AP-2β Is a Downstream Effector of PITX2 Required to Specify Endothelium and Establish Angiogenic Privilege During Corneal Development

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Purpose. The homeodomain transcription factor, PITX2, is at the apex of a genetic pathway required for corneal development, but the critical effector genes regulated by the PITX2 remain unknown. The purpose of this study was to discover and validate PITX2-dependent mechanisms required for specifying cell lineages and establishing angiogenic privilege within the developing cornea.

Methods. Microarrays were used to compare gene expression in corneas isolated from temporal *Pitx2* knockout embryos and control littermates. Quantitative RT-PCR and immunohistochemistry was used to further validate *Tfap2b* expression differences in *Pitx2* knockout versus control corneas. In situ hybridization and protein immunohistochemistry were used to assay eyes of a *Tfap2b* allelic series of embryos to identify differentiated cellular lineages in the cornea, blood vessel endothelium, or lymphatic vessel endothelium.

Results. We show that PITX2 is required for the expression of *Tfap2b*, encoding the AP-2β transcription factor, in the neural crest during corneal development. Markers of differentiated corneal epithelium and stroma are expressed in the absence of AP-2β. In contrast, markers of differentiated corneal endothelium are not expressed in the absence of AP-2β. Endomucin$^+$ blood vessels are present throughout the developing corneal stroma in the absence of AP-2β, whereas LYVE1$^+$ lymphatic vessels are not found.

Conclusions. The AP-2β transcription factor is an important effector of PITX2 function during corneal development, required for differentiation of corneal endothelium and establishment of angiogenic privilege. Unlike PITX2, AP-2β is not required for the early expression of available lineage specific markers for the corneal epithelium and stroma during embryogenesis, nor establishment of lymphangiogenic privilege. Therefore, additional PITX2-dependent factors likely regulate these latter processes during embryonic development. These results extend our understanding of the genetic mechanisms regulating cornea development.

Keywords: homeodomain transcription factor, neovascularization, genetic network

The cornea is an essential tissue required for vision. The properties of the cornea include transparency, refraction, and protection of inner components of the eye from external environmental factors. The mature cornea consists of three cellular layers—a 6- to 7-cell thick corneal epithelium that comprises the outer surface, a monolayer of endothelium that forms the inner surface, and a stroma layer that lies between the epithelium and the endothelium—and a highly ordered lamellae of keratocytes and specialized collagen bundles.[1–3] The endothelium is the first mature corneal lineage to differentiate, and it arises from migrating neural crest stem cells as they contact the epithelium of the anterior lens. Subsequently, neural crest stem cells migrate between the endothelium and the overlying surface ectoderm, where they compact and adopt an ordered, lamellar arrangement to form the corneal stroma.[4–6] Subsequently, the overlying ocular surface ectoderm differentiates into corneal epithelium in response to signaling from the underlying mesenchyme.[7] Understanding the molecular mechanisms that control cell fate decisions and gene expression in each corneal lineage during embryogenesis is likely to enhance the development of new and improved strategies for treating corneal diseases.

Normal vision also requires the complete absence of blood and lymphatic vessels from the cornea, states referred to as angiogenic and lymphangiogenic privilege, respectively. A loss of these states as a consequence of developmental abnormalities or postnatal insult results in corneal neovascularization, a sight-threatening condition.[8–10] Blood vessels never appear in the developing cornea, indicating that angiogenic and lymphangiogenic privilege are established early during corneal

Investigative Ophthalmology & Visual Science

morphogenesis. Apart from the recent demonstration that in humans and mice *FOXC1/Foxc1* is required to prevent vessel growth during cornea development, the mechanisms required for establishing angiogenic and lymphangiogenic privilege remain unknown.[11] Therefore, a detailed understanding of how angiogenic privilege is initially established during development, and subsequently preserved in the mature cornea, is important for expanding our knowledge of corneal development and adult corneal homeostasis and for identifying new, more effective therapies to treat corneal neovascularization.

Heterozygous mutations in human *PITX2* cause Axenfeld-Rieger syndrome, which includes dysgenesis of multiple anterior segment structures within the eye and a significant risk for glaucoma.[12] In mice and humans, central corneal thickness is sensitive to the *Pitx2/PITX2* gene dose.[13] The PITX2 transcription factor is present in the neural crest stem cells that give rise to the corneal stroma and endothelium prior to the initiation of corneal development and is required for differentiation of the corneal epithelium, stroma, and endothelium.[4,14] PITX2 is also required to establish angiogenic and lymphangiogenic privilege in the developing cornea.[14] The activation of *Dkk2* and the resulting suppression of canonical Wnt signaling activity is one essential mechanism by which PITX2 regulates specification of all three layers in the developing cornea.[14] However, additional downstream effectors are likely required. PITX2 is also required to establish angiogenic and lymphangiogenic privilege, however, the mechanism(s) by which this occurs are unknown.

The activating protein-2 (AP-2) transcription factors are a developmentally important family of genes that have been shown to play important roles in eye development.[15–21] *Tfap2a* is expressed in the corneal epithelium and is required for normal differentiation during development and repair in the adult.[22,23] *Tfap2b*, which encodes AP-2β, one of a highly related family of transcription factors that bind to specific DNA sequences as homo- or heterodimers, is expressed in the neural crest during corneal development.[16,21] AP-2 proteins can function as either transcriptional activators or repressors depending on context[24] and have been associated with both suppressing and promoting angiogenesis. For example, a loss of AP-2α permits angiogenesis and metastasis in cancers such as nasopharyngeal carcincoma and melanoma, whereas the overexpression of AP-2β promotes angiogenesis in lung adenocarcinomas.[25,26] Whether these opposing effects represent a true functional divergence between the two proteins or result instead from contextual differences between the different tumor types has not been established. Although encoded by different genes, members of the AP-2 protein family are known to have overlapping expression patterns and functional redundancy.[16] However, to date, AP-2β has not been associated with repressing angiogenesis in any system. In addition, a role for AP-2β in differentiation of cell lineages within the developing cornea has not been clearly defined.

In the current study, we determined that within the developing cornea, *Tfap2b* is an essential genetic target downstream of PITX2 during corneal development. Our findings demonstrate that AP-2β function is required to establish angiogenic but not lymphangiogenic privilege during corneal development. AP-2β function is also required for normal specification of the corneal endothelium. We conclude that AP-2β is an important downstream effector of PITX2 during corneal development that is capable of mediating the suppression of angiogenesis given the appropriate biological contexts.

Materials and Methods

Mouse Strains and Animal Husbandry

All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals, and all procedures involving mice were preapproved by the Committee on Use and Care of Animals at the University of Michigan. Generation of the *Pitx2^{null}*, *Pitx2^{flo}*,[27] *UBC-CreER^{T2}*,[28,29] and *Tfap2b^{null}* strains has been described previously.[30] Mice were mated to generate timed pregnancies, and the morning that a plug was identified was designated embryonic day 0.5 (e0.5). The relevant crosses were *UBC-CreER^{T2}; Pitx2$^{+/null}$* × *Pitx2$^{flox/flox}$; R26R/R26R*, and *Tfap2b$^{+/null}$* × *Tfap2b$^{+/null}$*. When indicated, a single intraperitoneal injection of tamoxifen (Sigma-Aldrich Corp., St. Louis, MO, USA) suspended in corn oil at a dose of 100 mg per gram body weight was administered to the pregnant dam at noon on the day noted. The resulting embryos were genotyped as appropriate using PCR-based methods.[27–30]

Laser Micro Dissection and Quantitative RT-PCR

Timed pregnant dams from mating *UBC-CreER^{T2}; Pitx2$^{+/null}$* × *Pitx2$^{flox/flox}$; R26R/R26R* were injected with tamoxifen at e10.5, and embryos were collected at e12.5, e13.5, or 16.5. Embryos were also collected at e12.5 from timed pregnant dams following the mating *Tfap2b$^{\pm}$* × *Tfap2b$^{\pm}$*. Embryos were flash frozen on dry ice and processed for cryosectioning. Corneal tissue was micro dissected from frozen sections using a Leica LMD7000 workstation (Buffalo Grove, IL, USA). Corneal tissue from the pair of eyes from each embryo was collected and considered as one sample. Total RNA was isolated from each sample using the RNeasy Micro kit (Qiagen, Hilden, Germany) and used to generate cDNA by the Ovation Pico WTA System V2 method (NuGEN, San Carlos, CA, USA). Relative expression of *Tfap2b*, *Vegfa*, *Foxc1*, *Lmx1b*, *Mmp2*, *Mmp9*, and *Flt1* were measured in replicate samples (N = 3-6/genotype) using TaqMan Gene Expression Assays (Life Sciences Technologies, Carlsbad, CA, USA).

Microarray Analysis

Amplification and labeling of RNA was performed using 200 pg of total RNA for each sample and the TargetAmp 2-Round Aminoallyl-aRNA Amplification Kit 1.0 (Epicentre, Madison, WI, USA). Hybridization of Alexa555 labeled RNA to microarrays (Mouse Gene Expression V.2, 8X60K) was performed per the protocol of Agilent Technologies (Santa Clara, CA, USA). Microarrays were scanned with an Agilent dual-laser scanner, and images were analyzed using Agilent Technologies' feature extraction software version 11.0.1.1.

Microarray data were imported into GeneSpring (Agilent Technologies) for analysis. Expression values were quantile normalized for each array. For each pair of conditions compared, genes were first filtered to select those having detectable expression in at least 75% of the samples for either condition. A moderated *t*-test and the Benjamini and Hochberg multiple test correction was used to identify differentially expressed genes. Minimal criteria were 2-fold change in expression and a false discovery rate of $\leq$ 5%. Microarray data are available from the NCBI Gene Expression Omnibus, accession GSE76047.

Embryo Processing and Histochemistry

Embryos generated for histology were fixed in 4% paraformaldehyde diluted in PBS, washed in PBS, dehydrated through graded alcohols, and processed into Paraplast Plus (McCormick

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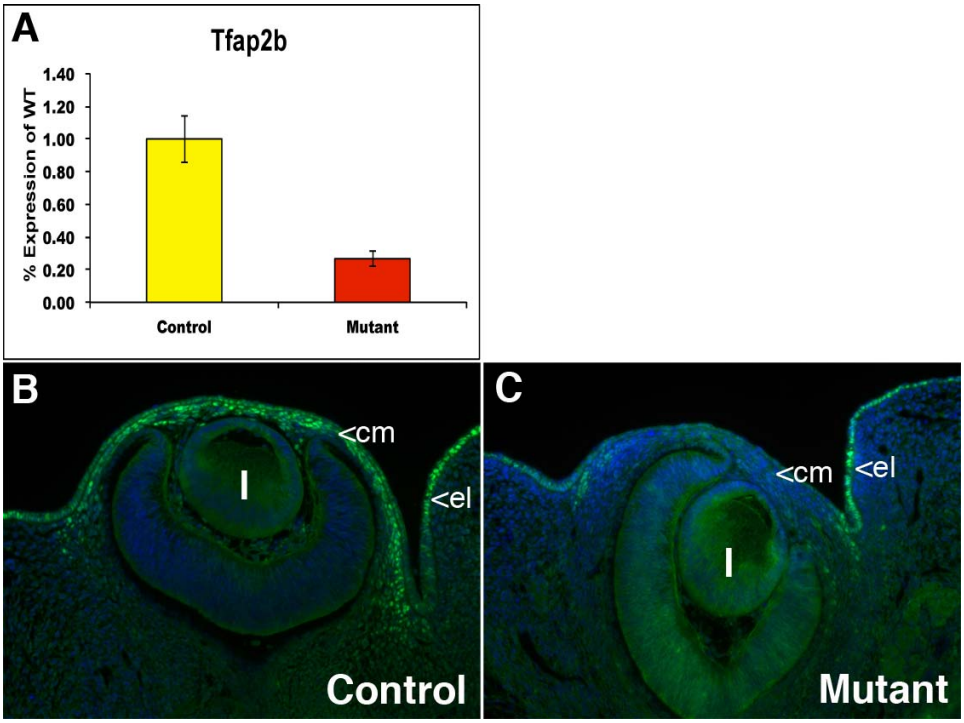

Figure 1. *Tfap2b* is a genetic target of *Pitx2* in neural crest during corneal development. (**A**) Quantitative RT-PCR assays were used to compare relative *Tfap2b* expression levels in corneas isolated from e12.5 control and *Pitx2-tko* mutant corneas by laser microdissection. *Tfap2b* expression is significantly ($P = 0.002$) decreased in *Pitx2-tko* mutant corneas. (**B**, **C**) Immunohistochemistry was used to detect AP-2β protein (*green*) in e12.5 control and *Pitx2-tko* mutant eyes. All nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Expression is specifically lost from the corneal mesenchyme in *Pitx2-tko* mutant eyes; expression is identical in eyelid ectoderm of eyes from each genotype. Key: cm, corneal mesenchyme; el, eyelid, eyelid ectoderm; l, lens.

Scientific, St. Louis, MO, USA) for paraffin sectioning. Mounted paraffin sections for morphologic analysis were dewaxed, rehydrated, and stained with hematoxylin and eosin.

Immunostaining and RNA In Situ Hybridization

Paraffin sections were immunostained as previously described.[31] Primary antibodies against PITX2 (gift from Tord Hjalt), AP-2β (Abnova, Walnut, CA, USA), cytokeratin 4 (cK4, Abcam, Cambridge, CA, USA), cytokeratin 12 (cK12, gift from Winston Kao), Endomucin (Affymetrix eBioscience, San Diego, CA, USA), ZO-1 (Invitrogen, Carlsbad, CA, USA), and LYVE-1 (Abcam) were used. Digoxigenin-labeled riboprobes against *Keratocan* (gift from Winston Kao) and *Dkk1* (gift from Christoff Niehrs) were generated from donated plasmid templates and used to stain paraffin sections as previously described.[32,33]

Results

Tfap2b Expression During Corneal Development Requires PITX2

We combined laser microdissection and microarray analysis using e12.5 embryos to identify genes regulated by *Pitx2* during corneal development. Because anterior segment morphogenesis and early corneal development are blocked prior to initiation of corneal development in both global and neural crest-specific *Pitx2-null* embryos,[27,33] we employed a temporal knockout strategy described previously to selectively ablate *Pitx2* at the onset of corneal development.[34] Briefly, we crossed mice carrying our conditional $Pitx2^{flox}$ allele with mice carrying the $UBC\text{-}CreER^{t2}$ transgene, which ubiquitously expresses a Cre fusion protein that is activated by tamoxifen.[28,29] Females carrying prospective mutants ($UBC\text{-}CreER^{t2}\text{:}Pitx2^{flox/null}$) and controls ($Pitx2^{flox/null}$) were injected with tamoxifen at e10.5 to ablate *Pitx2* in the prospective mutant (*Pitx2-tko*) embryos.[14] Our initial comparison of the resulting expression profiles identified 1917 genes whose expression was predicted to be different in wild-type versus *Pitx2-tko* corneas. Of these genes, 53 were encode transcription factors (Supplemental Fig. S1). We chose *Tfap2b* as an attractive candidate for further analysis because it encodes AP-2β, a member of the AP-2 family of transcription factors that are widely required for normal eye development, and it is expressed at the appropriate time in neural crest mesenchyme of the developing cornea.[15,21] Furthermore, the loss of AP-2 transcription factors is associated with pathologic angiogenesis, a prominent phenotype in developing corneas of *Pitx2*-deficient embryos.[14,25,26]

We used quantitative RT-PCR to confirm that *Tfap2b* expression in *Pitx2-tko* corneas taken from e12.5 and e16.5 eyes is significantly reduced when compared with corneas taken from wild-type *Pitx2* control embryos (Fig. 1A and data not shown). Immunohistochemistry confirmed that, in control mice at e12.5, AP-2β protein has a restricted pattern of expression in mesenchymal cells that will ultimately contribute to the cornea and subsequently the limbus and structures within the iridocorneal angle. AP-2β is also present weakly in the presumptive corneal ectoderm. Notably, AP-2β is not present in the primordia to other ocular structures that derive from the mesenchyme such as the sclera (Fig. 1B).[15] AP-2β is also expressed in the surface ectoderm of the presumptive eyelid conjunctiva of control mice at this timepoint (Fig. 1B).[15] In *Pitx2-tko* embryos, AP-2β is present in surface ectoderm of the eyelid conjunctiva, but in contrast to control embryos, AP-2β expression is absent in mesenchyme of the presumptive

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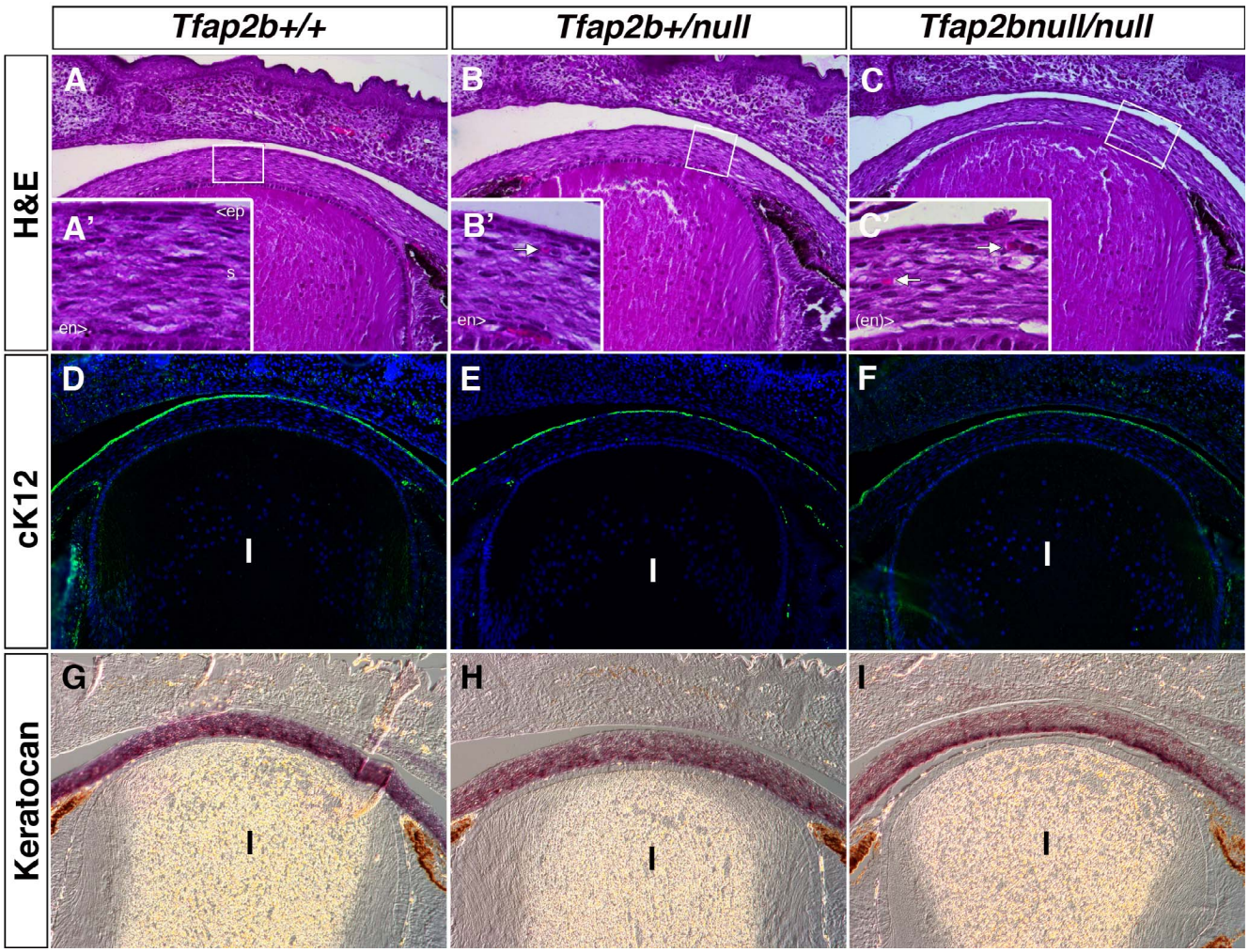

Figure 2. Differentiated corneal ectoderm and stroma do not require AP-2β. (**A–C**) Evaluation of eyes from e18.5 embryos of the indicated genotypes following staining with H&E revealed disorganized lamination within the stroma layer and the presence of blood vessels containing red blood cells in all homozygous eyes examined. Blood vessels were also present in 25% (2/8) of heterozygous eyes examined. Blood vessels were never observed in wild type eyes. (**D–F**) Immunohistochemistry was used to detect cK12, a specific marker of differentiated corneal ectoderm in e18.5 embryos of the indicated genotypes. All nuclei were counterstained with DAPI. The pattern of cK12 staining (*green*) is equivalent regardless of genotype. (**G–I**) In situ hybridization was used to detect *Keratocan*, a specific marker of differentiated corneal stroma in e18.5 embryos. *Keratocan* is expressed in the eyes of all three genotypes. However, in contrast to the uniform staining pattern found in eye of wild-type and heterozygous individuals, the staining pattern in homozygous-mutant individuals defines two layers based on differential expression levels. *White* arrows indicate blood vessels in B' and C'. en, endothelium; ep, epithelium; l, len; s, stroma.

cornea and limbus as well as the presumptive corneal ectoderm (Fig. 1C). Collectively, these results identify *Tfap2b* as a genetic target of *Pitx2* in the neural crest of the developing cornea and potentially additional anterior segment structures that differentiate from neural crest later during eye development.

AP-2β Is Required for Normal Development of Corneal Cell Lineages From the Neural Crest

To determine if the AP-2β transcription factor is essential for corneal development, we examined eye morphogenesis in wild type, *Tfap2b*$^{+/null}$, and *Tfap2b*$^{null/null}$ embryos. The initial steps in corneal morphogenesis, including the migration of periocular mesenchyme into the space between the newly formed lens vesicle and the overlying presumptive corneal ectoderm, appear to occur normally in the absence of AP-2β (data not shown). However, notable differences are apparent in the corneas of *Tfap2b*$^{null/null}$ mice during later gestation. By e16.5 in control eyes, the corneal endothelium is readily visible as a single monolayer overlying the anterior lens epithelium. In contrast, the monolayer of endothelium is not apparent in the corneas of *Tfap2b*$^{null/null}$ eyes (Figs. 2A–C). Patent blood vessels containing red blood cells are readily visible extending into the central cornea of all *Tfap2b*$^{null/null}$ eyes examined (Fig. 2C'). Interestingly, blood vessels containing red blood cells are also present in a subset of corneas (2/8) from *Tfap2b*$^{+/null}$ eyes (Fig. 2B'). These data suggested that AP-2β is an important effector downstream of PITX2 during corneal development.

Pitx2 is required for differentiation of all three major cell lineages during corneal development.[14] To definitively determine whether the loss of the AP-2β transcription factor is a contributing mechanism underlying these defects in *Pitx2-tko* mice, we assessed the expression of lineage-specific protein markers in *Tfap2b* mice. In wild-type mice, the intermediate filament protein cK12 is a specific marker of the differentiated corneal ectoderm (Fig. 2D),[35] and the related protein cK4 is expressed in the adjacent conjunctival ectoderm (Supplemen-

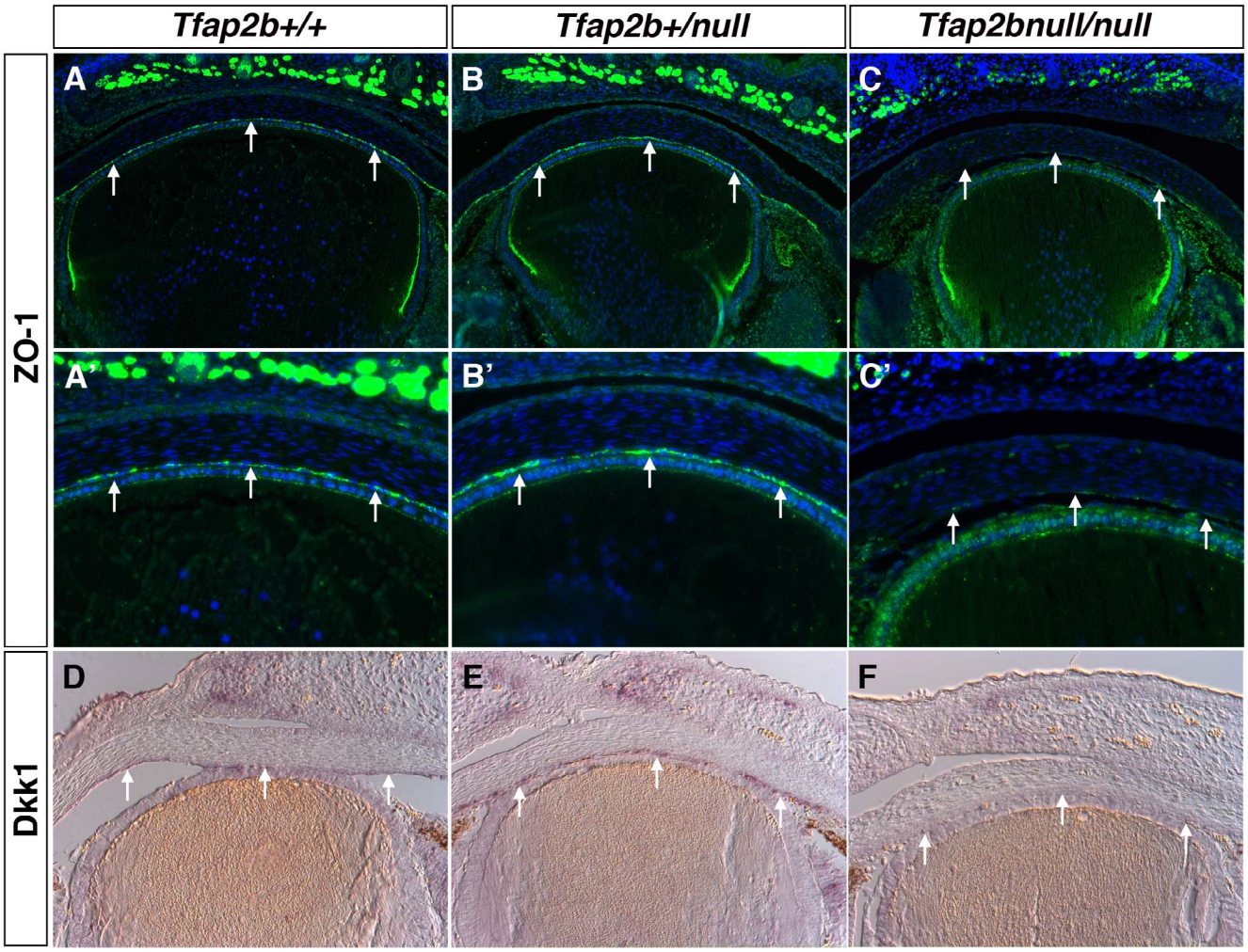

Figure 3. Normal differentiation of corneal endothelium requires AP-2β function. (**A–C**) Immunohistochemistry was used to detect ZO-1 (*green*), a specific marker of differentiated corneal endothelium in e16.5 embryos of the indicated genotypes. All nuclei were counterstained with DAPI. (**D–F**) In situ hybridization was used to detect *Dkk1*, a second specific marker of differentiated corneal endothelium in the same embryos. Expression of both markers was specifically lost from the eyes of homozygous null embryos. *White* arrows point to location of presumptive epithelial layer.

tal Fig. S2).[35] In heterozygous and homozygous *Tfap2b* mutant embryos, cK12 expression in the corneas is comparable with wild-type eyes, suggesting that the corneal ectoderm is specified normally in these mice (Figs. 2E, 2F). cK4 expression is also normal in *Tfap2b* mutant eyes (Supplemental Fig. S2). Collectively, these data indicate that AP-2β function in the presumptive corneal neural crest is not required for correct specification of the overlying corneal ectoderm during embryogenesis.

Stromal keratocytes are marked by the uniform expression of *Keratocan* by late gestation, and we observed an analogous expression pattern in *Tfap2b* heterozygous eyes as well (Figs. 2G, 2H). In contrast, *Keratocan* expression in the corneas of *Tfap2b* homozygous mutants defines two distinct layers based on apparent signal intensity. Expression is high within the posterior stroma adjacent to the lens and appears to match expression in wild-type eyes, whereas expression is notably reduced in the anterior stroma located adjacent to the corneal ectoderm (Fig. 2I). This location corresponds to the cells that show the greatest degree of disorganization in histologic sections (Fig. 2C'). Collectively, these data suggest that AP-2β function is likely not required for the differentiation of stromal keratocytes.

Finally, we sought to confirm the absence of a corneal endothelium in *Tfap2b*$^{null/null}$ eyes by examining markers specific for this layer. Expression of the tight junction protein ZO-1 specifically marks the corneal endothelium in the eyes of wild-type and *Tfap2b* heterozygous mice (Figs. 3A, 3B). In contrast, ZO-1 expression in the corneas of *Tfap2b*$^{null/null}$ mice is either discontinuous or absent (Fig. 3C). We also examined expression of the *Dkk1,* encoding an inhibitor of canonical Wnt signaling. *Dkk1* expression labels the corneal endothelium in wild-type and heterozygous *Tfpap2b* mice but is missing from the cornea in *Tfap2b*$^{null/null}$ eyes (Figs. 3D–F). Collectively, these data suggest that AP-2β is required for normal differentiation of the corneal endothelium and that this layer is likely absent or undifferentiated in *Tfap2b*$^{null/null}$ mice.

AP-2β Is Required for Establishment of Angiogenic but Not Lymphangiogenic Privilege During Corneal Development

The appearance of patent blood vessels extending to the central cornea of *Tfap2b* mutant mice is reminiscent of *Pitx2-tko* eyes, where both angiogenic and lymphangiogenic privilege is lost in corneas following temporal ablation of

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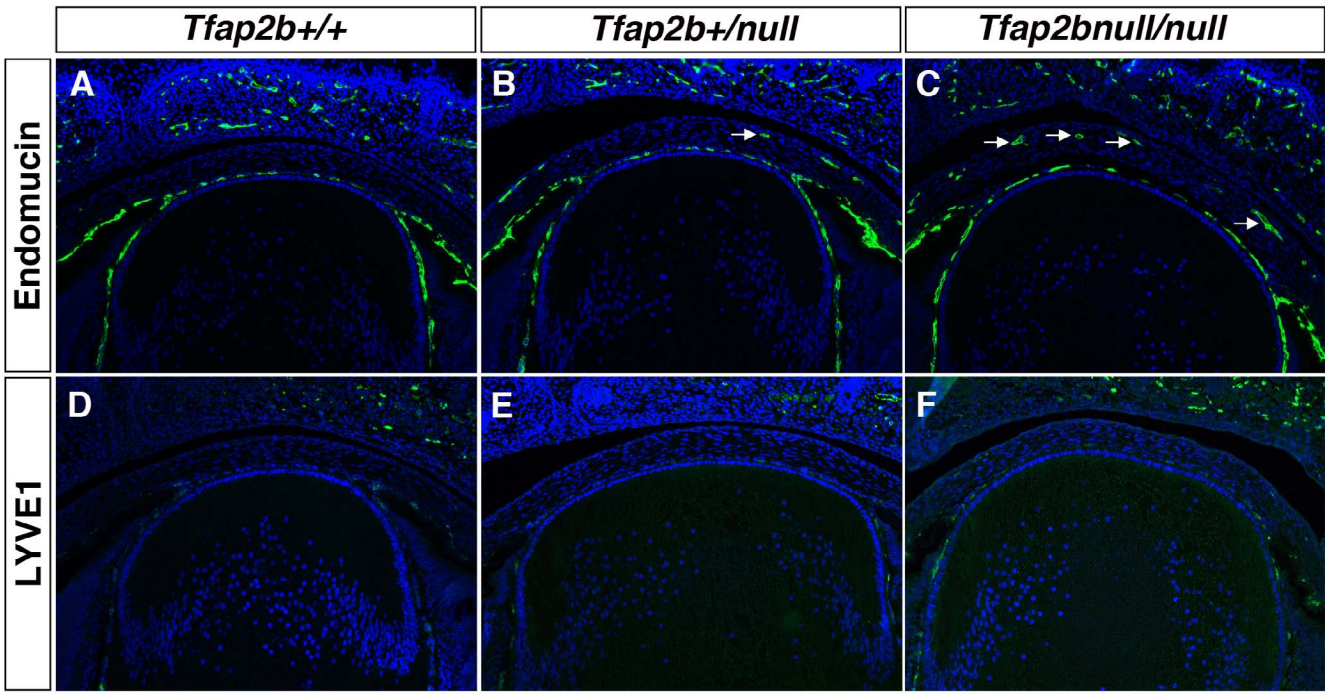

Figure 4. AP-2β is required for establishing angiogenic privilege during corneal development. (**A–F**) Immunohistochemistry revealed that all vessels found in e16.5 eyes of homozygous and heterozygous individuals stained positively for a specific marker of blood vessel endothelium (Endomucin) (*green*) but negative for a specific marker of lymphatic vessel endothelium (LYVE-1) (*green*). Note: Although corneas of all genotypes were negative for a specific marker of lymphatic vessel endothelium (LYVE1), positive staining of lymphatic vessels (*upper right* [**B**]) in the eyelids of *Tfap2b*$^{+/+}$ eyes provides a positive control for this marker. All nuclei were counterstained with DAPI.

Pitx2.[14] To further classify the blood vessels present in the corneas of heterozygous and homozygous *Tfap2b* mutant corneas, we stained sections for the presence of Endomucin, a specific marker of blood vessel endothelium, and LYVE-1, a specific marker of lymphatic vessels endothelium. Endomucin staining was absent from the corneas of all wild-type eyes, as expected (Fig. 4A). Endomucin-positive blood vessels were present in a subset (2/8 eyes scored) of *Tfap2b*$^{+/null}$ corneas as well as in the corneas of all *Tfap2b*$^{null/null}$ embryos examined (Figs. 4B, 4C). Strikingly, although present in the eyelids of *Tfap2b*$^{+/+}$ embryos as a positive control, LYVE-1 staining was absent from the corneas of all *Tfap2b* mutant embryos, irrespective of genotype (Figs. 4D–F). Collectively, these data indicate that AP-2β is an essential effector downstream of PITX2 that is required to establish angiogenic but not lymphangiogenic privilege during corneal development.

Loss of AP-2β Alters Expression of a Subset of Genes Required for Normal Corneal Development and Establishment of Angiogenic Privilege

Alterations in the expression of several genes have previously been associated with phenotypes observed in *Tfap2b*-null mice. Therefore, we used quantitative RT-PCR to analyze the expression of these genes in RNA samples isolated from developing e12.5 wild-type and mutant corneas to gain further insight into the role of AP-2β. The homeodomain gene, *Lmx1b* and the forkhead gene, *Foxc1*, each encode transcription factors that are expressed in neural crest during corneal development and are required for normal differentiation of the endothelium layer.[36,37] We found that *Lmx1b* expression is not significantly altered in the developing corneas of *Tfap2b*$^{-/-}$ when compared with wild-type littermates (Fig. 5). In contrast, *Foxc1* expression is significantly reduced in the absence of AP-2β. These results suggest that the normal expression of *Foxc1*, but not *Lmx1b*, is dependent on AP-2β and that reduced FOXC1 levels may contribute to altered development of the corneal endothelium in *Tfap2b* mutant eyes.

Vegfa encodes the signaling molecule VEGF, a potent inducer of angiogenesis, including in the developing cornea.[38] We assessed *Vegfa* expression but found no significant difference between wild-type and *Tfap2b* mutant corneas. To prevent abnormal angiogenesis, the levels of biologically available VEGFα protein are posttranscriptionally regulated at several levels, including sequestration by the extracellular matrix or soluble inhibitory proteins that prevent binding to and subsequent activation of cognate receptors. *Mmp2* and *Mmp9* encode matrix metalloproteinases that promote the release of biologically active VEGFα from the extracellular matrix. Interestingly, AP-2α is required to activate *Mmp2* expression in the cornea,[39] whereas both *Mmp2* and *Mmp9* are derepressed in *Foxc1*-mutant mice, leading to VEGFα-dependent loss of angiogenic privilege in the cornea.[11] *Mmp2* expression is significantly reduced in the absence of AP-2β, whereas *Mmp9* expression could not be reliably detected in the corneas of either wild-type or *Tfap2b*-null embryos. These data suggest that activation of *Mmp2* or *Mmp9* is unlikely to contribute to a loss of angiogenic privilege in the corneas of *Tfap2b*-null mice. Finally, we assessed the expression of the secreted isoform of VEGF receptor 1 (*sFlt1*), which prevents the binding of VEGFα to the membrane-bound form of the receptor and is essential for maintaining angiogenic privilege in the adult cornea.[40] We found that the expression of *sFlt1* is not reduced in developing *Tfap2b*-null corneas, making it unlikely that the loss of this protein accounts for the loss of angiogenic privilege in these eyes.

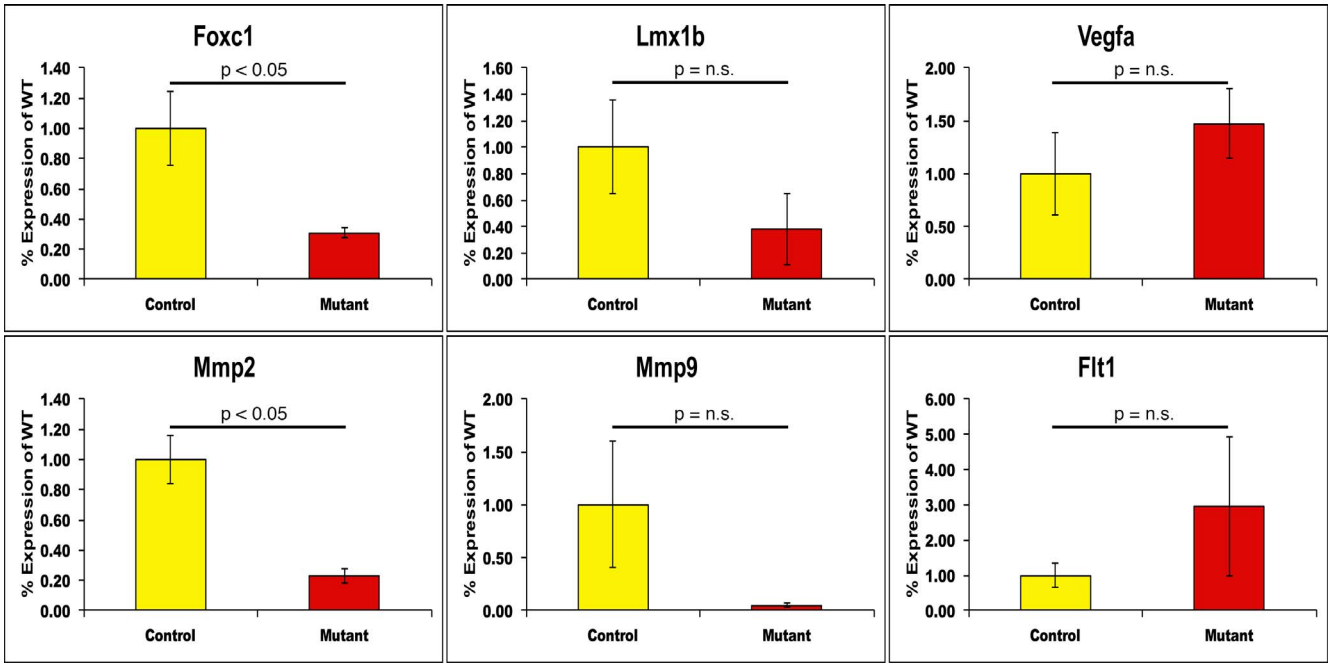

Figure 5. Comparison of gene expression in wild-type versus *Tfap2b*$^{-/-}$ corneas. Quantitative RT-PCR was used to assess relative expression of the indicated genes in total RNA from corneal tissue isolated from e14.5 wild-type control and *Tfap2b*$^{-/-}$ embryos ($n = 3$-4 embryos/genotype) by laser microdissection. n.s., not significant.

Discussion

The correct development of the cornea is essential for normal vision, but the genes critical for differentiation of the three major cell lineages and establishment of an avascular environment during corneal development remain largely unknown. A detailed understanding of the molecular mechanisms regulating these processes could in the future contribute to more effective treatments of corneal diseases. Recently, we demonstrated that the homeodomain transcription factor PITX2 is essential for multiple developmental events in the cornea, but the essential downstream effectors of PITX2 function remained to be identified. In this report, we examined gene expression changes in the cornea caused by a loss of PITX2 and determined there was a dramatic reduction in *Tfap2b* transcripts. We subsequently demonstrated that *Tfap2b*, encoding the AP-2β transcription factor, functions downstream of PITX2 and is required for correct differentiation of corneal endothelium and for establishing angiogenic privilege.

AP-2β Contributes to a Transcriptional Code Required for Specification of Corneal Endothelium

The endothelium is unique among mature corneal cell lineages because cells lost to disease or trauma normally are not replaced. Loss of the barrier function provided by the endothelium leads to swelling and opacity within the adjacent stroma, which leads to vision loss. Transplantation is the only currently available therapy with the potential to replace lost endothelium cells. However, because sources of endothelium for engraftment are extremely limited, there is considerable interest in the development of stem cell–based therapies. A detailed understanding of the genes and mechanisms required for embryonic development of the endothelium is likely to aid in the design of strategies to program stem cells to differentiate into this lineage.

Specification of the endothelium from the neural crest located between the newly formed lens vesicle and the overlying surface ectoderm requires inductive signaling from the adjacent anterior lens epithelium and the anterior rim of the optic cup.[41–44] The complete composition of the signal remains to be determined but it includes retinoic acid.[42–44] A key molecular response to retinoic acid signaling within the neural crest is the induction of *Pitx2* expression, which is subsequently required for the differentiation of all three major corneal cell lineages.[14] Previously, we have shown that PITX2-dependent expression of *Dkk2*, and the resulting suppression of canonical Wnt signaling within the adjacent surface ectoderm, is essential for the differentiation of the corneal epithelium.[14] In contrast, the differentiation of cell lineages from the neural crest is relatively unaffected by loss of *Dkk2*.[14] In the present report, we establish that PITX2 is required for the expression of *Tfap2b* throughout the corneal neural crest and that AP-2β is required for specification of the corneal endothelium.

Two additional transcription factors that are required for specification of the corneal endothelium have been identified. Retinoic acid signaling also induces expression of the gene encoding the forkhead transcription factor FOXC1 within the neural crest. Mutations in human *FOXC1* are a second cause of Axenfeld-Rieger syndrome, suggesting that PITX2 and FOXC1 likely coregulate common molecular pathways during anterior segment development.[45,46] Like PITX2, FOXC1 is also required for normal specification of the corneal endothelium as well as the stroma.[36] Although a dependence on retinoic acid signaling has not been reported, the homeobox gene *Lmx1b* encodes a fourth transcription factor that is expressed within the neural crest and is required for specification of the endothelium.[37] Our data suggest that full expression of *Foxc1*, but not *Lmx1b*, depends on AP-2β and that FOXC1 may act downstream effector of AP-2β required for normal development of the corneal endothelium.

Ultimately, specification of the corneal endothelium must depend on the unique combinatorial expression of transcription factors in this lineage. Although these four genes are each individually required for differentiation of the corneal endo-

Investigative Ophthalmology & Visual Science

thelium, they are also coexpressed in the ocular neural crest in cells not fated to form the endothelium, and similar to PITX2, FOXC1 and LMX1B are required for specification of additional lineages during eye development. These observations provide compelling evidence that our understanding of the transcription factor code required for specification of the corneal endothelium remains incomplete and that additional components remain to be identified. Completely elucidating additional upstream transcription factors essential for *Tfap2b* expression, as well as cofactors that function together with AP-2β, will provide important opportunities to discover additional components of the code. In addition, the identification of the cascade of molecular events located downstream of AP-2β will help uncover additional components of the regulatory cascade required for differentiation of the corneal endothelium. In this context, at present it is not clear if PITX2 regulates the expression of *Tfap2b* via direct or indirect mechanisms and will require the identification and characterization of the *Tfap2b cis*-acting sequences responsible for expression in the developing cornea. Therefore, further studies will be required to identify the regulatory interactions between these four transcriptional regulators of corneal endothelium development.

AP-2β Is Required for Establishment of Angiogenic Privilege During Corneal Development

The genes and mechanisms required for establishing angiogenic and lymphangiogenic privilege during development also remain largely unknown despite the importance of these properties for normal vision and the likelihood that elucidation of these pathways could have a positive impact on the development of new therapeutic strategies. FOXC1 is required for the establishment of both properties through a mechanism that includes the repression of genes encoding matrix metalloproteinases 2 and 9, which enhance the generation of available bioactive forms of VEGF.[11] Recently, we showed that PITX2 is also essential for both properties but downstream effectors were not identified.[14] We now extend the knowledge of this pathway by establishing the gene encoding AP-2β as a required PITX2-dependent mediator of angiogenic but not lymphangiogenic privilege during corneal development.

Previously, overexpression of the gene encoding AP-2β had been shown to correlate with angiogenesis and poor prognosis in human lung adenocarcinomas through mechanisms that include modulation of VEGF/PDGF signaling.[47] Our current data now establish that AP-2β is also capable of also suppressing angiogenesis. These observations imply that the effects of AP-2β on blood vessel growth versus nongrowth are context dependent. This property is consistent with the established functions of the highly related family member AP-2α, which has been demonstrated to promote or suppress angiogenesis depending on context. The activator and repressor functions of AP-2 transcription factors are generally dictated by the identity of essential interacting partners,[24] suggesting that the differences in angiogenic responses are likely to result from the complement of cofactors available in each context. The need to understand AP-2β mediated suppression of angiogenesis during corneal development highlights the importance of identifying the complement of available cofactors during this process.

Our data demonstrate that AP-2β is required for the establishment of angiogenic privilege during corneal development, but the underlying mechanism remains unknown. In the cornea, as elsewhere, the growth or nongrowth of new blood vessels is ultimately the net result of a tightly regulated balance between the local availability of proangiogenic and antiangiogenic factors. The *Vegfa* promoter is a direct target of both AP-2α and AP-2β in certain tissues, with the net effect dependent on context.[47,48] Furthermore, the overexpression of AP-2α correlates with increased levels of HIF-1α, a potent modulator of the *Vegfa* promoter, in certain contexts.[49] AP-2α suppresses the expression of *Mmp2* and *Mmp9* in some tissues[50] but activates *Mmp2* expression in the cornea.[22] Interestingly, although the expression levels of *Vegfa*, *Mmp2*, and *Mmp9* are all elevated in corneas from *Pitx2-tko* mice, our data suggest that enhanced transcription of these genes does not account for a loss of angiogenic privilege in developing *Tfap2b* corneas. Therefore, additional experiments are required to identify the factor(s) required downstream of AP-2β during the establishment of angiogenic privilege in the cornea.

Summary

Our current results identify AP-2β as an important downstream effect of PITX2 in corneas during embryogenesis but a number of critical questions remain regarding the roles of both proteins in developing and mature ocular anterior segment structures. For PITX2, although we have identified AP-2β as an important factor that accounts at least in part for the requirement for PITX2 in differentiation of the endothelium and establishment of angiogenic privilege, other factors must be required for PITX2-dependent processes such as differentiation of the stroma and for lymphangiogenic privilege. For AP-2β, persistent expression throughout corneal development and in the mature cornea suggests additional potential roles at later timepoints. Both PITX2 and AP-2β are present at high levels in neural-crest derived structures within in the iridocorneal angle, highlighting the possibility that these factors may also serve important functions in the development and/or maintenance of these structures as well. Conditional alleles for both genes together with suitable Cre transgenic mouse lines will be essential for future experiments addressing the tissue-specific and temporal requirements of *Pitx2* and *Tfap2b* in anterior segment development.

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Investigative Ophthalmology & Visual Science

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