Conditional Deletion of the Mouse *Klf4* Gene Results in Corneal Epithelial Fragility, Stromal Edema, and Loss of Conjunctival Goblet Cells^{∇†}

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The Krüppel-like transcription factor KLF4 is among the most highly expressed transcription factors in the mouse cornea (B. Norman, J. Davis, and J. Piatigorsky, *Investig. Ophthalmol. Vis. Sci.* 45:429–440, 2004). Here, we deleted the *Klf4* gene selectively in the surface ectoderm-derived structures of the eye (cornea, conjunctiva, eyelids, and lens) by mating *Klf4-LoxP* mice (J. P. Katz, N. Perreault, B. G. Goldstein, C. S. Lee, P. A. Labosky, V. W. Yang, and K. H. Kaestner, *Development* 129:2619–2628, 2002) with *Le-Cre* mice (R. Ashery-Padan, T. Marquardt, X. Zhou, and P. Gruss, *Genes Dev.* 14:2701–2711, 2000). *Klf4* conditional null (*Klf4*CN) embryos developed normally, and the adult mice were viable and fertile. Unlike the wild type, the *Klf4*CN cornea consisted of three to four epithelial cell layers; swollen, vacuolated basal epithelial and endothelial cells; and edematous stroma. The conjunctiva lacked goblet cells, and the anterior cortical lens was vacuolated in *Klf4*CN mice. Excessive cell sloughing resulted in fewer epithelial cell layers in spite of increased cell proliferation at the *Klf4*CN ocular surface. Expression of the keratin-12 and aquaporin-5 genes was downregulated, consistent with the *Klf4*CN corneal epithelial fragility and stromal edema, respectively. These observations provide new insights into the role of KLF4 in postnatal maturation and maintenance of the ocular surface and suggest that the *Klf4*CN mouse is a useful model for investigating ocular surface pathologies such as dry eye, Meesmann's dystrophy, and Steven's-Johnson syndrome.

The transparent cornea is the most anterior and chief refractive tissue of the mammalian eye and also serves as a barrier against environmental insults. It is a multilayered tissue comprising an outer stratified squamous epithelium, an inner monolayered endothelium, and a central stroma with regularly arranged collagen lamellae sparsely populated by keratocytes (30). Defects in development and/or maintenance of the cornea result in severe defects in vision (38, 68). In the mouse, following eye opening around 2 weeks after birth, cells in the one- to two-cell-layered epithelium proliferate and differentiate to form a four- to five-cell-layered stratified squamous epithelium by 20 days after birth (74). The mature corneal epithelium containing five to eight cell layers is present by 6 to 8 weeks after birth (15, 30). This program of proliferation and differentiation continues in the adult mouse, allowing the most superficial corneal epithelial cells that are sloughed off at a regular basis to be continually replaced by the differentiating basal cells moving up from the underlying layers, which in turn are replaced by the centripetal migration of the newly formed basal epithelial cells originating from the limbal stem cells (6, 15, 16, 40, 45, 64).

Relatively little is known about the genetic network of transcription factors required for embryonic morphogenesis, postnatal maturation, and maintenance of cornea (1, 2, 10, 12, 20, 23, 26, 32, 39, 46, 47, 55, 59, 60, 65). Serial analysis of gene expression identified KLF4, a member of the Krüppel-like transcription factor (KLF) subfamily of Cys2-His2 zinc finger proteins, as one of the most highly expressed transcription factors in both 9-day-old and 6-week-old mouse cornea (50). At least 15 members of the KLF family, all capable of binding the “GT box” or “CACCC” element and expressed in a tissue-selective manner, have been identified in mammals (7, 17). Different KLFs are expressed in the mouse and the human cornea and conjunctiva (11, 46, 50). Beginning at embryonic day 10, KLF4 is expressed in a stripe of mesenchymal cells extending from the forelimb bud to the developing eye (25). In the adult mouse, KLF4 is expressed in differentiated postmitotic epithelial cells of the skin and gastrointestinal tract (27, 57), as well as cornea (50). *Klf4* null mice die within 15 h after birth due to late-stage defects in skin barrier formation (56). *Klf4* is considered a tumor suppressor, inasmuch as ectopic expression induces cell cycle arrest (34). *Klf4* is frequently silenced or deleted in human gastrointestinal tumors (71), and deletion of *Klf4* in the gastric epithelium results in polyps and hyperplasia in mice (36). KLF4 inhibits cell proliferation by promoting the expression of p21 (53, 54). KLF4 also plays a critical role in colonic epithelial goblet cell differentiation (37).

Even though KLF4 is among the most highly expressed transcription factors in the cornea, its role in postnatal matu-

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ration of cornea is not known due to perinatal lethality of *Klf4* null mice (56). Genetic mosaics generated through tissue-selective expression of Cre recombinase provide a viable alternative in cases where knockouts result in either premature lethality or complex phenotype (9, 15). In this study, we have investigated the role of KLF4 in ocular surface morphogenesis by conditionally deleting the *Klf4* gene by mating *KLF4-LoxP* mice (37) with *Le-Cre* mice (5, 23). By this approach, *Klf4* expression was abolished in the surface ectoderm-derived structures of the eye, including cornea, lens, and conjunctiva, while normal expression levels were maintained in the rest of the body. The resultant *Klf4* conditional null (*Klf4*CN) mice were viable and fertile. While at postnatal day 1 *Klf4*CN mice looked normal, 8-week-old *Klf4*CN mice exhibited multiple ocular defects, including corneal epithelial fragility, stromal edema, defective lens, and loss of conjunctival goblet cells. These observations establish that the Krüppel-like transcription factor KLF4 has a critical role in postnatal ocular surface maturation and maintenance.

MATERIALS AND METHODS

Conditional disruption of *Klf4*. The derivation and use of *Klf4-LoxP* (37) and *Le-Cre* (5) mice has been described previously. *Klf4^{loxP/loxP} Le-Cre^{-/-}* mice were mated with *Klf4^{loxP/loxP}* mice to obtain equal proportions of *Klf4^{loxP/loxP} Le-Cre^{-/-}* (*Klf4*CN) and *Klf4^{loxP/loxP}* (control) offspring. Genomic DNA isolated from tail clippings of these mice was assayed for the presence of the *Klf4-LoxP* and *Le-Cre* transgenes by PCR using specific primers. Mice studied here were on a mixed genetic background and maintained in accordance with the guidelines set forth by the Animal Care and Use Committee of the National Eye Institute, NIH.

Histology. Eyeballs from carbon dioxide-asphyxiated mice were fixed in freshly prepared 4% paraformaldehyde (Sigma Chemical Company, St. Louis, MO) in phosphate-buffered saline (PBS) for 24 h at 4°C, embedded in glycol methacrylate (Polysciences, Warrington, PA), sectioned, and stained with hematoxylin and eosin or by the periodic acid-Schiff stain (PAS) procedure. For staining with alcian blue, paraformaldehyde-fixed heads were decalcified and embedded in paraffin (Sigma Chemical Company, St. Louis, MO). Light microscopy was performed with a Zeiss Axioplan 2 microscope and the images captured using a Spot RT color camera (Diagnostic Instruments, Inc., San Diego, CA). Midsections from four different eyeballs each were used for ocular measurements in the wild-type and *Klf4*CN corneas. For transmission electron microscopy (TEM), ultrathin sections were collected on 300-mesh grids (copper discs of 3 mm in diameter, with 300 squares in each grid) from the eyeballs fixed in a solution containing 2.5% glutaraldehyde, 6% sucrose, and 50 mM sodium cacodylate buffer (pH 7.2) for a minimum of 24 h at room temperature and stained with uranyl acetate and lead citrate. Images were captured with a JEM-100CX electron microscope (JEOL USA, Inc., Peabody, MA). Scanning electron microscopy (SEM) was performed on eyes fixed in 4% formaldehyde and 2% glutaraldehyde for a minimum of 24 h at the NCI-Frederick electron microscopy core facility, using a Hitachi S-570 scanning electron microscope equipped with a GW backscatter detector.

Measurement of cell proliferation by BrdU incorporation. Cryosections of eyes from age-matched wild-type or *Klf4*CN littermates intraperitoneally injected with 100 µg 5-bromo-2'-deoxyuridine (BrdU) per gram of body weight and sacrificed 24 h later were fixed in buffered 4% paraformaldehyde for 30 min, treated with 2 N HCl in 0.5% Triton X-100 for 30 min, washed thrice with PBS containing 0.1% Tween 20 (PBST) for 10 min each, blocked in 10% sheep serum in a humidified chamber, incubated with a 1:100 dilution of anti-BrdU monoclonal antibody (Sigma Chemical Company, St. Louis, MO) for 2 h at room temperature, washed thrice for 10 min each, and incubated with 1:300 dilution of Alexafluor-conjugated rabbit anti-mouse antibody (Molecular Probes, Carlsbad, CA) for 1 h. Following three washes of 10 min each, these sections were mounted with Prolong Gold antifade reagent with DAPI (4',6'-diamidino-2-phenylindole) (Molecular Probes, Carlsbad, CA) and observed with a Zeiss Axioplan 2 fluorescence microscope.

Isolation of total RNA, RT-PCR, and real-time Q-RT-PCR. Total RNA was isolated from dissected corneas by using the RNeasy minikit (QIAGEN, Valencia, CA). Eluted RNA was quantified, the concentration adjusted with RNase-free water to 100 ng/µl, and one step reverse transcription-PCR (RT-PCR)

performed using 100 ng total RNA and Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ). The forward and reverse primers used were located on adjacent exons such that the amplification products from contaminating genomic DNA, if any, could be distinguished from those originating from the mRNA. *Klf4* forward (5'-TGCCAGACCAGATGCAGTCAC-3'), *Klf4* reverse (5'-GTAGTGCTGGTTCAGTTCATC-3'), RNA polymerase II forward (5'-GCCATGCAGAAGTCTGGCCGTCCTCCCTCAAG-3'), and RNA polymerase II reverse (5'-CTTATAGCCAGTCTGCAGATGAAGGTAC-3') primers were used to amplify the 260-bp *Klf4* and the 354-bp RNA polymerase II gene products, respectively. The RT-PCR products were separated on a 1.5% agarose gel using Tris-borate-EDTA buffer.

The reagents, equipment, and software for TaqMan gene expression real-time quantitative RT-PCR (Q-RT-PCR) assays were obtained from Applied Biosystems, Foster City, CA. The High Capacity cDNA Archive Kit was used to generate cDNA, using total RNA isolated from pooled corneas of 10 wild-type or *Klf4*CN mice. Q-RT-PCR assays with prestandardized gene-specific probes for the KLF4, keratin-12, and aquaporin-5 genes were performed in a 7900HT thermocycler with 18S rRNA as an endogenous control, and the results were analyzed using SDS software version 2.1.

Immunoblots and immunohistochemistry. Equal amounts of total protein extracted by homogenizing dissected corneas in 8.0 M urea, 0.08% Triton X-100, 0.2% sodium dodecyl sulfate, 3% β-mercaptoethanol, and proteinase inhibitors and quantified by the bicinchoninic acid method (Pierce, Rockford, IL) were separated by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and subjected to immunoblot analysis. Rabbit anti-keratin-12 (a kind gift from W. W. Kao, University of Cincinnati) (35), anti-aquaporin-5 (Calbiochem, La Jolla, CA), anti-KLF4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and antiactin antibody (Sigma Chemical Company, St. Louis, MO) were used as primary antibodies at a 1:1,000 dilution in PBST. Horseradish peroxidase-coupled anti-rabbit immunoglobulin G (Amersham Biosciences, Piscataway, NJ) was used as a secondary antibody at a 1:5,000 dilution. Immunoreactive bands were identified by chemiluminescence following incubation with Super Signal West Pico solutions (Pierce, Rockford, IL).

For immunohistochemistry, 10-µm-thick cryosections from OCT-embedded eyeballs were fixed in freshly prepared buffered 4% paraformaldehyde for 30 min, blocked with 10% sheep serum in PBST for 1 h at room temperature in a humidified chamber, washed twice with PBST for 5 min each, incubated with a 1:100 dilution of the primary antibody for 1 h at room temperature, washed thrice with PBST for 10 min each, incubated with secondary antibody (Alexafluor 555-coupled goat anti-rabbit IgG antibody; Molecular Probes, Carlsbad, CA) at a 1:300 dilution for 1 h at room temperature, washed thrice with PBST for 10 min each, mounted with Prolong Gold antifade reagent with DAPI (Molecular Probes, Carlsbad, CA), and observed with a Zeiss Axioplan 2 fluorescence microscope.

Construction of reporter vectors, cell culture, and analysis of promoter activities. Mouse genomic DNA was used to amplify the *Aqp5* -502/+22-bp promoter by using the downstream +22/+2C (+22ATGCAAGCTTCGAGTCTGGAAGTCCCTCTC+2C) and upstream -502/-482 (ATGCCTCGAGGACCAACAGGGACAAGAAGC) primers and to amplify the *Krt12* -531/+49-bp promoter by using the downstream +49/+27C (ATGCAAGCTTAAGCGACATGCTGTGTCTGGAGA) and upstream -531/-509 (ATGCCTCGAGGCAGATGCTCTCAGAGCCTTGC) primers. These promoter fragments were cloned upstream of the luciferase reporter gene in pGL3Basic vector (Promega, Madison, WI) digested with HindIII and XhoI to generate reporter vectors pAqp5-Luc and pKrt12-Luc, respectively. The plasmid pCI-Klf4, in which the full-length *Klf4* gene is expressed under the control of the cytomegalovirus promoter, was a kind gift of Janine Davis, NEI. Simian virus 40-transformed human corneal epithelial (HCE) cells (3) were grown at 37°C in Dulbecco's modified Eagle medium-Ham's F-12 supplemented with 10% fetal bovine serum, 0.5% (vol/vol) dimethyl sulfoxide, cholera toxin (0.1 µg/ml), epidermal growth factor (10 ng/ml), insulin (5 µg/ml), gentamicin (40 µg/ml), and glutamine (20 mM) in a humidified chamber containing 5% CO₂ in air. Cells in six-well plates in mid-log phase of growth were transfected with 0.5 µg of pAqp5-Luc or pKrt12-Luc along with 10 ng pRL-SV40 (Promega, Madison WI) for normalization of transfection efficiency and 0.5 µg of pCI or pCI-Klf4, using 3 µl of Eugene 6 reagent (Roche Molecular Biochemicals). After 2 days, cells were washed with cold PBS and lysed with 500 µl of passive lysis buffer (Promega, Madison, WI). The lysate was clarified, and 50 µg lysate was analyzed using a dual-luciferase assay kit (Promega, Madison WI) and a Victor microplate luminometer (Perkin-Elmer). The measurement was integrated over 10 seconds with a delay of 2 seconds. Results from at least three independent experiments, normalized for transfection effi-

FIG. 1. Conditional deletion of the mouse *Klf4* gene. (A) Structure of the *Le-Cre* and *Klf4-LoxP* transgenes used. *Cre* recombinase is under the control of *Pax6* P0 promoter and lens/pancreas specific enhancer (top). NLS-Cre, Nuclear localization signal fused to *Cre* recombinase; IRES-GFP, internal ribosome entry site fused to the gene encoding green fluorescent protein. The *Klf4-LoxP* transgene contains *LoxP* sites (triangles) inserted in the first and third introns. In *Klf4CN* tissues expressing *Cre*, the second and third exons are excised out, fusing the first exon out of frame with the fourth exon of the *Klf4* gene. (B) RT-PCR of *Klf4* and RNA polymerase II transcripts in the total RNA from wild-type and *Klf4CN* corneas. (C) Relative quantities of *Klf4* transcripts in the wild-type (WT) and *Klf4CN* corneas as measured by Q-RT-PCR analysis. (D) Immunohistochemistry with anti-KLF4 antibody in wild-type and *Klf4CN* corneas. Reactions using wild-type corneas without primary antibody served as controls. Left, DAPI-stained nuclei. Right, fluorescence from secondary antibody against anti-KLF4 antibody.

ciency using the simian virus 40 promoter-driven *Renilla* luciferase activity, were used to obtain mean promoter activities.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was performed following the EZ-ChIP protocol suggested by Upstate USA, Inc. (Charlottesville, VA). DNA-bound proteins were cross-linked to DNA by treatment with 1% paraformaldehyde. The chromatin was then purified and sonicated to generate 200- to 1,000-bp-long fragments and immunoprecipitated with either preimmune serum or anti-KLF4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and protein G-Sepharose. Following the reversal of cross-linking by heating overnight at 65°C in the presence of NaCl and purification of eluted DNA, Krt12 and Aqp5 promoter fragments were detected by PCR with hKrt12 +48/+23C (CATGGTGTGTTGTTGGAGAGATCCATG) and hKrt12 -378/-355 (AGCATAAGGTTTAGGAAGAAGTAT) primers and with hAqp5 +9/

-15C (CGTCTAGCTCCGCCGCTTTACCGCG) and hAqp5 -370/-347 (GATCCGTTGCCTAGTCCAGGTACT) primers, respectively.

RESULTS

Conditional disruption of *Klf4* in the surface ectoderm-derived tissues of the eye. The structure of the transgenes used to generate *Klf4CN* mice is displayed in Fig. 1A. The utility of *Klf4-LoxP* and *Le-Cre* mice for generating mice that are conditional null in specific target tissues has been demonstrated (5, 23, 37). *Klf4^{loxP/loxP} Le-Cre/-* mice were mated with *Klf4^{loxP/loxP}*

FIG. 2. Morphology and histology of *Klf4CN* eyes, showing comparisons of wild-type and *Klf4CN* external appearance (A and B, respectively), eyes (C and D), eyeballs (E and F), dissected cornea and iris viewed from the position of the lens (G and H), midsection of the whole eye (I and J) (magnification, $\times 25$), central cornea (K and L) (magnification, $\times 400$), lens anterior (M and N) (magnification, $\times 400$), and lens equator (O and P) (magnification, $\times 400$). Arrows in panels E and G indicate a well-formed pupil in the wild-type eye; arrows in panels F and H indicate the iris hypertrophy and absence of pupil in the *Klf4CN* eye. An epithelial bullus in the *Klf4CN* cornea is indicated (arrowhead in panel L). The whorl-shaped rosette in the wild-type retina to the left of the optic nerve is an artifact of sectioning.

mice to generate roughly equal numbers of *Klf4*CN (*Klf4^{loxP/loxP} Le-Cre/-*) and wild-type control (*Klf4^{loxP/loxP}*) littermates. Unlike the *Klf4* null mice, which die soon after birth, the *Klf4*CN mice displayed normal viability and fertility. RT-PCR using total RNA from corneas of 8-week-old mice detected *Klf4* transcripts in the wild-type but not the *Klf4*CN cornea (Fig. 1B). Amplification of equal amounts of RNA polymerase II transcripts from the wild-type and *Klf4*CN corneas served as a control for the amount and integrity of total RNA used in these reactions (Fig. 1B). Q-RT-PCR using 18S rRNA as an endogenous control showed that the *Klf4* transcripts are roughly 10-fold reduced in the *Klf4*CN compared to the wild-type cornea (Fig. 1C). Immunohistochemistry with cryosections of eyeballs from 8-week-old mice demonstrated that the wild-type but not the *Klf4*CN cornea contained KLF4 protein (Fig. 1D). Reactions where no primary antibody was used served as control in these experiments (Fig. 1D). Taken together, these results demonstrate that the conditional disruption of the *Klf4* gene provided us with a viable and fertile mouse model system to study the role of KLF4 in postnatal maturation of the ocular surface.

Defects in the morphology and histology of the *Klf4*CN eyes.

Comparison of the 1-day-old wild-type and *Klf4*CN pups by visual examination and histology revealed no major difference in the eyelid fusion or the structure of the developing eye (see Fig. S1 in the supplemental material). Visual examination of the 8-week-old adult mice indicated inflammation of the *Klf4*CN eyelids, unlike in the wild type (Fig. 2A through D). Enucleated *Klf4*CN eyeballs (Fig. 2F) were marginally smaller and displayed a rough and speckled surface compared to the wild-type counterparts (Fig. 2E). In roughly 20% of the *Klf4*CN eyes, the iris appeared hyperplastic, causing the pupil to be smaller or absent in some eyes (Fig. 2G and H). Histological examination of the hematoxylin-eosin-stained sections revealed abnormalities associated with the cornea and the lens in *Klf4*CN eyes (Fig. 2I through P). The *Klf4*CN corneal epithelium possessed three or four instead of the normal five to eight cell layers, with abnormally swollen and vacuolated, relatively less eosinophilic basal epithelial cells. Additionally, epithelial bulli were occasionally visible in the *Klf4*CN cornea (Fig. 2L). The *Klf4*CN stroma was edematous, and endothelial cells were swollen compared to those of the wild-type littermates (Fig. 2K and L). Measurements from four different sections each from the wild-type and the *Klf4*CN eyes showed that while the mean size of the *Klf4*CN eye and the lens decreased by about 12%, the mean *Klf4*CN stromal thickness increased by 45% (Table 1). Unlike the compactly packed fiber cells in the wild-type lens, the *Klf4*CN lens displayed vacuolated anterior cortical lens fiber cells beneath the epithelium, extending to the bow region (Fig. 2M, N, O, and P).

In addition to confirming the structural features described above determined by light microscopy (Fig. 2K and L), TEM revealed that the most superficial cells of the *Klf4*CN corneal epithelium contained fewer microvilli and showed a higher frequency of delamination than the wild-type cells (Fig. 3A and B). SEM, whose applicability for analysis of corneal surface is well documented (21), revealed a mix of variably electron-dense and light cells on the *Klf4*CN corneal surface, compared to the uniformly light surface on the wild-type corneas, at low magnification ($\times 500$) (Fig. 3C and D). At high magnification

TABLE 1. Ocular measurements from sections of wild-type and *Klf4*CN eyes

Measurement	Mean (SD) value (mm) in:	
	Wild-type mice	<i>Klf4</i> CN mice
Central cornea to optic nerve head	3.30 (0.05)	2.93 (0.18)
Anterior-posterior depth of lens	1.81 (0.08)	1.59 (0.32)
Equatorial width of lens	2.23 (0.13)	1.99 (0.28)
Thickness of corneal stroma	0.134 (0.0047)	0.092 (0.0045)

($\times 10,000$), a variable density of microvilli was observed on the *Klf4*CN corneal surface, with fewer microvilli on the darker cells than on the lighter cells, unlike the uniformly dense microvilli on the wild-type corneal surface (Fig. 3E and F). As the surface density of microvilli reflects the age of the cells, with the highest density of microvilli seen on older and lighter cells (21), a mix of variably electron-dense and lighter cells provides evidence of excessive cell sloughing in the *Klf4*CN corneas, unlike in the wild-type corneas. Furthermore, the darker cells in the *Klf4*CN corneas were at a relatively lower depth than the neighboring lighter cells, providing additional proof that they have been exposed due to premature sloughing off of the overlying cells (Fig. 3E and F). This higher frequency of appearance of electron-dense cells in SEM provides an indirect measure of the higher frequency of delamination at the *Klf4*CN epithelial surface, supporting our observation with TEM (Fig. 3A and B) and confirming that the structural integrity of the *Klf4*CN corneal epithelium is compromised.

The reduced number of epithelial cell layers in the *Klf4*CN cornea is not due to a reduced rate of cell proliferation. In order to determine if the reduced number of epithelial cell layers in the *Klf4*CN cornea is due to a reduced rate of cell proliferation, we compared the proportion of actively cycling cells in the wild-type and *Klf4*CN corneas by BrdU incorporation assay. The eyeballs from mice sacrificed 24 h after BrdU injection were cryosectioned in OCT compound, and BrdU incorporation was measured by immunohistochemistry with anti-BrdU antibody. We observed an approximately twofold increase in the number of BrdU-incorporating cells, from 40 in the wild-type to 77 in the *Klf4*CN corneal epithelium (Fig. 4). This increase in cell proliferation is consistent with the known antiproliferative property of KLF4 and suggests that the reduced number of cells at the *Klf4*CN corneal surface is due to excessive cell sloughing rather than a reduced rate of cell proliferation. In addition to the absence of the antiproliferative influence of KLF4, excessive cell sloughing at the apical surface could possibly stimulate compensatory cell proliferation in the *Klf4*CN corneal epithelium (14, 16).

Conjunctival goblet cell development is compromised in *Klf4*CN mice. Conjunctival goblet cells play a critical role in the physiology of the ocular surface by secreting the soluble, gel-forming acidic mucin-5 and other glycoproteins such as peroxidase, trefoil peptides, and defensins into the tear film (18). Because goblet cells do not develop in the *Klf4* null colon (33), we examined the development of conjunctival goblet cells in the *Klf4*CN mice. Paraformaldehyde-fixed, paraffin-embedded heads from 8-week-old wild-type and *Klf4*CN mice were decalcified, sectioned, and stained with alcian blue for acidic mucins or with PAS for a variety of glycoproteins and carbohydrates,

FIG. 3. Ultrastructural analysis of wild-type and *Klf4CN* corneas. (A and B) Transmission electron microscopy of wild-type (A) and *Klf4CN* (B) corneas. *Klf4CN* corneal epithelium contains fewer cell layers, fewer microvilli on the superficial cells (arrows), and swollen, spherical, and vacuolated (arrowheads) basal cells. Delamination at the surface of the *Klf4CN* corneas is indicated (long arrow in panel B). Magnification: $\times 5,000$. (C to F) Surface scanning electron microscopy at low (C and D) (magnification, $\times 500$) and high (E and F) (magnification, $\times 10,000$) magnifications. Unlike the uniformly stained cells at the wild-type corneal surface (C), the *Klf4CN* corneal surface contained a mix of electron-dense (arrows in panel D) and light cells.

including mucins. Alcian blue- or PAS-positive goblet cell clusters were detected in the conjunctival fornix of the wild-type but not the *Klf4CN* mice (Fig. 5). Therefore, conjunctival goblet cells fail to develop at the *Klf4CN* ocular surface, which in

turn may alter the tear film composition, causing epithelial fragility and inflammation.

Defects in the basement membranes of *Klf4CN* mice. Examination of the PAS-stained corneas revealed that the basement

FIG. 4. Increased cell proliferation in the *Klf4CN* corneal epithelium. Cryosections of eyeballs from BrdU-injected wild-type and *Klf4CN* mice probed by immunohistochemistry with anti-BrdU antibody (right panels) are shown. Bright-field images of the corresponding corneal sections are shown on the left.

layer beneath the epithelial cells was stained in the wild-type but not the *Klf4CN* corneas (Fig. 6A). Further examination by TEM confirmed that the basement layer beneath the epithelial cells is thinner in the *Klf4CN* corneas (Fig. 6B). There were fewer and less-electron-dense hemidesmosomes connecting the basal epithelial cells to the basement membrane in the *Klf4CN* corneas than in the wild-type corneas. In the *Klf4CN* anterior stroma, the mean number of collagen fibrils per unit cross-sectional area, counted from four different sections ($814 \text{ fibrils}/\mu\text{m}^2$; standard deviation, 23) was reduced to about 70% of the wild-type number ($1,130 \text{ fibrils}/\mu\text{m}^2$; standard deviation, 22), resulting in higher interfibrillar space, consistent with the *Klf4CN* stromal edema. Even though the *Klf4CN* endothelial cells were highly vacuolated, no major defects could be seen in the Descemet's membranes of *Klf4CN* corneas (Fig. 6A).

Downregulation of the keratin-12 (*Krt12*) gene in the *Klf4CN* cornea. Keratin-12 is a corneal epithelium-specific keratin expressed in a differentiation-dependent, developmentally regulated manner, which forms the cytoskeletal intermediate filaments linked to the intercellular desmosomes and hemidesmosomes bound to the basement membrane (41, 42, 62). Inasmuch as keratin-12 gene-deficient mice develop fragile corneal epithelia with fewer epithelial cell layers (35) resembling the *Klf4CN* corneal epithelium described above (Fig. 2 and 3), we examined the expression levels of the keratin-12 gene in the *Klf4CN* corneal epithelial cells. Real-time Q-RT-PCR analyses with 18S rRNA as an endogenous control showed that the *Klf4CN* corneal epithelial cells contained threefold fewer keratin-12 gene transcripts than the wild type (Fig. 7A). In immunoblot analysis, the 55-kDa keratin-12 protein in the *Klf4CN*

corneal extracts was reduced to approximately 30 to 40% of that in the wild type, confirming that the keratin-12 gene expression is downregulated in the *Klf4CN* corneas (Fig. 7B). Equal loading of proteins was ensured by stripping the membrane of antibodies and reprobing with an antiactin antibody, which did not show any difference between wild-type and *Klf4CN* corneas (Fig. 7B). In addition, keratin-12 expression detected by immunohistochemistry was greatly reduced in the *Klf4CN* corneal epithelial cells (Fig. 7C). A reaction in which no primary antibody was used served as a control in these experiments (Fig. 7C).

Examination of the mouse *Krt12* proximal promoter sequence revealed the presence of potential KLF4 binding sites similar to the consensus KLF4 recognition site (see Fig. S2 in the supplemental material) (58). In cotransfection experiments, KLF4 dose-dependent upregulation of the *Krt12* promoter activity was observed in HCE cells with increasing amounts of the KLF4 expression plasmid pCI-Klf4 (Fig. 7D). Chromatin immunoprecipitation of cross-linked HCE cell chromatin with anti-KLF4 antibody revealed that KLF4 occupies the *Krt12* promoter in vivo in these cells (Fig. 7E). Taken together, these results indicate that KLF4 upregulates the *Krt12* promoter activity by binding the KLF4-responsive *cis* elements and that the *Klf4CN* corneal epithelial fragility is in part due to the reduced expression of the keratin-12 gene.

Downregulation of the aquaporin-5 (*Aqp5*) gene in the *Klf4CN* cornea. Aquaporins regulate the pressure, volume, and hydration levels of different tissues in the eye by facilitating fluid transport (29, 67). Considering the similar increase in stromal thickness of *Aqp5*-deficient (63) and *Klf4CN* corneas (Fig. 2), we examined the expression level of *Aqp5* in *Klf4CN*

FIG. 5. Defective goblet cell development in the *Klf4CN* conjunctiva. Sections from 10-week-old wild-type (A, C, E, and G) or *Klf4CN* (B, D, F, and H) mouse heads stained with Alcian blue (A to D) or PAS (E to H) at low (A, B, E, and F) (magnification, $\times 100$) or high (C, D, G, and H) (magnification, $\times 400$) magnification. Dark blue (alcian blue)- or purple (PAS)-stained goblet cell clusters were observed in the wild-type (arrows) but not the *Klf4CN* conjunctiva. BC, bulbar conjunctiva; PC, palpebral conjunctiva; CF, conjunctival fornix.

corneal epithelium. Real-time Q-RT-PCR with 18S rRNA as an endogenous control showed that *Aqp5* was expressed at an approximately fourfold-lower level in the *Klf4CN* than in the wild-type corneal epithelial cells (Fig. 8A). In immunoblot analysis, the 30-kDa Aqp5 protein was detected in the wild-type but not the *Klf4CN* corneal whole-cell extracts, confirming that the *Klf4CN* corneas contain very little, if any, Aqp5 (Fig.

8B). Equal loading of proteins in these experiments was ensured by stripping the membrane of antibodies and reprobing with an antiactin antibody, which did not show any difference between wild-type and *Klf4CN* corneas (Fig. 8B). Immunohistochemistry revealed that the expression of Aqp5, localized to the corneal epithelial cell membranes in the wild-type mice, was greatly reduced in the *Klf4CN* mice

FIG. 6. Defects in basement layer deposition in *Klf4CN* mice. (A) Wild-type (left) or *Klf4CN* (right) mouse central cornea from midsections stained with PAS. Magnification, $\times 400$. (B) Transmission electron microscopy of thin midsections from the wild-type (left) or *Klf4CN* (right) mouse central corneas at high magnification (magnification, $\times 25,000$).

(Fig. 8C). A reaction with wild-type cornea in which no primary antibody was used served as control in these experiments.

The mouse *Aqp5* proximal promoter contains several *cis* elements similar to the consensus KLF4 recognition site (see Fig. S3 in the supplemental material) (58). KLF4 dose-dependent upregulation of the *Aqp5* promoter activity was observed in cotransfection experiments with HCE cells with increasing amounts of the KLF4 expression plasmid pCI-Klf4 (Fig. 8D). Chromatin immunoprecipitation of cross-linked HCE cell chromatin with anti-KLF4 antibody revealed that KLF4 occupies the *Aqp5* promoter in vivo in these cells (Fig. 8E). Taken together, these results indicate that KLF4 activates the *Aqp5* promoter by binding the KLF4-responsive *cis* elements and that the reduced expression of *Aqp5* is responsible for the *Klf4CN* stromal edema.

DISCUSSION

By using the Cre-lox approach for selective ablation of the *Klf4* gene, we have demonstrated that KLF4 plays a critical role in the postnatal maturation and maintenance of the adult ocular surface. This role of KLF4 does not extend to the embryonic morphogenesis of the eye, as we did not observe significant differences between the wild-type and *Klf4CN* eyes at postnatal day 1, consistent with the earlier report that the *Klf4* null mice developed normally (56). The adult *Klf4CN* corneal epithelium possessed fewer cell layers in spite of the increased cell proliferation, presumably due to the reduced intercellular adhesion resulting in increased cell sloughing at the surface. Thus, *Klf4* appears to be an integral part of the genetic network that regulates the fine balance between cell proliferation and stratification at the ocular surface. Our re-

FIG. 7. Downregulation of keratin-12 gene expression in the *Klf4CN* corneal epithelium. (A) Relative quantities of keratin-12 gene transcripts in total RNA from wild-type (WT) and *Klf4CN* corneas as measured by Q-RT-PCR analysis. Error bars indicate standard deviations. (B) Immunoblot of total proteins from wild-type or *Klf4CN* cornea, probed with anti-keratin-12 antibody. The blot was stripped and reprobed with antiactin antibody to ensure equal protein loading from wild-type and *Klf4CN* corneas. (C) Immunohistochemistry with anti-keratin-12 antibody in the wild-type or *Klf4CN* corneas. Reactions without primary antibody served as controls. Left, DAPI-stained nuclei. Right, fluorescence from secondary antibody against anti-keratin-12 antibody. (D) Relative activity of the *Krt12* promoter driving the luciferase reporter gene, measured by cotransfection of increasing amounts of KLF4 expression plasmid pCI-Klf4 in human corneal epithelial cells. (E) Chromatin immunoprecipitation of *Krt12* promoter-bound KLF4 by anti-KLF4 antibody, detected by PCR of *Krt12* promoter fragment. The negative control is a reaction in which preimmune rabbit serum was used in place of anti-KLF4 antibody.

sults, taken together with other reports, demonstrate that the structural integrity of the corneal epithelium is governed by a complex set of transcription factors, such as KLF4, Pax6, and AP-2 α (20, 23, 59, 60, 72) as well as chromosomal protein HMGN1 (8). It is surprising that in spite of multiple abnormalities associated with the *Klf4CN* ocular surface in the mouse, the human *KLF4* locus has not yet been associated with any ocular dystrophies. While this may reflect the complexity of the genetic networks that govern ocular surface development and maintenance, it is possible that the spontaneous human *Klf4* mutations are lethal, thus evading detection.

The vertebrate eye is a complex organ, with multiple tissues and cell types influencing the development and functions of each other (13). By our approach, *Klf4* was deleted in the developing lens, conjunctiva, and eyelids in addition to the cornea. It is therefore conceivable that while some of the corneal phenotypes we have described are direct consequences of the absence of KLF4 in the cornea, others may arise as secondary or indirect results of the absence of KLF4 in the neighboring

tissues. The expression of *Klf4* in the mouse embryonic ocular surface begins at embryonic day 10 (25), preceding that of *Krt12*, which begins at embryonic day 15.5 (62), making it plausible that the *in vivo* expression of *Krt12* is regulated by KLF4. The present study shows that KLF4 occupies and activates the *Krt12* and *Aqp5* promoters. Moreover, *Klf4CN* corneal epithelium and stroma recapitulate the *Krt12* and *Aqp5* null corneal epithelial and stromal phenotypes, respectively (35, 63). We thus conclude that the *Klf4CN* corneal epithelial fragility and stromal edema are direct effects of the loss of *Klf4* in the cornea. On the other hand, it is likely that the hyperplastic iris observed in about 20% of the *Klf4CN* mice is due to an indirect, noncell autonomous effect, because the expression of the transgene *Le-Cre* has never been detected in the iris or its developmental precursors. The smaller pupil size may be a consequence of the smaller lens in *Klf4CN* mice (Fig. 2; Table 1), as the size of the rodent eye is affected by the size of the central lens.

Disruption of the *Klf4* gene in the ocular surface resulted in

FIG. 8. Downregulation of aquaporin-5 gene expression in the *Klf4CN* corneal epithelium. (A) Relative quantities of *Aqp5* transcripts in the wild-type (WT) and *Klf4CN* corneas as measured by Q-RT-PCR analysis. Error bars indicate standard deviations. (B) Immunoblot of total proteins from wild-type or *Klf4CN* cornea, probed with anti-Aqp5 antibody. The blot was stripped and reprobed with antiactin antibody to ensure equal protein loading from wild-type and *Klf4CN* corneas. (C) Immunohistochemistry with anti-Aqp5 antibody in the wild-type or *Klf4CN* corneas. Reactions without primary antibody served as controls. Left, DAPI-stained nuclei. Right, fluorescence from secondary antibody against anti-Aqp5 antibody. D. Relative activity of the *Aqp5* promoter driving the luciferase reporter gene, measured by cotransfection of increasing amounts of KLF4 expression plasmid pCI-Klf4 in human corneal epithelial cells. E. Chromatin immunoprecipitation of *Aqp5* promoter-bound KLF4 by anti-KLF4 antibody, detected by PCR of *Aqp5* promoter fragment. The negative control is a reaction in which preimmune rabbit serum was used in place of anti-KLF4 antibody.

corneal phenotypes overlapping with different dystrophies associated with eye development. Mutations in *FoxC1*, *PitX2*, and *Pax6* (31) and the collagen $\alpha 1(\text{IV})$ gene (66) have been associated with Axenfeld-Rieger anomaly, a genetically heterogeneous disease with iridocorneal adhesions and defects in basement membrane, similar to those observed in the *Klf4CN* mice. Mutations in cornea-specific keratin-3 or keratin-12 gene are associated with fragility of the corneal epithelium, a feature of Meesmann's corneal dystrophy observed in the *Klf4CN* mice (Fig. 2 and 3) (33, 35, 49). We have shown that keratin-12 gene expression is downregulated in the *Klf4CN* corneas and that KLF4 binds and activates the keratin-12 gene promoter (Fig. 7). It is interesting to note here that keratin-12 gene expression is regulated by KLF6 as well (10, 69). Since different KLFs with diverse regulatory domains bind similar DNA sequences (7, 17), it is possible that the interplay between activities of different KLFs such as KLF4 and KLF6 is involved in the spatiotemporal regulation of expression of specific target genes such as the keratin-12 gene.

The transparency of the cornea depends on, among many factors, the hydration level of the stroma. An increased level of hydration results in increased light scattering, affecting vision (24, 43, 44). Aquaporin 5 expressed in the corneal epithelium plays a critical role in maintenance of proper hydration levels of the corneal stroma (29, 67). The stromal edema observed in *Klf4CN* mice is reminiscent of the ocular phenotypes developed by the *Aqp5* knockout mice (63). We have shown that KLF4 binds and activates the *Aqp5* promoter and that the expression of *Aqp5* in the *Klf4CN* corneal epithelium is downregulated, presumably reducing the osmotic water efflux from the stroma to the external tear film and causing stromal edema (Fig. 8). It is interesting to note here that *Aqp5* mRNA is downregulated in the keratoconus cornea, a noninflammatory thinning of the corneal epithelium leading to visual defects through ectasia, astigmatism, and opacity (52).

Our observations extend the earlier-demonstrated requirement of KLF4 for development of colonic goblet cells (37) to conjunctival goblet cells secreting mucins into the tear film (18,

19, 70). It is noteworthy that goblet cells are lost in human ocular surface disorders such as Stevens-Johnson syndrome and ocular cicatricial pemphigoid (48, 51), suggesting that the *Klf4*CN mouse may be a useful model for these ocular pathologies. Moreover, acidic mucin-5 levels are decreased in conjunctival cells and tears of patients with dry eye compared to normal controls (4, 73), raising the possibility that defects in *Klf4* function may be associated with dry eye conditions. In support of the dry eye connection, a mouse model of keratoconjunctivitis sicca demonstrated an increased number of proliferating epithelial cells and a significantly decreased goblet cell density, which parallel our observations for *Klf4*CN mice (22). Additional symptoms of dry eye such as a reduced number of microvilli on the *Klf4*CN corneal surface along with the corneal epithelial bulli strengthen the link between dry eye syndrome and *Klf4*. A reduced number of microvilli on the superficial corneal epithelial cells and a reduced number of conjunctival goblet cells were also observed in rats fed a zinc-deficient diet, providing additional circumstantial evidence for the involvement of zinc finger transcription factors such as KLF4 in ocular surface development and maintenance (28). Considering that neurturin and *IκBζ* gene-deficient mice provide the only other mouse models deficient for conjunctival goblet cells (61, 65), the *Klf4*CN mice may be a valuable resource for studying dry eye-related inflammation at the ocular surface.

To summarize, the results presented in this report demonstrate that the structural integrity of the corneal epithelium, maintenance of proper hydration levels of the stroma, and development of the conjunctival goblet cells are affected in the absence of KLF4. We have also demonstrated that the expression levels of *Krt12* and *Aqp5* are reduced, consistent with the epithelial fragility and stromal edema, respectively, in the *Klf4*CN cornea and have provided direct evidence that KLF4 binds and activates *Krt12* and *Aqp5* promoters. In view of the wide range of *Klf4*CN ocular surface phenotypes, identification of additional KLF4 target genes is a worthwhile challenge for understanding the role of this transcription factor in maturation and maintenance of the ocular surface.

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