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Lmx1b is required for murine trabecular meshwork formation and for maintenance of corneal transparency

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Abstract

Studies of Lmx1b have shown that it is required for anterior segment formation during embryonic development and that reduction of Lmx1b may contribute to elevated intraocular pressure in the adult. However, whether Lmx1b is required for formation of anterior segment tissues that are associated with regulation of intraocular pressure has not been addressed due to the perinatal lethality of Lmx1b null allele. Here we use conditional deletion strategies to circumvent perinatal lethality. Our results indicate that Lmx1b is required in neural crest-derived periocular mesenchyme for formation of anterior segment tissues, including trabecular meshwork, a critical regulator of intraocular pressure. Furthermore, we show that Lmx1b is essential to maintain proper functioning of those tissues in the adult. Taken together, our results are the first to link a specific transcription factor to trabecular meshwork formation and the first to demonstrate specific requirements for Lmx1b in maintaining the integrity of adult anterior segment.

Keywords

Lmx1b; Neural Crest; Trabecular meshwork; Cornea; Mouse

Introduction

The anterior segment of the vertebrate eye is composed of the cornea, the trabecular meshwork, the iris, and the ciliary body (reviewed in (Gould et al., 2004)). The activities of these tissues are integrated to ensure proper functioning of the visual system as a whole. One essential function of anterior segment tissues is to regulate intraocular pressure, accomplished largely through the opponent activities of the ciliary epithelium and the trabecular meshwork. The ciliary epithelium secretes aqueous humor that fills the anterior chamber while the trabecular meshwork's main function is to act as a drainage pathway for the aqueous humor. Increased production and/or decreased aqueous humor outflow via the trabecular meshwork leads to elevated intraocular pressure, a key risk factor in glaucoma.

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Decreased aqueous humor outflow through the trabecular meshwork may result from intrinsic defects in the trabecular meshwork or from physical blockage of the outflow pathway as is seen in pigmentary dispersion glaucoma and closed angle glaucoma.

A second critical function of anterior segment tissues is in maintaining corneal transparency (reviewed in (Edelhauser, 2006)). This is accomplished by dual functions of the corneal stroma that contains extracellular matrix (ECM) secreting corneal keratocytes and the corneal endothelium. A major component of the corneal ECM is collagen, arranged in orthogonal bundles. When the integrity of the cornea is compromised, for example, through injury or disease to the corneal endothelium, corneal edema can ensue, leading to disruption of the ordered arrangement of collagen fibrils ultimately resulting in loss of corneal transparency and impaired visual function. A second important function of the corneal stroma is to maintain an avascular region that is essential for corneal transparency (Azar, 2006; Beebe, 2008). Again, in cases of injury or disease, this function can be compromised leading to neovascularization and attendant loss of corneal transparency and visual function.

The complex arrangement and diverse tissue types of the anterior segment are specified and laid out during embryonic and early postnatal development (reviewed in (Gould et al., 2004)). Chief contributors to anterior segment tissues are the cranial mesoderm and the cranial neural crest. These tissues contribute to many anterior segment tissues, such as the corneal stroma and endothelium, the trabecular meshwork, the ciliary body, and the anterior iris stroma. In addition several anterior segment tissues are derived from non-neural crest and non-cranial mesoderm sources. For example, the posterior portion of the iris is derived from neuroectodermal tissue via the developing retina while the corneal epithelium is a derivative of the surface ectoderm.

Despite the importance of the anterior segment in proper visual function, relatively little is known about embryological mechanisms that orchestrate its development in mammals. For example, while fate mapping experiments were conducted by the Noden laboratory in the 1970s in the chick to demonstrate contributions of neural crest and cranial mesoderm to anterior segment tissues (Johnston et al., 1979), only in the past several years has genetic techniques been applied to fate map the mammalian neural crest and cranial mesoderm (Gage et al., 2005). Likewise, although the lens has been suggested to be an organizing tissue in avian anterior segment development (Beebe and Coats, 2000), whether this is the case in mammals has not been examined in detail despite evidence that communication between the lens and periocular mesenchyme is critical during murine embryogenesis (Breitman, 1989). Some attention has been paid to understanding developmental mechanisms that lead to proper specification and morphogenesis of selected anterior segment tissues, most notably the cornea (Hay, 1979), but again, these studies have limited to descriptive studies in mammals and the relevant experimental embryology has largely been carried out in non-mammalian species, such as the chick. Hence, developmental mechanisms that direct proper anterior segment development remain largely unknown, especially in mammalian systems.

The periocular mesenchyme, a population of mesenchymal cells that lie in close apposition to the developing retina and lens during development, plays a critical role in anterior

segment development. Fate mapping studies in the chick (Johnston et al., 1979) and mouse (Gage et al., 2005) have defined the contributions of the periocular mesenchyme to anterior segment structures, including the sclera, ciliary body, trabecular meshwork, anterior portion of the iris, corneal endothelium and stroma. That the periocular mesenchyme is a key tissue in murine anterior segment development has been demonstrated by genetic analysis of signaling molecules such as transforming growth factor beta (Tgfβ), bone morphogenetic protein 4 (Bmp4), and retinoic acid. Each of these pathways plays a prominent role in murine anterior segment development, highlighting the role of the periocular mesenchyme in that process. In the case of Tgfβ, both gain (Flugel-Koch et al., 2002) and loss of function (Saika et al., 2001) affects corneal, iris, and ciliary body development and the likely target tissue is neural crest derived periocular mesenchyme (Ittner et al., 2005). Likewise, the periocular mesenchyme has been determined to be the retinoic acid responsive tissue (Matt et. al., 2005, 2008). Mice heterozygous for *Bmp4* display a range of anterior segment anomalies, including to blockage of trabecular meshwork outflow and glaucoma (Chang et al., 2001).

Additional progress has been made in the identification of transcription factors that are essential for proper anterior segment development and function through studies of human congenital glaucoma. Most prominent among these transcription factors are Pax6, Pitx2, Foxc1, and Lmx1b. In each case mutations have been identified in humans (reviewed in (Idrees et al., 2006)) that are associated with open angle glaucoma and each of these transcription factors is expressed during development in progenitors of anterior segment tissues. Of particular note is that three of these genes: *Pitx2, Foxc1*, and *Lmx1b* are transcribed in the periocular mesenchyme (Gage and Camper, 1997; Kidson et al., 1999; Lu et al., 1999; Pressman et al., 2000), Targeted disruption of each of these genes in mice has demonstrated their critical importance in early steps of anterior segment development (Gage et al., 1999; Kidson et al., 1999; Lu et al., 1999; Pressman et al., 2000).

Our laboratory has focused on the requirements for the LIM-homeodomain transcription factor Lmx1b in the proper development of ocular and non-ocular tissues. Lmx1b was first identified as a transcription factor that controls dorsal-ventral patterning during limb development and was subsequently shown to have essential functions in the development of the podocytes of the kidney, the mid-hindbrain organizer, and in specification of specific neuronal subtypes of the CNS (reviewed in (Dai et al., 2009)). In ocular tissues, Lmx1b is expressed in the periocular mesenchyme and expression persists in a subset of adult derivatives of the periocular mesenchyme most prominently the trabecular meshwork, the corneal stroma and endothelium and the anterior iris stroma. Analysis of mice that completely lack Lmx1b function revealed an essential role for Lmx1b in anterior segment development (Pressman et al., 2000). Multiple anterior segment tissues are affected in *Lmx1b* homozygotes, with prominent phenotypic features being decreased anterior chamber thickness, a thin cornea with abnormal arrangement of collagen fibrils, and lack of ciliary body and anterior iris stroma formation. Hence, Lmx1b is an essential factor in anterior segment development and in its absence these tissues either fail to develop properly, likely due to an inability to be specified properly.

These initial studies carried out with the *Lmx1b* null allele were informative in the sense that they showed a critical requirement for Lmx1b activity for proper anterior segment formation. However, since Lmx1b is absent in all tissues, questions related to precisely which tissues require Lmx1b during development could not be addressed using the null allele. Furthermore, since *Lmx1b* null mice die soon after birth (Chen et al., 1998), the role of Lmx1b in postnatal development has not been addressed. Finally, although Lmx1b expression continues in adult tissues, whether that expression is required to maintain anterior segment integrity and to prevent ocular disease has not been examined. To test requirements for Lmx1b in specific tissues during development, and to assay the role of Lmx1b in postnatal ocular development and in the adult, we have employed a conditional allele of *Lmx1b* (Ding et al., 2003) in conjunction with Cre recombinase lines that are active in neural crest cells, in periocular mesenchyme cells, and a tamoxifen-inducible Cre line that allows for normal development and systemic Lmx1b deletion in the adult. Our findings demonstrate that Lmx1b is required in neural crest-derived periocular mesenchyme cells during embryonic development and that expression is required for formation of the trabecular meshwork postnatally. Additionally, we demonstrate that Lmx1b is required in adult tissues to maintain corneal transparency and to prevent corneal neovascularization. Taken together, our results indicate multiple essential functions for Lmx1b in orchestrating anterior segment development and in maintaining proper homeostatic mechanisms in the adult cornea and trabecular meshwork.

Materials and Methods

Animal and genotyping

All studies involving animals were carried out in accordance with regulations of UT MD Anderson Cancer Center Animal Care and Use Committee. To generate Lmx1b-*GFP* mouse strain, an *IRES-GFP-PGKneo* cassette was inserted into a HindIII site of the 3′-UTR of Lmx1b locus by homologous recombination using the same strategy for Lmx1b-*lacZ* allele (Schweizer et al., 2004). Linearized vectors were electroporated into RJ2.2 cells (unpublished). Targeted ES clones were screened by Southern blot with an external probe. Chimeric mice were generated by blastocyst injection (GEMF, UT MD Anderson Cancer Center). All mice were bred on a B6/129 mixed background and housed in a conventional facility. Genotyping was performed by PCR with tail biopsies.

Immunofluorescence

Embryos and adult eyeballs were fixed in 4% paraformaldehyde (PFA) for 15 minutes, cryo-preserved in 30% sucrose, and embedded in OCT for frozen section. Immunofluorescent staining was performed according to standard method. Briefly, slides were washed with PBST (phosphate-buffered saline, 0.1% Triton), blocked with 2% BSA for 1 hour, and incubated with primary antibodies (rabbit GFP (1:1000, Molecular Probes, Invitrogen), mouse β-galactosidase (1:1000, Promega), rabbit NCAM (1:400, Chemicon), mouse monoclonal α-smooth muscle actin-Cy3 (1:500, Sigma)) for 2 hours. After wash with PBST, slides were incubated with fluorescent-conjugated species-specific secondary antibodies (1:400, Molecular Probes, Invitrogen) for 2 hours, washed again with PBST, and counterstained with propidium iodide.

RNA in situ hybridization

In situ hybridization on frozen sections was performed as described (Qiu et al., 2009). To generate Lmx1b homeodomain-specific probe, a 1.8 kb genomic DNA fragment encoding the homeodomain of Lmx1b was subcloned into Bluescript and in vitro transcribed according to manufacturer's instructions (Roche, Mannheim, Germany). Riboprobes for *Keratocan* and *Myocilin* were as described (Kim et al., 1999, Pressman et al., 2000). *Opticin* riboprobe was in vitro transcribed using a linearized EST clone (IMAGE: 40104738).

Histology

Adult kidneys and eyes of newborn mice were fixed in 4% PFA overnight at 4 °C and processed for paraffin embedding. Tissue block was sectioned at 7 μm. Sections were dewaxed in xylene, rehydrated and stained with hematoxylin and eosin (H&E). Eyes of P10 or older mice were fixed 4% PFA and processed for 1.5 μm semi-thin plastic section as described (Kim et al., 1999). Slides were stained with 0.1% toluidine blue solution for 5-15 minutes, de-stained with ddH_2O, baked dry, and mounted with permount medium.

Quantitative analysis of pupil shape irregularity, microphthalmia and small lens

Top-view image of newborn eyeballs (with pupil facing upward) were taken with a Zeiss imaging system. On the image, the center point of the eyeball was used as the center of a circle, and 12 equally spaced lines were drawn starting from the center to measure the radius of the pupil. For each eyeball, 12 values of the radius were obtained and calculated for standard deviation, which reflected the regularity of the pupil shape. The averages of standard deviations were compared between wild-type and mutant. At least 8 eyeballs from each group were measured.

To quantify microphalmia and small lens, 12 wild-type and 12 mutant eyes from P21 animals were enucleated and widths of each lens and eyeball were measured. Ratio of lens/eye width was calculated. Statistical significance was calculated by t-test.

Tamoxifen administration and X-gal staining

Tamoxifen (T-5648, Sigma) was administered by either intraperitoneal (IP) injection or eye drops. For IP injection, tamoxifen was dissolved in sunflower oil to a concentration of 10 mg/ml and injected into *CAGGs-CreER(T2); R26R* adult mice at the amount of 3 or 7 mg/40 g body weight for 5 consecutive days. Eyeballs were enucleated 5 days after the last injection and subject to X-gal staining. For eye drops administration, tamoxifen was dissolved in sunflower oil at 5 mg/ml and 15 μl drops were dropped onto the lower eyelids of each eyeball 3 times a day for 5 days. Eyes of treated mice were subject to X-gal staining 5 days later. To delete Lmx1b in *CAGGs-CreER(T2);*Lmx1b$^{neo/fl}$ adult, 6 mg/40g of tamoxifen was injected for 5 consecutive days, and eyeballs and kidneys were harvested in about 1 month for immunostaining, TEM and histological analysis.

X-gal staining of whole mount embryos and tissue sections was carried out as described (Schweizer et al., 2004). Slides were counterstained with nuclear fast red (Vector Laboratories, Berlingame, CA).

Transmission electron microcopy (TEM)

TEM procedures were performed as described (Pressman et al., 2000). Micrographs were taken on a JEOL JEM-1200 EX electron microscope by Kenneth Dunner Jr. (UT MD Anderson Cancer Center Electron Microscopy Core facility).

Results

Lmx1b is expressed in neural crest-derived periocular mesenchyme

Our previous analysis of Lmx1b expression in murine ocular tissues by radioactive in situ hybridization (Pressman et al., 2000) indicated that it is initially transcribed in the periocular mesenchyme beginning around E10.5 and that this expression continues largely unchanged until E15.5 whereupon expression is maintained in the anterior periocular mesenchyme and lost from the posterior periocular mesenchyme. Subsequently, by postnatal day 21, Lmx1b expression is further restricted to the trabecular meshwork, corneal stroma and endothelium, and anterior iris stroma.

To further analyze the onset and localization of Lmx1b expression in ocular tissues we employed a nuclear-localized *lacZ* knock-in allele (Schweizer et al., 2004) that allows for greater sensitivity and cellular resolution over radioactive in situ hybridization. The Lmx1b-*lacZ* knock-in allele faithfully recapitulates Lmx1b expression in ocular tissues as evidenced by the concordance of lacZ activity and transcript localization (cf. Supplementary Fig. S1 this report and Pressman et al. (2000)). Consistent with previous reports, Lmx1b transcription (as assayed by lacZ activity) is first detected in mesenchyme posterior to the optic cup at E10.0-E10.5 and lacZ activity intensifies over the next day so that by E11.5 intense lacZ staining is visible throughout the entire periocular mesenchyme. This staining pattern persists until E15.5 whereupon lacZ activity is progressively lost in the posterior periocular mesenchyme. Using the *Lmx1b-lacZ* allele we also extended our studies to examine postnatal expression of Lmx1b and our results indicate that Lmx1b expression is maintained in the trabecular meshwork, corneal stroma and endothelium, and anterior iris stroma in adult mice at least to postnatal day 40, the latest stage examined.

The periocular mesenchyme receives dual contributions from cranial mesoderm and neural crest cells (Evans and Noden, 2006; Gage et al., 2005; Johnston et al., 1979). As a first step to determine whether Lmx1b is expressed in one or both of these tissue compartments we compared the lacZ activity from the Lmx1b knock-in allele to the neural crest component of the periocular mesenchyme in age-matched tissue sections. The neural crest component of the periocular mesenchyme was marked according to (Gage et al., 2005) using *Wnt1-Cre* (Danielian and McMahon, 1998) in conjunction with the *R26R-lacZ (R26R)* reporter (Soriano, 1999). In bigenic animals robust lacZ activity is detected in periocular mesenchyme tissues as well as a much broader region of cranial mesenchyme at E10.5 (Supplementary Fig. S1), the earliest stage examined in this study. This broad pattern of staining that reflects neural crest contribution to ocular tissues persists into late stages of embryogenesis and into the adult and is consistent with that previously reported (Gage et al., 2005). In contrast, Lmx1b is consistently expressed in a subset of neural crest-derived

mesenchyme in ocular tissues. Hence, these results are consistent with Lmx1b being expressed in neural crest-derived ocular tissues during development and in the adult.

While comparison of neural crest localization and Lmx1b expression in age-matched sections of ocular tissues suggests that some or perhaps a majority of Lmx1b expressing cells are neural crest-derived, these experiments cannot establish whether all cells that express Lmx1b are neural crest-derived or whether a portion of Lmx1b expressing cells are derived from non-neural crest progenitors such as the cranial mesoderm. To address this question we first built reagents that would allow for simultaneous detection of Lmx1b and neural crest cells. To that end we introduced an *IRES-GFP* cassette into the 3′ UTR of *Lmx1b*, employing the same strategy used for generation of the *Lmx1b-lacZ* allele (Schweizer et al., 2004). GFP protein expressed in the Lmx1b pattern within the periocular mesenchyme can be readily detected by fluorescent antibody staining using highly specific anti-GFP antibodies. Using tissue from triple transgenic mice harboring the *Lmx1b-GFP*, *Wnt1-Cre*, and *R26R* alleles, we were able to co-localize GFP and lacZ activities (Supplementary Fig. S2). Since the ß-galactosidase produced from the recombined *R26R* allele and the GFP produced from the *Lmx1b-GFP* allele are both cytoplasmic, overlap is indicated by yellow fluorescence whereas non-overlap is indicated either by green for GFP or red for lacZ. Careful examination of multiple sections from E11.5 (Supplementary Fig. S2) and E13.5 dpc (data not shown) embryos demonstrates that a majority of cells positive for lacZ expression are positive for GFP in the anterior periocular mesenchyme suggesting that most of these cells are neural crest-derived, although we cannot rule out the possibility that there are a limited number of Lmx1b-positive periocular mesenchymal cells that are not derived from the neural crest. In contrast, some mesenchymal cells in the posterior periocular mesenchyme label for GFP, but not for lacZ. This is especially apparent in the vicinity of the developing periocular muscles where Lmx1b-GFP expressing cells appear to contribute to muscle connective tissue that is derived from the cranial mesoderm (Supplementary Fig. 2D-I). Hence, neural crest contributes to most Lmx1b expressing cells in the anterior periocular mesenchyme, and to many, but not all, of the Lmx1b expressing cells in the posterior periocular mesenchyme.

Lmx1b activity is required in neural crest-derived periocular mesenchyme

After establishing that Lmx1b is expressed in the neural crest component of the periocular mesenchyme we sought to address whether its activity was required in that tissue to direct proper development of periocular mesenchyme derivatives. To accomplish this objective we made use of a previously generated conditional allele at the *Lmx1b* locus where the homeodomain is flanked by *loxP* sites (Ding et al., 2003). Recombination is confined to neural crest-derived cells by using *Wnt1-Cre* (Jiang et al., 2000) in conjunction with the *Lmx1b* conditional allele. Initially to ascertain whether *Wnt1-Cre* is effective in removal of Lmx1b in periocular mesenchymal tissues, we compared the expression pattern of Lmx1b in wild-type embryos with Lmx1b expression in embryos that contained *Wnt1-Cre* and were compound heterozygous for the *Lmx1b* conditional and null alleles (*Lmx1bneo*) using a probe derived from the presumptive deleted region (Supplementary Fig. S3). Although this probe is significantly less effective in detection of *Lmx1b* transcripts as compared to a full-length probe (data not shown) transcripts can be readily detected in wild-type periocular

mesenchyme and CNS of E11.5 embryos. In contrast *Lmx1b* conditional mutants lack detectible staining in the periocular mesenchyme, while retaining expression in non-ocular tissues such as the CNS. Hence, *Wnt1-Cre* is effective in deleting Lmx1b specifically in neural crest-derived periocular mesenchyme.

To determine the consequences of removal of Lmx1b in neural crest-derived periocular mesenchyme we initially compared the phenotypes of conditional deletion to the *Lmx1b* null mutant and to wild-type embryos at a gross and histological level (Fig. 1). In E18.5 whole eyes that completely lack Lmx1b activity a reduction of size relative to wild-type is clearly evident as well as a characteristic irregular pupil, indicative of abnormal iris development. The conditional mutant eye exhibits a similar, although not identical phenotype. To better quantify the iris irregularity in the wild-type and conditional mutant embryos, we examined the standard deviation from the twelve equally-spaced diagonals (Fig. 2). The length of diagonals from wild-type eyes showed little variation while diagonals from null mutants exhibited considerable variation. The conditional null mutants exhibited a phenotype intermediate to that of wild-type and the null.

The similarity of null and conditional phenotypes extends to the histological level where the null mutant displays several significant anterior segment defects, including stromal iris and ciliary body hypoplasia, reduction of depth of the anterior chamber, and less dense stromal component of the cornea (Fig. 1). Additionally, in both the null and conditional mutants, there is a thickening of the lens epithelium (white arrows in Fig. 1, K, L) and presence of blood vessels in the corneal stroma (black arrows in Figure 1, H, I). The histological phenotypes of the null and conditional mutant are identical at our level of resolution. To further compare the null and conditional phenotypes, we examined the expression of Keratocan, a heparin-sulfate glycoprotein, that we had previously shown depends on Lmx1b expression (Pressman et al., 2000). Consistent with the histological comparisons, Keratocan expression is absent from the conditional mutants in a manner identical to that of the null mutants (Fig. 3). To gain insight into the integrity of the corneal endothelium of null and conditional mutants, we examined the expression of NCAM by immunofluorescence. NCAM is normally present in the corneal endothelium and lens epithelium but is absent in corneal tissues of *Lmx1b* null and conditional mutants (Fig. 3). Also apparent in these sections is the narrow depth of the anterior chamber in both null and conditional mutants. Taken together, comparison of the gross, histological, and molecular phenotypes in ocular tissues in embryos that lack Lmx1b specifically in the neural crest-derived periocular mesenchyme to the phenotype of ocular tissues that completely lack Lmx1b activity are highly similar and/or identical indicating that Lmx1b activity is required in neural crest-derived periocular mesenchyme for proper anterior segment development.

Lmx1b is not required for proper migration of periocular mesenchyme cells

In principle, several factors could contribute to the hypoplastic anterior segment defects that we observe in both *Lmx1b* null and neural crest-specific conditional deletion mutants. Decreased proliferation and/or increased apoptosis of the periocular mesenchyme are attractive possibilities to explain the observed phenotypes. However, since previous studies failed to reveal any obvious differences in proliferation and apoptosis within the periocular

mesenchyme of Lmx1bnull mutants relative to the wild-type, it is unlikely that any difference would be observed the *Wnt1-Cre* conditional null mutants. Another possibility is that Lmx1b is essential for proper migration of neural crest cells, especially to the anterior portion of the periocular mesenchyme. The availability of *Wnt1-Cre* and *R26R* lines enabled us to address this question by following the fate of neural crest cells in an *Lmx1b* conditional null background (Supplementary Fig. S4). No difference was observed between neural crest contribution to ocular tissues in wild-type and conditional null mutants suggesting that a gross defect in neural crest migration is unlikely to cause the observed hypoplasia in periocular mesenchyme-derived tissues in Lmx1b mutants.

Lmx1b is required for trabecular meshwork formation

The trabecular meshwork is a fenestrated endothelial-like tissue situated at the intersection of the cornea and the iris. The main function of the trabecular meshwork is to provide drainage for the aqueous humor. Although the trabecular meshwork is known to derive from neural crest cells, there is little known about genetic and developmental programs that direct its formation and differentiation. Since the trabecular meshwork forms in the mouse postnatally, being first detected morphologically at around two weeks after birth (Smith et al., 2001), it has not previously been possible to address requirements for genes that when mutated result in embryonic or perinatal lethality. To circumvent perinatal lethality in the case of Lmx1b, we used a knock-in line at the *Pitx2* locus (Liu et al., 2002) allowing for deletion of Lmx1b in periocular mesenchyme while preserving expression in the CNS. Expression of Lmx1b and Pitx2 overlap in the periocular mesenchyme (Supplementary Fig. S5) and this strategy should be effective in removal of *Lmx1b* transcripts during the embryonic period. Conditional deletion of Lmx1b in periocular mesenchyme was confirmed by in situ hybridization (Supplementary Fig. S6). Mice that were heterozygous for *Pitx2-Cre* and compound heterozygotes for the *Lmx1bneo* and *Lmx1b* floxed alleles (*Pitx2-Cre;Lmx1b*$^{neo/fl}$) were born at expected Mendelian frequencies and exhibited normal growth in the perinatal period. Gross examination of ocular tissues from P13 mice just prior to eye opening revealed irregularly shaped pupil and corneal opacity (Fig. 4). At three weeks of age, about 25% of the mutants developed microphthalmia (data not shown). These defects are accompanied by a modest (approximately 20%) reduction in lens size relative to overall eye size in the conditional mutants (Supplementary Fig. S7). Further histological examination indicated a hypoplastic iris associated with angle closure, thickened lens epithelium and a notable reduction of corneal endothelial tissue. Immunofluorescence for α-smooth muscle actin indicated absence of sphincter and dilator muscles in mutant iris (Fig. 5). The ciliary epithelium was normal as assayed by NCAM immunostaining and *opticin* mRNA expression, although the ciliary body was not detectible in mutant tissues. Trabecular meshwork tissue was absent at the light microscopy level and this observation was confirmed by in situ hybridization to *Myocilin*, an extracellular matrix protein abundantly produced by the murine trabecular meshwork (Fig. 5). In contrast to wild-type animals, Myocilin expression was absent in angle tissues from *Pitx2-Cre;Lmx1b*$^{neo/fl}$ mutants. Hence, Lmx1b is required in periocular mesenchyme tissues for proper formation of the trabecular meshwork.

Lmx1b is required in the adult to maintain corneal transparency and to prevent corneal neovascularization

To address whether Lmx1b expression is required in adult tissues, we generated animals that maintained normal expression of Lmx1b in embryonic and perinatal periods, but allowed for removal of Lmx1b in the adult. This was accomplished by breeding *CAGGs-CreER(T2)* mice that contain a ubiquitously expressed fusion of Cre recombinase with a modified form of the estrogen hormone receptor ligand binding region (Hayashi and McMahon, 2002). In the unstimulated state, this fusion protein is retained in the cytoplasm. Administration of tamoxifen results in translocation of the CreER(T2) fusion protein to the nucleus where it can recombine the *Lmx1b* floxed allele thereby generating animals that have reduced or absent Lmx1b expression in an inducible manner. Tamoxifen injection is effective in inducing Cre-mediated recombination in anterior segment tissues and the retina, but is unlikely to approach complete recombination, even at the maximal doses employed (Supplementary Fig. S8). Despite incomplete excision of the floxed *Lmx1b* allele, examination of ocular tissues from *CAGGs-CreER(T2)*; $Lmx1b^{neo/fl}$ animals four weeks after they had been injected with tamoxifen (7mg/40 g body weight) for five consecutive days revealed significant alterations (Fig. 6). Mutant animals had cloudy corneas accompanied by abundant neovascularization. To further examine the consequence of removal of Lmx1b in the adult we compared wild-type and mutant ocular tissues at the light and EM level. Mutant corneas exhibited a thinning of the corneal epithelium, more sparsely distributed corneal keratocytes, and a significant reduction of NCAM immunoreactivity in the corneal endothelium and trabecular meshwork (Fig. 7). Transmission electron microscopy revealed a disorganized organization of collagen fibrils in the mutant accompanied by wavy lamellae (Fig. 8). The orthogonal alignment of collagen fibrils was disrupted in the mutants with more electron lucent space present. The corneal endothelium was present and the thickness of Descemet's membrane was unaltered. Cross sections through collagen fibrils showed a slight increase in fibril diameter and in the interfibrillar spaces. In addition to the ocular defects we observed, mutant animals became lethargic several weeks post-injection. Examination of kidneys from mutant animals revealed a severe degenerative phenotype with dilated tubules filled with eosinophilic material indicating renal failure (Supplementary Fig. S9). Taken together, our observations demonstrate a significant postnatal requirement for Lmx1b in maintaining anterior segment function and in preventing corneal opacification and neovascularization.

Discussion

In this report we have examined the expression and function of Lmx1b within the neural crest-derived component of the periocular mesenchyme. The contribution of the neural crest to Lmx1b expressing periocular mesenchyme cells differs in the anterior and posterior portion of the eye. In the anterior periocular mesenchyme, neural crest cells contribute to most, if not all of the Lmx1b expressing cells, whereas in the posterior periocular mesenchyme there is a population of Lmx1b expressing cells that is not derived from neural crest. These cells appear in and around the nascent periocular muscle masses and probably represent muscle connective tissue. Our studies that employed *Wnt1-cre* mediated conditional deletion of Lmx1b in neural crest-derived tissues suggest that although Lmx1b

may be expressed in both neural crest and to some extent in non-neural crest tissues, its activity is required autonomously in the neural crest-derived component.

Although we have demonstrated an autonomous requirement for Lmx1b in neural crest-derived periocular mesenchyme, this does not preclude interactions between neural crest and cranial mesoderm in the development of the anterior segment. Additionally, we have noted several non-cell autonomous effects of Lmx1b inactivation, including a thickened lens epithelium and micropthalmia. As the development of the anterior segment is regulated by multiple tissue interactions, it is conceivable that these non cell-autonomous effects impact anterior segment development and function. Our current studies have not directly addressed this possibility, but in principle these questions could be addressed further using chimeric embryos composed of mixtures of wild-type and Lmx1b mutant cells.

In both the null and *Wnt1-cre* conditional null mutants, Lmx1b is essential for proper formation of multiple anterior segment tissues. In the case of the corneal stroma, it appears that this tissue is specified properly in that the tissue contains cells that morphologically resemble corneal keratocytes, there are abundant collagen fibrils present, and the tissue is transparent. However, as evidenced by the lack of Keratocan expression, the corneal keratocytes are not functioning properly in the absence of Lmx1b. In contrast, the stromal portion of the iris appears hypoplastic and these cells have either failed to differentiate, undergone an altered migration pattern, or have undergone apoptosis. Additional studies will need to be done to differentiate between these possibilities, but the differences in requirements for Lmx1b in different anterior segment tissues (loss/hyperplasia vs. abnormal function) highlights the possibility that Lmx1b might regulate specification, survival, and differentiation independently in different anterior segment tissues.

A considerable amount of development of the anterior chamber occurs postnatally (Gould et al., 2004). At birth the cornea and iris have formed distinct layers, but their final adult morphologies are not achieved until several weeks later. Other tissues, such as the trabecular meshwork are not present at birth, but begin to differentiate during the second week of postnatal life. Lmx1b is expressed in all of these anterior segment tissues after birth through adulthood, suggesting that Lmx1b might have important functions in regulating their differentiation and/or function during postnatal stages. We have used two approaches to demonstrate essential functions for Lmx1b in the postnatal period. Using *Pitx2-Cre* we have shown that Lmx1b is required for trabecular meshwork formation and using *CAGGs-CreER(T2)* we have shown that Lmx1b is required in the adult for maintenance of corneal transparency and to prevent corneal neovascularization. The molecular mechanisms leading to these phenotypes are not currently known, but likely involve the Lmx1b-dependent transcriptional regulation of genes, including those involved in maintaining normal activities of the corneal endothelium and corneal stroma. Mutant corneas are thicker and the spacing of corneal keratocytes is expanded indicating edema. This may result from an abnormal Descemet’s membrane allowing water to infiltrate the corneal stroma resulting in opacification, inflammation, followed by neovascularization. Further studies will be required to address these issues.

Lmx1b is mutated in individuals with nail patella syndrome (NPS), a congenital disorder that affects the limbs, kidneys, and is associated with an elevated risk of open angle glaucoma (Dreyer et al., 1998; Vollrath et al., 1998). Clinical examination of NPS patients reveals no significant optical anomalies and the angle is open in these individuals (Mimiwati et al., 2006). Hence, it is unlikely that congenital anterior segment dysgenesis plays a significant role in the pathogenesis of glaucoma in NPS. Rather, it is likely that reduced Lmx1b activity contributes to a decreased outflow through the trabecular meshwork thereby elevating intraocular pressure and eventually leading to disease. Although the ocular manifestations of acute elimination of Lmx1b function in adult mice differ from chronic haploinsufficiency in humans, they demonstrate a critical requirement for Lmx1b in maintenance of anterior segment tissues in the adult.

In summary, we have used conditional mutagenesis strategies to determine the spatial and temporal requirements for Lmx1b in ocular development in the mouse. Our findings have extend earlier observations carried out with the Lmx1b null allele that show requirements for Lmx1b in neural crest-derived periocular mesenchyme for proper anterior segment formation, an essential role for Lmx1b in trabecular meshwork formation, and highlight that persistent Lmx1b expression in adult ocular tissues is necessary to maintain corneal transparency and to prevent neovascularization. These are the first studies to define an essential transcription factor required for trabecular meshwork formation and the first to demonstrate a necessary requirement for Lmx1b expression in the adult anterior segment. Future definition of transcriptional targets of Lmx1b during the embryonic, postnatal, and adult periods will yield further insight into the molecular mechanisms that regulate anterior segment development and maintain proper anterior segment function in the adult.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Azar DT. Corneal angiogenic privilege: angiogenic and antiangiogenic factors in corneal avascularity, vasculogenesis, and wound healing (an American Ophthalmological Society thesis). Trans Am Ophthalmol Soc. 2006; 104:264–302. [PubMed: 17471348]

Beebe DC. Maintaining transparency: a review of the developmental physiology and pathophysiology of two avascular tissues. Semin Cell Dev Biol. 2008; 19:125–133. [PubMed: 17920963]

Beebe DC, Coats JM. The lens organizes the anterior segment: specification of neural crest cell differentiation in the avian eye. Dev Biol. 2000; 220:424–431. [PubMed: 10753528]

Breitman ML, Bryce DM, Giddens E, Clapoff S, Goring D, Tsui LC, Klintworth GK, Bernstein A. Analysis of lens cell fate and eye morphogenesis in transgenic mice ablated for cells of the lens lineage. Development. 1989; 106:457–463. [PubMed: 2598819]

Chang B, Smith RS, Peters M, Savinova OV, Hawes NL, Zabaleta A, Nusinowitz S, Martin JE, Davisson ML, Cepko CL, Hogan BL, John SW. Haploinsufficient Bmp4 ocular phenotypes include

anterior segment dysgenesis with elevated intraocular pressure. BMC Genet. 2001; 2:18. [PubMed: 11722794]

Chen H, Lun Y, Ovchinnikov D, Kokubo H, Oberg KC, Pepicelli CV, Gan L, Lee B, Johnson RL. Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. Nat Genet. 1998; 19:51–55. [PubMed: 9590288]

Dai JX, Johnson RL, Ding YQ. Manifold functions of the Nail-Patella Syndrome gene Lmx1b in vertebrate development. Dev Growth Differ. 2009; 51:241–250. [PubMed: 19222527]

Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. Curr Biol. 1998; 8:1323–1326. [PubMed: 9843687]

Ding YQ, Marklund U, Yuan W, Yin J, Wegman L, Ericson J, Deneris E, Johnson RL, Chen ZF. Lmx1b is essential for the development of serotonergic neurons. Nat Neurosci. 2003; 6:933–938. [PubMed: 12897786]

Dreyer SD, Zhou G, Baldini A, Winterpacht A, Zabel B, Cole W, Johnson RL, Lee B. Mutations in LMX1B cause abnormal skeletal patterning and renal dysplasia in nail patella syndrome. Nat Genet. 1998; 19:47–50. [PubMed: 9590287]

Edelhauser HF. The balance between corneal transparency and edema: the Proctor Lecture. Invest Ophthalmol Vis Sci. 2006; 47:1754–1767. [PubMed: 16638979]

Evans DJ, Noden DM. Spatial relations between avian craniofacial neural crest and paraxial mesoderm cells. Dev Dyn. 2006; 235:1310–1325. [PubMed: 16395689]

Flugel-Koch C, Ohlmann A, Piatigorsky J, Tamm ER. Disruption of anterior segment development by TGF-beta1 overexpression in the eyes of transgenic mice. Dev Dyn. 2002; 225:111–125. [PubMed: 12242711]

Gage PJ, Camper SA. Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. Hum Mol Genet. 1997; 6:457–464. [PubMed: 9147650]

Gage PJ, Rhoades W, Prucka SK, Hjalt T. Fate maps of neural crest and mesoderm in the mammalian eye. Invest Ophthalmol Vis Sci. 2005; 46:4200–4208. [PubMed: 16249499]

Gage PJ, Suh H, Camper SA. Dosage requirement of Pitx2 for development of multiple organs. Development. 1999; 126:4643–4651. [PubMed: 10498698]

Gould DB, Smith RS, John SW. Anterior segment development relevant to glaucoma. Int J Dev Biol. 2004; 48:1015–1029. [PubMed: 15558492]

Hay ED. Development of the vertebrate cornea. Int Rev Cytol. 1979; 63:263–322.

Hayashi S, McMahon AP. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol. 2002; 244:305–318. [PubMed: 11944939]

Idrees F, Vaideanu D, Fraser SG, Sowden JC, Khaw PT. A review of anterior segment dysgeneses. Surv Ophthalmol. 2006; 51:213–231. [PubMed: 16644364]

Ittner LM, Wurdak H, Schwerdtfeger K, Kunz T, Ille F, Leveen P, Hjalt TA, Suter U, Karlsson S, Hafezi F, Born W, Sommer L. Compound developmental eye disorders following inactivation of TGFbeta signaling in neural-crest stem cells. J Biol. 2005; 4:11. [PubMed: 16403239]

Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cardiac neural crest. Development. 2000; 127:1607–1616. [PubMed: 10725237]

Johnston MC, Noden DM, Hazelton RD, Coulombre JL, Coulombre AJ. Origins of avian ocular and periocular tissues. Exp Eye Res. 1979; 29:27–43. [PubMed: 510425]

Kidson SH, Kume T, Deng K, Winfrey V, Hogan BL. The forkhead/winged-helix gene, Mf1, is necessary for the normal development of the cornea and formation of the anterior chamber in the mouse eye. Dev Biol. 1999; 211:306–322. [PubMed: 10395790]

Kim BS, Savinova OV, Reedy MV, Martin J, Lun Y, Gan L, Smith RS, Tomarev SI, John SW, Johnson RL. Targeted Disruption of the Myocilin Gene (Myoc) Suggests that Human Glaucoma-Causing Mutations Are Gain of Function. Mol Cell Biol. 2001; 21:7707–7713. [PubMed: 11604506]

Liu C, Liu W, Palie J, Lu MF, Brown NA, Martin JF. Pitx2c patterns anterior myocardium and aortic arch vessels and is required for local cell movement into atrioventricular cushions. Development. 2002; 129:5081–5091. [PubMed: 12397115]

Lu MF, Pressman C, Dyer R, Johnson RL, Martin JF. Function of Rieger syndrome gene in left-right asymmetry and craniofacial development. Nature. 1999; 401:276–278. [PubMed: 10499585]

Matt N, Dupe V, Garnier JM, Dennefeld C, Chambon P, Mark M, Ghyselinck NB. Retinoic acid-dependent eye morphogenesis is orchestrated by neural crest cells. Development. 2005; 132:4789–4800. [PubMed: 16207763]

Matt N, Ghyselinck NB, Pellerin I, Dupe V. Impairing retinoic acid signalling in the neural crest cells is sufficient to alter entire eye morphogenesis. Dev Biol. 2008; 320:140–148. [PubMed: 18539269]

Mimiwati Z, Mackey DA, Craig JE, Mackinnon JR, Rait JL, Liebelt JE, Ayala-Lugo R, Vollrath D, Richards JE. Nail-patella syndrome and its association with glaucoma: a review of eight families. Br J Ophthalmol. 2006; 90:1505–1509. [PubMed: 16825280]

Okada Y, Saika S, Shirai K, Hashizume N, Yamanaka O, Ohnishi Y, Senba E. Disappearance of desmosomal components in rat corneal epithelium during wound healing. Ophthalmologica. 2001; 215:61–65. [PubMed: 11125272]

Pressman CL, Chen H, Johnson RL. LMX1B, a LIM homeodomain class transcription factor, is necessary for normal development of multiple tissues in the anterior segment of the murine eye. Genesis. 2000; 26:15–25. [PubMed: 10660670]

Qiu Q, Chen H, Johnson RL. Lmx1b-expressing cells in the mouse limb bud define a dorsal mesenchymal lineage compartment. Genesis. 2009; 47:224–233. [PubMed: 19298015]

Saika S, Liu CY, Azhar M, Sanford LP, Doetschman T, Gendron RL, Kao CW, Kao WW. TGFbeta2 in corneal morphogenesis during mouse embryonic development. Dev Biol. 2001; 240:419–432. [PubMed: 11784073]

Schweizer H, Johnson RL, Brand-Saberi B. Characterization of migration behavior of myogenic precursor cells in the limb bud with respect to Lmx1b expression. Anat Embryol (Berl). 2004; 208:7–18. [PubMed: 15007643]

Smith RS, Zabaleta A, Savinova OV, John SW. The mouse anterior chamber angle and trabecular meshwork develop without cell death. BMC Dev Biol. 2001; 1:3. [PubMed: 11228591]

Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999; 21:70–71. [PubMed: 9916792]

Vollrath D, Jaramillo-Babb VL, Clough MV, McIntosh I, Scott KM, Lichter PR, Richards JE. Loss-of-function mutations in the LIM-homeodomain gene, LMX1B, in nail-patella syndrome. Hum Mol Genet. 1998; 7:1091–1098. [PubMed: 9618165]

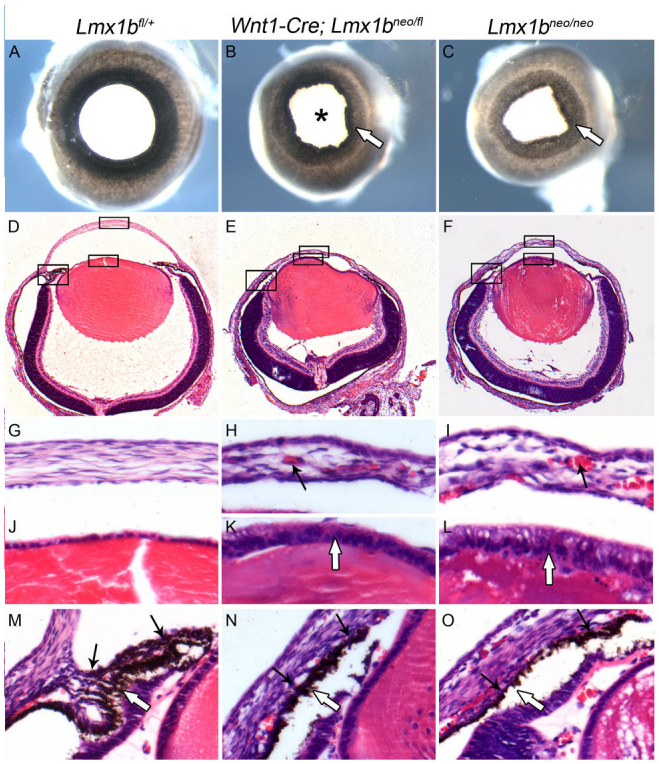

Figure 1. *Wnt1-Cre* conditional null mutants display multiple defects in ocular anterior segment

(A-C) Whole mount view of E18.5 eyes demonstrates iris hypoplasia (B, C arrows) and irregularlly shaped pupil (B asterisk) in mutants. H&E stained sections (boxed areas in D to F are enlarged in G to O) of conditional mutants show reduced depth of anterior chamber, vascularized cornea (blood cells indicated by arrows in H, I), and less dense corneal stroma as compared to wild-type cornea. Lens epithelium is thickened and lens epithelial cells are elongated in shape (K, L arrows). Presumptive iris and ciliary body stroma are hypoplastic (N, O arrows), and ciliary processes (N, O white arrow) are not present in mutants.

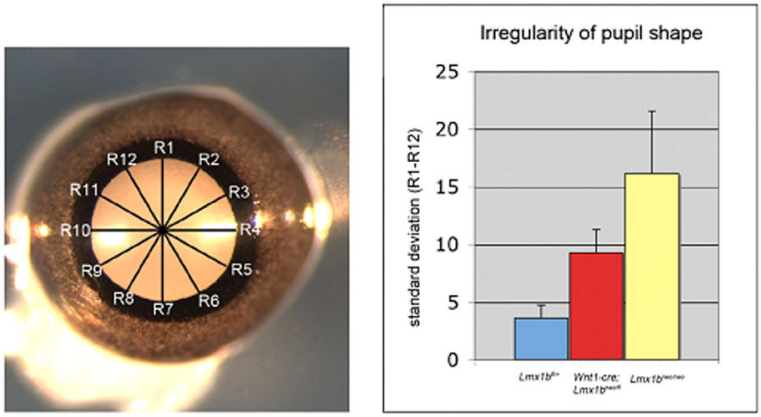

Figure 2. Quantification of irregularity of pupil shape in *Wnt1-Cre* conditional mutants

Irregularity of pupil shape is quantified by calculating the standard deviation of 12 radii (R1-R12) of a pupil. The angle between neighboring radii is 30°. For each group four mice (8 eyeballs) were analyzed.

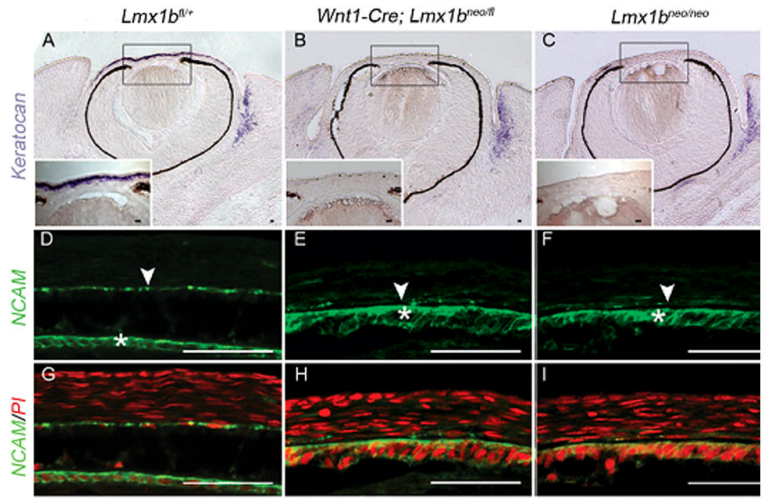

Figure 3. Absence of Keratocan and NCAM in the cornea of *Wnt1-Cre* conditional mutants

Section in situ hybridization shows that ECM molecule Keratocan is expressed in the developing cornea of wild-type at E15.5 (A), whereas no transcript is detected in the conditional mutants (B) and *Lmx1b*$^{neo/neo}$ (C). Boxed areas in A to C are enlarged in the insets. Adhesion molecule NCAM is expressed in wild-type corneal endothelium (D, arrowhead) and lens epthithelium (D, asterisk) at E18.5, but absent in the corneal endothelium of conditional and null mutants (E and F, arrowheads). NCAM immunostaining is counterstained with propidium iodide (G to I). Scale bar, 50 μm.

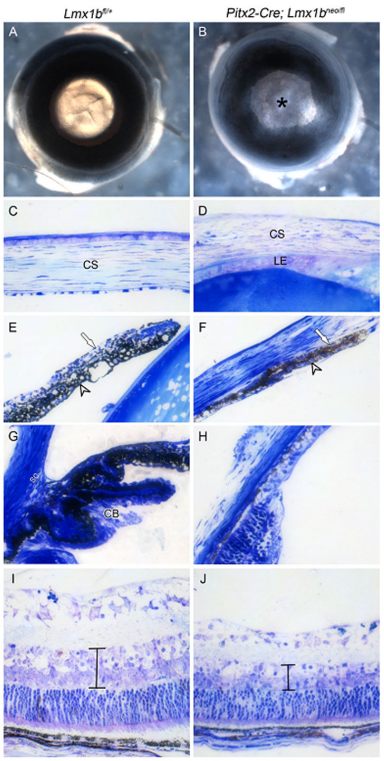

Figure 4. Deletion of Lmx1b in the periocular mesenchyme leads to multiple phenotypes in ocular anterior segment

(A, B) Whole mount view of P13 eyes demonstrates that mutant cornea is opacified and the shape of pupil is irregular (B, asterisk). Toluidine blue staining of plastic sections shows corneal stroma (CS) is edematous and corneal endothelium may be absent. Cornea and lens epithelium are attached (D), and lens epithelium (LE) is substantially thickened. Iris stroma is atrophic (F, arrow) and iris pigmented epithelium is flattened (F, arrowhead). Ciliary processes are completely absent. In addition, the angle is closed due to anterior synechia and no trabecular meshwork or Schlemm's canal (SC) can be detected (G, H). Secondarily, the inner nuclear layer of developing retina of mutants is thinner in comparison with wild-type indicated by scale bar in I and J. Retinal pigmented epithelium and sclera appear to be normal.

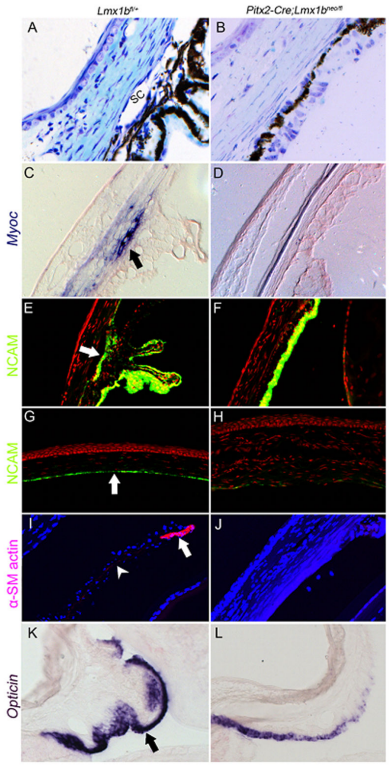

Figure 5. Altered expression of molecular markers for anterior segment in *Pitx2-Cre* conditional mutants

(A, B) Semi-thin plastic sections of P30 eye were stained with toluidine blue. No structures of trabecular meshwork or Schlemm's canal can be identified in mutant eyes, as compared with control (A, arrow). Marker of trabecular meshwork *Myocilin* is expressed abundantly in the wild-type (C), but absent in the mutant (D). NCAM protein is present in the wild-type trabecular meshwork (E arrow) and corneal endothelium (G arrow), but not in the mutant (F and H). α-smooth muscle actin stains wild-type iris sphincter muscle (I arrow) and dilator muscle (I arrowhead). Opticin transcript is detected in non-pigmented ciliary epithelium (K arrow) by in situ hybridization, however, the expression level is much lower in the mutant (L).

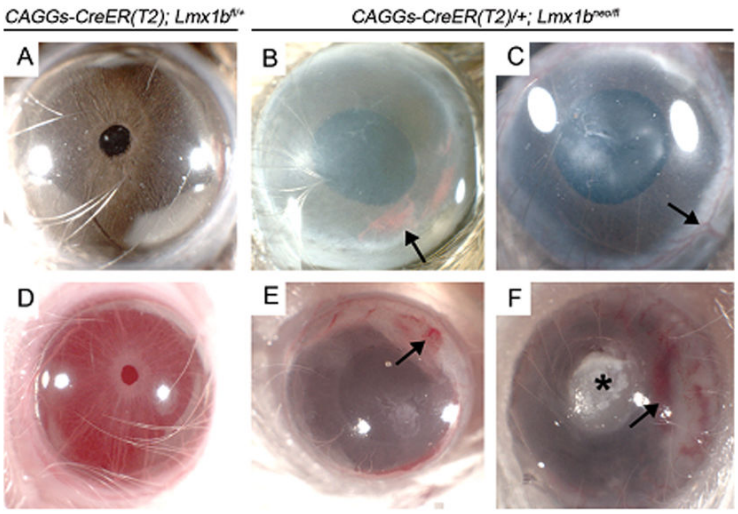

Figure 6. Global inactivation of Lmx1b in adult mice results in phenotypic changes in the cornea

CAGGsCre-ER(T2); Lmx1b$^{neo/fl}$ mice were injected with tamoxifen (7 mg/40g body weight) and sacrificed four weeks post-injection. Conditional deleted mutants exhibit corneal opacification (B, C, E, F), neovascularization (arrows) and local mineralization (F, asterisk).

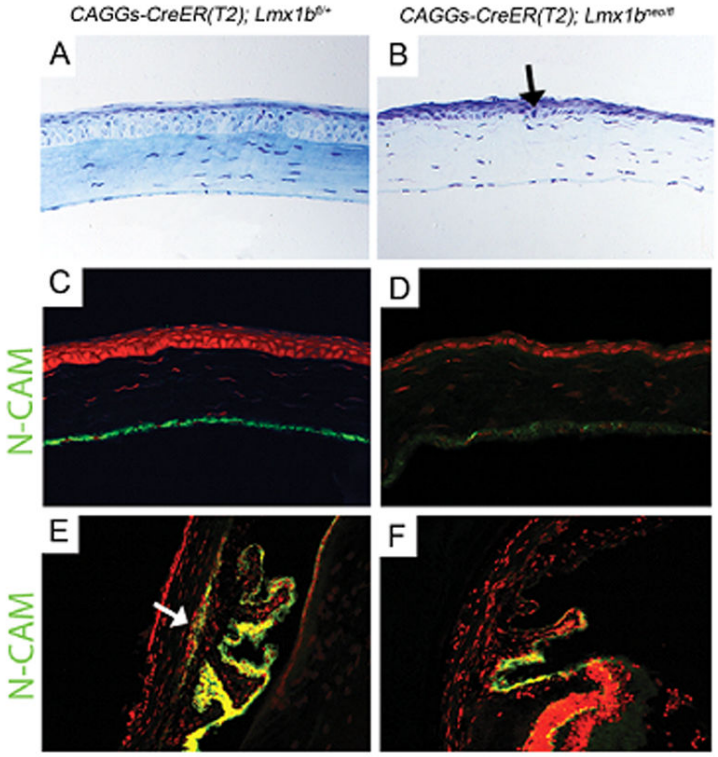

Figure 7. Alterations in the cornea at histological and molecular level upon deletion of Lmx1b in adult

(A, B) Eyes of 2-month-old *CAGGsCre-ER(T2); Lmx1b*$^{neo/fl}$ mice injected with tamoxifen were sectioned and stained with toluidine blue. Mutant cornea has thinner epithelium (B, arrow) and more sparsely distributed keratocytes. (C to F) Frozen sections were immunostained for NCAM. No NCAM is detected in the corneal endothelium (H) or trabecular meshwork (J) in the mutant

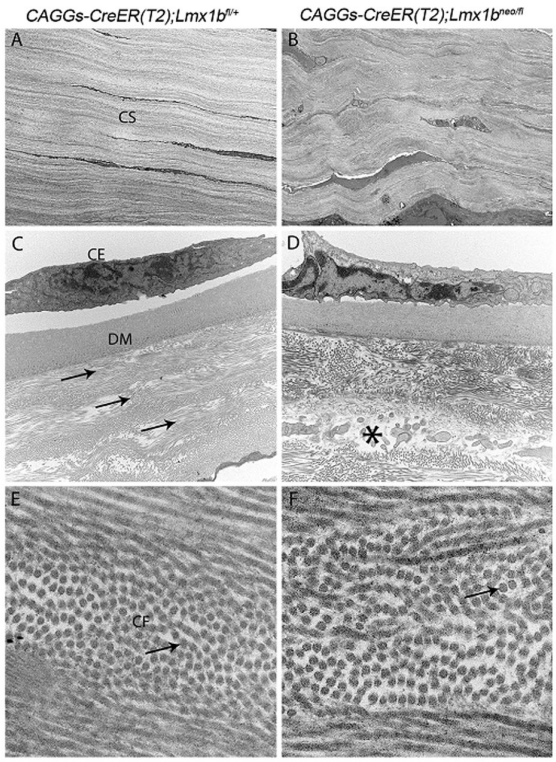

Figure 8. Disrupted ultrastructure in the cornea of *CAGGs-CreER(T2)* conditional mutant

Transmission electron microscopy reveals disturbed organization of collagen fibrils in the mutant shown by the wavy lamellae (B 5000x). The orthogonal alignment of collagen fibrils (C, arrows) is disrupted in the mutant and more electron lucent space is present in the mutant stroma (D, asterisk). Corneal endothelium is present and the thickness of Descemet membrane is not affected in the mutant (C, D 10000x). Cross sections through collagen fibrils show slight increase of the diameter of fibrils and interfibril spaces (E, F 100000x). CS, corneal stroma; CE, corneal endothelium; DM, Descemet membrane; CF, collagen fibril.