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

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PURPOSE. Klf4, 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 interbifilar 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

The cornea consists of a connective tissue stroma of multiple, superficially 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. Collagen fibril diameter may be controlled during fibrillogenesis in the embryo by interaction of different collagen types to form hybrid fibrils. 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 dysorphies and pathologic conditions, leading to disruption of the ordered ultrastructure, clouding or opacity of the tissue and accompanying loss of vision.

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. 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. 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. KLF4 null mice do not survive beyond 15 hours postpartum owing to loss of fluid that is directly attributable to increased epithelial permeability. 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

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approach.\(^{14}\) Klf4\(\text{loxP/loxP}\) 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\(\text{loxP/loxP}\) 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\(\text{loxP/loxP}\) stromal edema.

**MATERIALS AND METHODS**

**Animals and Tissue Acquisition**

Klf4\(\text{loxP/loxP}\) 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\(\text{loxP/loxP}\)) and WT control sibling mice used in our assays. Klf4\(\text{loxP/loxP}\), LeCre/\(\text{loxP/loxP}\) mice were mated with Klf4\(\text{loxP/loxP}\), LeCre/\(\text{loxP/loxP}\) mice to obtain a roughly equal proportion of Klf4\(\text{loxP/loxP}\), LeCre/\(\text{loxP/loxP}\) (Klf4/CN), and Klf4\(\text{loxP/loxP}\), LeCre/\(\text{loxP/loxP}\) (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 euthanized 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 mm\(^2\). 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\(\text{loxP/loxP}\) 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\(\text{loxP/loxP}\) 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\(\text{loxP/loxP}\) corneas in triplicate using 6 M urea with protease inhibitors as described previously\(^ {17}\) and PGs were isolated using NH\(_4\)I-exchange micromolumn.\(^ {18}\) Triplicate samples containing equal amounts of protein were digested with 100 mM/\(\mu\)L chondroitinase ABC or keratanase (Sigma-Aldrich, St. Louis, MO) in 0.1 M ammonium acetate pH 7.4, at 37\(^\circ\) 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 24kDa cutoff membrane filter and repeated drying before derivatization with 2-amino-5-methoxynaphthalene and analysis by 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\(\text{loxP/loxP}\) corneas was quantified, and the concentration adjusted with RNase-free water to 100 ng/\(\mu\)L 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\(\text{loxP/loxP}\) 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\(\text{loxP/loxP}\) 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\(\text{loxP/loxP}\) 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\(\text{loxP/loxP}\) 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\(\text{loxP/loxP}\) 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\(\text{loxP/loxP}\) cornea (Figs. 2E–H), compared with the WT (Figs. 2A–D). Even
though the increased collagen fibril spacing and diameter were both evident throughout the Klf4CN 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 Klf4CN 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 Klf4CN (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

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<th>Mouse ID</th>
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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 Klf4CN (n = 8) mice.
Data from the earlier microarray\textsuperscript{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\textsuperscript{−/−} 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\textsuperscript{−/−} 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,\textsuperscript{20} and MMP3 and -13 are indeed upregulated in the Klf4\textsuperscript{−/−} compared with the WT cornea, by 1.76, 3.5, 2.9, and 4.3-fold, respectively.\textsuperscript{14,16} The upregulation of MMP-2, -3, and -9 expression indicated by microarray analysis was confirmed by RT-PCR (Fig. 6).

\section*{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.\textsuperscript{1} Fibre diameter and spacing are influenced by several interacting factors, including the collagen-type composition of the fibrils,\textsuperscript{2,3} nature, and abundance of the PGs that interact with the fibrils\textsuperscript{21–24} and the level of stromal hydration.\textsuperscript{24} In this study, we used x-ray fiber diffraction to measure the collagen fibril spacing and diameter in WT and Klf4\textsuperscript{−/−} mouse corneas, which develop significant stromal edema.\textsuperscript{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.\textsuperscript{25} Moreover, Klf4\textsuperscript{−/−} 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\textsuperscript{−/−} 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,\textsuperscript{26} keratocan,\textsuperscript{27,28} and osteoglycin,\textsuperscript{29}) and the CS/DS PG rich PG (SLRP) family which includes three KS-linked proteins (lumican,\textsuperscript{26} keratocan,\textsuperscript{27,28} and osteoglycin,\textsuperscript{29}) of KS chains), do not vary significantly between WT and Klf4\textsuperscript{−/−} corneas.

Alterations in stromal PGs of Klf4\textsuperscript{−/−} corneas were confirmed by biochemical analyses of both core proteins and their GAG chains in total protein extracts of WT and Klf4\textsuperscript{−/−} corneas. CS/DS and KS were detected by using FACE after digestion with specific endoglycosidases. FACE analysis showed that Klf4\textsuperscript{−/−} 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\textsuperscript{−/−} 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\textsuperscript{−/−} 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\textsuperscript{−/−} cornea.

We then compared the expression levels of the transcripts encoding the PGs tested above, in the WT and Klf4\textsuperscript{−/−} corneas.
of degrading stromal PGs, and which may also contribute to the thinning of the epithelial basement membrane in Klf4^CN corneas described previously.\textsuperscript{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,\textsuperscript{37,38} as well as human corneas in pseudophakic eyes.\textsuperscript{39} In view of the fact that elevated expression of MMPs is associated with tissue remodeling and wound healing,\textsuperscript{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 indicates that, rather than solely regulating matrix turnover, they potentially control many complex aspects of cell behavior and homeostasis in the extracellular matrix.\textsuperscript{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.\textsuperscript{41} KS PG-null mice, deficient in lumican, keratocan, or osteoglycin, all to some extent exhibit increased diameters of collagen fibrils in corneal stroma.\textsuperscript{41–44} Decorin has also been shown to influence fibril diameter and alignment in studies on skin and tendon.\textsuperscript{21–23,45} Consistent with our observation that lumican is almost absent from the Klf4^CN cornea, increased fibril diameters and dis-
ruptured fibril spacing were especially prominent in posterior regions of the stroma, where lumican concentrations are highest.44 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 ingress after perturbation of epithelial and/or endothelial barrier spacing throughout the stroma, presumably the result of water fluctuations.41–44

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.

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

References


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.

FIGURE 4. Quantitative expression analysis of Klf4 target genes reveals the molecular basis of the diverse regulatory roles of Klf4 in the mouse cornea.


