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Distinctive localization and function for lumican, fibromodulin and decorin to regulate collagen fibril organization in periodontal tissues

S. Matheson, H. Larjava, L. Häkkinen
Department of Oral Biological and Medical Sciences, Laboratory of Periodontal Biology, Faculty of Dentistry, University of British Columbia, Vancouver, British Columbia, Canada

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Background: Small leucine-rich proteoglycans (SLRPs) decorin, biglycan, fibromodulin and lumican are secreted extracellular matrix molecules that associate with fibrillar collagens and regulate collagen fibrillogenesis. Collagens are the major extracellular matrix components of periodontal connective tissues where they provide mechanical attachment of the tooth to the bone and gingiva and mediate signals that regulate cell functions, including remodeling of the periodontal ligament and bone. Structural organization of collagen may also be important for the defense against periodontal disease, because in certain conditions abnormal collagen fibrils associate with increased susceptibility to periodontal disease.

Objectives: The purpose of this study was to find out the role of SLRPs to regulate collagen fibril and fibril bundle formation in periodontal tissues.

Methods: The localization of SLRPs in human and mouse periodontal tissues was studied using immunohistochemical methods. To assess the function of SLRPs we studied periodontal tissues of mice harboring targeted deletions of decorin, fibromodulin or lumican genes and lumican and fibromodulin double knockout mice using histological and electronmicroscopical methods.

Results: The SLRPs were coexpressed in human and mouse gingival and periodontal ligament connective tissues where they colocalized with collagen fibril bundles. Teeth in the knockout animals were fully erupted and showed normal gross morphology. Targeted deletion of decorin, fibromodulin, lumican or both lumican and fibromodulin resulted in abnormal collagen fibril and fibril bundle morphology that was most evident in the periodontal ligament. Each of the gene deletions resulted in a unique fibril and fibril bundle phenotype.

Conclusions: These findings indicate that decorin, fibromodulin and lumican coordinately regulate the fibrillar and suprafibrillar organization of collagen in the periodontal ligament.

Lari Häkkinen, Department of Oral Biological and Medical Sciences, Laboratory of Periodontal Biology, Faculty of Dentistry, University of British Columbia, 2199 Wesbrook Mall, JB Macdonald Bldg, Room 344, Vancouver, B.C., Canada V6T 1Z3
Tel (office): +1 604 822 0096
Tel (lab): +1 604 822 0744
Fax: +1 604 822 3562
e-mail: lhakkine@interchange.ubc.ca

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Periodontal diseases affect one-third to one-half of the American population (1), with the most severe forms affecting relatively fewer people (2). They cause inflammatory destruction of the collagen-rich extracellular matrix of the periodontal tissues and lead to detachment of the gingival adhesion to the tooth and eventually to tooth loss. Periodontal diseases have been linked to a number of causes, including virulent bacteria and host susceptibility. In some relatively rare cases, this increased susceptibility is attributed to a certain gene defect (3), as with Papillon Lefevre syndrome or Kindler's syndrome (4–6). However, in most cases, the underlying reason for rapidly progressing disease is unknown. Recent advances in the understanding of biological functions of extracellular matrix molecules have provided novel information about their possible role in the development and maintenance of periodontal tissues.

Collagens, particularly type I collagen, are the major extracellular matrix components of the periodontal connective tissues and provide mechanical attachment of the tooth to the bone and gingiva (7). In addition, collagen fibrils mediate mechanical forces between teeth and surrounding tissues and cells, which regulates remodeling of the periodontal ligament and bone. Collagens regulate cell functions also by providing substrates for cell adhesion and migration and mediate signaling events by binding to specific cell surface receptors (8–10). The turnover rate of collagen in the gingiva and periodontal ligament is among the fastest in the body (11–13), indicating a need for rapid remodeling for changing functional demands. Certain forms of Ehlers–Danlos syndrome, which cause abnormal collagen fibrils, associate with severe periodontal disease at an early age (14–20). Therefore, ideal organization of collagen in periodontal tissues seems to be critical for the maintenance and defense of periodontal tissues and altered collagen fibrils may predispose to periodontal disease. Although some changes in the collagen structure in Ehlers–Danlos syndrome have been attributed to heterogeneous genetic changes in collagen or collagen processing enzyme genes, other still unknown genes regulating collagen fibrillogenesis are likely involved (21).

Fibrillogenesis of type I collagen is regulated at various levels *in vivo*. It involves both cell-independent and dependent mechanisms including self-assembly of procollagen monomers after secretion and cell-mediated fibrillogenesis that requires fibronectin (8, 22–24). Additionally, fibrillogenesis is regulated by other extracellular matrix molecules. Small leucine-rich proteoglycans (SLRPs) comprise a group of at least 13 different gene products in the extracellular matrix of hard and soft tissues (25). They are, like larger proteoglycans, comprised of a protein core and one or more glycosaminoglycan side chains. The distinguishing feature of SLRPs is the presence of a central domain containing leucine-rich repeats in the protein core. This domain is responsible for most of the functional activity of these molecules. Members of the SLRP family differ in their numbers of leucine-rich repeats, amino acid substitutions and glycosylation (26). Among SLRPs, class I SLRP decorin and class II SLRPs, fibromodulin and lumican, bind to type I collagen and regulate fibrillogenesis *in vivo* (25). Targeted deletion of any of these SLRPs lead to altered collagen fibrils in skin, cornea, or tendons (25, 27, 28). We showed recently that absence of decorin leads to altered collagen fibrils also in the periodontal ligament (29). Although it is not completely clear whether biglycan, a closely related class I SLRP with decorin, can bind directly to type I collagen, targeted disruption of the biglycan gene also caused morphological changes in collagen fibrils (30–32). Based on the current model (33), it is the binding of the SLRP core protein to the collagen molecule that regulates the organization of the fibrils (28). It is not known whether collagen-associated SLRPs also regulate cell-mediated collagen fibrillogenesis. The binding site of decorin core protein to type I collagen molecule is different from that of fibromodulin and lumican, whereas the latter two SLRPs share a common binding site (34–36). The relative abundance of SLRPs in tissues varies during development and by location. Therefore, it is not surprising that the phenotypes of various SLRP knockout mice were unique (25), suggesting a tissue-specific role for each SLRP in the development and maintenance of tissue structure (37).

We have shown previously that decorin and biglycan associate with collagen in human gingival and periodontal ligament (38). However, it is not known in detail whether decorin, biglycan, lumican and fibromodulin coordinately associate with collagen and what their role is to regulate organization of collagen fibrils and fibril bundles in periodontal tissues. To this end, we have compared the expression and localization of these SLRPs in human and mouse periodontal tissues. To assess the function of these molecules we also studied collagen organization in the periodontal tissues of mice harboring targeted deletions of decorin, fibromodulin or lumican genes and in mice with deletion of both lumican and fibromodulin genes. The findings showed that these SLRPs were coexpressed in human and mouse gingiva and periodontal ligament where they colocalized with collagen fibril bundles. Targeted deletion of decorin, fibromodulin, lumican or both lumican and fibromodulin resulted in abnormal fibril and fibril bundle morphology that was most evident in the periodontal ligament. Each of the gene deletions resulted in a unique fibril and fibril bundle phenotype. These findings indicate that decorin, fibromodulin and lumican coordinately regulate collagen fibrillogenesis and fibril bundle formation in the periodontal ligament.

Material and methods

Human samples

A set of frozen sections from four healthy human marginal gingival biopsies was obtained from the tissue collection from the Laboratory of Periodontal Biology at the University of British Columbia. The biopsies were from marginal gingiva around erupted maxillary third molars undergoing

routine extraction. The subjects were 20–28 years of age with no history of smoking or periodontal disease and they did not use any medication. Immediately after collection, gingival samples were mounted in Tissue-Tek® (Sakura Finetek USA, Inc., Torrence, CA, USA) and snap frozen in liquid nitrogen. In order to collect samples of healthy periodontal ligament, teeth were collected after careful extraction of erupted maxillary third molars from three healthy males (age 18–24 years). Immediately after extraction, the teeth were fixed for 2 h in 4% neutral buffered formaldehyde in phosphate buffered saline (PBS) and then decalcified in 12.5% EDTA (ethylene-diamine-tetra-acetic acid) and 2% formaldehyde in distilled water at 4°C. The samples were washed with phosphate-buffered saline and incubated in 2.3 mol/l sucrose in phosphate-buffered saline for 18 h followed by mounting in Tissue-Tek® and snap frozen in liquid nitrogen. The samples were stored at −86°C until sectioning and immunostaining. All the procedures were approved by the University of British Columbia Research Ethics Board.

Animals

Samples from five adult decorin (DCN –/–), seven lumican (LUM –/–), eight fibromodulin (FM –/–) knockout mice and eight lumican and fibromodulin (LUM-FM –/–) double knockout mice (gifts from Drs R. Iozzo and D. Birk, Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA, USA, and from Dr S. Chakravarti, Department of Medicine, Johns Hopkins University, Baltimore, Maryland, USA, and Dr A. Oldberg, Department of Cell and Molecular Biology, University of Lund, Sweden) and from 10 CD-1 out-bred wild-type mice were used in this study (Table 1). The generation and characterization of these mice have been described in detail elsewhere (39–42). After the mice were killed, the mouse heads were immediately fixed in 10% formalin. The maxillae and mandibles were dissected out, separated and divided in half at the midline. Half of each maxilla was defleshed in 2% potassium hydroxide for 2 weeks and examined under a dissecting microscope for gross morphological changes in jaws and teeth, and presence or absence of alveolar bone loss (see below). The mandibular and remaining half maxillary samples were decalcified in 5% formic acid containing 0.9% sodium chloride. Then, one half of the mandible from each animal was embedded in paraffin in a mesio-distal orientation and the other half in a bucco-lingual orientation and sectioned at 6 μm. Standardized sections were chosen for the analyses from each mouse so that the widest diameter of the pulp chamber was visible in each section, the length and shape of the roots and number of teeth per section (for mesio-distal sections) were the same to ensure that similar areas of the teeth were being compared. One half of all defleshed maxillae was analyzed under a dissecting microscope for alveolar bone loss indicative of periodontal disease. Bone loss was classified as described previously: Grade I, horizontal component of bone loss in the furcation; Grade II, through-and-through furcations, and Grade III, through-and-through furcations with alveolar bone loss into the apical third of the tooth root (43).

Table 1. Sex (M = male; F = female) and age (months) of wild-type (CD-1) and decorin (DCN –/–), fibromodulin (FM –/–), lumican (LUM –/–) and lumican and fibromodulin double knockout (LUM-FM –/–) mice used in this study

	Wild-type		DCN –/–		FM –/–		LUM –/–		LUM-FM –/–	
Mouse	Sex	Age	Sex	Age	Sex	Age	Sex	Age	Sex	Age
1	M	5	F	3	F	5.5	M	4.4	F	8
2	M	5	F	3	F	5.5	M	4.5	F	8
3	M	5	M	3	F	5.5	M	6	F	8
4	F	6	M	5	F	5.5	M	6	F	8
5	F	6	F	13	F	5.5	F	6	F	8
6	F	6			M	5.5	F	6	M	8
7	F	8			M	5	F	6	M	8
8	F	8			M	5			M	4.5
9	M	8								
10	M	8								

Antibodies

Mouse sections were incubated with polyclonal antibodies against mouse decorin (LF-113) and biglycan (LF-106) (gifts from Dr L. Fisher, National Institutes of Health, NIDR, Bethesda, MD, USA) (44), mouse lumican core protein (gift from A. Oldberg, Department of Cell and Molecular Biology, University of Lund, Sweden) (41), bovine fibromodulin (gift from Dr A. Plaas, Shriner's Hospital for Crippled Children, Tampa, FL, USA) (45) or mouse type I collagen (Chemicon, Temecula, CA, USA) at 4°C for 16 h. Human frozen sections were incubated with polyclonal antibodies against human decorin core protein (46), human biglycan core protein (LF-15; gift from Dr L. Fisher) (47), human lumican core protein (ab353; gift from Dr P. Roughley, Genetics Unit, Shriners Hospital for Crippled Children, and the Department of Surgery, McGill University, Montreal, QE, Canada) (48), human fibromodulin (gift from Dr A. Plaas) (45) and type I collagen (Biodesign International, Kennebunk, ME, USA).

Immunohistochemical staining

For immunohistochemical staining, bucco-lingual and mesio-distal sections of the mandibular first molars from mice were deparaffinized. Human frozen sections were shortly thawed at room temperature before 10-min fixation with ice-cold acetone. Then samples were incubated in 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity, followed by rinsing in phosphate-buffered saline and incubation with normal blocking serum (Vectastain; Vector

Laboratories Inc., Burlingame, CA, USA) for 60 min at room temperature. Then, samples were incubated with the primary antibody at 4°C for 18 h followed by rinsing and incubation with biotinylated anti-rabbit antibody for 1 h. After rinsing, the sections were incubated with ABC avidin-peroxidase reagent (Vectastain Elite kit, Vector Laboratories Inc.) for 30 min. For color development, sections were reacted with the Vector VIP substrate (Vector Laboratories Inc.) for a standardized time. Control stainings were performed without primary antibody or with non-immune rabbit serum instead of the primary antibody. These samples did not show any immunoreactivity (not shown). All sections were mounted with Entellan mounting medium (Merck, Darmstadt, Germany) before being examined using the Axiolab E light microscope (Carl Zeiss, Jena, Germany) with 10 ×, 20 × and 40 × objectives. The relative immunostaining intensity within the mouse dental and periodontal tissues was determined by two independent examiners using bucco-lingual and mesiodistal sections and by using the following scale: + + + (most intense), + + (moderately intense), + (least intense), or – (no immunoreactivity).

Histological analysis, polarizing light microscopy and scanning electron microscopy

Paraffin sections were stained with hematoxylin and eosin to analyze general histology, morphologic changes in the dental and periodontal tissues, presence or absence of bone loss and cellular infiltrate indicative of inflammation. In order to study collagen organization, sections were stained with phosphotungstic acid hematoxylin (49) or picrosirus red stain. For picrosirus red staining, deparaffinized sections were immersed in 10% Sirus red (F3B Gurr, BDH Chemicals, Ltd, Poole, England) in saturated aqueous picric acid (BDH Chemicals) for 1 h and then washed in 1% acetic acid for 15 min. Stained sections were mounted using Entellan. Standardized areas of the hematoxylin and eosin and phosphotungstic acid hematoxylin-stained mandibular first molar sections were analyzed using an Axiolab E light microscope (Carl Zeiss) equipped with a 20 × and 40 × objective. Images were recorded with a Nikon Coolpix 995 digital camera attached to a Nikon Eclipse TS 100 microscope (Nikon, Richmond, BC, Canada) equipped with a 20 × objective. The picrosirus red-stained mandibular first molar sections were examined using a Jenapol Polarizing Light Microscope (Carl Zeiss) equipped with a 20 × objective. Angles on the polarizing light microscope were set so that at 0 degree light angle the sections were aligned with the long axis of the periodontal ligament spaces. Standardized images were recorded at 0, 45 and 90 degree light angle using a Canon EOS D60 digital camera.

Portions of periodontal ligament, alveolar bone and dentin at the mid root area of maxillary molars from two mice from each mouse line were microdissected from the paraffin-embedded tissue blocks and processed for transmission electron microscopy (TEM) as we have described previously (29). For scanning electron microscopy (SEM), 6 μm thick serial bucco-lingual paraffin sections including mandibular first molars from each mouse were processed using standard procedures as we have described previously (29).

Results

Immunohistochemical localization of small leucine-rich proteoglycans in human gingiva and periodontal ligament

Immunohistochemical staining of frozen sections from healthy human marginal gingiva showed that lumican, fibromodulin, decorin and biglycan localized abundantly in the subepithelial extracellular matrix on collagen fibril bundles (Fig. 1). Similarly, these SLRPs localized on the collagen fibril bundles in the periodontal ligament (Fig. 1). Immunoreactivity for lumican localized uniformly on collagen fibril bundles throughout the periodontal ligament and on the bone and cementum surface (precementum) (Fig. 1C), whereas fibromodulin, decorin and biglycan showed most intense staining next to the tooth surface. They also localized strongly on the surface of acellular root cementum (precementum) (Figs 1F, I and L). Additionally, lumican, fibromodulin, decorin and biglycan showed weak immunoreactivity in the acellular cementum layer covering the root surface. This staining appeared to colocalize with collagen fibril bundles extending from the periodontal ligament into the acellular cementum (Sharpey's fibers).

Immunohistochemical localization of small leucine-rich proteoglycans in mouse dental and periodontal tissues

In order to study the expression and localization of the SLRPs in mouse dental and periodontal tissues we performed immunohistochemical stainings on decalcified paraffin sections from adult wild-type CD-1 mice. All of the studied SLRPs were expressed in mouse dental and periodontal tissues but they showed individual variations in the distribution between different tissue locations (Fig. 2 and Table 2). In the soft connective tissues, SLRPs localized on collagen fiber bundles. Decorin and lumican showed the most intense staining in the gingiva and oral mucosa, whereas biglycan staining was the strongest in the gingiva and periodontal ligament. The relative staining intensity for fibromodulin was equally intense in the connective tissues of lingual oral mucosa, gingiva, periodontal ligament and dental pulp, whereas the labial oral mucosa showed the strongest staining intensity. The immunostaining for all SLRPs was relatively uniform throughout the whole width of the periodontal ligament (Figs 2G–J). All of the SLRPs showed discrete immunostaining at the predentin. No staining of any of the SLRPs in the extracellular matrix in dentin, or in acellular or cellular cementum was noted (Table 2). However, cementocytes in the apical cellular cementum were positive for biglycan, fibromodulin and lumican (Table 2). Additionally, the surface of cementum (precementum) stained relatively strongly for all of the SLRPs (Table 2).

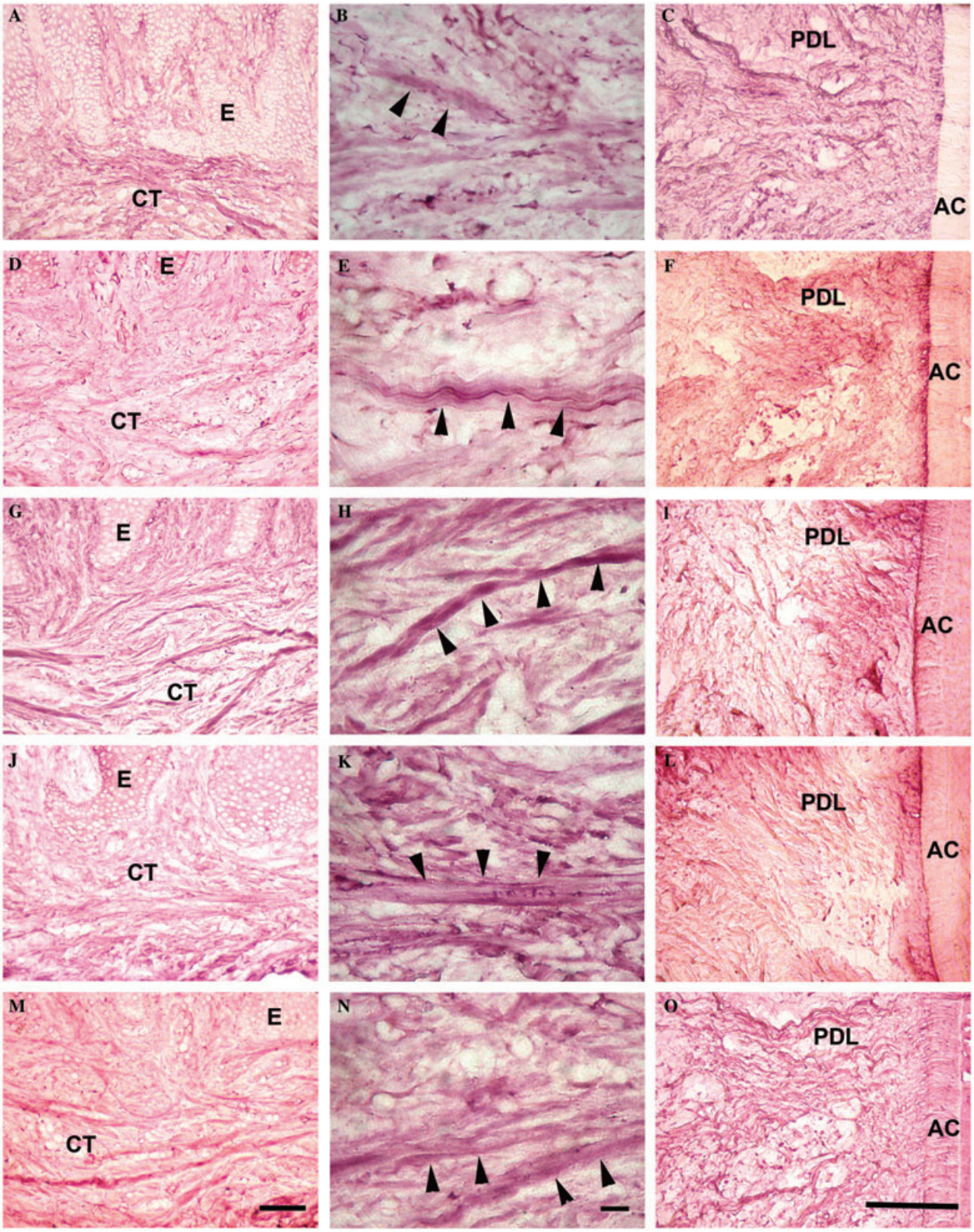

Fig. 1. Localization of small leucine-rich proteoglycans and type I collagen in human gingiva and periodontal ligament. Immunohistochemical staining of lumican (A–C), fibromodulin (D–F), decorin (G–I), biglycan (J–L) and type I collagen (M–O) in representative samples from human marginal gingiva (A, B, D, E, G, H, J, K, M and N) and periodontal ligament (C, F, I, L and O). CT: connective tissue; E: epithelium; PDL: periodontal ligament; AC: acellular cementum. Arrowheads indicate the collagen fibril bundles. A, C, D, F, G, I, J, L, M, O: bar = 50 μm; B, E, H, K, N: bar = 10 μm.

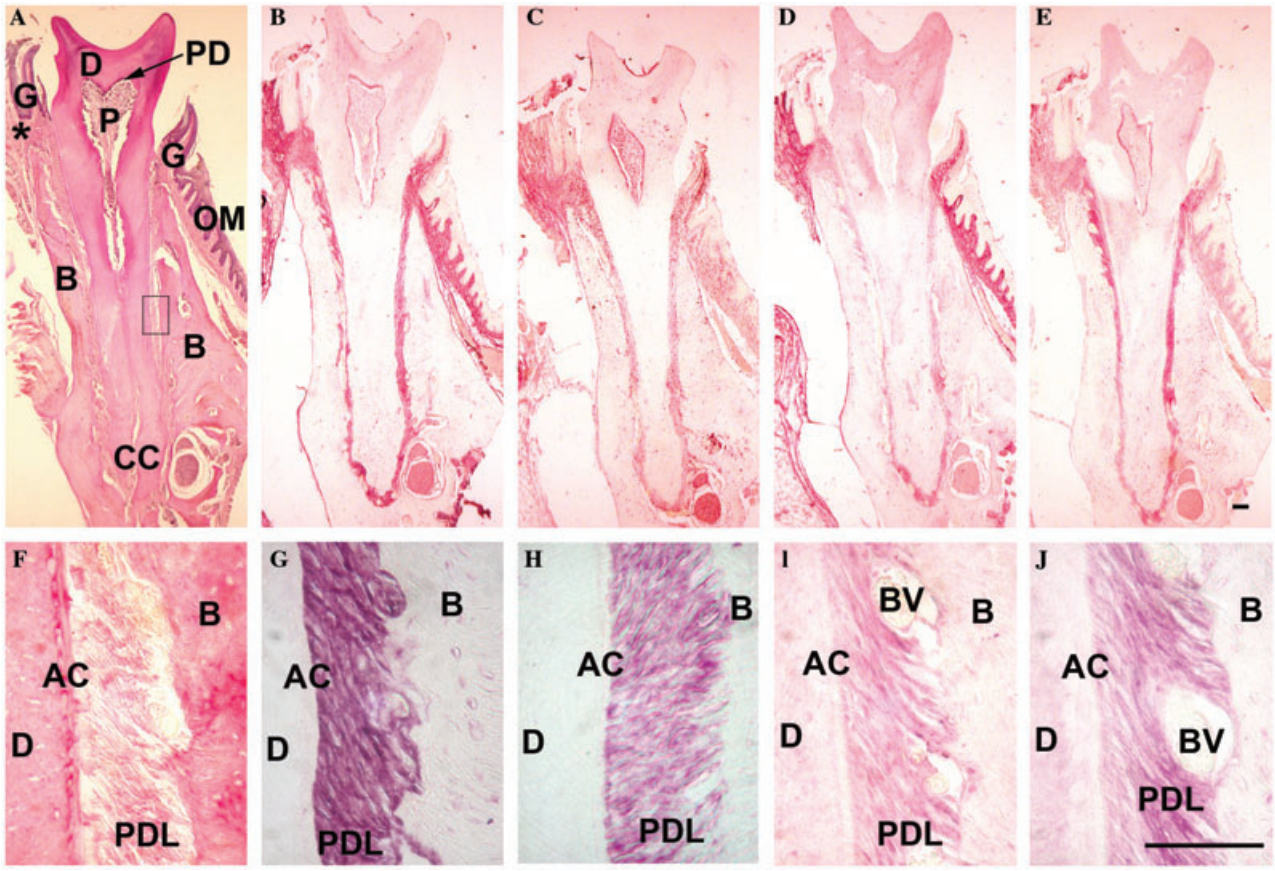

Fig. 2. Localization of small leucine-rich proteoglycans in wild-type mouse dental and periodontal tissues. Phosphotungstic acid hematoxylin staining of a representative bucco-lingual section (A). Parallel sections stained with an antibody against lumican (B), fibromodulin (C), decorin (D) and biglycan (E). Organization of collagen fibril bundles and localization of small leucine-rich proteoglycans in the periodontal ligament demonstrated by immunostaining with an antibody against type I collagen (F), lumican (G), fibromodulin (H), decorin (I) and biglycan (J). The rectangle in A indicates the area of the periodontal ligament that is shown in F–J. G: gingiva; OM: oral mucosa; B: bone; PD: predentin; P: pulp; PDL: periodontal ligament; CC: cellular cementum: AC: acellular cementum; D: dentin; BV: blood vessel. *Indicates the labial oral mucosa. Bar = 50 μm.

Table 2. Relative immunostaining intensity of decorin, biglycan, lumican and fibromodulin in dental and periodontal tissues of wild-type CD-1 mice

	Relative staining intensity[a]			
Tissue	Decorin	Biglycan	Fibromodulin	Lumican
Periodontal ligament	+ +	+ + +	+ +	+ +
Gingival connective tissue	+ + +	+ + +	+ +	+ + +
Mucosal connective tissue	+ + +	+ +	+ +/+ + +[b]	+ + +
Pulp	+	+	+ +	+
Bone matrix/osteocytes	+/–	+/+	+/+	+/+
Acellular cementum matrix	–	–	–	–
Cellular cementum matrix/cementocytes	–/–	–/+	–/+	–/+
Precementum	+ +	+	+ +	+ +
Dentin/predentin	–/+ +	–/+ +	–/+ + +	–/+ +

[a]Relative staining intensity measures are relative to staining intensity between different tissue locations within the same sample. Intensity of immunostaining: + + + (most intense), + + (moderately intense) or + (least intense); – indicates no immunostaining detected.
[b]Relative staining intensity in lingual and labial connective tissue, respectively.

The bone matrix was weakly positive for all of the SLRPs, whereas osteocytes in bone were positive for biglycan, fibromodulin and lumican (Table 2).

Characterization of the small leucine-rich proteoglycas knockout mice

In order to study the function of SLRPs in the periodontal tissues we compared macroscopically and histologically the dental and periodontal tissues in decorin, fibromodulin, lumican knockout mice and in fibromodulin and lumican double knockout mice with wild-type CD-1 mice (Table 1 and Fig. 3). In all of the study groups, all teeth were present, fully erupted and

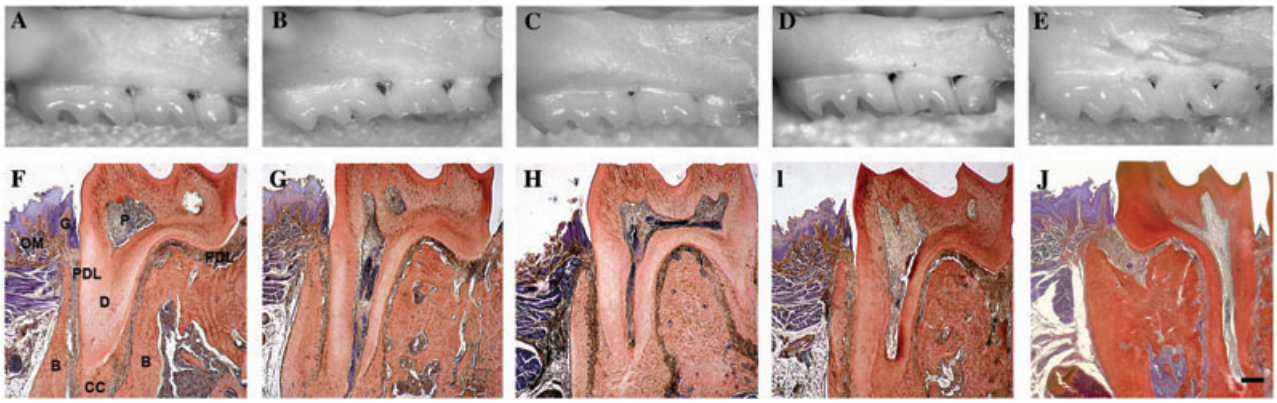

Fig. 3. Teeth in the proteoglycan knockout mice are fully developed and erupted and periodontal tissues show no signs of periodontal disease. Defleshed maxillae showing molar teeth in a representative wild-type mouse (A) and lumican (B), fibromodulin (C), lumican and fibromodulin (D), and decorin (E) knockout mice. Representative demineralized mesio-distal paraffin section from mandibular first molars from the wild-type mouse (F), and from lumican (G), fibromodulin (H), lumican and fibromodulin (I), and decorin (J) knockout mice stained with phosphotungstic acid hematoxylin are shown. Sections show only one of the two roots in the teeth because of the angle of the sample processing. OM: oral mucosa; G: gingiva; PDL: periodontal ligament; D: dentin; B: bone; CC: cellular cementum. F–J: bar = 100 μm.

appeared to have no gross anatomical changes (Fig. 3). Class I furcation defects (46) were detected in all groups but there was no statistically significantly difference among the groups (Bonferroni's post test) (data not shown). Only one specimen, fibromodulin knockout mouse #1, showed any appreciable amount of bone loss, exhibiting class II furcation defects. Histologically, the dental and periodontal tissues including bone and root cementum appeared healthy and there was no inflammation or signs of periodontal disease.

In order to confirm the absence of lumican, fibromodulin, and lumican and fibromodulin in the dental and periodontal tissues in the corresponding knockout mice, we used immunostaining. We have shown previously that decorin knockout mice do not express any decorin (29). Lumican, fibromodulin and double knockout mice showed no immunoreactivity for the corresponding proteoglycans (Fig. 4). To determine if targeted deletion of lumican or fibromodulin or both was associated with a compensatory up-regulation of the expression of

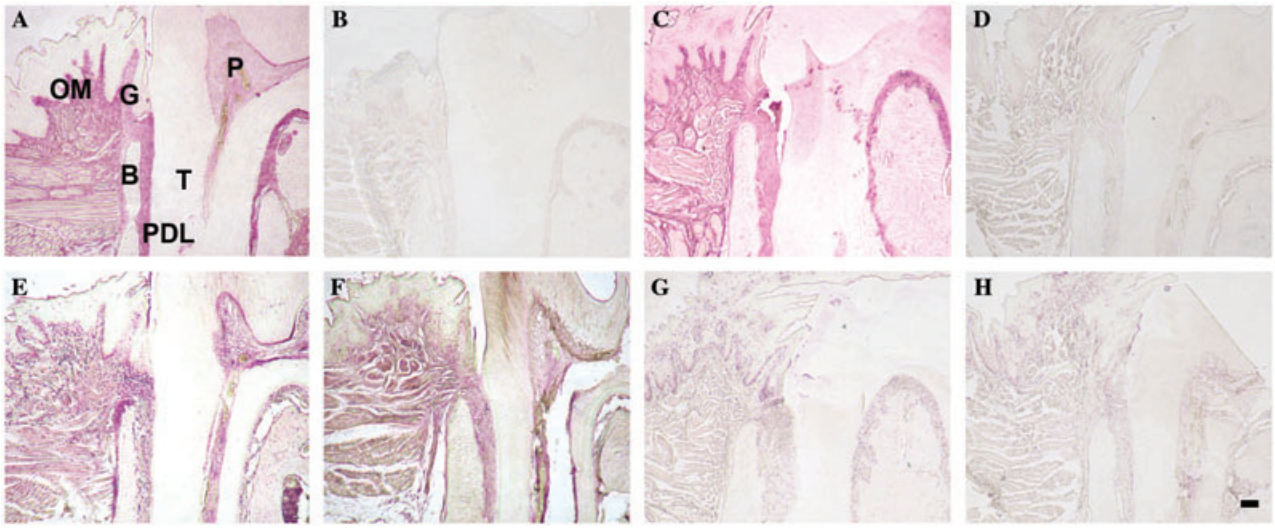

Fig. 4. Relative expression of fibromodulin and lumican in the knockout mice. Representative mandibular first molar sections immunostained using a standardized protocol with an antibody against lumican (A–D) and fibromodulin (E–H) are shown. A and E: wild-type mouse; B and F: lumican knockout mouse; C and G: fibromodulin knockout mouse; D and H: lumican and fibromodulin double knockout mouse. Lumican, fibromodulin, and lumican and fibromodulin knockout mice do not show any immunoreactivity for the corresponding knocked out proteoglycan/proteoglycans in the dental and periodontal tissues. OM: oral mucosa; G: gingiva; PDL: periodontal ligament; T: tooth; B: bone; P: pulp. Bar = 50 μm.

the other members of the SLRP family, four sets of mesio-distal sections (one per mouse group) of lumican, fibromodulin and double knockout and CD-1 wild-type mice were stained with anti-lumican, anti-fibromodulin (Fig. 4), anti-biglycan or anti-decorin antibodies using a standardized immunostaining protocol. The findings showed that there was no apparent compensatory up-regulation of any of the other SLRPs in the knockout mice (Fig. 4 and data not shown).

The small leucine-rich proteoglycan knockout mice have abnormal collagen fibrils and fibril bundles

In the periodontal tissues, SLRPs colocalized with collagen fibril bundles. Because SLRPs can regulate collagen fibrillogenesis we compared collagen fibril bundle morphology and organization in the periodontal ligament (Fig. 5) and in oral mucosa and gingiva (Fig. 6) in the knockout and wild-type mice.

The periodontal ligament of wild-type mice was filled with well-organized collagen fibril bundles with small, evenly distributed interbundle spaces (Figs 5A–D). The fibril bundles were homogenous in thickness and they were oriented parallel to each other and at a 45 degree angle to the root surface. Moreover, the fibril bundles could easily be traced from tooth to bone surface (Figs 5A and B). When examined by SEM, the fibril bundles showed smooth outlines and surface texture. The fibril bundles had a sheet-like appearance in the middle zone where they anastomosed with each other to form an intermediate plexus (Figs 5C and D). The individual collagen fibrils were of relatively uniform size and shape with even interfibrillar spacing and fibril outline in cross sections (Fig. 5D).

Lumican knockout mice expressed a periodontal ligament phenotype different from that of wild-type and other knockout mice (Figs 5E–H). In some occasions it was difficult to trace individual fibril bundles from tooth to bone surface (Fig. 5E). There was also an increase in the space between the fibril bundles, and the fibril bundles often were non-homogenous in thickness along the length of the bundles or compared to other bundles within the ligament (Figs 5E–G). Thin fibril bundles predominated throughout the ligament, giving the periodontal ligament fiber bundles a stringy appearance (Figs 5F and G). Characteristically, the fibril bundles showed very uneven outlines with numerous thin fibrils projecting out from the bundles (Fig. 5G). In TEM examination, individual collagen fibrils had irregular cross sections and showed heterogeneity in size. Overall the fibril diameters were smaller than in other mice. Interfibrillar spaces were in some locations almost absent, whereas in other locations they were increased as compared with wild-type mice (Fig. 5H).

The periodontal ligament of fibromodulin knockout mice displayed also collagen fibril bundles that were heterogeneous in thickness along the length of the bundles or among the different bundles in the ligament (Fig. 5I–K). However, in contrast to lumican knockout mice, there appeared to be a relative increase in the number of thick fibril bundles, giving the ligament fiber bundles a clumpy appearance. The fibril bundles had a poorly defined outline and often appeared blurry and when examined with SEM showed some disruptions (Figs 5J and K). Although the basic orientation of the fiber bundles was apparent, running at 45 degree angle from tooth to bone surface, it was difficult to consistently trace the fibril bundles from tooth to bone surface (Fig. 5I). Also, the spaces between the fibril bundles were not evenly distributed throughout the ligament, with narrow and wide spaces scattered throughout. Overall, similar to lumican knockout mice, there appeared to be more spacing between the fibril bundles than that found in wild-type mice (Figs 5I–K). In TEM examination, individual collagen fibrils displayed generally enlarged cross-sectional areas but there was also heterogeneity in the fibril diameter, with small fibrils dispersed between larger ones. The interfibrillar spaces were also variable and enlarged. These mice also showed uneven fibril outlines as compared with wild-type mice (Fig. 5L).

The lumican and fibromodulin double knockout mice expressed a periodontal ligament phenotype that contained some of the attributes of both single knockout mice (Figs 5M–P). The space between collagen fibril bundles was usually increased as compared to wild-type mice (Figs 5M–O). The collagen fibril bundles were heterogeneous in thickness and had an uneven surface texture (Figs 5N and O). Also, it was difficult to trace the fibril bundles from tooth to bone surface and the basic orientation of 45 degree angle from tooth to bone was not always obvious (Fig. 5M). The cross sections of individual collagen fibrils showed greatly abnormal fibril outlines, heterogeneity in the fibril diameters and increased interfibrillar spacing (Fig. 5P).

In the decorin knockout mice, the periodontal ligament collagen fibril bundles showed the typical 45 degree angle orientation seen in the wild-type mice but the fibril bundles were heterogeneous in size and showed increased spaces between the bundles (Fig. 5Q–S). Notably, the fibril bundles were wider as compared to other knockout animals and non-uniform in shape (Fig. 5S). Like in the other knockout animals, the fibril bundles displayed very thin filamentous structures branching out from the main bundles (Fig. 5S). These thin branches were not apparent in the wild-type mouse ligament fiber bundles (Fig. 5C). In TEM examination the fibrils were heterogeneous in size, with numerous small diameter fibrils among larger fibrils. The fibrils displayed also slightly more uneven outlines as compared with the wild-type animals. The interfibrillar spacing was more variable as compared with control mice (Fig. 5T).

The altered characteristics of the collagen fibrils (not shown) and fibril bundle morphology (Fig. 6) in the knockout mice were also apparent in the gingival and oral mucosal connective tissue. However, the changes in fibril bundles were not as obvious as in the periodontal ligament.

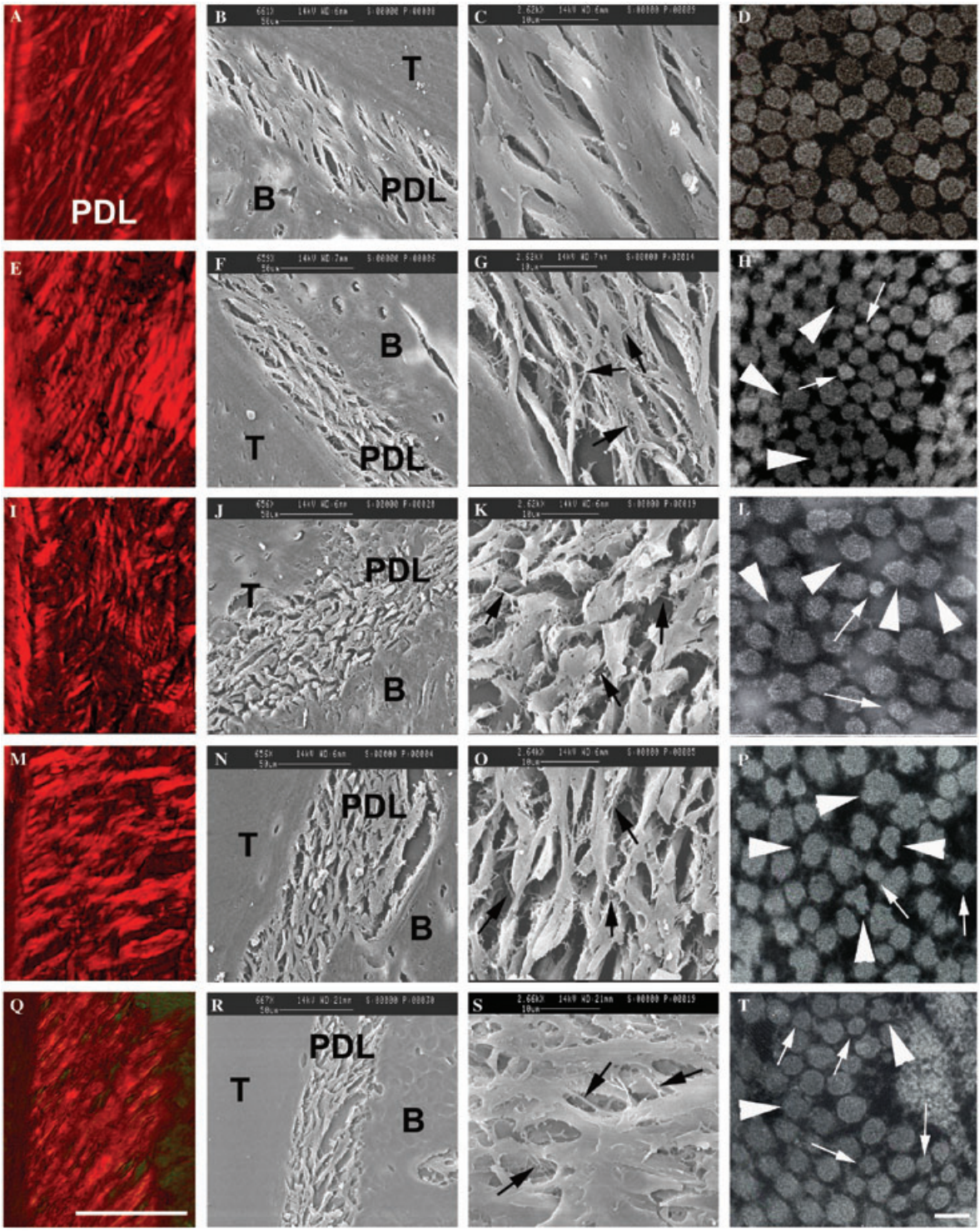

Fig. 5. Collagen fibril and fibril bundle organization and morphology in the periodontal ligament of proteoglycan knockout mice are altered. Representative samples from the coronal third of periodontal ligament from mandibular first molars from wild-type mice (A–D) and lumican (E–H), fibromodulin (I–L), lumican and fibromodulin (M–P) and decorin (Q–T) knockout mice are shown. A, E, I, M and Q: Sections stained with picrosirus red and examined under polarizing light microscope with 45 degree light angle. B, C, F, G, J, K, N, O, R and S: scanning electron microscopy (SEM) images of periodontal ligament collagen fibril bundles. D, H, L, P, and T: representative transmission electron microscopy (TEM) micrographs of individual periodontal collagen fibrils. T: tooth; PDL: periodontal ligament; B: bone. Bar in picrosirus red stained samples = 50 μm. Bar in TEM micrographs = 100 nm. Black arrows: fibrils projecting out of the collagen fibril bundles; white arrowheads: collagen fibrils with irregular outlines; white arrows: small diameter collagen fibrils.

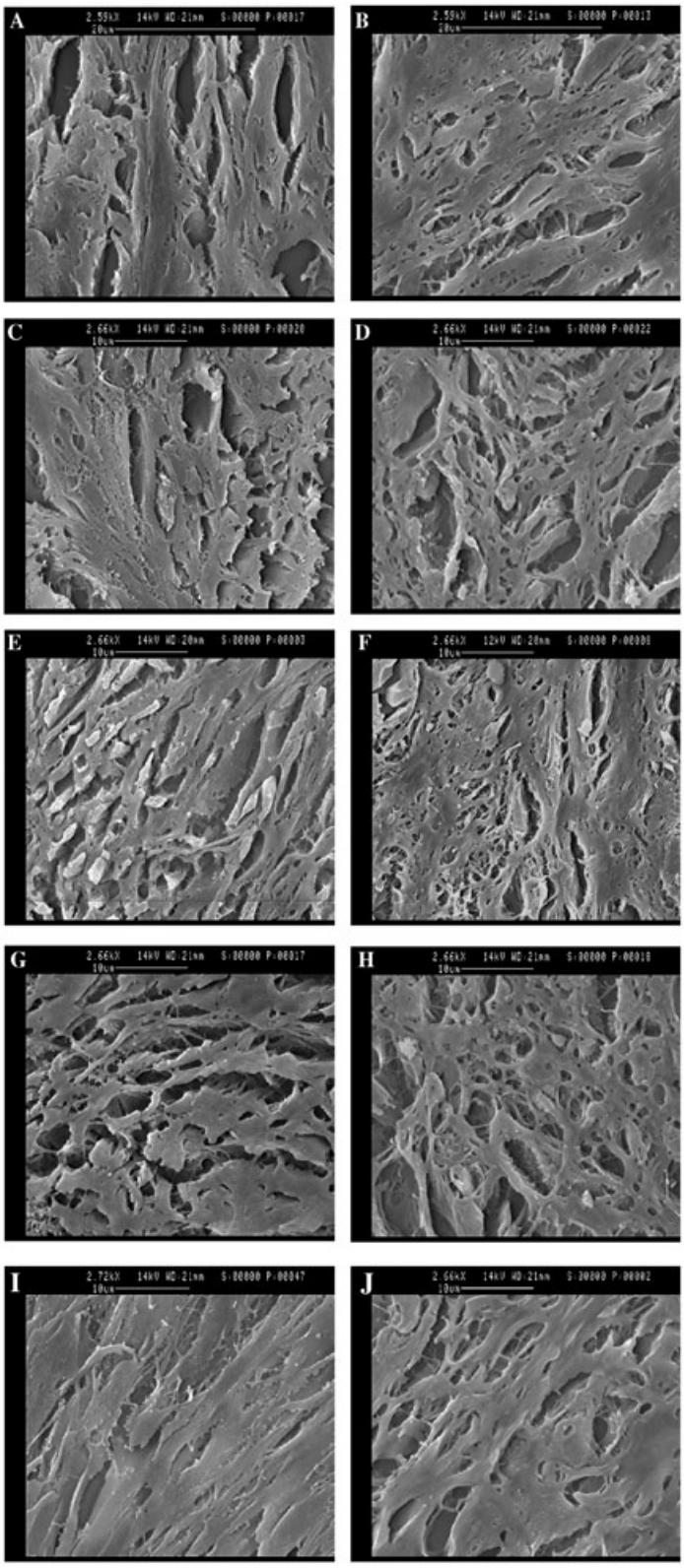

Fig. 6. Scanning electron microscopy (SEM) analysis of collagen fibril bundles in gingiva and oral mucosa. Representative samples from mandibular gingiva (A, C, E, G and I) and oral mucosa (B, D, F, H and J) from wild-type mice (A and B), and lumican (C and D), fibromodulin (E and F), lumican and fibromodulin (G and H) and decorin (I and J) knockout mice are shown.

Discussion

The present study showed for the first time that lumican, fibromodulin, decorin and biglycan are coexpressed in human and mouse periodontal tissues where they colocalized with collagen fibril bundles. Of the examined SLRPs, biglycan and fibromodulin showed equal expression level in periodontal ligament, gingiva and oral mucosa, whereas the staining intensity of decorin and lumican was lower in the periodontal ligament. This different relative expression pattern suggests that these molecules have tissue-specific functions. The SLRPs also showed relatively strong staining in predentin and precementum, suggesting that they regulate dentin and cementum formation. In fact, biglycan knockout mice displayed with abnormal dentin and enamel development (50). No apparent expression of these SLRPs was found in hard tissue matrix of dentin, cementum and bone, although in the human samples, the SLRPs localized on collagen fibril bundles that inserted from the periodontal ligament (Sharpey's fibers) into the acellular cementum. Our findings agree with previous studies showing lumican, fibromodulin, decorin and biglycan localization at the predentin in human or murine teeth (30, 50–52). Our findings also agree with the general SLRP expression pattern reported in bovine, human or rat cementum, showing no expression in cementum matrix but relatively strong expression on cementum surface (precementum) (53–56). Expression of these SLRPs in bone has been described previously (57–60). Interestingly, none of the four SLRPs localized to the acellullar cementum in the mouse samples, whereas human acellular cementum showed weak immunoreactivity. Therefore, these molecules appear to be differently expressed in human and mouse acellular cementum. However, we cannot rule out that different decalcification and processing methods of human (frozen samples decalcified with EDTA) and mouse (paraffin embedded samples decalcified using formic acid) samples may differentially affect access or interaction of antibodies with these molecules.

Previous studies have demonstrated several changes in the morphology, diameter and spacing of individual collagen fibrils in various tissues, including skin, tendon and bone in the SLRP deficient mice (25). In periodontal ligament, individual collagen fibrils are organized into groups that form collagen fibril bundles. In the present study, we compared for the first time, the role of SLRPs to regulate the morphology of these fibril bundles and individual collagen fibrils. Interestingly, all SLRP knockout mouse lines displayed abnormal but unique collagen fibrils and fibril bundles. In periodontal ligament, collagen fibril bundles showed a very distinctive organization as compared with the more random fibril bundle organization in gingiva and oral mucosa. Therefore, it is not surprising that collagen fibril bundle morphology was most clearly affected by the gene deletions in the periodontal ligament. A common property to all of the knockout mouse lines was that periodontal ligament displayed fibril bundles that were disorganized, varied in width and showed relatively increased amount of space between them. Periodontal ligament in lumican, fibromodulin, and lumican and fibromodulin knockout mice had relatively thinner bundles than wild-type mice, whereas in decorin knockout mice, the fibril bundles were more heterogeneous in size and shape. Moreover, there were several small fibril branches protruding from the main collagen fibril bundles present in all knockout groups. These branching structures were not present in wild-type mice. The filamentous structures may represent collagen fibrils that have broken away or failed to fuse with the rest of the bundle and are suggestive of altered lateral fusion of the fibrils within the fibril bundle. Interestingly, TEM findings showed altered fibril outlines and heterogeneity in size and spacing of the fibrils in all knockout animals. This suggests aberrant lateral fusion of individual collagen molecules. In particular, the lumican and fibromodulin double knockout animals showed strikingly irregular fibril cross sections. Thus, SLRPs regulate both assembly of individual collagen fibrils and their organization into collagen fibril bundles.

Lumican and fibromodulin share the same major binding site in the type I collagen molecule, with fibromodulin having a higher affinity, whereas decorin binds to a separate site (34–36). Decorin, lumican and fibromodulin seem to have different roles to regulate collagen fibril and fibril bundle assembly because the decorin, lumican, fibromodulin and the double knockout mice had a distinctive and different fibril and fibril bundle morphology. It appears that lumican and fibromodulin have differential expression during mouse tendon development, with lumican being expressed early and fibromodulin expression increasing with age (61). Therefore, lumican may be required for early fibril development, whereas fibromodulin is needed for maturation of collagen fibrils (61). This also suggests that lumican and fibromodulin cannot compensate for the function of each other if one of them is missing. This is supported by our findings showing uniquely altered collagen fibrils and fibril bundles in lumican and fibromodulin single knockout mice. In TEM examination, collagen fibrils were strikingly abnormal in the lumican and fibromodulin double knockout mice, whereas the single knockouts displayed milder but still obvious changes. This suggests that fibromodulin and lumican also collaborate to regulate the fibril assembly. Interestingly, previous studies have reported increased lumican expression in fibromodulin knockout mouse tendons using protein analysis (41, 42). On the other hand, fibromodulin was less expressed in the lumican knockout animals (42). We did not find any compensatory up-regulation of the expression of any of the other SLRPs in the knockout mice as determined by relative immunostaining intensity. The difference in our study to previous reports may be explained by different analysis methods or proteoglycan expression may be intrinsically different in periodontal tissues and tendons.

The turnover rate of the periodontal ligament is high and has been estimated to be 15 times that of skin and five times that of alveolar bone with respect to its collagenous components (11). Turnover and remodeling in the periodontal ligament involves continuous synthesis and removal of matrix components, most notably the meshwork of type I collagen (62). Therefore, the role of SLRPs may be accentuated in the periodontal ligament. Collagen removal that is important for fast turnover happens through endocytosis and phagocytosis by fibroblasts. This involves specific interactions of collagen with cell surface receptors (63–65). Undoubtedly, SLRPs regulate self-assembly of collagen molecules (25, 28). However, it is possible that the altered morphology of collagen fibril bundles in the knockout mice may, at least partially, result also from altered cell interactions with collagen that are important for cell-mediated collagen fibril organization and phagocytosis. It remains to be shown if SLRPs regulate also these processes.

In addition to documented structural changes in collagen, mice deficient in SLRPs exhibit some functional phenotypes. Biglycan knockout mice exhibit decreased growth rate characterized by decreased bone mass (66). However, they also have thin skin but no apparent functional abnormality (32). Decorin knockout mice exhibit increased skin fragility associated with thinning of the skin and abnormal collagen fibers (39). They also show decreased average fibril diameter and size range in bone compared to wild-type mice but no macroscopic phenotype as a result of this (32). These variable phenotypes might be explained by the different abundance of biglycan in bone relative to decorin and decorin in skin relative to biglycan. Similarly, lumican deficiency resulted in a macroscopic phenotype characterized by decreased transparency of the cornea and fragility of skin (40), the tissues where it is abundant. Mice deficient in fibromodulin were susceptible to ectopic tendon ossification and developed osteoarthritis (31). Lumican and fibromodulin double knockout mice were smaller than wild-type mice, had gait abnormality, joint laxity and age-dependent osteoarthritis (67). In the present study, despite of structural changes in collagen fibril

bundles in soft connective tissues, the mice did not show any apparent changes in bone or teeth. All teeth of the knockout mice were present and fully erupted. The apparent morphological changes in the periodontal ligament collagen fibril bundles were not associated with evident functional defects. Mice grew normally, indicating that they were able to eat normally. Additionally, there was no evidence of bone loss or inflammation that would indicate periodontitis. Periodontitis does not usually occur naturally in mice (68), although some studies have reported it in some wild mouse strains (43, 69). To this extent, it is not surprising that no alveolar bone loss indicative of periodontal disease was found. Perhaps if the animals are experimentally induced to develop periodontal disease, the significance of the lack of SLRPs for susceptibility to periodontal disease could be better answered.

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