Col8a2 and cohesiveness of the anterior segment during development

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Introduction

Glaucoma

Glaucoma, a type of optic neuropathy, refers to a group of neurodegenerative diseases that cause retinal ganglion cell death and changes in the optic nerve resulting in visual defects and eventually total blindness if left untreated. It is the leading cause of irreversible blindness worldwide with an estimated 67 million people in 2000 suffering from bilateral blindness due to glaucoma (Coleman 1999). Most treatment options available to the estimated 67 million glaucoma sufferers worldwide focus on reducing intraocular pressure, with varying degrees of effectiveness depending on the individual. The damage caused by glaucoma before detection, however, is irreversible and treatment focuses simply on preventing further vision loss. Unfortunately, for some treatment is never particularly effective and vision loss persists unabated. (Coleman,1999). There is currently little understanding of the disease process of glaucoma, and how elevated levels of intraocular pressure are related to the characteristic retinal ganglion cell death and optic nerve changes (See figure 1). Additionally, it has been observed that elevated intraocular pressure is not always necessary or sufficient for development of the disease. Some individuals with significantly elevated intraocular pressure never develop glaucoma, while others with normal intraocular pressure have been observed to develop the disease (Ray 2003). Going forward, to improve treatment and enhance early detection, a better understanding of the underlying biological basis of the disease will be crucial.

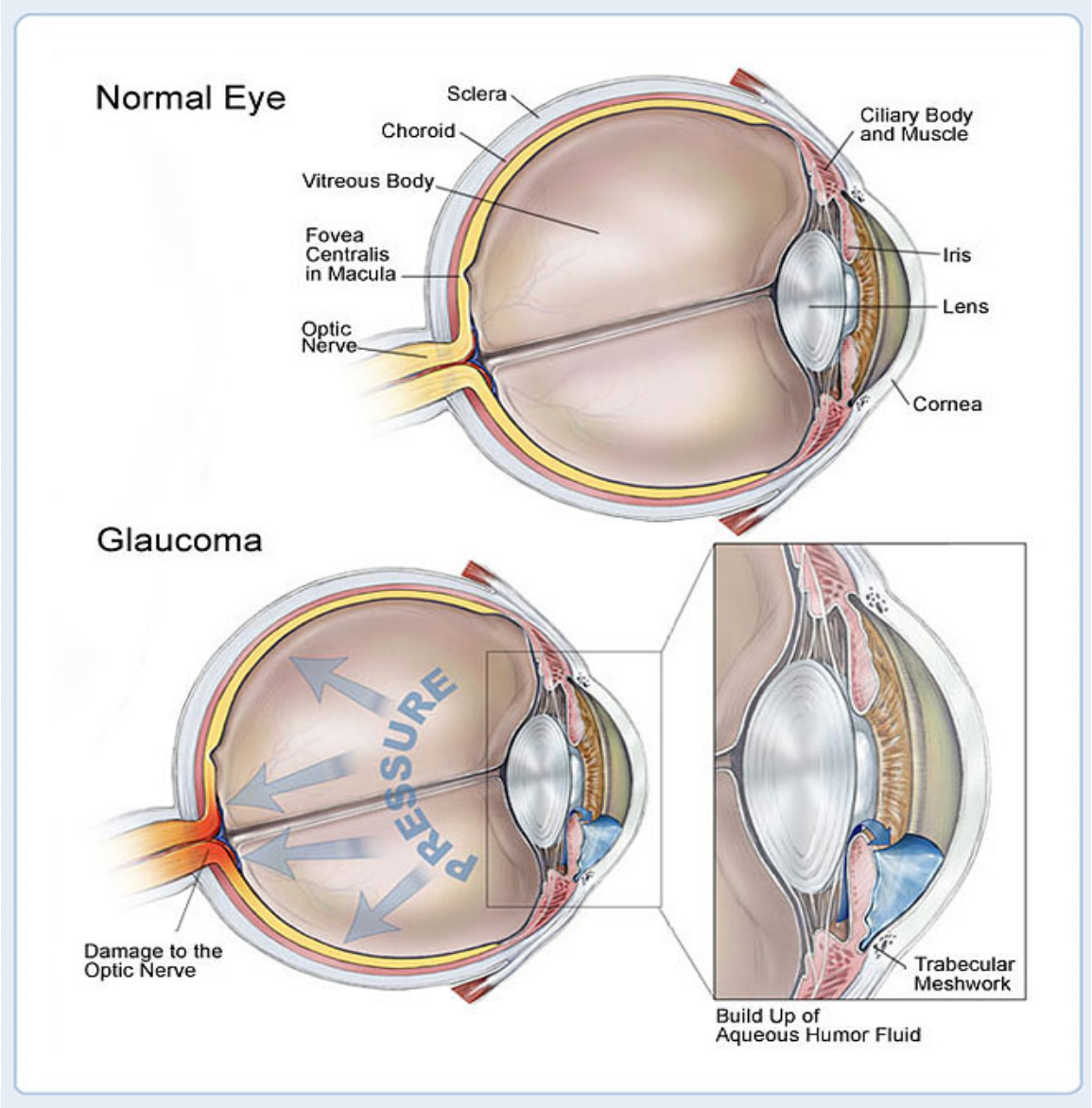


Figure 1. Reproduced from

http://www.ahaf.org/glaucoma/about/understanding/build-up-of-aqueous.html

Illustration of increased intraocular pressure resulting in damage to the optic nerve, a characteristic of glaucoma.

While in rare cases neither necessary nor sufficient, monitoring of intraocular pressure is one of the best ways for clinicians to assess a patient's risk for glaucoma (Ray 2003). It is determined by the pressure of aqueous humor, a thick fluid that is produced in the ciliary body that flows between the lens and cornea in the anterior segment of the eye. It is important in maintaining healthy functioning, through nutrient delivery and waste removal. Aqueous humor drains out of the eye through the trabecular meshwork and next through Schlemm's canal, after which it rejoins the bloodstream. The coupling of production of aqueous humor in the ciliary body and drainage through the trabecular meshwork should result in properly balanced intraocular pressure (Ray 2003). Defects in the anterior segment however, however, can result in elevated pressures. (See Figure 2)

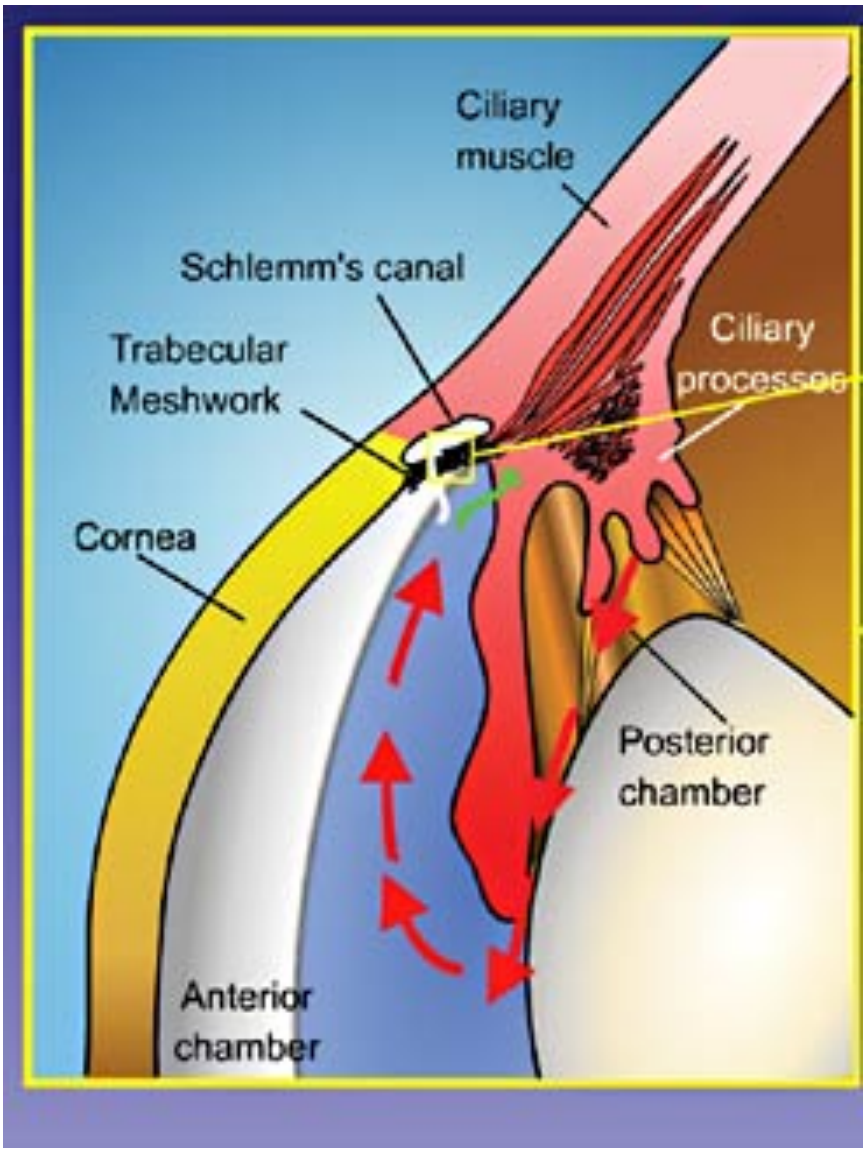


Figure 2 Reproduced from http://journals.prous.com/journals/servlet/xmlxsl/pk_journals.xml_summary_pr?p_JournalId=3&p_RefId=3140&p_IsPs=Y. Aqueous humor is produced in the ciliary body, flows between the lens and cornea and drains out the trabecular meshwork and Schlemm's canal.

Glaucoma can be grouped into two categories, late-onset seen in elderly individuals which develops late in life, and the much less common developmental form which becomes prevalent between birth and the teen years in humans (Gould 2002). Both forms exhibit the same retinal ganglion cell death and optic nerve changes as well as increased intraocular pressure, suggesting common biological mechanisms are shared between the two. Because of the likelihood of shared mechanisms between the two, a good understanding of the developmental form of the disease will help in future diagnosis and treatment of the much more common late-onset form.

Eye Development

The anterior segment of the eye, consisting of the cornea, iris, lens, ciliary body, trabecular meshwork, and Schlemm's canal, is formed during development through a complicated series of events involving predominantly surface ectoderm, neural ectoderm and periocular mesenchyme (Gould 2004). The surface ectoderm differentiates into corneal epithelium and lens. The neural ectoderm will form the retina as well as the epithelia of the iris and ciliary body. The periocular mesenchyme differentiates to form many structures of the mature eye including the corneal stroma and endothelium, sclera, iris stroma, ciliary muscles, ciliary stroma and trabecular meshwork. (Gould 2004) In mice the first trace of what will become the mature eye is observed at approximately embryonic day e8.5, located on the anterior part of the forebrain. Shortly thereafter, by embryonic day e9.0 the optic pit begins to deepen as the optic vesicles form. These optic vesicles must migrate through a layer of mesynchymal tissue until they touch the surface ectoderm, which runs along the outside of the developing head (Figure 3A). The surface ectoderm and optic vesicle next interact causing formation of the lens placode and retinal placode. By e10.5 the neural ectoderm has folded, forming the optic cup (Figure 3B). The lens placode differentiates into the lens vesicle, which will stay connected to the surface ectoderm until e11.0, when it detaches to allow formation of the cornea to begin (Figure 3C). Later the optic cup will contribute to the anterior segment, becoming the epithelium of the iris and ciliary body. By embryonic day e12.5 the cornea has gone through significant development, consisting of two cell layers of surface ectoderm. As the eye continues to develop by e14.5 the corneal endothelium is present, and the anterior chamber becomes present as a small space opens between the lens and corneal

endothelium (Figure 3D). By embryonic day e16.5 the anterior chamber is fully formed. Finally between embryonic days e17.0-e19.0, mesenchymal cells migrate to the iridocorneal angle to form the trabecular meshwork and Schlemm's canal, important drainage structures for maintaining appropriate IOP levels (Figure 3E). (Gould 2004)

Throughout the complicated series of events that result in the development of the mature eye, the anterior segment maintains a cohesive structure. As the moving parts of the anterior segment develop they must stay attached to each other, and to the exterior of the head. This implicates one or many important cellular adhesion molecules in the development of the anterior segment, which would function to maintain this cohesiveness.

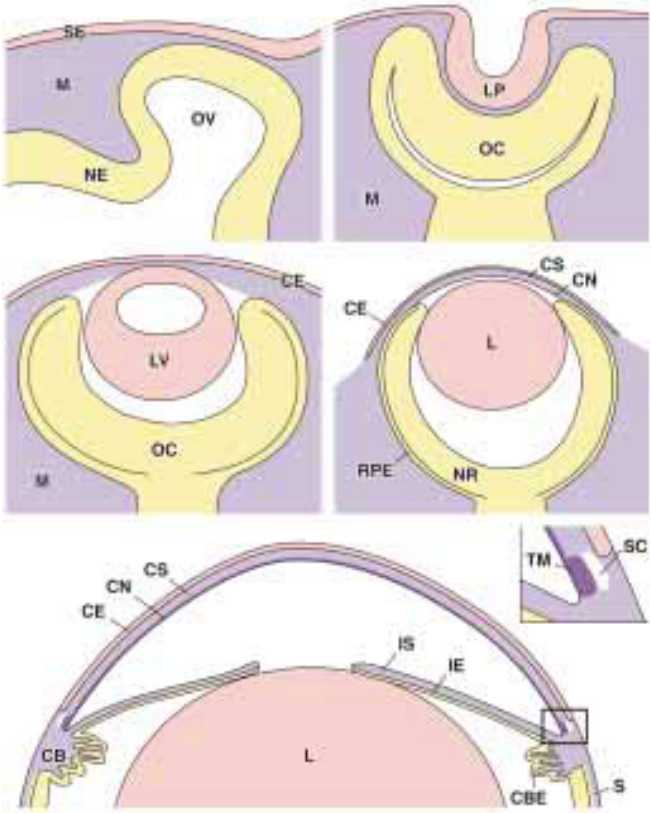


Fig. 3. Tissue derivations of developing ocular structures. Moving left to right, A-E. A colorscheme is used to represent tissue derivations. For clarity, periocular mesenchyme derived tissues are represented by two shades of purple. **(A)** Neural ectoderm (NE, yellow) of the emerging optic vesicle (OV) moves through periocular mesenchyme (M, purple) until it reaches the surface ectoderm (SE, pink). **(B)** The surface ectoderm thickens and invaginates to form the lens pit (LP) as the optic vesicle forms the optic cup (OC). **(C)** The lens vesicle (LV) detaches from the surface ectoderm. The surface ectoderm becomes the epithelial layer of the future cornea (CE). Periocular mesenchyme migrates between the surface ectoderm of the corneal epithelium and the lens vesicle. **(D)** Lens fibers fill the lens vesicle. Mesenchyme that has migrated between the corneal epithelium and the lens forms corneal stroma(CS) and corneal endothelium (CN). Neural ectoderm in the inner layer of the optic cup will form neural retina (NR) and the outer layer will form retinal pigmented epithelium (RPE). **(E)** In the mature eye, the anterior rim of the optic cup has moved centrally and forms the epithelia of the iris (IE) and ciliary body (CBE). Iris stroma (IS), stroma and muscle of the ciliary body (CB), trabecular meshwork (TM) and Schlemm's canal (SC) are formed from periocular mesenchyme. Condensed periocular mesenchyme forms the sclera (S) and surrounds the posterior of the eye.
Reproduced from Gould (2002)

Pitx2 and Eye Development

PITX2 is a paired-like homeodomain transcription factor that has been shown to be crucial for normal eye development (Semina 1996) (Gage 1999). Heterozygous mutations of *PITX2* in humans cause Axenfeld Rieger Syndrome (ARS). ARS symptoms include hearing loss, teeth malformations, heart defects, and skeletal malformations as well as number of anterior segment defects. These include malformation of the anterior chamber angle and drainage structures crucial for maintaining optimal intraocular pressure as well as iris hypoplasia, corectopia (asymmetric pupils), polycoria (iris tears), iridocorneal tissue adhesions and possible absence of the corneal endothelium and Descemet's membrane. As a result of these defects, which lead to increased intraocular pressure, 50% of patients with the ARS develop glaucoma, making *PITX2* an ideal gene to study in a mouse model to further understand the disease. (Lines 2002)

In mice, *Pitx2* expression begins in the neural crest at developmental day e9.5, where its activity is crucial for development of the anterior segment. It is also expressed in the mesoderm derived periocular mesenchyme from e8.5-e13.5. By e13.5 it is also seen in the stroma of the presumptive cornea. After the early stages of development its role becomes less significant and by e18.5 it is expressed only in the presumptive iris and iridocorneal angle. (Gould 2004) (Gage 2005)

Studies of *Pitx2* global knockout mice (*Pitx2* -/-) have confirmed the gene's importance in normal eye development. Not viable at birth due to other developmental deficiencies, these embryos show problems with anterior segment development as well as problems in development of other parts of the eye. Notably, the cohesiveness that is so crucial to normal eye development is gone in several important ways. First, severe

agenesis of the anterior segment including the cornea is observed, likely related to the elevated IOP seen in human *PITX2* mutations. The most notable lack of cohesion during eye development in these mutants occurs as the optic cup is displaced towards the midline (specifically towards the ventral hypothalamus). With the cohesion between the developing eye and outside of the head absent, by developmental day e16.5 an extremely foreshortened optic stalk is present, with the eyes located in the center of the head. Problems outside of the anterior segment begin with a severely dysmorphic optic stalk, suggesting *PITX2* may be crucial for normal development of the neurons this structure houses. Agenesis of the extraocular muscles is observed as well. (Gage 1999). *Pitx2* +/- mice, while viable, exhibit a varying degree of anterior segment defects as well including corectopia, anisocoria (uneven pupil size), clouded lenses and multiple pupillary openings. (Gould 2004)

Because it was discovered that *Pitx2* is expressed in neural crest and not the cells making up the optic stalk itself, it was concluded that an essential function of *Pitx2* is to regulate extrinsic signaling factors that are required for normal development of these cells. This original research had implications in optic nerve development as well as in treatment of ARS patients with developmental glaucoma. It suggested in addition to the elevated intraocular pressure seen in developmental glaucoma that other factors such as subtle abnormalities in optic nerve as well as anterior segment development may occur in ARS patients leading to the retinal ganglion cell axon damage and death seen in glaucoma (Gould 2004) (Gage 1999) (Semina 1996).

It is in the exploration of further downstream signaling effectors of *Pitx2* that will be the focus of my thesis. The lack anterior segment cohesion observed in *Pitx2* -/-

embryos suggests that one or many of these downstream effectors may be cellular adhesion molecules, vital for maintaining cohesiveness. To investigate this possibility a neural crest specific Beta-catenin knockout strain (*Ctnnb1*-ncko) will be utilized where only a transient burst of *Pitx2* expression is seen from developmental days 10-12 in the neural crest. This *Pitx2* expression pattern is in contrast to the sustained *Pitx2* expression seen in wild type embryos and complete lack of expression seen in *Pitx2* -/- embryos. The knockout of *Ctnnb1* activity interrupts one of the pathways through which *Pitx2* functions. The transient burst observed in the neural crest is the result of another signaling pathway, probably originating with retinoic acid, an upstream effector of *Pitx2*. In the *Ctnnb1*-ncko strain cohesiveness of the anterior segment is maintained as the eye is found in its normal peripheral position attached to the surface ectoderm. In contrast, the eye in the *Pitx2* -/- strain recedes more and more to the center of the head. Comparison of these two strains suggests a mechanism for bonding of structures through cellular adhesion that is promoted by the transient burst of *Pitx2* observed in the *Ctnnb1*-ncko stain.

Comparing the optic nerve housing optic stalks of the *Pitx2* -/- embryos and *Ctnnb1*-ncko embryos they are both severely deformed but in different ways. In *Ctnnb1*-ncko embryos as the eye stays attached to the surface ectoderm the brain stretches out to meet the eye resulting in a thick neuroblastic structure as opposed to the normal slender, elongated optic nerve seen in wild type. In the *Pitx2* -/- embryos the optic nerve is deformed in that given the recession of the eye to the center of the head it becomes extremely foreshortened. (See Figure 4)

Wiltype *Pitx2* -/- *Ctnnb1*-ncko

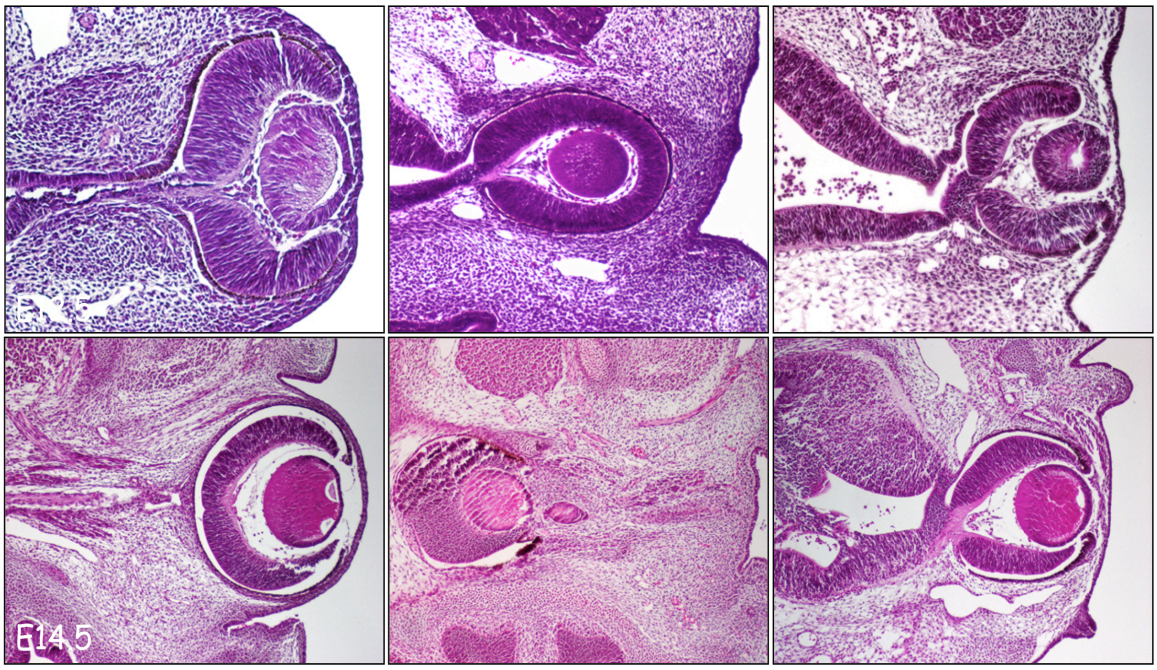


Figure 4. In *Pitx2* -/- embryos the eye migrates to the center of the head. In *Ctnnb1*-ncko mutant embryos the eye remains attached to the surface ectoderm while the optic nerve becomes severely deformed compared to wildtype.

The observation of the transient burst of *Pitx2* expression along with the adhesion of the eyes to the surface ectoderm seen in the *Ctnnb1*-ncko embryos suggests that the transient burst of *Pitx2* may have downstream targets of cell adhesion genes that cause the adhesion of the eye to the surface ectoderm and gross deformation of the optic nerve in *Ctnnb1*-ncko embryos. The primary goal of my research was to determine the nature of this adhesion to the surface ectoderm, with the hypothesis that *Pitx2* has one or more downstream targets of cell adhesion genes. Good candidate genes should show loss of expression in *Pitx2* -/- eyes but be retained in *Ctnnb1*-ncko and *Pitx2* +/+ eyes.

Identification and description of cellular adhesion candidates

Microarray data from *Pitx2* -/- mice suggests a significant number of possible cellular adhesion genes that appear to lose expression in *PITX2* -/- eyes. Of these genes we have selected three best possible downstream cellular adhesion gene targets of the transient *Pitx2* burst, collagen 8 alpha 2 (*Col8a2*), fibulin 5 (*Fbln5*), and dermatopontin (*Dpt*).

Col8a2 encodes a protein that is a major component of the basement membrane of the corneal endothelium, where it forms trimers with other collagens. An important study of this gene's role in anterior segment development was performed using a homozygous *Col8a2*G257D mutant human transgene mouse. Deficits in these mice include enlarged anterior chambers, thin corneas, and a reduction in size of the corneal epithelium, stroma, and Descemet's membrane (Puk 2009). These defects may result in increased intraocular pressure, consistent with another finding that these mice exhibit protrusion of the anterior segment. Point mutations of the *COL8A2* gene in humans have been associated with two types of corneal dystrophies, which share features seen in the mutant mouse model including thinning of Descemet's membrane and the corneal endothelium. These patients also have been observed to be at an increased risk for glaucoma (Puk 2009). These data, when considered in conjunction with the microarray data from the Gage lab, implicates *Col8a2* as a potentially important downstream target of *Pitx2*.

Fbln5 encodes a matricellular glycoprotein, important in forming cell-extracellular matrix interactions through the use of integrins. It is expressed in elastin-rich tissues, including the eye. *Fbln5* knockout mice have been found to exhibit heart

vessel defects, aneurisms, and lung defects suggesting a role in vascular development and remodeling (Yanagisawa 2009) (Chapman 2010). The eyes of the *Fbln5* knockout mice have not been fully examined so its role in eye development is not yet adequately understood. Its reduced expression has, however, been associated with human Age Related Macular Degeneration Type 3, an eye disease in which vision is impaired due to damage to the retina. Cutis laxa, a connective tissue disorder in which the skin becomes inelastic and hangs from the body in folds, has also been associated with mutant fbln5 (Jones 2010). Research into eye defects associated with these diseases has provided inconsistent results.

The third potential target of *Pitx2* examined was *Dpt*, an extracellular matrix protein that interacts with collagens. It is expressed in mesenchyme and mediates collagen fibrillogenesis, the process by which the fibrils of collagen are produced, an important aspect of corneal assembly (Takeda 2002). Proper development of the cornea depends on the organization and maintenance of the stromal extracellular matrix, which must include uniform small collagen fibrils and constant interfibrillar spacing. Presumably due to the role of *Dpt* as a mediator of fibrillogenesis, *Dpt* knockout mice have been found to exhibit corneal defects. These mice on average show a 24% thinner corneal stroma compared to wildtype mice as well as disorganization of the fibrils making up the stroma (Cooper 2006).

Experimental outline for each gene and hypothesis

1) Confirm predicted expression loss in *Pitx2* -/- predicted by DNA microarray

It was first necessary to confirm expression loss of *Col8a2*, *Fbln5* and *Dpt*. Utilizing qRT-PCR allowed comparison of relative mRNA levels of these genes in wildtype and *Pitx2* -/- eyes, giving confirmation to the changes predicted by the microarray. Confirmation of expression loss in the *Pitx2* -/- eye tissue led to step 2 for *Col8a2*.

2) Develop riboprobes for each gene that has a confirmed change in expression.

I developed several riboprobes for *Col8a2* using PCR to generate a DNA fragment that served as the template for subsequently synthesizing digoxigenin (DIG)-tagged RNA copies of the DNA template. The DIG labeled probes were then tested using RNA in-situ hybridization on paraffin sections of wild type eye tissues to confirm that they worked. The presence of annealed probe was detected by immunohistochemistry directed against the DIG tags on the probe. Because several probes I designed did not adhere to their target mRNAs on wildtype tissue where the mRNAs are known to be present, I redesigned new probes until a successful one was developed.

3) Obtain antibodies for genes found to be present in in-situ experiments for use in immunohistochemistry experiments to increase assay specificity.

To increase specificity of detection, once the *Col8a2* transcript was found to be present in the developing anterior segment, an antibody was obtained for use in immunohistochemistry antibody stainings.

4) Determine each gene's expression pattern in wild type eyes through the relevant time window

The probes and antibodies for each gene were then used in RNA in-situ hybridization and antibody staining experiments, respectively, on wildtype eye tissues from embryonic days e10.5-14.5 to determine wild type expression patterns. Because antibody staining proved to be more effective, the focus of my work was in using this technique. The goal was to determine the expression pattern of *Col8a2* relative to *Pitx2* in wild type eyes where we know *Pitx2* is expressed in neural crest and is fully functional.

5) Determine expression differences in wild type vs. *Pitx2* -/- and *Ctnnb1*-ncko eyes

A combination of RNA in-situ hybridizations and antibody stainings were used on e10.5-14.5 embryos to determine relative expression patterns of cell adhesion genes and *Pitx2* in our two knockout models. One being *Pitx2* -/-, where *Pitx2* is never expressed anywhere and the eyes migrate to the center of the head leading to an extremely foreshortened optic nerve by e 16.5. The second knockout model was the *Ctnnb1*-ncko strain, where a transient burst of *Pitx2* is seen from e10-12 and the eyes adhere to the surface ectoderm with a deformed, thick, neuroblastic optic nerve present.

Hypothesis and possible outcomes

We hypothesized two possible outcomes to these experiments. The first is that *Pitx2* directly activates one or more cellular adhesion genes, which contributes adherence of the optic cup to the surface ectoderm during development. In this scenario the cellular adhesion gene would turn on after *Pitx2* expression begins in the same cells in wild type eyes and will not be expressed at all in the *Pitx2* -/- eyes. We would expect to see expression of the cellular adhesion gene after the transient burst of *Pitx2* expression in the *Ctnnb1*-ncko eyes. The other possible outcome is that *Pitx2* is required to maintain the expression of the cell adhesion gene(s). In this scenario, the cell adhesion gene will be on before and maintained after expression of *Pitx2* in the wild type and *Ctnnb1*-ncko eyes. It would be on initially in *Pitx2* -/- eyes but its expression would be lost after *Pitx2* never turns on.

Materials and methods

Mouse Husbandry

Timed pregnant breeding cages were set up to generate embryos for *Pitx2* -/-, *Ctnnb1*-ncko, and *Pitx2* +/+ embryos. Females were monitored each day for copulation plugs at 12 pm. The day a plug was found corresponds to day embryonic development day e0.5. Various litters were harvested between days e10.5-e14.5. Pregnant females were euthanized using cervical dislocation, after which embryos were immediately harvested and processed. Some of the embryos used for the experiments were generated specifically for this project, while others were harvested and processed at an earlier date.

Embryo fixing and sectioning

After harvesting, embryos were fixed in 4% PFA, which was followed by a wash in PBS and dehydration through an ethanol gradient after which they were embedded in paraffin in preparation for sectioning. Embryos were sectioned using a microtome producing tissue sections of 7 microns in thickness. These sections were mounted onto slides on a hot plate and dried overnight in an incubator.

Generation of PCR primers for qRT-PCR and in-situ hybridization templates

Complete mRNA sequences for each gene of interest were obtained from the MGI- Mouse Genome Informatics website at http://www.informatics.jax.org. Next, the Primer3 program at http://frodo.wi.mit.edu/primer3/ was used to develop two sets of primers for each gene, one for use in qRT-PCR experiments and one for use in the generation of an in-situ hybridization probe. The in-situ hybridization primers required further modification while the qRT-PCR primers did not. To each reverse primer of an in-situ hybridization set a T7 promoter sequence was added to facilitate T7 RNA

polymerase binding. To test the primers and determine their optimal annealing temperatures gradient PCR experiments were performed with annealing temperatures ranging from 55-70 degrees Celsius.

qRT-PCR experiments

qRT-PCR experiments were used to confirm expression changes between e12.5 wildtype and e12.5 Pitx2 -/- cDNA and e14.5 wildtype and Pitx2 -/- cDNA. Experiments were run and analyzed by a iCycler PCR machine, using CyBr green reaction mix.

Generation of in-situ hybridization probes

In-situ hybridization probes were generated from amplifying a DNA template. cDNA templates were used for those primers which were designed across introns. After the PCR was complete, a small portion of the reaction mix was run on an agarose gel to confirm successful amplification. Next the product was purified using QIAquick PCR cleanup. Integrity of this purified DNA product was confirmed in two ways. First, a Nanodrop machine was used to check the concentration of the product. If the product was found to be sufficiently concentrated (above 40 ng/ul of DNA) it was next sequenced at the University of Michigan DNA Sequencing Core to confirm its identity.

Once found to be sufficiently concentrated and to be the proper sequence, the purified DNA product was used to generate a digoxigenin (DIG)-labeled riboprobe. A reaction mix was created with 500 ng of this template along with T7 RNA polymerase, which could recognize the T7 binding site designed into the DNA sequence. The RNA product obtained from this reaction was purified using an RNeasy kit and protocol. Finally before being tested on tissue sections the purified product was dot blotted on a nylon membrane to quantify its strength.

In-situ hybridization

During the first day of the hybridization RNAse free conditions were maintained. First sections were dewaxed in xylene, followed by rehydration through a gradient of ethanol washes and DEPC-treated H2O and next DEPC-PBS. The sections were then treated with 100 mM Glycine in DEPC-PBS followed by 0.3% Triton-X in DEPC-PBS. Next the sections were washed in DEPC-PBS to remove remnants of the previous washes. The sections were then incubated at 37 degrees Celsius in a 15 µg/ml of proteinase K in proteinase K buffer (100 mM Tris•HCl, 50 mM EDTA, pH 8.0) to permeabalize the tissue allowing reagents better access to the transcripts. Next the slides were washed briefly in DEPC-H2O, followed by fixing in 4% paraformaldehyde. Following fixation, the slides were then washed with DEPC-PBS and next were acetylated in TEA/acetic anhydride to neutralize positive charge and reduce non-specific background signal. Next, while the slides were washed in DEPC-PBS the probes were denatured at 95 degrees Celsius and then applied to the sections and incubated overnight at temperatures ranging from 50 to 60 degrees Celsius.

During the second day, RNAse free conditions were no longer required. To begin this day the sections were washed with 50% Formamide/0.5X diluted in H_2O at the overnight hybridization temperature to remove any remaining non-specifically bound probe. This was followed by a wash of 0.5X SSC. Next the slides were incubated in a humidified tupperware box with antibody blocking solution, followed by an antibody to digoxigenin diluted in this solution. Next, alkaline phospatase was used to label the antibody during incubation. Following this were washes of PBS and a chromagen buffer,

after which an NBT/BCIP solution diluted in chromagen was applied to the slides for 24-48 hours during which time the alkaline phospatase used to label the antibody to digoxigenin underwent an enzymatic reaction producing a purple stain. After this stain had adequately developed the slides were washed with PBS, fixed in 4% paraformaldehyde, and then mounted with pro-long gold under coverslips.

Antibody staining

Col8a2 antibodies were obtained from Paul Davis in New Zealand. Tissues used in antibody stainings were first dewaxed in Xylene, and next rehydrated in 100% and 95% ethanol washes, followed by washes in PBST to remove traces of ethanol. Next the tissues were boiled in 0.1 M citric acid, to expose epitopes necessary for antibody binding. After further PBST washes, the sections were washed in 3% H_2O_2 to quench endogenous peroxidases. Left unquenched these peroxidases may increase non-specific background signal. An image iT incubation was next utilized to enhance the quality of the photographs to be taken at the conclusion of the experiment. To finish the first day of the experiment, the sections were incubated in TSA block followed by an overnight incubation with the primary *Col8a2* antibody diluted in TSA block. The optimal primary antibody dilution was found to be 1:6400. Day two of the experiment began with an incubation of a secondary antibody directed against the rabbit epitopes of the first antibody. Next the slides were incubated with a tertiary reagent, streptavidin horseradish peroxidase (SA-HRP), directed against the secondary antibody, to label the proteins for fluorescent detection. Finally the sections were incubated with a mixture of flourophores and a H_2O_2 dilution that catalyzes a reaction between the flourophores and SA-HRP, producing fluorescently labeled protein.

Image analysis and photography

Tissues stained with the in-situ hybridization protocol were imaged under bright-field microscopy. Tissues stained with the antibody staining protocol were imaged with fluorescent microscopy. All photographs were taken using the Nikon ACT-1 program and were edited with Adobe Photoshop.

Results

qRT-PCR results

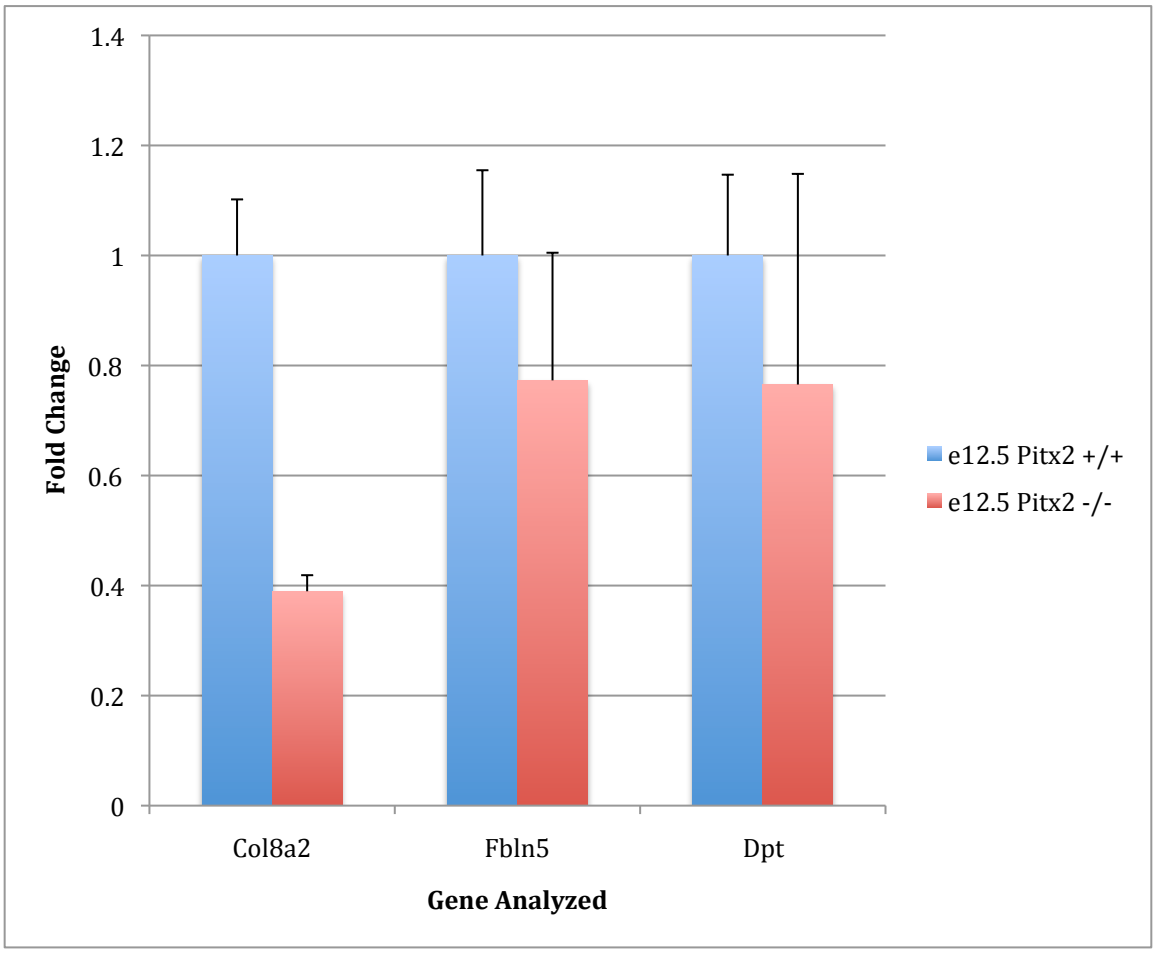
qRT-PCR experiments were performed to compare relative levels of transcripts of interest in wildtype and *Pitx2* -/- eyes at both e12.5 and e14.5 developmental time points. These experiments were performed to examine the fold change of transcript levels of *Col8a2*, *Dpt*, and *Fbln5*. (See graphs 1 and 2)

At e12.5 a decrease in transcript levels was observed in all three genes of interest. *Col8a2* exhibited a 0.389 fold change in *Pitx2* -/- as compared to wildtype, *Fbln5* exhibited a 0.773 fold change and *Dpt* exhibited a 0.765 fold change. A paired T-test of the data was performed which showed only the fold change observed in *Col8a2* levels to be statistically significant, giving a P value of .0275. The fold changes of *Fbln5* and *Dpt* levels were not statistically significant, giving P values of .432 and .577, respectively.

At e14.5 a decrease in transcript levels was observed in all three genes of interest as well. *Col8a2* exhibited a 0.526 fold change in *Pitx2* -/- eyes as compared to wildtype, *Fbln5* exhibited a 0.620 fold change and *Dpt* exhibited a 0.709 fold change. A paired T-test found the *Col8a2* fold change to be very near statistical significance, giving a P value of .112. The fold changes observed for *Fbln5* and *Dpt* were also not statistically significant at .144 and .322, respectively.

These results confirmed that *Col8a2* levels are indeed decreased at e12.5 in *Pitx2* -/- eyes, and probably in e14.5 Pitx2 -/- eyes, however the results at e14.5 were not statistically significant. *Fbln5* and *Dpt* levels may be decreased, however as these results lacked statistical significance their decrease was not confirmed.

Because *Col8a2* levels were confirmed as affected by the loss of *Pitx2*, while *Dpt* and *Fbln5* were not, *Col8a2* was singled out as the gene I would further investigate through in-situ hybridization and immunostaining experiments.



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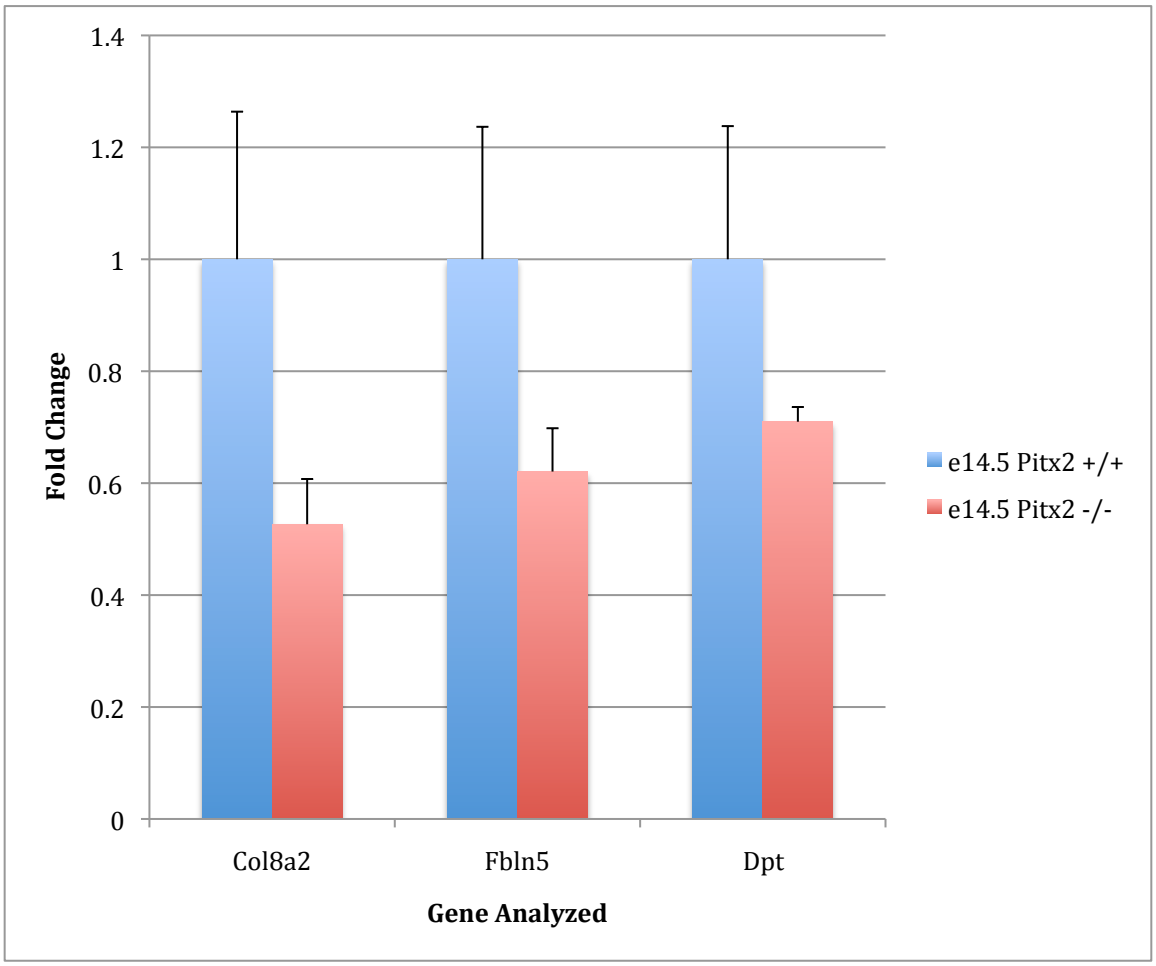


Graph 2. At e14.5 a decrease in transcript levels was observed in all three genes of interest as well. *Col8a2* exhibited a 0.526 fold change in *Pitx2* -/- eyes as compared to wildtype, *Fbln5* exhibited a 0.620 fold change and *Dpt* exhibited a 0.709 fold change. In these experiments none of the fold changes were found to be statistically significant using a paired T-test.

Col8a2 in-situ hybridization results

After confirmation of a change in *Col8a2* levels in *Pitx2* -/- eyes, an in-situ hybridization probe was developed to detect the transcript in embryonic eye sections. The probe was tested extensively on e10.5- e14.5 embryos and was found to only be effective on e14.5 samples. When functioning properly, it gave very specific signal in the developing cornea. (See Figure 5) Detection of transcripts was very inconsistent from slide to slide and experiment to experiment. Because of the inconsistency with which the probe proved to function, to further examine *Col8a2* expression an antibody to the protein was obtained for use in immunohistochemistry experiments.

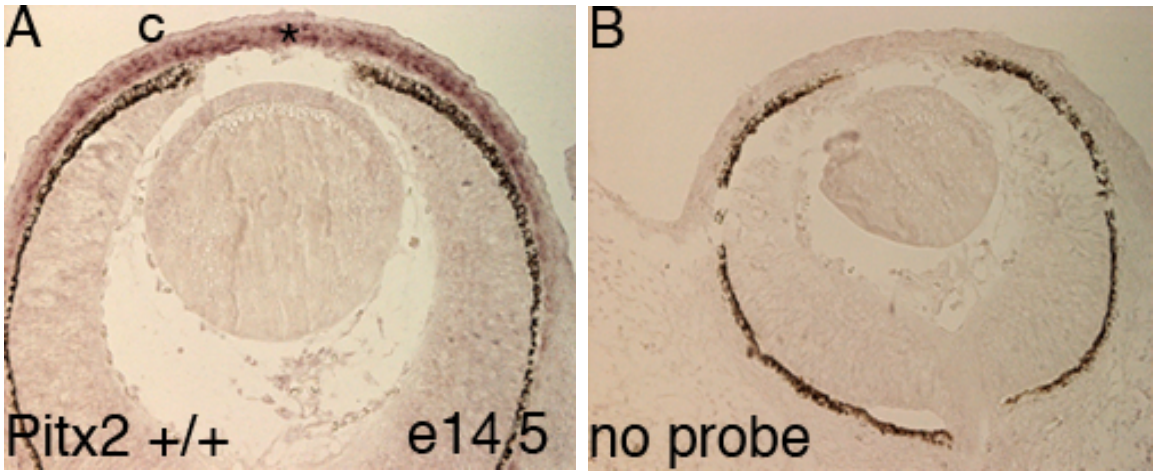


Figure 5. ***Col8a2*** **expression in e14.5 wild type eyes. (A) Col8a2 expression (denoted with *) located in the developing cornea (denoted by c). 1:50 dilution. (B) Negative control.**

Col8a2 Immunostaining Results

After localizing the location of *Col8a2* transcripts in the developing mouse eye, two antibodies against the *COL8A2* protein were obtained from Paul Davis and used to further examine its expression. Each of the antibodies was developed against the *COL8A2* protein, however, in different rabbits. The two antibodies are referred to as *COL8A2* M and *COL8A2* S.

Col8a2 expression during the relevant time window in wildtype eyes

First, it was necessary to establish the expression pattern of *Col8a2* in wildtype eyes from e12.5-e14.5. In e12.5 eyes *Col8a2* appears to be expressed in the developing cornea. *COL8A2* M and *COL8A2* S stain slightly differently at e12.5 with S giving a slightly broader and stronger signal (Figure 6A, B). Similar results were observed at e13.5 where *Col8a2* again appears to be expressed in the developing cornea. Again, *COL8A2* S gives a slightly broader and stronger signal. *COL8A2* M gives more signal in epithelial cells (Figure 6C, D). Finally at e14.5, again *Col8a2* appears to be expressed in the developing cornea. At e14.5 *COL8A2* M gives the stronger single staining the anterior portion of the cornea (Fig 6F). *COL8A2* S on the other hand gives a weaker signal in the posterior portion of the cornea (Fig 6E).

After testing the two antibodies across the relevant developmental time points it became clear that they stain somewhat differently. However, both stain the developing cornea, as expected from my in-situ results. From this evidence their stain appears to be specific, the two antibodies may simply be recognizing different forms or different parts of the *COL8A2* protein. As there are multiple splice variants of the *COL8A2* protein the possibility that the two antibodies recognize different forms of the protein seems likely.

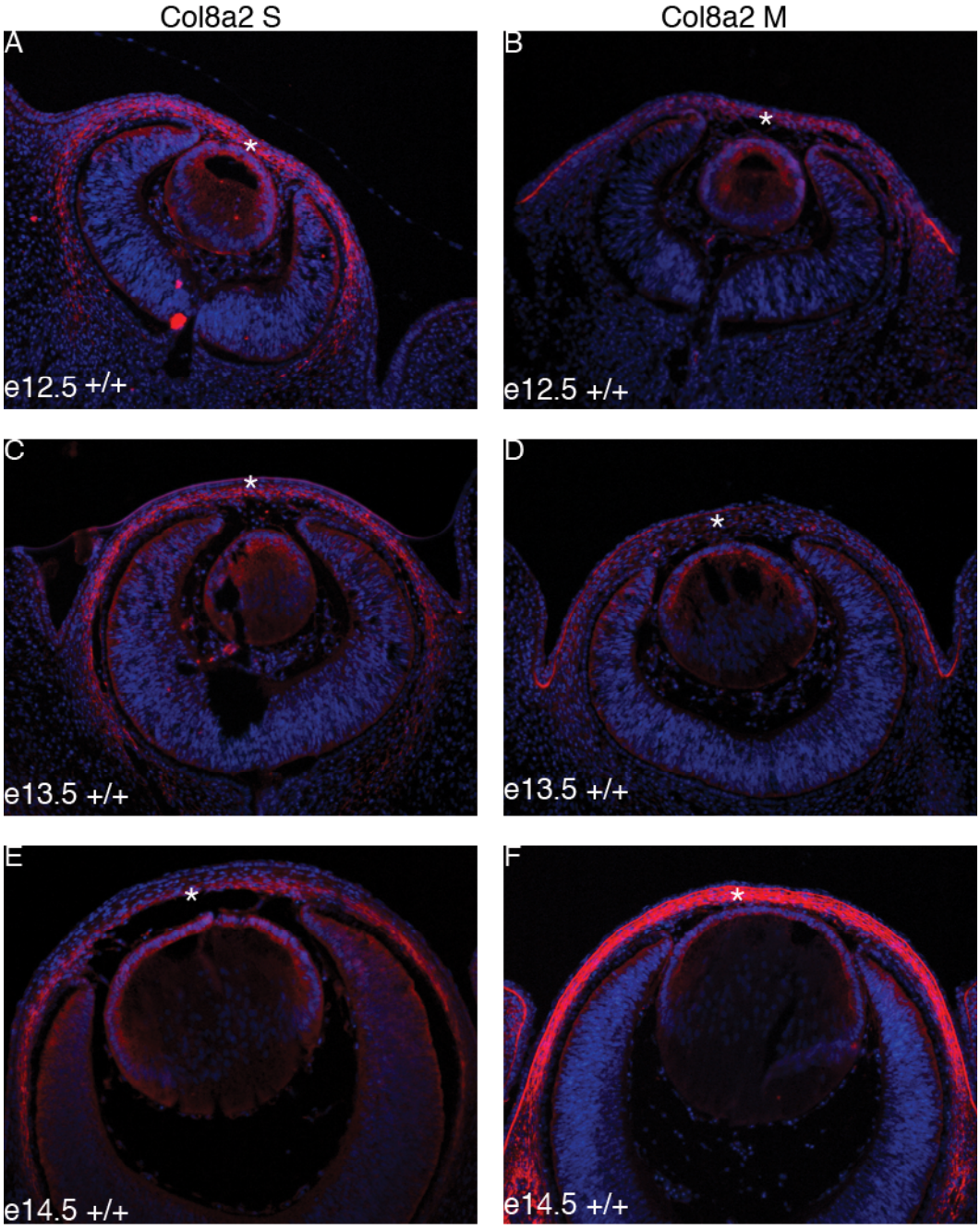


Figure 6. *Col8a2* expression in wildtype eyes. *Col8a2* expression is shown in red, DAPI staining is in blue. *Col8a2* expression is denoted with *. (A-F) *Col8a2* appears to be expressed in the developing cornea from developmental time points e12.5-e14.5. Antibody dilution 1:6400.

Col8a2 expression in *Pitx2* -/- eyes mutant

After localizing *COL8A2* in the developing cornea the next step was to determine the extent to which it is expressed in *Pitx2* -/- embryos. As in the wildtype experiments, *COL8A2* M and *COL8A2* S were found to exhibit similar staining patterns. The experiments could only be performed on e13.5 and e14.5 *Pitx2* -/- embryos because e12.5 *Pitx2*- /- embryos were not available. At both e13.5 and e14.5 *Col8a2* expression was absent from the mutant eyes in the area where the cornea should have formed (see figure 7 and 8 B-D, F-H). Very little *Col8a2* expression is seen in these mutants, and that which is observed is likely non-specific background. Both *COL8A2* S signal and *COL8A2* M signal disappears in the same manner, providing further evidence that both specifically recognize the *COL8A2* protein.

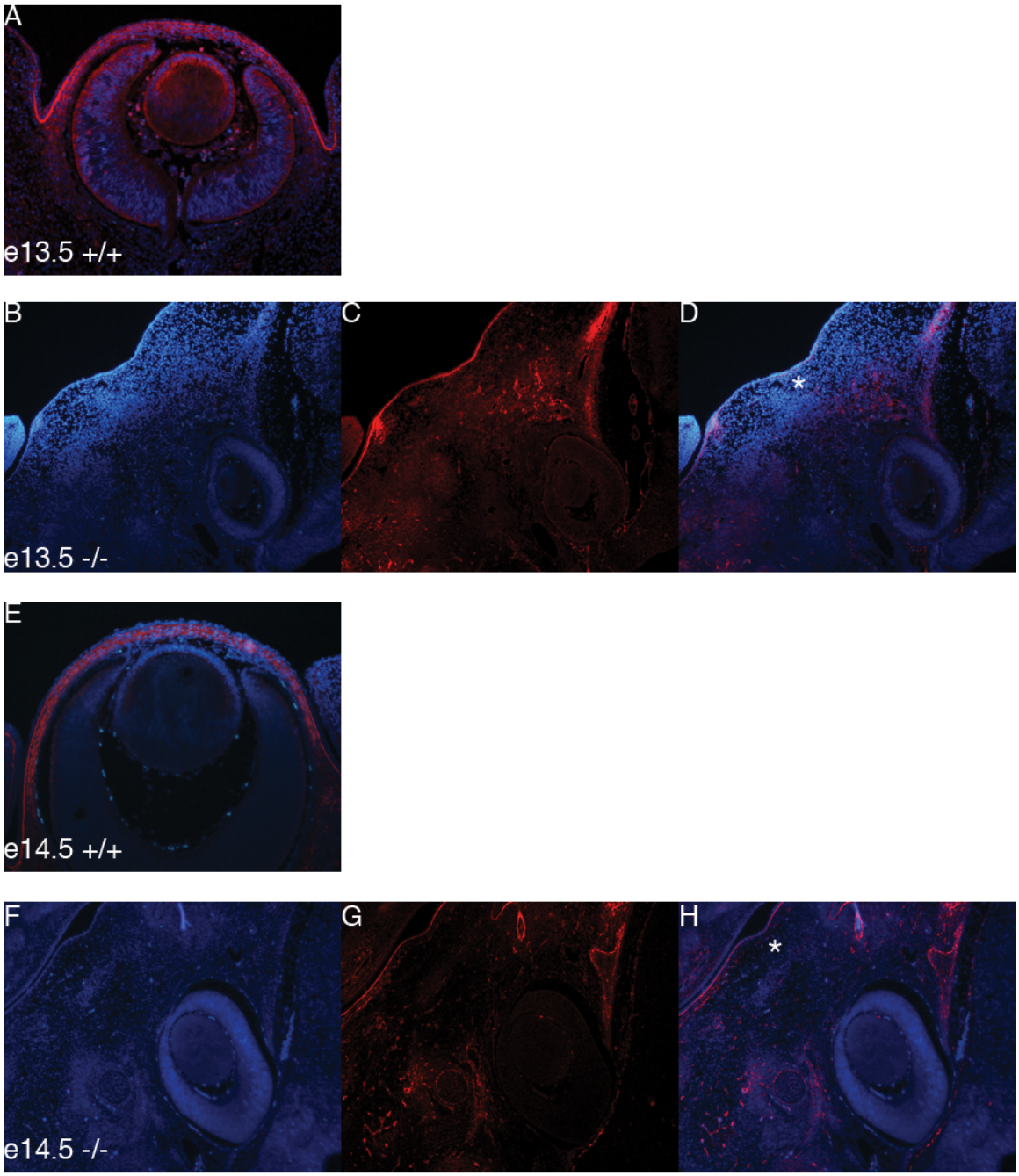


Figure 7. *COL8A2* M expression in *Pitx2* +/+ and *Pitx2* -/- eyes at e13.5 and e14.5. *Col8a2* in red, DAPI in blue. (A) Wildtype control. (B) e13.5 -/- DAPI only. (C) *Col8a2* only. (D) Merge. The area in which the cornea should have developed is denoted with *. *Col8a2* expression is largely absent in this area. (E) e14.5 wildtype

control. (F) e14.5 -/- DAPI only. (G) *Col8a2* only. (H) Merge. The area in which the cornea would have been expected to develop is denoted with *. *Col8a2* expression is largely absent in this area.

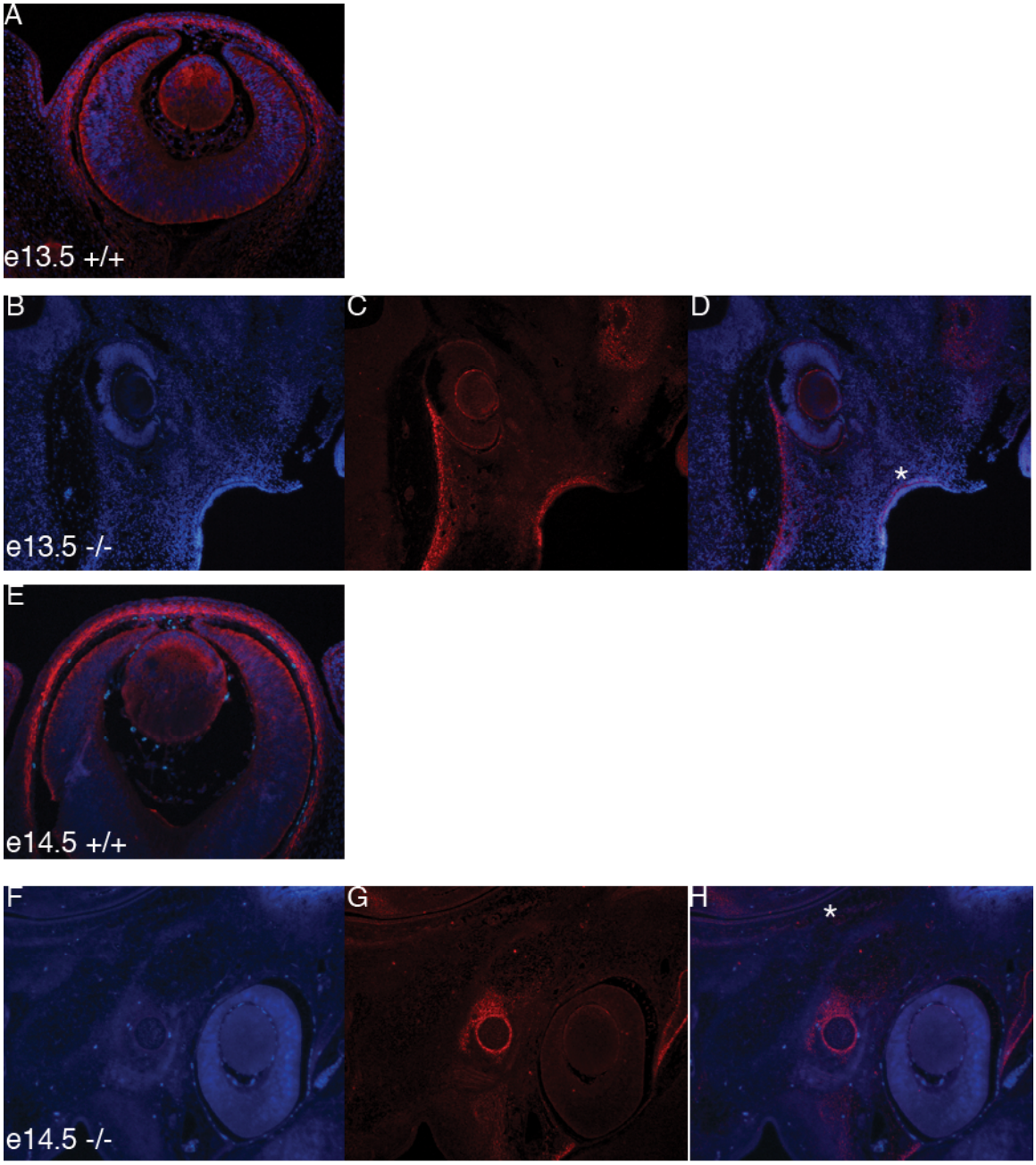


Figure 8. *COL8A2* S expression in *Pitx2* +/+ and *Pitx2* -/- eyes at e13.5 and e14.5. *Col8a2* in red, DAPI in blue. (A) Wildtype control. *Col8a2* expression is seen in the developing cornea. (B) e13.5 -/- DAPI only. (C) *Col8a2* only. (D) Merge. The area in which the cornea would have been expected to develop is denoted with *. *Col8a2* expression is largely absent in this area. (E) e14.5 wildtype control. (F) e14.5 -/- DAPI only. (G) *Col8a2* only. (H) Merge. The area in which the cornea should have developed is denoted with *. *Col8a2* expression is largely absent in these areas.

The experiment was later repeated on an additional e14.5 *Pitx2* -/- mutant, which showed a similar staining pattern. *COL8A2* M and *COL8A2* S signal is largely absent in the area where the cornea would have been expected to develop. The signal that is present appears to be non-specific background. See Figure 9.

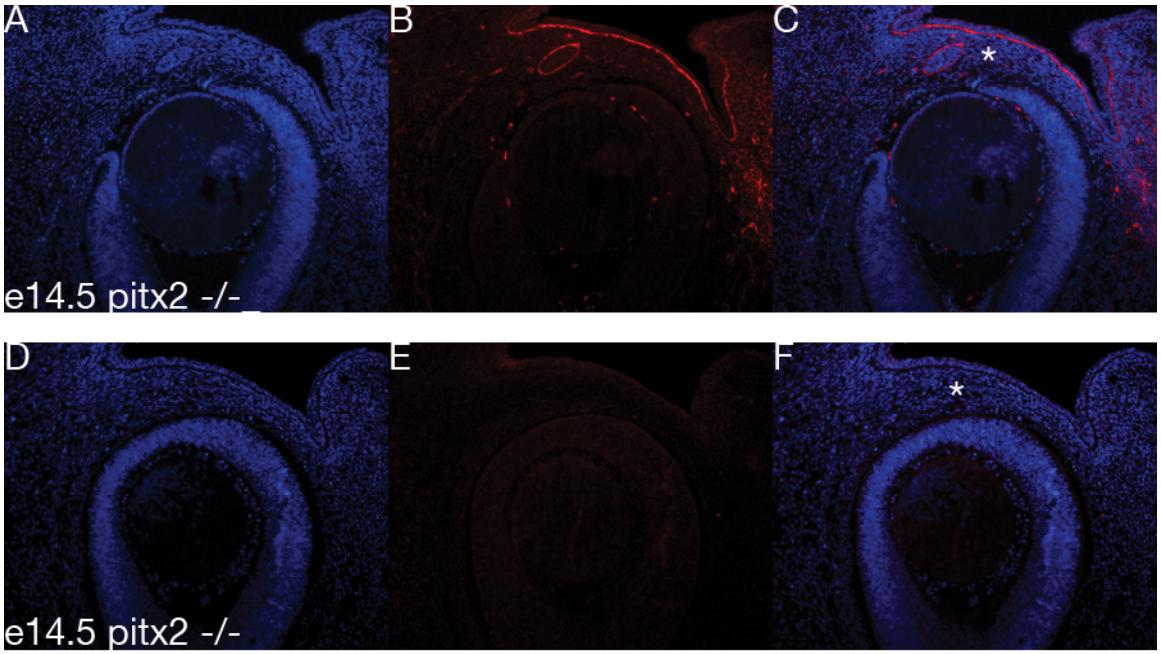


Figure 9. *Col8a2* expression e14.5 Pitx2 -/- (A-C) *COL8A2* M (A) DAPI only (B) *Col8a2* only (C) Merge. . The area in which the cornea would have been expected to develop is denoted with *. *Col8a2* expression is largely absent in this area. That which is present appears to be non-specific background (D-F) *COL8A2* S (D) DAPI Only (E) *Col8a2* only (F) Merge. The area in which the cornea developed is denoted with *. *Col8a2* expression is largely absent in these areas.

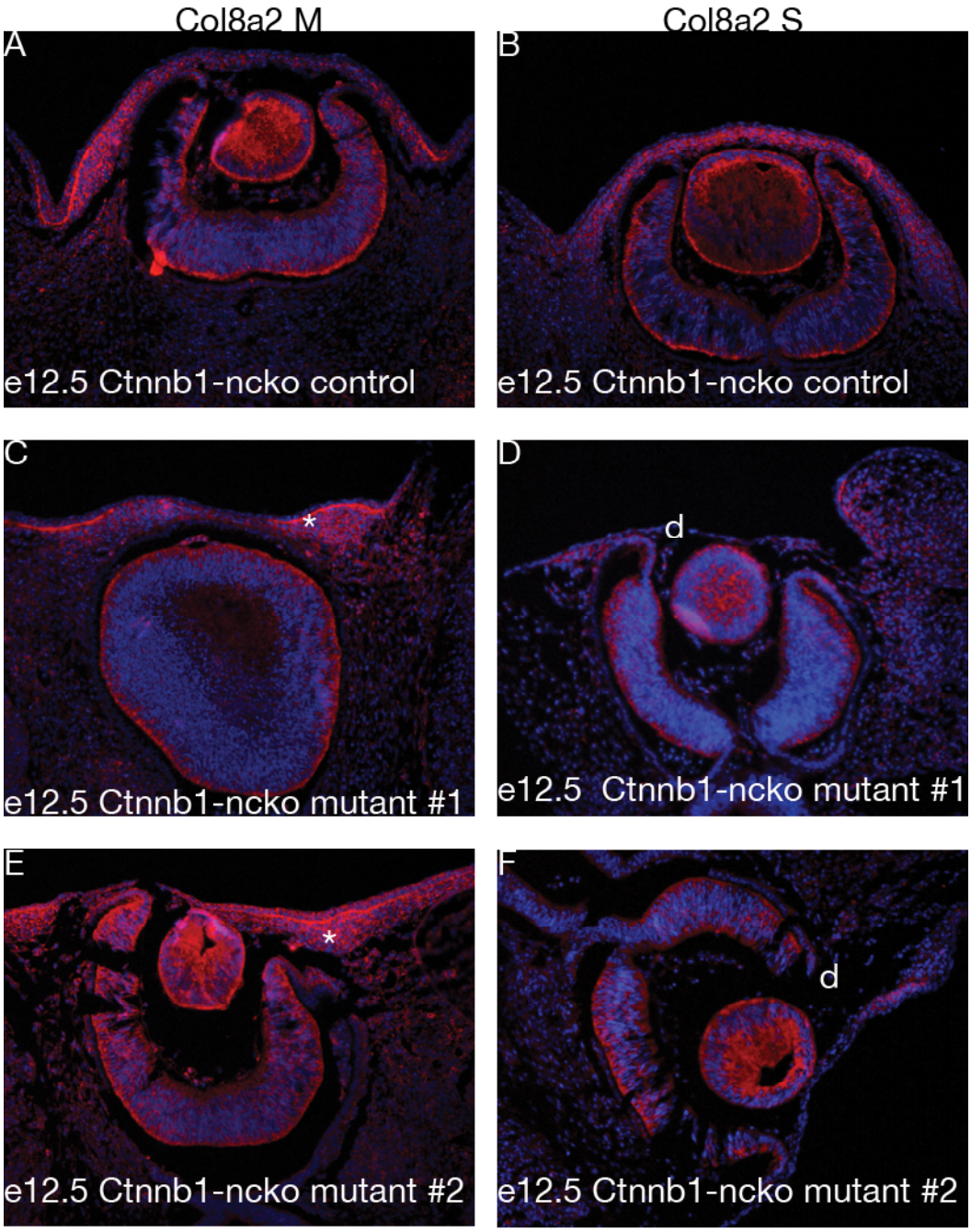
Col8a2 expression appears to be down in mutants

Comparing wildtype eyes to *Pitx2* -/- eyes in these experiments, *Col8a2* levels appear to be decreased in the mutants. As the eye recedes more towards the center of the developing brain, *Col8a2* staining in the area where the cornea would have developed is largely absent. This suggests that *Col8a2* may indeed have the function of adhering the optic cup to the surface ectoderm during developmental and maintaining cohesiveness of the anterior segment during development. To further investigate this hypothesis, *Col8a2* expression was analyzed in *Ctnnb1*-ncko mutants at e12.5 and e14.5.

Col8a2 expression in e12.5 *Ctnnb1*-ncko mutants

The next step was to determine if *Col8a2* expression is observed in *Ctnnb1*-ncko mice, potentially contributing to the rescuing of the proper eye placement. phenotype. Unfortunately, high levels of background signal, insufficient number of cells in the anterior segment, and highly fragile sections plagued these experiments. See Figure 10.

These problems made *Col8a2* expression in e12.5 *Ctnnb1*-ncko embryos very difficult to analyze for several reasons. First, the small number of cells in the developing cornea made for a very small population of cells that can show signal. Second, because the eyes of these sections were very fragile, they often became damaged during the staining procedure (Figure 10D, F, G, H). Third, when signal was observed it appeared to be non-specific background signal (Figure 10C, E, G). After repeating the experiment several times, results at e12.5 remained inconclusive.


Col8a2 M
Col8a2 S
A
B
e12.5 Ctnnb1-ncko control
e12.5 Ctnnb1-ncko control
C
D
*
d
e12.5 Ctnnb1-ncko mutant #1
e12.5 Ctnnb1-ncko mutant #1
E
F
*
d
e12.5 Ctnnb1-ncko mutant #2
e12.5 Ctnnb1-ncko mutant #2

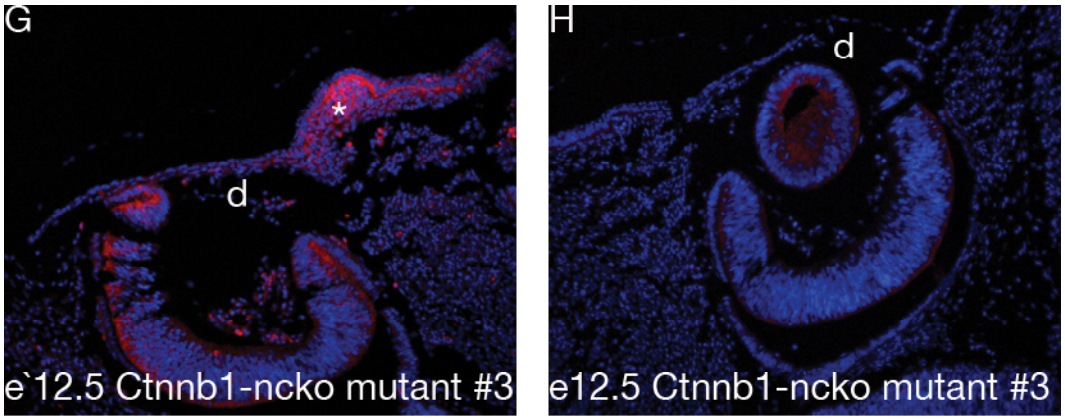
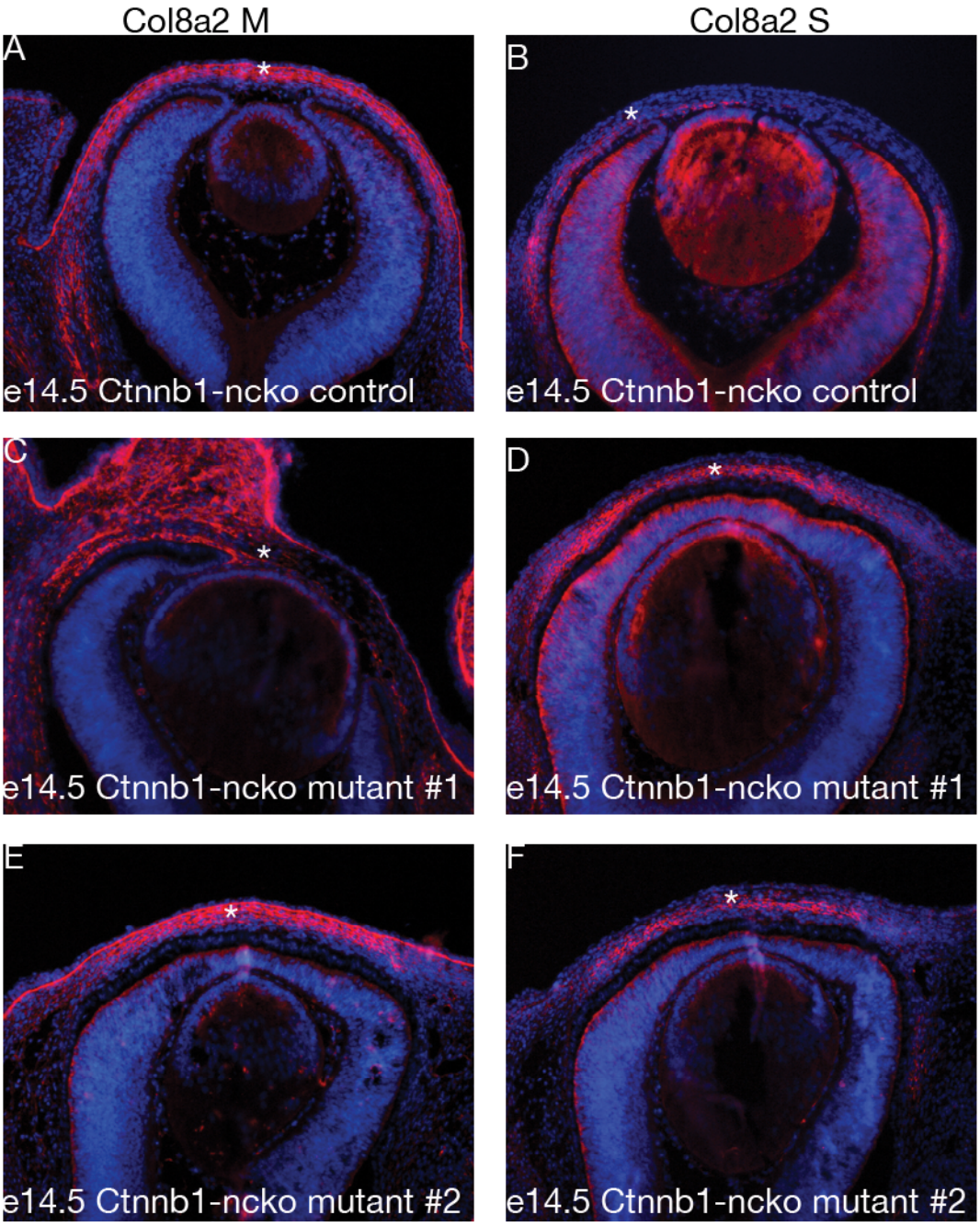


Figure 10. *Col8a2* expression in e12.5 *Ctnnb1*-ncko mutants. DAPI shown in blue, *Col8a2* shown in red. Potentially non-specific background signal is denoted with *. Damage to sections is denoted with d. (A-B) e12.5 wildtype control. (C-D) *Ctnnb1*-ncko mutant #1: Some staining is observed in the developing cornea, however it may be non-specific. Damage occurred in D. (E-F) *Ctnnb1*-ncko mutant #2: Again, *Col8a2* signal is present, however it appears to be somewhat non-specific. (G-H) *Ctnnb1*-ncko mutant #3: Again damage to sections plagued these results. Signal observed was potentially non-specific background.

Col8a2 expression in e14.5 *Ctnnb1*-ncko mutants

After e12.5 *Ctnnb1*-ncko results proved inconclusive, I moved to e14.5 embryos to test *Col8a2* expression. In *Ctnnb1*-ncko mutants, correct eye placement is rescued, as the eye remains as a cohesive unit during development and adheres to the surface ectoderm. In these mutants *COL8A2* M and *COL8A2* S expression is consistently seen in an area where the cornea seems likely to have developed. The signal is often less robust than in wildtype, and more randomly located. In some mutants who seem to have developed a somewhat normal eye, it highly resembles the stain seen in a normally developing cornea (Figure 11 D-F). In other mutants in which the eye mutation is more severe, the stain is less robust and often more limited in expression (Figure 11C, G-H) Despite these differences, each *Ctnnb1*-ncko mutant observed gave some type of signal in the developing anterior segment. See Figure 11.


Col8a2 M
Col8a2 S
A
B
C
D
E
F
e14.5 Ctnnb1-ncko control
e14.5 Ctnnb1-ncko control
e14.5 Ctnnb1-ncko mutant #1
e14.5 Ctnnb1-ncko mutant #1
e14.5 Ctnnb1-ncko mutant #2
e14.5 Ctnnb1-ncko mutant #2

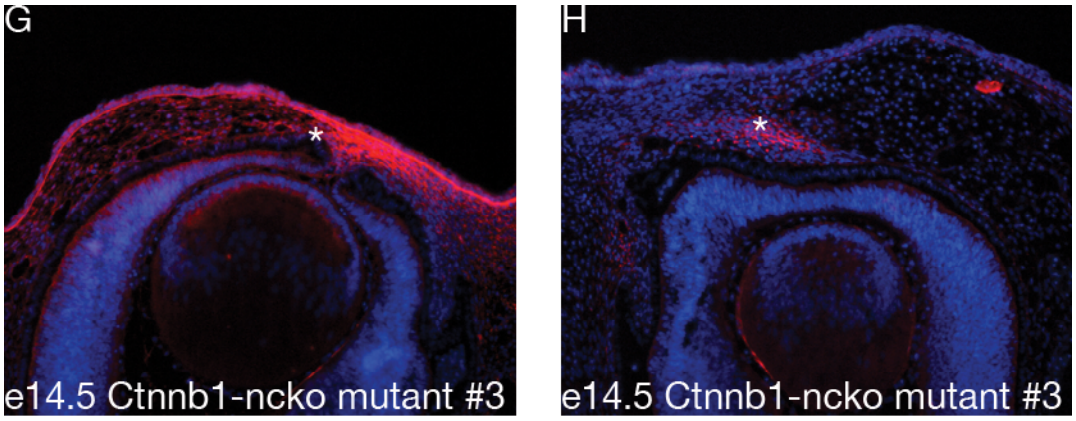
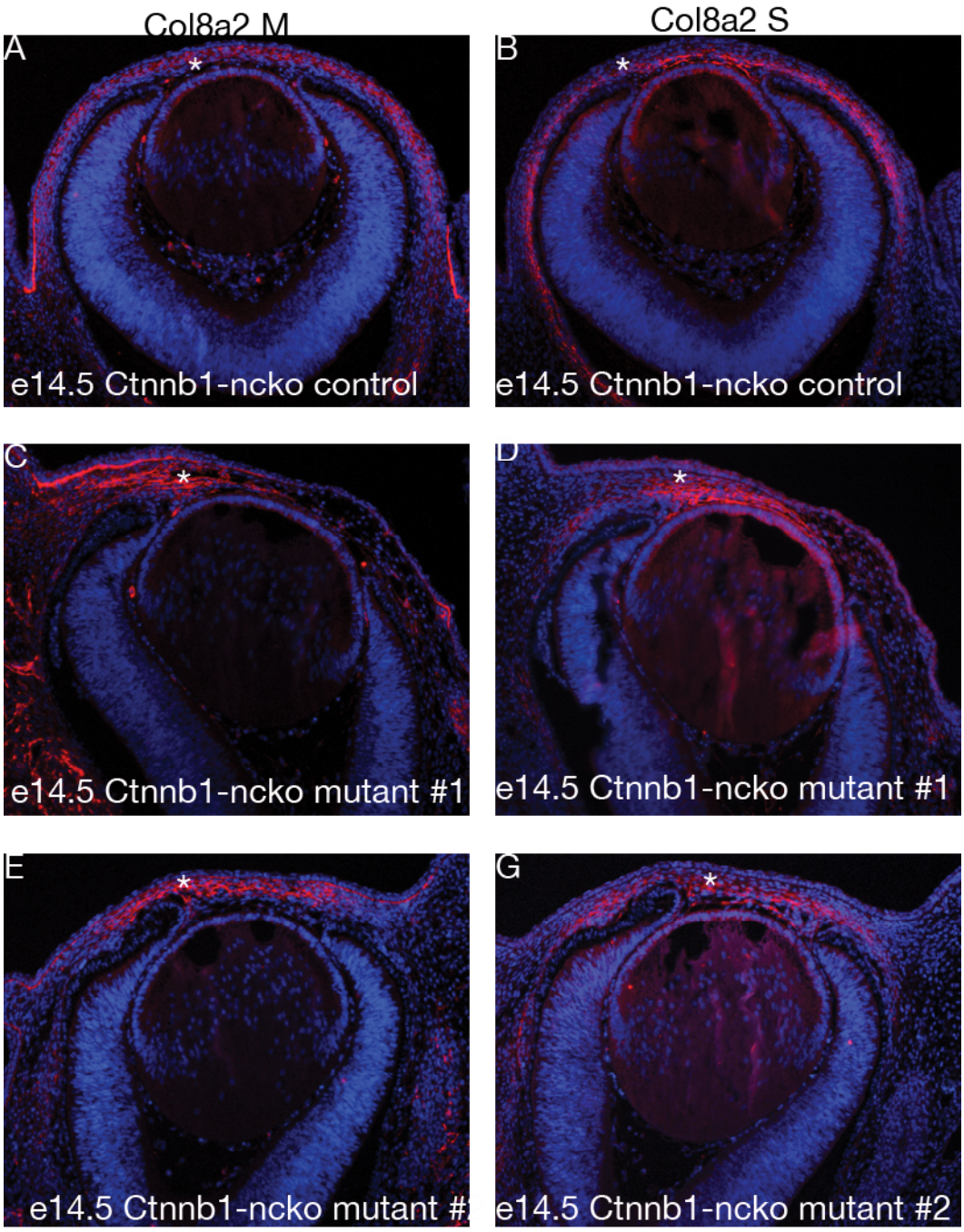


Figure 11. *Col8a2* expression in e14.5 *Ctnnb1*-ncko mutants. *Col8a2* in red, DAPI in blue. *Col8a2* expression is denoted with * (A-B) Wildtype controls: *Col8a2* is observed in the developing cornea. (C-D) *Ctnnb1*-ncko mutant #1: Mutant #1 has developed one eye which appears similar to a wildtype eye (D) and one eye which is more deformed (C). *Col8a2* expression is present in both but highly resembles wildtype in the more normal eye. (E-F) *Ctnnb1*-ncko mutant #2. *Col8a2* expression highly resembles wildtype expression. (G-H) *Ctnnb1*-ncko mutant #3: The eyes are highly deformed, however, some *Col8a2* expression persists.

Next, I repeated this experiment to further verify the *Col8a2* expression observed in these mutants is specific and reproducible. This experiment gave similar results. All *Ctnnb1*-ncko mutants gave some signal in the developing anterior segment. In some mutants this signal highly resembled the pattern observed in a wildtype cornea (Figure 12 D-F) while in others it differed somewhat (Figure 12 C, G-H).


Col8a2 M
Col8a2 S
A
B
C
D
E
G
e14.5 Ctnnb1-ncko control
e14.5 Ctnnb1-ncko control
e14.5 Ctnnb1-ncko mutant #1
e14.5 Ctnnb1-ncko mutant #1
e14.5 Ctnnb1-ncko mutant #
e14.5 Ctnnb1-ncko mutant #2

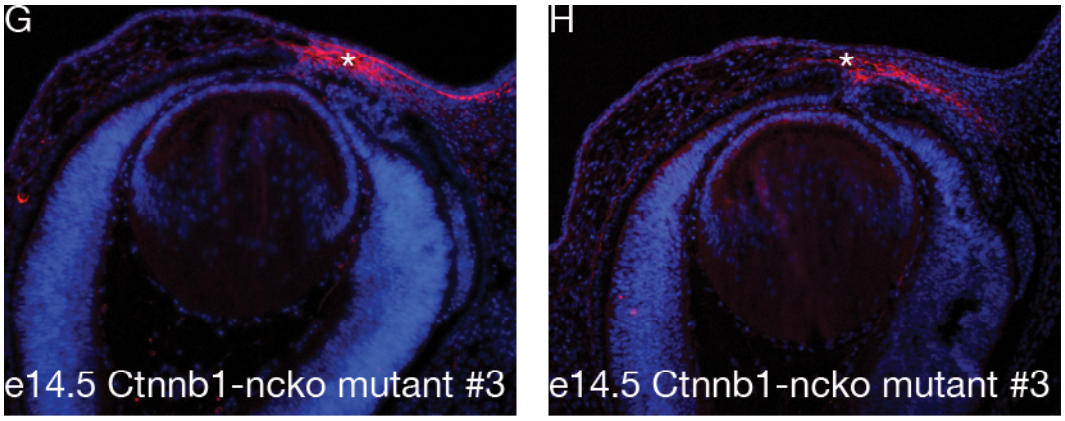


Figure 12. *Col8a2* expression in e14.5 *Ctnnb1*-ncko mutants. *Col8a2* in red, DAPI in blue. *Col8a2* expression is denoted with * (A-B) Wildtype controls: *Col8a2* is observed in the developing cornea. (C-D) *Ctnnb1*-ncko mutant #1: *Col8a2* is expressed in a location where a cornea should have developed (C). Expression is close to wildtype expression (D). (E-F) Both *COL8A2* M and *COL8A2* S stain in a pattern very similar to wildtype. (G-H) *Col8a2* expression is observed, however it is somewhat altered in pattern when compared to wildtype controls.

Col8a2 expression is restored in e14.5 *Ctnnb1*-ncko mutants

Analyzing the expression of *Col8a2* in these embryos, it reappears in the area of the cornea by e14.5. The absence of expression in *Pitx2* -/- embryos at this time point suggests *Col8a2* may have some function in adhering the optic cup to the surface ectoderm and maintaining cohesiveness during development of the anterior segment.

Discussion

I have found that the *COL8A2* protein is expressed in the developing mouse cornea from e12.5-e14.5. It is most strongly at expressed at e14.5. qRT-PCR results first showed this expression is decreased in a *Pitx2* -/- mutant. In antibody staining experiments, *Col8a2* is not expressed in the area where the cornea should have formed. Cohesiveness of the anterior segment is lost as the eye migrates towards the center of the developing mouse brain. At e14.5 in a *Ctnnb1*-ncko mutant, where cohesiveness of the anterior segment during development is rescued, *Col8a2* is again expressed in an area where the cornea would be expected to develop. These results suggest that *Col8a2* may contribute to the cohesive development of the anterior segment, and may be important for normal corneal development. It appears that *Col8a2* may be activated by *Pitx2*, as hypothesized, however a direct link has not been established. However, *Col8a2* has held up as a gene that is important for cohesiveness of the developing anterior segment. Temporally, it remains unclear from my work when *Col8a2* is activated relative to *Pitx2* because I was only able to monitor *Col8a2* expression between e12.5-e14.5.

Throughout my research, I encountered several difficulties. First, the data I collected for *Fbln5* and *Dpt* in qRT-PCR experiments did not prove to be statistically significant. Although expression of these genes appeared altered at both e12.5 and e14.5 in a *Pitx2* -/- mutant, these results are meaningless without statistical significance. If these genes were to be investigated again in the future, a higher number of samples would be important for ensuring more reliable results. Due to constraints on time as well as resources, only one *Pitx2* -/- mutant was examined at both e12.5 and e14.5 in qRT-PCR. Second, in-situ hybridizations experiments proved very unreliable in monitoring

expression of *Col8a2*. Even after sequencing of the riboprobe to confirm its identity as well as localization of *Col8a2* in the cornea with antibody stainings, in-situ hybridizations still failed to reliably detect the *Col8a2* transcripts. It is possible that problems with the in-situ hybridization protocol I used contributed to these difficulties. Differences in annealing temperature, reagent concentrations, and incubation time all must be controlled and established for each new probe that is developed. While I performed many experiments to establish these parameters for my *Col8a2* probe, it is possible more would have been necessary to achieve an optimal protocol. This explanation seems especially plausible given the sporadic nature of the probe's effectiveness. Another possibility for my difficulty in localizing *Col8a2* transcripts is that the transcripts could be short lived in comparison to the *COL8A2* protein. It is possible that relatively short-lived *Col8a2* transcripts give rise to long-lived *COL8A2* protein, causing an insufficient number of transcripts in the cornea for detection. This would explain why antibody stainings, which recognize proteins, were more effective in monitoring *Col8a2* expression. A third problem I encountered was the slight differences in staining pattern exhibited by my two antibodies *COL8A2* M and *COL8A2* S. Slight variations between results from these two antibodies is seen throughout my results. However, I am confident that both antibodies specifically recognize the *COL8A2* protein for several reasons. First, both antibodies gave signal in the same area as my riboprobe signal during in-situ hybridizations, when it functioned properly. Second, signal from both antibodies showed the same behavior as it disappeared in *Pitx2* -/- mutants and reappeared in similar areas in *Ctnnb1*-ncko mutants. It seems likely that the two antibodies may simply recognize different forms or parts of *COL8A2*.

At this time, the relationship between *Col8a2* and *Pitx2* is still somewhat unclear. The evidence I have provided suggests that *Col8a2* expression may rely on *Pitx2* activation, however the link has not been established directly. My localization of *Col8a2* in the developing cornea supports previous findings that *Col8a2* mutations have been observed in corneal dystrophies in humans (Puk 2009). Human *Col8a2* patients have also been observed to be at a higher risk for glaucoma (Puk 2009). The potential relationship I have established between *Col8a2* and *Pitx2* supports this finding as well, as *Pitx2* is a gene which is also known to increase risk for glaucoma in humans. Finally, my work has begun to elucidate further downstream effectors of *Pitx2* in normal eye developmental. Evidence for *Col8a2* as a downstream effector of *Pitx2* is high, while *Dpt* and *Fbln5* remain open as possibilities to be further investigated. Further work into the relationship of *Pitx2* and *Col8a2* will help to further elucidate their connection, as well as the mechanisms through which *Col8a2* may maintain cohesiveness of the anterior segment during development.

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References

Chapman, S. L., F. X. Sicot, et al. (2010) "Fibulin-2 and fibulin-5 cooperatively function to form the internal elastic lamina and protect from vascular injury." Arteriorscler Thromb Vasc Biol **30**(1): 68-74

Coleman, A.L. (1999). "Glaucoma" Lancet. **354**(9192):1803-10.

Cooper, L.J., A.J. Bentley, et al. (2006) "The role of dermatopontin in the stromal organization of the cornea." Invest Ophthalmol Vis Sci **47**(8):3303-10

Gage, P.J., A. Evans. (2005) Expression of the homeobox gene Pitx2 in neural crest is required for optic stalk and ocular anterior segment development. Hum Mol Gen **14**(22):3347-5

Gage, P.J., Suh, et al. (1999). "Dosage requirement of Pitx2 for development of multiple organs." Development **126**(20): 4643-51

Gould, D.B., S. W. John (2002). "Anterior segment dysgenesis and the developmental glaucoma's are complex traits." Hum Mol Genet **11**(10): 1185-93

Gould, D.B., R.S. Smith et al. (2004) "Anterior segment development relative to glaucoma." Int J Dev Biol **48** (8-9): 1015-1029

Jones, R.P., C. Ridley, et al. (2010) "Structural effects of fibulin 5 missense mutations associated with age-related macular degeneration and cutis laxa." Invest Ophthalmol Vis Sci **51**(5): 2356-62

Lines, M. A., K. Kozlowski, et al. "Molecular genetics of Axenfeld-Rieger malformations." Hum Mol Genet **11**(10): 1177-84

Puk, O., C. Dalke, et al. (2009) **"**Reduced corneal thickness and enlarged anterior chamber in a novel ColVIIIa2G257D mutant mouse." Invest Ophthalmol Vis Sci **50**(12):5653-61

Ray, K., Mukhopadhyay, A. et al. (2003) "Recent advances in molecular genetics of glaucoma." Mol Cell Biochem. **253**: 223-31.

Semina, E. V., R. Reiter, et al. (1996). "Cloning and characterization of a novel bicoid-related homeobox transcription factor, RIEG, involved in Rieger Syndrome." Nat Genet **14**(4): 392-9.

Takeda, U., A. Utani, et al. (2002) "Targeted disruption of dermatopontin causes abnormal collagen fibrillogenesis." J Invest Dermatol **119**(3):678-83

Yanagisawa, H., M.K. Schluterman, et al. (2009). "Fibulin 5, an integrin-binding matricellular protein: its function in development and disease." J Cell Commun Signal **3**(3-4):337-47