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Colocalization of the collagen-binding proteoglycans decorin, biglycan, fibromodulin and lumican with different cells in human gingiva

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Background and objective: Decorin, biglycan, fibromodulin and lumican are structurally related molecules that belong to the family of small leucine-rich proteoglycans (SLRPs). These SLRPs are secreted extracellular matrix molecules that interact with type I collagen and regulate collagen fibrillogenesis. They may also modulate cell functions that are important in maintenance of connective tissue structure. The aim of this study was to localize decorin, biglycan, fibromodulin and lumican in human gingiva.

Methods: Localization of decorin and its proform (prodecorin), biglycan, fibromodulin and lumican and mature and proform of type I collagen was studied by immunohistochemical staining of frozen tissue sections from healthy human attached gingiva. Double immunostaining with anti-SLRP or anti-type I procollagen antibodies and specific markers for different connective tissue cells was used to study association of these molecules with cells.

Results: The mature and proforms of decorin and collagen and biglycan, fibromodulin and lumican showed distinct localization in the extracellular matrix, where they associated with type I collagen fiber bundles. Prodecorin also localized to the epithelial basement membrane zone. Fibroblasts, myofibroblasts, endothelial cells and pericytes showed immunoreactivity for procollagen, prodecorin, biglycan and fibromodulin, whereas lumican associated with fibroblasts and myofibroblasts only. Biglycan and fibromodulin were also associated with macrophages. Basal epithelial cells of the gingival epithelium showed immunoreactivity for biglycan, fibromodulin and lumican.

Conclusions: Decorin, biglycan, fibromodulin and lumican associate with type I collagen and may collaborate to regulate collagen fibrillogenesis in human gingiva. Each of the SLRPs showed a distinct association with different connective tissue cells, suggesting that the cells produce these molecules and/or that the cells interact with them. Localization of biglycan, fibromodulin and lumican at the epithelial cells suggests novel functions for these SLRPs in human gingival epithelium.

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The family of small leucine-rich proteoglycans (SLRPs) consists of at least 13 structurally related molecules that have evolved from different genes (1). They are widely expressed in the extracellular matrix (ECM) of various hard and soft connective tissues, where they are involved in different specialized functions. For example, SLRPs are important in the maintenance of the mineralized matrix of bones and teeth, the transparency of the cornea, the tensile strength of the skin and tendons, and the viscoelasticity of blood vessels. All members of the SLRP family consist of a protein core and one or more glycosaminoglycan side chains that can be chondroitin, dermatan or keratan sulphate. The core protein of SLRPs has a characteristic central domain with multiple leucine-rich repeats. This domain is responsible for most of the functional activities of these molecules, although some of the functions are also mediated by the glycosaminoglycan chains (2).

The most studied characteristic of SLRPs is the property of class I SLRPs decorin and biglycan and class II SLRPs lumican and fibromodulin to regulate collagen fibrillogenesis. Gene knockout studies showed that targeted deletion of one or more of these SLRP genes caused abnormal collagen fibril morphology (1, 3). For example, altered collagen fibrils were found in skin and periodontal ligament in the absence of decorin (4, 5). Fibromodulin-null mice also had structurally abnormal collagen fibrils and tissue organization in tendons (6), and lumican-null mice had opaque corneas with abnormally thick collagen fibrils (7). In decorin- and lumican-null mice the abnormal collagen fibril morphology resulted in functional defects evidenced by reduced tensile strength and fragility of skin (4, 8). Targeted disruption of biglycan also caused abnormal type I collagen fibrils in mice (9, 10). Thus, decorin, biglycan, fibromodulin and lumican appear to regulate collagen fibrillogenesis *in vivo*.

The regulation of collagen fibrillogenesis by decorin, fibromodulin and lumican has been attributed to their property to bind type I collagen. However, it is not clear whether biglycan interacts directly or indirectly with type I collagen. Binding of the core protein of decorin to type I collagen regulates the kinetics of collagen fibrillogenesis and the diameter of and the distance between the fibrils (11). Binding of fibromodulin to collagen also retards the formation of collagen fibrils (12).

Besides influencing the assembly of the ECM, SLRPs regulate cell functions, including cell adhesion, proliferation, differentiation and apoptosis. These effects are cell-type specific and are mediated by binding of the SLRPs to certain growth factors, by SLRP-induced intracellular signaling cascades or by regulation of cell–ECM interactions (1). For example, decorin, biglycan and fibromodulin regulated activity of transforming growth factor beta (TGF-β) by binding to it and decorin also modulated TGF-β-induced signaling cascades (13–16). Overexpression of decorin regulated expression of several matrix metalloproteinases and cytokines in gingival fibroblasts (17), and decorin also reduced growth of periodontal fibroblasts *in vitro* and *in vivo* by a novel mechanism (5). Also biglycan and lumican regulate cell growth. In pancreatic cancer cells, biglycan inhibited cell growth by a mechanism that may have involved SMAD4 signaling pathway (18, 19). Overexpression of lumican suppressed anchorage-independent growth of melanoma cells *in vitro* and *in vivo* (20). Decorin, biglycan and lumican also regulate cell adhesion and migration (21–25).

The gingival connective tissue consists of a dense network of type I collagen fibril bundles that provide firmness to the gingiva and attach the gingiva to the tooth and alveolar bone (26). Type I collagen is also one of the major cell adhesion molecules that regulates functions of the connective tissue cells (27). Gingiva has a fast turnover rate of collagen, allowing it to adapt to changing functional demands (28, 29). Given the multiple functions of SLRPs in the regulation of collagen fibrillogenesis and cell functions, they may play an important role in gingival homeostasis. Previous studies have shown that decorin and biglycan are expressed in human gingiva (30). Human gingival fibroblasts also express fibromodulin in culture (31), but localization of fibromodulin or lumican in human gingiva is not known. The purpose of this study was to analyze the localization and which cell types in human gingiva express collagen binding proteoglycans decorin, biglycan, fibromodulin and lumican. The findings showed that decorin, biglycan, fibromodulin and lumican associated with type I collagen fiber bundles, suggesting that they collaborate to regulate collagen fibrillogenesis in gingiva. As these SLRPs cover the collagen fibers, they may also modulate cell interactions with type I collagen. The SLRPs also showed association with distinct connective tissue and epithelial cells, suggesting that various gingival cells participate to produce or metabolize these molecules and/or that these molecules interact with cells to regulate their function.

Material and methods

Tissue samples

Tissue samples from palatal attached gingiva were obtained from seven healthy individuals between 23 and 43 years of age with the approval from the Office of Research Services, Behavioral Research Ethics Board, Biosafety and Animal Care, University of British Columbia. After collection, tissues were immediately embedded in optimal cutting temperature compound (Tissue-Tek®, Miles Inc., Elkhart, IA, USA), snap frozen in liquid nitrogen, and stored at −86°C until sectioning. After cutting, cryostat sections (6 μm) were transferred to 3-aminopropyltriethoxysilane-coated slides, air dried, and kept at −86°C until use. The absence of inflammation was confirmed by hematoxylin and eosin staining of representative sections from each sample (not shown).

Immunohistochemical staining

Tissue samples were shortly thawed at room temperature before fixation using −20°C acetone for 5 min. Samples were then rehydrated with phosphate-buffered

saline for 5 min. For immunostaining using ABC avidin/peroxidase system (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, CA, USA), sections were incubated in 0.3% hydrogen peroxide in methanol for 30 min in order to quench endogenous peroxidase activity. After washing with phosphate-buffered saline, sections were incubated with appropriate normal blocking serum (Vectastain ABC Kit) for 60 min at room temperature and then incubated with a primary antibody against the core protein of the SLRP, collagen, procollagen or various cell specific markers (Table 1) in phosphate-buffered saline containing bovine serum albumin (1 mg/ml) and Triton X-100 (0.01%) at 4°C overnight. After washing with phosphate-buffered saline, sections were incubated with biotinylated anti-rabbit or anti-mouse antibodies for 60 min, rinsed again and then reacted with ABC avidin/peroxidase reagent (Vectastain Elite Kit, Vector Laboratories Inc.). Once optimal color intensity was obtained, reaction was halted by immersing the tissue sections in distilled water. The sections were then air-dried and mounted using Vecta-Mount™ (Vector Laboratories Inc.). The slides were examined using Axiolab E light microscope (Carl Zeiss, Jena, Germany).

For double immunofluorescence staining, sections were blocked with phosphate-buffered saline containing bovine serum albumin (10 mg/ml) and Triton X-100 (0.01%) for 30 min at room temperature followed by overnight incubation with the primary antibody (Table 1) as above. The sections were then washed and incubated with second primary antibody (Table 1) for 1 h at room temperature. After washing, the samples were incubated with appropriate Alexa-conjugated secondary antibodies (Alexa 488 and Alexa 596; Molecular Probes Inc., Eugene, OR, USA) against both primary antibodies for 1 h at room temperature. The control samples were only exposed to secondary antibodies. The slides were mounted using Immuno-mount solution (Thermo Shadon, Pittsburgh, PA, USA), examined by a Zeiss Laser Confocal Scanning Microscope 10 (LSM 10) (Carl Zeiss), and images were captured using Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada). The relative staining intensity was recorded for each molecule at different tissue locations by using two to three representative sections stained with both immunoperoxide and immunofluorescence methods from three subjects. Relative immunostaining intensity was scored –, +, + +, or + + + by two examiners (–, represented no immunoreactivity; + + +, represented the most intense reaction).

Table 1. List of antibodies used for immunostaining

Antibody	Dilution	Reference/manufacturer
Anti-decorin	1 : 100	Krusius and Ruoslahti, 1986 (32)
Anti-prodecorin (LF 122)	1 : 2000	Fisher *et al.* 1995 (33)
Anti-fibromodulin	1 : 2000	Plaas and Wong Palms, 1993 (34)
Anti-lumican	1 : 2000	Grover *et al.* 1995 (35)
Anti-collagen type I	1 : 300	Biodesign International, Kennebunk, ME, USA
Anti-procollagen type I	1 : 10,000	Chemicon International, Temecula, CA, USA
Anti-biglycan (LF 106)	1 : 1000	Fisher *et al.* 1989 (36)
Anti-fibroblast antigen (Ab-1)	1 : 10	Oncogene Research Products, San Diego, CA, USA; Saalbach *et al.* 1998 (37)
Anti-α-smooth muscle actin	1 : 100	Sigma, Saint Louis, MO, USA
Anti-CD31, Endothelial cells	1 : 200	BD Biosciences, Mississauga, ON, Canada
Anti-CD68, Macrophages	1 : 100	DakoCytomation Inc., Mississauga, ON, Canada

Results

Localization of various connective tissue cells in human gingiva

In order to localize different connective tissue cells in healthy human attached gingiva, we immunostained tissue sections with antibodies that recognize all fibroblasts [fibroblast antigen (Ab-1)], myofibroblasts and perivascular cells [Ab-1 and α-smooth muscle actin (α-SMA)], endothelial cells (CD31) and macrophages (CD68) (Table 1). Ab-1 positive fibroblasts were numerous throughout the connective tissue (Fig. 1A, arrowheads). Additionally, blood vessels showed positive immunoreaction for the Ab-1 antigen (Fig. 1A, arrows). α-SMA positive myofibroblasts were also evenly scattered throughout the connective tissue (Fig. 1B, arrowheads). The density of myofibroblasts appeared less than that of fibroblasts expressing the Ab-1 marker. α-SMA positive cells also localized at many of the blood vessels (Fig. 1B, arrows). Because both the anti-Ab-1 and anti-α-SMA antibodies were produced in mouse, we were not able to perform double immunolocalization experiments using these two antibodies in the same sections. However, by comparing parallel serial sections it was possible to differentiate the Ab-1 positive and α-SMA negative (Ab-1 + /α-SMA–) cells from Ab-1 and α-SMA positive (Ab-1 + /α-SMA +) cells. Macrophages were most abundant at the subepithelial connective tissue, although some cells were noted also in the deeper areas (Fig. 1C, arrowheads). CD31 positive blood vessels localized in the papillary and deep connective tissue (Fig. 1D).

The proform and mature form of type I collagen show distinct localization in human gingiva

The proform of type I collagen (procollagen) was present throughout the entire connective tissue, where it localized in the ECM (Fig. 2 and Table 2) and associated with cells (Fig. 3 and Table 3). Compared to deep connective tissue, more intense staining was localized in the ECM of papillary connective

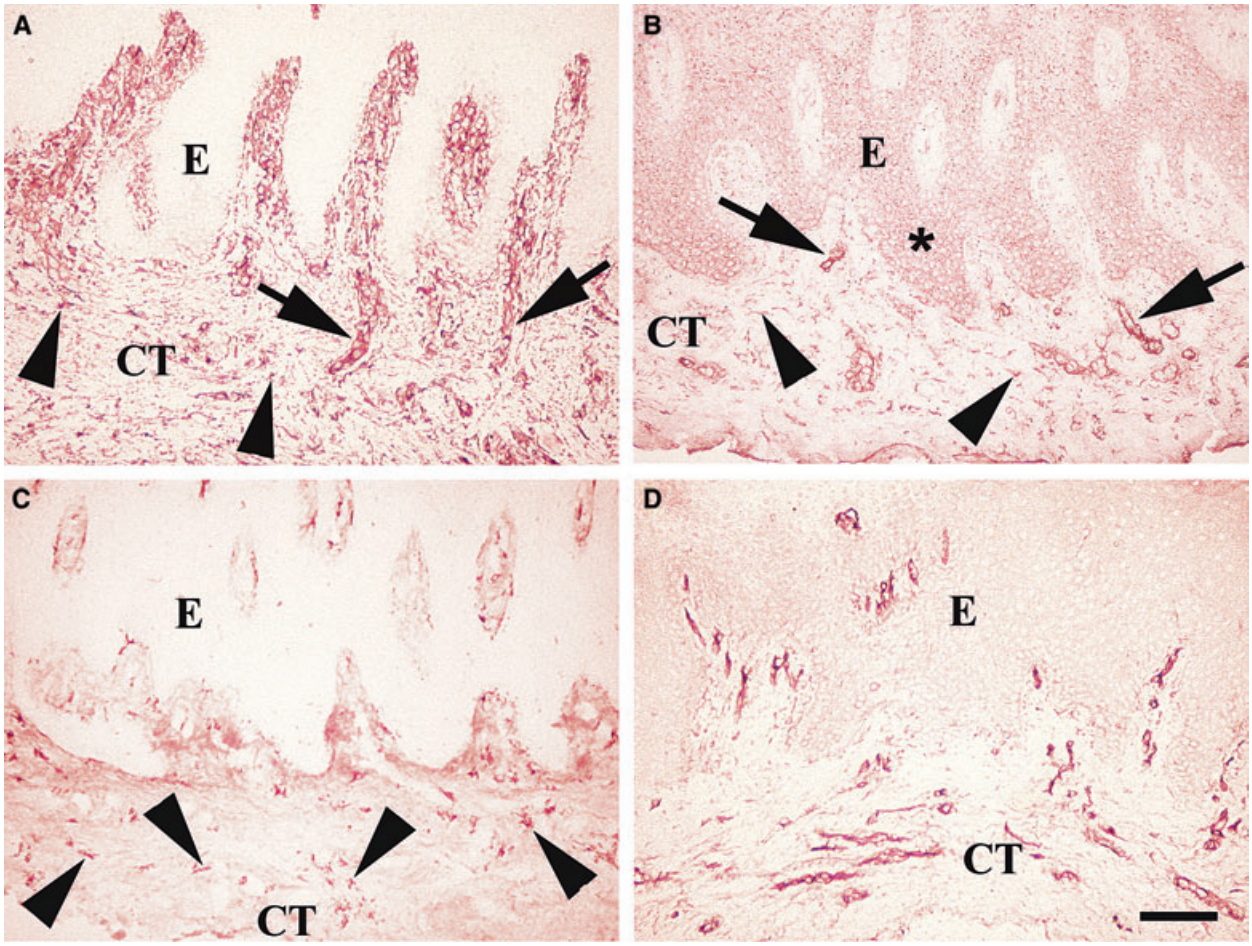

Fig. 1. Immunolocalization of connective tissue cells in gingiva. (A) Gingival connective tissue contained abundantly Ab-1-positive fibroblasts throughout the connective tissue (arrowheads). Also blood vessels showed immunoreactivity for the Ab-1 antibody (arrows). (B) α-Smooth muscle actin (α-SMA) positive myofibroblasts were scattered throughout the connective tissue (arrowheads). α-SMA positive perivascular cells were also noted in many of the blood vessels (arrows). (C) Macrophages were most abundant at the subepithelial connective tissue, although some cells were noted also in the deeper areas (arrowheads). (D) CD31 positive cells localized at blood vessels in the papillary and deep connective tissue. *Non-specific background staining; E, epithelium; CT, connective tissue. Magnification bar = 50 μm.

tissue, where a band-like staining was localized most abundantly below the epithelial basement membrane zone (Figs 2A and B and Table 2). This expression pattern was different from the fully processed (mature) form of type I collagen that showed relatively even distribution throughout the connective tissue and was organized into thick collagen fiber bundles (Fig. 2C and Table 2). Type I procollagen colocalized with Ab-1 + /α-SMA–

Fig. 2. Immunolocalization of small leucine-rich proteoglycans (SLRPs) and type I collagen in gingiva. (A and B) Type I pro-collagen showed most intense staining in the papillary connective tissue (arrowheads in A). A band-like staining was localized most abundantly below the basement membrane zone of epithelium (arrows in B). (C) Type I collagen showed even distribution throughout the connective tissue. (D and F) A distinct line of prodecorin staining was localized at the basement membrane zone (arrows). (E) Prodecorin also localized on the collagen bundle fibers (arrows) and around certain blood vessels (arrowhead). (G and H) Decorin was widely present on collagen fiber bundles throughout the connective tissue (arrows). (I) Papillary connective tissue and areas close to epithelium stained strongly for decorin. (J–L) Biglycan showed most abundant staining in the deep connective tissue (J) and on collagen bundle fibers (arrows in K). Biglycan was also present around blood vessels (arrowheads in J). Biglycan showed cell membrane-associated staining in basal epithelial cells (arrows in L). (M and N) Fibromodulin localized most abundantly in the deep connective tissue (M) where it localized on collagen fiber bundles (arrows in N). (O) Basal epithelial cells showed distinct immunoreactivity of fibromodulin around the cell membranes (arrows). (P and Q) Lumican localized also most abundantly in the deep connective tissue (P) on collagen fiber bundles (arrows in Q). (R) In the rete ridge areas, lumican localized on the cell membranes of basal epithelial cells. On these cells most abundant staining localized against the basement membrane (arrowheads). At the connective tissue papilla area, lumican was localized around the basal cells (arrows). E, epithelium; DCT, deep connective tissue; PCT, papillary connective tissue; *non-specific background staining. Magnification bar = 50 μm.

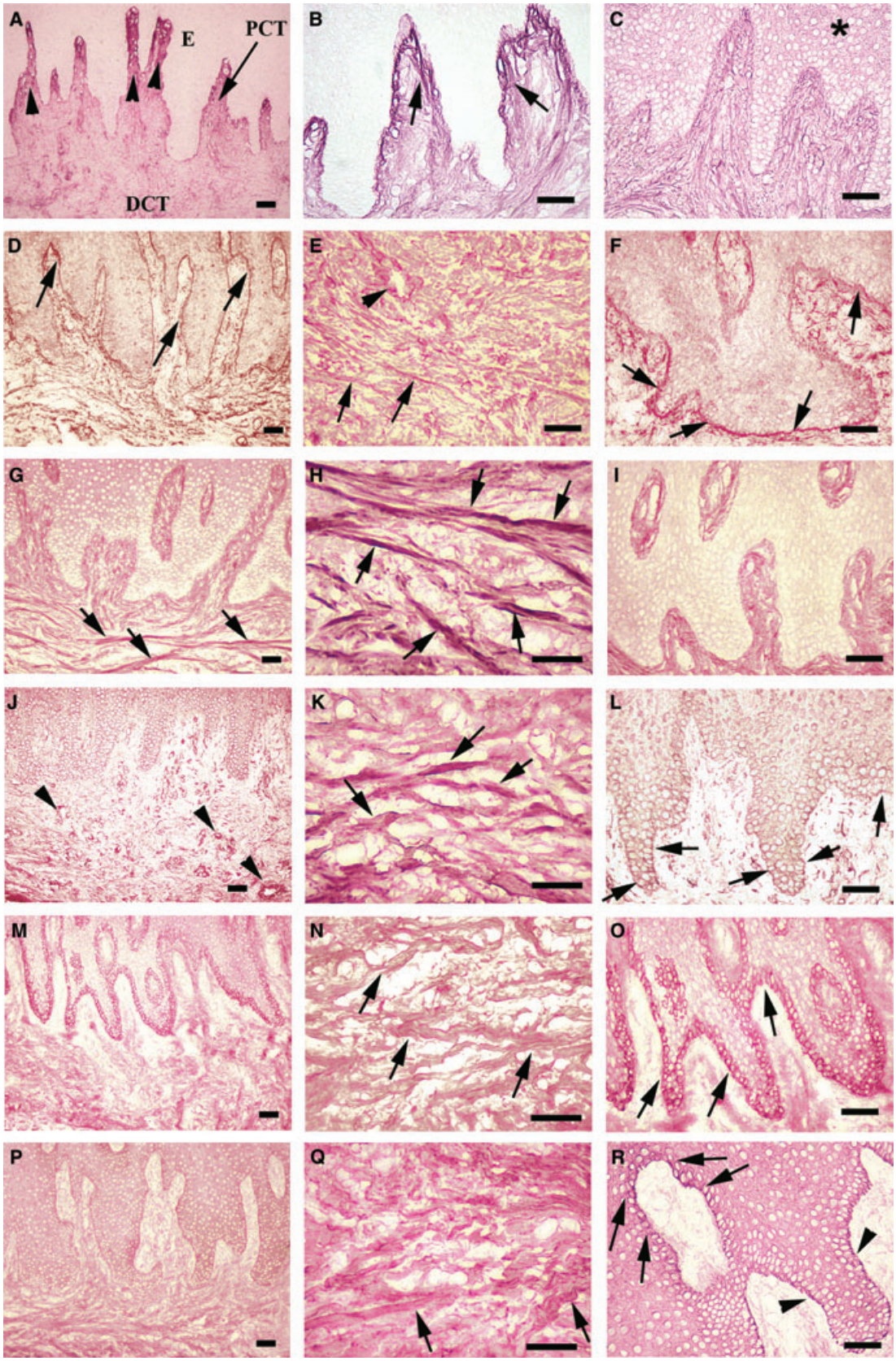
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Table 2. Relative immunostaining intensity of small leucine-rich proteoglycans and collagen in human gingiva

Molecule	Tissue area			
	Basal epithelial cells	Basement membrane zone	Papillary connective tissue	Deep connective tissue
Procollagen	–	–	+ + +	+ +
Collagen	–	–	+ +	+ +
Prodecorin	–	+ +	+	+ +
Decorin	–	–	+ + +	+ +
Biglycan	+	–	+ +	+ + +
Fibromodulin	+ + +	–	+	+ +
Lumican	+	–	+	+ +

–, no immunoreactivity; +, weakest; + +. intermediate; + + +, strongest immunostaining intensity. The relative intensity compares staining of each molecule between different tissue sites, not between different molecules.

fibroblasts and all α-SMA + myofibroblasts and perivascular cells (Figs 3A–C and Table 3). It also colocalized with capillary endothelial cells (Figs 3D–F and Table 3). However, macrophages did not show any colocalization (Figs 3G–I and Table 3). The association of procollagen and SLRPs with distinct cells and epithelium were consistent among all the subjects used for the study.

Distinct localization of the proform and mature form of decorin in human gingiva

The proform of decorin (prodecorin) localized in the connective tissue ECM (Fig. 2 and Table 2) and associated with

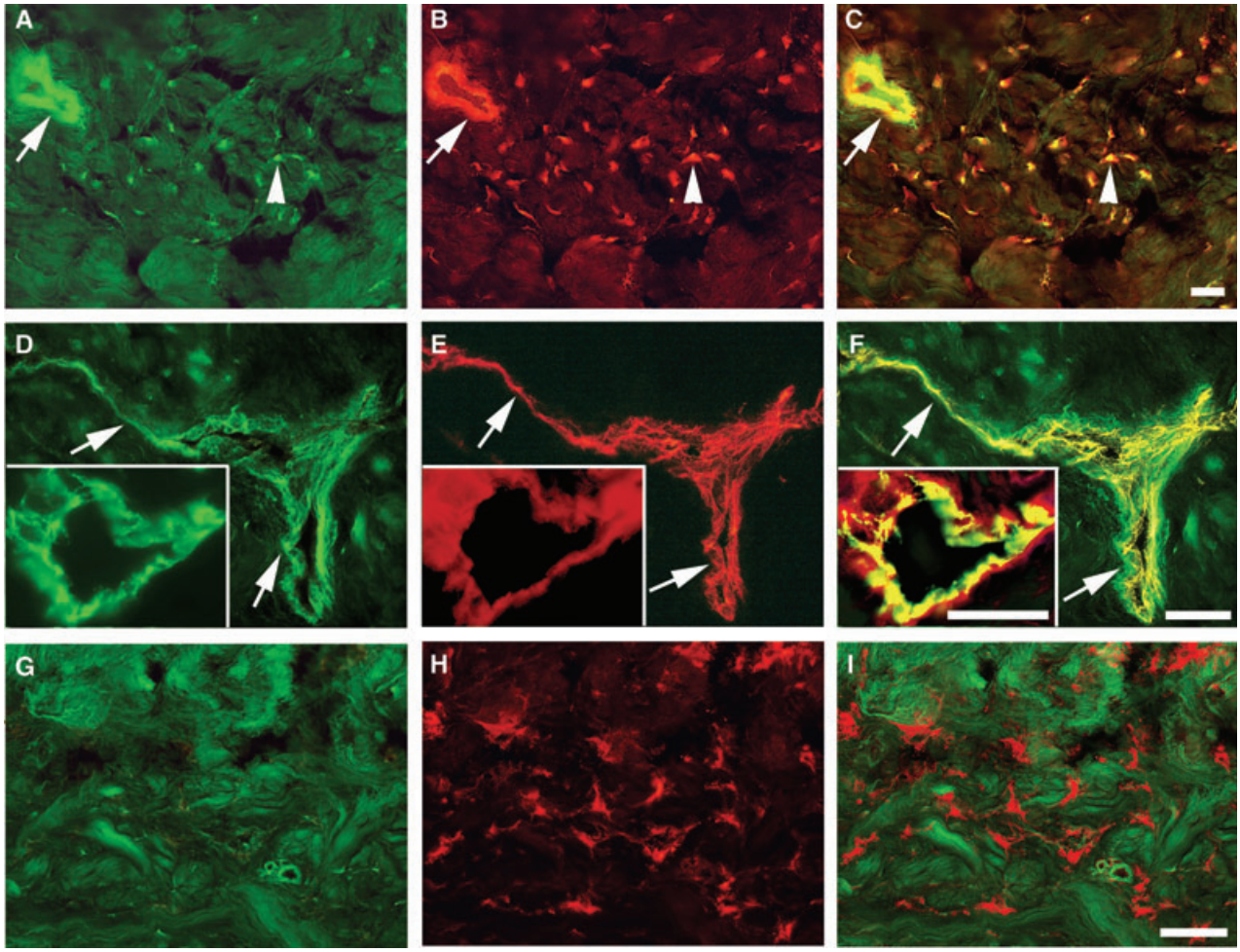

Fig. 3. Type I procollagen associates with endothelial and α-smooth muscle actin (α-SMA) positive cells. (A–C) Type I procollagen colocalized with α-SMA positive fibroblasts located in the extracellular matrix (arrowheads) and around blood vessels (arrows). (D–F) Procollagen also colocalized with capillary endothelial cells (arrows). Inserts show higher magnification of cross sections of representative blood vessels. (G–I) Macrophages did not show any colocalization with type I procollagen. (A, D and G) Type I procollagen staining. (B) α-SMA staining. (E) CD31 (endothelial cell) staining. (H) CD68 (macrophage) staining. (C, F and I) Corresponding merged images with yellow color indicating colocalization. Magnification bar = 50 μm.

Table 3. Association of small leucine-rich proteoglycans with different connective tissue cells in human gingiva

Molecules	Cell markers			
	Fibroblasts (Ab-1+/α-SMA–)	Myofibroblasts and pericytes (α-SMA+)	Endothelial cells (CD31+)	Macrophages (CD68+)
Procollagen	+	+*	+	–
Decorin	–	–	–	–
Prodecorin	+	+*	+	–
Biglycan	+	+*	+	+
Fibromodulin	+	+*	+	+
Lumican	+	+†	–	–

–, no colocalization; +, colocalization.
*Myofibroblasts and perivascular cells.
†Myofibroblasts only.
Ab-1, fibroblast antigen; α-SMA, α-smooth muscle actin.

certain cells (Figs 2 and 4 and Table 3). In the ECM it showed the most intense staining in the deep connective tissue (Fig. 2D and Table 2), where it localized on the collagen fiber bundles (Fig. 2E). Also, a strong, distinct line of prodecorin staining was noted at the epithelial basement membrane zone, whereas epithelium did not show any specific staining (Figs 2D and F and Table 2). Double immunostaining showed that prodecorin colocalized with Ab-1+/α-SMA– fibroblasts and some of the α-SMA+ myofibroblasts and perivascular cells (Figs 4A–F and Table 3). It also associated with endothelial cells (Figs 4G–I and Table 3) but no colocalization with macrophages was noted (Figs 4J–L and Table 3). Immunostaining of fully processed decorin using an antibody that recognizes the mature protein showed that decorin localized on collagen fiber bundles throughout the connective tissue (Figs 2G–I). In contrast to prodecorin, papillary connective tissue and areas close to epithelium stained most strongly for mature decorin (Fig. 2I and Table 2). No decorin immunoreactivity localized in the epithelium (Figs 2G and I and Table 2) and no colocalization with Ab-1, α-SMA, CD31 or CD68 positive cells was noted (Table 3).

Biglycan associates with collagen and connective tissue and epithelial cells in human gingiva

Biglycan showed immunoreactivity throughout the connective tissue but the most abundant staining localized to the deep connective tissue (Fig. 2J and Table 2) and colocalized with collagen fiber bundles (Figs 2J and K). Additionally, basal epithelial cells showed cell membrane-associated biglycan staining (Fig. 2L and Table 2). In these cells, most abundant staining localized against the basement membrane zone. Double immunostaining revealed that biglycan also associated with Ab-1+/α-SMA– fibroblasts and α-SMA+ perivascular cells (Figs 5A–C and Table 3) and myofibroblasts (Figs 5D–F and Table 3). In addition, a distinct colocalization of biglycan with endothelial cells (Figs 5G–I and Table 3) and macrophages was observed (Figs 5J–L and Table 3).

Fibromodulin associates with collagen and connective tissue and epithelial cells in human gingiva

Fibromodulin was most abundantly localized in the deep connective tissue where it localized on collagen fiber bundles (Figs 2M and N and Table 2). Basal epithelial cells also showed strong immunoreactivity of fibromodulin around the cell membranes (Figs 2M and O and Table 2). Double immunostaining showed that fibromodulin colocalized with Ab-1+/α-SMA– fibroblasts and αSMA+ perivascular cells (Figs 6A–C and Table 3) and myofibroblasts (Figs 6D–F and Table 3). It also colocalized with endothelial cells (Figs 6G–I and Table 3) and macrophages (Figs 6J–L and Table 3).

Lumican associates with collagen, fibroblasts, myofibroblasts and epithelial cells in human gingiva

Lumican localized abundantly throughout the connective tissue. The expression of lumican appeared to be less intense in the papillary than in the deeper connective tissue (Fig. 2P and Table 2). In the connective tissue, lumican was localized on collagen fiber bundles but not with blood vessels (Fig. 2Q). In the rete ridge areas of the epithelium, lumican localized on the cell membranes of basal cells (Fig. 2R and Table 2). This staining appeared most intense in the cell membrane facing the basement membrane. At the connective tissue papilla area, lumican staining was noted around the basal cells (Fig. 2R). Lumican also associated with Ab-1+/α-SMA– fibroblasts and α-SMA+ myofibroblasts (Figs 7D–F and Table 3) but no colocalization with perivascular α-SMA+ cells (Figs 7A–C and Table 3), endothelial cells (Figs 7G–I and Table 3) or macrophages was noted (Figs 7J–L and Table 3).

Discussion

Previous studies have shown the distribution and expression of SLRPs in dental and periodontal tissues of different animal species. For example, matrix deposition of decorin and biglycan was investigated during odontogenesis in the rat incisor. Administration of growth hormone to the dwarf rats markedly elevated the expression of both proteoglycans (38). In addition, a recent study on the adult rat periodontium has localized fibromodulin in the gingival epithelium and gingival and periodontal fibroblasts, as well as their surrounding ECM. Strong expression of fibromodulin was noted in the palatal gingival tissues and at the interfaces of the periodontal ligament with alveolar bone and cementum (39). In bovine cementum, lumican and fibromodulin were found to be located almost exclusively in non-mineralized portions such as precementum and the pericementocyte area (40). Although SLRPs have been studied in periodontal tissues of animal models, there

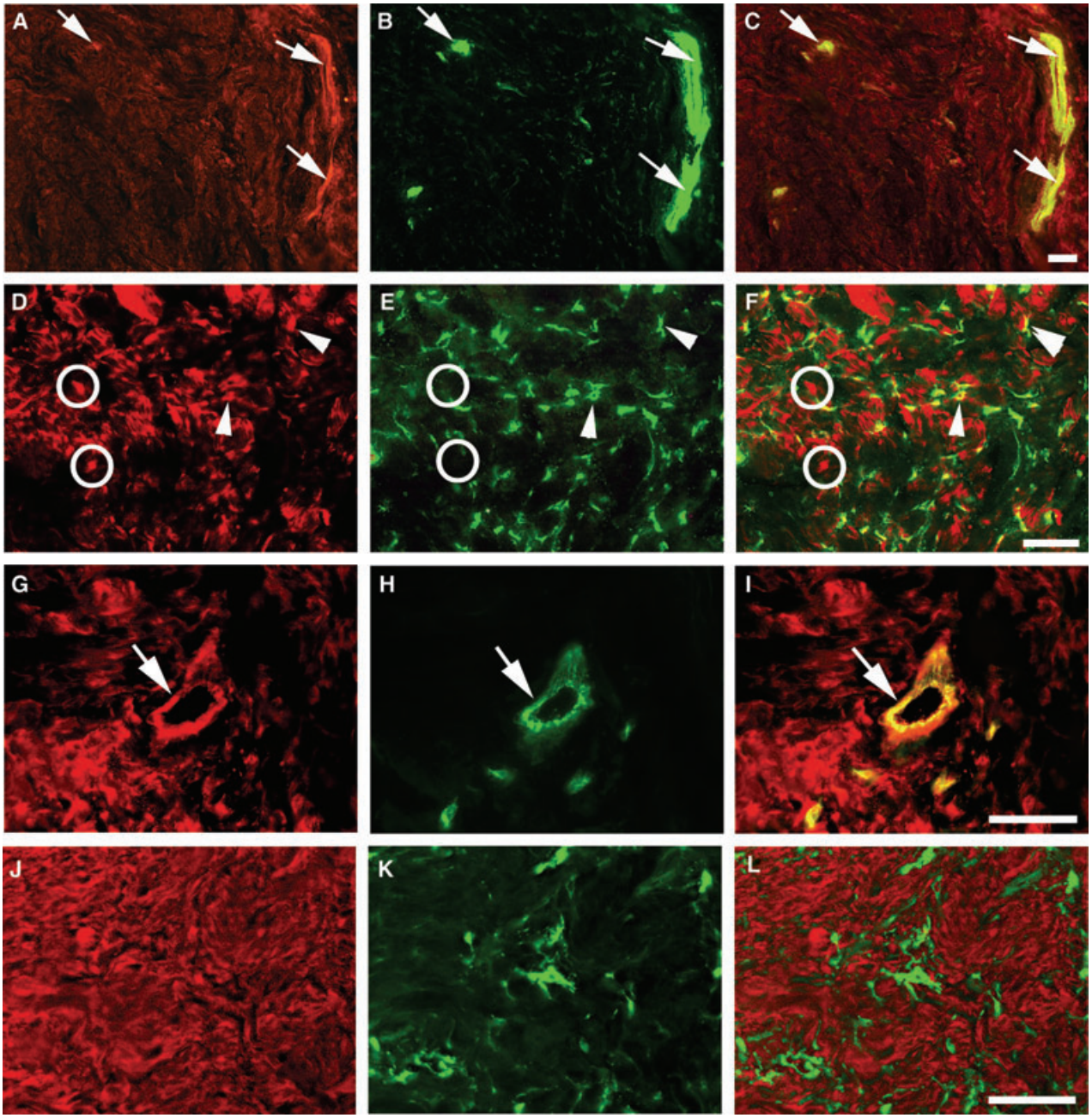

Fig. 4. Prodecorin associates with α-smooth muscle actin (α-SMA) positive cells. (A–C) Prodecorin colocalized with α-SMA expressing perivascular cells (arrows). (D–F) Some α-SMA positive myofibroblasts in the connective tissue (arrowheads) showed colocalization with prodecorin, whereas some cells showed no staining (circles). (G–L) Prodecorin also colocalized with endothelial cells (G–I) but did not associate with macrophages (J–L). (A, D, G and J) Prodecorin staining. (B and E) α-SMA staining. (H) CD31 (endothelial cells) staining. (K) CD68 (macrophage) staining. (C, F, I and L) Corresponding merged images with yellow color indicating colocalization. Magnification bar = 50 μm.

is lack of evidence about the localization of these molecules and their interactions with collagen fibers in human gingiva. Previously, we demonstrated that decorin and biglycan are expressed in human gingiva and periodontal ligament (30). In the present study, we showed that fibromodulin and lumican are also expressed in human gingiva. In the ECM, decorin showed the most abundant staining in the papillary connective tissue, whereas biglycan, fibromodulin and lumican were more abundant in the deep connective tissue, suggesting that they may serve for different functions in the areas where they accumulate.

These SLRPs associated with type I collagen fiber bundles in the ECM. Type I collagen is a cell adhesion molecule for connective tissue cells and

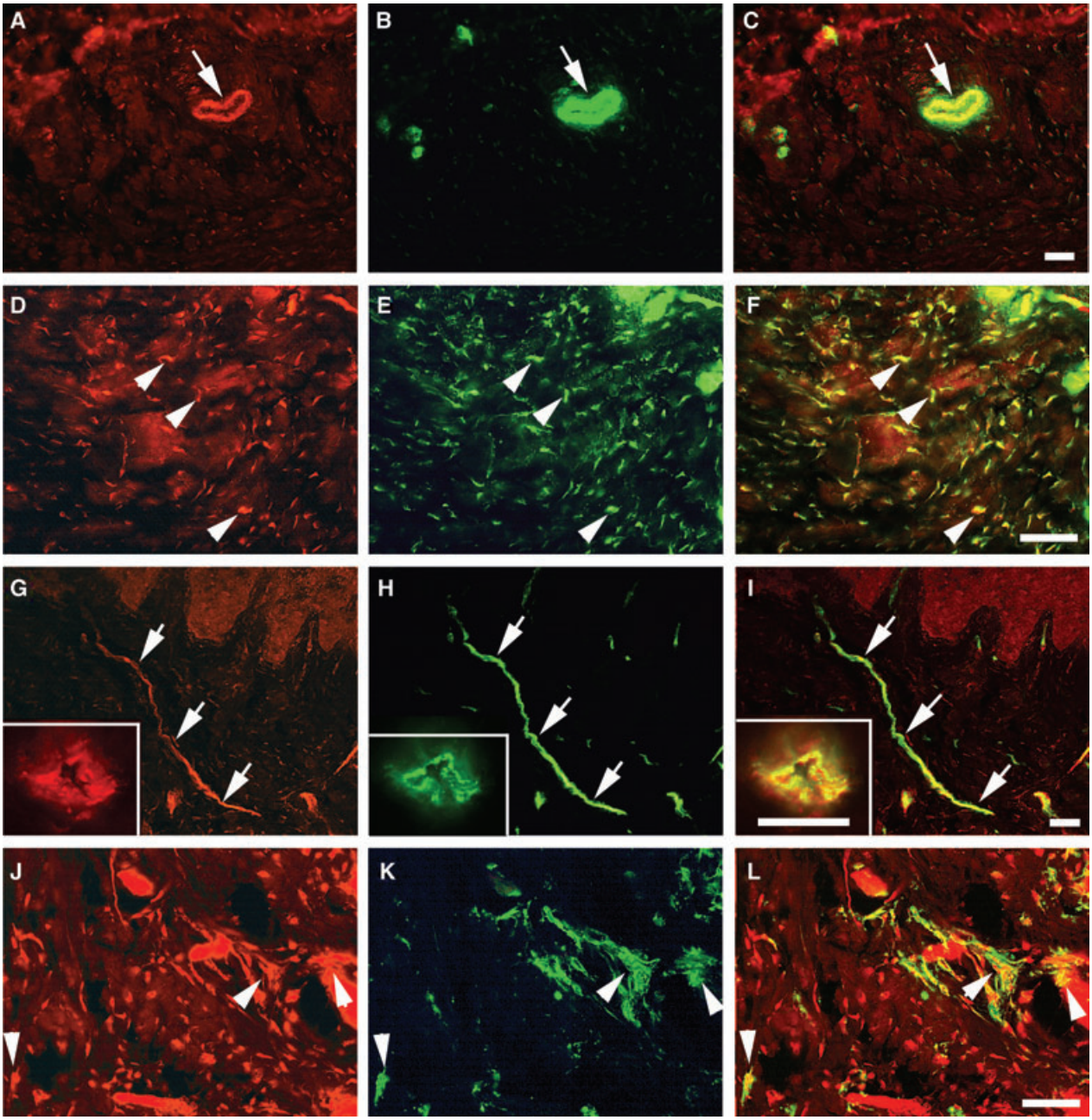

Fig. 5. Biglycan associates with α-smooth muscle actin (α-SMA) positive cells, endothelial cells and macrophages. (A–C) Biglycan colocalized with α-SMA positive perivascular cells (arrows). (D–F) Biglycan also colocalized with α-SMA positive myofibroblasts (arrowheads). (G–L) Biglycan colocalized with capillary endothelial cells (arrows in G–I) and macrophages (arrowheads in J–L). In (G)–(I), inserts show higher magnification of cross sections of representative blood vessels. (A, D, G and J) Biglycan staining. (B and E) α-SMA staining. (H) CD31 (endothelial cell) staining. (K) CD68 (macrophage) staining. (C, F, I and L) Corresponding merged images with yellow color indicating colocalization. Magnification bar = 50 μm.

interaction of cells with collagen regulates several key signaling pathways involved in cell proliferation, survival, gene expression and collagen turnover (41–43). It is not known, however, whether SLRPs modulate these interactions in gingival cells. SLRPs are also important regulators of collagen fibrillogenesis *in vitro* (11, 12, 44). Consequently, single gene knockout of each of the SLRPs leads to altered collagen fibrillogenesis *in vivo* (1, 3–7). Thus, our findings that the four SLRPs colocalized to collagen fiber bundles suggest that these SLRPs collaborate to regulate collagen fibrillogenesis in human gingiva. There is evidence that these four SLRPs may have distinct but also synergistic functions (1). Therefore, collagen fibrillogenesis is likely tissue and tissue site specific and depends on multiple variables including level of expression of individual SLRPs and collaboration between the SLRPs.

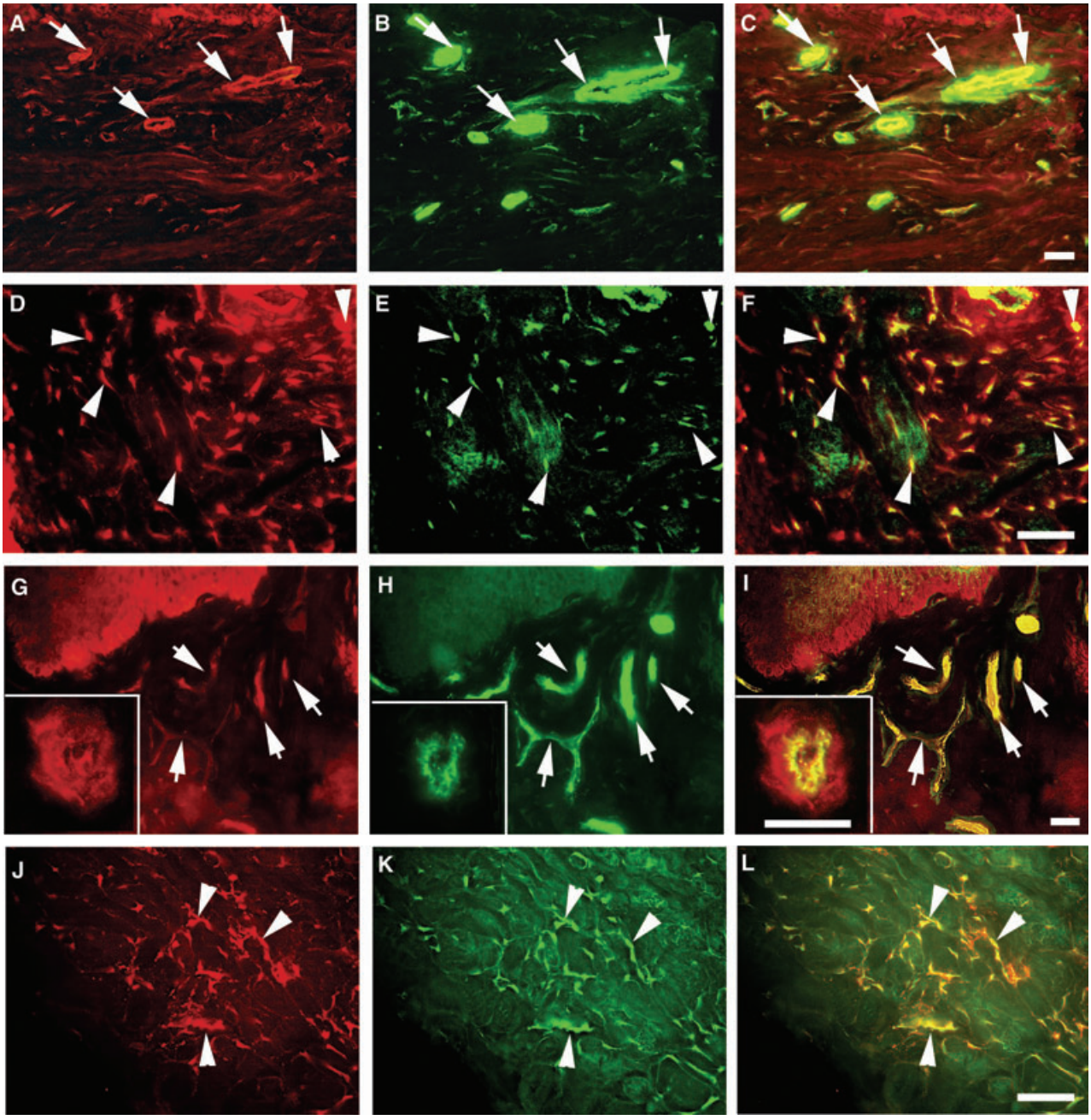

Fig. 6. Fibromodulin associates with α-smooth muscle actin (α-SMA) positive cells, endothelial cells and macrophages. (A–F) Fibromodulin colocalized with α-SMA positive perivascular cells (arrows) and α-SMA positive myofibroblasts (arrowheads). (G–L) Fibromodulin also colocalized with capillary endothelial cells (arrows in G–I) and macrophages (arrowheads in J–L). In (G)–(I), inserts show higher magnification of cross sections of representative blood vessels. (A, D, G and J) Fibromodulin staining. (B and E) α-SMA staining. (H) CD31 (endothelial cells) staining. (K) CD68 (macrophage) staining. (C, F, I and L) Corresponding merged images with yellow color indicating colocalization. Magnification bar = 50 μm.

In order to find out whether the SLRPs could interact with gingival cells, we studied whether immunoreactivity for the SLRPs associated with different cells in gingiva. To identify different connective tissue cells, we used antibodies that recognize specific molecules expressed by fibroblasts (Ab-1), myofibroblasts and pericytes (α-SMA), macrophages (CD68) and endothelial cells (CD31). The anti-fibroblast antigen (Ab-1) antibody recognizes CD90/Thy-1 that is expressed by fibroblasts, neurons, certain CD34+ blood stem cells and activated endothelial cells (37, 45–47). α-SMA is a cytoskeletal protein that is expressed by myofibroblasts and perivascular cells (pericytes) (48). Differentiation of myofibroblasts and expression of α-SMA is induced during

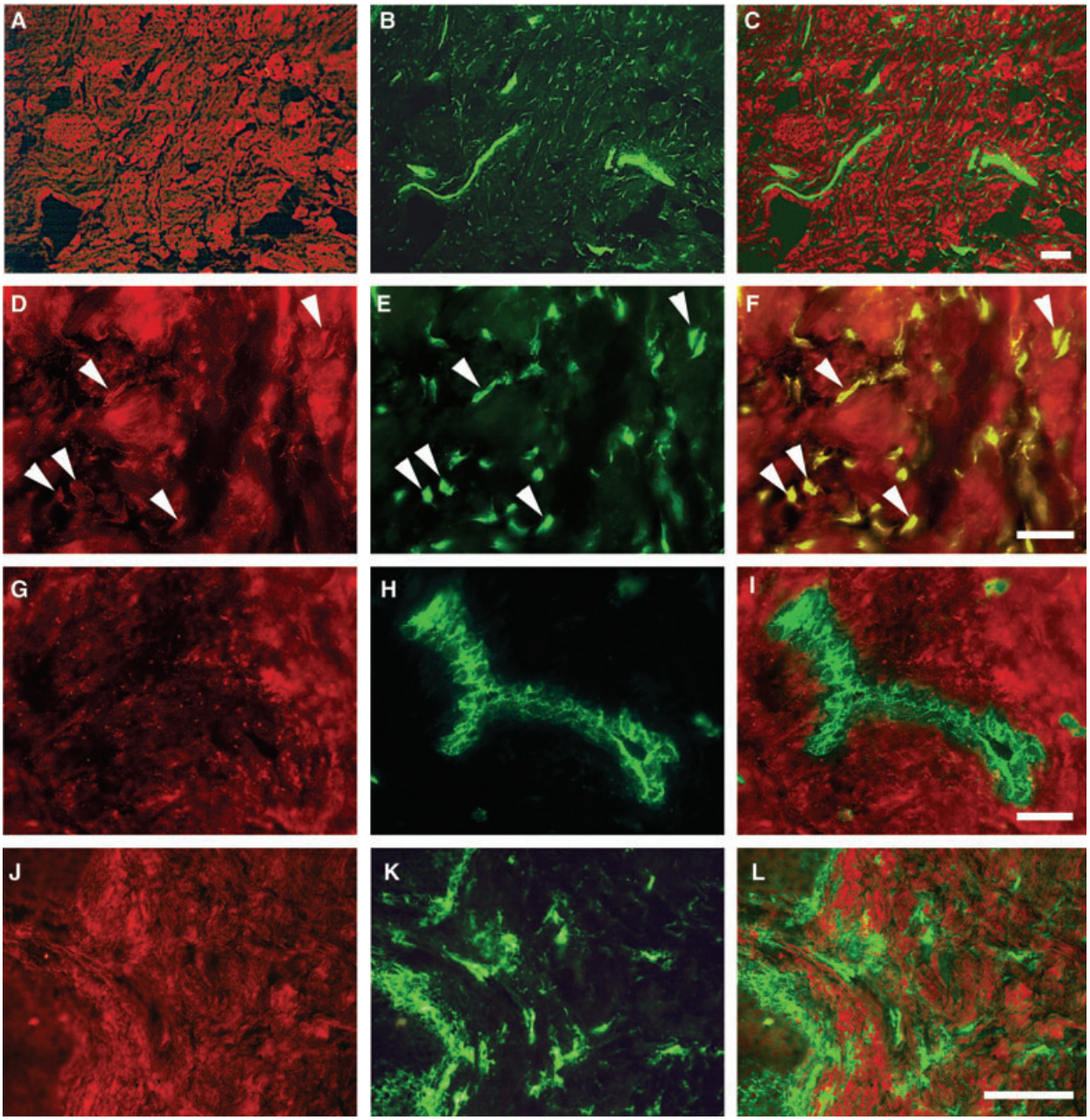

Fig. 7. Lumican associates with myofibroblasts. (A–C) Lumican did not show colocalization with α-smooth muscle actin (α-SMA) expressing perivascular cells. (D–F) However, α-SMA positive myofibroblasts in the connective tissue (arrowheads) showed colocalization with lumican. (G–L) No colocalization of lumican with endothelial cells (G–I) or macrophages (J–L) was noted. (A, D, G and J) Lumican staining. (B and E) α-SMA staining. (H) CD31 (endothelial cells) staining. (K) CD68 (macrophage) staining. (C, F, I and L) Corresponding merged images with yellow color indicating colocalization. Magnification bar = 50 μm.

organogenesis, wound healing and in certain pathological conditions including fibrosis, where these cells are involved in secretion and organization of ECM molecules (49). Pericytes exist in all organs, where they associate abluminally with all vascular capillaries and postcapillary venules and regulate local blood flow (50). In healthy human gingiva, α-SMA positive cells were located both within the ECM and in association with blood vessels. Therefore, the former cell population represents myofibroblasts, whereas the latter population represents pericytes. By comparing parallel serial sections it was possible to differentiate the Ab-1+/α-SMA– fibroblasts from Ab-1+/α-SMA+ myofibroblasts and to show that the fibroblasts were more abundant than myofibroblasts. Interestingly, myofibroblasts do not usually reside in, for example, normal skin connective tissue (data not shown). Therefore, a unique property of human gingiva appears to be the presence of myofibroblasts in the healthy tissue.

Our findings also support previous findings showing that a proportion of fibroblasts isolated from healthy human gingiva express α-SMA in culture (31, 51, 52).

By using double immunostaining, we showed that distinct SLRPs associated with various connective tissue cells. Cell association of SLRP immunoreactivity can have several explanations. First, it suggests that the cells participate to secrete these molecules to the ECM. Previous findings have shown that human periodontal fibroblasts and myofibroblasts from wound or chronically inflamed granulation tissue produce at least decorin, biglycan and fibromodulin in culture (31, 53). Second, cells may bind SLRPs to remove them from the ECM by endocytosis and/or phagocytosis. This would facilitate ECM turnover taking place in gingiva. Comparable turnover mechanisms have been described for collagens, decorin and biglycan in fibroblasts (54–57). Third, SLRPs may interact with the cells to modulate their functions. This is supported by findings showing that decorin regulated growth and gene expression of human periodontal fibroblasts (5, 17).

Fibroblasts are considered to be the major cell type responsible for the maintenance and turnover of the connective tissue ECM in tissue homeostasis. Our findings suggest that in human gingiva, myofibroblasts, macrophages, pericytes and endothelial cells also participate in this process. The double immunostainings showed that procollagen, prodecorin, biglycan, fibromodulin and lumican associated with both fibroblasts and myofibroblasts. In contrast to myofibroblasts, pericytes did not show colocalization with lumican, although they showed immunoreactivity for the other ECM molecules. Macrophages showed colocalization with biglycan and fibromodulin, whereas endothelial cells of gingival blood vessels associated with procollagen, prodecorin, biglycan and fibromodulin. Previous studies of other tissues have shown that pericytes synthesize decorin (58), whereas decorin is absent from resting capillaries (59). However, decorin appears to be involved in neovascularization because endothelial cells express decorin during angiogenesis *in vitro* and *in vivo* (60, 61). Corneal endothelial cells and cultured endothelial cells undergoing angiogenesis *in vitro* also synthesize type I collagen (60, 62). Based on these findings, localization of prodecorin and procollagen to gingival blood vessels suggests that these vessels are undergoing constant remodeling. This is further supported by our finding that gingival blood vessels showed immunoreactivity for the anti-Ab-1 antibody that recognized CD90/Thy-1 that is expressed by activated endothelial cells (46). The finding that biglycan and fibromodulin also associated with endothelial cells is novel and their function remains to be shown.

Our findings showing different tissue distribution and cell association of prodecorin and procollagen in gingiva suggest that there are site-specific variations in the processing of procollagen and prodecorin molecules. Gingiva, like other connective tissues, is composed of phenotypically and functionally different fibroblast populations (63, 64). Differences in, for example, synthesis of collagen, glycosaminoglycans and decorin have been noted in gingival fibroblast clones and subpopulations (53, 65, 66). Thus, the spatial differences in the abundance, localization and processing of the SLRPs and procollagen may result from the presence of phenotypically different fibroblast subpopulations in the tissue. Furthermore, our findings suggest that fibroblasts, myofibroblasts, pericytes, macrophages and endothelial cells produce distinct SLRPs, which can lead to variable expression of these molecules at different tissue sites. Epithelial cells were recently shown to regulate collagen and decorin expression by fibroblasts (67), which may also account for the increased localization of decorin, prodecorin and procollagen in the subepithelial connective tissue. The abundant immunoreactivity of prodecorin underneath the epithelium found in the present study is the first demonstration that prodecorin associates with the basement membrane zone.

Our findings also showed for the first time that SLRPs were localized in human gingival epithelium. Previous studies have localized lumican to the basal epithelial cells of injured mouse corneal epithelium and biglycan to developing human skin epithelium (68, 69). In the present study biglycan, fibromodulin and lumican showed distinct and partially different localization in basement membrane zone and basal epithelium, suggesting that these molecules may serve for different functions in these locations. The specific function of these molecules in the gingival epithelium remains to be shown.

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References

1. Ameye L, Young MF. Mice deficient in small leucine-rich proteoglycans: novel in vivo models for osteoporosis, osteoarthritis, Ehlers–Danlos syndrome, muscular dystrophy, and corneal diseases. *Glycobiology* 2002;**12:**107R–16R.
2. Iozzo RV. Matrix proteoglycans: from molecular design to cellular function. *Annu Rev Biochem* 1998;**67:**609–652.
3. Reed CC, Iozzo RV. The role of decorin in collagen fibrillogenesis and skin homeostasis. *Glycoconj J* 2002;**19:**249–255.
4. Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. *J Cell Biol* 1997;**136:**729–743.
5. Häkkinen L, Strassburger S, Kähäri VM *et al.* A role for decorin in the structural organization of periodontal ligament. *Lab Invest* 2000;**80:**1869–1880.
6. Svensson L, Aszodi A, Reinholt FP, Fassler R, Heinegard D, Oldberg A. Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and

altered lumican deposition in tendon. *J Biol Chem* 1999;**274:**9636–9647.
7. Chakravarti S, Petroll WM, Hassell JR *et al*. Corneal opacity in lumican-null mice: defects in collagen fibril structure and packing in the posterior stroma. *Invest Ophthalmol Vis Sci* 2000;**41:**3365–3373.
8. Chakravarti S, Magnuson T, Lass JH, Jepsen KJ, LaMantia C, Carroll H. Lumican regulates collagen fibril assembly. skin fragility and corneal opacity in the absence of lumican. *J Cell Biol* 1998;**141:**1277–1286.
9. Ameye L, Aria D, Jepsen K, Oldberg A, Xu T, Young MF. Abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis. *FASEB J* 2002;**16:**673–680.
10. Corsi A, Xu T, Chen XD *et al*. Phenotypic effects of biglycan deficiency are linked to collagen fibril abnormalities, are synergized by decorin deficiency, and mimic Ehlers-danlos-like changes in bone and other connective tissues. *J Bone Miner Res* 2002;**17:**1180–1189.
11. Vogel KG, Paulsson M, Heinegard D. Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. *Biochem J* 1984;**223:**587–597.
12. Hedbom E, Heinegard D. Interaction of a 59-kDa connective tissue matrix protein with collagen I and collagen II. *J Biol Chem* 1989;**264:**6898–6905.
13. Yamaguchi Y, Mann DM, Ruoslahti E. Negative regulation of transforming growth factor-beta by the proteoglycan decorin. *Nature* 1990;**346:**281–284.
14. Hildebrand A, Romaris M, Rasmussen LM *et al*. Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. *Biochem J* 1994;**302:**527–534.
15. Zhao M, Takata T, Ogawa I, Yada T, Kimata K, Nikai H. Immunohistochemical evaluation of the small and large proteoglycans in pleomorphic adenoma of salivary glands. *J Oral Pathol Med* 1999;**28:**37–42.
16. Abdel-Wahab N, Wicks SJ, Mason RM, Chantry A. Decorin suppresses transforming growth factor-beta-induced expression of plasminogen activator inhibitor-1 in human mesangial cells through a mechanism that involves Ca2+-dependent phosphorylation of Smad2 at serine-240. *Biochem J* 2002;**362:**643–649.
17. Al Haj Zen A, Lafont A, Durand E *et al*. Effect of adenovirus-mediated overexpression of decorin on metalloproteinases, tissue inhibitors of metalloproteinases and cytokines secretion by human gingival fibroblasts. *Matrix Biol* 2003;**22:**251–258.
18. Weber CK, Sommer G, Michl P *et al*. Biglycan is overexpressed in pancreatic cancer and induces G1-arrest in pancreatic cancer cell lines. *Gastroenterology* 2001;**121:**657–667.
19. Chen WB, Lenschow W, Tiede K, Fischer JW, Kalthoff H, Ungefroren H. Smad4/DPC4-dependent regulation of biglycan gene expression by transforming growth factor-beta in pancreatic tumor cells. *J Biol Chem* 2002;**277:**36118–36128.
20. Vuillermoz B, Khoruzhenko A, D'Onofrio MF *et al*. The small leucine-rich proteoglycan lumican inhibits melanoma progression. *Exp Cell Res* 2004;**296:**294–306.
21. Winnemoller M, Schmidt G, Kresse H. Influence of decorin on fibroblast adhesion to fibronectin. *Eur J Cell Biol* 1991;**54:**10–17.
22. Winnemoller M, Schon P, Vischer P, Kresse H. Interactions between thrombospondin and the small proteoglycan decorin: interference with cell attachment. *Eur J Cell Biol* 1992;**59:**47–55.
23. Saika S, Ohnishi Y, Ooshima A, Liu CY, Kao WW. Epithelial repair. roles of ECM. *Cornea* 2002;**21:**S23–S29.
24. Schaefer L, Beck KF, Raslik I *et al*. Biglycan, a nitric oxide-regulated gene, affects adhesion, growth, and survival of mesangial cells. *J Biol Chem* 2003;**278:**26227–26237.
25. Tufvesson E, Westergren-Thorsson G. Biglycan and decorin induce morphological and cytoskeletal changes involving signalling by the small GTPases RhoA and Rac1 resulting in lung fibroblast migration. *J Cell Sci* 2003;**116:**4857–4864.
26. Narayanan AS, Page RC. Synthesis of type V collagen by fibroblasts derived from normal, inflamed and hyperplastic human connective tissues. *Coll Relat Res* 1985;**5:**297–304.
27. White DJ, Puranen S, Johnson MS, Heino J. The collagen receptor subfamily of the integrins. *Int J Biochem Cell Biol* 2004;**36:**1405–1410.
28. Sodek J, Ferrier JM. Collagen remodelling in rat periodontal tissues: compensation for precursor reutilization confirms rapid turnover of collagen. *Coll Relat Res* 1988;**8:**11–21.
29. Sodek J. A comparison of the rates of synthesis and turnover of collagen and non-collagen proteins in adult rat periodontal tissues and skin using a microassay. *Arch Oral Biol* 1977;**22:**655–665.
30. Häkkinen L, Oksala O, Salo T, Rahemtulla F, Larjava H. Immunohistochemical localization of proteoglycans in human periodontium. *J Histochem Cytochem* 1993;**41:**1689–1699.
31. Häkkinen L, Westermarck J, Kähäri VM, Larjava H. Human granulation-tissue fibroblasts show enhanced proteoglycan gene expression and altered response to TGF-beta 1. *J Dent Res* 1996;**75:**1767–1778.
32. Krusius T, Ruoslahti E. Primary structure of an ECM proteoglycan core protein deduced from cloned cDNA. *Proc Natl Acad Sci USA* 1986;**83:**7683–7687.
33. Fisher LW, Stubbs JT III, Young MF. Antisera and cDNA probes to human and certain animal model bone matrix noncollagenous proteins. *Acta Orthop Scand* 1995;**66:**61–65.
34. Plaas AH, Wong-Palms S. Biosynthetic mechanisms for the addition of polylactosamine to chondrocyte fibromodulin. *J Biol Chem* 1993;**268:**26634–26644.
35. Grover J, Chen XN, Korenberg JR, Roughley PJ. The human lumican gene. Organization, chromosomal location, and expression in articular cartilage. *J Biol Chem* 1995;**270:**21942–21949.
36. Fisher LW, Termine JD, Young MF. Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) and several nonconnective tissue proteins in a variety of species. *J Biol Chem* 1989;**264:**4571–4576.
37. Saalbach A, Kraft R, Herrmann K, Haustein UF, Anderegg U. The monoclonal antibody AS02 recognizes a protein on human fibroblasts being highly homologous to Thy-1. *Arch Dermatol Res* 1998;**290:**360–366.
38. Zhang CZ, Li H, Bartold PM, Young WG, Waters MJ. Effect of growth hormone on the distribution of decorin and biglycan during odontogenesis in the rat incisor. *J Dent Res* 1995;**74:**1636–1643.
39. Qian H, Xiao Y, Bartold PM. Immunohistochemical localization and expression of fibromodulin in adult rat periodontium and inflamed human gingiva. *Oral Dis* 2004;**10:**233–239.
40. Cheng H, Caterson B, Neame PJ, Lester GE, Yamauchi M. Differential distribution of lumican and fibromodulin in tooth cementum. *Connect Tissue Res* 1996;**34:**87–96.
41. Lee W, Sodek J, McCulloch CA. Role of integrins in regulation of collagen phagocytosis by human fibroblasts. *J Cell Physiol* 1996;**168:**695–704.
42. Ivaska J, Heino J. Adhesion receptors and cell invasion. mechanisms of integrin-guided degradation of ECM. *Cell Mol Life Sci* 2000;**57:**16–24.
43. East L, Isacke CM. The mannose receptor family. *Biochim Biophys Acta* 2002;**1572:**364–386.
44. Rada JA, Cornuet PK, Hassell JR. Regulation of corneal collagen fibrillogenesis in vitro by corneal proteoglycan (lumican and decorin) core proteins. *Exp Eye Res* 1993;**56:**635–648.

45. Saalbach A, Aneregg U, Bruns M, Schnabel E, Herrmann K, Haustein UF. Novel fibroblast-specific monoclonal antibodies: properties and specificities. *J Invest Dermatol* 1996;**106:**1314–1319.
46. Saalbach A, Wetzig T, Haustein UF, Anderegg U. Detection of human soluble Thy-1 in serum by ELISA. Fibroblasts and activated endothelial cells are a possible source of soluble Thy-1 in serum. *Cell Tissue Res* 1999;**298:**307–315.
47. Saalbach A, Haustein UF, Anderegg U. A ligand of human thy-1 is localized on polymorphonuclear leukocytes and monocytes and mediates the binding to activated thy-1-positive microvascular endothelial cells and fibroblasts. *J Invest Dermatol* 2000;**115:**882–888.
48. Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 2003;**200:**500–503.
49. Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol* 1999;**277:** C183–C201.
50. Hirschi KK, D'Amore PA. Pericytes in the microvasculature. *Cardiovasc Res* 1996;**32:**687–698.
51. Arora PD, McCulloch CA. Dependence of collagen remodelling on alpha-smooth muscle actin expression by fibroblasts. *J Cell Physiol* 1994;**159:**161–175.
52. Giannopoulou C, Cimasoni G. Functional characteristics of gingival and periodontal ligament fibroblasts. *J Dent Res* 1996; **75:**895–902.
53. Larjava H, Häkkinen L, Rahemtulla F. A biochemical analysis of human periodontal tissue proteoglycans. *Biochem J* 1992; **284:**267–274.
54. Hausser H, Ober B, Quentin-Hoffmann E, Schmidt B, Kresse H. Endocytosis of different members of the small chondroitin/dermatan sulfate proteoglycan family. *J Biol Chem* 1992;**267:**11559–11564.
55. Wienke D, MacFadyen JR, Isacke CM. Identification and characterization of the endocytic transmembrane glycoprotein Endo180 as a novel collagen receptor. *Mol Biol Cell* 2003;**14:**3592–3604.
56. Everts V, van der Zee E, Creemers L, Beertsen W. Phagocytosis and intracellular digestion of collagen, its role in turnover and remodelling. *Histochem J* 1996;**28:**229–245.
57. Engelholm LH, List K, Netzel-Arnett S *et al*. uPARAP/Endo180 is essential for cellular uptake of collagen and promotes fibroblast collagen adhesion. *J Cell Biol* 2003;**160:**1009–1015.
58. Ungefroren H, Ergun S, Krull NB, Holstein AF. Expression of the small proteoglycans biglycan and decorin in the adult human testis. *Biol Reprod* 1995;**52:**1095–1105.
59. Bosse A, Schwarz K, Vollmer E, Kresse H. Divergent and co-localization of the two small proteoglycans decorin and proteoglycan-100 in human skeletal tissues and tumors. *J Histochem Cytochem* 1993; **41:**13–19.
60. Järvelainen HT, Iruela-Arispe ML, Kinsella MG, Sandell LJ, Sage EH, Wight TN. Expression of decorin by sprouting bovine aortic endothelial cells exhibiting angiogenesis in vitro. *Exp Cell Res* 1992;**203:**395–401.
61. Nelimarkka L, Salminen H, Kuopio T *et al*. Decorin is produced by capillary endothelial cells in inflammation-associated angiogenesis. *Am J Pathol* 2001;**158:** 345–353.
62. Kay EP, Gu X, Choi SH, Ninomiya Y. Posttranslational regulation of type I collagen in corneal endothelial cells. *Invest Ophthalmol Vis Sci* 1996;**37:**11–19.
63. Bordin S, Narayanan AS, Reddy J, Cleveland D, Page RC. Fibroblast subtypes in the periodontium. A possible role in connective tissue regeneration and periodontal reattachment. *J Periodont Res* 1984;**19:**642–644.
64. Häkkinen L, Larjava H. Characterization of fibroblast clones from periodontal granulation tissue in vitro. *J Dent Res* 1992;**71:**1901–1907.
65. Hassell TM, Stanek EJ 3rd. Evidence that healthy human gingiva contains functionally heterogeneous fibroblast subpopulations. *Arch Oral Biol* 1983;**28:**617–625.
66. Fries KM, Blieden T, Looney RJ *et al*. Evidence of fibroblast heterogeneity and the role of fibroblast subpopulations in fibrosis. *Clin Immunol Immunopathol* 1994;**72:**283–292.
67. Shephard P, Martin G, Smola-Hess S, Brunner G, Krieg T, Smola H. Myofibroblast differentiation is induced in keratinocyte-fibroblast co-cultures and is antagonistically regulated by endogenous transforming growth factor-beta and interleukin-1. *Am J Pathol* 2004;**164:**2055–2066.
68. Bianco P, Fisher LW, Young MF, Termine JD, Robey PG. Expression and localization of the two small proteoglycans biglycan and decorin in developing human skeletal and non-skeletal tissues. *J Histochem Cytochem* 1990;**38:**1549–1563.
69. Saika S, Shiraishi A, Liu CY *et al*. Role of lumican in the corneal epithelium during wound healing. *J Biol Chem* 2000;**275:** 2607–2612.