THE EFFECTS OF BETAB2-CRYSTALLIN MUTATION ON TGFBETA SIGNALING LEADING TO EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The mammalian eye lens consists of fiber cells covered on the anterior surface by a monolayer of cuboidal epithelial cells. The most abundant protein in adult lens fibers is βB2-crystallin. It has recently been demonstrated that in mice homozygous for a 12 nucleotide deletion in the βB2-crystallin gene (Crybb2^{Phil}), the lens epithelium undergoes an epithelial-mesenchymal transition (EMT) leading to severe lens abnormalities. The TGFβ signaling pathway is involved in other cases of EMT in the lens and other systems. Therefore, a SuperArray real time RT-PCR gene expression panel was used to test the hypothesis that TGFβ signaling is activated in the Crybb2^{Phil} homozygous lens. It was found that of the 84 genes represented on the panel, 15 were significantly upregulated in the Crybb2^{Phil} lens epithelium at 4 months of age. These results were used to select genes of interest for further investigation. The mRNA expression of several genes was studied at earlier points in development. This showed that expression of several of these genes changed over time in a manner indicating TGFβ activity between 3 weeks and 2 months of age.

Chapter 1

INTRODUCTION

1.1 Structure, function, and development of the mammalian lens

The main function of the mammalian eye lens is to focus light on the retina. The lens contributes approximately 33% of the refractive power of the eye, while the cornea provides the other 67% (Hung 2001). However, unlike the cornea, the shape of the human lens can change, changing the focal depth of the eye. This ability of the lens, called accommodation, allows humans to focus on objects at a wide range of distances. In order to perform its function of refracting light, the lens must have a high refractive index and be as transparent as possible; this is achieved by the highly ordered cellular and molecular arrangement of the lens (Donaldson, Kistler et al. 2001). Disturbances in this ordered state, which can result from inherited genetic defect, injury to the lens, or simple aging, can lead to opacification of the lens, known as cataract (Oyster 1999).

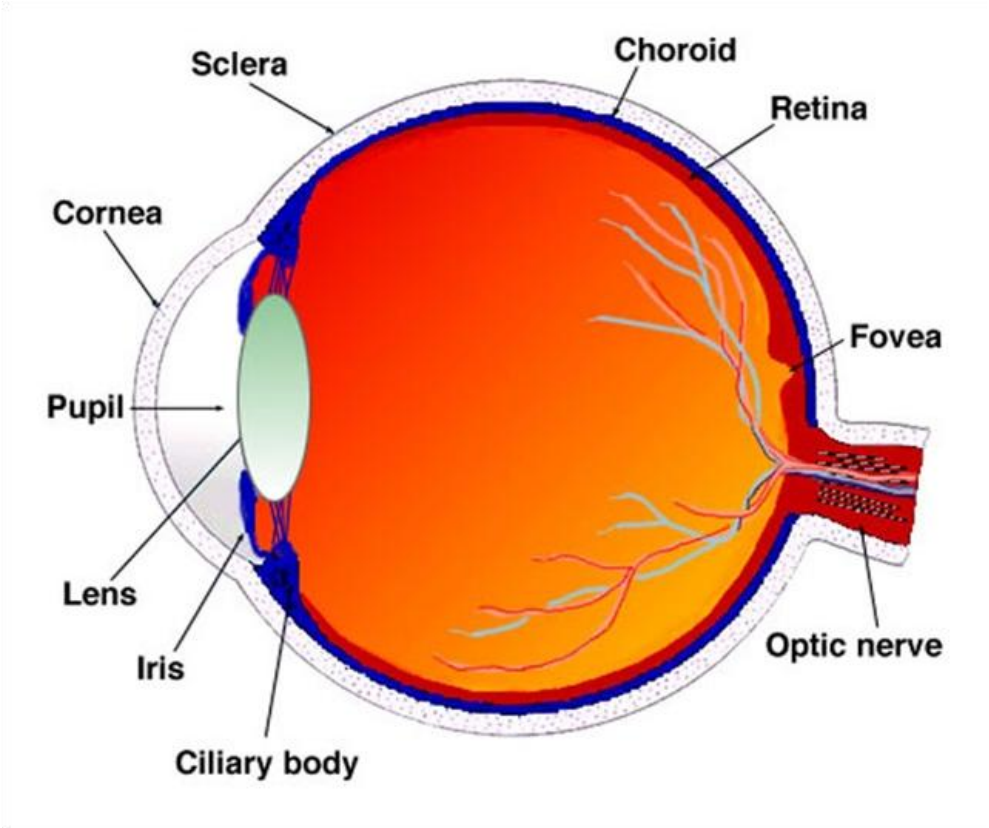

Figure 1. **Structures of the human eye.** Light passes through the cornea and the lens before being focused on the retina. (Kolb, Fernandez et al. 2008)

The mammalian lens consists of two cells types. Fiber cells form the bulk of the lens, while the anterior surface is covered by a monolayer of cuboidal epithelial cells (Craig and Paton 1974). The entire lens is surrounded by the lens capsule, a basement membrane secreted by the lens epithelium (de Iongh, Wederell et al. 2005).

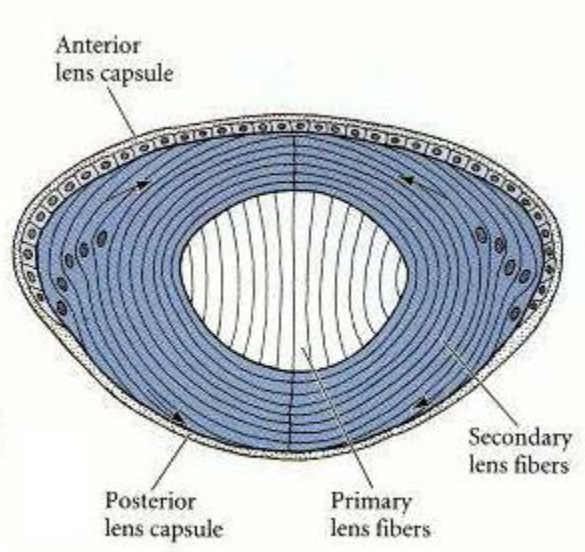

Figure 2. **The human lens.** The bulk of the lens consists of fiber cells. The lens epithelium covers the anterior portion of the lens, and the lens capsule surrounds the entire lens (Craig and Paton 1974).

During embryonic development, the lens starts as a hollow ball of epithelial cells, surrounded by a basement membrane (the lens capsule). Upon stimulation with signals from the retina, epithelial cells in the posterior portion of the lens vesicle terminally differentiate into primary fiber cells (Oyster 1999). These cells elongate until their apical surfaces reach the apical surfaces of the anterior epithelial cells (Zampighi, Eskandari et al. 2000). This differentiation is also marked by the loss of organelles, including nuclei, and by high expression of β/γ crystallins (Lovicu, Ang et al. 2004). Eventually these fiber cells lose their basal connection to the basement membrane, and form the center of a lens "nucleus" (Gwon 2006).

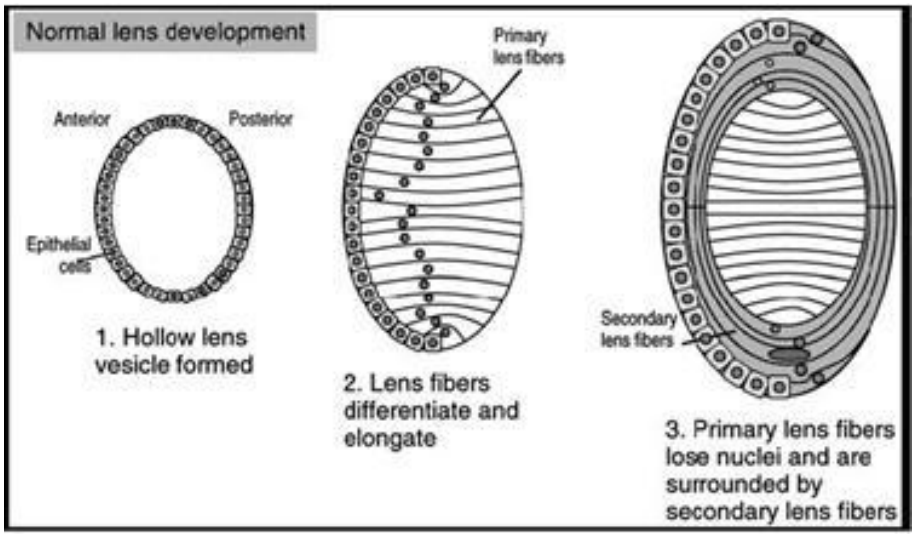

Figure 3. **Prenatal development of the lens.** The lens develops from the lens vesicle. The anterior epithelial cells elongate and lose their organelles, becoming the primary fiber cells (Gwon 2006).

At this point in development, the basic morphological features of the lens (fiber cells, epithelium, and capsule) are all present. Throughout the life of organism, epithelial cells at the equatorial region of the lens, the transition zone, continue to terminally differentiate into new fiber cells, with new cells elongating and forming successive layers over old cells, so that the lens continues to grow throughout life. The anterior lens epithelium remains throughout the life of the organism (Menko 2002).

1.2 Crystallins in the lens

In order to create the transparency and high refractive index necessary in the lens, lens cells have an unusually high concentration of proteins; 30-35% w/w, compared to an average of 15% w/w in other body tissues (Purves, Sadava et al. 2004; Hoehenwarter, Klose et al. 2006). The concentration of crystallins is greatest in the central fiber cells, creating a gradient refractive index (Donaldson, Kistler et al. 2001).

90% of the water soluble proteins of the lens are crystallins (Andley 2007). There are two superfamilies of crystallin proteins, α and β/γ.

1.3 α-crystallins in the lens and other tissues

There are two α-crystallins, αA and αB, both of which are expressed in the lens as well as other tissues. In the lens, the α-crystallins form large complexes and act as molecular chaperones for other lens proteins, preventing abnormal protein aggregation and therefore maintaining lens transparency. α-crystallins are expressed in both lens epithelial and fiber cells. Structurally, they are very similar to small heat shock proteins, and they are believed to act as chaperones to help stabilize the other proteins of the lens. αB has been shown to inhibit apoptosis by slowing the maturation of caspase-3, and may be involved in preventing cell death in stressed cells (Andley 2007).

1.4 The β/γ crystallin superfamily

The other major class of crystallins is the β/γ superfamily. This group is further subdivided into β and γ crystallins, which have similar protein folds. The characteristic domain is a "β sheet sandwich" composed of two Greek key motifs arranged to form a wedge. One Greek key is composed of a 4 stranded antiparallel β sheet. A schematic diagram of this arrangement is shown in Figure 4. While γ crystallins are monomers, β crystallins exist as homo- or hetero- dimers, as well as large oligomeric complexes (Slingsby and Clout 1999).

Because the crystallins found in lens fiber cells do not turn over, these proteins must be extraordinarily stable. The Greek key motif is a very stable motif found in microbial stress proteins (Jaenicke and Slingsby 2001).

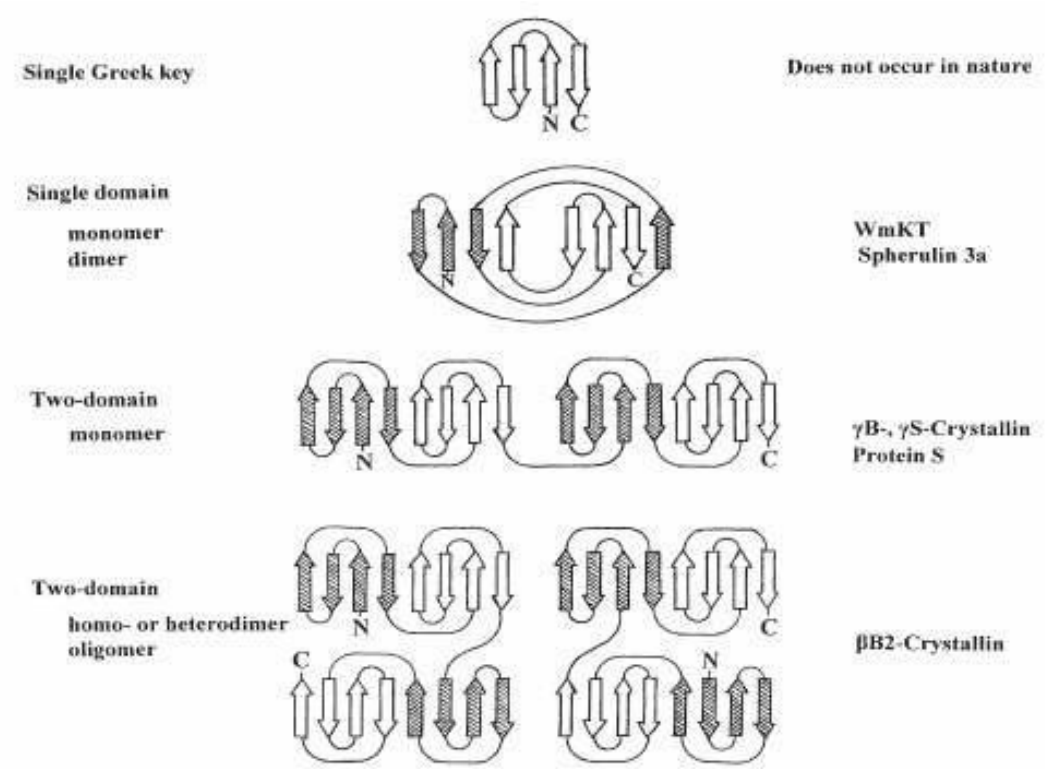

Figure 4. Schematic diagram of several protein folds involving Greek key domains. The Greek key domain is a very stable structure and the basic component of β/γ crystallins. (Jaenicke and Slingsby 2001)

Although it was previously believed that β/γ crystallins were only expressed in fiber cells, recent results have shown that these proteins appear in lens epithelial cells (Wang, Garcia et al. 2004) and extralenticularly (Andley 2007). For example, βB2-crystallin is expressed in ovaries and testes, and may play a role in both male and female fertility (Duprey, Robinson et al. 2007). In the lens, β/γ crystallins are considered structural proteins; other functions are suspected but currently unclear. One proposed function of β crystallins is regulating calcium levels in the lens and other tissues (Jobby and Sharma 2007).

1.5 βB2-crystallin

The most abundant protein in the fiber cells of the adult human lens is βB2-crystallin (Jobby and Sharma 2007). Figure 5 shows the 3rd and 4th Greek key motifs of normal human βB2-crystallin, folded in the characteristic sandwich. The entire protein consists of two sandwiches like that shown in Figure 5, and a C–terminal tail. βB2 is classified as a basic β-crystallin, due to its basic pI; this class is also characterized by a long C-terminal domain (Slingsby and Clout 1999).

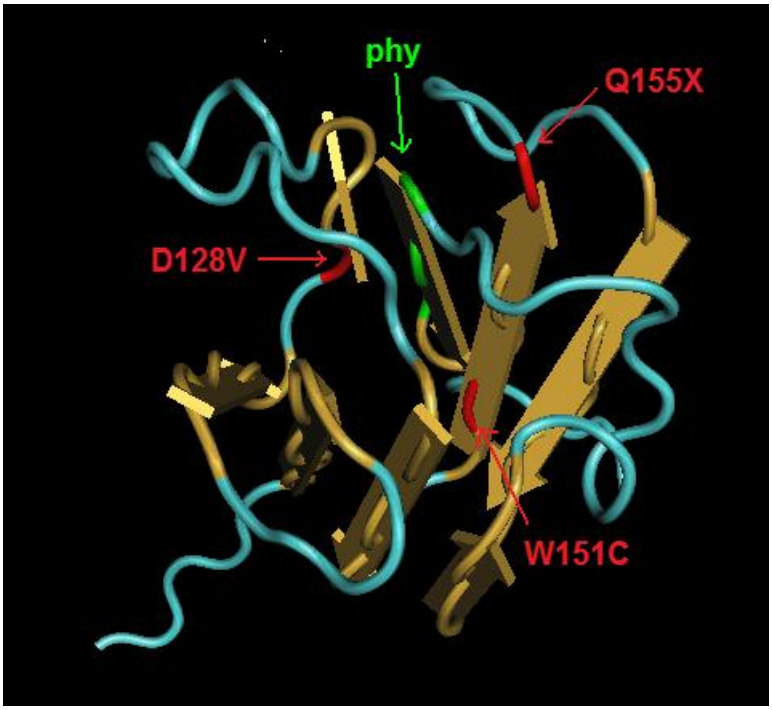

Figure 5. **A β-pleated sheet "sandwich". β**B2-crystallin contains two of these sandwiches joined by a flexible linker strand. This diagram shows the 3rd and 4th Greek key motifs of βB2-crystallin. (Slingsby, Smith et al. 2005) Amino acids which have been found mutated in humans are shown in red. The amino acids deleted in the Crybb2^{Phil} mutant are shown in green.

In the rat lens, the expression of βB2-crystallin mRNA in the lens is low during prenatal development, but rises quickly after birth and peaks approximately 4-5 months postnatally (Aarts, Lubsen et al. 1989).

In the lens, β-crystallins form large oligomeric complexes; at the lower protein concentrations present in other tissues, they may exist as dimers (Slingsby and Clout 1999). The discovery that βB2 crystallin binds calcium raises the possibility that it (as well as other β-crystallins) acts as a calcium buffer in the lens, actively protecting the lens from calcium-induced cataract (Jobby and Sharma 2007). In general, βB2 is the most resistant to modification of the β-crystallins (Duprey, Robinson et al. 2007). Cataract may arise in the lens due to the age-related degradation and insolubilization of certain crystallins, including βB2 (Ueda, Duncan et al. 2002).

1.6 Mutations of CRYBB2 lead to cataracts in humans

Mutations in CRYBB2 has been associated with cataract formation in humans (Bateman, von-Bischhoffshaunsen et al. 2007). The areas of the protein affected by several of these mutations are shown in red in Figure 2. The Q155X mutation is the best studied CRYBB2 mutation known to cause human cataracts. This allele 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. In some cases, an additional C → T mutation is found in association with the first mutation, but because the mutation occurs downstream of the new stop codon, it causes no additional phenotypic effect (Bateman, von-Bischhoffshaunsen et al. 2007). This mutation appears to have occurred by a gene conversion event between CRYBB2 and the closely related pseudogene CRYBB2P1 (Vanita, Sarhadi et al. 2001).

The Q155X mutation has been found in several unrelated families, causing a variety of cataract phenotypes. The mutation was first identified in a family exhibiting autosomal dominant cerulean cataract, with cataract formation beginning prenatally or during childhood, but usually becoming severe only during adulthood (Litt, Carrero-Valenzuela et al. 1997). Another study found this mutation to be one cause of autosomal dominant Coppock-like cataract, in which case visual impairment began in the teens and generally became severe in the 40s (Gill, Klose et al. 2000). One family with the mutation exhibits sutural cataract with cerulean opacities, with easily observable opacities forming during childhood (Vanita, Sarhadi et al. 2001).Yet another study described a family exhibiting highly variable cataract phenotypes usually appearing after birth or during childhood, and requiring surgery by 40 years of age (Yao, Tang et al. 2005). The highly variable phenotypes associated with this single mutation suggest the existence of unidentified genetic modifiers.

Further mutations in CRYBB2 associated with cataract formation have recently been observed. An Indian study identified several mutations in this gene which are believed to cause cataracts, including one causing the substitution of cystine for tryptophan at position 151. This mutation is believed to interfere with the folding of the Greek key domains (Santhiya, Manisastry et al. 2004). Another study found a mutation involving A → T mutation at amino acid 383, causing the substitution of valine for asparagine in the midst of a region of amino acids highly conserved among species. This mutation causes congenital cataract (Pauli, Soker et al. 2007).

1.7 The Philly mutant

The Philly mouse is a model for studying congenital cataract and the role of βB2 crystallin in the lens. This animal carries a mutant Crybb2 gene with a 12

nucleotide deletion in the coding region and a separate point mutation (Crybb2^{Phil}). This gene produces an altered peptide lacking 4 amino acids (shown in green in Figure 5) and with serine substituted for isoleucine at position 166. These mutations prevent the correct folding of the protein. It is speculated that this alteration prevents the normal formation of β-crystallin complexes and promotes improper aggregation and insolubilization (Chambers and Russell 1991).

Initial studies of the Crybb2^{Phil} phenotype showed that the lens developed normally through the first week postnatal. Opacities began to appear in the anterior subcapsular region during the second postnatal week. By the second postnatal week, differentiating fiber cells were observed to abnormally retain their nuclei. By the sixth week, the entire lens was opaque (Uga, Kador et al. 1980).

Results obtained in our lab indicate severe disruption of the cells of adult Crybb2^{Phil} homozygotes. F-actin appears to be lost in the fiber cells of adult homozygotes. The intermediate filament protein filensin seems to be slightly upregulated in the fiber cells of adult Crybb2^{Phil} homozygotes (Decker 2008).

1.8 EMT in Crybb2^{Phil} homozygotes

In homozygous Crybb2^{Phil} mutants, the lens epithelium inappropriately undergoes an epithelial mesenchymal transition (EMT) during development (Decker 2008). EMT occurs naturally in the development of several tissues, but in this case, it restricts the growth of the lens and causes small, non-transparent lenses. EMT occurs when epithelial cells respond to some sort of signal and differentiate into myofibroblast-like cells. When this occurs, certain molecular markers associated with epithelial cells, such as Pax6 expression, are turned off; certain molecular matrix

proteins, including αSMA, are upregulated in the differentiated cells (de Iongh, Wederell et al. 2005).

In the Crybb2^{Phil} homozygous mouse, plaques of fibrotic cells begin to form by 4 weeks postnatal. αSMA expression increases in the lens epithelium, E-cadherin expression is lower and not localized to cell-cell junctions. These findings indicate that the cells have lost their epithelial character. However, changes in αSMA and E-cadherin expression occur after fibrosis is evident, indicating that these are an effect rather than a cause the EMT observed in this model (Decker 2008).

Furthermore, expression of the epithelial transcription factor Pax6 is not downregulated as would be expected; in fact Pax6 expression is increased in the homozygous mutant epithelium. Therefore, although epithelial cells in this system clearly lose characteristics of normal epithelial cells and acquire myofibroblast-like properties, this is not a "classic" EMT (Decker 2008).

1.9 Posterior capsule opacification and EMT

EMT also occurs frequently after cataract surgery, when lens cells respond to the surgical wound by proliferating and creating scar tissue. If these cells spread into the visual axis, they cause opacity and obstruct vision. Soemmering's ring, a ring of proliferating cells around the periphery of the lens, can also form (Chew, Werner et al. 2006). Both cases necessitate further surgery, making prevention desirable (Medvedovic, Tomlinson et al. 2006). It is hoped that a better understanding the triggers of lens EMT will eventually lead to treatments which can prevent the necessity for further surgery. Therefore, it is important to understand the role of βB2-crystallin in lens EMT.

1.10 TGFβ/Smad signaling

The Transforming Growth Factor β (TGFβ) pathway is known to be involved in lens EMT following lens injury (Saika, Okada et al. 2001) and in the subcapsular cataracts of a transgenic mouse model (Lovicu, Ang et al. 2004). TGFβ signaling is normally involved in the differentiation of lens fiber cells (de Iongh, Wederell et al. 2005). The classical pathway is initiated when the signaling molecule transforming growth factor (TGFβ) binds to a receptor complex, which leads to activation by phosphorylation of Smad 2 or 3. The activated Smad then binds Smad4, and the complex moves to the nucleus where it acts as a transcription factor (Miyazono, ten Dijke et al. 2000).

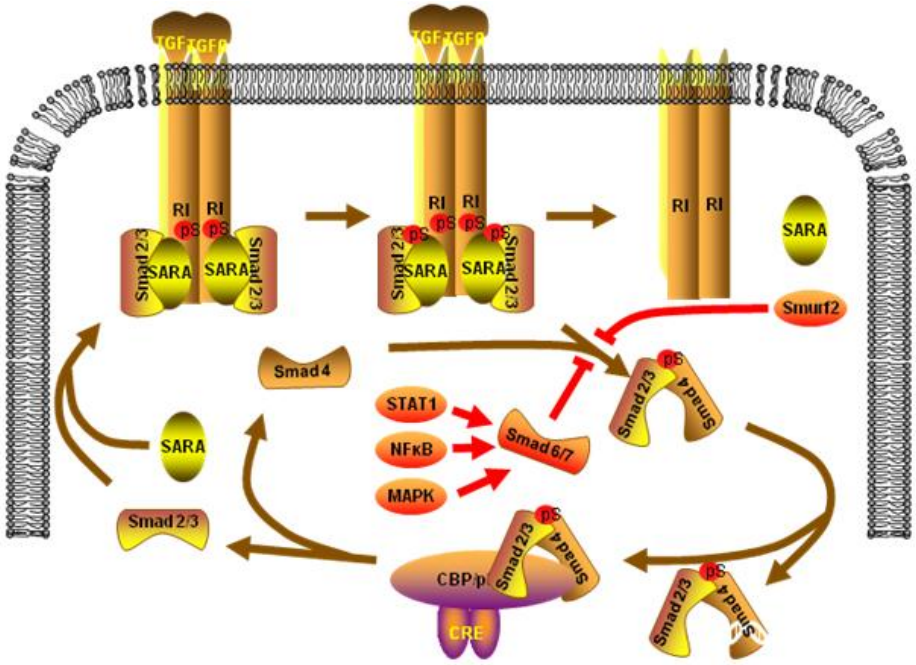

Figure 6. **TGFβ/Smad signaling.** Signaling is initiated when TGFβ binds to the receptor dimer. This leads to the phosphorylation of Smad 2 or 3, which binds to Smad 4. The Smad complex translocates to the nucleus and acts as a transcription factor. (Duncan 2008)

Stimulation of lens epithelial cells with TGFβ has been shown to induce the loss of epithelial markers such as E-cadherin and connexin 43, and to stimulate the expression of myofibroblast markers such as αSMA (Lovicu, Ang et al. 2004). In anterior polar cataracts, mRNA expression of both TGFβ and the type I and II receptors has been shown to be upregulated, indicating that abnormal autocrine stimulation in lens epithelial cells may be one method of pathenogenesis (Lee, Seomun et al. 2000). TGFβ is well established as a cause of EMT in cultured and *in vivo* lens epithelial cells (de Iongh, Wederell et al. 2005). If the Crybb2^{Phil} mutation activates TGFβ/Smad signaling in the lens epithelium, this may connect βB2-crystallin to TGFβ-induced lens EMT in other systems, such as PCO.

Chapter 2

MATERIALS AND METHODS

2.1 Crybb2 Genotyping

After weaning, mice from Crybb2 heterozygous matings were labeled with numbered ear tags and 1 cm tail snips were taken. Tail snips were stored at -80ºC. DNA for genotyping was isolated from these tail snips using the PUREGENE DNA Isolation Kit (Gentra Systems). Cells were lysed in 600 μL Cell Lysis Solution, (a Tris, EDTA, SDS solution) and 10 μL Proteinase K Solution. This preparation was mixed by inversion and then incubated overnight in a shaking water bath at 55ºC. The samples were then cooled to room temperature.

200 μL protein precipitation solution (ammonium acetate) was added to the lysate. The samples were mixed by vortexing at high speed for 10-20 seconds, and then centrifuged at 13,000 rpm for 6 minutes to pellet the protein. The DNA-containing supernatant was poured into a new 1.5 mL tube containing 600 μL 100% isopropanol, and samples were mixed by inversion. The samples were centrifuged for 1 minute at 13,000 rpm to pellet the DNA. The supernatant was discarded and the pellet was washed with 600 μL 70% ethanol. Tubes were air dried for 15 minutes on a clean paper towel. The DNA was then rehydrated overnight in 100 μL DNA Hydration Solution (containing Tris, EDTA), at room temperature. The DNA was stored at 4ºC.

DNA was amplified by PCR using the forward primer (5’ CTA CCG TGG GCT GCA CCT GC 3’) and the reverse primer (5’ GTG GAA GGC ACC TCG CTG

GTG C 3'). The region amplified by these primers includes the 12 bases deleted in the *Crybb2*^{Phil} gene. A 132 bp product is expected using wild-type DNA, while a 120 bp product is expected using *Crybb2*^{Phil} DNA. The cycling conditions for the PCR reaction were as follows: 30 second initial denaturation at 95ºC followed by 30 cycles of 94ºC, 30 seconds; 60ºC, 30 seconds; 72ºC, 30 seconds; and a final 10 minute extension at 72ºC. Finally, samples were cooled to 4ºC.

The amplified DNA was resolved on a 10% polyacrylamide gel. 10 μL DNA samples were mixed with 1 μL gel running dye. 10 μL of a 100 bp ladder were also mixed with 1 μL running dye. 10 μL of sample were pipetted into each well, or 5 μL of ladder. The gel was run for approximately 2 hrs at 150V. The gel was then stained with ethidium bromide for 10 minutes, and the gel was observed under UV light. By this method, the 120 bp product of the *Crybb2*^{Phil} gene could be easily distinguished from the 132 bp product of the wild-type gene, and the mice could be classified as wild-type, heterozygous, or homozygous mutants.

2.2 Tissue Harvesting for Immunofluorescence

Eyes were harvested from C57Bl/6 +/+ and Crybb2^{Phil} -/- mice of appropriate ages. Mice were euthanized by asphyxiation in a CO_2 chamber (Fisher), and the necks broken. Whole eyes were frozen in TissueTek OCT Compound in 10mm x 10mm x 5 mm TissueTek Crymold Biopsy molds on dry ice and then stored at -80ºC. 16 μm sections were collected on Fisher Colorfrost/Plus slides using a Leica CM3050 S Cryostat at -15 to-17ºC and stored at -80ºC.

2.3 Igfbp3/αSMA Immunofluorescence

Sections were fixed in a 1:1 acetone-methanol solution, then were blocked for 1 hour in 1% BSA in PBS. Igfbp3 antibody (R&D Systems, AF775) was diluted to a concentration of 20 μg/mL in 1% BSA and applied to blocked sections. Antibody was allowed to incubate for 1 hour at room temperature. 3 10-minute washes were performed in 1x PBS. Slides were then incubated for 1 hour at room temperature with a second antibody solution containing: AlexaFlour® 568 donkey anti-goat IgG (Invitrogen, A-11057) diluted to 8 μg/mL; monoclonal anti-actin, α-Smooth Muscle-FITC conjugated monoclonal αSMA antibody (Sigma, F3777) diluted 1:250; and DRAQ5^{TM} nuclear stain (Biostatus Lmd., (1:3000), diluted in 1% BSA. Slides were washed again, and examined using a Zeiss LSM 510 Confocal Microscope.

2.4 RNA Isolation

RNA for reverse-transcription PCR was isolated using SV Total RNA Isolation System (Invitrogen). Tissue was submerged in 175 μL Lysis buffer and ground in a 1.5 mL Eppendorf tube. 350 μL of SV RNA Dilution Buffer was added. Samples were mixed by inversion and then incubated in a 70°C water bath for up to 3 minutes. Samples were then centrifuged at 13000 rcf for 10-15 minutes to pellet protein. The supernatant was transferred to a fresh 1.5 mL Eppendorf tube, and 200 μL of 95% ethanol was added. Samples were mixed by flicking the tubes, and then transferred to a spin column assembly containing a silica glass membrane for binding RNA. Samples were centrifuged at 13000 rcf for 1 minute and the liquid in the collection tube was discarded. The membrane was washed with 600 μL of SV RNA Wash solution and centrifuged for 1 minute at 13000 rcf; the liquid in the collection tube was discarded. The membrane was then incubated for 15 minutes at room

temperature with a DNase solution containing: 40 µL Yellow Core Buffer; 5 µL 0.09M $MnCl_2$; and 5 µL DNase I enzyme. At the end of the incubation, 200 µL of SV DNase Stop Solution were added to the membrane, which was centrifuged at 13000 rcf for 1 minute, and the liquid in the collection tube was discarded. The membrane was washed with 600 µL SV RNA Wash solution and centrifuged, and then washed again with 250 µL SV RNA Wash solution; liquid was discarded. Finally, RNA was eluted from the membrane with 70-100 µL of Nuclease-Free water. Isolated RNA was stored at -80°C.

2.5 cDNA Synthesis

cDNA was synthesized using a SuperScript® III First Strand Synthesis kit (Invitrogen). A RNA mixture was prepared consisting of the following: 1 µL random hexamers; 1 µL 10 mM dNTP mix; up to 5 µg total RNA; and nuclease-free H_2O to 10 µL. This mixture was incubated for 5 minutes at 65°C, then chilled on ice for 1 minute. A cDNA synthesis mixture was prepared containing: 2 µL 10 RT buffer; 4 µL 25 mM $MgCl_2$; 2 µL 0.1 M DTT; 1 µL RNaseOUT™ (40 U/µL); 1 µL SuperScript™ III RT (200 U/µL). This reaction mixture was added to the RNA mixture, and mixed, and incubated for 10 minutes at room temperature, then for 50 minutes at 42°C. The reaction was terminated for 5 minutes at 70°C. 1 µL RNase H was added to the mixture, which was incubated for 20 minutes at 37°C. When not used immediately, cDNA was stored at -80°C.

2.6 RT^2 *Profiler*^{TM} PCR Array

A RT^2 Profiler^{TM} PCR Array System (SA Bioscience) was used to investigate the expression of 84 genes involved in TGFβ signaling in the mutant and wild type adult lens epithelium.

The experimental mixture was prepared by mixing 1275 µL of Master Mix with 81 µL of cDNA and 1194 µL of ddH_2O. 25 µL of this solution was added to each of the 96 wells of the SuperArray plate. The real-time PCR reaction was performed using an ABI Prism 7000 Sequence Detection System. 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. After PCR, the results were loaded into an Excel template file and 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 2 sample groups. The program uses the ΔΔCT method described in section 2.8.

2.7 Primer Design for Real Time PCR

All primers were designed using the Primer3 program at http://frodo.wi.mit.edu/. Default settings were used with the following exceptions: optimal product size was set to 100-150 base pairs; max Poly-X was set to 3; optimal primer size was set to 21 base pairs. Appropriate target areas were determined using exon structures obtained from the Ensembl Genome Database. Primer specificity was checked using NCBI's Primer BLAST function; primers were checked for unintended products in both mRNA and genomic DNA.

2.8 Real-Time RT-PCR

Real-Time PCR was performed using an ABI Prism 7000 Sequence Detection System. Samples were prepared in a MicroAmp® Optical 96-Well Reaction Plate. Each well contained: 4 μL of cDNA, 12.5 μL of SYBR Green Master Mix, 1.5 μL each of forward and reverse primers, and H_2O to 25 μL. The primers used for these experiments are shown in Table 1. The following cycling conditions were used: 45 cycles of 30 seconds at 95°C, 15 seconds at 58°C, and 15 seconds at 68°C.

The machine returns data in the form of C_T for each well. The C_T of a sample is the cycle number at which fluorescence reached a certain threshold. Ideally, the amount of double stranded DNA doubles in each cycle, as each targeted strand in the sample serves as a template for 1 new strand. Therefore, the C_T will depend of the amount of target template in the original sample.

Sample C_Ts were normalized by subtracting the average C_T values of control genes, whose expression should be constant through development in the mutant and wild type. This gives the ΔC_T value.

$$\Delta C_T = C_T^{exp} - C_T^{control}$$

To find the level of expression of the gene compared to the control genes, the following calculation is used, taking advantage of the fact that DNA concentration doubles with each cycle:

$$expression = 2^{\Delta C_T}$$

To compare the expression of a gene in the mutant and wild type samples the $\Delta\Delta C_T$ is found.

$$\Delta\Delta C_T = \Delta C_T^{mutant} - \Delta C_T^{wild\ type}$$

The $\Delta\Delta C_T$ is then used to find a ratio of expression in the mutant to the wild type, according to the following equation:

relative expression = $2^{-\Delta\Delta C_T}$

Table 1 Primers for Real-Time RT-PCR

Gene	Forward Primer	Reverse Primer
αSma	CCGAGATCTCACCGACTACCT	GCACAGCTTCTCCTTGATGTC
Col1α1	AGGAGCTAGAGGCTCTGAAGG	AGCAATACCAGGAGCACCATT
Crybb2	AGGACAGACTCCCTCAGCTCT	GGCACATCGTCGTCTACAATC
Fst	TGGATAGCCTATGAGGGAAAG	GACACAGCTCATCGCAGAGA
Igfbp3	CTAAGCGGGAGACAGAATACG	GTCACAGTTTGGGATGTGGAC
Ltbp2	CTGGCTCCTACACTTGTCTGG	AAAGGAGCCTTCCATGTTGAT
Smad2	AAGATGGAGAAACAAGTGACCAA	CCAAGCTGTGATTAACAGGAGA
Smad4	CTAATTTGCCTCACCACCAAA	CCAGTACTCAGGAGCAGGATG
Tgfbi	CCTCACCTCCATGTACCAGAA	TGGAAATGACCTTGTCAATGAG
TR2	TGTGTGCCTGTAACATGGAAG	GGTGGACACGGTAGCAGTAGA
TR5	TGGATGAGCATACCATCAACA	AGATCCTGTCGTCAATGTCGT
Loading Controls:		
β2m	TACGCCTGCAGAGTTAAGCAT	TCAAATGAATCTTCAGAGCATCA
Gusb	GGGTCAATAAGCACGAGGATT	AGTGGCTGGTACGAAAGGAAT
Tbp	CCGTGAATCTTGGCTGTAAAC	TCCGTGGCTCTCTTATTCTCA

Chapter 3

RESULTS

3.1 TGFβ Signaling Pathway SuperArray

Due to the known importance of TGFβ signaling in other instances of lens EMT, a TGFβ BMP Signaling Pathway RT^2 *Profiler*^{TM} PCR Array (SA Biosciences, cat. #PAMM-035) was used to examine the mRNA expression of genes involved in this pathway. Of the 84 genes on the array, 20 were found to be significantly upregulated in the adult Crybb2^{Phil} mutant lens as compared to the wild type C57Bl6 lens (defined as upregulation > 5 fold and $p \leq 0.01$). These results are summarized in Table 2. ΔC_T values refer to the cycle number at which double stranded DNA levels in a particular sample passed the detection threshold, relative to the control gene samples; a lower ΔC_T indicates higher mRNA expression in the sample. Fold upregulation refers to the ratio of expression in the mutant lens epithelium to expression in the wild type lens epithelium.

Table 2. Results of TGFβ SuperArray

Gene	Abbrev.	wt ΔC_T	Crybb2^{Phil} ΔC_T	Fold Up Regulated
Follistatin	Fst	9.98	2.89	136
Transforming growth factor, beta induced	Tgfbi	6.58	-0.51	136
Insulin-like growth factor binding protein 3	Igfbp3	8.52	2.43	68
Interleukin 6	Il6	9.38	4.41	31
Platelet derived growth factor, B polypeptide	Pdgfb	7.49	2.77	26
Latent transforming growth factor beta binding protein 4	Ltbp4	7.42	2.84	24
Cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	6.98	2.64	20
Collagen, type I, alpha 2	Col1a2	5	0.8	18
Collagen, type III, alpha 1	Col3a1	8.27	4.4	15
Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	Cdkn2b	4.52	0.74	14
Bone morphogenetic protein 1	Bmp1	1.83	-1.94	14
Latent transforming growth factor beta binding protein 2	Ltbp2	5.59	1.94	13
Runt related transcription factor 1	Runx1	6.94	3.38	12
FBJ osteosarcoma oncogene	Fos	5.43	2.25	9
Transforming growth factor, beta receptor II	Tgfbr2	8.12	5.36	7

3.2 Real-Time RT-PCR

The mRNA for the initial array was obtained from pooled samples of 4-5 month old mice. However, lens EMT is observed in the Crybb2^{Phil} mutant mouse well before this age. Therefore, the initial array data was used as a guide in selecting genes for further study. The lens epithelium mRNA expression of selected genes was studied was studied for lenses of the following ages: 1 week, 3 weeks, 2 months, and 4 months. βB2-crystallin expression begins at approximately the time of birth (Ueda, Duncan et al. 2002); the abnormal phenotype is first observed at approximately 3

weeks of age; by 2 months, the lens of the Crybb2^{Phil} homozygote has generally undergone extensive EMT (Decker 2008).

For the following Real Time PCR results, the tables show the expression of each gene in the wild type and mutant lens epithelium over time. These values represent the fold expression above or below the average expression of the control genes. The control genes used were β2-microglobulin, TATA-binding protein, and β-glucuronidase. Rather than using decimals in cases where expression of a particular gene is lower than expression of the controls, these values are shown as negative numbers. Because the numbers are expressed this way, there is actually difference between -1 and 1, and no values can occur between these two values.

The graphs show the ratio of expression in the mutant to expression in the wild type lens epithelium. Therefore, an apparent increase or decrease in expression over time is only a relative increase, compared to the wild type expression at the same age. In other words, a relative increase could result from either increased expression of a gene in the Crybb2^{Phil} lens at a particular age, or decreased expression in the wild type, or from both. Negative values indicate that expression is higher in the wild type epithelium. As above, values between -1 and 1 are meaningless.

3.2.1 βB2-Crystallin

As Crybb2 is the mutated gene in this model, its expression was of interest. Protein expression of βB2-crystallin in the lens begins around the time of birth, and gradually increases during development (Ueda, Duncan et al. 2002).

Table 3 **Crybb2 mRNA expression in wild type and mutant lens epithelium over time**

Crybb2	1 w	±	3 w	±	2 mo	±	4 mo	±
wild type	25		505	265	1438		1335	276
Crybb2^{Phil}	1050	673	212	48	41	30	73	46

In the wild type, Crybb2 mRNA expression was fairly high at 1 week, and increased from that point. By 2 months of age mRNA expression seemed to have plateaued at a level more than 1000x that of the control genes.

In the mutant, mRNA expression was very high in the 1 week old epithelium, but then decreased. Although the mRNA expression did decrease, there was still fairly high expression by 4 months of age.

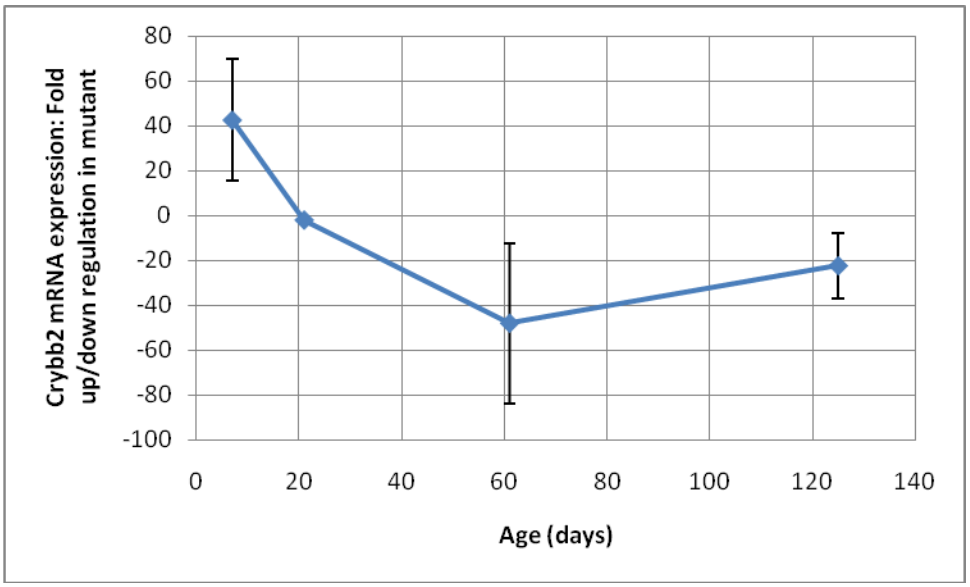

Figure 8. **Relative expression of βB2-Crystallin mRNA in lens epithelium over time.** Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

In very young mice (1 week), the expression of Crybb2 mRNA was found to be upregulated approximately 45 fold in the mutant lens epithelium compared to the wild type. By 3 weeks of age, expression in the mutant is slightly lower than in the wild type, and this decline continues, leveling off at an approximately 40 fold downregulation by 2 months of age. However, there was significant deviation in the mRNA levels measured for this gene, and the extent of up- or down-regulation may not actually be as great as these average results indicate.

3.2.2 Transforming Growth Factor β-induced

Transforming Growth Factor β-induced (TGFBi, Big-H3) is a secreted protein known to be induced by normal TGFβ signaling. It forms long fibrillar structures *in vitro*, and interacts with extracellular matrix components (Kim, Park et al. 2002). The relationship of this molecule to cataract formation has been previously studied. One study showed linked increased TGFBi mRNA and protein expression in the lens epithelial cells to anterior polar cataract (Lee, Seomun et al. 2000). In another study, transgenic mice overexpressing human TGFBi showed corneal and lens abnormalities, including abnormal proliferation of lens epithelial cells (Kim, Han et al. 2007).

Table 4 Tgfbi mRNA expression in wild type and mutant lens epithelium over time

Tgfbi	**1 w**	±	**3 w**	±	**2 mo**	±	**4 mo**	±
wild type	-14		-92	11	-25		-42	1
Crybb2^{Phil}	-53	22	-50	5	2.2	1.6	2.0	1.4

Wild type expression of Tgfbi mRNA appears to fluctuate. At 1 week, expression is approximately 15 fold below that of the control genes, and by 3 weeks it

decreases to almost 100 fold lower than control gene expression. After this point, expression increases again, although it is still 25-50 fold below expression of the control genes.

In the mutant lens epithelium, expression at 1 week seems to be even lower than in the wild type. By 3 weeks, expression has not changed, but by 2 months, expression increases approximately 100 fold. This relatively high expression continues through 4 months. Again, there is fairly high deviation in the data, but in this case it seems clear that by 2 months, there is significant upregulation in Tgfbi mRNA.

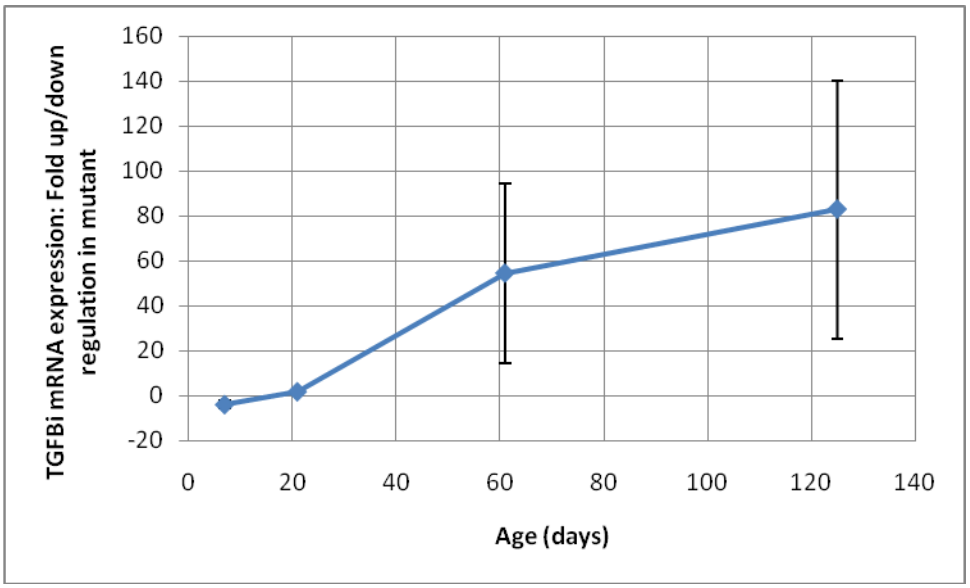

Figure 9. Relative expression of Transforming Growth Factor β-induced Protein mRNA in lens epithelium over time. Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

Figure 9 shows that TGFBi mRNA levels are approximately equal in the mutant and wild type lens at 1 and 3 weeks of age. By 2 months, mRNA levels are

approximately 50 fold higher in the mutant lens than in the wild type. Although increased expression might be expected in the 3 week samples, by which point EMT can sometimes be observed, it is possible that EMT has simply not begun to occur in this sample, or that it occurs in a relatively small number of the epithelial cells, and therefore is not detectable by PCR. The significant upregulation in the 2 month sample is as would be expected.

3.2.3 Follistatin

The extracellular protein follistatin has been implicated as an inhibitor of TGFβ3-mediated EMT which acts by binding the TGFβ molecule (Nogai, Rosowski et al. 2008). Follistatin is also an antagonist of the TGFβ family member activin (Sidis 2001). Recent studies have shown that in some cell types, Smad signaling through activin can upregulate follistatin mRNA expression (Blount, Vaughan et al. 2008).

Table 5 Fst mRNA expression in wild type and mutant lens epithelium over time

Fst	**1 w**	±	**3 w**	±	**2 mo**	±	**4 mo**	±
wild type	-21		-1647	830	-32		-2142	
Crybb2^{Phil}	-75	35	-331	49	1.7	0.8	1.8	1.0

In the wild type lens epithelium, follistatin levels appear to fluctuate. However, it should be noted that most of the wild type data does not include a standard deviation. This was because in most cases, only 1 set of usable data was obtained for expression of follistatin mRNA. The data is sufficient to show that the mRNA expression for this gene is low to very low in the wild type lens epithelium.

In the mutant epithelium, the major change in expression seems to occur between 3 weeks and 2 months of age. During this time, expression rises from several

hundred fold below the controls, to a level slightly higher than control gene expression.

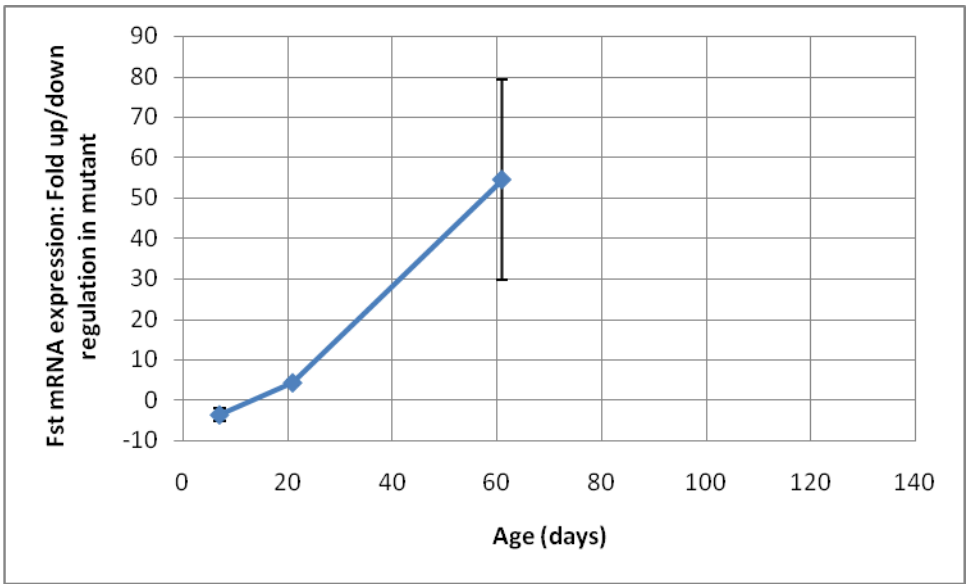

Figure 10. Relative expression of Follistatin mRNA in lens epithelium over time. Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

As follistatin expression is induced by TGFβ signaling, its expression level would be expected to be normal initially, and then increase. This is in fact what occurs. It should be noted that in Figure 10, the point for 4 months would be even higher than that for 4 months; it was not included, as this would extend the scale of the graph to over 2000, and obscure the significance of earlier points.

3.2.4 Smads 2 & 4

Smad 4 is the "adaptor Smad", to which activated "receptor-regulated" Smads (Smad 2 or 3) bind, and through which gene transcription activity is mediated. Smads 2 and 3 are activated through phosphorylation by the TGFβ receptors (Miyazono, ten Dijke et al. 2000). As such, they play a key role in TGFβ signaling.

Table 6 Smad2 mRNA expression in wild type and mutant lens epithelium over time

Smad2	1 w	±	3 w	±	2 mo	±	4 mo	±
wild type	1.0	0.6	1.6	0.0	1.8	0.5	1.9	0.3
Crybb2^{Phil}	5.7	0.1	1.3	0.5	-1.4	0.5	-2.8	0.5

Smad2 mRNA expression in the wild type lens epithelium is maintained at a fairly constant level from 1 week through 4 months of age. Expression is fairly high, comparable to the average expression of the control genes.

In the mutant lens epithelium, Smad 2 expression is somewhat high at 1 week of age (approximately 5 times higher than in the average of control gene expression). By 2 weeks of age, expression decreases to a level equivalent to the wild type expression. By 2 months, expression again decreases slightly, and again by 4 months.

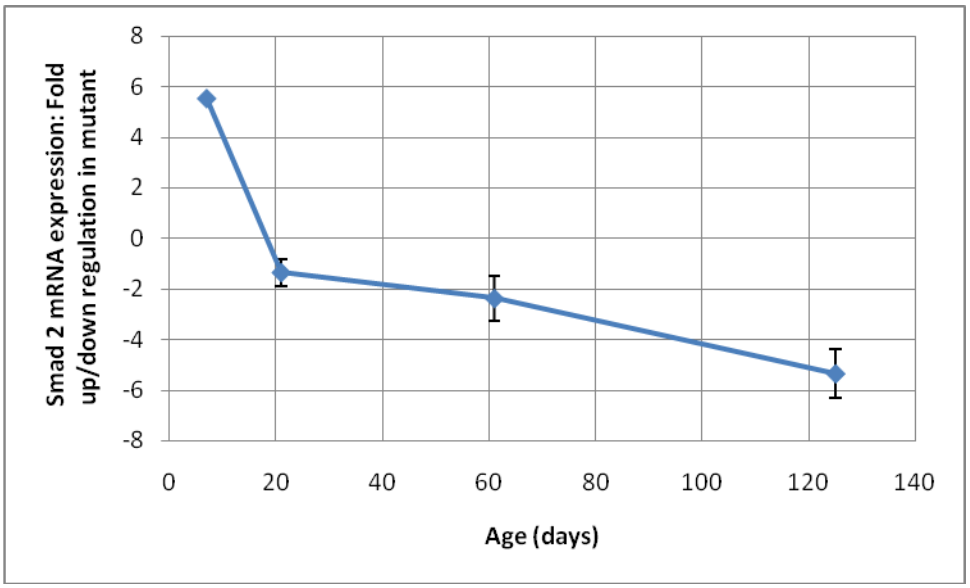

Figure 11. Relative expression of Smad 2 mRNA in lens epithelium over time. Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

As Figure 11 shows, the ratio of expression of Smad2 mRNA in the mutant lens to expression in the wild type lens decreases slowly but consistently throughout development.

Table 7 Smad4 mRNA expression in wild type and mutant lens epithelium over time

Smad4	**1 w**	±	**3 w**	±	**2 mo**	±	**4 mo**	±
wild type	-2.0		-1.4	0.3	1.1		1.6	0.0
Crybb2^{Phil}	2.5	1.5	1.1	0.2	-2.3	0.6	-2.2	1.5

The expression of Smad 4 mRNA in the wild type lens epithelium does not change much between 1 week and 4 months. If anything, there is a slight increase in expression occurring between 1 week and 2 months.

Expression in the mutant also does not change much, although there does appear to be a slight decrease in expression between 1 week and 2 months. From 1 to 3 weeks, expression appears to decrease 1-2 fold, and a similar decrease occurs between 3 weeks and 2 months.

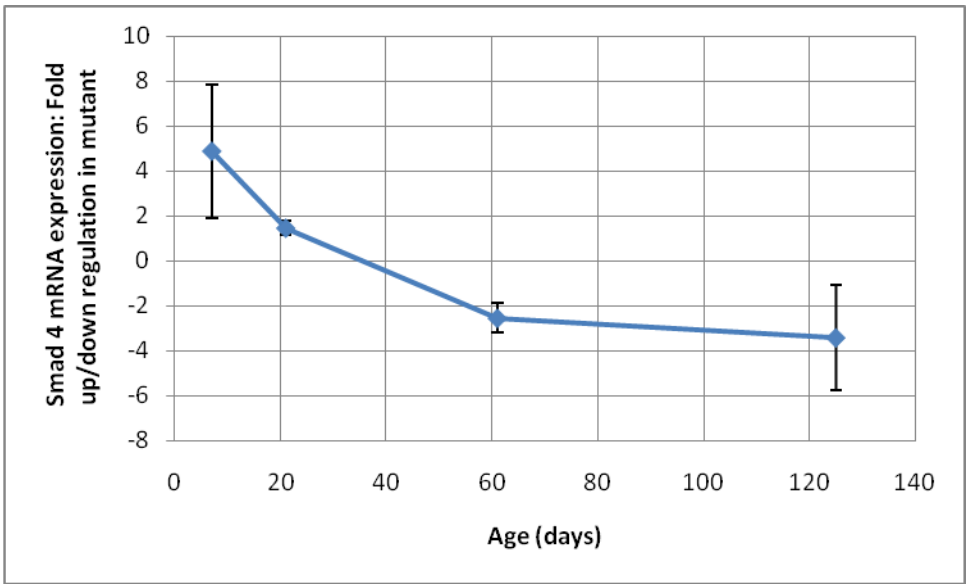

Figure 12. Relative expression of Smad 4 mRNA in lens epithelium over time. Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

As Figure 12 shows, Smad 4 mRNA levels in the mutant compared to the wild type decrease steadily from 1 week to 2 months, at which point the ratio remains fairly steady. However, at no point is expression of the Smad4 mRNA drastically affected in the mutant.

3.2.5 α-Smooth Muscle Actin

αSMA is expressed in myofibroblasts but not in most epithelial cells, and is therefore a useful marker of EMT. αSMA is normally expressed in the lens epithelium; however, expression in greatly increased in cases of lens EMT.

Table 8 αSMA mRNA expression in wild type and mutant lens epithelium over time

αSma	**1 w**	±	**3 w**	±	**2 mo**	±	**4 mo**	±
wild type	2.1	0.4	17	1	8.6	0.5	19	4
Crybb2^{Phil}	15	1	5.3	0.6	23	10	17	10

At 1 week in the wild type lens epithelium, mRNA for αSMA is expressed at a moderate level, comparable to expression of the control genes. By 3 weeks, expression appears to increase almost 10 fold. At 2 months, expression decreases somewhat, and then rises again. This is an unexpected pattern of expression. It may be worthwhile to repeat this experiment several more times to see if this pattern holds.

The mutant lens epithelium also shows an unexpected pattern of αSMA mRNA expression. Expression is fairly constant, at a level comparable to the apparent final expression in the wild type. The only exception is a dip in expression at 3 weeks. This dip occurs exactly where a peak would be expected, based on protein expression data previously reported (Decker 2008).

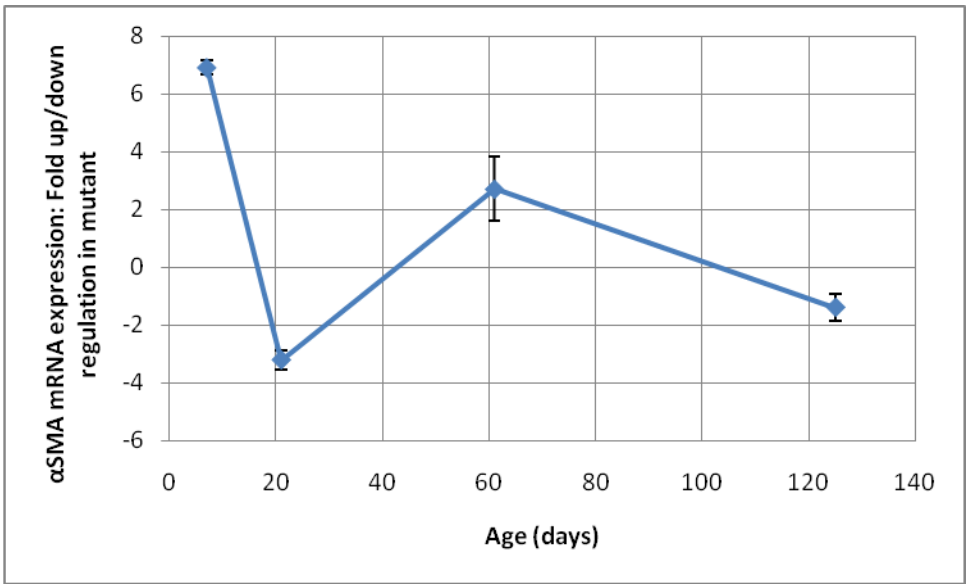

Figure 13. Relative expression of α-Smooth Muscle Actin mRNA in lens epithelium over time. Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

The ratio of expressions shown in Figure 13 shows this unexpected pattern of expression. Expression seems to be higher in the mutant at 1 week and higher in the wild type at 3 weeks. At 2 and 4 months, the expression may actually be fairly stable, as neither shows a statistically significant difference from the expression of the control genes.

3.2.6 Collagen 1α1

Before EMT, the collagen secreted by lens epithelial cells is predominantly type IV collagen. With EMT is a switch to collagen type I (Gotoh, Perdue et al. 2007).

Table 9 Col1α1 mRNA expression in wild type and mutant lens epithelium over time

Col1α1	**1 w**	±	**3 w**	±	**2 mo**	±	**4 mo**	±
wild type	-290		-229	42	-5.0		-176	83
Crybb2^{Phil}	-1006		-162	109	3.1	1.6	4.2	1.0

In the wild type, expression of Col1α1 mRNA is consistently low, as would be expected. The only exception occurs at 2 months of age, at which point expression appears to rise to a level only a few fold lower than expression of the control genes. However, this experiment should be repeated, as only 1 set of data for this point was obtained. It is possible that the level at this point is actually consistent with the other time points.

In the mutant lens epithelium, expression is initially very low, and gradually increases. The most dramatic increase is between 3 weeks and 2 months of age.

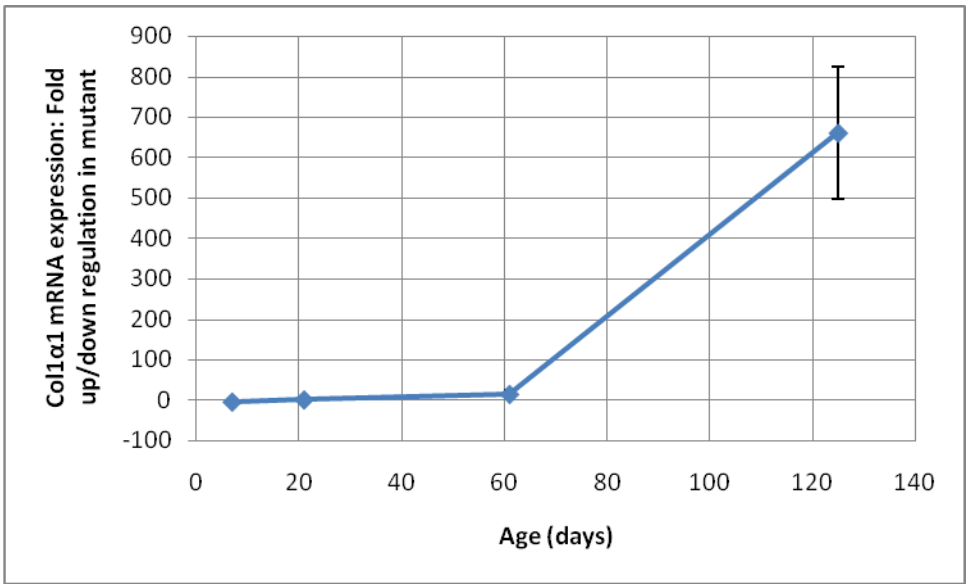

Figure 14. Relative expression of Collagen 1α1 mRNA in lens epithelium over time. Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

Figure 14 clearly shows the gradually increasing expression of collagen 1α1 mRNA in the mutant lens epithelium. For the first 3 weeks after birth, col1α1 mRNA levels are approximately equal in the mutant and wild type. By 2 months of age, mRNA expression is significantly higher in the mutant, and be 4 months, expression is several hundred fold higher in the mutant than in the wild type. It should be noted that the result at 4 months shows a much greater upregulation than was found using the SuperArray.

3.2.6 Latent TGFβ-Binding Protein 2

Latent Transforming Growth Factor Binding Proteins (LTBPs) bind TGFβ before it is secreted and regulate its activity. They can target TGFβ to a particular location, or prevent binding to the receptor (Saharinen, Hyytiainen et al. 1999).

Table 10 Ltbp2 mRNA expression in wild type and mutant lens epithelium over time

Ltbp2	**1 w**	±	**3 w**	±	**2 mo**	±	**4 mo**	±
wild type	-100	81	-48		-46	24	-3.8	0.3
Crybb2^{Phil}	not detected		-9.1	3.3	2.1	0.4	2.0	1.0

In the wild type lens epithelium, expression of Ltbp2 mRNA is fairly low initially, and increases as the lens ages. By 4 months of age, expression reaches a level comparable to the expression of the control genes.

In the mutant epithelium, this mRNA was not detectable at 1 week of age. However, by 3 weeks of age it showed a moderate level of expression, which further increased by 2 months. At this point, expression appears to plateau in the mutant lens epithelium.

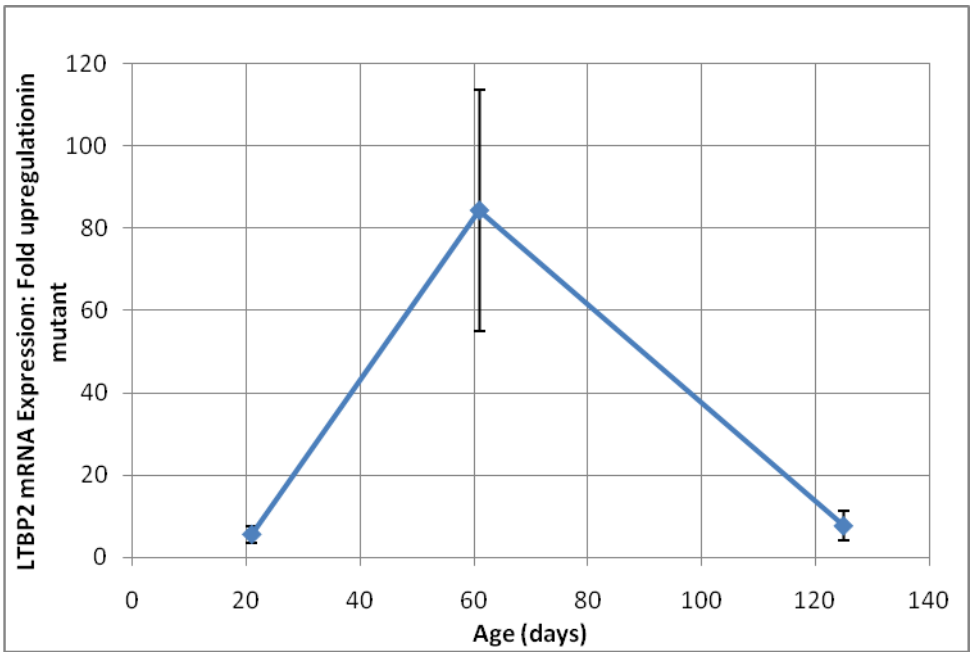

Figure 15. Relative expression of Latent TGFβ-Binding Protein 2 mRNA in lens epithelium over time. Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

Figure 15 shows no data for 1 week, because at 1 week, the mRNA was undetectable in the mutant; expression can be considered 0. By 3 weeks, mRNA levels are somewhat higher in the mutant, and by 2 months of age, mRNA levels are 80 fold higher in the mutant than in the wild type. By 4 months, relative levels decrease, but are still higher in the mutant lens epithelium than in the wild type. The decrease in comparative expression is actually due to the increase in wild type expression at this age, rather than a decrease in mutant expression.

3.2.7 TGFβ Receptor, type II

TR2 is part of the receptor dimer responsible for classical TGFβ signaling.

Table 11 TR2 mRNA expression in wild type and mutant lens epithelium over time

TR2	**1 w**	±	**3 w**	±	**2 mo**	±	**4 mo**	±
wild type	-10	4	-30	10	-16	2	3.9	1.6
Crybb2^{Phil}	-17	10	-10	4.5	-1.0	0.1	1.4	0.3

In the wild type epithelium, TGFβ type 2 receptor mRNA is expressed at a moderate level throughout the first 4 months of life. There may be some fluctuation within the first 2 months, but the greatest increase seems to occur between 2 and 4 months of age.

In the mutant epithelium, expression levels are fairly similar to those of the wild type, but the increase in TR2 mRNA level seems to occur earlier, by 2 months, and possibly starting by 3 weeks.

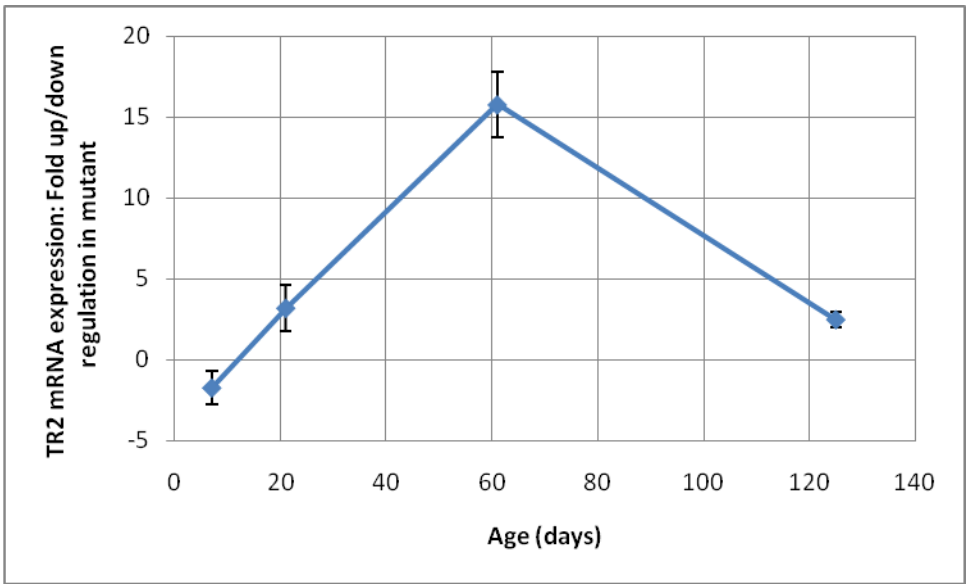

Figure 16. Relative expression of TGFβ Receptor, type II mRNA in lens epithelium over time. Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

When the ratio of expression in the mutant to the wild type is examined, a pattern of increasing expression during the first 2 months of life is observed. TR2 levels in the mutant are normal at 1 week and possibly slightly elevated by 3 weeks. By 2 months, there is moderate but clear upregulation. By 4 months, TR2 levels approach normal.

3.2.8 TGFβ Receptor, type V

TR5 is another TGFβ receptor, but it is not part of the classical signaling pathway, and its expression in the lens epithelium had not been previously reported. TGFβ signaling through TR5 leads to growth inhibition in epithelial cells. TR5 is also the receptor for IGFBP3 (Huang and Huang 2005).

Table 12 TR5 mRNA expression in wild type and mutant lens epithelium over time

TR5	1 w	±	3 w	±	2 mo	±	4 mo	±
wild type	1.4		1.9	0.5	5.1		7.3	4.6
Crybb2^{Phil}	1.1	0.5	2.5	1.3	5.4	2.4	5.6	2.3

In both the wild type and the mutant, the TGFβ type V receptor is expressed at a moderate level at all of the ages examined. In both, expression appears to increase slightly with age.

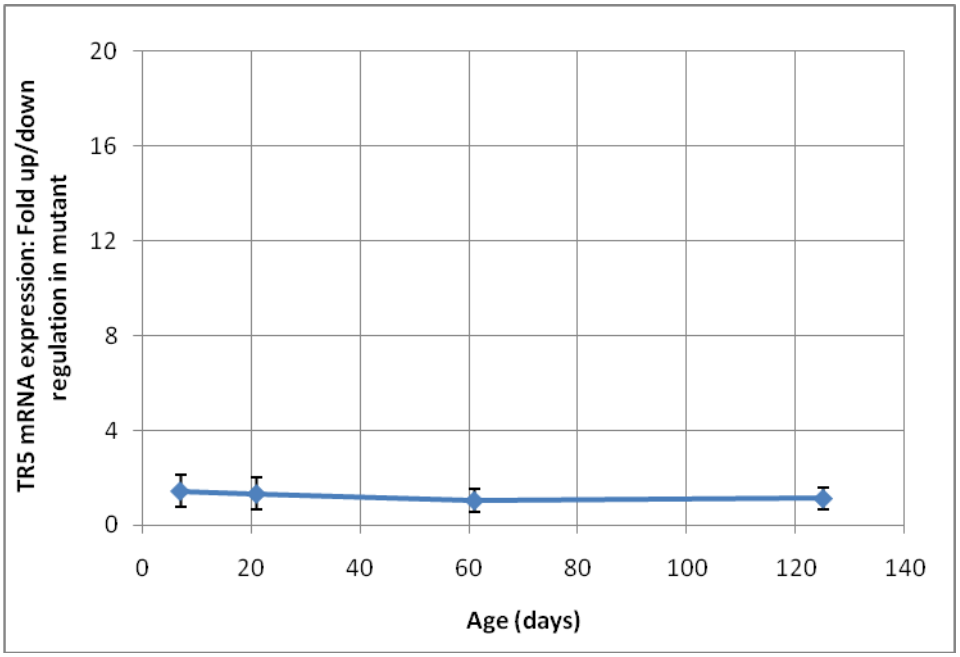

Figure 17. Relative expression of TGFβ Receptor, type V mRNA in lens epithelium over time. Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

TR5 mRNA levels are generally normal in the mutant. This is somewhat surprising, because as will be shown below, Igfbp3 mRNA levels rise drastically in the mutant lens epithelium. However, TR5 mRNA is relatively highly expressed in both the wild type and mutant lens epithelium. This can be seen by comparing the expression of the types II and V receptors shown in Tables 11 and 12. Expression of TR2 mRNA reaches levels comparable to TR5 expression only by 4 months of age.

3.2.9 Insulin-like Growth Factor-Binding Protein 3

Insulin-like Growth Factor Binding Protein (IGFBP3), as its name suggests, binds and regulates the insulin-like growth factors, inhibiting their mitogenic effects (Yan, Forbes et al. 2004). However, the molecule also has another mechanism of regulating cell growth; it can interact with the type V TGFβ receptor (TR5), inhibiting epithelial cell growth or promoting fibroblast cell growth (Leal, Liu et al. 1997; Huang and Huang 2005). IGFBP3 signaling through TR5 is believed to act through insulin receptor substrate (IRS) proteins, as well as crosstalk with signaling pathways mediated by other TGFβ receptors (Huang and Huang 2005). IGFBP3 upregulation has been previously described in cases of non-lens EMT (Andersen, Mejlvang et al. 2005).

Table 13 Igfbp3 mRNA expression in wild type and mutant lens epithelium over time

Igfbp3	1 w	±	3 w	±	2 mo	±	4 mo	±
wild type	-26	15	-52	14	-236	187	-57	58
Crybb2^{Phil}	-3.5	0.1	-15	3.1	1.8	0.9	1.3	0.8

The expression of Igfbp3 mRNA in the wild type lens epithelium seems to be consistently low during the first 4 months after birth. There seems to be a decrease at 2 months of age, although the large amount of deviation in the data should be noted.

In the mutant epithelium, Igfbp3 mRNA is expressed at a moderate level at 1 week post natal. By 3 weeks, expression decreases somewhat, but by 2 months of age, expression rises again. Expression remains fairly stable for the next 2 months.

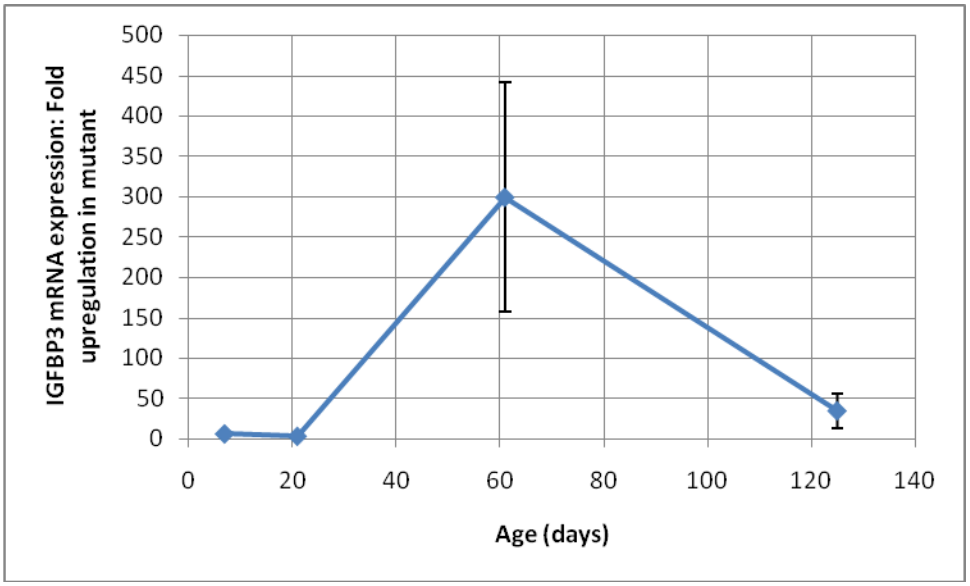

Figure 18. Relative expression of Insulin-like Growth Factor-Binding Protein 3 mRNA in lens epithelium over time. Each data point represents the ratio of expression in the mutant to expression in the wild type; negative values indicate that expression is higher in the wild type.

Figure 18 shows that levels of Igfbp3 mRNA are similar in the mutant and wild type lens epithelium for the first 3 weeks after birth. However, by 2 months, mRNA levels in the mutant are much higher than in the wild type, and by 4 months, return to almost normal levels.

3.3 Immunofluorescence imaging of IGFBP3 and αSMA

Previous work has established that increased αSMA protein expression is seen in the fibrotic plaques observed in the mutant lens, while the wild type epithelium stains weakly for αSMA; therefore, increased αSMA expression is a useful marker of lens EMT. Sections from adult lenses were double stained with anti-IGFBP3 and anti-αSMA. The wild type lens stained weakly for both IGFBP3 and αSMA in the epithelium; the fiber cells were not stained. In the mutant, both fiber and epithelial cells show IGFBP3 expression. However, cells in the fibrotic plaque express lower levels of IGFBP3.

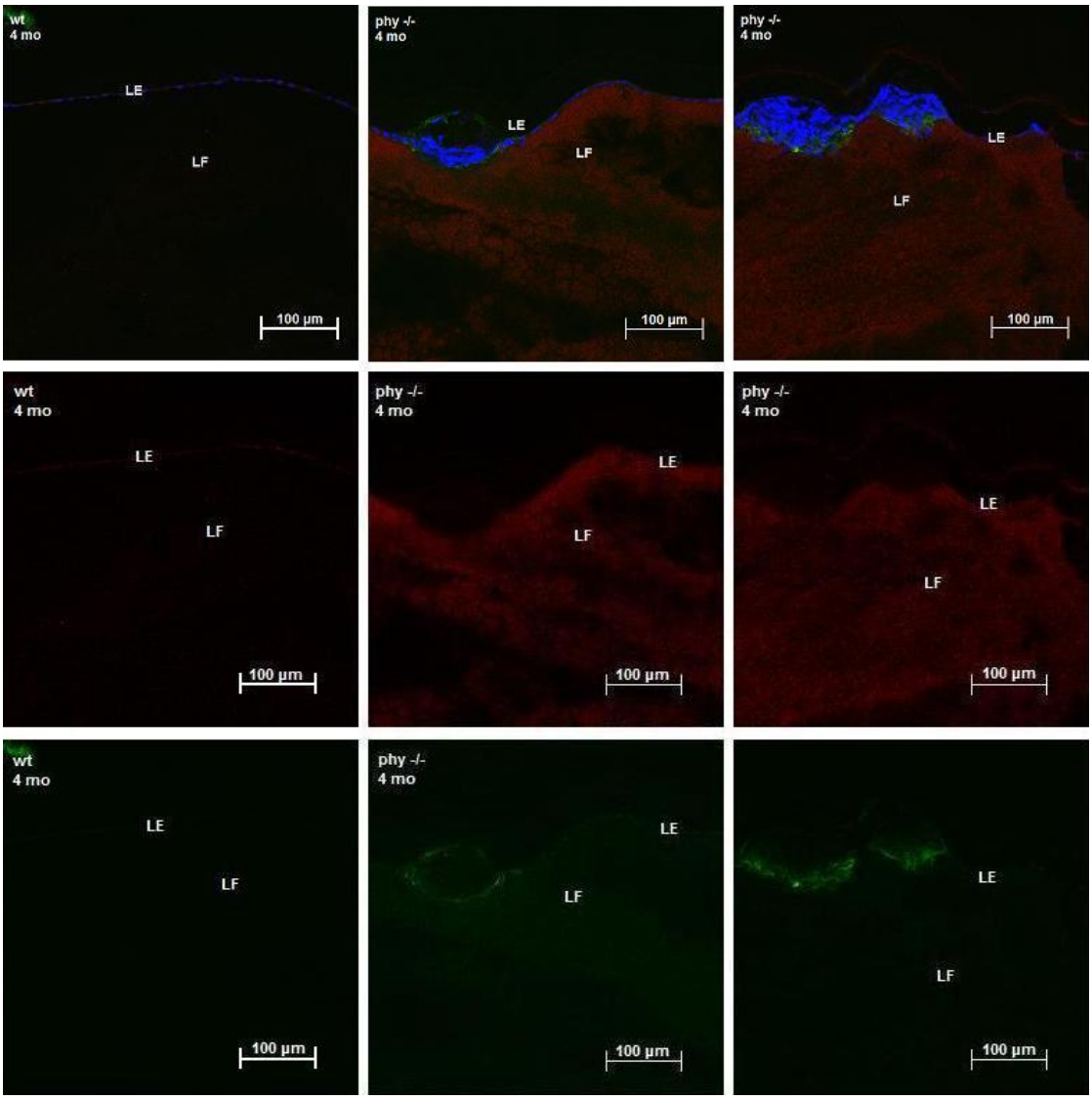

Figure 19. Immunofluorescence image showing Igfbp3 and αSMA expression in adult wild type and mutant lens. blue = nuclei; red = IGFBP3; green = αSMA

Chapter 4

DISCUSSION

4.1 TGFβ Signaling is affected by the Crybb2^{Phil} mutation.

These results show that mRNA levels for several molecules involved in TGFβ signaling are affected by the Crybb2^{Phil} mutation.

In the initial array, significant upregulation of several genes was observed, but none were observed to be downregulated. This was somewhat unexpected, as several of the genes on the array were actually inhibitors of EMT (for example, follistatin and IGFBP3). This was believed to be due to the fact that in this experiment, epithelia from relatively old mice were studied. Although markers of EMT can be observed in mice as young as 3 weeks, epithelia from 4 month old mice were used for this array. The array data suggests that by this point in development, the signaling that initially caused EMT has been largely turned off by negative feedback signals.

This supported by the time point data. In the adult, high expression of several genes which inhibit EMT or are often upregulated in response to EMT was found. These genes include follistatin, TGFβ-induced protein, and insulin-like growth factor-binding protein. When earlier time points were studied, it was found that mRNA for these genes begins to increase between 3 weeks and 2 months of age, or later. Certain genes would be expected to be active during EMT, but were not upregulated or were only slightly upregulated in the initial array. These include TGFβ type 2 receptor and latent TGFβ-binding protein 2. For both of these genes, a peak of

upregulation was observed by 2 months of age, after which point expression decreased; this was the reason little upregulation was observed in the initial array.

It was expected that mRNA for genes associated with EMT would show at least some upregulation by 3 weeks of age. However, this was not generally the case. As EMT is just beginning at this point, transcriptional regulation of relevant genes may not yet be affected. Another possibility is that has only begun in a small minority of the lens epithelial cells, so that upregulation of mRNAs in these cells is masked by the normal expression in the rest of the epithelium.

4.2 The role of Smads in EMT

The classic TGFβ signaling pathway signals through Smads as described in section 1.10. Briefly, the legend-bound receptor dimer phosphorylates Smad2 or 3. The activated Smad2 or 3 binds Smad4; the Smad complex translocates to the nucleus and acts as a transcription factor (Miyazono, ten Dijke et al. 2000).

The real-time RT-PCR results show that mRNA levels of Smad2 and 4 are not very different in the Crybb2^{Phil} mutant and the wild type at any of the time points studied. Therefore, there does not seem to be significant regulation of Smad expression at the level of transcription (although mRNA levels of Smad3, the other receptor-regulated Smad, were not studied). However, it is possible that protein expression of Smads is controlled by other methods. Ubiquitination is one important method of controlling receptor-Smad protein levels; certain signaling molecules, for example Smurf1 and 2, can target receptor-Smads for degradation. However, in normal tissue, ubiquitination of receptor-Smads seems to be more important for BMP signaling (Bone Morphogenic Protein, a member of the TGFβ superfamily) (Derynck and Zhang 2003)

The activity of Smads can also be regulated by inhibitor-Smads, Smads6 and 7 (Miyazono, ten Dijke et al. 2000). Smad 7 specifically interferes with TGFβ/Smad signaling. The inhibitor-Smads function by competing with receptor-Smads for binding to the activated receptor (Itoh, Itoh et al. 2000). Smad7 in association with Smurf1 can also cause ubiquitination of the TGFβ receptor (Derynck and Zhang 2003).

4.3 The role of Igfbp3 in EMT

Igfbp3 has been shown at both the mRNA and protein expression levels in the Crybb2^{Phil} lens epithelium. In other systems, this protein has been primarily implicated in inhibiting growth of epithelial and other cell types. Igfbp3 signaling through TR5 can cross-talk with the canonical TGFβ pathway (Huang and Huang 2005). IGFBP3 levels have been reported to rise in response to TGFβ signaling in several cell types (Izumi, Kurosaka et al. 2006). In cancer, IGFBP3 has been shown to promote cell proliferation and metastasis in some cases (Xi, Nakajima et al. 2006) and suppress proliferation and motility in other cases (Ullmannova and Popescu 2007), while in other cases IGFBP3 retarded cancer growth and progression (Fuchs, Goldberg et al. 2008). Igfbp3 has also been shown to induce apoptosis in the corneal epithelium as well as other systems (Robertson, Ho et al. 2007). As Igfbp3 is primarily known as an inhibitor of cell proliferation, it is possible that Igfbp3 upregulation is a response by which cells try (inadequately) to prevent the pathological process of EMT. It would be interesting to see whether this protein also plays a role in other cases of lens EMT.

4.4 The role of TGFBi in EMT

Transforming Growth Factor β-induced protein is a secreted protein whose expression is induced, as its name implies, by TGFβ signaling. It forms long fibrillar structures *in vitro*, and interacts with extracellular matrix components (Kim, Park et al. 2002). The relationship of this molecule to cataract formation has been previously studied. One study showed linked increased TGFBi mRNA and protein expression in the lens epithelial cells to anterior polar cataract (Lee, Seomun et al. 2000). In another study, transgenic mice overexpressing human TGFBi showed corneal and lens abnormalities, including abnormal proliferation of lens epithelial cells (Kim, Han et al. 2007).

TGFBi has been shown to promote cell adhesion and motility in a variety of cell types, including corneal epithelial cells. It performs this function by binding various integrins (Thapa, Lee et al. 2007). Interestingly, a recent paper describes the effect of TGFBI in human colon cancer cells, in which the protein appears to disrupt VE-cadherin junctions (Ma, Rong et al. 2008). A loss of E-cadherin at cell-cell contacts occurs in the Crybb2^{Phil} (Decker 2008); it is possible that Tgfbi plays a role in this loss.

4.5 A Crybb2 knockout mouse displays a fairly mild phenotype.

Zhang et. al. recently created a Crybb2 knockout mouse. Surprisingly, this mouse exhibited only mild morphological problems. The knock out and wild type lenses exhibited similar morphologies at birth, which is not surprising because high βB2-crystallin expression begins at birth. Postnatal lens growth is slower in the knockout than the wild type, and there is a significant difference in lens mass by 4

months of age. Protein extracts from knockout lenses are less resistant to denaturation by heat and oxidative stress.

However, while the Crybb2^{Phil} mutant lens shows abnormalities as early as 3 weeks postnatal, the knockout only begins to develop cataracts 6-8 weeks postnatal (Zhang, Li et al. 2008). The relative mildness of this phenotype is surprising, both because βB2-crystallin is the most highly expressed protein in the adult lens, and because as this paper has shown, βB2-crystallin seems to have a role in signaling. It is possible that the mutant actively interferes with normal processes, rather than simply causing a loss of βB2-crystallin function. Alternatively, related proteins such as other βB-crystallins, might partially compensate for the loss of βB2-crystallin in the knockout. Possibly, the expression of some of these proteins is upregulated in the knockout.

4.6 How does βB2-crystallin affect TGFβ signaling?

The mechanism by which βB2-crystallin might affect TGFβ signaling is currently unclear. β crystallins are generally considered structural rather that signaling proteins (Jobby and Sharma 2007)

One possible mechanism by which βB2 crystallin could affect signaling is through regulation of cellular calcium levels. βB2, as well as other crystallins, is a calcium-binding protein. Given the high concentration of crystallins in the lens, it is possible that these proteins act as a calcium buffer in the lens (Jobby and Sharma 2007). Recently, the expression of calpains, calcium-dependent proteases, was shown to be upregulated in the lens epithelium of mice with a novel mutation in βB2-crystallin (Ganguly, Favor et al. 2008).

TGFβ signaling leads to an increase in cytoplasmic Ca^{2+} concentration (Alevizopoulos, Dusserre et al. 1997). One possibility is that the Crybb2^{Phil} mutation leads to decreased calcium binding by βB2-crystallin. This might sensitize cells to the effects of TGFβ. Previous studies have shown that the TGFβ1 and 2 are present in the normal post-natal lens and are potentially available to initiate signaling (Gordon-Thomson, de Iongh et al. 1998).

4.7 Relevance to other systems

These results indicate several proteins whose role in lens EMT should be further investigated. Proteins such as follistatin, Igfbp3, and Tgfbi may play a role in the EMT observed not only in the Crybb2^{Phil} mutant, but also in other cases of lens EMT. These proteins could have clinical relevance in Posterior Capsule Opacification, and possibly in cataract caused by mutations in βB2-crystallin.

Chapter 5

CONCLUSIONS

- mRNA expression of several genes involved in TGFβ signaling is affected by the Crybb2^{Phil} mutation. Several of these genes are known to be activated in response to TGFβ/Smad signaling. This indicates a role for the βB2-crystallin protein in signaling.
- The expression of Smad 2 and 4 mRNA does not appear to be altered in the Crybb2^{Phil} mutant lens compared to wild-type.
- Igfbp3 is unevenly expressed in fibrotic plaques of the adult Crybb2^{Phil} lens epithelium, but is highly upregulated in the lens fiber cells.

Chapter 6

FUTURE WORK

The real-time PCR data has provided evidence that TGFβ signaling occurs in the homozygous Crybb2^{Phil} lens epithelium. However, the activity of the pathway has not been quantified. Western Blotting could be used to determine protein expression of several key factors should be investigated, including TGFβ, TGFβ receptors, and Smads. Furthermore, the phosphorylation state of Smads 2 and 3 should be examined in order to determine whether there is a greater proportion of active Smads in the mutant lens epithelium. Finally, the localization of Smads 2, 3, and 4 in the nucleus or cytoplasm could be investigated by immunofluorescence imaging. If there is increased signaling through Smads, increased localization to the nucleus would be expected, as the Smad complexes act as transcription factors.

Another interesting area of study would by the role of Igfbp3 in signaling after EMT has occurred in the lens. An important first step would be to study the protein expression and localization of its receptor (TGFβ type V receptor) through immunofluorescence imaging. A more precise understanding of when Igfbp3 protein levels are upregulated could also be found by Western Blotting; this could be useful in determining its role in Crybb2^{Phil} mutation-induced EMT. Most importantly, the expression of molecules associated with Igfbp3 signaling should be studied, in order to determine how this molecule affects the mutant lens epithelium.

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