Stromal Edema in *Klf4* Conditional Null Mouse Cornea Is Associated with Altered Collagen Fibril Organization and Reduced Proteoglycans

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PURPOSE. *Klf*4, one of the highly expressed transcription factors in the mouse cornea, plays an important role in maturation and maintenance of the ocular surface. In this study, the structure and proteoglycan composition of the *Klf4* conditional null (*Klf4*CN) corneal stroma was investigated, to further characterize the previously reported *Klf4*CN stromal edema.

METHODS. Collagen fibril spacing and diameter were calculated from scattering intensity profiles from small angle synchrotron x-ray scattering patterns obtained across the cornea along a vertical meridian at 0.5-mm intervals. Collagen fibril organization and proteoglycans were visualized by electron microscopy (EM), with or without the cationic dye cuprolinic blue. Proteoglycans and glycosaminoglycans were further analyzed by fluorophore-assisted carbohydrate electrophoresis (FACE) and immunoblot analysis. Q-RT-PCR was used to measure the transcript levels.

RESULTS. In the central cornea, the average collagen interfibrillar Bragg spacing increased from 44.5 nm (SD ±1.8) in wild-type to 66.5 nm (SD ±2.3) in *Klf4*CN, as measured by x-ray scattering and confirmed by EM. Mean collagen fibril diameter increased from 32 nm (SD ±0.4) in wild-type to 42.3 nm (SD ±4.8) in *Klf4*CN corneal stroma. Downregulation of proteoglycans detected by EM in the *Klf4*CN stroma was confirmed by FACE and immunoblot analysis. Q-RT-PCR showed that, whereas the *Klf4*CN corneal proteoglycan transcript levels remained unchanged, matrix metalloproteinase (MMP) transcript levels were significantly upregulated.

CONCLUSIONS. The *Klf4*CN corneal stromal edema is characterized by increased collagen interfibrillar spacing and increased diameter of individual fibrils. The stroma also exhibits reduced interfibrillar proteoglycans throughout, which is possibly caused by increased expression of MMPs. (*Invest Ophthalmol Vis Sci.* 2009;50:4155–4161) DOI:10.1167/iovs.09-3561

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The cornea consists of a connective tissue stroma of multiple, superimposed lamellae each formed from collagen fibrils with highly regular structure and orientation. The stroma is covered by a stratified epithelium on its outer boundary and a monolayer of endothelial cells lining the inner surface, both cellular populations fulfilling an important barrier function, which influences movement of fluid into and out of the stroma. Corneal transparency is essential for vision and consequently, tight regulation of molecular interactions governing structural integrity and hydration of matrix components is thought to be important in tissue homeostasis, both in development and in the adult. Highly ordered collagen architecture with uniformity of fibril diameter and spacing is central to corneal transparency.[1] Collagen fibril diameter may be controlled during fibrillogenesis in the embryo by interaction of different collagen types to form hybrid fibrils.[2–5] Proper hydration of interfibrillar proteoglycans (PGs) appears to be equally important in maintaining appropriate fibril diameter and spacing consistent with optimal light transmission. Overhydration (edema) of stroma is a consequence of impaired function of epithelial or endothelial limiting cell layers, and can occur in a range of corneal dystrophies and pathologic conditions, leading to disruption of the ordered ultrastructure, clouding or opacity of the tissue and accompanying loss of vision.[6,7]

Maturation of the fully functional transparent cornea is driven by complex signaling interactions as well as extrinsic environmental stimuli during development. In rodents, important developmental events take place in association with eyelid opening.[8] In a study conducted to identify changes in gene expression during postnatal maturation of the mouse cornea, serial analysis of gene expression was recently used.[9] Transcription factors associated with barrier function were among the most highly expressed transcription factors in the maturing as well as adult mouse cornea. Foremost among these was KLF4, a member of the Krüppel-like transcription factor (KLF) family of zinc finger-containing proteins previously identified as an important regulator of epithelial differentiation in skin, lung, and gastrointestinal tract.[10–13] *Klf4* null mice do not survive beyond 15 hours postpartum owing to loss of fluid that is directly attributable to increased epithelial permeability.[13] The lethal effect of *Klf4* deletion made it impossible to investigate its role in the cornea until recently, after the successful production of hybrid mice with conditional deletion of the *Klf4* gene in the developing ocular surface, using the Cre-Lox

approach.[14] *Klf4*CN mice are viable and express KLF4 normally, except in the ocular tissues of ectodermal origin including cornea, conjunctiva, and lens, where the *Klf4* gene is disrupted. *Klf4*CN corneas contain fewer epithelial cell layers with vacuolated cells, swollen endothelium, and edematous stroma, than do wild-type (WT) corneas.[14]

Corneal stromal edema is considered to result from malfunctioning of one or both of the limiting cellular layers,[15] although endothelial abnormality is normally regarded as the primary cause of stromal changes. An understanding of the factors leading to stromal edema remains important from a clinical standpoint, yet structural matrix changes at the molecular and fibrillar levels which underlie the clinical signs of stromal edema have not been clearly characterized. Mice with defective KLF4 expression thus represent an important model for investigations of mechanisms and molecular interactions involved in this process. We report the results of a study using x-ray scattering, electron microscopy, and PG analysis to define the changes in macromolecular composition and organization associated with *Klf4*CN stromal edema.

MATERIALS AND METHODS

Animals and Tissue Acquisition

*Klf4*CN mice with selective disruption of the *Klf4* gene in the cornea, conjunctiva, eyelids, and lens were generated as described previously.[14] The following breeding scheme was used to generate the *Klf4* conditional null (*Klf4*CN) and WT control sibling mice used in our assays: *Klf4loxP/loxP, LeCre/⁻* mice were mated with *Klf4loxP/loxP* mice to obtain a roughly equal proportion of *Klf4loxP/loxP, LeCre/⁻* (*Klf4*CN), and *Klf4loxP/loxP* (WT control siblings) offspring, as described before.[14,16] The mice studied herein were age matched (12-week-old) on a mixed genetic background and maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The eyes were enucleated from mice euthanatized by carbon dioxide asphyxiation and the corneas, together with a rim of sclera, were dissected within 10 minutes of death and transferred to 4% paraformaldehyde fixative in 0.1 M phosphate buffer.

X-Ray Scattering

Corneas were transported in fixative to the Synchrotron Radiation Source (Daresbury, UK) and small angle x-ray scattering was performed with a beam approximately 0.5 mm². Corneas were rinsed briefly in buffer and enveloped in plastic film to prevent dehydration, and the beam was passed through full tissue thickness at the center. Exposures were made along a vertical meridian at 0.5-mm intervals with a computer-operated translation stage. Patterns were analyzed to produce scattering intensity plots from which mean center-to-center collagen fibril Bragg spacing and fibril diameter were calculated.

Electron Microscopy

After removal from the x-ray beam, some corneas of WT and *Klf4*CN mice were fixed for electron microscopy by immersion in either 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, followed by aqueous 1% osmium tetroxide for routine electron microscopy, or 2.5% glutaraldehyde in 25 mM sodium acetate buffer containing 0.1 M magnesium chloride and 0.05% cuprolinic blue for PG localization. Specimens were dehydrated in a graded ethanol series and embedded in Araldite CY212 resin. Sections, 90-to 100-nm thick, were cut from the polymerized resin blocks, collected on uncoated copper grids and stained with uranyl acetate and lead citrate, or uranyl acetate alone for examination in an electron microscope (EM model 208; Philips, Eindhoven, The Netherlands).

Statistical Analysis

Collagen fibril spacing in the WT and *Klf4*CN corneal stroma was calculated for mean ± SD and tested for normality and equal variance before analysis by Student's two-sample *t*-test (Minitab Statistical Software; Minitab Ltd., Coventry, UK). When data were not normally distributed or of unequal variance, the Mann-Whitney test was performed.

PG Analysis

Total protein was extracted from two to three WT or *Klf4*CN corneas in triplicate using 6 M urea with protease inhibitors as described previously[17] and PGs were isolated using NH_2-ion exchange microcolumn.[18] Triplicate samples containing equal amounts of protein were digested with 100 mU/mL chondroitinase ABC or keratanase (Sigma-Aldrich, St. Louis, MO) in 0.1 M ammonium acetate pH 7.4, at 37° for 16 hours to digest chondroitin sulfate/dermatan sulfate (CS/DS), or keratan sulfate (KS) glycosaminoglycans (GAGs), respectively. The GAG fragments were recovered by passage through a 2-kDa cutoff membrane filter and repeated drying before derivatization with 2-aminoacridone and analysis by using fluorophore-assisted carbohydrate electrophoresis (FACE), as previously described.[18] PG core proteins in the digests were identified by immunoblot analysis with an antibody to keratocan, Kera-C (provided by Winston Kao, University of Cincinnati, Cincinnati, OH), a monoclonal antibody to lumican, Lum-1 (from Bruce Caterson, Cardiff University, Wales, UK), or to decorin (Sigma-Aldrich), as previously described.[19]

Isolation of Total RNA and Q-RT-PCR

Total RNA isolated from the WT or *Klf4*CN corneas was quantified, and the concentration adjusted with RNase-free water to 100 ng/μL RT-PCR, and quantitative real time RT-PCR (Q-RT-PCR) assays were performed with cDNA generated by high-capacity cDNA archive kit and total RNA isolated from pooled corneas of 10 WT or *Klf4*CN mice. The RT-PCR products were separated on a 2% agarose gel with TBE buffer. Q-RT-PCR assays for different transcripts were performed in a thermocycler (model 7700; Applied Biosystems [ABI], Foster City, CA) using 18S rRNA as endogenous control. The results were then analyzed (SDS software; ABI). To distinguish the products originating from the mRNA from those amplified from the contaminating genomic DNA, if any, the forward and reverse primers used in RT-PCR were picked from adjacent exons. The sequence of primers used for RT-PCR and Q-RT-PCR is provided in Supplementary Table S1, http://www.iovs.org/cgi/content/full/50/9/4155/DC1. Data are expressed as the mean (±SD).

RESULTS

As measured by synchrotron x-ray diffraction, average collagen fibril spacing in WT corneas increased from the corneal center toward the periphery (Fig. 1). *Klf4*CN mouse corneas also exhibited a center-to-periphery increase in fibril spacing (Fig. 1), but with the spacing in these animals always exceeding that recorded in WTs. Average collagen interfibrillar Bragg spacing in the central cornea increased by approximately 50%, from 44.5 nm (±1.8) in the WT (*n* = 5) to 66.5 nm (±2.3) in the *Klf4*CN stroma (*n* = 8; Fig. 1, Table 1). A similar proportional increase was observed in the periphery of the cornea, with the interfibrillar Bragg spacing measuring 65.8 nm (±11.9) in the WT and 88.7 nm (±14.5) in the *Klf4*CN corneas (Fig. 1, Table 1). These studies also revealed that the average diameter of individual collagen fibrils increased by approximately 32%, from 32 nm (±0.4) in WT to 42.3 nm (±4.8) in the *Klf4*CN corneas (Table 1).

Consistent with the x-ray scattering data, electron microscopy also illustrated increased collagen fibril diameter and spacing throughout the depth of the central *Klf4*CN cornea (Figs. 2E–H), compared with the WT (Figs. 2A–D). Even
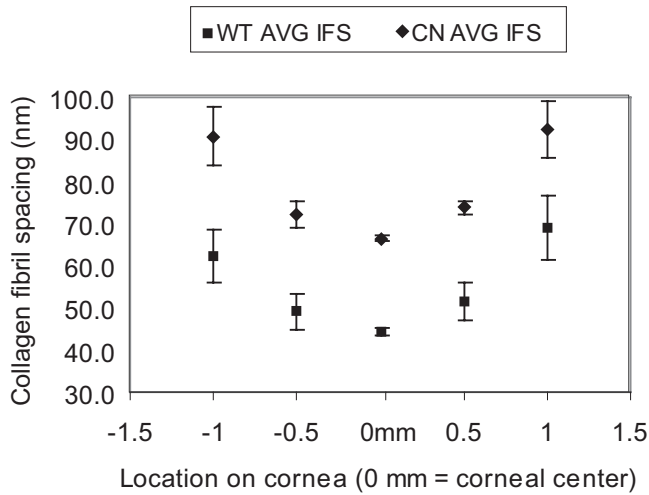
FIGURE 1. Graphic representation of average collagen interfibrillar (Bragg) spacing (*y*-axis) from x-ray scattering patterns obtained at five sites at 0.5-mm intervals (*x*-axis) along a vertical meridian across the cornea in five WT and eight *Klf4*CN mice. In both WT and *Klf4*CN stroma, the interfibrillar spacing became increasingly greater on moving further toward the periphery from the corneal center (represented by 0 mm). Fibril spacing in the *Klf4*CN mouse cornea exceeded that in WT across the meridian.

though the increased collagen fibril spacing and diameter were both evident throughout the *Klf4*CN cornea (compare Figs. 2B–D and 2F–H), this effect was much more striking in the posterior stroma (compare Figs. 2D and 2H), than the middle or anterior stroma. Electron microscopy also revealed that the *Klf4*CN subepithelial stroma was disrupted with randomly organized collagen fibrils, unlike the regular organization of subepithelial extracellular matrix in the WT (compare Figs. 2E and 2A).

Electron microscopy of corneal specimens fixed in the presence of cuprolinic blue showed PGs as electron-dense filaments associated with collagen fibrils. PGs appeared considerably reduced in size and abundance in the *Klf4*CN (Figs. 3G–L) compared with those in WT (Figs. 3A–F) cornea, in both longitudinal (Figs. 3A, 3C, 3E, 3G, 3I, 3K) and transverse sections (Figs. 3B, 3D, 3F, 3H, 3J, 3L). This reduction in PGs was observed in the anterior (Figs. 3A, 3B, 3G, 3H), middle
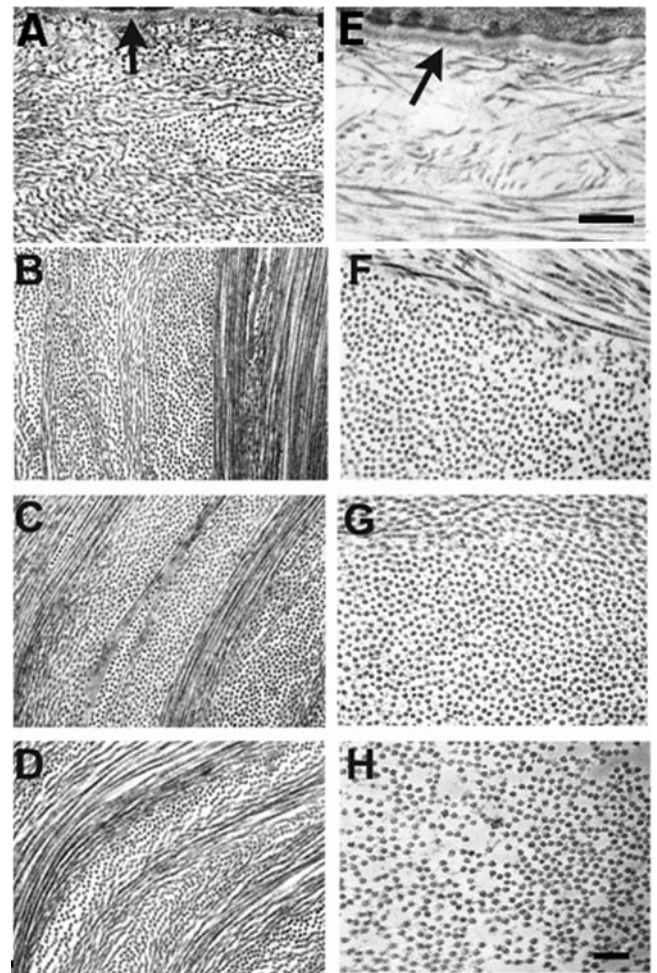

FIGURE 2. Electron micrographs of corneal lamellae in WT (**A–D**) and *Klf4*CN corneal stroma (**E–H**). Subepithelial stroma appeared disrupted in mutant cornea (**E**). *Arrows:* basal lamina below the epithelium (**A**, **E**); anterior stroma (**B**, **F**); mid-stroma (**C**, **G**); posterior stroma (**D**, **H**). Bar: (**A**, **E**) 480 nm; (**B–D**, **F–H**) 300 nm.

TABLE 1. Summary of Collagen Interfibrillar (Bragg) Spacing

	Wild-Type				**Klf4CN**		
	Interfibrillar Bragg Spacing (nm)				**Interfibrillar Bragg Spacing (nm)**		
Mouse ID	**Center**	**Periphery**	**Fibril Diameter (nm) Center**	**Mouse ID**	**Center**	**Periphery**	**Fibril Diameter (nm) Center**
425	44	76.7	32.2	426	65.5	109.4	38.6
425A	42.1	51	31.5	426A	65.5	75.6	37.7
437	45.4	59	31.7	436	64.1	67	38.6
437A	44	79	31.9	424	65.5	92.1	47.3
423	46.8	63.2	32.6	432	65.5	75.6	43.8
				435	70.2	100	51.3
				429	70.2	95.1	40.9
				433	65.5	95.1	40.2
Mean	44.5	65.8	32.0		66.5	88.7	42.3
SD	1.8	11.9	0.4		2.3	14.5	4.8
SE	0.8	5.3	0.2		0.8	5.1	1.7

Data were collected at the at corneal center and periphery and the fibril diameter at the corneal center was calculated from X-ray scatter patterns obtained in wild-type (*n* = 5) and *Klf4*CN (*n* = 8) mice.
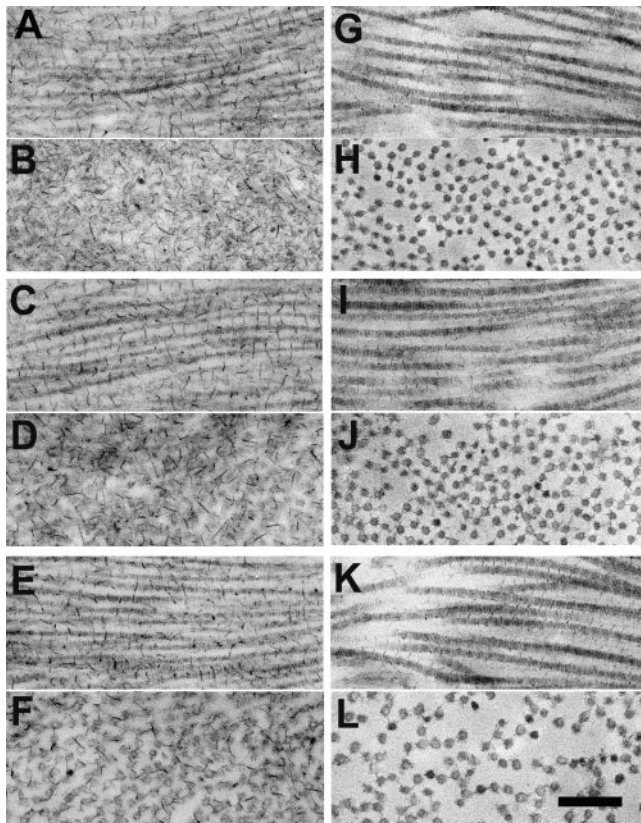
FIGURE 3. Electron micrographs of cornea in anterior, mid-, and posterior stroma from WT (**A–F**) and *Klf4*CN (**G–L**) corneas, prepared with contrast-enhanced fixation of PGs with cuprolinic blue. Collagen fibrils appeared in longitudinal (**A, C, E, G, I, K**) and transverse (**B, D, F, H, J, L**) sections. PGs were seen as fine filaments decorating the collagen fibrils and were more prominent in WT than in *Klf4*CN mouse corneas, where interfibrillar spacing was also seen to be increased. Bar, 300 nm.

(Figs. 3C, 3D, 3I, 3J), and posterior (Figs. 3E, 3F, 3K, 3L) cornea. Consistent with the previous results (Figs. 2), the heightened diameter and spacing of collagen fibrils in *Klf4*CN stroma was also evident at higher magnification in the posterior compared with the central or anterior locations (Figs. 3 K, 3L compared with 3G–J).

Alterations in stromal PGs of *Klf4*CN corneas were confirmed by biochemical analyses of both core proteins and their GAG chains in total protein extracts of WT and *Klf4*CN corneas. CS/DS and KS were detected by using FACE after digestion with specific endoglycosidases. FACE analysis showed that *Klf4*CN corneas contained approximately 35% of the WT levels of CS/DS (both nonsulfated and monosulfated disaccharides), and only 15% of the KS (Figs. 4A, 4C). Immunoblots with antibodies specific for the PG core proteins, decorin, lumican, and keratocan indicated a significant downregulation of all three components in *Klf4*CN compared with WT corneas (Fig. 4B). Densitometric scans of these blots showed that decorin, keratocan, and lumican were reduced to approximately 63%, 50%, and 6% of the WT levels, respectively, in *Klf4*CN corneas (Fig. 4D). These results imply that lumican and KS GAGs exhibit a greater reduction pro rata than decorin and CS/DS GAGs (Fig. 4C). Consistent with the results of cuprolinic blue contrast-enhanced electron microscopy (Fig. 3), these biochemical analyses provided quantitative estimates of the extent of reduction of GAGs in the *Klf4*CN cornea.

We then compared the expression levels of the transcripts encoding the PGs tested above, in the WT and *Klf4*CN corneas.

Data from the earlier microarray[16] and the current Q-RT-PCR analyses indicated that the transcript levels of decorin, lumican, keratocan, and Chst5 (an enzyme involved in the sulfation of KS chains), do not vary significantly between WT and *Klf4*CN corneas (Fig. 5). To test the possibility that increased degradation of corneal PGs by elevated expression of MMPs may be responsible for the observed reduction in PGs in the *Klf4*CN stroma, we examined the microarray data for the levels of MMPs. We found that MMP2 and -9 known to play an active role in corneal extracellular matrix rearrangement during wound healing,[20] and MMP3 and -13 are indeed upregulated in the *Klf4*CN cornea compared with the WT cornea, by 1.76-, 3.5-, 2.9- and 4.3-fold, respectively.[16] The upregulation of MMP-2, -3, and -9 expression indicated by microarray analysis was confirmed by RT-PCR (Fig. 6).

DISCUSSION

Maintenance of precise spacing between collagen fibrils with highly regular diameter is considered to be critically important for the transparency of the corneal stroma.[1] Fibril diameter and spacing are influenced by several interacting factors, including the collagen-type composition of the fibrils,[2,3] nature, and abundance of the PGs that interact with the fibrils[21–23] and the level of stromal hydration.[24] In this study, we used x-ray fiber diffraction to measure the collagen fibril spacing and diameter in WT and *Klf4*CN mouse corneas, which develop significant stromal edema.[14] Use of an x-ray beam focused to 0.5 mm has permitted collection of data from multiple sites across mouse cornea, showing that average interfibrillar spacing of collagen fibrils is higher in the corneal periphery, as previously described in human cornea.[25] Moreover, *Klf4*CN corneas demonstrated significantly increased collagen interfibrillar spacing across the cornea compared with WT. The collagen fibrils were spread wider apart at all depths in the *Klf4*CN corneal stroma, but with especially increased spacing in posterior regions.

Factors regulating collagen fibril spacing are not fully understood, but evidence suggests that interfibrillar PGs, consisting of a core protein and attached GAG chains, fulfill an important role. Stromal PGs are members of the small leucine-rich PG (SLRP) family which includes three KS-linked proteins lumican,[26] keratocan,[27,28] and osteoglycin,[29] and the CS/DS PG decorin.[30] PGs interact with collagen fibrils at specific sites via their core protein domains,[31] with the sulfated, highly-hydrophilic GAG chains extending into the interfibrillar space where they bind water and thus influence stromal hydration.[32] We found that GAGs, visualized by electron microscopy with cuprolinic blue contrast, were markedly reduced in the edematous *Klf4*CN corneal stroma. Biochemical analysis confirmed this reduction using FACE, which revealed that both KS and CS/DS GAGs were significantly reduced. The presence of reduced GAGs initially seems difficult to reconcile with tissue edema and increased water content in the *Klf4*CN stroma. Similar decreases in GAG levels have been reported previously, in response to postsurgical and experimentally induced corneal edema.[33,34] In other situations, however, reduced sulfated KS is associated with a more compact stroma with reduced spacing between collagen fibrils—for example, in the *Chst5*-knockout mouse cornea,[35] and also in corneas from patients with macular corneal dystrophy.[36] It therefore seems more likely that the PG loss in *Klf4*CN corneas is a secondary effect of tissue edema rather than its cause.

PG transcript levels in *Klf4*CN corneas were similar to WT despite a reduction in PG and GAG levels, suggesting increased degradation of these molecules in the mutant. Accordingly, in *Klf4*CN corneas we observed an upregulation in MMPs capable
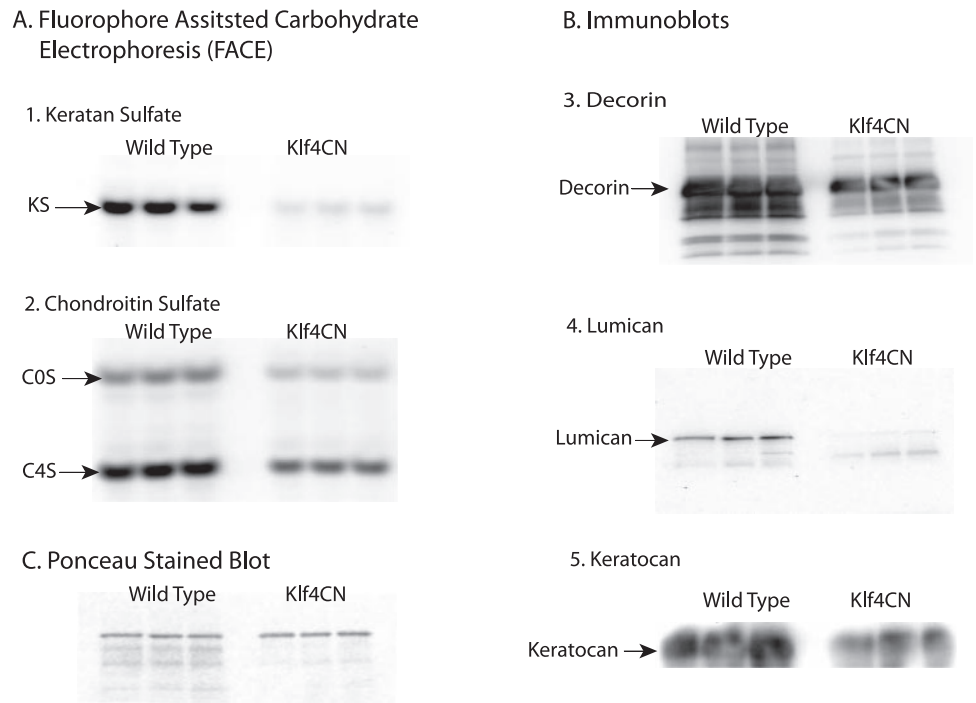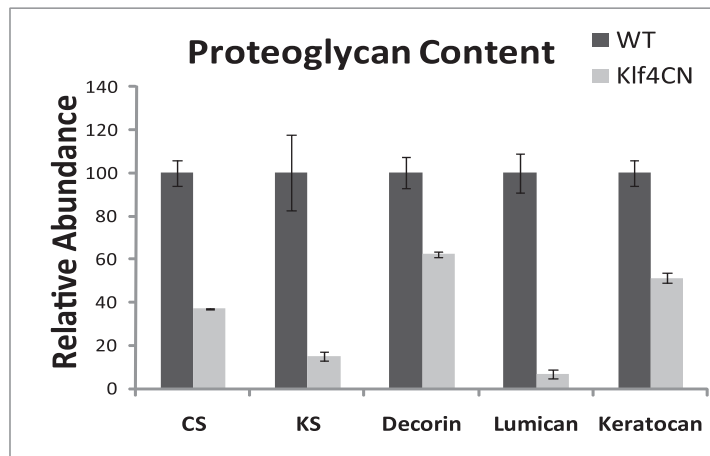
A. Fluorophore Assitsted Carbohydrate Electrophoresis (FACE)

1. Keratan Sulfate

2. Chondroitin Sulfate

B. Immunoblots

3. Decorin

4. Lumican

5. Keratocan

C. Ponceau Stained Blot

D. Quantitative Densitometric Scan of the above blots with error bars

Proteoglycan Content

FIGURE 4. Analysis of PGs extracted from WT and *Klf4*CN mouse corneas. (**A**) Fluorophore-assisted carbohydrate electrophoresis analysis of (**A1**) keratan sulfate-(KS) monosulfated disaccharides generated by keratanase digestion. (**A2**) Nonsulfated (COS) and monosulfated (C4S) CS/DS disaccharides generated by chondroitinase ABC digestion. (**B**) Western blot identification of (**B3**) decorin core protein; (**B4**) lumican core protein; (**B5**) keratocan core protein. (**C**) Ponceau-stained blot showing equal protein loading. (**D**) For each of these analyses, quantitative analysis of three replicates from WT and *Klf4*CN corneal extracts are displayed with the SE. In each case, the differences were statistically significant ($P < 0.05$).

of degrading stromal PGs, and which may also contribute to the thinning of the epithelial basement membrane in *Klf4*CN corneas described previously.[14] The results presented here do not allow us to determine whether the increased expression of MMPs is due to activation of their promoter activities, or increased stability of their corresponding transcripts. It is possible that the signals generated in response to the *Klf4*CN corneal epithelial fragility or stromal swelling simulate a corneal wounding response and elicit the observed increase in *Klf4*CN corneal MMP expression.

Upregulation of MMPs appears to be a consistent feature concomitant with edema, reported in diverse tissues, including skin, brain, and the vascular system,[37,38] as well as human corneas in pseudophakic eyes.[39] In view of the fact that elevated expression of MMPs is associated with tissue remodeling and wound healing,[20] exploring the role of KLF4 in regulating the expression of MMPs in the cornea may reveal if KLF4 influences corneal wound healing by regulating MMPs. The broad spectrum of known activities of the MMP family indi-

cates that, rather than solely regulating matrix turnover, they potentially control many complex aspects of cell behavior and homeostasis in the extracellular matrix.[40] Thus, upregulation of MMPs probably has far-reaching consequences in the *Klf4*CN phenotype through additional effects on many nonmatrix substrates such as cell receptors, signaling, and adhesion molecules.

Besides increased interfibrillar spacing in edematous *Klf4*CN corneas, a surprising finding was the increased diameter of stromal collagen fibrils. This may be directly related to the reduction in the amount of PGs, as stromal PGs are known to bind to fibrillar collagen in vitro and influence the lateral association of collagen during fibrillogenesis.[22] KS PG-null mice, deficient in lumican, keratocan, or osteoglycin, all to some extent exhibit increased diameters of collagen fibrils in corneal stroma.[41–44] Decorin has also been shown to influence fibril diameter and alignment in studies on skin and tendon.[21–23,45] Consistent with our observation that lumican is almost absent from the *Klf4*CN cornea, increased fibril diameters and dis-
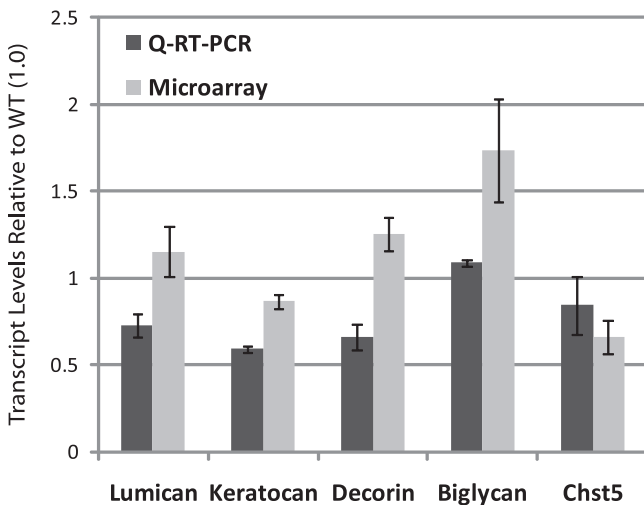
FIGURE 5. Expression of PG-related transcripts in the *Klf4*CN compared with WT cornea. Relative expression of different PG transcripts in the *Klf4*CN compared with WT corneas, measured by Q-RT-PCR and microarray analyses is depicted.

rupted fibril spacing were especially prominent in posterior regions of the stroma, where lumican concentrations are highest.[46] However, unlike in our findings, fibril changes were not uniform in PG-knockout mice. Rather, clusters of atypical fibrils appeared, scattered among relatively normal fibril populations.[41–44]

Edema in *Klf4*CN corneas is manifest as increased fibril spacing throughout the stroma, presumably the result of water ingress after perturbation of epithelial and/or endothelial barriers. Swamynathan et al.[14,16] have shown downregulation of the epithelial water channel proteins, aquaporins-3 and -5 in *Klf4*CN corneas, whereas aquaporin-1 in endothelium is unaltered, and suggested that disturbance of aquaporin levels may be the reason for stromal edema. This notion implies a more important role for the epithelium in the control of stromal hydration than recognized hitherto. The epithelium is instigative in stromal morphogenesis from an early stage in corneal development.[47] *Klf4* expression was detectable in the developing mouse eye from embryonic day 10,[48] at the threshold of major morphogenetic changes involving tissue compaction, loss of water, and increasing transparency.[5,46] Perturbation of *Klf4*-driven epithelial differentiation, disregulation of water channel components and subsequent MMP overexpression appear to contribute to an altered matrix structure which persists in the adult animal.

In summary, the results presented in this report provide quantitative measurements of matrix changes that occur with
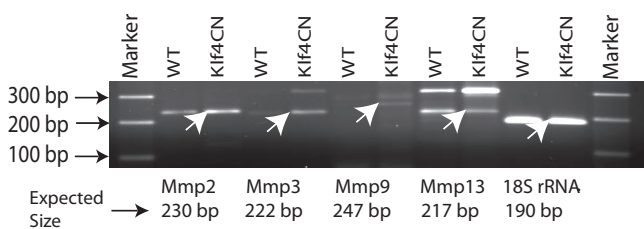

FIGURE 6. Elevated expression of MMPs in the *Klf4*CN cornea. RT-PCR analysis of expression levels of transcripts encoding MMP2, -3 -9 and -13, in the WT and *Klf4*CN corneas. Expression levels of 18S rRNA transcripts were used as the endogenous controls. *Arrows*: the expected amplicons, with the corresponding expected amplicon sizes are indicated below the gel photo for each MMP.

stromal edema in *Klf4*CN corneas and indicate that an increased expression of MMPs is probably responsible for reduced corneal PGs in *Klf4*CN. Thus, *Klf4*CN mice represent a useful model for further investigations into the physiological mechanisms underlying stromal edema in the cornea.

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