ **No**

   If no, please state Funding Source and Award Number:

2. **Record of Animal Use:**

Common Name	Genus Species	Total Number Previously Approved	Number Used To Date
1. mouse	*Mus musculus*	12,000	3309
2. rat	*Rattus rattus*	30	none
3. chicken (embryo)	*Gallus gallus*	9000	60
4. chicken (post hatch)	*Gallus gallus*	60	none
5.			

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

   Request for Protocol Continuance:

   X☐ A. Active: Project ongoing

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

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

   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? X☐ Yes ☐ No

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

Project Personnel Deletions:

Name	Effective Date
1. Abby Grabitz	5/2013
2. Jocelyn Zajac	5/2013
3. Cullen Worsh	2/2013
4. Anne Terrell	6/2013

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

We have been making substantial progress on the work leading to the following publications either accepted or under review. We are still actively working on the function of beta crystallins in the lens, the function of Zeb proteins in EMT, the Function of Bin3 in the lens, the function of Prox1 in the lens, the function of beta1 integrins in the lens both early and late in development and the function of fibronectin in the developing lens and during EMT.

Bhagwat V. Alapure*, Jaime K. Stull*, Zeynep Firtina and **Melinda K. Duncan** (2012) The Unfolded Protein Response is activated in Connexin50 mutant mouse lenses *Experimental Eye Research* **102,** 28-37.

Sharmila Chatterjee, Yan Wang, **Melinda K. Duncan** and Ulhas P. Naik (2013) Junctional adhesion molecule-A regulates vascular endothelial growth factor receptor-2 signaling-dependent mouse corneal wound healing. *PLOS one* 8(5):e63674. doi: 10.1371/journal.pone.0063674

Abby L. Manthey, Salil A. Lachke, Paul G. FitzGerald, Robert W. Mason, David A. Scheiblin, John H. McDonald, and **Melinda K. Duncan** (2013) Loss of Sip1 leads to migration defects and retention of ectodermal markers during lens development *Mechanisms of Development, in press.*

Fahmy A. Mamuya, Yan Wang, Victoria H. Roop, David A. Scheiblin, Jocelyn C. Zajac and **Melinda K. Duncan** (2013) The Roles of α_V Integrin in Posterior Capsular Opacification *Journal of Cellular and Molecular Medicine, in revision*

David A. Scheiblin, Junyuan Gao, Jeffrey L. Caplan, Vladimir N. Simirskii, Kirk J. Czymmek, Richard T. Mathias and **Melinda K. Duncan** (2013) Beta-1 integrin is important for the structural maintenance and homeostasis of differentiating fiber cells *International Journal of Biochemistry and Cell Biology, submitted#1039-2014-1 MDuncan*

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