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Regulation of Corneal Stroma Extracellular Matrix Assembly

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Abstract

The transparent cornea is the major refractive element of the eye. A finely controlled assembly of the stromal extracellular matrix is critical to corneal function, as well as in establishing the appropriate mechanical stability required to maintain corneal shape and curvature. In the stroma, homogeneous, small diameter collagen fibrils, regularly packed with a highly ordered hierarchical organization, are essential for function. This review focuses on corneal stroma assembly and the regulation of collagen fibrillogenesis. Corneal collagen fibrillogenesis involves multiple molecules interacting in sequential steps, as well as interactions between keratocytes and stroma matrix components. The stroma has the highest collagen V:I ratio in the body. Collagen V regulates the nucleation of protofibril assembly, thus controlling the number of fibrils and assembly of smaller diameter fibrils in the stroma. The corneal stroma is also enriched in small leucine-rich proteoglycans (SLRPs) that cooperate in a temporal and spatial manner to regulate linear and lateral collagen fibril growth. In addition, the fibril-associated collagens (FACITs) such as collagen XII and collagen XIV have roles in the regulation of fibril packing and inter-lamellar interactions. A communicating keratocyte network contributes to the overall and long-range regulation of stromal extracellular matrix assembly, by creating micro-domains where the sequential steps in stromal matrix assembly are controlled. Keratocytes control the synthesis of extracellular matrix components, which interact with the keratocytes dynamically to coordinate the regulatory steps into a cohesive process. Mutations or deficiencies in stromal regulatory molecules result in altered interactions and deficiencies in both transparency and refraction, leading to corneal stroma pathobiology such as stromal dystrophies, cornea plana and keratoconus.

Keywords

Corneal Stroma; Small Leucine-Rich Proteoglycans; Collagens; Fibrillogenesis; Extracellular Matrix; Stromal Organization

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Introduction

The cornea is the transparent tissue at the anterior of the eye. Transparency is its main function, allowing passage of the light, and the cornea is the major refractive structure of the eye. The cornea, together with the sclera, also provides mechanical stability and a protective barrier. The cornea is composed of three layers: an outer epithelial layer; a middle layer – the stroma that is the focus of this review, and an inner endothelial layer. The corneal stroma comprises about 90% of the thickness of the cornea. It is a collagen-rich extracellular matrix (ECM) assembled to provide transparency and maintain the structure required for refraction of light. The main structural features of the stroma are dense, regularly packed collagen fibrils. The collagen fibrils have a homogeneous distribution of small (~25 nm) diameters. The fibrils are organized as regularly packed bundles or fibers. The fibers coalesce and form stacked layers or lamellae (Fig. 1). The organization of lamellae has been and continues to be described as orthogonal which indicates a 90° offset of adjacent lamellae. Individual sections support a description of adjacent lamellae being offset roughly 90° from each other. However, inter-lamellar angles ranging from 1° to 90° have been found associated with different species and stromal locations (Radner et al., 1998; Thomasy et al., 2014). Therefore, the over generalized used of “orthogonal” misrepresents structural differences that are undoubtedly critical determinants of functional requirements. However, the majority of lamellae are offset from adjacent lamellae forming a plywood-like structure and terms such as ‘roughly orthogonal’ are likely to be perpetuated even as work detailing the structure-function relationships of this level of the stromal hierarchy continues. Finally, based on the physical properties of light, the regular, stacked lamellar structure of the corneal stroma produces minimal light scattering and therefore maximal transparency (Benedek, 1971; Farrell et al., 1973; Maurice, 1957). The structural features provide the molecular basis required for stromal transparency; therefore, regulation of stromal extracellular matrix assembly is essential for corneal transparency.

The cornea has a different curvature from the rest of the ocular surface. In addition, there is a difference in the thickness between the central and peripheral cornea. In the central cornea, the majority of collagen fibrils are oriented superior-inferior or nasal-temporal, orthogonally. At the periphery, however, collagen fibril bundles are branched and interlacing gradually into a circular orientation at the cornea and sclera interface (Boote et al., 2008). There are also differences in fibril organization between anterior and posterior stroma in the nasal and temporal regions. These regional differences in collagen fibril organization provide mechanical strength and underlie the structural features critical for maintaining corneal shape and its refractive properties.

Extracellular matrix assembly in the corneal stroma is tightly regulated during development. This review will focus on the regulation of stromal extracellular matrix assembly during development and maintenance of stromal structure. However, the concepts discussed will provide a foundation for studies of corneal stromal pathophysiology, as well as application to the corneal stroma of the approaches utilized in regenerative medicine.

Corneal Stromal Assembly During Development

The development of the corneal stroma has been extensively studied in the chicken (Cornuet et al., 1994; Coulombre and Coulmbre, 1958; Coulombre and Coulombre, 1958; Funderburgh et al., 1986; Hay, 1977; Hay and Revel, 1969; Quantock and Young, 2008; Toole and Trelstad, 1971) and the rabbit (Cintron and Covington, 1990; Cintron et al., 1988a; Cintron et al., 1984; Cintron and Hong, 1988; Cintron et al., 1988b; Takahashi et al., 1993; Zhan et al., 1995). In avian species, neural crest cells migrate into the space between the corneal epithelium and the lens epithelium, and they are loosely aggregated into several layers filling the space. The first wave of neural crest cells condenses to form the corneal endothelium. Additional neural crest cells between the corneal epithelium and endothelium become keratoblasts. In avian development, corneal epithelial cells secrete a structured acellular matrix as a rudimentary primary stroma, before the neural crest cells that will form keratoblasts migrate into the stroma. There are numerous variations in stromal development between different species. In mammalian species there is no primary stroma, but overall the two waves of neural crest invasion, endothelial and keratoblast are comparable (Hay, 1980).

Keratoblasts are the major cells that synthesize the molecular components of the embryonic corneal stroma. They continue to proliferate, and differentiate into keratocytes. Keratocytes proliferate slower, but synthesize high levels of collagens and proteoglycans (Cintron et al., 1983; Cornuet et al., 1994; Funderburgh et al., 1986; Young et al., 2007). As corneal stromal development progresses, the keratocytes maintain long-range associations with assembled collagen fibrils through an extended network of cytoplasmic filopodia. These cellular protrusions/processes are distinct and common during the course of stromal development and particularly lamella formation (Birk and Trelstad, 1984; Young et al., 2014). Corneal development continues in the postnatal period, yielding complete transparency (Chakravarti et al., 2006; Coulombre and Coulmbre, 1958; Coulombre and Coulombre, 1958; Song et al., 2003). Analysis of stromal development in embryonic chickens demonstrated that collagen fibrillogenesis occurred within small surface recesses of the keratocytes (Birk and Trelstad, 1984). Folds in the cell surface continued to fuse as the cell surface retracted, thus forming large surface-associated compartments in which fibril bundles coalesced to form lamellae (Fig. 2). During stromal development, keratocytes and their processes have two major axes at approximately right angles to one another (Fig. 3). The surface compartments involved in the production of the corneal stroma are aligned along two axes that are ~90° to one another. Thus, the cells are capable of forming ECM with fibril bundles heading in two directions perpendicularly. Using a 3-D reconstruction technique, it has been demonstrated that by volume, keratocytes occupy more than 20% of the stroma, and the filopodia of the keratocytes can extend more than 30 μm into the extracellular space. Moreover, there is a clear orthogonal organization in both cell and matrix organization (Young et al., 2014).

Regulation of Corneal Stroma Extracellular Matrix Assembly

Intracellular Compartments and Extracellular Micro-Domains

Within the corneal stroma, keratocytes are the cells responsible for the synthesis and secretion of stromal ECM constituents. The subsequent assembly of the stromal matrix provides attachment sites for keratocytes, which contributes to their stratification.

Extracellular matrix deposition between keratocytes generates cell-separating lamellae. Keratocytes have a compact cell body with numerous filopodia, giving them a dendritic morphology. Keratocytes are interconnected in a three-dimensional network by these filopodia (Hahnel et al., 2000; Poole et al., 1993). The compact cell body minimizes the surface area of the keratocyte exposed to light; this would serve to reduce light scattering while the extended processes provide for gap junction-mediated cell-cell communication and a communicating keratocyte network (Watsky, 1995). Corneal crystallines, which are accumulations of high levels of enzymes such as aldehyde dehydrogenase and transketolase that reduce light scattering in ocular cells in the visual axis, like lens and corneal epithelial cells also accumulate in the keratocyte cytoplasm and reduce light scattering (Jester et al., 1999; Piatigorsky, 1998, 2000; Jester, 2008).

The primary structural building block of the stroma is the collagen fibril. Assembly and deposition of corneal collagen fibrils involve a sequence of events that occurs in both intracellular and extracellular compartments. Procollagen molecules are synthesized, hydroxylated, glycosylated, assembled from three polypeptides, and folded in the rough endoplasmic reticulum. Packaging occurs in the Golgi, and the polypeptides are transported via specialized and elongated intracellular transport vesicles with secretion at the cell surface. The assembly of collagen molecules is regulated by interactions with other ECM components, i.e., different fibril-forming collagens, small leucine-rich proteoglycans (SLRPs) and FACIT collagens. The control of these interactions begins in the intracellular compartments and during trafficking. Observations in a mouse model of human congenital stromal corneal dystrophy suggest that the expression and secretion of extracellular components also is affected by ER status (Chen et al., 2013).

Collagen fibril assembly requires the removal of the procollagen N- and C-propeptides after secretion. Collagen molecules assemble into striated protofibrils with a periodic banding pattern. The D period is usually defined as 67nm. However, variations have been described that given differences in fibril-forming collagen composition of fibrils as well as spatial and temporal differences in fibril-associated molecules is not surprising (Birk et al., 1995; Birk et al., 1989b; Daxer et al., 1998; Graham et al., 2000; Kadler et al., 1996; Marchini et al., 1986; Rawe et al., 1994). These protofibrils are immature fibrils with distinctly tapered ends that have short lengths (4–12 μm) and small and uniform diameters (~20 nm) when compared to mature fibrils. In mature tissues, collagen fibrils are functionally continuous; that is, they are long with lengths that are difficult to measure and therefore, have not been fully characterized, as well as diameters in the range 20–500 nm, depending on the tissue and developmental stage (Birk et al., 1989a; Birk and Trelstad, 1985; Birk et al., 1989b; Canty and Kadler, 2002; Mienaltowski and Birk, 2014b). The mature fibril is assembled by end-to-end and lateral association of protofibrils. A model for the multi-step assembly of mature fibrils is presented in Fig. 4.

Collagen fibril assembly thus begins when procollagen is processed into collagen, which assembles into protofibrils that are closely associated with the keratocyte surface. Once the protofibrils are deposited into the extracellular matrix, assembly into higher order structures occurs in a series of compartmentalized micro-domains (Figs. 2,3). That is, fibrils first form small fibers (fibril bundles) and then form larger structures characteristic of the specific

tissues, e.g., lamellae in the corneal stroma. The establishment of a hierarchy of micro-domains within the extracellular space provides a mechanism for the fibroblast to exert control over the extracellular steps in extracellular matrix assembly, whether it is the sequestering procollagen processing enzymes at the sites of initial fibril assembly, or the addition of fibril-associated molecules during or after assembly of the protofibril.

The relationship between extracellular domain structure and collagen fibril assembly was initially described in the avian cornea (Birk and Trelstad, 1986) and has since been extensively studied in the uniaxial tendon. Much of the knowledge gained can be applied generally to other connective tissues (Birk and Linsenmayer, 1994; Canty and Kadler, 2002; Zhang et al., 2005). Fibril assembly begins in deep recesses or channels defined by the fibroblast surface (Birk and Trelstad, 1986; Canty and Kadler, 2005; Canty et al., 2004; Trelstad and Hayashi, 1979). These extracellular channels form at the time of secretion as specialized post-Golgi secretory compartments. The compartments then fuse with the fibroblast membrane and are maintained due to slow membrane recycling associated with the presence of the assembled protofibrils (Birk and Trelstad, 1986; Birk et al., 1989b). However, other studies suggest that limited procollagen processing could also occur intracellularly within elongated Golgi-to-plasma membrane compartments (Canty and Kadler, 2005; Canty et al., 2004). These compartments represent sites of protofibril assembly and deposition in developing connective tissues, including cornea, tendon, and dermis (Banos et al., 2008; Birk, 2001; Birk and Bruckner, 2005, 2011; Birk et al., 1990a; Birk et al., 1986; Birk et al., 1996; Birk et al., 1995; Birk and Trelstad, 1984, 1985, 1986; Birk and Zycband, 1993; Birk et al., 1990b; Ploetz et al., 1991).

Corneal Fibril Formation

The mature corneal stroma is composed of a single, homogeneous population of collagen fibrils with small diameters that are regularly packed. These collagen fibrils are heterotypic; that is, they are co-assemblies of collagens I and V. These heterotypic collagen fibrils also have fibril-associated molecules on the fibril surface, which alter the fibril surface properties, and change depending on the developmental stage. These fibril-associated molecules include the small leucine-rich proteoglycans (SLRPs) and FACIT collagens (fibril-associated collagens with interrupted triple helices). This results in a complex composite fibril with a heterotypic core composed of fibril-forming collagens I and V with fibril surface-associated molecules at the fibril periphery, therefore providing for the modulation of fibril surface properties (Birk and Bruckner, 2011; Mienaltowski and Birk, 2014b).

Interactions of Collagen V and I

The first step in fibrillogenesis involves nucleation of protofibril assembly. In the cornea, nucleation and initial assembly of a protofibril is dependent on collagen-collagen interactions. Corneal keratocytes synthesize two fibril-forming collagens. Collagen I is the predominant fibrillar collagen, making up 80–90 percent of the total, while the $\alpha1(V)_2\alpha2(V)_1$ isoform of collagen V is the quantitatively minor regulatory fibril-forming collagen. When collagens I and V co-assemble, the collagen V triple helix is internalized within the fibril, while the non-collagenous N-terminal domain projects through the gap

region to the fibril surface (Fig. 5) (Birk, 2001; Linsenmayer et al., 1993). The helical domain of collagen V is approximately 10% longer than the collagen I domain, and does not perfectly fit a quarter-stagger arrangement with collagen I (Fessler et al., 1982; Silver and Birk, 1984). Thus, properties intrinsic to these collagen-collagen interactions regulate the nucleation and assembly of the protofibril. Because corneal collagen V content is an order of magnitude greater than in most other tissues rich in collagen I, this feature leads to a larger number of nucleation events, thus producing a large number of small diameter protofibrils (Sun et al., 2011). This fibril assembly characteristic is essential to the cornea. In vitro and mouse model-based fibril assembly assays, in which levels of corneal collagen V are modulated, demonstrate that reduction in the percentage of collagen V relative to collagen I results in larger diameter fibrils (Birk et al., 1990a; Birk et al., 1988; Sun et al., 2011). Moreover, reductions in collagen V also cause decreased numbers of collagen protofibrils assembled overall in cell-based analyses (Marchant et al., 1996; Segev et al., 2006; Sun et al., 2011; Wenstrup et al., 2004) This, coupled with the lack of fibril assembly in collagen V-null embryonic mice, indicates a major regulatory role in nucleation of protofibril assembly (Wenstrup et al., 2004). It is also possible that the increased relative amounts of collagen V incorporated into corneal fibrils have an additional regulatory role on the corneal fibril surface. For example, the N-terminal domain, which contains sulfated tyrosines, may play a role in modulating steric and/or electrostatic interactions along the fibril surface.

Regulatory roles of small leucine-rich proteoglycans

The second stage in corneal fibrillogenesis involves linear fibril growth, needed to establish the tensile properties required to maintain the structure of the anterior eye and resist compressive forces due to hydration in the highly charged inter-fibrillar matrix. Additionally, there is an inhibition of lateral fibril growth, which is essential because large diameter fibrils are incompatible with transparency.

The corneal stroma is enriched with small leucine-rich proteoglycans (SLRPs). There are two major classes of SLRPs in the corneal stroma. Decorin and biglycan are class I SLRPs; lumican, keratocan and fibomodulin are class II SLRPs (Chen and Birk, 2011, 2013; Schaefer and Iozzo, 2008). All SLRPs have a similar or homologous core protein structure with a similar size (35–40 kDa), and they are members of the leucine-rich repeat (LRR) superfamily. To that end, they contain as their major structural feature ~9 leucine-rich regions that are homologous in structure, similar in length, and equally spaced within the central region of the core protein. Within this region of the core protein, the polypeptide chain is coiled into a tight spiral so that every 360 degrees there is a leucine-rich region, thus aligning all leucine-rich regions on one surface of the coil, and the non-leucine-rich regions on the opposite surface of the coil. The aligned leucine-rich regions are thought to interact with the collagen molecules to regulate fibril formation, while the non-leucine-rich surface contains asparagine amino acid attachment sites for keratan sulfate chains (Dunlevy et al., 1998). Decorin and biglycan compete for a binding site on collagen I, while fibromodulin and lumican compete for another different binding site (Pringle and Dodd, 1990; Svensson et al., 2000). Glycosaminoglycan content varies among SLRPs. Decorin has a single 55–60 kDa chondroitin/dermatan sulfate chain; lumican, keratocan and osteoglycin/mimican have 2–3 keratan sulfate chains of 10–15 kDa each (Dunlevy et al., 1998; Hassell et al., 1979;

Midura and Hascall, 1989). Thus, the SLRPs are relatively small, fit well in the spaces between the collagen fibrils, and play a role in the regulation of their spacing (Beecher et al., 2005; Chakravarti et al., 1998; Cintron et al., 1978; Hassell et al., 1983; Liu et al., 2003).

As previously discussed, class I SLRPs have chondroitin sulfate/dermatan sulfate glycosaminoglycan (GAG) chains, while class II SLRPs have keratan sulfate GAGs (Chen and Birk, 2013; Schaefer and Iozzo, 2008). However, their GAG chains are under-sulfated and they are mostly in glycoprotein form (Cornuet et al., 1994) at the embryonic stage. Glycosaminoglycans (GAGs) affect SLRP protein core conformation during biosynthesis (Krishnan et al., 1999); however, SLRP protein cores are critical for the regulation of fibrillogenesis, independent of the GAGs (Rada et al., 1993). The SLRP protein cores bind collagen fibrils via their concave face, while GAGs extend outward into the inter-fibrillar space where they play roles in regulating matrix hydration (Liu et al., 2003) and interacting with adjacent collagen fibrils. Periodic interactions between GAGs and collagen fibrils have been observed, and GAGs can tether two or more collagen fibrils to form a network (Henninger et al., 2007; Lewis et al., 2010; Raspanti et al., 2008). Computational studies also have proposed that GAGs bridge and transfer force between adjacent fibrils, thus improving the mechanical integrity of the tissue (Redaelli et al., 2003). Interestingly, a correlation has been detailed between the appearance of metachromatic glycosaminoglycan staining in stromal ECM and the appearance of the highly organized collagen fibril packing during the acquisition of corneal transparency in the developing embryo(Coulombre and Coulmbre, 1958; Coulombre and Coulombre, 1958) In contrast, there is a loss of such metachromatic staining in opaque corneal wounds, which coincides with a reduction in the levels of corneal keratan sulfate (Anseth, 1961; Dunnington and Smelser, 1958). Consequently, the GAG chains on SLRPs can not only be involved in the regulation of fibrillogenesis, but can also influence inter-fibril spacing as well as organization during matrix assembly (Fig. 6).

Corneal SLRPs demonstrate specific temporal and spatial differences in their expression patterns in the developing and mature stroma (Chen and Birk, 2011; Schaefer and Iozzo, 2008). Expression of both class I SLRPs is homogeneous across the corneal stroma. However, biglycan expression is low in the mature stroma, while decorin expression remains high (Zhang et al., 2009). Class II SLRPs show both temporal and spatial differences. While lumican and keratocan are homogeneous across the cornea stroma at birth, the expression of lumican is restricted to the posterior stroma after maturation (Chakravarti et al., 2006; Chen et al., 2010; Zhang et al., 2009). Fibromodulin expression in the corneal stroma is strongest at P14, but is decreased and restricted to the peripheral cornea with maturation (Chen et al., 2010). These differential temporal and spatial expression patterns may contribute to disparities in collagen fibril structure and organization between the central and peripheral cornea, as well as between anterior and posterior stroma during development.

Mouse models have demonstrated that SLRPs are critical regulators of collagen fibrillogenesis, particularly the linear and lateral growth of protofibrils into mature fibrils (Chakravarti et al., 1998; Chakravarti et al., 2003; Chakravarti et al., 2006; Chen and Birk, 2013; Chen et al., 2010; Ezura et al., 2000; Mienaltowski and Birk, 2014a; Zhang et al.,

2009). Mice that are deficient in decorin or biglycan only have a mild corneal phenotype. However, compound mutant mice deficient in both decorin and biglycan demonstrate a severe phenotype with increased numbers of large diameter fibrils, a very heterogeneous diameter distribution, and irregular, cauliflower-like contours in both the anterior and the posterior stroma. Moreover, the spatial distribution of the abnormal phenotype was coincident with decorin expression. That is, both in vivo and in vitro studies demonstrated that biglycan is up-regulated in the absence of decorin to functionally compensate for the loss of Class I SLRP decorin (Zhang et al., 2009). In the absence of Class II SLRP keratocan, null-mice have thinner corneal stromas and a narrower cornea-iris angle, indicating that keratocan regulates stroma hydration and shape during development (Liu et al., 2003). Fibromodulin is also involved in the regulation of corneal stroma development; it is expressed within a very narrow window of time during postnatal stromal development and contributes to cornea-sclera integration (Chen et al., 2010). Yet a deficiency in either keratocan or fibromodulin in the corneal stroma does not result in changes to fibril structure. In contrast, lumican-deficient mice exhibit progressive corneal opacity with age, which is associated with irregularly packed, large diameter collagen fibrils and irregular, cauliflower-like contours in the posterior stroma. The altered fibril characteristics are consistent with the dysfunctional regulation of lateral fibril growth steps. Thus, because transparency requires a homogeneous population of small diameter fibrils, this abnormal phenotype, caused by the lack of lumican in the posterior stroma, results in increased light scattering and opacity in the region (Chakravarti et al., 2000; Song et al., 2003). Interestingly, a deficiency of lumican in the corneal stroma influences the expression of both keratocan and fibromodulin (Shao et al., 2011).

Coordinated expression of SLRPs is likely due to gene clustering, where class I decorin and class II lumican and keratocan are cis-clustered on mouse chromosome 10 (Chakravarti and Magnuson, 1995; Danielson et al., 1999) and human chromosome 12 (Schaefer and Iozzo, 2008). Additionally, SLRP expression can be altered by the presence or absence of other class members. For example, in the absence of decorin, biglycan expression can be increased (Zhang et al., 2009), and the expression of keratocan can be driven by the expression of lumican (Carlson et al., 2005). Furthermore, in a mutant decorin transgenic mouse model, altered expression of both class I and class II SLRPs also was observed (Chen and Birk, 2011). Moreover, regulatory interactions across classes also have been demonstrated by an increased severity of the corneal stromal fibril structural phenotype in the absence of both biglycan and lumican, compared to either one alone (Chen et al., 2014). This is evidence of inter-class cooperation in the regulation of fibril growth. Defining the network of regulatory SLRP interactions is essential to elucidate the roles in development, maintenance and regeneration of tissue-specific structures and functions.

In regard to linear fibril growth within the corneal stroma, little evidence exists to address its regulation. Prior to dehydration in the non-compacted cornea, it is possible that the protofibrils are so suitably separated that the resulting frequency of interaction is therefore low. It has been shown ex vivo that when opportunities for interaction are increased, end-to-end growth then occurs (Graham et al., 2000). It is possible that the end-to-end interactions occur more frequently in development as the charge densities of the SLRP GAG chains increase and induce order to the inter-fibrillar environment. Finally, it is also possible that

the tapered ends of stromal protofibrils alter binding properties, thus providing stabilization to the ends, while the normal turnover of bound molecules provides increased opportunities for interaction in a controlled manner.

FACIT collagens

Fibril-associated collagens with interrupted triple helices (FACIT) also affect collagen fibrillogenesis in development, maturation, and maintenance of the corneal stroma. FACIT collagens interact with collagen fibril surfaces and other ECM components, as well as molecules associated with cell membrane-bound proteins. Thus, FACIT collagens serve to modulate fibril surface properties, affect fibril packing, and maintain the ECM niche via matrix molecule and ECM-cell interactions. Hence, because FACIT collagens regulate linear and lateral fibril growth in ECM organization, they have the potential to impact corneal transparency and corneal refraction.

The fibril-associated collagens detected in mammalian corneas include collagens XII and XIV (Akimoto et al., 2002; Gordon et al., 1996; Marchant et al., 2002; Young et al., 2002). In the avian cornea, collagen IX also has been implicated (Akimoto et al., 2002; Fitch et al., 1998; Fitch et al., 1994; Young et al., 2002). FACIT collagens have several short collagenous (COL) domains interrupted by non-collagenous (NC) domains. At the N-terminus of each FACIT collagen, a large globular NC domain protrudes from the fibril surface into the inter-fibrillar space (Fig. 7), demonstrating FACIT collagen interaction with collagen (Birk and Bruckner, 2005).

In mammals, the roles of FACIT collagens in corneal stromal development have yet to be investigated. However, the roles of collagens IX, XII, and XIV in corneal stromal development have been investigated in the chicken (Akimoto et al., 2002; Fitch et al., 1998; Fitch et al., 1994; Linsenmayer et al., 1998; Marchant et al., 2002; Young et al., 2002). Immature forms of fibrillar collagen II and FACIT collagen IX are deposited in the subepithelial corneal zone, which will become the corneal stroma and at this time is termed primary stroma, until day 5 of embryonic chicken development (Fitch et al., 1994). At day 6, the stroma begins to swell, and expression of collagen IX decreases until absent by day 8 (Fitch et al., 1998; Fitch et al., 1994). A correlation between the increased swelling of the corneal stroma and the reduction of expression of collagen IX has led to speculation that collagen IX must be a primary factor in maintaining the cornea’s initial compact state, by forming cross-interactions between collagen fibrils and other matrix components (Linsenmayer et al., 1998). Thus, the removal of collagen IX would essentially open the ECM to allow for predominant hyaluronic acid to draw water and expand the stroma. Collagen IX also impacts the cell adhesion properties of collagen II fibrils (Kapyla et al., 2004). While less has been described about the roles of collagens XII and XIV in corneal development and maturation, some basic details have been determined that suggest that these two FACIT collagens also regulate ECM organization in the corneal stroma. Two isoforms have been recognized for collagen XII: a long form (340 kDa) and a short form (220 kDa) (Chiquet et al., 2014; Koch et al., 1995) While for most systems, where typically the long form is present during embryonic development and the short form is present in maturation and adulthood, the long form appears in late embryonic development and

remains in the corneal stroma of adult chickens, rabbits, and humans (Akimoto et al., 2002; Anderson et al., 2000; Oh et al., 1993; Wessel et al., 1997; Young et al., 2002). While ultimately present throughout the stroma in adulthood, collagen XII localizes along the Descemet's Membrane-stroma interface, Bowman's Layer-stroma interface, and sclera-stroma interface during early development, in correlation with increases in keratocyte density (Akimoto et al., 2002; Anderson et al., 2000; Marchant et al., 2002). This localization is suggestive of a role in stabilizing interfacial matrices and fibrillar collagen structure throughout, as well as cell organization within the corneal stroma (Marchant et al., 2002). In the mature corneal stroma, collagen XII is believed to stabilize associations between fibrils, particularly along interfaces, and maintain fibril packing throughout the stroma lamellae (Linsenmayer et al., 1998). Though collagen XII is thought to play an important role in the maintenance of corneal stromal organization, its overexpression in opaque corneal scars both in human patients and in mouse injury models serves as an example of the complex association between this molecule, and balances its role between homeostatic regulator in health and detriment to fibrillogenesis in injury (Massoudi et al., 2012). Mutations in *COL12A1* in humans and *Col12a1* in mice have also led to phenotypes overlapping those of collagen VI related to myopathies and Ehlers–Danlos Syndrome, although alterations from the norm in corneal stroma are still to be determined (Chiquet et al., 2014; Hicks et al., 2014; Zou et al., 2014).

Collagen XIV is found throughout the corneal stroma during early development, but levels decrease as the stroma matures. In avian species, collagen XIV is greatly reduced in late development (day 19), and never returns post-hatching nor in adulthood (Young et al., 2002). Collagen XIV is found throughout the corneal stroma in mice in adulthood at low levels (Hemmavanh et al., 2013). In the mature corneal stroma, collagen XIV is believed to stabilize associations between fibrils once they have undergone coalescence (Linsenmayer et al., 1998). The roles of collagen XIV in corneal stroma development and maintenance remain to be characterized.

Collagen VI

Collagen VI also plays a role in the regulation of corneal stromal development. Collagen VI is ubiquitous within connective tissues, including within the corneal stroma (Cho et al., 1990; Cintron and Hong, 1988; Zimmermann et al., 1986). Collagen VI can be assembled into several forms: beaded microfibrils, broad-banded structures, and hexagonal networks (Bruns et al., 1986; Furthmayr et al., 1983; von der Mark et al., 1984). It interacts with many extracellular molecules, including integrins, SLRPs, hyaluronan, heparin, fibronectin, and collagens I, II, IV, and XIV. Collagen VI integrates ECM with cells, and it can influence cell proliferation, apoptosis, migration, and differentiation. In the corneal stroma, collagen VI is found throughout all stages. It is localized to filaments forming an extensive network around the small diameter fibrils of the stroma, as well as adjacent to corneal fibroblasts (Linsenmayer et al., 1986a; Linsenmayer et al., 1985; Linsenmayer et al., 1986b). Given the network-forming structure, it is thought to influence matrix integrity by interconnecting levels of ECM in a stable mesh within the corneal stroma (Linsenmayer et al., 1985).

Collagen VI is formed as a heterotrimer composed of α1(VI), α2(VI) and α3(VI) chains (Fleischmajer et al., 1997; Kielty and Grant, 2002). Each monomer has a 105-nm triple helical domain with flanking N- and C- terminal globular domains. Intracellular collagen VI begins to assemble; two monomers assemble laterally in an antiparallel fashion to form a dimer. Then, intracellularly, two dimers align to form tetramers, which are secreted extracellularly and associate end-to-end to form beaded filaments as well as larger scale beaded microfibrils and hexagonal lattices (Bruns et al., 1986; Wiberg et al., 2002). As with fibrillar collagens, Collagen-VI rich supramolecular aggregates are composite structures containing other integrated molecules, including SLRPs (Wiberg et al., 2002). Association of collagen VI with molecules like SLRPs thus influences tissue organization, structure, and function.

Corneal stromal diseases

Collagen fibrillogenesis is tightly regulated to generate tissue-specific structures and therefore highly specialized functions. In the corneal stroma, homogeneous small diameter fibrils, are regularly packed and arranged as stacked lamellae with adjacent layers roughly orthogonal, are required for corneal transparency. Mutations in genes that regulate collagen fibrillogenesis at any level of the structural hierarchy thus could affect expression, and consequently the proper structure and function of the corneal stroma. Stromal dystrophies also can be seen in the clinic as a result of other etiologies related to the deposition of transparency-affecting molecules and cells; for a review, see (Klintworth, 2009). Here, we focus on those diseases associated with developmental changes in corneal stromal collagen fibrillogenesis and its regulation.

In regard to cornea-specific temporal and spatial expression etiologies for disease, proteoglycans are well-represented examples. Proteoglycans are critical for precisely-regulated matrix assembly during development. Defective proteoglycan synthesis has been shown to result in blindness in humans, due to the subsequent disruption in the organization of the collagen fibrils. Two inherited corneal dystrophies are caused by faulty keratan sulfate proteoglycan production. Cornea plana results from mutations in the gene that codes for the core protein of keratocan (Pellegata et al., 2000). Mutations in a gene that codes for one of the sulfotransferases that puts sulfate esters on keratan sulfate cause macular corneal dystrophy (Hassell et al., 1980; Hayashida et al., 2006; Midura et al., 1990; Musselmann and Hassell, 2006). Thus, the presence of sulfate groups on keratan sulfate chains is essential for the function of the proteoglycan, mostly likely because the sulfate groups on proteoglycan GAG chains bind water. In the normal cornea, hydration levels are achieved when chondroitin/dermatan sulfate chains are fully hydrated while the keratan sulfate chains are not; this characteristic is suggestive of a hydration reserve role for keratan sulfate (Bettelheim and Plessy, 1975). Moreover, the sulfate esters on keratan sulfate are important for maintaining the solubility of the proteoglycan in an aqueous environment (for review on keratan sulfate see (Funderburgh, 2000).

Human congenital stromal corneal dystrophy is the only human disease that has been associated with a mutant decorin gene. Three different frame-shift mutations (c.947delG; c. 967delT; c.941delC) have been reported at the C-terminus of decorin; all of these deletion

mutations lead to identical truncations of decorin, lacking a 33 amino acid segment that includes the SLRP-specific “ear” repeat (Bouhenni et al., 2011; Bredrup et al., 2010; Kim et al., 2011; Rodahl et al., 2006). Besides the deletion mutations, a family has been described with a decorin-associated CSCD involving a novel nucleotide substitution (c.1036T>G) within the C-terminus of decorin, which has resulted in a milder phenotype than in the truncated mutations (Lee et al., 2012). Presumably, the loss of the cys residue prevents disulfide bond formations in this critical region, resulting in altered conformation rather than a truncation, and a plausible explanation for the milder functional consequences. A novel animal model that recapitulates human CSCD was generated in our lab, where mice expressed both wild-type and mutant decorin (Chen et al., 2011). Several phenotypic features were described, including: early onset corneal opacities throughout, increased severity toward the posterior stroma, and disrupted lamellae architecture with relatively normal lamellae separated by regions of abnormal fibril organization. Moreover, within abnormal zones, the inter-fibrillar spacing and the fibril diameters were increased (Chen et al., 2011). Furthermore, the presence of truncated decorin negatively affected the expression of endogenous decorin, biglycan, lumican, and keratocan, while being associated with increased levels of fibromodulin (Chen et al., 2011). In vitro studies demonstrate that the C-terminal truncation of decorin results in misfolded proteins that get retained in the ER. Consequently, the mutant decorin alters cytoplasmic trafficking, induces ER stress, and leads to dysregulated homeostasis of extracellular matrix components, as well as disrupted matrix assembly in corneal stroma (Chen et al., 2014).

Changes in expression of some genes can also affect the proper curvature of the cornea required for the controlled refraction of light. For proper resistance to intraocular pressure and to withstand sheer stress, the non-extensile mechanical strength from the collagen fibrils, as well as the adhesiveness and compaction of the lamellae, are important. Thus, changes in composition and organization can lead to structural and shape changes. For example, keratoconus is a non-inflammatory thinning of the cornea due to undefined etiologies producing changes in corneal curvature and visual acuity. It has been suggested that regional weakness of the cornea in keratoconus is associated with proteoglycan changes, resulting in reduced adhesion between lamellae (McMonnies and Schief, 2006). Increased keratocan expression is observed in the stroma of keratoconus corneas (Wentz-Hunter et al., 2001). Moreover, high myopia is associated with intronic variations and single nucleotide polymorphisms (SNPs) in *fibromodulin*, *PRELP* and *opticin* genes (Majava et al., 2007; Wang et al., 2009). In addition, rare autosomal dominant disorder Posterior Amorphous Corneal Dystrophy (PACD) – which typically causes patients to demonstrate posterior lamellar corneal opacification, decreased corneal thickness, and corneal flattening – has been associated with haploinsufficiency of SLRPs, keratocan, lumican, decorin, and epiphycan, via differences in copy number caused by the deletion of a region of Chromosome 12 containing several SLRPs (Kim et al., 2014). Also, SNPs within and near *COL5A1* are associated with corneal thinning related to keratoconus and glaucoma (Hoehn et al., 2012; Li et al., 2013; Sahebjada et al., 2013).

In summary, the coordinated synthesis and controlled interactions of several different collagen types and the core proteins of several different leucine-rich type proteoglycans, as

well as post-translational modifications of the collagens and the proteoglycans, is the basis for collagen fibrillogenesis, fibril organization, lamellae stacking, and stroma assembly and packing. These molecules interact and are integrated and interwoven. This promotes finely tuned temporal-spatial regulation in corneal stroma matrix assembly, producing a functional cornea.

Future Directions

In recent years, stem cells and engineered bio-scaffolds for the treatment of corneal diseases have been studied extensively. Understanding the mechanisms regulating corneal stroma development is the basis for this component of ophthalmic regenerative medicine. In this review, we outlined how corneal stroma development relies not only upon the assembly of collagen fibrils, but instead, how dependent development is upon tightly regulated temporal-spatial expression and incorporation of several components that cohesively integrate to form a functional cornea. The components include cells and molecules that are essential through development, through maturation, or throughout the functional life of the corneal stroma. A keen awareness of each player's role in each step allows for improvements in understanding corneal physiology and pathology, as well as in regenerative strategies to restore function to a cornea.

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Highlights

- The corneal stroma is a collagen-rich extracellular matrix
- Corneal stroma transparency is dependent on a hierarchical organization of extracellular matrix
- Keratocytes define micro-domains to regulate extracellular steps in stromal matrix assembly
- Collagen fibril assembly is regulated by extracellular interactions of matrix molecules
- Gene mutations altering regulatory interactions result in stromal pathobiologies

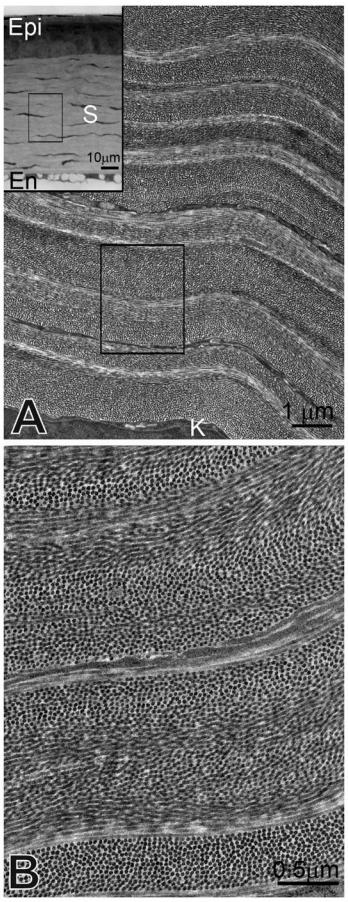

Figure 1. The ultrastructure of the cornea stroma

Three layers specifically define the cornea: the outer epithelium, the stroma, and the inner endothelium. All three layers can be seen; the stroma comprises more than 90% of the corneal thickness **(A, inset). (A)** The stroma contains keratocytes (K) positioned parallel to the corneal surface, between the stromal lamellae. **(B)**. The lamellae are composed of small diameter collagen fibrils with regular packing; adjacent layers are at approximately right angles to one another (B, is an enlarged area of the rectangle in A). P30 mouse cornea; A, inset light micrograph; A and B transmission electron micrographs. Figure modified from (Hassell and Birk, 2010).

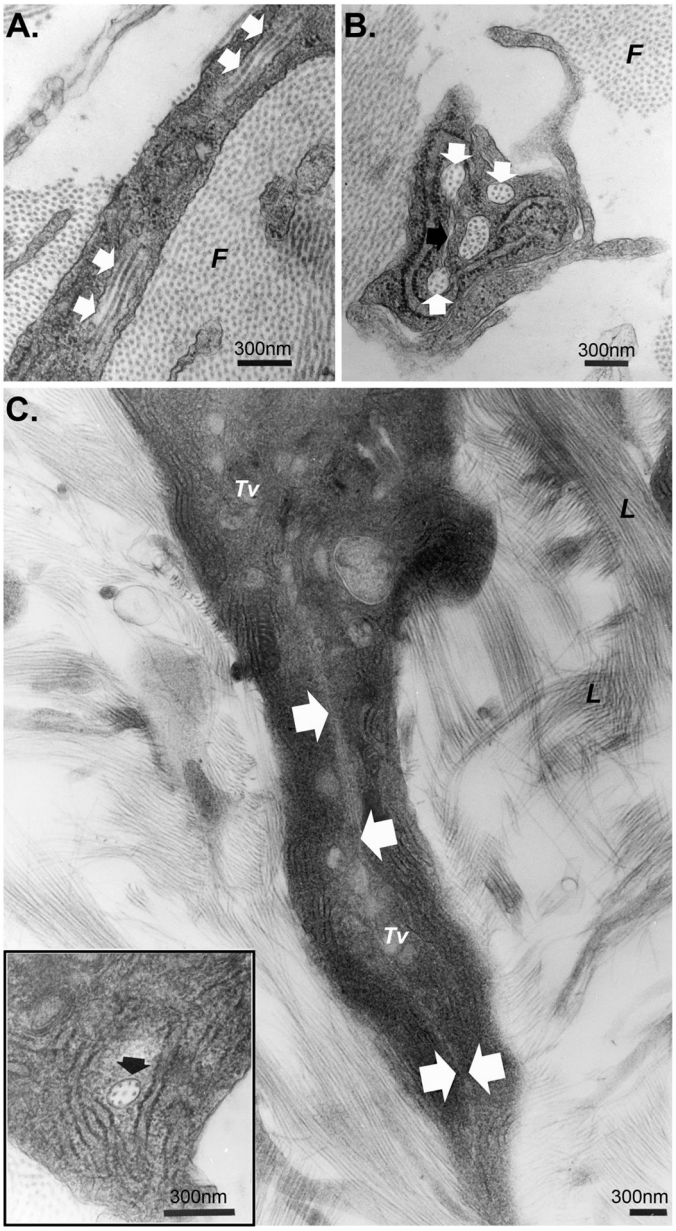

Figure 2. Keratoblast micro-domains during stromal embryonic development

(A,B) Transmission electron microscopy of keratoblasts from 14 d (stage 40) chicken embryos cut perpendicular to the cornea surface. **(A)** A keratoblast process contains an extracellular micro-domain, termed channels with several collagen fibrils in longitudinal section (white arrows). The fibrils in the in the process of being deposited, are perpendicular to the orientation of the fibrils (*F*) on either side of the cell process. **(B)** A corneal keratoblast that contains 4 small extracellular micro-domains. Three of them are similar in size and contain 7–10 collagen fibrils (white arrows) while the fourth one contains 19 collagen fibrils. Two of the smaller micro-domains are joining as indicated by the membranous connection (black arrow). **(C)** High voltage electron microscopy of the corneal keratoblasts from 14 d (stage 40) chicken embryo corneas cut parallel to the corneal surface

(0.5 μm thick section). An extracellular micro-domain (indicated by white arrows) is seen running through most of this section, indicating how extensive this first level of extracellular micro-domains can be; running from deep with the keratoblast, opening at the cell surface and continuing into the extracellular environment. The inset shows a transverse section of the micro-domain within the keratoblast (black arrow) that contains eight collagen fibrils. (*Tv*: Transport Vesicle) Figure modified from Birk and Trelstad, 1984.

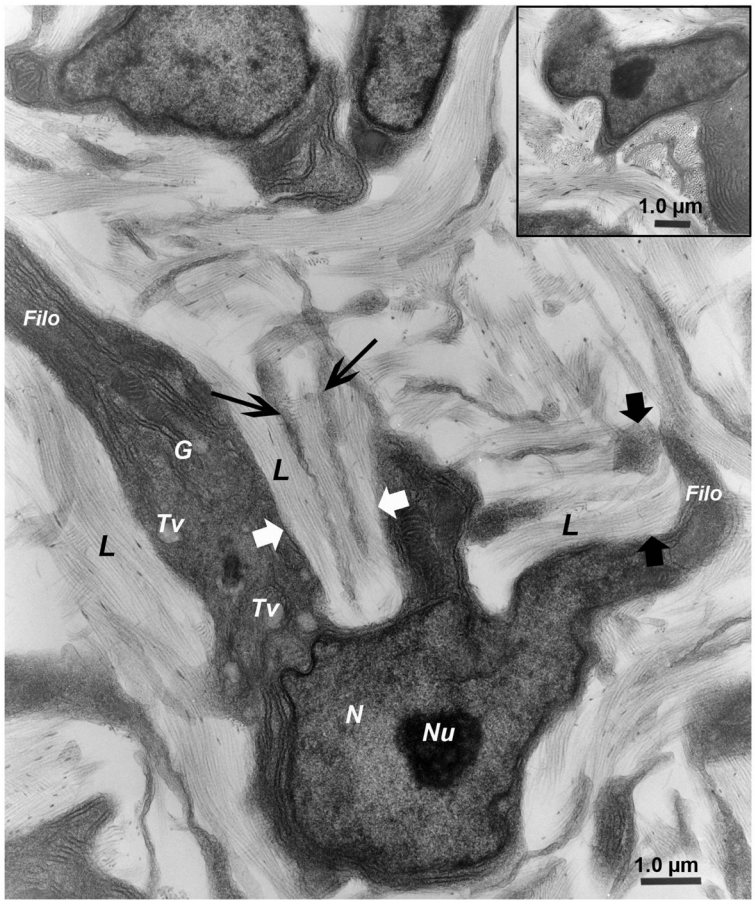

Figure 3. The keratoblast surface compartments and collagen fibrils deposited have an orthogonal organization

A high voltage electron microscopy of a 0.5 μm-thick-section from a 14 d (stage 40) chicken embryo cut parallel to the cornea surface. The corneal keratoblast and its processes demonstrate a roughly orthogonal organization. Bundles of the collagen fibrils, fibers are seen within cell surface foldings. Cell processes are seen separating fibers. A fusion of these compartments and a retraction of cell surface forms large surface associated compartments as seen at the black thin arrows. The compartment is seen along two major axes at approximately right angles to one another (white vs. black thick arrows). It is within these keratoblast-associated compartments that collagen fibril, fiber, and lamellar (*L*) formation occur. The inset shows a section cut perpendicular to a similar region within which fibers coalesce to form large bundles and lamellae (*L*) in a large compartment. (*Tv*: Transport vescicle; *G*: Golgi; *N*: Nucleus; *Nu*: Nucleolus; *Fil*: Filopodia.) Figure modified from Birk and Trelstad, 1984.

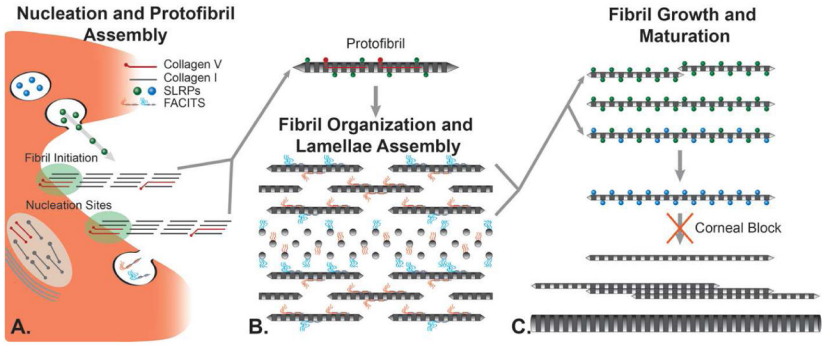

Figure 4. Corneal stroma collagen fibril assembly model

Collagen fibrillogenesis is a multiple-step process that is tightly regulated by the interaction of many molecules. **(A)** Initially, fibrils nucleate at the cell surface, due to interaction of collagen I and collage V, to form a heterotypic fibril. **(B)** Then fibril-associated collagens with interrupted triple helices (FACITs) interact with protofibrils to regulate fibril packing, lamellar assembly, and organization into the stroma. **(C)** Moreover, small leucine-rich proteoglycans (SLRPs) bind to the protofibrils' surface to regulate linear and lateral fibril growth as protofibrils mature to collagen fibrils, thus resulting in a "corneal block," wherein fibril diameters are limited and organization occurs in such a way to allow for proper refraction of light and transparency. Figure adapted from (Chen and Birk, 2013).

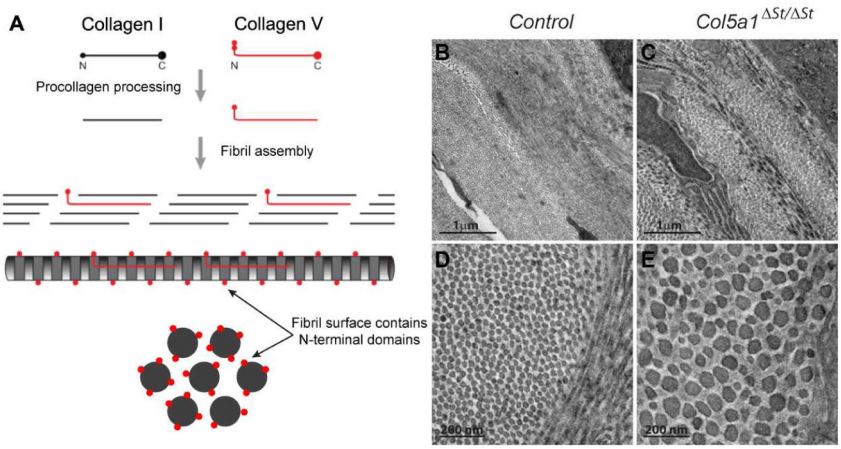

Figure 5. Heterotypic collagen fibrils

Collagen fibrils are heterotypic. **(A)** They are co-assemblages of quantitatively major fibril-forming collagens like collagen I, and regulatory fibril-forming collagens like collagen V or XI. Regulatory fibril-forming collagens have a partially processed N-terminal propeptide, retaining a non-collagenous domain that must be in/on the gap region/fibril surface. The heterotypic interaction is involved in nucleation during fibril assembly; typically these interactions promote regular packing of the lamellae, as in wild-type mice **(B, D)**. However, an absence of collagen V, as in *Col5a1*$^{\Delta st/\Delta st}$ cornea stroma-specific conditional null mice, causes disorganized lamellae packing associated with very large fibrils and abnormal structures **(C, E)**. Panel A has been adapted from (Birk and Bruckner, 2011); panels B–E have been adapted from (Sun et al., 2011).

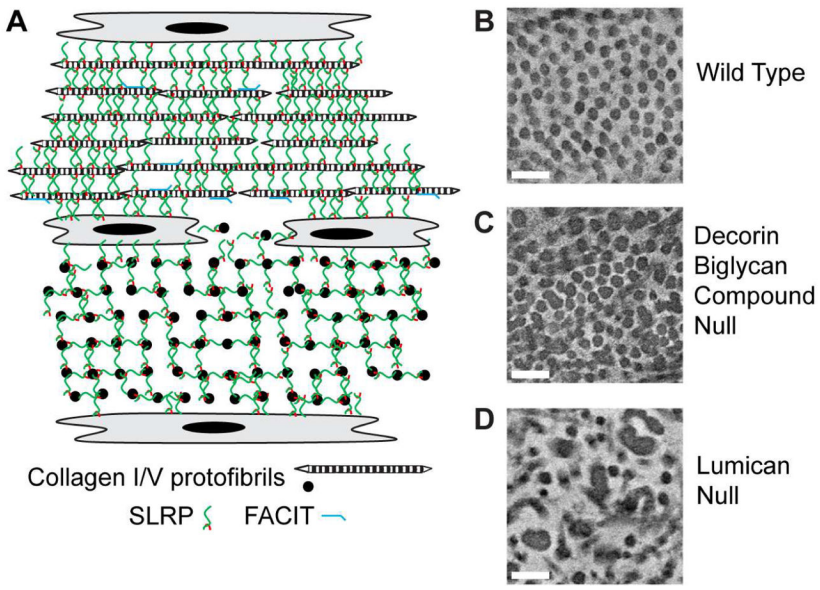

Figure 6. SLRP roles in regulating fibril assembly

Small leucine-rich repeat proteoglycans (SLRPs) regulate extracellular matrix (ECM) assembly, particularly linear and lateral fibril growth, by binding to collagen fibril surface **(A)**. SLRPs also interact with ECM components besides collagens; these include cytokines and cell surface receptors. **(B)** SLRPs affect fibril diameter and spacing in the corneal stroma. **(C, D)** When SLRPs are absent, as in null mice or in gene mutations in human patients, the fibril structure, organization and spacing are impacted. Panel A has been adapted from (Chen and Birk, 2013).

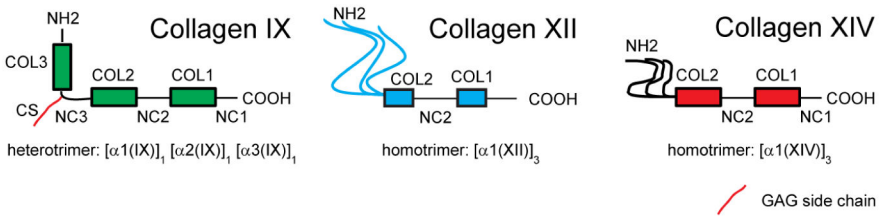

Figure 7. FACIT roles in regulating fibril assembly

Fibril-Associated Collagens with Interrupted Triple Helices (FACIT) have 2–3 COL domains and 3–4 NC domains with a large N-terminal NC domain that projects into the inter-fibrillar space. The FACIT collagens all associate with the surface of collagen fibrils. Figure adapted from (Birk and Bruckner, 2011) and (Linsenmayer et al., 1998).