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RESEARCH ARTICLE

Downregulation of collagen XI during late postnatal corneal development is followed by upregulation after injury

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ABSTRACT

Collagen XI plays a role in nucleating collagen fibrils and in controlling fibril diameter. The aim of this research was to elucidate the role that collagen XI plays in corneal fibrillogenesis during development and following injury. The temporal and spatial expression of collagen XI was evaluated in C57BL/6 wild-type mice. For wound-healing studies in adult mice, stromal injuries were created using techniques that avoid caustic chemicals. The temporal expression and spatial localization of collagen XI was studied following injury in a *Col11a1* inducible knockout mouse model. We found that collagen XI expression occurs during early maturation and is upregulated after stromal injury in areas of regeneration and remodeling. Abnormal fibrillogenesis with new fibrils of heterogeneous size and shape occurs after injury in a decreased collagen XI matrix. In conclusion, collagen XI is expressed in the stroma during development and following injury in adults, and is a regulator of collagen fibrillogenesis in regenerating corneal tissue.

KEY WORDS: Regeneration, Stroma, Fibroblasts, Wound, Collagen XI

INTRODUCTION

Loss of corneal stroma integrity caused by surgical procedures, infections or trauma increases the risk of scar formation that can, in turn, adversely affect corneal curvature, transparency and vision. In the stroma, the unique structure and hierarchical organization of the extracellular matrix is a major contributor to its clarity (Espana and Birk, 2020; Hassell and Birk, 2010; Maurice, 1957; Meek and Knupp, 2015). The stroma comprises 90% of the cornea and consists of water, collagens and proteoglycans (Espana and Birk, 2020; Hassell and Birk, 2010; Maurice, 1957; Meek and Knupp, 2015).

The structural unit of the stroma is the collagen fibril. In the cornea, collagens I and V are the main fibril-forming collagens. Collagen I is the most abundant protein while collagen V, $\alpha1(V)_2\alpha2(V)_1$ isoform, makes up 10–20% of fibril-forming collagens (McLaughlin et al., 1989; Pöschl and von der Mark, 1980; Tseng et al., 1982). In the cornea, where the smallest-diameter collagen fibrils in the body are assembled, collagen V is responsible for controlling collagen fibril diameter at ∼25–35 nm (Espana and Birk, 2020; Komai and Ushiki, 1991). These uniquely small fibrils are essential for minimizing light scatter.

Collagen XI is a fibril-forming collagen found in collagen II-containing tissues like cartilage, where it assembles with collagen II to form heterotypic fibrils (Blaschke et al., 2000; Fichard et al., 1995; Li et al., 1995). In the eye, collagens II and XI are known components of the vitreous gel, but, to our knowledge, there are no reports of collagen XI expression in the cornea (Mayne et al., 1993).

Collagens V and XI are fibril-forming collagens (Blaschke et al., 2000; Fichard et al., 1995; Smith and Birk, 2012) that can be considered different molecular forms of a single collagen type due to their structural similarities (Fichard et al., 1995; Hoffman et al., 2010; Smith and Birk, 2012). Collagens V and XI are characterized by a large globular region of the terminal NC3 that projects outward on the surface of the heterotypic fibril (Hoffman et al., 2010). This N-terminal domain, in the case of collagen V, contains sulfated tyrosine and is believed to regulate steric and/or electrostatic interactions at the fibril surface and, ultimately, fibril size (Birk, 2001; Linsenmayer et al., 1993). In this study, we investigated the expression of collagen XI in the corneal stroma during development. We further explored the roles of α1(XI) collagen chain in normally developed adult corneas after injury, using a conditional-null mouse model with floxed *Col11a1* alleles.

RESULTS

Collagen XI is only expressed in the immature stroma

To assess the role of collagen XI in corneal development, temporal and spatial expression of collagen XI was examined at different stages of life: immature (days 1–14), pre-adult (day 30) and adult (days 90 and 160). *Col11a1* mRNA expression was measured in the stroma using quantitative real-time PCR, and temporal changes in expression were noticeable. *Col11a1* mRNA was expressed at immature stages (Fig. 1A), peaking at day 4. There was a statistically significant trend demonstrating decreased *Col11a1* mRNA expression with tissue maturation (Cuzick's test, $P<0.0001$). Corneal collagen XI protein was also present in the stroma in immature stages when analyzed using the Wes western system and measuring the α1(XI) collagen chain (Fig. 1B,C). Collagen XI protein was barely detectable in pre-adult tissue. There was a statistically significant trend demonstrating decreased collagen XI expression with tissue maturation. Once the temporal expression of collagen XI had been determined, the patterns of spatial expression were investigated using immunofluorescence microscopy (Fig. 2). We found homogeneous collagen XI expression throughout the cornea only during immature stages of development. Quantitation of

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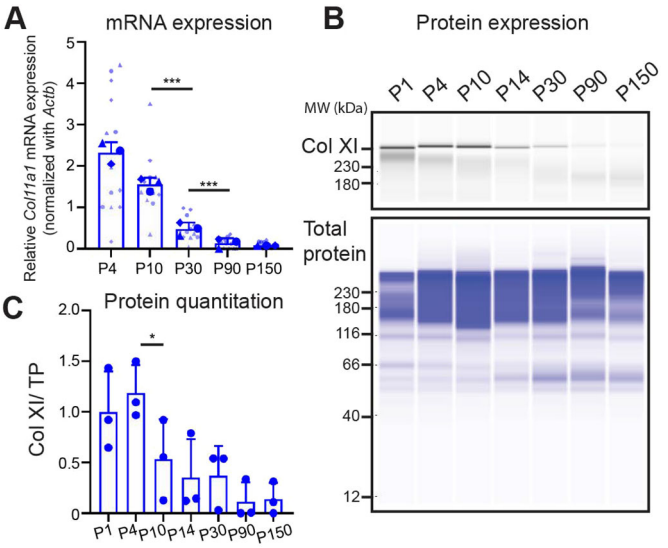

Fig. 1. Temporal expression of collagen XI in wild-type (WT) mice. (A) *Col11a1* mRNA expression was higher during stromal development and decreased sharply after postnatal day (P)10. Circles, triangles and diamonds represent sets of replicates. Each replicate consists of three to six individual corneas from different animals. Relative *Col11a1* levels normalized to β-actin (*Actb*). (B) Collagen XI protein was present in the maturing stroma. Representative image shows that collagen XI was present early in the maturing stroma but declined after P10. Total protein was used as a protein loading control. (C) Relative collagen XI protein quantitation normalized to total protein (TP). The standard deviation was obtained by Wes analysis of three sets of independent protein samples. Each set was obtained from pooling tissue from three to 12 corneas from different animals. **P*<0.05; ****P*<0.005 (Cuzick's test).

pixel intensity in the obtained images suggested decreased collagen XI expression with stromal maturation (Fig. S1). A statistically significant trend was demonstrated showing decreased collagen XI expression with tissue maturation (Cuzick's test, $P<0.0001$). The temporal and spatial expression patterns suggest a role for collagen XI in regulating early corneal development.

Stromal healing occurs with re-expression of collagen XI in the regenerated tissue

Stromal healing was assessed by evaluating deposition of new matrix above the stromal injury bed that was labeled with 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein (DTAF) at the time of surgery. The stromal injury model followed a previously published T3 injury model (Cogswell et al., 2021). We created a central partial thickness keratotomy followed by stromal debridement on the sides of the keratotomy (Fig. 3A). When analyzed 3 weeks post-injury, using fluorescence microscopy, areas of surgical keratotomy and stromal debridement remained strongly labeled with DTAF (Fig. 3C). Immunofluorescence microscopy of histology sections showed the DTAF-labeled stromal bed in green and absence of DTAF labeling in the newly regenerated stroma in the keratotomy injury area (Fig. 4A) and debridement injury area (Fig. 4B), demonstrating that new extracellular matrix had formed and suggesting that new stromal tissue formation took place. Immunofluorescence microscopy showed reactivity against collagen XI in newly formed stromal matrix within areas of keratotomy (Fig. 4C) and debridement injury (Fig. 4D).

Col11a1 mRNA expression measured in the stroma, following injury, using quantitative real-time PCR confirmed re-expression of collagen XI. *Col11a1* mRNA upregulation was observed in the early stages of injury and continued up to 6 weeks after injury

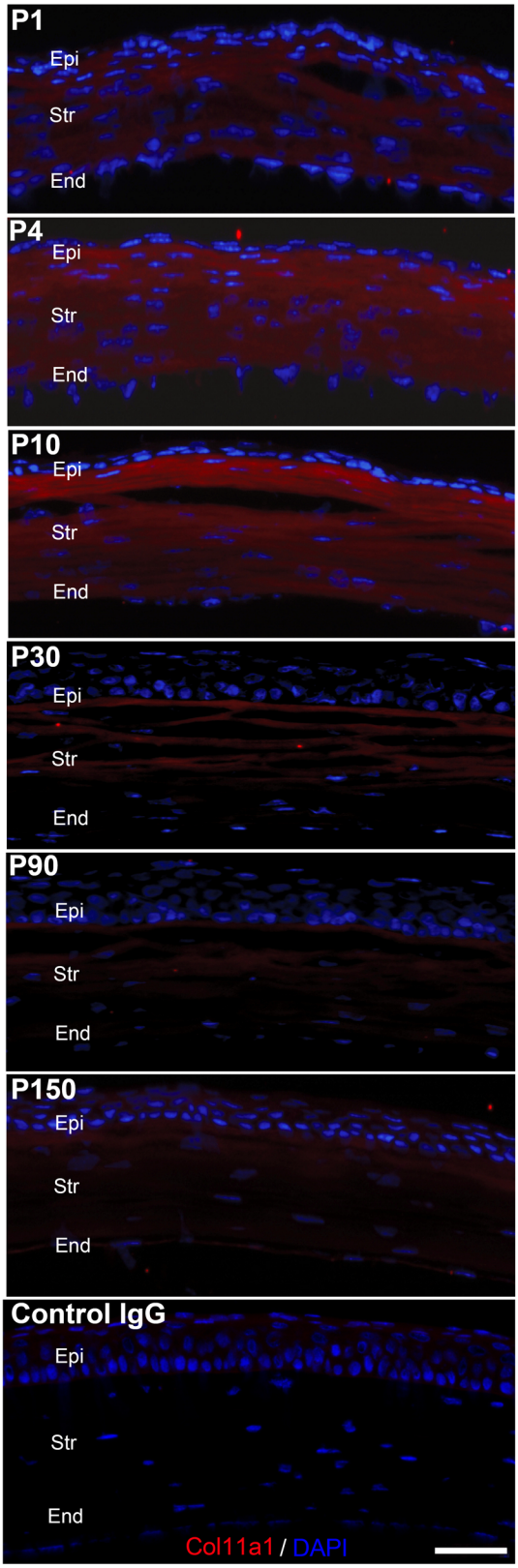

Fig. 2. Spatial expression of collagen XI in WT mice. Immunofluorescence localization images showed expression of collagen XI restricted to stromal maturation only (red) in the WT cornea. No corneal epithelial or endothelial staining was noted. Negative controls showed no reactivity. Nuclei were stained with DAPI (blue). Scale bar: 50 µm. Corneal tissue was obtained from three to four animals per age. Epi, epithelium; Str, stroma; End, endothelium.

Journal of Cell Science (2022) 135, jcs258694. doi:10.1242/jcs.258694

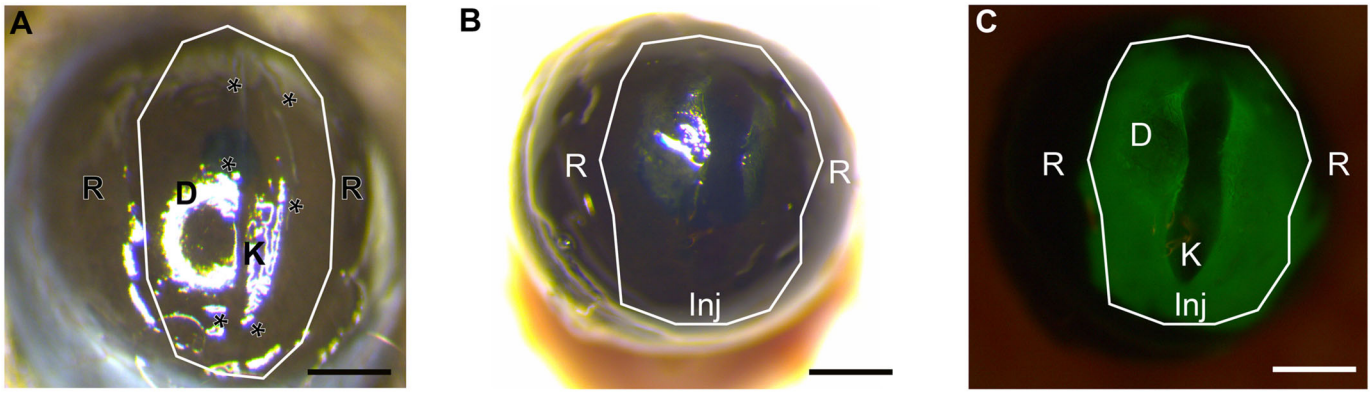

Fig. 3. Injury model and scar formation as well as DTAF labeling seen 3 weeks post-injury. (A) Surgical microscope image obtained after creating an injury. Injury area delimitated by the white line is divided into the debridement area of the wound (D) and the keratotomy (K), delineated by asterisks. (B) Photographs obtained 3 weeks after injury show the area of injury (Inj) and the remote area (R) that was not injured. (C) Corneal stroma DTAF labeling in the injured area is demonstrated by fluorescence microscopy. Scale bars: 500 µm.

(Fig. 5). There was a significant decreasing trend in *Col11a1* expression over time from day 1 to week 6 in the injured group (Cuzick's test, P=0.0264). However, no significant trend was observed in the control group (P=0.1981). Taken together, these findings demonstrate that collagen XI expression was recapitulated in the regenerated stroma.

Inducible mouse model significantly decreases stromal collagen XI synthesis

To evaluate whether efficient Cre excision was obtained after induction with Tamoxifen (Tm), stromal tissue was obtained 3 weeks after injury in three different groups of mice including a bi-transgenic conditional $Rosa^{Cre\text{-}ERT2/Cre\text{-}ERT2}/Col11a1^{flox/flox}$ mouse line that we name I-$Col11a1^{-/-}$ induced with intraperitoneal Tm. A control group of conditional $Rosa^{Cre\text{-}ERT2/Cre\text{-}ERT2}/Col11a1^{flox/flox}$ mice was treated with intraperitoneal vehicle (corn oil). A second wild-type (WT) control group was also given intraperitoneal Tm. When injured stromas were collected, they were divided into two regions: a central injured (Inj) area, where injured tissue was located, and a remote area (R) in the peripheral cornea, where no injury occurred (Fig. 3B,C). Quantification of $Col11a1^{-/-}$ expression was done using quantitative PCR in the Inj and R sampled areas. This quantification demonstrated a statistically significant decrease in *Col11a1* expression in the injured area of the induced mouse group compared to controls, 3 weeks after injury, and validated the inducible mouse model used in this paper (Fig. 6A). Similarly, immunofluorescence assay demonstrated decreased collagen XI synthesis only in the I-$Col11a1^{-/-}$ group 3 weeks post-injury (Fig. 6B,C).

Collagen XI is a regulator of fibril morphology after injury

To further evaluate the function of collagen XI in corneal fibrillogenesis, fibril morphology was assessed 6 weeks after

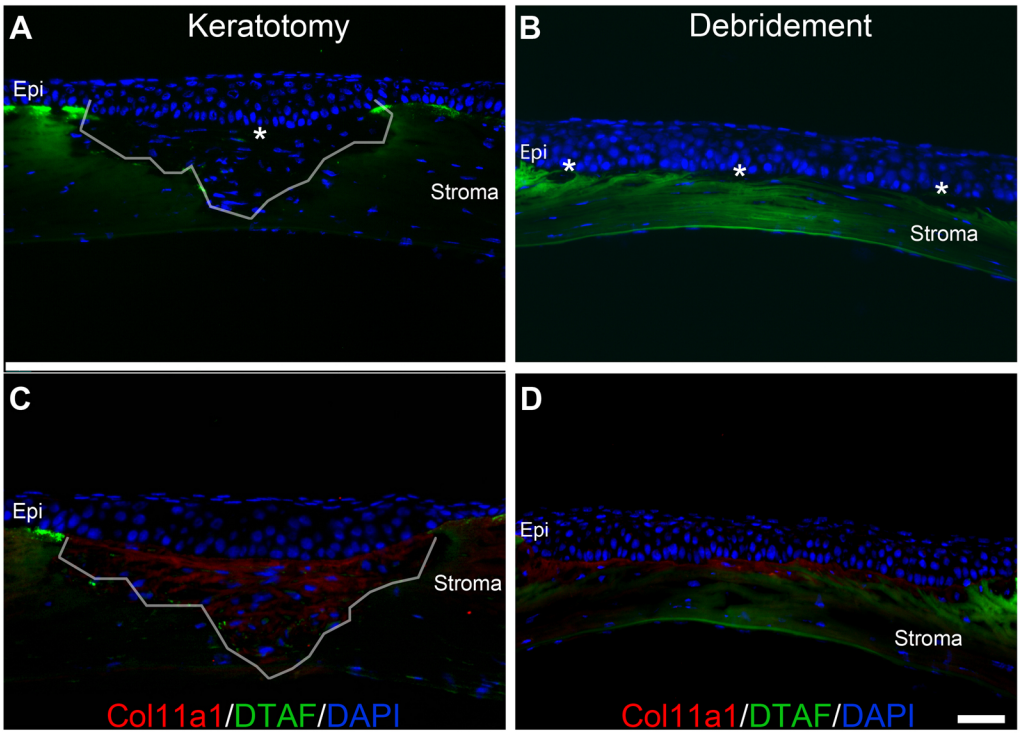

Fig. 4. Collagen XI re-expression following injury as shown in histology slides 3 weeks post-injury. (A) DTAF-labeled stroma and area of newly formed matrix after keratotomy injury not labeled by DTAF, demarcated by the white line and marked with an asterisk. (B) DTAF-labeled stroma and area of newly formed matrix after debridement injury not labeled by DTAF and marked with asterisks. (C,D) There is re-expression of collagen XI in both types of injuries (C, keratotomy; D, debridement) shown in red. Scale bar: 50 µm. Re-expression was noted in seven different eyes in which injury was created and tested. Epi, epithelium.

Journal of Cell Science (2022) 135, jcs258694. doi:10.1242/jcs.258694

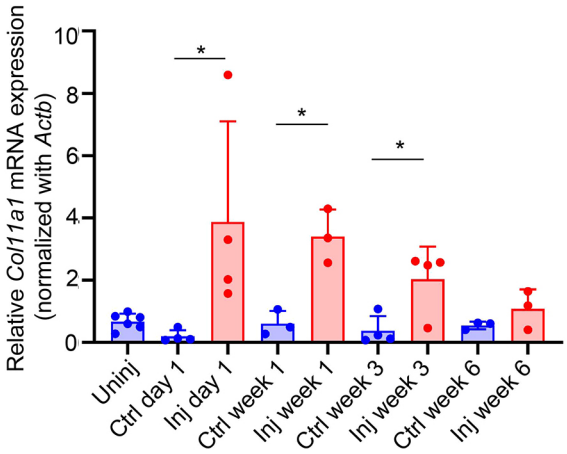

Fig. 5. Collagen XI mRNA expression is upregulated following injury. Uninj, normal adult corneal stroma; Ctrl, contralateral uninjured stroma; Inj, injured stroma. A significant trend for decreasing *Col11a1* mRNA expression over time was observed in the injured group (P=0.0264). Relative *Col11a1* levels normalized to *Actb*. n=36. *P<0.05 (Cuzick's test).

stromal injury. Control groups showed formation of new fibrils of similar size and shape to those in uninjured areas in the areas of stromal remodeling and regeneration (Fig. 7A,B). The uninjured corneal stroma was made of fibrils with diameter similar to that of controls, with no significant difference in fibril diameter size or shape (Fig. 7C). In contrast, in the I-*Col11a1*$^{-/-}$ group, in which fibril formation occurred in a collagen XI-deficient matrix, newly formed fibrils were of heterogeneous shape and size (Fig. 7D; see Fig. S2 for a comparison of fibril diameter distributions). Overall, these findings suggest that collagen XI plays a role in regulating fibrillogenesis after stromal injury and that the regulatory roles of collagen XI are important in determining lateral fibril growth after injury.

DISCUSSION

Stromal scars that increase the scattering of light and compromise vision often require corneal transplantation. Understanding how fibrillogenesis is regulated in stromal tissue after injury is an important step towards reducing scar formation and re-attaining corneal transparency. Understanding how specific matrix components contribute to corneal transparency is an evolving area of investigation with significant translational potential. In this study, we demonstrate that collagen XI is expressed by the corneal keratocyte during development and that re-expression of collagen XI synthesis plays a major role in the regulation of stromal structure after injury.

We consider the findings in this study to have three significant translational research implications. First, we demonstrate that collagen XI is expressed in the cornea during development, a stage of active fibrillogenesis. This is a novel finding. Second, we show that tissue regeneration occurs in the stroma during wound healing and that collagen XI contributes to fibrillogenesis in this new matrix. Third, we establish collagen XI as a regulator of fibril assembly as demonstrated by abnormal fibril morphology following injury in a decreased collagen XI matrix.

The unexpected finding that collagen XI, or at least a1(XI) chain, is expressed in the cornea during corneal development is novel and

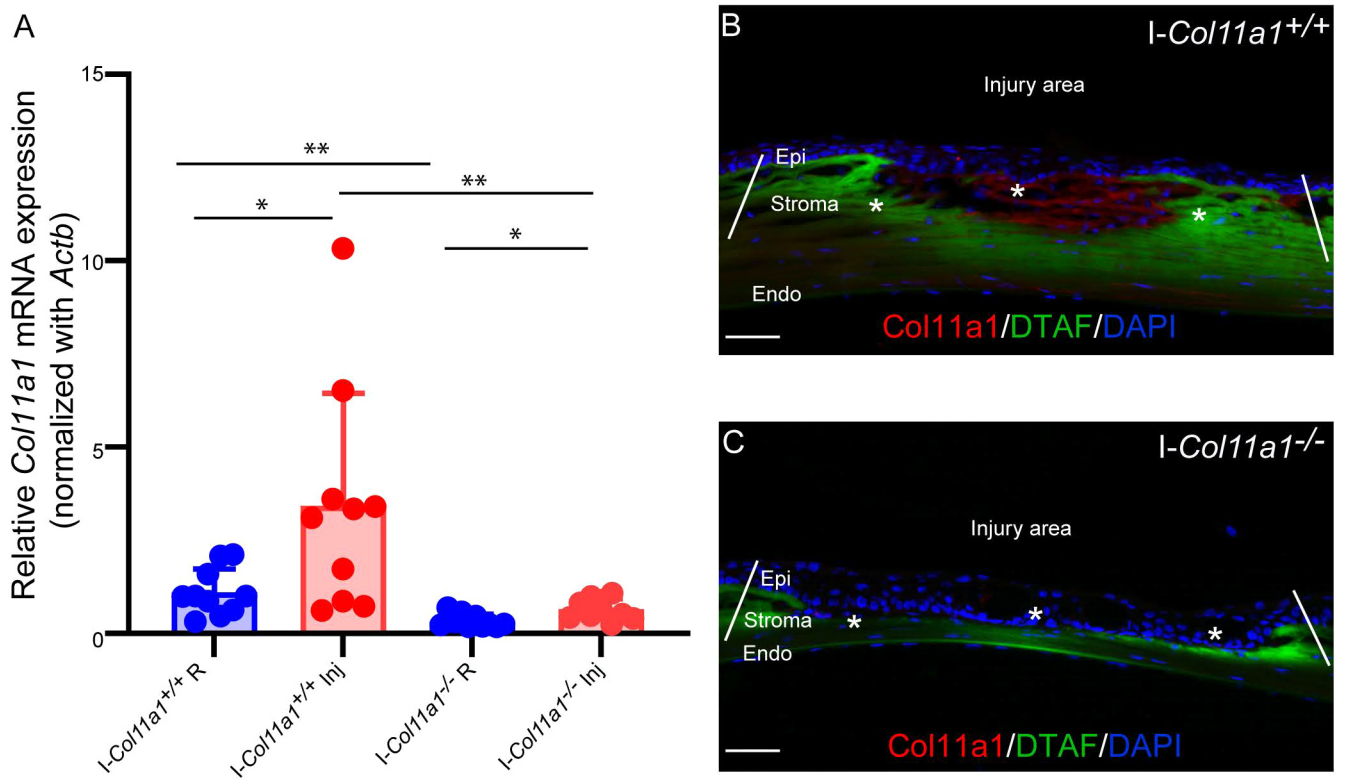

Fig. 6. An inducible mouse model with decreased collagen XI expression following injury. (A) *Col11a1* mRNA expression was decreased significantly after Tamoxifen (Tm) injection compared to a control group injected with intraperitoneal vehicle (corn oil). R, remote area; Inj, injured area. (B) Immunofluorescence microscopy shows collagen XI (red) expression in the stroma at 3 weeks in control cornea. (C) Decreased to absent collagen XI expression is observed in the injured model after induction with Tm. Tissue was procured 3 weeks after injury. Injury area demarcated by white lines and asterisks. Relative *Col11a1* levels normalized to *Actb*. n=10 per condition, experiments were performed three times. *P<0.05; **P<0.01 (one-tailed unpaired Student's t-test). Scale bars: 50 µm.

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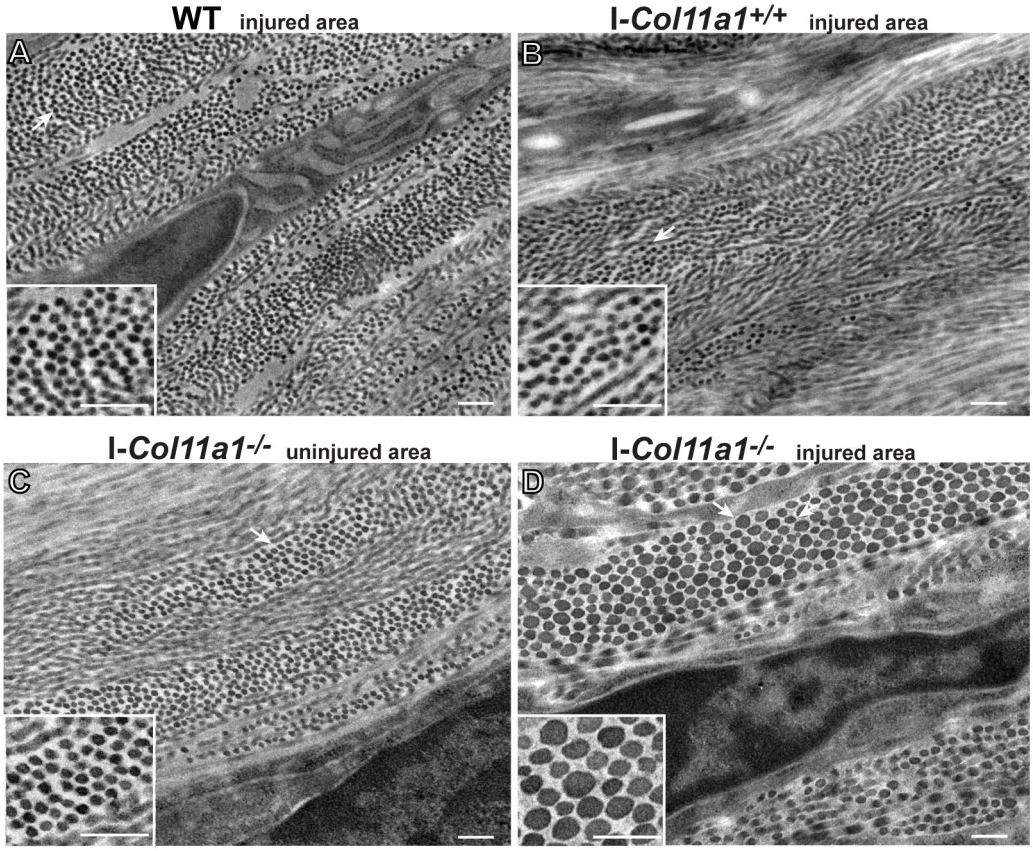

Fig. 7. Collagen XI is a regulator of corneal fibrillogenesis 6 weeks after injury. (A) Microphotographs from control WT mice injected with intraperitoneal Tm show typical small corneal fibrils of homogenous size in the injured area. (B) A second control group, *Rosa*$^{Cre\text{-}ERT2/Cre\text{-}ERT2}$/*Col11a1*$^{flox/flox}$ mice, injected with intraperitoneal oil show typical small corneal fibrils of homogenous size in the injured area. (C) Similarly, I-*Col11a1*$^{-/-}$ mice injected with intraperitoneal Tm show typical small corneal fibrils of homogenous size in the uninjured area. (D) I-*Col11a1*$^{-/-}$ mice injected with intraperitoneal Tm show an abnormal group of regenerated fibrils of different size and shape in the injured area. Insets show fibrils at higher magnification. Three to five corneas were studied per condition. Scale bars: 200 nm.

suggests that collagen XI can play a role as regulator of fibrillogenesis. How collagen XI might regulate fibrillogenesis is unknown. Clearly, more research is needed to explore the temporal role that collagen XI plays in early stromal development and the mechanism by which collagen XI could affect fibrillogenesis. We speculate that collagen XI could regulate fibrillogenesis per se or by forming V/XI hybrid molecules {[a1(XI)]2a2(V) and a1(XI)a2(V)a3(XI)}, both of which contain the a1(XI) chain (Smith and Birk, 2012). In other words, collagen XI could have similar regulatory functions to collagen V. Our laboratory is currently exploring the role of collagen XI in stromal development using a cornea-specific keratocan-driven mouse model.

Does collagen XI play additional roles besides regulating fibril spacing and diameter? Interestingly, collagen XI is highly expressed in avascular tissues (i.e. cartilage, vitreous gel and now in the cornea). One hypothesis proposes a functional role for collagen XI in keeping the stroma avascular.

A second implication of this paper is that we demonstrated that collagen XI re-expression occurs during fibrillogenesis after injury, confirming previous reports that showed stromal regeneration in rabbits even after excimer laser ablation (Cintron and Kublin, 1977; Kivanany et al., 2018). Corneal stromal regeneration after corneal injury is not supported by published studies. The entire field of laser refractive surgery of the cornea is based on the dogma that laser ablation of stromal tissue creates a predictable and permanent change in corneal stromal shape without a clinically substantial scar (Erie et al., 2002; Patel et al., 2007). It is not clear if stromal regeneration occurs following all mechanisms of injury or if regeneration only occurs in some mammals and not in humans.

Third, we have shown that re-expression of collagen XI is an essential step regulating stromal fibrillogenesis after injury. Microphotographs obtained from the regenerated stroma of WT and control mice show collagen fibrils of normal size and shape. In contrast, normal adult corneas from inducible mouse models in which the expression of collagen XI was decreased at the time of injury clearly display abnormal fibrillogenesis. In addition, this occurs with collagen fibrils of different and irregular diameters. These findings suggest that collagen XI plays a significant role in the regulation of corneal fibrillogenesis including fibril size and shape. It also establishes collagen XI as an important fibril nucleator in the cornea.

Collagen XI is a topic of interest in oncology research. Collagen XI was the most overexpressed gene product in solid cancers in a meta-analysis of microarray data from multiple solid tumors (Kim et al., 2010). It is considered a biomarker for poor clinical outcomes in various solid tumors (Vázquez-Villa et al., 2015). Collagen XI is highly expressed by activated stromal cells occurring within the desmoplasmic reaction found in different solid tumors (Galván et al., 2014; Vázquez-Villa et al., 2015). Interestingly, some authors believe that collagen XI-expressing cancer-associated fibroblasts originate from bone marrow and not from tissue-specific fibroblasts (Galván et al., 2014). *In vitro* studies of bone marrow-expanded

Journal of Cell Science (2022) 135, jcs258694. doi:10.1242/jcs.258694

myofibroblasts and corneal-expanded myofibroblasts showed that collagen XI is highly expressed by myofibroblasts derived from bone marrow cells. In contrast, myofibroblasts phenotypically converted from resident stromal keratocytes did not express significant levels of collagen XI (Saikia et al., 2020). These are interesting observations that could implicate bone marrow-derived cells in modulating stromal fibrillogenesis (Wilson et al., 2021). Further work is needed to evaluate whether the irregularly shaped fibrils assembled in a collagen XI-deficient environment are produced by stromal-resident cells or by bone marrow-derived cells that home to the cornea after injury. The question of what type of cells is synthesizing collagen XI is not inconsequential. Could collagen XI be a marker for progenitor stromal cells or immature keratocytes or keratocyte-derived myofibroblasts? Or is it possible that a severe injury in the stroma sends signals for reinforcements from bone marrow-derived cells? We are currently exploring the role of collagen XI in scar formation and evaluating if bone marrow-derived cells can synthesize collagen XI *in vivo*.

In conclusion, we show that collagen XI is expressed in the cornea during development and that collagen XI expression is recapitulated during wound healing. The role of collagen XI in stromal function and regeneration needs further exploration.

MATERIALS AND METHODS

Animals

WT C57BL/6 mice were used for studies evaluating the expression of collagen XI in development of the corneal stroma. The dissected whole corneas from mice at postnatal day (P)4, P10, P30, P90 and P150 were examined. To evaluate the function of collagen XI in stromal wound healing, a conditional *Col11a1*$^{flox/flox}$ mouse model (Sun et al., 2020) was bred with the knock-in Tm-inducible Cre (*Rosa*$^{Cre\text{-}ERT2/Cre\text{-}ERT2}$) mouse (Stock 008463, The Jackson Laboratory, Bar Harbor, ME) and generated a bi-transgenic conditional *Rosa*$^{Cre\text{-}ERT2/Cre\text{-}ERT2}$/*Col11a1*$^{flox/flox}$ mouse line that we name I-*Col11a1*$^{-/-}$ when injected with intraperitoneal Tm. Excision of *Col11a1* sequences was then induced with Tm dissolved in corn oil (Sigma-Aldrich, St Louis, MO), administered into the peritoneum of male I-*Col11a1*$^{-/-}$ mice aged 60 days (100 mg/kg body weight), for a total of 3 days. A control group of *Rosa*$^{Cre\text{-}ERT2/Cre\text{-}ERT2}$/*Col11a1*$^{flox/flox}$ mice was injected with vehicle only (corn oil), and a second control group (WT mice) was injected with intraperitoneal Tm dissolved in vehicle. Tm or control corn oil vehicle treatments were given for two consecutive days prior to surgery and a third injection was given the day of surgery. Pilot studies for time and dose inhibition showed effectiveness of *Col11a1* inhibition with three doses of intraperitoneal Tm in 72 h. All mice were housed and treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD).

Stromal injury model

Only left corneas were subjected to injury. All experiments conformed to the use of Laboratory Animals and 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. I-*Col11a1*$^{-/-}$ and control groups were treated for 3 days with Tm or vehicle. Animals were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Under microscope visualization, a partial thickness keratotomy was performed from limbus to limbus using a custom-made diamond blade with a 70 µm guarded depth (Mastel, Rapid City, SD) followed by stromal debridement as previously described using a diamond burr (Cogswell et al., 2021). The stromal debridement was done on both sides of the keratotomy. In a group of mice, DTAF (Sigma-Aldrich) was applied immediately to the corneal surface after injury to evaluate deposition of new matrix because DTAF labels collagen in the stromal residual bed and not in the regenerated tissue (Jester et al., 1995; Kivanany et al., 2018). Eight mice per condition were used.

RNA isolation and quantification of mRNA

Whole corneas were dissected from WT mice at ages P4, P10, P30, P90 and P150, and in a group of adult mice that underwent eye injury, 1 day, 1, 3 and 6 weeks after creating the injury as described above. In another group of injured corneas, the tissue was separated into two regions: the area of injury (Inj), where the wound was created, and the remote area (R), where no injury existed. Isolated corneal tissues were incubated with Dispase II (Roche, Indianapolis, IN) to remove the cornea epithelium. The stroma of cornea was cut into small pieces, and total RNA was extracted using QIAzol Lysis Reagent (Qiagen, Venlo, Limburg, The Netherlands) and an RNeasy MinElute Cleanup Kit (Qiagen). Reverse transcription and quantitative real-time PCR analysis was performed as described previously (Sun et al., 2020). The following primer sequences were used: *Col11a1* FW, GACCAGAA-GACACACTGAAAGCA; *Col11a1* RV, TCCATGCCATCTGAGTAGT-CAAGA; *Actb* FW, AGATGACCCAGATCATGTTTGAGA; *Actb* RV, CACAGCCTGGATGGCTACGT. Each sample was run in duplicate PCR reaction, and statistical analysis was performed from three to six corneas of different mice at each time point.

Quantitation of protein expression

Corneas from WT mice at P1, P4, P10, P14, P30, P90 and P150 were cut into small pieces so that three to 12 corneas were pooled for protein extraction using lysis buffer containing 50 mM Tris-HCl, pH 6.8, 1% SDS and fresh added proteinase inhibitor cocktail (Thermo Fisher Scientific). After agitation at 4°C overnight, the cornea lysate was dispersed by repeating three cycles of vortexing for 30 s, boiling for 1 min and cooling on ice briefly. Protein concentration was determined through a bicinchoninic acid assay (Thermo Scientific Pierce). Collagen XI protein expression was analyzed using a Wes (ProteinSimple, San Jose, CA) simple western system following the manufacturer's instructions, applying 12–230 kDa separation module, anti-rabbit detection module and total protein detection module. Briefly, samples diluted with 0.1× sample buffer (ProteinSimple) at a concentration of 0.5 µg/µl were loaded and hybridized with anti-mouse collagen XI antibody (1:50 dilution) (Sun et al., 2020). Total protein was used as a loading control. Quantification by densitometry was performed using the area of the targeted protein and normalized to total protein amount, which was analyzed by loading an equal amount of protein to a separate capillary cartridge and detected with the Wes total protein detection module. Data analyses were performed using the Compass Software (ProteinSimple).

Immunofluorescence microscopy

Fresh eyes were harvested from C57BL/6 mice at ages P1, P4, P10, P30, P90 and P150, and specimens collected 3–4 weeks after stromal injury. They were then embedded in OCT medium frozen with isopentane (Sigma-Aldrich) on dry ice. Corneal sections of 5–7 µm were blocked using 10% donkey serum (Sigma-Aldrich) and then pre-treated with testicular hyaluronidase (EMD Millipore, Burlington, MA). Sections were incubated with rabbit anti-mouse collagen XI antibody (1:100 dilution) (Sun et al., 2020) for 1 h at room temperature. The secondary antibody was Alexa Fluor 568 donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA) used at 1:200 dilution. DAPI Fluoromount-G® clear mounting solution (SouthernBiotech, Birmingham, AL) with DAPI was used as a nuclear marker. DTAF-labeled samples were also examined. Images were captured using a fluorescence microscope (Leica DM5500B). Identical conditions and negative controls were used to facilitate comparisons between samples. Images obtained from the corneal stroma at different ages were analyzed using ImageJ (National Institutes of Health). A mean pixel intensity value was obtained from different images as an approximation of collagen XI expression (Sun et al., 2021). Inferences in the differences in collagen XI expression during development were quantified, and Cuzick's test for temporal changes in collagen XI expression was used.

Transmission electron microscopy

Eyes were enucleated 6 weeks after injury, and stromal morphology was analyzed using transmission electron microscopy as previously described (Sun et al., 2020). Briefly, at least three corneas per group were dissected and fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium

cacodylate, pH 7.4, with 8.0 mM $CaCl_2$, post-fixed in 1% osmium tetroxide. The corneas were dehydrated in a graded ethanol series, followed by propylene oxide. The tissue samples were infiltrated and embedded in a mixture of Embed 812, nadic methyl anhydride, dodecenyl succinic anhydride and DMP-30 (Electron Microscopy Sciences, Hatfield, PA). Thin sections (~80 nm) were cut with a Leica ultramicrotome and post-stained with 2% aqueous uranyl acetate and 1% phosphotungstic acid, pH 3.2. The sections were examined at 80 kV with a JEOL 1400 transmission electron microscope equipped with a Gatan Ultrascan US1000 2K digital camera. Three to five mice per condition were used.

Fibril diameter distribution

Digital images were taken from non-overlapping regions in the remote and injured stroma of I-*Col11a1*$^{-/-}$ mice injected with intraperitoneal Tm and compared to an injured region of a control group, I-C*ol11a1*$^{+/+}$, at a magnification of 60,000×. Images were randomly masked, and fibril diameter and density were measured using ImageJ software. A region of interest of appropriate size was chosen within each image in which fibrils were perpendicular/cross-sectional to the viewing plane (Donovan et al., 2021; Sun et al., 2020).

Statistical analysis

Prism version 9.1.2 (GraphPad, San Diego, CA) and Stata version 17 (StataCorp, College Station, TX) were used for all statistical analyses, and all data are shown as means±s.d. Statistical analyses were done by the Research Methodology and Biostatistics Core, Morsani College of Medicine, University of South Florida, Tampa, FL. Statistical significance between two conditions was evaluated by non-parametric, two-tailed Kruskal–Wallis test. Trend analysis was performed using non-parametric Cuzick's analysis. Values with $P<0.05$ were considered statistically significant.

Competing interests

The authors declare no competing or financial interests.

Author contributions

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References

Birk, D. E. (2001). Type V collagen: heterotypic type I/V collagen interactions in the regulation of fibril assembly. *Micron* **32**, 223-237. doi:10.1016/S0968-4328(00)00043-3

Blaschke, U. K., Eikenberry, E. F., Hulmes, D. J. S., Galla, H.-J. and Bruckner, P. (2000). Collagen XI nucleates self-assembly and limits lateral growth of cartilage fibrils. *J. Biol. Chem.* **275**, 10370-10378. doi:10.1074/jbc.275.14.10370

Cintron, C. and Kublin, C. L. (1977). Regeneration of corneal tissue. *Dev. Biol.* **61**, 346-357. doi:10.1016/0012-1606(77)90304-9

Cogswell, D., Sun, M., Greenberg, E., Margo, C. E. and Espana, E. M. (2021). Creation and grading of experimental corneal scars in mice models. *Ocul. Surf.* **19**, 53-62. doi:10.1016/j.jtos.2020.11.008

Donovan, C., Koudouna, E., Margo, C. E., Avila, M. Y. and Espana, E. M. (2021). Genipin delays corneal stromal enzymatic digestion. *Transl. Vis. Sci. Technol.* **10**, 25. doi:10.1167/tvst.10.9.25

Erie, J. C., Patel, S. V., McLaren, J. W., Ramirez, M., Hodge, D. O., Maguire, L. J. and Bourne, W. M. (2002). Effect of myopic laser in situ keratomileusis on epithelial and stromal thickness: a confocal microscopy study. *Ophthalmology* **109**, 1447-1452. doi:10.1016/S0161-6420(02)01106-5

Espana, E. M. and Birk, D. E. (2020). Composition, structure and function of the corneal stroma. *Exp. Eye Res.* **198**, 108137. doi:10.1016/j.exer.2020.108137

Fichard, A., Kleman, J.-P. and Ruggiero, F. (1995). Another look at collagen V and XI molecules. *Matrix Biol.* **14**, 515-531. doi:10.1016/S0945-053X(05)80001-0

Galván, J. A., García-Martínez, J., Vázquez-Villa, F., García-Ocaña, M., García-Pravia, C., Menéndez-Rodríguez, P., González-del Rey, C., Barneo-Serra, L. and de los Toyos, J. R. (2014). Validation of COL11A1/procollagen 11A1 expression in TGF-β1-activated immortalised human mesenchymal cells and in stromal cells of human colon adenocarcinoma. *BMC Cancer* **14**, 867. doi:10.1186/1471-2407-14-867

Hassell, J. R. and Birk, D. E. (2010). The molecular basis of corneal transparency. *Exp. Eye Res.* **91**, 326-335. doi:10.1016/j.exer.2010.06.021

Hoffman, G. G., Branam, A. M., Huang, G., Pelegri, F., Cole, W. G., Wenstrup, R. M. and Greenspan, D. S. (2010). Characterization of the six zebrafish clade B fibrillar procollagen genes, with evidence for evolutionarily conserved alternative splicing within the pro-α1(V) C-propeptide. *Matrix Biol.* **29**, 261-275. doi:10.1016/j.matbio.2010.01.006

Jester, J. V., Petroll, W. M., Barry, P. A. and Cavanagh, H. D. (1995). Expression of alpha-smooth muscle (alpha-SM) actin during corneal stromal wound healing. *Invest. Ophthalmol. Vis. Sci.* **36**, 809-819.

Kim, H., Watkinson, J., Varadan, V. and Anastassiou, D. (2010). Multi-cancer computational analysis reveals invasion-associated variant of desmoplastic reaction involving INHBA, THBS2 and COL11A1. *BMC Med. Genomics* **3**, 51. doi:10.1186/1755-8794-3-51

Kivanany, P. B., Grose, K. C., Tippani, M., Su, S. and Petroll, W. M. (2018). Assessment of corneal stromal remodeling and regeneration after photorefractive keratectomy. *Sci. Rep.* **8**, 12580. doi:10.1038/s41598-018-30372-2

Komai, Y. and Ushiki, T. (1991). The three-dimensional organization of collagen fibrils in the human cornea and sclera. *Invest. Ophthalmol. Vis. Sci.* **32**, 2244-2258.

Li, Y., Lacerda, D. A., Warman, M. L., Beier, D. R., Yoshioka, H., Ninomiya, Y., Oxford, J. T., Morris, N. P., Andrikopoulos, K., Ramirez, F. et al. (1995). A fibrillar collagen gene, *Col11a1*, is essential for skeletal morphogenesis. *Cell* **80**, 423-430. doi:10.1016/0092-8674(95)90492-1

Linsenmayer, T. F., Gibney, E., Igoe, F., Gordon, M. K., Fitch, J. M., Fessler, L. I. and Birk, D. E. (1993). Type V collagen: molecular structure and fibrillar organization of the chicken alpha 1(V) NH2-terminal domain, a putative regulator of corneal fibrillogenesis. *J. Cell Biol.* **121**, 1181-1189. doi:10.1083/jcb.121.5.1181

Maurice, D. M. (1957). The structure and transparency of the cornea. *J. Physiol.* **136**, 263-286. doi:10.1113/jphysiol.1957.sp005758

Mayne, R., Brewton, R. G., Mayne, P. M. and Baker, J. R. (1993). Isolation and characterization of the chains of type V/type XI collagen present in bovine vitreous. *J. Biol. Chem.* **268**, 9381-9386. doi:10.1016/S0021-9258(18)98361-4

McLaughlin, J. S., Linsenmayer, T. F. and Birk, D. E. (1989). Type V collagen synthesis and deposition by chicken embryo corneal fibroblasts in vitro. *J. Cell Sci.* **94**, 371-379. doi:10.1242/jcs.94.2.371

Meek, K. M. and Knupp, C. (2015). Corneal structure and transparency. *Prog. Retin. Eye Res.* **49**, 1-16. doi:10.1016/j.preteyeres.2015.07.001

Patel, S. V., Erie, J. C., McLaren, J. W. and Bourne, W. M. (2007). Confocal microscopy changes in epithelial and stromal thickness up to 7 years after LASIK and photorefractive keratectomy for myopia. *J. Refract. Surg.* **23**, 385-392. doi:10.3928/1081-597X-20070401-11

Pöschl, A. and von der Mark, K. (1980). Synthesis of type V collagen by chick corneal fibroblasts in vivo and in vitro. *FEBS Lett.* **115**, 100-104. doi:10.1016/0014-5793(80)80735-6

Saikia, P., Crabb, J. S., Dibbin, L. L., Juszczak, M. J., Willard, B., Jang, G.-F., Shiju, T. M., Crabb, J. W. and Wilson, S. E. (2020). Quantitative proteomic comparison of myofibroblasts derived from bone marrow and cornea. *Sci. Rep.* **10**, 16717. doi:10.1038/s41598-020-73686-w

Smith, S. M. and Birk, D. E. (2012). Focus on molecules: collagens V and XI. *Exp. Eye Res.* **98**, 105-106. doi:10.1016/j.exer.2010.08.003

Sun, M., Zafrullah, N., Devaux, F., Hemmavanh, C., Adams, S., Ziebarth, N. M., Koch, M., Birk, D. E. and Espana, E. M. (2020). Collagen XII is a regulator of corneal stroma structure and function. *Invest. Ophthalmol. Vis. Sci.* **61**, 61. doi:10.1167/iovs.61.5.61

Sun, M., Zafrullah, N., Adams, S., Devaux, F., Avila, M. Y., Ziebarth, N., Margo, C. E., Koch, M. and Espana, E. M. (2021). Collagen XIV is an intrinsic regulator of corneal stromal structure and function. *Am. J. Pathol.* **191**, 2184-2194. doi:10.1016/j.ajpath.2021.08.016

Tseng, S. C., Smuckler, D. and Stern, R. (1982). Comparison of collagen types in adult and fetal bovine corneas. *J. Biol. Chem.* **257**, 2627-2633. doi:10.1016/S0021-9258(18)34970-6

Vázquez-Villa, F., García-Ocaña, M., Galván, J. A., García-Martínez, J., García-Pravia, C., Menéndez-Rodríguez, P., Rey, C. G.-D., Barneo-Serra, L. and de los Toyos, J. R. (2015). COL11A1/(pro)collagen 11A1 expression is a remarkable biomarker of human invasive carcinoma-associated stromal cells and carcinoma progression. *Tumour Biol.* **36**, 2213-2222. doi:10.1007/s13277-015-3295-4

Wilson, S. E., Sampaio, L. P., Shiju, T. M. and Carlos de Oliveira, R. (2021). Fibroblastic and bone marrow-derived cellularity in the corneal stroma. *Exp. Eye Res.* **202**, 108303. doi:10.1016/j.exer.2020.108303