Abnormal Corneal Endothelial Maturation in Collagen XII and XIV Null Mice

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Purpose. Maturation of the endothelium and the adjacent matrix was characterized in wild-type (WT) mice. The influence of FACIT collagen XII and XIV deficiency on the morphology, maturation, and function of the corneal endothelium was examined.

Methods. Analysis of the endothelium and Descemet's membrane (DM) was performed using transmission electron microscopy at postnatal day (P)4, P14, and P30 in WT, *Col12a1*$^{-/-}$, *Col14a1*$^{-/-}$, and *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ mice. Endothelial junctions were analyzed using ZO-1. The presence of endothelial–stromal communications was evaluated with phalloidin staining as well as electron microscopy. Finally, corneal thickness was assessed.

Results. A thin DM, clefts between endothelial cells and DM, and large "vacuole-like" structures were present in the endothelial cells of WT mice at P4 but not noted at P30. The endothelia of *Col12a1*$^{-/-}$, *Col14a1*$^{-/-}$, and compound *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ in the P30 cornea maintained the vacuole-like structures seen at P4. A mature endothelial junction pattern was delayed in the null corneas. Expression of ZO-1 in WT endothelia at P14 was diffuse and localized to the basolateral and apical cell membrane. At P30, staining was localized to intercellular junctions. ZO-1 reactivity was patchy in *Col12a1*$^{-/-}$, *Col14a1*$^{-/-}$, and compound *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ corneas at P14 and P30. Stromal thickness was increased in P30 null corneas. Endothelial cell processes were demonstrated penetrating the DM and into the underlying stroma, throughout the entire endothelial layer in the P4 cornea.

Conclusions. Collagen XII and XIV null mice demonstrate delayed endothelial maturation. The structural alterations suggest functional changes in endothelial function resulting in increased corneal thickness. Endothelial–stromal interactions suggest a pathway for signal transduction.

Keywords: FACIT collagens, cornea, corneal endothelium, stroma, collagen XII, collagen XIV, knockout mouse models

The corneal endothelium is a monolayer of hexagonal cells essential to maintaining optimal corneal hydration and transparency. Corneal endothelial dysfunction is a common indication for corneal transplantation. Patients with advanced endothelial dysfunction present with eye discomfort and pain, blistering of the corneal surface, and decreased visual acuity. New surgical techniques for endothelial transplantation include Descemet's stripping endothelial keratoplasty and Descemet's membrane (DM) endothelial keratoplasty. The underlying basis and continued development of these new surgical techniques require a better understanding of the structure and function of the posterior corneal stroma, DM, and endothelium.[1–4]

The family of fibril-associated collagens with interrupted triple helical domains (FACIT collagens) is important in the assembly of collagen fibrils and organization of fibrillar matrices. It is suggested that FACIT collagens are essential for corneal transparency and corneal function.[5–7] Collagens XII and XIV, members of the FACIT family, are expressed in a variety of tissues including the cornea.[7–9] Both collagens XII and XIV have been shown to be expressed in the human cornea[10–12] indicating that the mouse cornea is a valid model for these studies. Collagens XII and XIV are associated collagen I–containing fibrils. Collagen XII and XIV also interact with different extracellular matrix components like proteoglycans and are found in basement membrane zones.[8,9]

Collagen XII is expressed at interfaces where tissues interact. Although the function of collagen XII is poorly understood, it is believed that collagen XII is a stress molecule secreted in response to stress and shear. It was reported that the *Col12a1* gene could be directly stimulated by mechanical forces in fibroblasts[13,14] and endothelial cells[15] as well as osteoblasts.[16] In the chicken, collagen XII is expressed in interfacial regions of the cornea, Bowman membrane, and DM until hatching, suggesting a major role in the integration of different corneal layers.[17] Collagen XIV expression is homogeneous during stromal development and decreases in the mature stroma, suggesting a major role for collagen XIV during initial stages of fibrillogenesis and early stromal development and compaction.[6,7] In avian species, collagen XIV is expressed throughout the entire stroma as well as Bowman membrane and DM during development. However, mature corneas are not reactive against collagen XIV.[7]

DM is composed of collagen VIII assembled as a hexagonal lattice.[18] In contrast to the human DM, not much is known

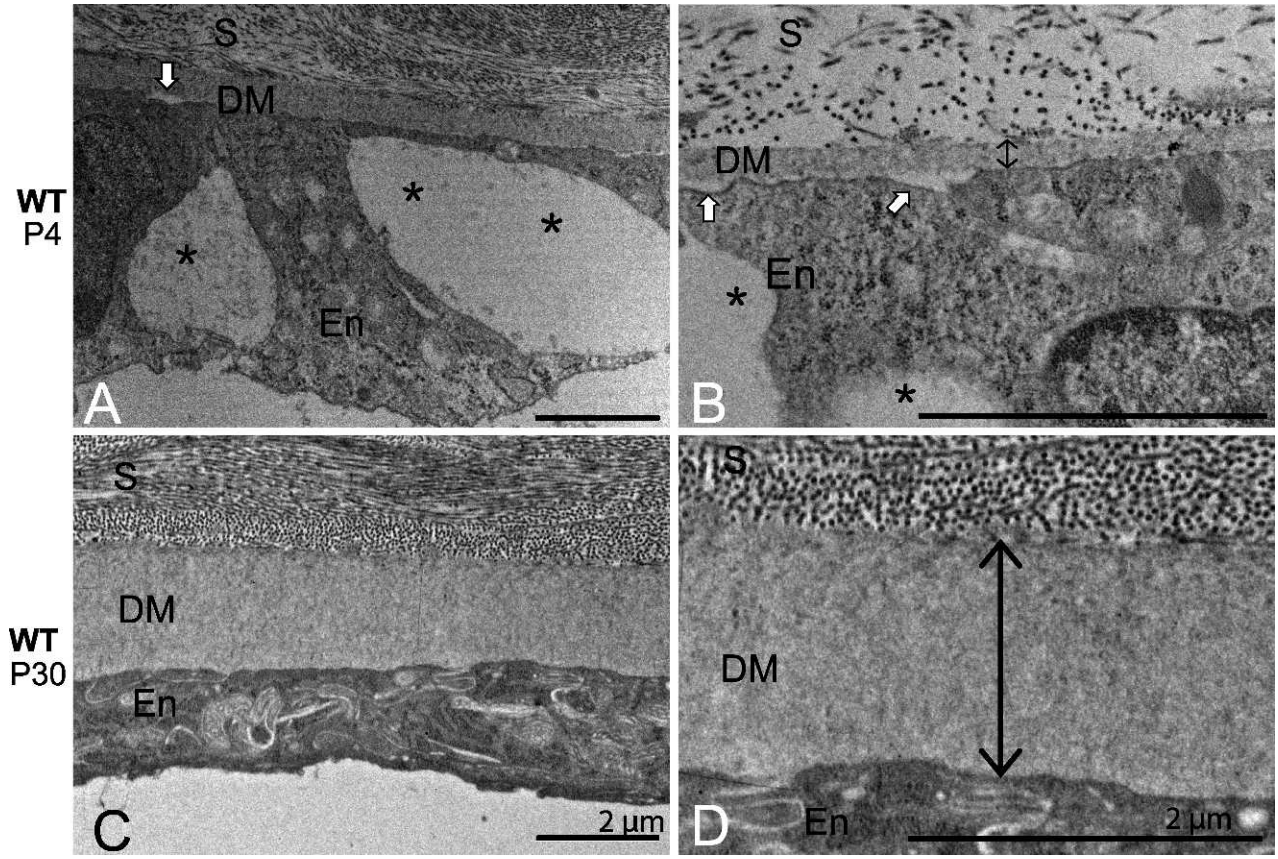

Figure 1. Corneal endothelial maturation. Normal maturation of the corneal endothelium (En) and DM was studied using transmission electron microscopy. (**A**, **B**) Immature (P4) and (**C**, **D**) mature (P30) WT corneas were compared. Endothelial maturation is associated with a thickening of the DM, thinning of the endothelium. Immature endothelia have large endothelial vacuoles (*asterisks*) and clefts (*black arrows*) between the DM and the endothelium (**A**, **B**). These disappear during maturation and the corneal endothelial monolayer thins (**C**, **D**). S, stroma. *Bars*: 2 µm.

about the structure of DM in mice. However, recent work indicates that the mouse is a good model of the human DM. Hopfer et al.[19] studied 2- to 6-month-old wild-type (WT) mice and described abundant banded material within the posterior layer of DM. An uneven appearance with focal thickening in 2-year-old mice was also described. Jun et al.[20] studied 2- to 16-week-old mice and described a banded layer with 110-nm periodicity comprising 90% to 100% of the total DM thickness. An accumulation of banded material continued between 2 and 6 weeks of age, suggesting an attenuated endothelial cell biosynthetic activity. However, the specific effects of collagens XII and XIV in DM and endothelial function remain largely unknown. This report supports the hypothesis that collagens XII and XIV in the posterior corneal stroma have a functional role in the regulation of corneal endothelial maturation.

Materials and Methods

Animals

Gene-targeted mice null in *Col12a1*$^{-/-}$[21] or *Col14a1*$^{-/-}$,[22] compound *Col12a1/Col14a1* null mice, and WT control mice were used. Compound collagen *Col12a1/Col14a1* null mice were generated by cross-breeding the single null mice. Corneal tissue from mice at P4, P14, and P30 were examined. All experiments conformed to the use of Laboratory Animals and the ARVO statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the University of South Florida, College of Medicine.

Immunoblotting

Immunoblotting was performed as previously reported.[23] Briefly, protein extracts were prepared in extracting buffer containing 50 mM Tris-HCl (pH 6.8), 1% SDS, and proteinase inhibitor cocktail (Roche, Indianapolis, IN). Cornea extracts (5 µg) were separated on 4% to 12% Bis-Tris gels (Invitrogen, Indianapolis, IN) and transferred onto Hybond-C membranes (GE Healthcare, Piscataway, NJ). Anti–collagen XII antibody KR33[24] at 1:200 and anti–collagen XIV at 1:200[22] were used. Actin was used as a protein loading control. Actin antibody was purchased from Millipore (Billerica, MA). All experiments were performed at least three times in tissue obtained from three different animals.

Immunofluorescence Microscopy

Immunofluorescence analysis was performed as previously described.[23] Briefly, corneas were fixed with 4% paraformaldehyde in PBS, cryoprotected with sucrose-PBS in a series of dilutions (10%, 20%, and then 30%), and embedded and frozen in OCT medium (Sakura Finetek, Torrance, CA). Cross-sections of 6 µm were cut using a HM 505E cryostat (Microm International, Walldorf, Germany) followed by immunofluorescence localization. Sections were blocked in 5% BSA and then incubated overnight at 4°C in anti–collagen IV at 1:100

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(Southern Biotech, Birmingham, AL), anti–collagen VIII at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA), anti–collagen XII antibody KR33[24] at 1:200, anti–collagen XIV at 1:200,[22,24] and anti-ZO1 (Molecular Probes, Eugene, OR), followed by Alexa Fluor 488–conjugated goat antirabbit IgG (Molecular Probes) at 1:200. Alexa Fluor 594 phalloidin (Molecular Probes) also was used. Positive and negative controls were processed. The nuclei were counterstained using Vectashield mounting solution with DAPI (Vector Labs, Inc., Burlingame, CA). Images were captured using a confocal laser-scanning microscope (FV1000 MPE; Olympus, Center Valley, PA) with a ×60 1.42 NA oil immersion lens. To avoid bleedthrough between fluorescence emissions, samples were scanned sequentially with 488- and 543-nm lasers, and emissions were collected with appropriate spectral slit settings.

Scanning and Transmission Electron Microscopy

Corneas were analyzed using transmission electron microscopy as previously described.[22] Briefly, corneas were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate, pH 7.4, with 8.0 mM $CaCl_2$, followed by postfixation with 1% osmium tetroxide. After dehydration corneas were infiltrated and embedded in a mixture of EMbed 812, nadic methyl anhydride, dodecenyl succinic anhydride, and DMP-30 (Electron Microscopy Sciences, Hatfield, PA). Semithin (1 μm) cross-sections from the cornea were cut using a Leica UCT ultramicrotome (Buffalo Grove, IL), stained with methylene blue-azur B, and examined under light microscopy on an Olympus BX61 microscope and photographed with a DP72 digital color camera (Olympus). Ultrathin sections were cut using a Leica ultramicrotome and poststained with 2% aqueous uranyl acetate and 1% phosphotungstic acid, pH 3.2. Central cornea sections were examined and imaged at 80 kV using a JEOL 1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) equipped with a Gatan Orius widefield side mount CC Digital camera (Gatan, Inc., Pleasanton, CA). To evaluate the possible interaction between corneal endothelium and the adjacent matrix, P4 mouse corneas were dissected in PBS under an operating microscope immediately after harvesting and processed for scanning electron microscopy. Tissue was fixed overnight in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, washed three times for 10 minutes each in 0.1 M sodium cacodylate buffer, postfixed in osmium tetroxide for 1 hour, and rinsed three times for 5 minutes in 0.1 M sodium cacodylate buffer. Corneas were then dehydrated in an ethanol series, dried in an Electron Microscopy Sciences 850 critical point dryer (Hatfield, PA), mounted onto metal stubs. Scotch tape (3M, St. Paul, MN) was then used to remove endothelial cells from the surface, exposing the DM. The corneal endothelial surface was examined using a scanning electron microscopy (JEOL model 6490LV). All experiments were performed at least three times in tissues obtained from five different animals.

Optical Coherence Tomography and Corneal Thickness Estimation

Whole eyes from euthanized mice were enucleated, and measurements were obtained less than 15 minutes after animals were sacrificed. Each enucleated eye was placed in a custom-made holder and placed in the Spectral Dominium Cirrus HDT Optical Coherence Tomography (OCT) (Zeiss, San Francisco, CA) device for corneal thickness measurements. Measurements were taken in less than 3 minutes after enucleating the eye. The eyes were kept in balanced saline solution before being mounted on the holder. Five measurements were obtained on the vertical plane and five measurements on the horizontal plane of the central cornea. All experiments were performed at least three times in tissue obtained from three different animals at P30.

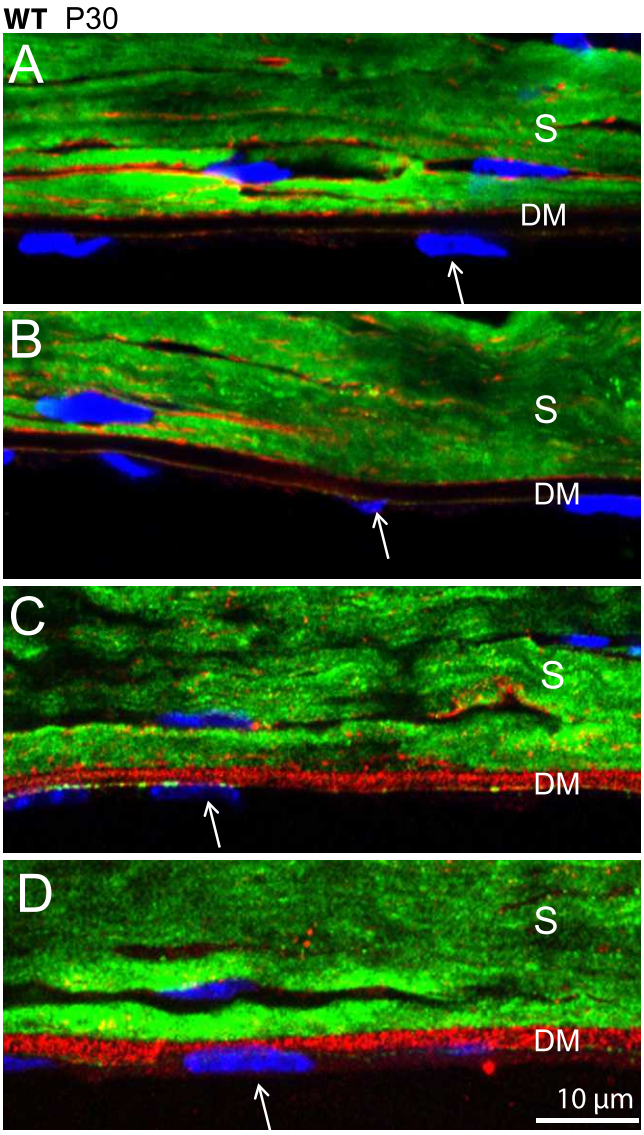

Figure 2. Localization of collagens XII and XIV in the posterior corneal stroma. (**A**, **C**) Collagen XII (*green*) is localized in the posterior stroma (S). (**B**, **D**) Collagen XIV (*green*) also is localized in the posterior stroma. DM separates the endothelium from the stroma. (**A**, **B**) Collagen IV (*red*) is located in the inner and outer borders of DM. (**C**, **D**) Collagen VII (*red*) is localized throughout DM. The arrows denote endothelial cells. *Bar*: 10 μm.

Statistical Analysis

An ANOVA test was performed with four different groups; each group represented a genotype. An ANOVA test was done against all four genotypes at P30 and at P60. If the ANOVA test reported a significant difference between the groups, *t*-tests were done pairing each null genotype against the control group.

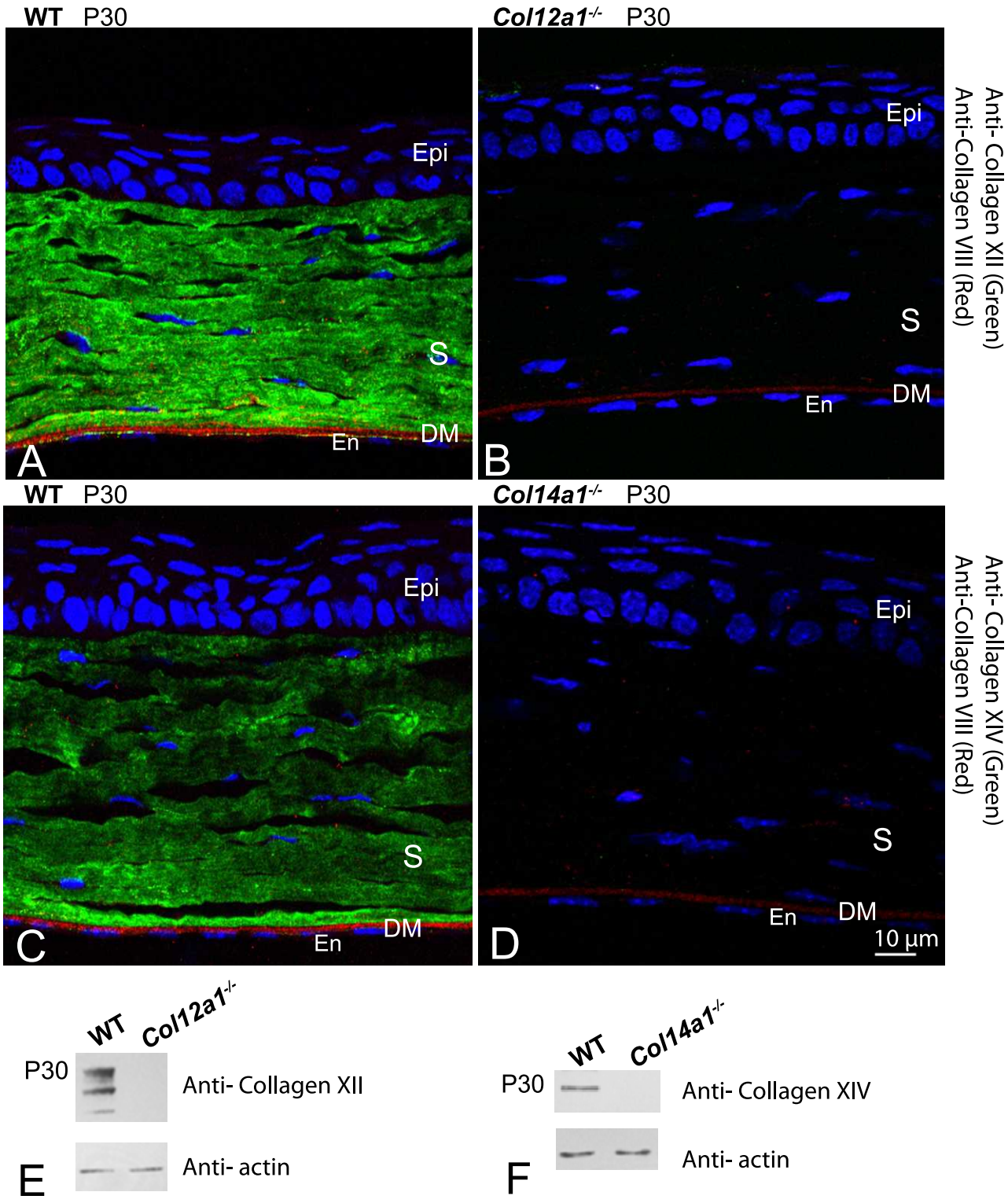

Figure 3. Mouse models null for corneal collagen XII and XIV. In the WT mouse, collagen XII expression localizes to the corneal stroma (**A**). In contrast, the *Col12a1*$^{-/-}$ mouse model did not express collagen XII (**B**). Immunofluorescence shows expression of collagen XIV in the full thickness corneal stroma (**C**). There is no immunoreactivity in the *Col14a1*$^{-/-}$ corneas (**D**). Epi, epithelium. Immunoblots confirmed the absence of collagen XII expression in the *Col12a1*$^{-/-}$ cornea (**E**) and collagen XIV expression in the *Col14a1*$^{-/-}$ (**F**). *Bar*: 10 µm.

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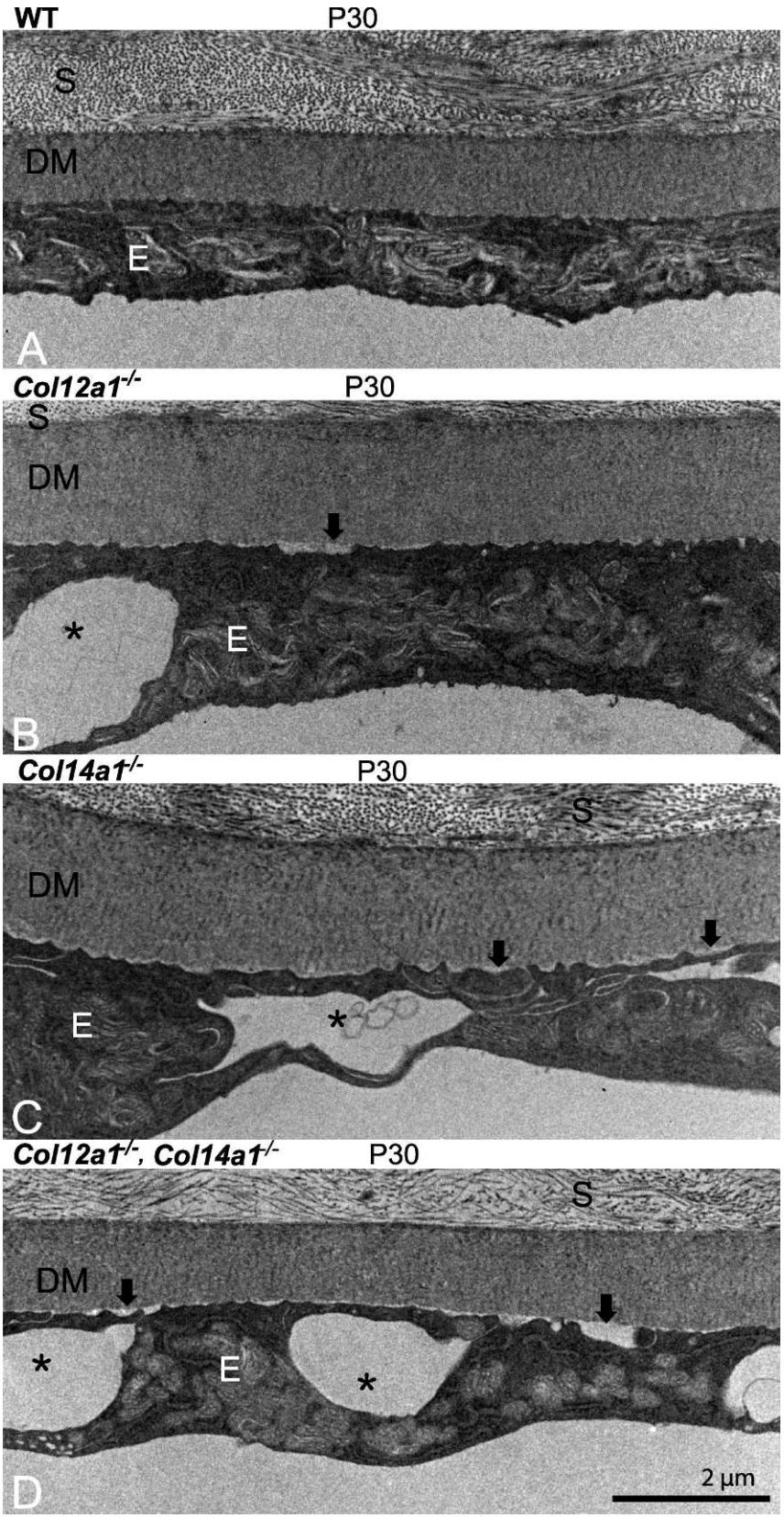

Figure 4. Delayed endothelial maturation in absence of collagen XII and/or XIV. (**A**) The mature WT endothelium (P30) is thin with an absence of vacuoles and clefts. The mice null for collagen XII and/or XIV all demonstrate features of immature endothelia. There is a retention of large vacuoles (*asterisks*) in the endothelium and clefts (*arrows*) between the endothelium and DM at P30 in (**B**) *Col12a1*$^{-/-}$, (**C**) *Col14a1*$^{-/-}$, and (**D**) *Col12a1*$^{-/-}$*/Col14a1*$^{-/-}$ corneas. *Bar*: 2 µm.

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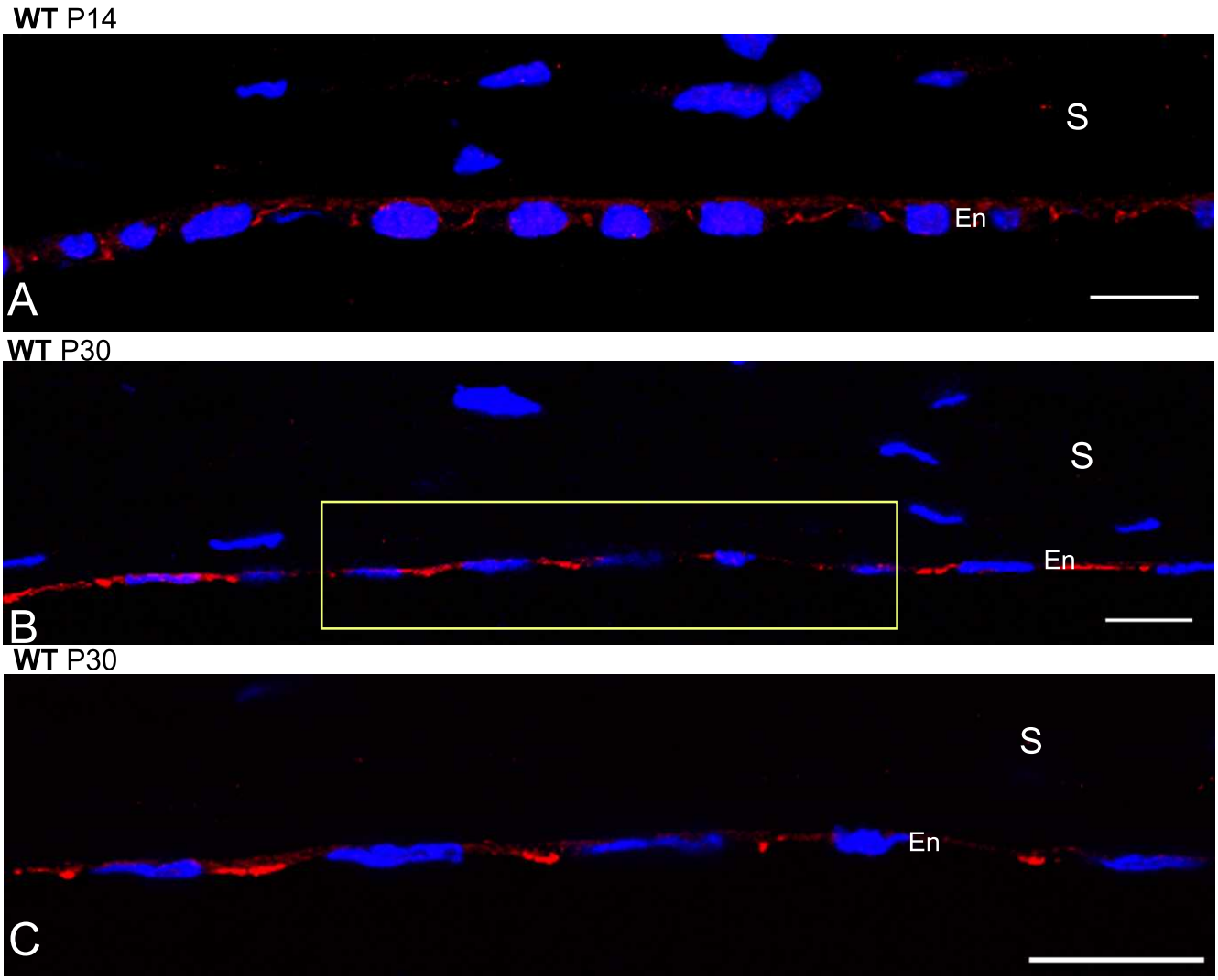

FIGURE 5. Endothelial junction maturation in immature and mature WT mice. ZO-1 (*red*) expression in the corneal endothelium of the WT mouse. (**A**) ZO-1 is expressed in the apical and lateral corneal endothelial membrane in immature (P14) mice. (**B**, **C**) The expression of ZO-1 becomes localized to the lateral membrane in mature (P30) WT corneas. The *yellow box* in (**B**) outlines the area in (**C**). *Bars*: 40 μm.

RESULTS

Normal Maturation of Corneal Endothelium and Adjacent Matrix

To establish the normal process of endothelial and adjacent extracellular matrix maturation in the WT cornea, an ultrastructural analysis was performed at P4, P14, and P30. Significant changes in the morphology of DM were observed from P4 to P30 (Fig. 1). DM was a thin single homogenous layer at P4 (Fig. 1B) that progressively became thicker at ages P14 (not shown) and P30 (Fig. 1D). In addition, clefts between the endothelium and DM were noted at P4 (Fig. 1B) and were not seen at P30 (Fig. 1D). A distinct basal lamina was not observed at either P14 or P30. The most striking findings with maturation were observed in the corneal endothelial monolayer. From P4 to P30, the endothelium became thinner with maturation. In addition, large endothelial vacuoles were noted at P4 (Fig. 1A) and not seen at P30 (Fig. 1C). There was also a disappearance of clefts between the endothelium and DM. In conclusion, three morphological findings were associated with normal maturation of the WT corneal endothelium: monolayer thinning, loss of vacuoles within the monolayer, and disappearance of clefts between the endothelium and thickening of DM.

Collagen XII and XIV Are Present in the WT Posterior Stroma

The potential roles of collagens XII and XIV in endothelial maturation was addressed using immunolocalization. Collagen XII was expressed in the posterior stroma and adjacent to DM (Figs. 2A, 2C). Collagen XII reactivity was excluded from the DM proper indicated by no collagen XII reactivity being colocalized with an anti–collagen VIII antibody in DM (Fig. 2C). Collagen XIV expression was restricted to the posterior stroma and no collagen XIV reactivity was colocalized with an anti–collagen VIII antibody in DM (Fig. 2D). The localization and limits of DM were defined by the expression of collagen IV and VIII. The outer limit of DM demonstrated a patchy pattern of reactivity with anti–collagen IV antibody as previously reported in the chicken.[25] In contrast, the collagen IV imminoreactivity along the inner layer of DM was continuous and linear (Figs.

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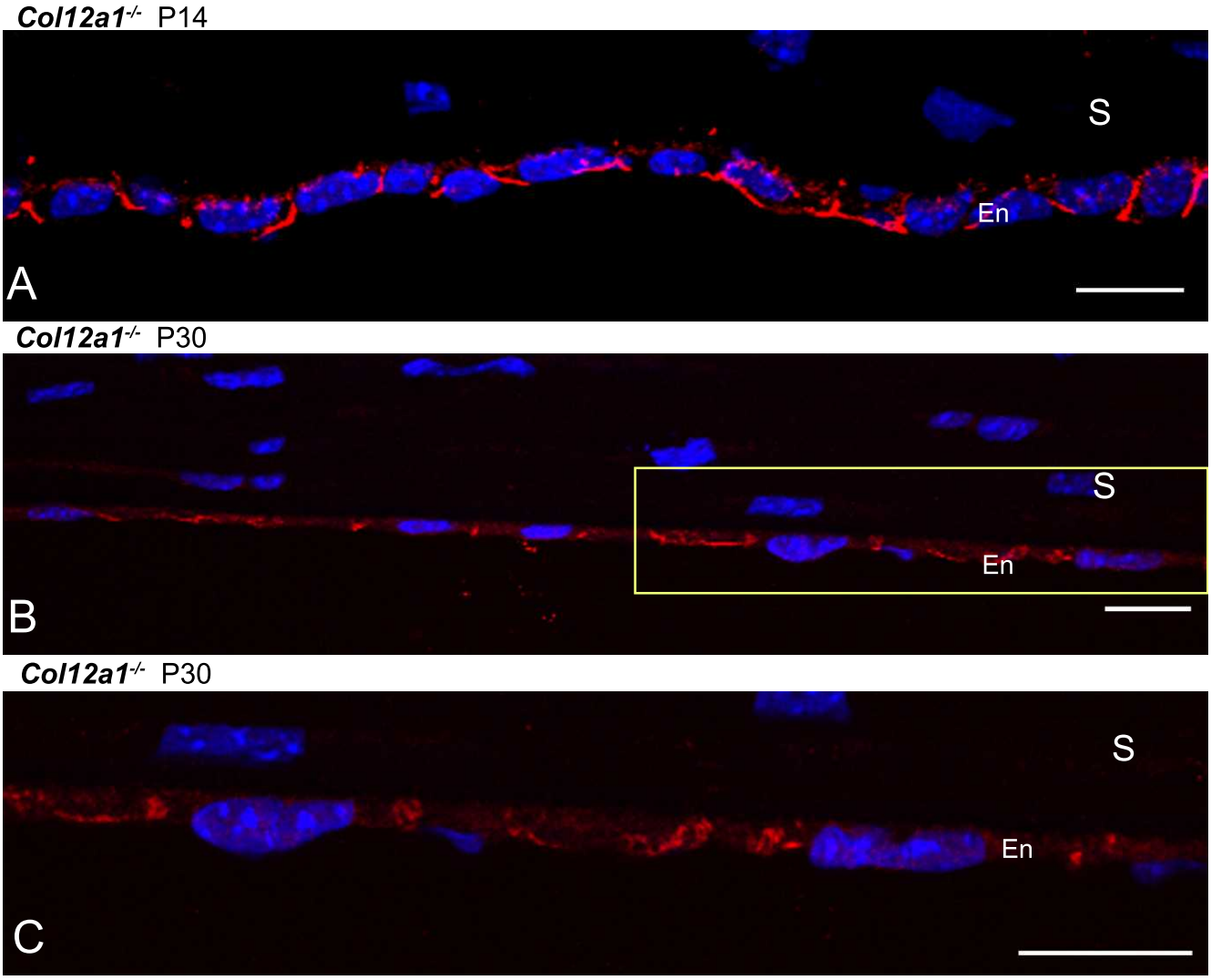

FIGURE 6. Delayed endothelial junction maturation in *Col12a1*$^{-/-}$ mice. ZO-1 (*red*) expression in the corneal endothelium of the *Col12a1*$^{-/-}$ mouse. (**A**) ZO-1 is expressed in the apical and lateral corneal endothelial membrane at P14. (**B**, **C**) The expression of ZO-1 does not localize exclusively to the lateral membrane in the endothelia of mature (P30) mice instead retaining the less mature localization. The *yellow box* in (**B**) outlines the area in (**C**). *Bars*: 40 μm.

2A, 2B). Collagen VIII immunoreactivity was confined to the DM proper (Figs. 2C, 2D).

Characterization of *Col12a1*$^{-/-}$ and *Col14a1*$^{-/-}$ Mouse Corneas

The *Col12a1*$^{-/-}$ and *Col14a1*$^{-/-}$ mouse models were previously characterized as null for collagens XII and XIV respectively.[21,22] The corneas from these mouse models were analyzed for collagen XII and XIV expression. Corneal expression of collagen XII (Fig. 3B) or collagen XIV (Fig. 2D) was not present by immunolocalization in the respective null mice. However, as expected, reactivity against collagen XII (Fig. 3A) and collagen XIV (Fig. 3C) was homogeneously localized throughout the WT stroma. These results were confirmed using immunoblots. Collagen XII was not present in the *Col12a1*$^{-/-}$ mouse corneal extracts (Fig. 3E), and there was no collagen XIV expression in the corneal extract of the *Col14a1*$^{-/-}$ mouse corneas (Fig. 3F). These data indicate that corneas obtained from the *Col12a1*$^{-/-}$ and *Col14a1*$^{-/-}$ mouse models do not express collagen XII or collagen XIV, respectively.

Delayed Corneal Endothelial Maturation in *Col12a1*$^{-/-}$, *Col14a1*$^{-/-}$, and Compound *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ Mice

The endothelium from *Col12a1*$^{-/-}$, *Col14a1*$^{-/-}$, and compound *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ mouse corneas was evaluated at P30 and compared to comparable WT endothelium ($n = 5$). Evaluation of the *Col12a1*$^{-/-}$ mice demonstrated intercellular vacuoles with subendothelial clefts (Fig. 4A). *Col14a1*$^{-/-}$ corneas also had intercellular vacuoles and subendothelial clefts that were moderately more numerous than in *Col12a1*$^{-/-}$ mice (Fig. 4B). Compared with *Col12a1*$^{-/-}$ and *Col14a1*$^{-/-}$ mice, compound deficient *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ mouse corneas had more numerous intercellular vacuoles as well as more subendothelial clefts than either of the single mutants (Fig. 4C). In contrast, WT corneas did not show any intercellular vacuoles in the mice examined (Fig. 4D). The presence of endothelial vacuoles was always associated with

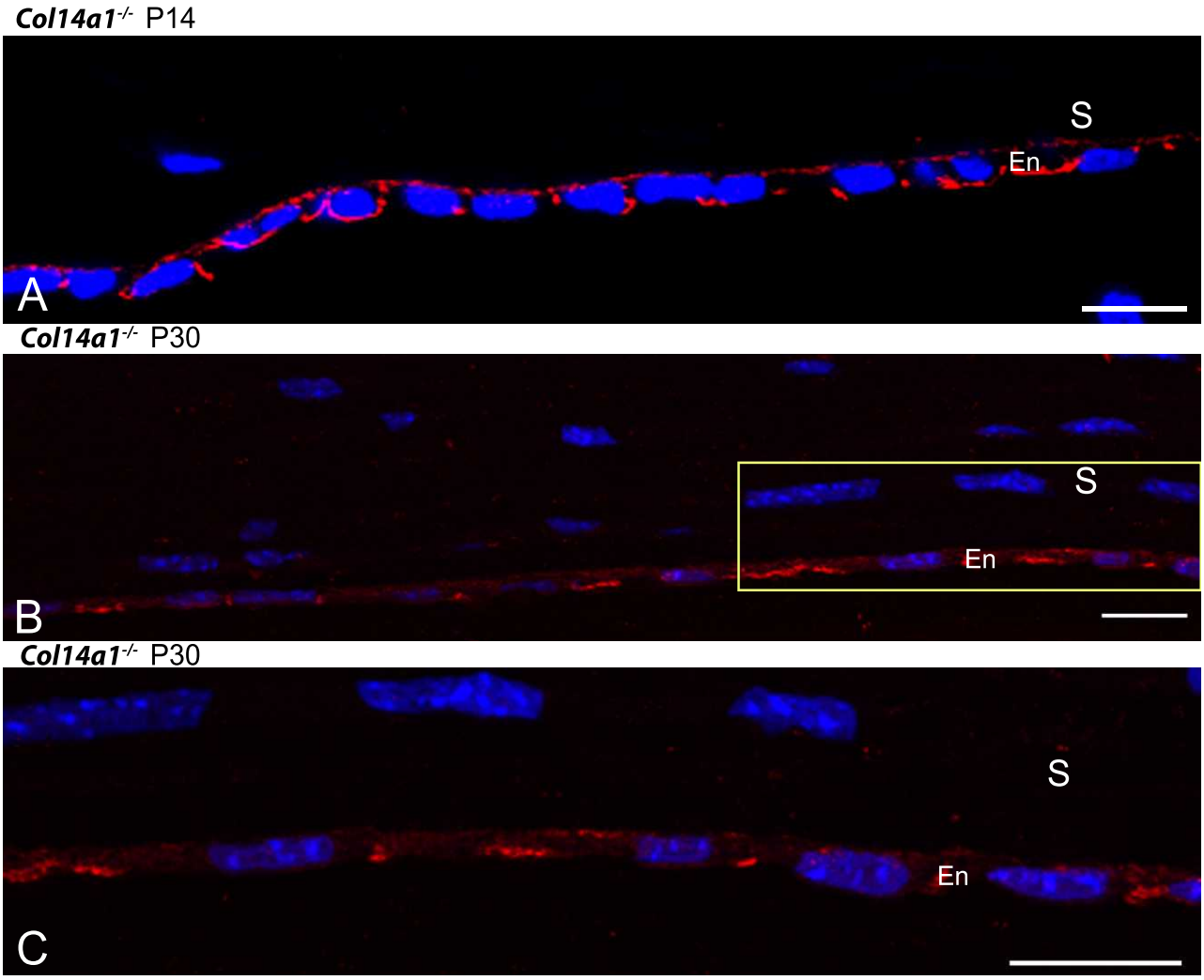

FIGURE 7. Delayed endothelial junction maturation in *Col14a1*$^{-/-}$ mice. ZO-1 (*red*) expression in the corneal endothelium of the *Col14a1*$^{-/-}$ mouse. (**A**) ZO-1 is expressed in the apical and lateral corneal endothelial membrane at P14. (**B**, **C**) The expression of ZO-1 does not localize exclusively to the lateral membrane in the endothelia of mature (P30) mice instead retaining the less mature localization. The yellow box in (**B**) outlines the area in (**C**). *Bars*: 40 μm.

the presence of subendothelial clefts between the endothelium and DM (Figs. 4B, 4D). The endothelial phenotype observed in the P30 FACIT collagen-null mice was comparable to the immature endothelium at P4 (Fig. 1). There was thickening of DM in the WT and mutant models. Taken together, these morphological findings were suggestive of delayed endothelial maturation in *Col12a1*$^{-/-}$/, *Col14a1*$^{-/-}$, and compound *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ mice.

Corneal Endothelial Barrier Maturation Is Delayed in Collagen XII and XIV Deficient Mice

ZO-1, a component of tight junctions, was use as a marker to follow endothelial barrier maturation. ZO-1 expression in the WT corneal endothelium was diffuse and localized to both the apical and lateral corneal endothelial membrane in immature WT mice at P14. The ZO-1 reactivity pattern was also irregularly spaced (Fig. 5A). The expression of ZO-1 became more localized to the lateral membrane and more regularly spaced at P30 in WT endothelia (Figs. 5B, 5C). In contrast, the expression of ZO-1 in *Col12a1*$^{-/-}$ and *Col14a1*$^{-/-}$ mice was noted in the apical and lateral endothelial cell membrane at P14 (Figs. 6A, 7A, respectively) and did not localize to the lateral cell membrane at P30. (Figs. 6B, 6C, 7B, 7C) The pattern of ZO-1 reactivity also remained irregularly spaced comparable to that observed in the immature P14 endothelium. This finding was suggestive of delayed endothelial barrier maturation and suggestive of impaired paracellular permeability in deficient mice.

Corneal Thickness Alterations in Collagen XII and XIV Deficient Mice

As an indication of corneal endothelial function, corneal thickness was analyzed in WT and mutant genotypes at P30 using OCT (Fig. 8). WT mice had a mean corneal thickness of 101.3 ± 3.8 μm. In contrast, all three mutant genotype had significantly thicker corneas compared to WT mice. The mean thicknesses were *Col12a1*$^{-/-}$, 132.1 ± 9.2 μm; *Col14a1*$^{-/-}$, 119.8 ± 9.1 μm; and compound *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$, 124.4 ± 11.3 μm. The ANOVA test at P30 reported a P value < 0.001 among all four genotypes. This signified that the means of all four genotypes were not equal (Fig. 8C). The following t-test done on the three null genotypes (*Col12a1*$^{-/-}$,

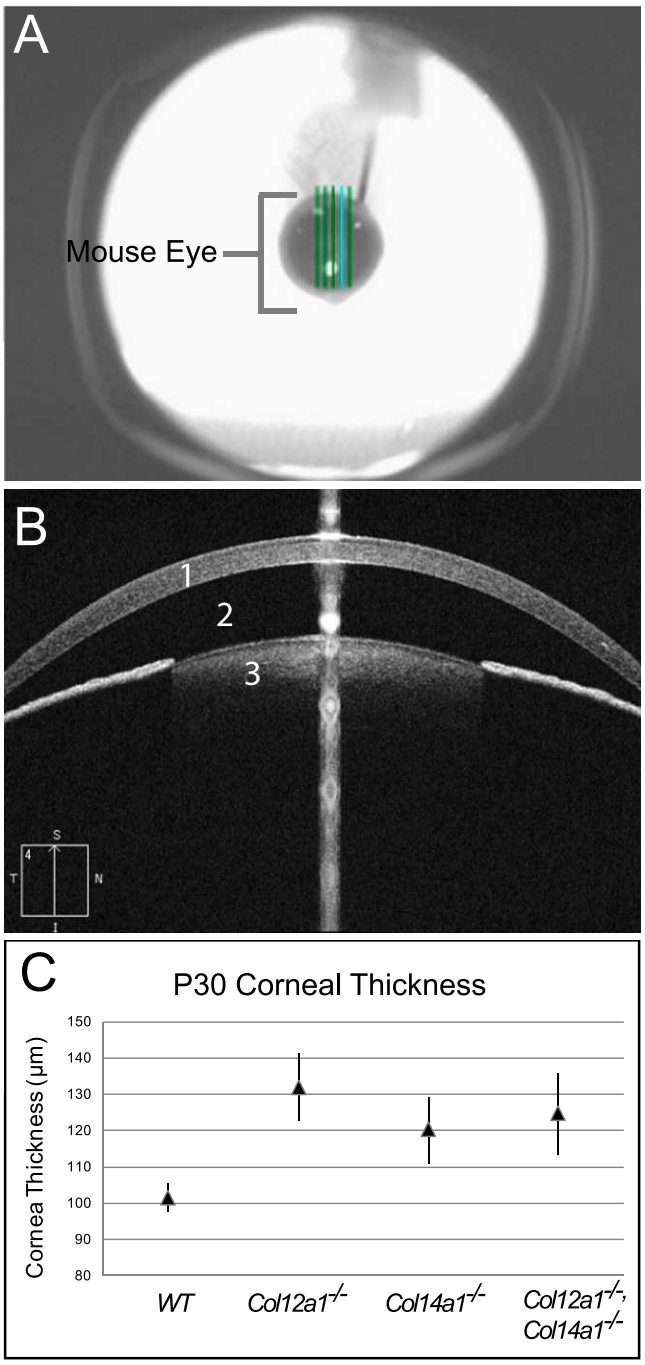

Figure 8. Increased corneal thickness in collagen XII and/or XIV deficient mice. (**A**) Five different measurements of central corneal thickness were taken. Each measurement is denoted by a *green* or *blue* line. (**B**) Example of a measured area: (1) cornea, (2) anterior chamber, (3) lens. The measured corneal thickness was taken from the apical end of the cornea to the posterior corneal border. (**C**) *Col12a1*$^{-/-}$, *Col14a1*$^{-/-}$, and *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ corneas were all significantly thicker than the WT cornea at a P value < 0.01.

Col14a1$^{-/-}$, and *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$) against the control (WT) corneal thickness reported that all null genotypes were significantly thicker than the control with a P value < 0.001. An ANOVA test done at P60 reported a P value < 0.001 among all four genotypes. The consequent t-test of the P60 null-genotypes against the control confirmed that only *Col12a1*$^{-/-}$ and *Col14a1*$^{-/-}$ P60 corneas were thicker than the control with a P value < 0.0001. The compound *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ had a P value $= 0.0018$, supporting the null hypothesis at $\alpha = 0.001$. These findings suggest delayed stromal compaction in the collagen XII and XIV deficient mice (Table).

Table. Differences in Corneal Thickness in P30 WT, *Col12a1*$^{-/-}$, *Col14a1*$^{-/-}$, and *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ Mice*

Source	SS	df	MS	*F*	*P*
Regression	3084.360	3	1028.120	13.284	0.000
Error	1547.900	20	77.395		
Total	4632.260	23			

* The table contains the ANOVA results against P30 corneal thickness in WT, *Col12a1*$^{-/-}$, *Col14a1*$^{-/-}$, and *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$. The P value of 0.000 was less than 0.05 so the resulting slope was significantly different from zero. The F statistical value of 13.284 shows that the slope is far from zero, because $F > 4$.

Corneal Endothelial–Stromal Interaction

Our data indicate that in the absence of collagens XII and XIV, endothelial maturation is abnormal. Corneal sections were evaluated after phalloidin staining and DAPI counterstaining. Phalloidin staining demonstrated endothelial processes penetrating the DM allowing communication between endothelial cells and the underlying stroma along the entire P4 corneal endothelium (Fig. 9A). Transmission electron microscopy also demonstrated the presence of cytoplasmic processes crossing DM (Fig. 9B). An analysis of the DM using scanning electron microscopy demonstrated the presence of pores through the DM allowing passage of endothelial processes and communication between the endothelium and posterior stroma (Figs. 9C, 9D). Similar endothelial–stromal interactions have been described in the rabbit and avian corneas.[25,26]

Discussion

The influence of FACIT collagens XII and XIV on corneal endothelium and adjacent matrix maturation and function is unknown and has not been previously investigated. In the current study, we demonstrate that in the absence of collagen XII and/or XIV normal corneal endothelial maturation is delayed. In addition, the normal corneal thinning that is essential for corneal clarity is altered. These data support our hypothesis that collagens XII and XIV influence corneal endothelial maturation and development of normal corneal function.

The molecular regulators or factors that dictate stromal thinning in the first days of life are unknown. Plausible mechanisms include at least one of the following two factors, or a combination of both: (1) deturgescense of the stroma by an activated and functional mature endothelium, and/or (2) subsequent compaction of collagen fibrils. Early corneal thinning in mammals has been previously studied in rabbits and was attributed to activation of the endothelial pump function that seems to correlate with the time of eyelid opening.[27,28] Our current work shows characteristic morphological and functional changes in the mouse corneal endothelium and adjacent matrix during maturation from P4 to P30 including thickening of DM and changes in the endothelial monolayer. A striking finding in the P4 mouse corneal endothelium is the presence of intracellular and subbasal vesicles in the immature mouse endothelium that are not present in the mature P30 cornea. These changes, suggestive of

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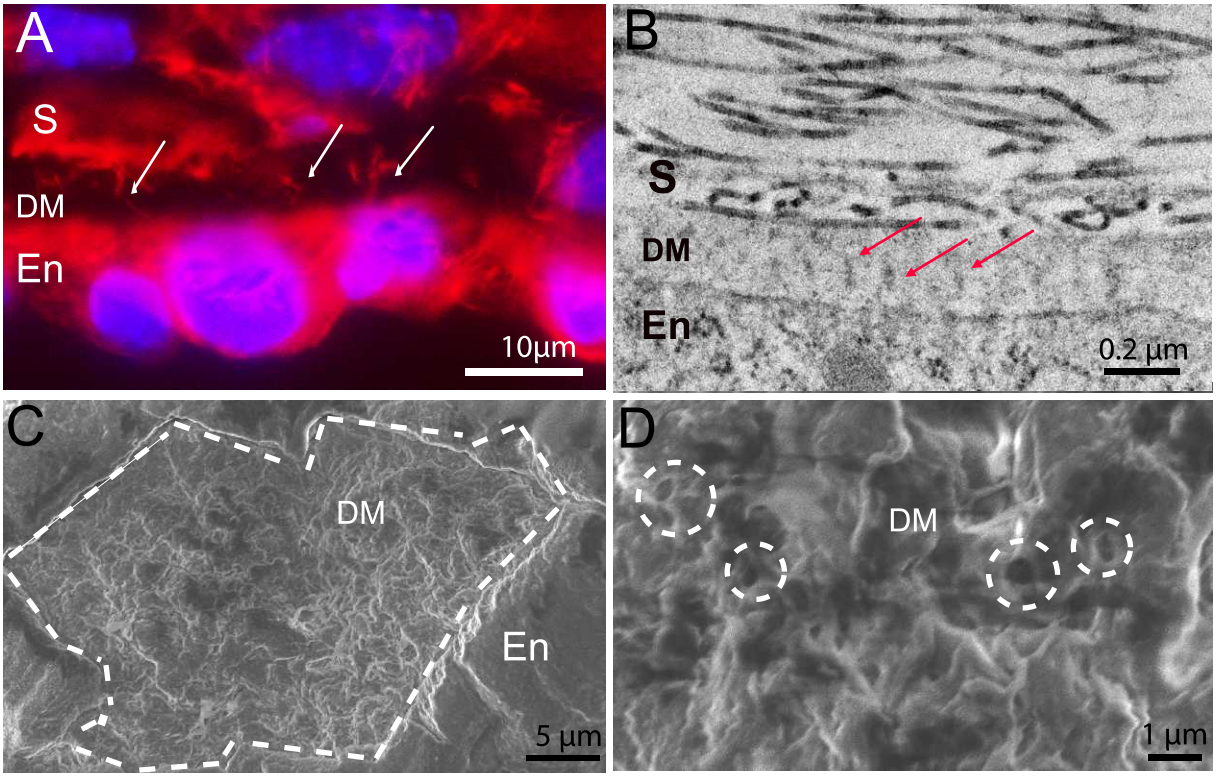

Figure 9. Corneal endothelial–stromal interactions. (**A**) Presence of endothelial processes extending through DM were observed using antiphalloidin staining (*arrows*) in the central cornea. (**B**) Transmission electron micrograph showing endothelial processes extending through DM (*arrows*). (**C**) Scanning electron micrograph, after stripping the endothelium, the DM is exposed within the area denoted by the *dotted white line*. (**D**) Scanning electron micrograph showing an enface (endothelial surface) view of DM. Pore-like structures are then exposed penetrating DM. The presence of pores in DM would allow communication between the stroma and the corneal endothelium. WT, P4 mice. *Bars*: 15 μm (**A**), 0. 2 μm (**B**), 5 μm (**C**), and 1 μm (**D**).

corneal endothelium and adjacent matrix immaturity, are similar to those described in the rabbit.[29,30]

To begin to evaluate paracellular permeability and corneal endothelial function, we used immunolocalization of ZO-1, a membrane protein associated with tight junctions (zonula occludens) in epithelial cells. The role of the endothelial tight junctions is of paramount importance in the maintenance of corneal transparency.[30] If the junctions are perturbed, stromal hydration increases causing loss of stromal proteoglycans, disarray of the collagen fibrils, and loss of transparency. Confocal microscopy of rat corneas on P1 and 10 demonstrated positive staining for ZO-1 on the apical and lateral aspects of the cells. By P21 and in the endothelium of adult rats, staining was restricted to the lateral aspect of the plasma membrane.[27] Similarly, we noted a diffuse pattern of ZO-1 staining in the P14 mice that became more organized and localized to the basolateral endothelial membrane in the P30 mouse. We believe that the basolateral membrane localization of ZO-1 together with the morphological changes noted in the endothelium at P30 are all synchronized and suggestive of a mature functional endothelium that is able to maintain corneal deturgescense.

To begin to define the influence of collagens XII and XIV on the function of the corneal endothelium, the corneal thickness was measured for all four genotypes. A normal P30 mouse cornea has reached its maturity and has a fully functional endothelium that works as an active pump that maintains corneal thickness. At P30, all null mice were significantly thicker than the control mice, signifying a delay of endothelial function and maturation. To determine if this thicker phenotype was from a delay of maturation, corneal thickness was measured again at P60 (data not shown). The *Col12a1*$^{-/-}$ and *Col14a1*$^{-/-}$ P60 corneas maintained a significantly thicker cornea than the control. The *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ was still thicker than the control thickness at P60 but failed the test for significance at $\alpha = 0.001$. The compound knockout cornea could be thicker because of structural weaknesses brought upon by the absence of both collagens XII and XIV. A synergistic interaction between collagen XII and XIV is also suggested by the more striking morphological endothelial changes noted in the compound *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ mice compared with the single mutants. We conclude that collagens XII and XIV influence the function of the corneal endothelium. In their absence the cornea is abnormally thicker. However, we cannot exclude the lack of structural interactions within the stroma as contributing to the increased thickness.

We have demonstrated that *Col12a1*$^{-/-}$, *Col14a1*$^{-/-}$, and compound *Col12a1*$^{-/-}$/*Col14a1*$^{-/-}$ mice had an immature endothelial phenotype at P30. The higher proportion of corneas with intercellular vacuoles and diffuse, interrupted Z0-1 staining, compared to WT and comparable to immature P14 WT endothelium, suggested that the corneal endothelium was immature in these mice. Although the exact mechanism of how FACIT collagens influence endothelial maturation is unknown, similar findings in the mouse corneal endothelium were recently noted in mutant mice at age 8 to 22 weeks old that either underexpressed or overexpressed PAX6.[31] In osteoblasts, collagen XII is involved in the regulation of differentiation and maturation, alignment, gap junction formation, and polarization. The deletion of collagen XII results in abnormal osteoblast differentiation and function, decreased bone matrix deposition,

and decreased bone quality. A similar effect can be suggested in the developing corneal endothelial cells.[21]

A potential mechanism whereby collagens XII and XIV may influence endothelial development and function is the presence of connections between the endothelium and stroma. These endothelial projections penetrate the DM. The existence of such trans-layer processes supports the hypothesis that the endothelium takes maturation cues from the developing stroma. The current understanding of the endothelial–DM–posterior stroma interaction is poor. The traditional view is that neural crest–derived endothelial cells are essential for corneal transparency.[30] Adult endothelial cells are described as quiescent cells without in vivo replicative capabilities, in most species,[27,32] that are isolated from the surrounding environment by DM. However, scientists have previously suggested that corneal endothelial cells interact directly with the underlying stroma. Cintron et al.[26] reported the presence of direct stroma–endothelial cell connections and the presence of pores in DM suggestive of direct endothelial–stromal cell interaction in the developing rabbit cornea. Similarly, Fitch et al.[25] showed the presence of collagen IV-rich matrix penetrating DM and connecting endothelial cells to the stroma at regular intervals in the chicken. Although the implications of such findings are unknown, the presence of direct endothelial–stromal contact contradicts the dogma that endothelial cells are isolated from the stroma by DM.

The statistically significant difference noted in corneal thickness between WT mice and the collagen XII and XIV deficient mice at P30 supports an immature endothelium in the deficient genotypes. Furthermore, mutant corneas become more compact at P60 although corneas were not as thin as the WT phenotype, suggesting a slow maturation of the endothelium. It could be hypothesized that poor endothelial function or maturation is responsible for increased hydration of these corneas, although no corneal edema was evident in these eyes and no quantification of corneal hydration was performed. Collagens XII and XIV in the cornea have been implicated in the assembly of collagen fibrils, and organization of fibrillar matrices and influences in this context require evaluation. Other extracellular matrix deficient mice models have shown stromal thinning. Lumican deficient corneas also showed stromal thinning after eyelid opening, suggesting that endothelial development and function may be normal in these mice. However, stromal compaction in the lumican deficient mouse led to increased light scattering and corneal opacification.[33]

In conclusion, our study revealed that in the absence of collagens XII and XIV, the maturation of the corneal endothelium is delayed and corneal compaction is abnormal. These FACIT collagens are critical for the development of normal corneal properties including hydration and thickness and therefore transparency. The data support our hypothesis that collagens XII and XIV are involved in the regulation of endothelial maturation and corneal function. However, the mechanisms whereby collagens XII and XIV affect the corneal endothelium are unknown. The mechanisms could be a direct interaction, involve macromolecular complexes, and/or feature sequestration of factors. The elucidation of specific mechanisms requires continued study.

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