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Targeted Deletion of *AP-2α* Leads to Disruption in Corneal Epithelial Cell Integrity and Defects in the Corneal Stroma

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Abstract

Purpose—The present study was undertaken to create a conditional knockout of *AP-2α* in the corneal epithelium.

Methods—A line of mice expressing Cre-recombinase specifically in the early lens placode was crossed with mice in which the *AP-2α* allele is flanked by two *loxP* sites. The resultant *Le-AP-2α* mutants exhibited a targeted deletion of *AP-2α* in lens placode derivatives, including the differentiating corneal epithelium.

Results—The *Le-AP-2α* mutant mice were viable and had a normal lifespan. The adult corneal epithelium exhibited a variation in the number of stratified epithelial layers, ranging from 2 to 10 cell layers. A substantial decrease in expression of the cell–cell adhesion molecule, E-cadherin, was observed in all layers of the *Le-AP-2α* mutant corneal epithelium. The basement membrane, or Bowman's layer, was thinner in the mutant cornea and in many regions was discontinuous. These defects corresponded with altered distribution of laminin and entactin, and to a lesser degree, type IV collagen. The *Le-AP-2α* mutant cornea also exhibited stromal defects, including disrupted organization of the collagen lamellae and accumulation of fibroblasts beneath the epithelium that showed increased immunoreactivity for proliferating cell nuclear antigen (PCNA), *α*-smooth muscle actin (*α*-SMA), p-Smad2, and TGF-*β*2.

Conclusions—In the absence of *AP-2α*, the corneal epithelium exhibits altered cell adhesion and integrity and defects in its underlying basement membrane. These defects likely caused the alterations in the corneal stroma.

Severe defects in vision are frequently associated with abnormalities of the cornea, a highly specialized transparent tissue responsible for the refraction of light.[1] The cornea comprises three tissue layers: the outer stratified squamous epithelium, the inner endothelium, and the intermediate stroma, the latter of which is composed of keratocytes (resident fibroblasts) separated by tightly packed, regularly arranged collagen lamellae. During development, the cranial neural crest cells give rise to the cells of the corneal stroma and endothelium,[2] whereas a region of the head ectoderm that is further defined as the lens placode gives rise to the corneal

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epithelium.[3,4] In mice, the corneal epithelium originates as a two layered structure that, after eyelid opening at 2 weeks of age, expands into a stratified epithelium consisting of 8 to 10 cell layers.[5,6]

The genetic programs responsible for early development of the cornea are governed by multiple transcription factors that act in a combinatorial fashion. Of significance is the homeobox and paired-box containing *Pax6* gene, a member of the *Pax* gene transcription factor family, which is expressed in the early lens placode and its derivatives and is essential for normal eye development.[7,8] Mutations in *Pax6* in humans and mice have been shown to lead to congenital defects that result in lens cataracts, microphthalmia (small eye), aniridia (iris defect), and multiple defects in the cornea.[3,9–12] In addition to its necessity during embryonic development, *Pax6* has been shown to be have a central role in regulating postnatal differentiation and maintenance of the adult cornea.[11,12] Specifically, the corneal epithelium of heterozygous *Pax6* Small eye ($Sey^{+/-}$) mice with half the levels of *Pax6*, exhibit a reduced number of stratified layers and compromised cellular adhesion.[12] After injury to the normal corneal epithelium, *Pax6* expression is induced at the wound edge where it has been shown to activate the *gelatinase B (matrix metalloproteinase [MMP]-9) promoter*.[13] In $Sey^{+/-}$ mice, corneal injury results in an increase in both inflammation and the rate of reepithelialization, compared with the effect in wild-type mice.[14] These observations indicate that appropriate levels of *Pax6* expression are necessary to suppress excess inflammation and control the rate of reepithelialization after corneal injury.

It is well known that Pax6 does not act alone in regulating gene expression during early eye development and, likewise, in the postnatal cornea, Pax6 has been shown to act in combination with additional regulators to activate or repress gene promoters. One of these regulators is AP-2*α* (activating protein-2*α*) transcription factor, which has a striking overlap in expression pattern with Pax6 in the developing and adult eye.[15] Specifically, in the adult corneal epithelium, AP-2*α* expression is confined to the more basally located cells of the corneal epithelium, similar to Pax6.[15,16] Earlier studies revealed a role for AP-2*α* in corneal epithelial repair in that its expression is upregulated at the leading edge of the migrating epithelium after wounding, and binds to and activates the MMP-9 promoter.[14,17,18] More recently, AP-2*α* has also been shown to interact directly with Pax6 and facilitate its binding to the MMP-9 promoter.[14] Further evidence for the requirement of *AP-2α* in corneal differentiation has been provided by the finding that overexpression of the *AP-2α* gene in a corneal epithelial cell line resulted in dramatic changes in cell phenotype, including a clumping growth behavior indicative of differentiation, as well as a change in cell adhesion expression.[16]

Requirements for *AP-2α* in ocular development have been revealed through studies of *AP-2α* knockout (KO) and chimeric mice.[15,19] *AP-2α* null mice exhibited multiple and complex ocular phenotypes ranging from anophthalmia (absence of eyes) to mutant eyes that exhibited an adhesion of the lens to the overlying surface ectoderm. Multiple defects in the developing optic cup (future retina) were also observed.[15] In the *AP-2α* null mice no corneas developed; this deficiency was attributed to secondary tissue defects. A proportion of *AP-2α* chimeric mice, composed of a mixture of *AP-2α* wild-type and null cells, exhibited a defect in separation of the lens from the overlying ectoderm and as a result had a persistent adhesion between the lens and corneal epithelium.[15,19] *AP-2α*-null cells, identified using a specific *β*-galactosidase cell lineage tracer, were localized in these regions. However, in the remaining part of the corneal epithelium, the *AP-2α*-null cells were excluded, and the corneal epithelium appeared relatively normal. Because of this finding and the fact that the *AP-2α*-null mice do not form a cornea at all, these mutant models could not be used to investigate the specific in vivo requirement(s) of *AP-2α* in postnatal corneal epithelial differentiation.

In the present study, we created a conditional KO mouse model of *AP-2α* (*Le-AP-2α* mutants), using the *Cre-loxP* approach, to examine the requirement for *AP-2α* in corneal epithelial differentiation. AP-2*α* expression in the *Le-AP-2α* mutant corneal epithelium was completely abolished, whereas other non–lens-placode–derived tissues maintained normal levels of AP-2*α*. Prominent defects in corneal epithelial stratification, cell adhesion, and basement membrane deposition were observed in the *Le-AP-2α* mutant corneas. In addition, the corneal stroma in these mutants exhibited an abnormal phenotype, including an activation of the resident fibroblasts. Together, these data reveal a specific requirement for *AP-2α* in the normal postnatal differentiation of the corneal epithelium.

Materials and Methods

Generation of *Le-AP-2α* Mice

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A conditional knockout of *AP-2α* in the lens placode was created, providing a model for examining the requirement of the *AP-2α* gene in differentiation of the corneal epithelium. The *Cre-loxP* approach was used for the specific knockout of *AP-2α* in the developing lens placode and its derivatives, including the corneal epithelium. Briefly, this approach involves breeding mice in which the gene of interest contains two *lox-P* sites flanking a critical region (a "floxed" gene) with transgenic mice that express Cre-recombinase in a specific cell type or lineage. The expression of Cre-recombinase in a particular cell or tissue type results in deletion of the genomic DNA located between the two *loxP* sites and generates a mutant allele in lineages derived from those cells. In contrast, normal gene function occurs outside the Cre expression–excision cell lineage. For our experiments, two lines of mice were interbred: (1) *Le-Cre* mice that expressed Cre-recombinase specifically in cells within the early lens placode.[20] These mice were also heterozygous for a null mutation of *AP-2α* caused by a *LacZ* knockin[21]; and (2) mice that are homozygous for an allele of *AP-2α* in which paired *loxP* sites flank a region encoding a critical area of the DNA binding domain.[21] In the absence of an expressed Cre transgene, such homozygous floxed *AP-2α* mice appeared normal. PCR analysis of tail biopsy specimens was used to identify the genotype of offspring from crosses between these two mouse lines. Subsequently, PCR analysis was also used to determine whether corneal biopsy specimens contain an intact or recombined floxed *AP-2α* allele. The primers *Alflp* (5′-CCT GCC TTG GAA CCA TGA CCC TCA G-3′), *Alflox4* (5′-CCC AAA GTG CCT GGG CTG AAT TGA C-3′), and *Alfscsq* (5′-GAA TCT AGC TTG GAG GCT TAT GTC-3′) were used to identify the *LacZ* knock-in allele (490 bp), an intact floxed allele (560 bp), and the correctly deleted floxed allele (185 bp). Similarly, primers *Cre1* (5′-GCT GGT TAG CAC CGC AGG TGT AGA G-3′) and *Cre3* (5′-CGC CAG CTT CCA GCA GGC GCA CC-3′) were used to identify the presence of the *Le-Cre* transgene. PCR analysis was performed in the presence of 4 mM magnesium chloride under the following experimental conditions: 1 cycle of 95°C for 2 minutes; 30 cycles of 95°C for 45 seconds, 65°C for 45 seconds, and 72°C for 1 minute 30 seconds; and 1 cycle of 72°C for 15 minutes. Littermates that contained a wild-type copy of *AP-2α* and/or lacked the Cre-recombinase transgene were used as wild-type control animals.

Histology

Adult *Le-AP-2α* mice and wild-type littermates were euthanatized by a CO_2 overdose, and whole eyes were dissected and either prepared for frozen sectioning or fixed in 10% neutral-buffered formalin overnight at 4°C, processed, and embedded in paraffin. Serial paraffin-embedded sections were cut at 5 μm and either used for immunohistochemical analysis or stained with hematoxylin and eosin (H&E). Periodic acid-Schiff (PAS) stain was also used to label carbohydrates in the corneal basement membrane. Specimens for frozen sectioning were embedded in optimal temperature cutting compound (Tissue-Tek; Sakura-Finetek, Torrance,

CA). Cryosections were cut at 5 to 7 μm and immediately placed on coated slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA).

Immunohistochemical Analysis

Paraffin-embedded sections (5 μm thick) to be used for immunohistochemical analysis were first deparaffinized, rehydrated, and then treated with a 10 mM sodium citrate buffer (pH 6.0; boiling for 20 minutes) to uncover masked antigens. Subsequently, sections were incubated in a 3% hydrogen peroxide solution (30 minutes, room temperature) to quench endogenous peroxidase activity. Next, sections were blocked with normal (goat or horse) serum for 20 minutes at room temperature followed by incubation with one of the following specific primary antibodies: Proliferating cellular nuclear antigen (PCNA, PC10; Dako Corp., Carpinteria, CA; 1:1000, 1 hour at room temperature [RT]); or phospho-Smad2 (p-Smad2, 465/467; Cell Signaling Technology Inc., Beverly, MA; 1:100, overnight at 4°C). Secondary antibody treatment included incubation with biotinylated anti-rabbit or anti-mouse antibodies followed by amplification with avidin-biotin complex (ABC) reagent (Vector Laboratories, Burlingame, CA). For chromogenic visualization, sections were incubated with a substrate for horseradish peroxidase (Fast DAB; Dako Corp.).

Indirect immunofluorescence was used to detect proteins on frozen sections. Sections were air dried for 1 hour and then fixed in acetone (10 minutes) on ice. The sections were blocked with normal goat serum for 20 minutes (room temperature; [RT]) followed by incubation with one of the following primary antibodies: AP-2α at 1:100 for 1 hour at RT(SC 8975; Santa Cruz Biotechnology Inc., Santa Cruz, CA), E-cadherin at 1:200 for 1 hour at RT (Zymed Laboratories, South San Francisco, CA); entactin at 1:400 for 1 hour at RT (Chemicon International, Temecula, CA); laminin at 1:200 for 1 hour at RT (Sigma-Aldrich, St. Louis, MO); type IV collagen at 1:100 for 1 hour at RT (Cortex Biochem, San Leandro, CA); or TGF-β2 at 1:200 for 1 hour at RT (SC-90; Santa Cruz Biotechnology Inc.). The presence of primary antibody was visualized with either a fluorescein isothiocyanate (FITC)-or rhodamine-conjugated secondary anti-mouse, anti-rabbit (1:50; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) or anti-rat antisera, (1:50; Chemicon International, Inc.) for 1 hour at RT. Direct immunofluorescence was used to detect α-smooth muscle actin (α-SMA), with a Cy3-conjugated monoclonal anti α-SMA antibody (1:100, overnight at 4°C; Sigma-Aldrich). All sections were mounted in antifade mounting medium with 4,6-diamino-2-phenylindole (DAPI; Vector Laboratories) to reveal nuclear staining. For each experiment, a section was stained without primary antibody to serve as a negative control. All staining was visualized with a microscope (Leica, Deerfield, IL) equipped with an immunofluorescence attachment, and images were captured with a high-resolution camera and associated software (Open-Lab; Improvision, Lexington, MA). Images were reproduced for publication with image-management software (Photoshop 7.0; Adobe Systems Inc., Mountain View, CA).

Statistical Analysis

Differences in the number of PCNA-stained corneal epithelial cells between wild-type and *Le-AP-2α* mutant littermates were analyzed with a two-tailed Student's *t*-test (Prism software; GraphPad, San Diego, CA). Six wild-type and six *Le-AP-2α* mutant mice were subjected to PCNA analyses. Five sections were counted per animal at an average area of 0.01 mm^2. Data are represented as the mean number of PCNA-positive cells per square millimeter ± SEM. Differences are defined as significant at $P < 0.05$.

Results

Targeted Deletion of *AP-2α* in the Corneal Epithelium

A conditional knockout of *AP-2α* in the corneal epithelium was created to further understand the requirement for *AP-2α* in differentiation and maintenance of the corneal epithelium. Mice expressing a lens-placode–specific Cre-recombinase,[20] that were also heterozygous for an *AP-2α*-null mutation[21] were crossed with mice that were homozygous for a floxed allele of *AP-2α*,[21] to generate *Le-AP-2α* mutant mice. PCR analysis confirmed the Cre-mediated deletion of the floxed *AP-2α* allele in the corneal epithelium and not in other nonplacode–derived tissues, such as epidermis of the ear, or in non-mutant tissues (Fig. 1A). The *Le-AP-2α* mutant mice were viable with a normal lifespan, and ocular defects were confined to lens placode derivatives, including the corneal epithelium. These mice therefore provided us with a model for examining the requirement of the *AP-2α* gene in corneal epithelial differentiation.

The localized loss of the AP-2*α* protein in the mutant corneal epithelium was confirmed by immunofluorescence (Fig. 1B). Immunofluorescent staining with an AP-2*α*-specific antibody showed that in wild-type littermates AP-2*α* expression was confined to the nuclei of the more basally located cells of the corneal epithelium, as previously reported.[15,16] In comparison, AP-2*α* expression was completely abolished in the *Le-AP-2α* mutant corneal epithelium.

Disruption in Epithelial Morphology and Adhesion in the *Le-AP-2α* Mutant Cornea

Histologic examination of the adult *Le-AP-2α* mutant eye revealed defects in the cornea and lens, including an unusual adhesion between the lens epithelium and the peripheral corneal epithelium (Fig. 2A). This corneal–lenticular adhesion has also been observed in the *Le-AP-2α* mutant mice during embryonic stages (Pontoriero G et al. *IOVS* 2004;45:ARVO Abstract 2641). In adults, this adhesion persisted in only a proportion of the adult mutants and frequently involved the iris (Fig. 2A). Further examination of the corneal epithelium in adult mutant mice lacking the adhesion or of epithelia away from the adhesion defect revealed that there was a noticeable variation in the number of stratified layers, ranging from 2 to 3 layers in some locations and 7 to 10 in others (Fig. 2B). This was unlike the wild-type corneal epithelium, which consisted of 8 to 10 layers across the entire corneal surface. In addition, the surface of the *Le-AP-2α* mutant corneal epithelium was rough and exhibited excessive sloughing. A disruption in the organization and packing of the cells was also observed in the mutant corneas. For example, the basal cells which are typically columnar shaped as observed in the wild-type littermates, were more squamous shaped and exhibited irregular shaped cell borders. The basement membrane, or Bowman's layer, as revealed by periodic acid-Schiff stain, was also thinner in the mutants than in wild-type mice and in many regions was discontinuous (Fig. 2B).

To determine whether the reduction in the cell layers observed in the mutant corneal epithelium was due to a reduced level of cellular proliferation, the expression of PCNA, a marker of cellular proliferation was examined (Fig. 3). The staining revealed that many more of the suprabasal epithelial cells were immunoreactive to PCNA in the mutant epithelium than in the wild-type (Fig. 3A). The number of PCNA-positive cells was determined for wild-type ($n = 6$) and *Le-AP-2α* mutant corneas ($n = 6$) and divided by the total area examined (0.01 mm^2), to determine the mean number of PCNA-positive epithelial cells per millimeter squared. This revealed a significant increase (approximately 50.1%) in the number of PCNA-positive epithelial cells in *Le-AP-2α* mutant corneas compared with those of the wild-type (Fig. 3B). In contrast, TUNEL analysis indicated that the relative levels of cell death between the wild-type and mutant corneas were similar (data not shown). Thus, our analyses demonstrated that the reduced number of layers in the corneal epithelium of the mutant was not caused by a major increase in cell death

or decrease in cell proliferation, and instead suggests that a loss of tissue integrity might be causative.

The altered organization of the epithelium in the *Le-AP-2α* mutant cornea, combined with earlier data showing a regulatory role for AP-2*α* in E-cadherin promoter activity,[16,22–25] suggested that E-cadherin-mediated cellular adhesion may be compromised. In wild-type mice, immunofluorescence with an antibody specific for E-cadherin revealed strong E-cadherin staining (red) along the cell–cell borders of all cells in the stratified epithelial cell layers of the corneal epithelium (Fig. 4). In contrast, E-cadherin staining in the *Le-AP-2α* mutant cornea was substantially suppressed in all layers of the corneal epithelium.

Alteration in Basement Membrane Deposition in the *Le-AP-2a* Mutant Cornea

Because the previous histologic examination of the *Le-AP-2α*-mutant cornea revealed regions of the epithelium lacking Bowman's layer (Fig. 2B), the nature of this defect was further explored by examining the expression of known individual basement membrane components, including laminin, entactin, and type IV collagen (Fig. 5). In both the *Le-AP-2α* mutant mice and wild-type littermates, these components localized as bright bands directly beneath the epithelium. However, in the *Le-AP-2α* mutant cornea, large gaps in laminin (Fig. 5A) and entactin (Fig. 5B) expression were evident. Moreover, aggregates of both laminin and entactin appeared in the anterior region of the stroma of the *Le-AP-2α* mutant cornea. Type IV collagen was localized as a band of intense staining directly beneath the corneal epithelium in the *Le-AP-2α* mutants, and unlike wild-type mice (Fig. 5C), exhibited small interruptions.

Evidence of Stromal Defects in the *Le-AP-2α* Mutant Cornea

In addition to the corneal epithelial defects, the *Le-AP-2α* mutants also exhibited defects in the corneal stroma. The lamellar alignment of the collagen fibers in the anterior stroma of the *Le-AP-2α* mutants was irregular (Fig. 2) and in regions where the basement membrane was absent, underlying stromal fibroblasts had begun to accumulate. These fibroblasts exhibited strong immunoreactivity for PCNA, indicating that they had entered the cell cycle (Fig. 6A). In contrast, stromal cells in the corneas of littermate, wild-type, mice did not exhibit PCNA staining (Fig. 6A). The activation in proliferation suggests that the stromal cells of the mutant cornea had acquired an activated phenotype, as previously reported for stromal cells of injured corneas.[26,27] To examine this further, we looked for another marker indicative of stromal cell activation, *α*-SMA[27] (Fig. 6B). Unlike the wild-type corneas (Fig. 6B), which did not express *α*-SMA, a small number of fibroblasts directly beneath the epithelium of the *Le-AP-2α* mutant cornea showed *α*-SMA immunoreactivity. Previous studies have shown that the appearance of myofibroblasts in the stroma is dependent on a release of TGF-*β*2 from the epithelium into the stroma.[27] We therefore examined the expression of TGF-*β*2 protein in the *Le-AP-2α* mutant. Strong TGF-*β*2 expression was detected in the stroma of the *Le-AP-2α* mutant cornea (Fig. 6C), whereas in the wild-type mice TGF-*β*2 expression was absent in the stroma, and only the corneal epithelium stained positively (Fig. 6C). Furthermore, immunostaining for pSmad2, a downstream effector of TGF-*β*2 signaling, also showed immunoreactivity in the *Le-AP-2α* mutant corneal epithelium and some stromal cells, whereas expression of wild-type littermates was confined to the corneal epithelium (Fig. 6D). Together, these data provide evidence for corneal stromal cell activation in the *Le-AP-2α* mutant.

Discussion

Studies from our laboratory have shown an in vivo requirement for *AP-2α* in early development of the eye and lens.[15] However, relatively little is known about the specific role of *AP-2α* in corneal epithelial differentiation in vivo, because of the embryonic lethality and defects in the *AP-2α*-KO mice. In this study, targeted deletion of *AP-2α* in the developing lens placode using

the *Cre-loxP* approach resulted in abolished expression of AP-2*α* in the differentiating corneal epithelium. Unlike the full *AP-2α*-KO mice, the resultant mutant mice (*Le-AP-2α*) are viable, live to adulthood, and exhibit defects confined to lens ectoderm derivatives. Thus, an in vivo model (*Le-AP-2α*) for investigating the role of AP-2*α* in the postnatal differentiating corneal epithelium has been created. With this model, we have shown that, in the absence of AP-2*α*, the corneal epithelium forms stratified layers, albeit the number of stratified layers varied widely across the corneal surface and the morphology of the epithelium was compromised. These phenotypic changes further correlated with a substantial reduction in E-cadherin protein expression, as well as aberrant deposition of basement membrane components and further defects in the corneal stroma.

AP-2*α* expression is normally restricted to the basal cell compartments of the corneal epithelium and other epithelial tissues, including the eyelid epidermis, epidermis of the skin, and the undifferentiated population of cells of the lens, suggesting that it is appropriately positioned for controlling epithelial differentiation. AP-2*α* has also been shown to bind to and regulate the promoters of multiple epithelial genes that correlate with differentiation.[15,25,28,29] One of these genes is E-cadherin, a calcium-dependent transmembrane protein that forms functional junctions (adherens) between cells.[30] In the cornea, E-cadherin is normally expressed in all cell layers except for the basal membrane of the basal cells and is important in maintaining corneal epithelial structural and functional integrity.[31–33] A substantial decrease in E-cadherin expression in the *Le-AP-2α* mutant corneal epithelium was observed and concurs with earlier findings in our laboratory, which showed intermittent E-cadherin expression in the eyelid epidermis of *AP-2α* chimeric mice.[15] The reduced E-cadherin expression probably caused the altered integrity of the epithelial cell layers including the reduced packing and disrupted cellular organization. Reduced expression of E-cadherin may also have contributed to the variation in the number of stratified layers observed in the *Le-AP-2α* mutant corneas. Excessive sloughing of the epithelium was observed, and indeed, the lack of proper cell–cell adhesion could promote abnormal shedding of the more basal layers. A reduction in cell layers and increased loss of cells from the superficial layer of the corneal epithelium was reported for heterozygous *Pax6* ($Sey^{+/-}$) mutant mice, and similar to our model, this was correlated with altered expression of cell adhesion proteins.[11] Finally, as we found an increase in cellular proliferation in *Le-AP-2α* mutants, the corneal epithelium of the heterozygote *Pax6* ($Sey^{+/-}$) mutant mice exhibited an increase in proliferative index over wild-type littermates. As hypothesized for the *Pax6* ($Sey^{+/-}$) mice, we believe the most likely cause for the increase is the excessive loss of cells from the upper layers and subsequent disruption of the epithelial barrier, factors known to stimulate proliferation.[34]

Coincident with the defects in the *Le-AP-2α* mutant corneal epithelium were aberrations in the deposition of the underlying basement membrane components, including gaps in laminin, entactin, and to some extent, type IV collagen localization. The altered pattern of expression of the basement membrane proteins may be because they are direct downstream targets of AP-2*α*. For example, the entactin promoter contains multiple AP-2*α* binding sites.[35] However, because intermittent expression of the basement membrane proteins was evident, it is more likely that the altered pattern is a secondary defect. The corneal epithelium is responsible for secreting its underlying basement membrane components.[36] Thus, the altered epithelial cell integrity and structure in the mutant cornea may have led to the defects in basement membrane deposition. Aggregates of laminin and entactin were also observed in the mutant corneal stroma. Stromal staining of entactin has been reported after corneal keratectomy wounds.[27] Thus, the stromal staining for entactin and laminin in the mutant cornea may reflect a transition of corneal stromal cells to an “activated” phenotype. Alternatively, aberrant deposition of the basement membrane components in the mutant cornea may have resulted in their displacement into the corneal stroma. The corneas of heterozygote *Pax6* ($Sey^{+/-}$) mice also exhibit aggregates

of laminin in the corneal stroma,[11] further demonstrating an overlap in the corneal phenotype of these mice with that of the *Le-AP-2α* mutants.

Disruptions in the corneal basement membrane, as occurs in corneal disorders and after clinical procedures such as photorefractive keratectomy (PRK) have been shown to lead to epithelial–stromal interactions that promote stromal cell activation. When the basement membrane is kept intact, as in the newer corrective procedure, laser in situ keratomileusis (LASIK), the interaction between the epithelium and the stroma is prevented or minimized and as a result, a fibrotic response does not ensue.[37] The *Le-AP-2α* mutant mice exhibited defects in the basement membrane of the cornea and correspondingly showed many features of corneal stromal cell activation, including an increase in proliferation of stromal cells and induction of a myofibroblast phenotype as shown by their immunoreactivity to α-SMA. Recent work in mice has shown that TGF-β2, a major serum cytokine expressed by corneal epithelial cells, is the fibrotic regulator and, after disturbance of the basement membrane, is released into the corneal stroma.[27] Once in the corneal stroma, TGF-β2 induces many aspects of the fibrotic repair phenotype including an induction in proliferation and morphologic transformation of the stromal cells into myofibroblasts. In agreement with this model, TGF-β2 protein was detected in the *Le-AP-2α* mutant in both the corneal stroma and epithelium, whereas it was confined to the epithelium in wild-type littermates. The αSMA expressing cells were localized to regions of the *Le-AP-2α* mutant cornea where the basement membrane appeared discontinuous (Fig. 6), further demonstrating the importance of an intact basement membrane in preventing these epithelial–mesenchymal interactions.

Mutations in the human *AP-2α* gene (*TFAP2A*) have yet to be directly correlated with human ocular disorders. However, the fact that *TFAP2A* maps to chromosome 6, region p24, a region associated with multiple ocular disorders,[38] suggests the likelihood of *AP-2α*'s being involved in ocular genetic disease in humans. A role for *AP-2α* in corneal development and differentiation in humans is supported by findings that hemizygous deletions of the 6p24-25 chromosomal region, including the *AP-2α* gene, resulted in ocular phenotypes that included corneal clouding and sclerocornea.[39] Similar to the *AP-2α* KO and chimeric mice, the patient with corneal clouding also exhibited craniofacial and limb anomalies,[39] providing further evidence that specific deletion of *AP-2α* may be responsible for these observed phenotypes.

In summary, our findings have revealed, through the use of a conditional KO model of *AP-2α*, that *AP-2α* is required for normal stratification and cellular organization of the corneal epithelium. In the absence of *AP-2α* in the *Le-AP-2α* mutant, we observed altered morphology of the corneal epithelium with a reduction in cell layers that was correlated with a substantial downregulation of E-cadherin expression. In addition, disruptions in the basement membrane were found, which probably led to epithelial–stromal interactions in the mutant cornea, that are not evident in wild-type littermates. Because we have shown in earlier studies that *AP-2α* participates in regulating genes required for corneal epithelial repair,[14,17] it would be of interest to employ the *Le-AP-2α* mutant model in regenerative studies to test the in vivo requirement for *AP-2α* in controlling reepithelialization and stratification of the corneal epithelium after injury.

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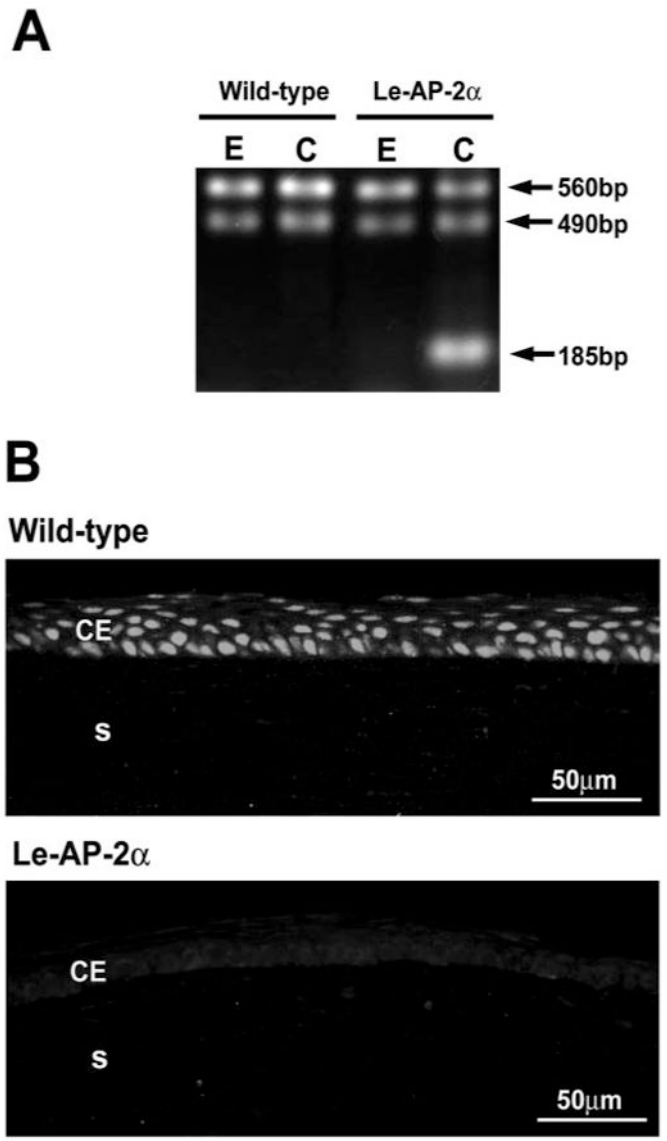

Figure 1.

Conditional knockout of *AP-2α* in the corneal epithelium. (**A**) PCR analysis of the *Le-Cre*-mediated deletion using the primers *Alflp, Alflox4*, and *Alfscsq*. The *Le-AP-2α* mouse tissues contain the *AP-2α* null allele and this generates a 490-bp product with primers *Alflox4* and *Alfscsq* (E, ear; C, cornea). In addition, these tissues also contain the undeleted allele that generates a band of 560 bp with primers *Alflox4* and *Alfscsq*. The deleted conditional allele produces a 185-bp band from primers *Alflp* and *Alfscsq* and is only detected in the cornea of *Le-AP-2α* mutants in the presence of *Le-Cre*. (**B**) Immunolocalization of AP-2*α* in the corneal epithelium of wild-type versus *Le-AP-2α* mutant mice. A FITC-conjugated secondary antibody was used to show specific staining for AP-2*α*. AP-2*α* was expressed in the more basally situated cells in the corneal epithelium of wild-type mice, whereas its expression was completely abolished in the corneal epithelium of the *Le-AP-2α* mutant mice. Some residual expression in the corneal epithelium is due to background in the *Le-AP-2α* mutant mice. CE, corneal epithelium; S, stroma.

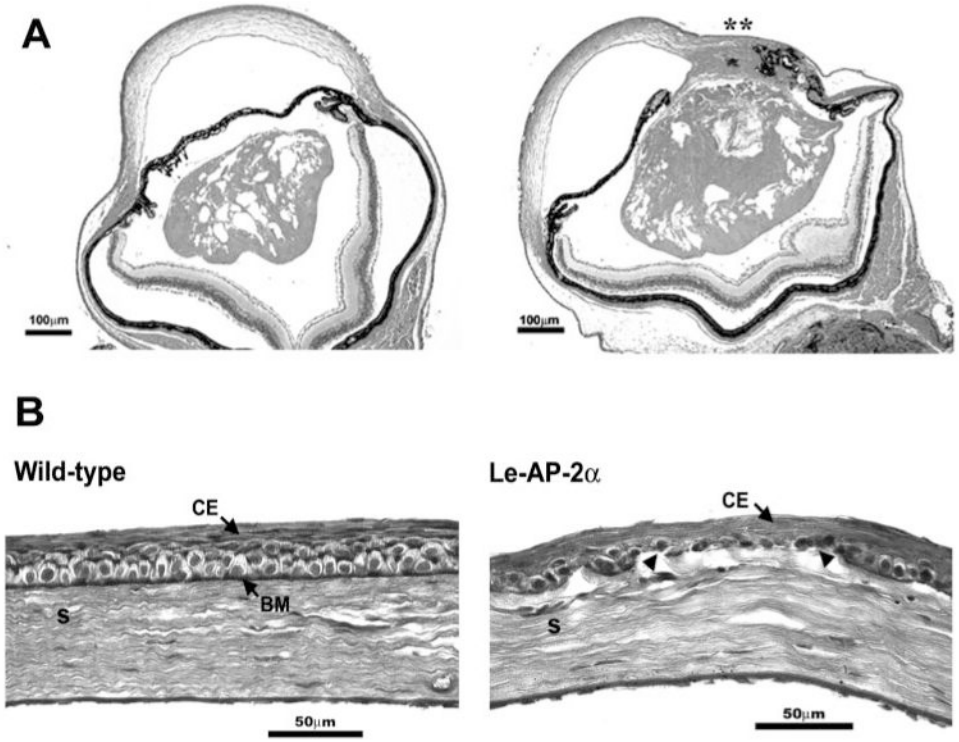

Figure 2.

(**A**) Hematoxylin and eosin-stained sections of adult *Le-AP-2α* mutant eyes. The mutant eye on the left exhibits a dysmorphic lens but no adhesion to the overlying cornea. The mutant eye on the right exhibits a severe adhesion of the lens to the peripheral cornea and the iris (**). (**B**) PAS staining of the basement membrane (Bowman's layer) in wild-type and *Le-AP-2α* mutant mouse corneas. The basement membranes of the corneas of wild-type mice were intact. In the *Le-AP-2α* mutant cornea, the basement membrane was thinner or absent (*arrowheads*) in some regions. CE, corneal epithelium, S, stroma.

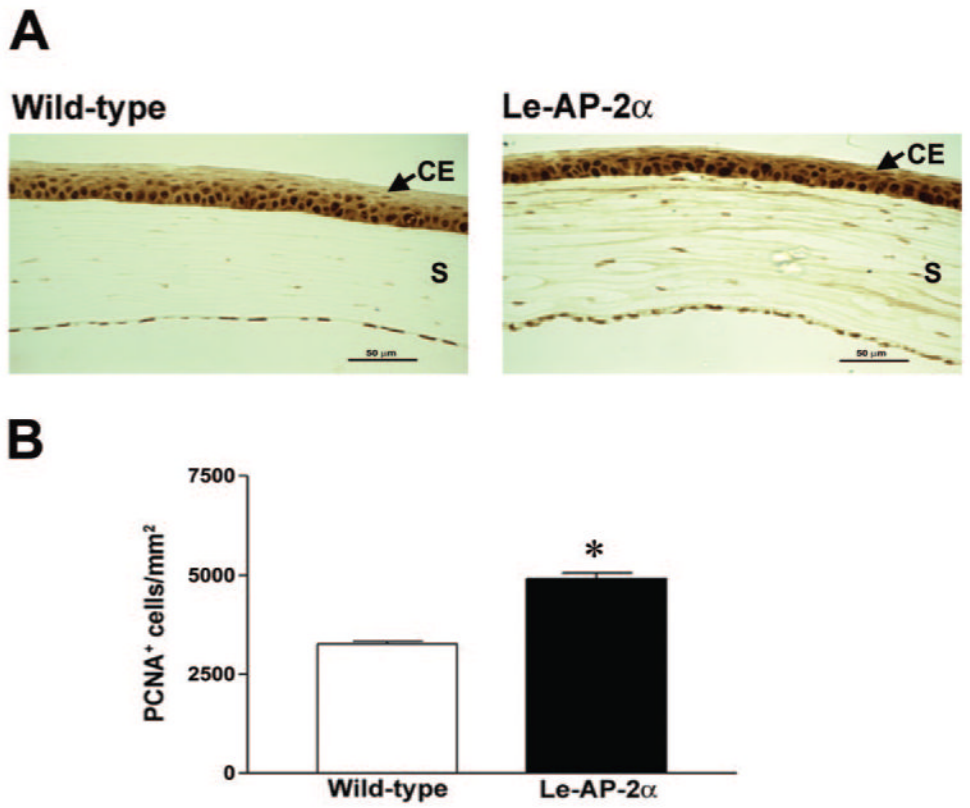

Figure 3.

(**A**) Immunostaining for PCNA in the corneal epithelium (CE) of wild-type and *Le-AP-2α* mutant mice. The corneal epithelium of *Le-AP-2α* mutant mice exhibited increased staining for PCNA in the suprabasal cell compartment compared with corneas of wild-type mice. (**B**) Statistical analysis of PCNA staining in the *Le-AP-2α* mutant versus wild-type corneal epithelium. The *Le-AP-2α* corneal epithelium showed a significant increase in the number of PCNA-positive corneal epithelial cells compared with wild-type littermates ($n = 6$; $^*P < 0.05$). S, stroma.

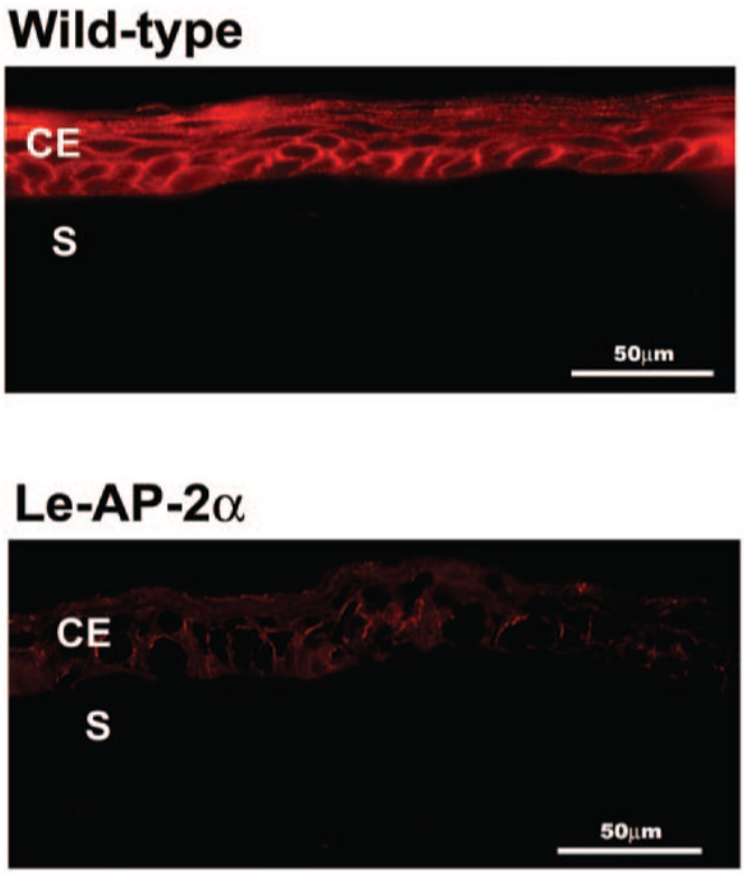

Figure 4.
Immunolocalization of E-cadherin in wild-type and *Le-AP-2α* mutant mouse corneas. E-cadherin staining (*red*, rhodamine-conjugated antibody) was observed along the cell–cell borders in all the stratified layers of the corneal epithelium (CE) of wild-type mice. In the *Le-AP-2α* mutant corneal epithelium, E-cadherin expression was substantially decreased. The integrity of the corneal epithelial layers was also altered in the *Le-AP-2α* mutant corneas. S, stroma.

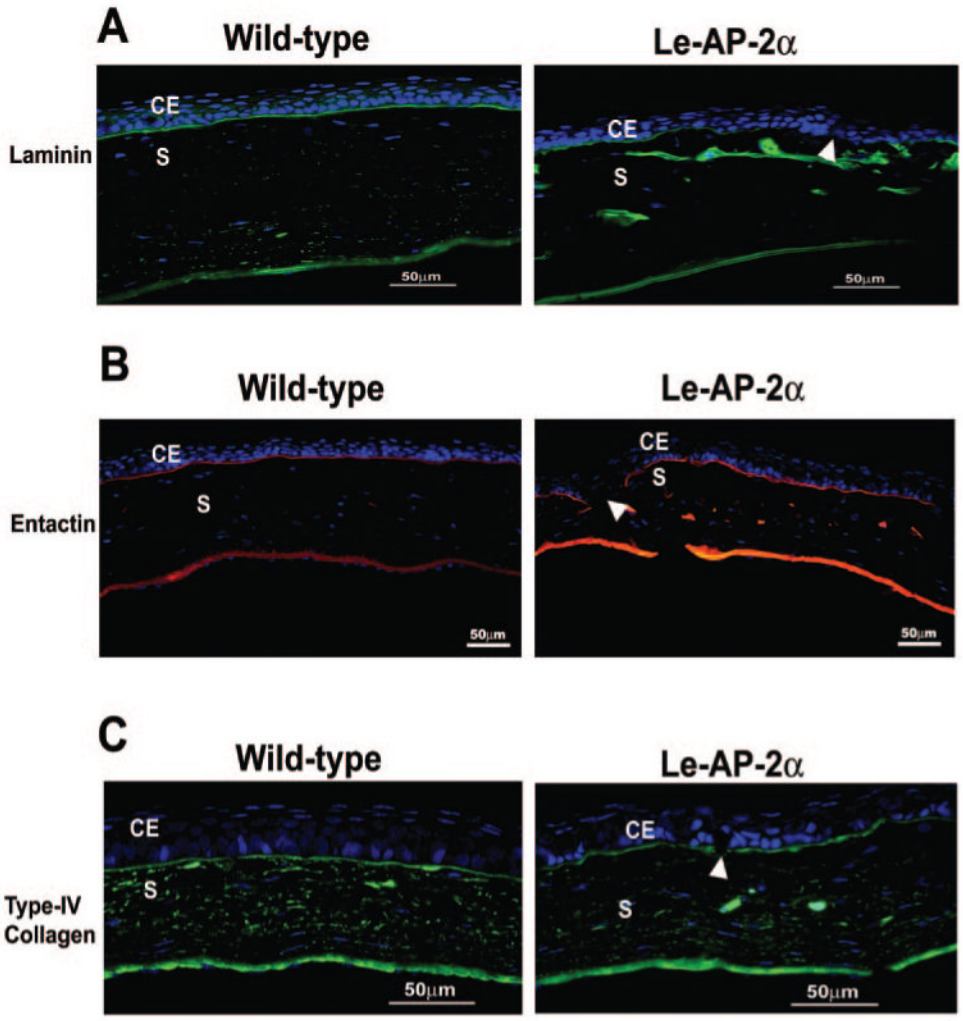

Figure 5.

Immunolocalization of basement membrane components (**A**) laminin, (**B**) entactin, and (**C**) type IV collagen in the *Le-AP-2α* mutant versus wild-type cornea. (**A**) Laminin was detected in the basement membrane underlying the corneal epithelium (CE) and the endothelium of wild-type and *Le-AP-2α* mutant mice by immunofluorescence with a FITC-conjugated secondary antibody (*green*). Note the discontinuous laminin staining (*arrowhead*) in the *Le-AP-2α*-mutant corneas and appearance of large aggregates of laminin in the anterior region of the stroma (S) in the *Le-AP-2α* mutant corneas. (**B**) Immunolocalization of entactin was observed in the basement membrane underlying the corneal epithelium and the endothelium of wild-type and *Le-AP-2α* mutant corneas using a rhodamine-conjugated secondary antibody (*red*). Note the discontinuous entactin staining in the *Le-AP-2α* mutant corneas with aggregates of entactin in the stroma (*arrowhead*). (**C**) Type IV collagen was localized with an FITC-conjugated secondary antibody (*green*). Type IV collagen was localized as a band of intense staining in the basement membrane underlying the corneal epithelium and the endothelium of both the wild-type and *Le-AP-2α* mutant corneas; however, note the small breaks in staining in the mutant (*arrowhead*). In all panels, the nuclei were colocalized by staining with DAPI (*blue*).

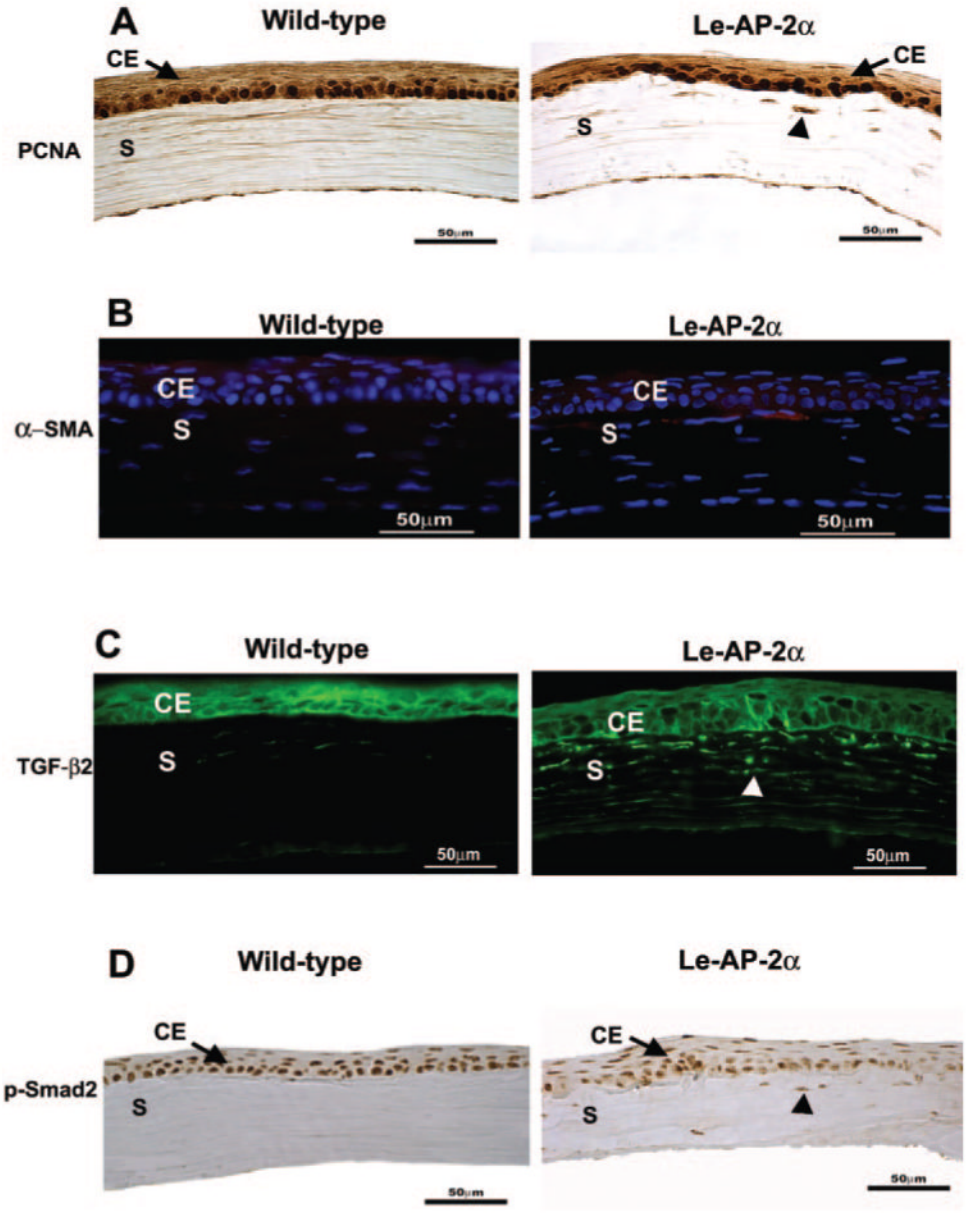

Figure 6.

Markers of fibroblast activation are evident in the *Le-AP-2α* corneal stroma (S). (**A**) The proliferating status of the corneal stromal cells was determined by immunostaining for PCNA. Little to no proliferative response was observed in the stroma of the wild-type cornea. In contrast, stromal fibroblasts (*arrowhead*) in the *Le-AP-2α* mutant cornea exhibited immunoreactivity for PCNA, particularly in regions where the basement membrane was absent. (**B**) The activated response was further examined by immunofluorescent localization of *α*-SMA, with a CY3-conjugated secondary antibody (*red*). Cell nuclei were stained with DAPI (*blue*) to delineate the location of cells. The wild-type corneas did not exhibit any *α*-SMA protein expression, whereas some fibroblasts beneath the epithelium in the *Le-AP-2α* mutant corneas showed immunoreactivity to *α*-SMA. (**C**) TGF-*β*2 was immunolocalized with a FITC-conjugated secondary antibody (*green*). In wild-type mice TGF-*β*2 staining was absent in the corneal stroma and only evident in the corneal epithelium (CE). In the *Le-AP-2α* mutant cornea,

both the corneal epithelium and stroma (S; *arrowhead*) stained positively for TGF-*β*2 (*green*). (**D**) Immunostaining for phospho-Smad2 (p-Smad2), a downstream effector of TGF-*β*2 signaling, showed that in wild-type mice, p-Smad2 was localized only in the epithelium, whereas in the *Le-AP-2α* mutant cornea both the stroma (*arrowhead*) and epithelium showed immunoreactivity to p-Smad2.