Lumican and Fibromodulin in the Periodontal Ligament: A Study in Knockout Mice

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ABSTRACT

Periodontal disease is one of the most common diseases of mankind and may present in otherwise healthy individuals or as part of a medical condition or syndrome. The cause of periodontitis seems to be multifactorial involving both periodontal pathogens and host response. Periodontal disease involves breakdown of collagen fibers of the periodontal ligament, which manifests as increased probing depths and attachment loss around teeth. The integrity of the periodontal ligament, then, seems important in this disease process. Small leucine-rich proteoglycans have been located in the periodontal ligament. These are a family of molecules in the extracellular matrix that, among other things, play a role in collagen fibrillogenesis. This blinded, controlled study investigates the location of lumican and fibromodulin, two class II small leucine-rich proteoglycans, in oral periodontal tissues and their role in the collagen fibrillogenesis using adult mice that are singly or doubly deficient in these two small leucine-rich proteoglycans.

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Chapter One – A Review of the Literature

1.1 Introduction. Small Leucine-Rich Proteoglycans Of Dental Tissues: Do They Play A Role In Dental Disease?

Small leucine-rich proteoglycans have been localized in several dental tissues. Deficiency or absence of small leucine-rich proteoglycans may be implicated in diseases associated with dental manifestations such as osteogenesis imperfecta, infantile progeroid syndrome, Ehlers-Danlos syndrome and Marfan syndrome (Beavan et al, 1993; Fedarko et al, 1992; Fushimi et al, 1989; Straub et al, 2002). It is suspected that these proteoglycans offer much in the way of organization and strength to the periodontal ligament as well as influence the development and mineralization of hard dental tissues, including enamel. It seems plausible that a deficiency or absence of these seemingly inconspicuous molecules may allow for greater susceptibility to periodontal breakdown and perhaps altered mineralization or disease of the hard dental tissues.

Periodontal disease, for example, is a very ubiquitous disease in our culture affecting one third to one half of the American population (Albandar, 2002), with severe forms affecting relatively few (Brown & Loe, 1993). Periodontal disease has 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 (Hart et al, 2000), as with Papillon Lefevre syndrome. However, in most cases, the underlying reason for rapidly progressing disease is unknown. Undoubtedly, type and virulence of particular microbiota play an important role in these cases. However, recent advances in the understanding of biological functions of extracellular matrix molecules have provided novel information about their critical role in the development and maintenance of

periodontal tissues. Cell culture and in vivo studies using gene knockout animals (animals targeted for specific gene deletion) have suggested that changes in the expression of extracellular matrix molecules may contribute to increased susceptibility to periodontal disease (discussed later).

In recent years, there have been very few reviews of small leucine-rich proteoglycans of oral tissues (Rahemtulla, 1984). Much of the knowledge that has been gained thus far in this area has been based mainly on connective tissue research, particularly in cartilage (Embery, 2001). This paper reviews the small leucine-rich proteoglycans that have been localized in dental tissues thus far and discusses how they may influence the biology and pathology of these tissues. It should be noted that not all small leucine-rich proteoglycans have been investigated in oral tissues and their exclusion in this review does not indicate their absence.

1.2 Proteoglycans

Proteoglycans are a group of macromolecules that are found in the extracellular matrix and associated with cell membranes of virtually all connective tissues in the body. For simplicity, the extracellular matrix proteoglycans can be divided into three groups (Iozzo, 1998). The hyalectans are a family of proteoglycans that interact with hyaluronan and lectin and include aggrecan and versican. They form the gelatinous matrix in which collagen fibrils are embedded. The basement membrane proteoglycans, such as perlecan, form the second group, and the small leucine-rich proteoglycans, the focus of this paper, form the third group.

Structurally, proteoglycans contain a protein core that has O- or N- linked oligosaccharides and covalently attached glycosaminoglycan side chains that are separate gene products (Fig. 1). Functionally, proteoglycans act to regulate cell growth (Yamaguchi and Ruoslahti, 1988), cell adhesion (Lewandowska et al, 1987), collagen fibril formation (Vogel et al, 1984), extracellular matrix formation (Gallagher, 1989), maintain the transparency of the cornea (Chakravarti et al, 1998), the tensile strength of skin and tendon (Danielson et al, 1997; Chakravarti et al, 1998), the viscoelasticity of blood vessels, the compressive properties of cartilage, the mineralized matrix of bone (Iozzo, 2000), and bind, store and regulate the activity of growth factors and cytokines (Yamaguchi et al, 1990). Some of the functions of proteoglycans are mediated by its protein core, while others by the glycosaminoglycan chain(s) that are attached to it. Other functions seem to require interactions with both the protein core and attached glycosaminoglycans.

1.3 Glycosaminoglycans

Glycosaminoglycans are linear hetero-polysaccharides of repeating disaccharide units of N-acetyl hexosamine and hexuronic acid. They are attached to the proteoglycan core proteins by trisaccharide galactose – galactose – xylose that recognize serine – glycine residues and various amino acids in the core protein (Bourdon et al, 1987). Glycosaminglycans sometimes play an important role in determining the functional characteristics of proteoglycans.

Seven species of glycosaminoglycans have been identified: hyaluronan, dermatan sulfate, chondroitin 4-sulphate, chondroitin 6-sulphate, keratan sulphate, heparan sulphate, and heparin. Selective hexuronic acid residues within dermatan sulfate and heparan sulfate and selective sulphation of the hexosamine residue within chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin and keratan sulfate allow for greater diversification, leading to the production of glycosaminoglycan chains with specific properties. An exception to this rule is hyaluronic acid, which is neither sulphated nor covalently bound to a protein core in connective tissue. It is found to aggregate with large proteoglycan aggregates like aggrecan and versican and with the cell surface proteoglycan, CD44 (Embery, 2001).

The predominant glycosaminoglycans of the periodontal tissues are hyaluronic acid, heparan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate, keratan sulfate and dermatan sulfate (Table 2). The number (from 1-100), size and type of the glycosaminoglycan chains attached to the protein core can vary greatly depending on the cell/tissue origin. The glycosaminoglycan side chains mediate many functions of proteoglycans. For example, glycosaminoglycans can potently bind and regulate the function of growth factors, they mediate some of the interactions of proteoglycans with cells and the extracellular matrix and they are responsible in maintaining the appropriate conformation of the proteoglycan molecule (Zimmermann and Ruoslahti, 1989; Sheikh et al, 1998; Toole, 1982; Comper and Laurent, 1978).

1.4 Small Leucine-Rich Proteoglycans

Small leucine-rich proteoglycans comprise a group of proteoglycans (containing chondroitin / dermatan sulfate or keratan sulfate glycosaminoglycan side chains) and glycoproteins (containing N-linked oligosaccharides) in the extracellular matrix of hard and soft tissues. They are, like larger proteoglycans, comprised of a protein core and glycosaminoglycan side chains. The distinguishing feature of small leucine-rich proteoglycans, however, is the presence of a central domain containing leucine-rich repeats in the protein core (Fig. 2). This domain, which is responsible for most of the functional activity of these molecules, is flanked by smaller, less conserved, N-terminal and C-terminal cysteine clusters in characteristic positions (Iozzo, 1997). The structure of these molecules can be divided into four domains: domain I containing a signal peptide and a propeptide; domain II containing four evenly spaced cysteine residues and the glycosaminoglycan attachment site(s); domain III containing the leucine-rich repeats; domain IV containing two cysteine residues (Iozzo, 1998). Members of the small leucine-rich proteoglycan family differ in their numbers of leucine-rich repeats, amino acid substitutions and glycosylation (Iozzo, 1997) (Fig. 3).

Small leucine-rich proteoglycans are involved in collagen fibrillogenesis, modulation of growth factor activity, and regulation of cell growth (Iozzo & Murdoch, 1996). They were first discovered as small proteoglycans of bone and were subsequently found in the extracellular matrix of most tissues. The relative abundance of small leucine-rich proteoglycans in tissues varies during development and by location. This suggests a role in the differentiation and maintenance of tissue structure (Johnson et al, 1997).

Knowledge in the field of small leucine-rich proteoglycans is expanding rapidly. In 1996, Iozzo noted in a review article the existence of only 5 small leucine-rich proteoglycans. Today, just 7 years later, there are 15 distinct gene products in this group (Table 1). It is only with the advent of molecular biological and immunohistological techniques that they are being identified and their ultrastructural distribution and possible biological roles are beginning to be clarified (Embery et al, 2001). Small leucine-rich proteoglycans are divided into 3 classes based on their similarities in structure (Iozzo, 1997).

1.4.1 Class I Small Leucine-Rich Proteoglycans – This group of molecules contains decorin, biglycan (Fisher et al, 1989) and asporin (Henry et al, 2001). Decorin and biglycan are the most homologous (~57% identical based on human amino acid sequences) of all the small leucine-rich proteoglycans and are the only small leucine-rich proteoglycans to contain a pro-peptide (Krusius & Ruoslahti, 1986; Fisher et al, 1989), the function of which remains unclear (Neame & Kay, 2000). Members of this group contain an N-terminal domain that is usually substituted with one (decorin) or two (biglycan) chondroitin sulfate/ dermatan sulfate side chains. They contain 10 leucine-rich repeats flanked by cysteine clusters. The cysteine residues have a unique spacing pattern and intervening amino acids, both of which are maintained within each subfamily. For instance, the cysteine consensus sequence for class I small leucine-rich proteoglycans is CX_3CXCX_6C and is different from that of the other classes. The class I members are encoded by genes composed of 8 exons with intron and exon junctions in highly conserved positions (Henry, 2001).

1.4.2 Class II Small Leucine-Rich Proteoglycans – This group contains fibromodulin (Oldberg et al, 1989), lumican (Blochberger et al, 1992), PRELP (Bengtsson et al, 1995), keratocan (Corpuz et al, 1996) and osteoadherin (Sommarin et al, 1998). Members of this group may exist as proteoglycans or glycoproteins (with no glycosaminoglycan chains) (Neame & Kay, 2000). They have a common gene structure of 3 exons with cysteine spacing in the N-terminal region of CX_3CXCX_9C. The core proteins contain 10 leucine-rich repeats (except PRELP) and can be substituted with N-linked keratan sulfate glycosaminoglycan side chains (Henry, 2001). As a group, they have several amino acids in common and share a number of conserved features (Corpuz et al, 1996). On the amino acid level fibromodulin and lumican are 50% identical (Blochberger et al, 1992) while lumican, fibromodulin and keratocan are 35% identical (Corpuz et al, 1996). PRELP is 36% similar to fibromodulin and 33% similar to lumican (Bengtsson et al, 1995), while osteoadherin is 42% similar to bovine keratocan, 38% similar to bovine fibromodulin, lumican and human PRELP-1 (Sommarin et al, 1998). Lumican and keratocan have a characteristic unlike other small leucine-rich proteoglycans in that outside of the cornea, where they both carry keratan sulfate, they are poorly sulfated or unsulfated glycoproteins (Corpuz et al, 1996).

1.4.3 Class III Small Leucine-Rich Proteoglycans – This group contains epiphycan/PG-Lb (Shinomura & Kimata, 1992), osteoglycin/mimecan (Madisen et al, 1990; Funderburgh et al, 1997) and opticin (Reardon et al, 2000). They have a common gene structure composed of 7-8 exons and only 6 leucine-rich repeats with cysteine spacing in the N-terminus region of CX_2CXCX_6C. Osteoglycin/mimecan and epiphycan/PG-Lb can

be substituted with N-linked keratan sulfate glycosaminoglycan side chains and O-linked chondroitin sulfate / dermatan sulfate side chains respectively while opticin is substituted with O-linked sialylated oligosaccharides making it a glycoprotein rather than a proteoglycan (Henry, 2001).

Nyctalopin and chonroadherin are considered to be members of the small leucine-rich proteoglycan family of proteoglycans but have not been classified in any of the above three classes. Although they have the leucine-rich repeat that shows some homology with the other members, their structure is quite different from the conventional small leucine-rich proteoglycans perhaps resulting from early divergence during evolution of this group of molecules (Henry et al, 2001).

Table 1 summarizes all members of the small leucine-rich proteoglycan family known to date. All members are very similar in structure (Fig. 3), but are different at both the protein and gene levels (Neame and Kay, 2000).

1.5 Small Leucine-Rich Proteoglycans Of Oral Tissues

Several small leucine-rich proteoglycans have been localized or associated with animal and human oral tissues. The expression pattern of small leucine-rich proteoglycans seen during development of body organs in mice (Wilda et al, 2000), suggests that they play a role in development. Body organs develop through epitheliomesenchymal interactions. Teeth also form through the interaction of these two entities such as when the epithelium invaginates into the mesenchyme of the maxillary and mandibular processes (Ten Cate,

1994) and thus it might be imagined that small leucine-rich proteoglycans play a role in tooth development as well. It is not surprising, then, to find many small leucine-rich proteoglycans in oral and related tissues or associated with their development. The following discusses all the small leucine-rich proteoglycans that have thus far been localized in oral tissues. It should be noted, however, that not all small leucine-rich proteoglycans have been investigated in the oral cavity and omission of some small leucine-rich proteoglycans here is not meant to suggest their lack of existence in oral tissues.

1.5.1 Class I Small Leucine-Rich Proteoglycans In Oral Tissues

Decorin

Decorin (AKA: DS-PG-II; PG II; PG-S2; PG40) is the most studied member of the small leucine-rich proteoglycan family. First detected in cartilage and bone (Rosenberg et al, 1985; Fisher et al, 1983), it has since been found in several body tissues such as articular cartilage, intervertebral discs (Neame & Kay, 2000), tendon, skin, gingiva (Fisher et al, 1989; Hakkinen et al, 1993), sclera (Cöster and Fransson, 1981), mouse heart, kidney, small intestine, testes and liver (Henry, 2001). It was so named for its microscopic appearance on the collagen network, "decorating" the collagen fibers (Hocking, 1998).

The decorin gene is located on human chromosome 12q23 (Danielson et al, 1993) and distal end of mouse chromosome 10 (Scholzen et al, 1994), the same site as fibromodulin and lumican (Chakravarti e& Magnuson, 1995) suggesting a possible overlap in function of these small leucine-rich proteoglycans. The mature protein is highly conserved across

species as shown in comparison of human, murine, bovine and avian forms (Weber et al, 1996).

Structurally, decorin is composed of a 36.5 kDa core protein that contains 10 leucine-rich repeats with disulfide bond stabilized loops on either side. Decorin is secreted in pro-forms that are proteolytically modified in the extracellular matrix during maturation (Neame & Kay, 2000). The propeptide is short, consisting of 14 amino acids and its functional role, if any, remains unclear. Decorin usually carries one glycosaminoglycan chain (chondroitin sulfate or dermatan sulfate) which is found at the fourth amino acid position near the N-terminus, but it has been reported to have two glycosaminoglycan substitutions in chicken (Blaschke et al, 1996) or none at all as in the case of human cartilage (Johnstone et al, 1993). Chondroitin sulfate is the predominating glycosaminoglycan in the mineralized tissue (Fisher et al, 1989) while dermatan sulfate predominates in soft tissue (Bratt et al, 1992). Recently, a catabolically truncated form of decorin has been identified in human skin. This truncated form in called decorunt and contains only 43% of the molecule - that containing the amino terminus (Carrino et al, 2003).

Based on the known functions of decorin, it likely takes part in development and maintenance of soft and hard tissues, mineralization of hard tissues, wound healing and neovascularization. The best-documented function of decorin is its ability to influence collagen organization and fibril formation through collagen binding (Vogel et al, 1984; Bidanset et al, 1992; Pogany et al, 1994; Schonherr et al, 1995). Targeted deletion of

decorin in animals leads to reduced tensile strength of the skin due to the development of abnormal collagen fibrils (Danielson et al, 1997). Additionally, decorin can bind growth factors (Yamaguchi et al, 1990; Yamaguchi & Ruoslahti, 1988), ions, including calcium (Embery et al, 1998) and zinc (Yang et al, 1999), epidermal growth factor receptor (EGFR) (Patel et al, 1998), and extracellular matrix molecules including fibronectin (Schmidt et al, 1991; Winnemoller et al, 1992), thrombospondin (Winnemoller et al, 1992) and complement component C1q (Krumdiek et al, 1992).

Aside from its very important role in collagen fibrillogenesis and organization, which will be discussed in greater detail later, decorin has been referred to as the naturally occurring inhibitor of transforming growth factor – beta (TGF-β) (Ruoslahti & Yamaguchi, 1991). TGF-β is a cytokine whose many functions include control of cell proliferation, differentiation, adhesion and deposition of the extracellular matrix, processes that are very important in tissue growth and repair (Hocking et al, 1998). Decorin interacts with TGF-β (Yamaguchi et al, 1990) via its core protein (Hildebrand et al, 1994). When binding occurs, the activity of TGF-β is modulated. This modulation may be through the blocking of TGF-β receptors on cell surfaces (Hocking et al, 1998), the sequestration of TGF-β in the extracellular matrix (Hildebrand et al, 1994) or by decreased production of TGF-β through a negative feedback mechanism (Yamaguchi et al, 1990).

The role of decorin as a TGF-β inhibitor is substantiated by studies in rats. Administration of decorin into rats with experimental glomerulonephritis (a fibrotic

disease characterized by increase extracellular matrix by mesangial cells due to an overproduction of TGF-β as a result of injury) results in a reduction of pathological matrix accumulation (Border et al, 1992; Noble et al, 1992). Similar therapeutic findings were found using decorin gene therapy in rats (Isaka et al, 1996).

Other studies have found that decorin only has an inhibitory effect on certain functions of TGF-β (Hausser et al, 1994) and that decorin-binding to TGF-β actually increases TGF-β's ability to bind to receptors thereby increasing it function (Takeuchi et al, 1994). Hausser et al (1994) showed in vitro that decorin added to osteosarcoma cells inactivated TGF-β's function to up-regulate biglycan synthesis but the down regulation of proteoglycan 100 remained unaltered and did nothing to decrease TGF-β's function in monocyte cell proliferation. As such, the exact role decorin has with regards to TGF-β binding remains unclear.

Decorin has also been shown to bind to the epidermal growth factor receptor (EGFR) with concurrent activation of the mitogen-activated protein kinase-signaling pathway. Patel et al (1998) noted that activation of this receptor results in elevation of intracellular calcium ion concentration ([Ca++]) in single A431 squamous carcinoma cells. As well, it has been shown that decorin may act as a natural inhibitor of the EGFR signaling pathway (Csordas et al, 2000) resulting in growth suppression. However, these effects seem to be cell type specific. In periodontal fibroblasts, decorin suppresses cell growth by another mechanism (Hakkinen et al, 2000).

Decorin may also play a role in the mineralization process, the precise nature of which remains unclear, although evidence suggests that its role may be that of an inhibitor (Hoshi et al, 1999). It's glycosaminoglycan components have been shown to bind Ca++, interact with hydroxyapatite (Embery et al, 1998) and have been shown to be present in pre-mineralized tissues such as osteoid (Robey, 1996) and predentin (Takagi et al, 1990; Yoshiba et al, 1996). Immunolocalization studies of mineralized tissues show that decorin concentration decreases around collagen fibers where calcification has started (Hoshi et al, 1999). As well, decorin, like biglycan, decreases in proportion during the development of rabbit knee ligaments and has a precise location in these tissues suggesting a developmental role. After injury, decorin, together with biglycan, must be reproduced if complete healing is to occur (Kavanagh & Ashhurst, 2001).

Previous in vitro and in vivo studies have shown that decorin plays a role in angiogenesis. Angiogenesis is the formation of new capillaries from preexisting vessels through sprouting that occurs in a variety of physiological processes (embryonic development, ovarian follicle maturation and tissue repair) as well as pathological processes (diabetic retinopathy, tumor growth and metastasis, chronic inflammation) (Nelimarkka et al, 2001). In vitro studies have shown that large vessels do not express decorin in detectable amounts but when they begin to grow new capillaries, the endothelial cells synthesize decorin (Jarvelainen et al, 1992). As well, it has been shown that when macrovasular endothelial cells are transduced to overexpress decorin in vitro, they form tubes in the collagen lattices while control cells do not (Schonnher et al, 1999). The presence of decorin in human atherosclerotic plaques (Gutierrez et al, 1997) and

granulomatous tissues (Schonherr et al, 1999) suggest an in vivo role as well. Inflammation may play a role in this process since decorin does not appear to be expressed by "resting endothelial cells" (Bosse et al, 1993) but is detectable when inflammation is present (Nelimarkka et al, 2001). How decorin is involved in the angiogenesis process can only be speculated at present. Whether it is decorin's ability to help organize and stabilize the extracellular matrix through it's binding of collagen or its ability to interact with TGF-β or EGFR or one of its other many interactions remains to be elucidated. Studies of tumor growth, an angiogenic process, raise further questions regarding decorin's exact role in the angiogenesis process. Controlled animal studies show that decorin-expressing tumor xenografts grow much slower than wild-type counterparts and that this decreased growth may be due to a decreased expression of vascular endothelial growth factor (Grant et al, 2002). Thus, although decorin seemingly has an important role in angiogenesis and tumor growth suppression, its specific role remains unclear and may involve interaction of other matrix constituents or be tissue or cell specific.

Biglycan

Biglycan (AKA: DS-PG-I; PG-I; PS-S1) is a dermatan / chondroitin sulfate proteoglycan whose gene is located on the X chromosome (McBride et al, 1990). It is found in articular cartilage, intervertebral discs, skin, tendon, gingiva (Hakkinen et al, 1993), bone (Fisher et al, 1987), mouse heart, kidney, small intestine, testes, spleen, lung, liver (Wegrowski et al, 1995; Henry, 2001), collateral and cruciate ligaments and menisci of rabbit knee joint, endothelial cells and smooth muscle cells (Stöcker et al, 1991; Marcum & Thompson,

1991). Biglycan is found frequently associated with the cell surface and peri-cellular matrix (Neame & Kay, 2000) and often associated with specialized cells like skeletal myofibers, keratinocytes, and endothelial cells (Bianco et al, 1990).

Structurally, biglycan is secreted in pro-forms and then proteolytically modified in the extracellular matrix during maturation. The propeptide is short, consisting of 21 amino acids and it is not clear whether it serves any functional role. Cleavage takes place prior to the aspartate residue (...MMN-DEE...) while the surrounding sites show considerable conservation suggesting that a common enzyme is involved in processing of biglycan and decorin. The degree to which processing occurs is both age and tissue dependent with unprocessed pro-forms being particularly abundant in adult articular cartilage where they can represent 20% of the molecules present (Neame & Kay, 2000). Biglycan possesses a domain structure similar to that of decorin. It usually carries two glycosaminoglycan side chains (and so named biglycan (Fisher et al, 1989) attached to amino acid 5 and 11 within the protein core (Iozzo, 1997). As with decorin, chondroitin sulfate-containing biglycan would appear in mineralized tissue (Fisher et al, 1989) while the dermatan sulfate - containing type occurs in soft tissue (Bratt et al, 1992). In addition, non-glycanated forms of biglycan have been found in adult cartilage (Johnstone et al, 1993).

Functionally, biglycan appears to play a role in the mineralization process, although, like decorin, the precise nature of its role remains to be determined. It has been suggested that the role of decorin in the mineralization process is one of an inhibitor (Hoshi et al, 1999) while that of biglycan is one of a mineralization nucleator (Xu et al, 1998). It may do this

through its known ability to bind collagen (Pogany et al, 1994; Schonherr et al, 1995), or interact with soluble growth factors, like TGF-β, to modulate their functional activity (Iozzo & Murdoch, 1996) or hydroxyapatite (Bosky et al, 1997). Its glycosaminoglycan components have been shown to bind Ca++ and interact with hydroxyapatite (Embery et al 1998) and are present in pre-mineralized tissues such as osteoid (Robey 1996) and predentin (Takagi et al 1990; Yoshiba et al 1996). Biglycan is also suspected of being a repressor of amelogenin expression and enamel formation (Goldberg et al, 2002). Along with the above-mentioned interactions, bliglycan also binds to Cq1 and therefore may play a role in inflammation (Cox et al, 1970).

Like decorin, biglycan binds TGF-β but with slightly different mechanisms. Through it's binding, biglycan modulates the activity of TGF-β (Hildebrand et al, 1994). Although how this modulation is carried out is unclear, it may be through sequestration of the TGF-β in extracellular matrices (Hildebrand et al, 1994).

Xu et al (1998) showed in a study of biglycan knockout mice that those deficient in biglycan presented with an osteoporotic phenotype characterized by reduced growth rate and decreased bone mass (decreased amount and density of trabecular bone and decreased cortex thickness confirmed with high resolution radiographic and microradiographic techniques) that becomes more obvious with age. These findings were associated with a relative decrease in osteoblast:osteoclast ratio, attributing the reduction of bone mass to decreased formation of bone. Previous studies have shown that biglycan levels may be related to stature in humans. Patients with Turners syndrome (XO) have

short stature and have decreased levels of biglycan whereas patients with increased number of sex chromosomes have increased limb length and increased levels of biglycan (Vetter, 1993; Geerkens et al, 1995).

Periodontal Ligament Associated Protein-1 (PLAP-1)

PLAP-1 was discovered by Yamada et al (2001) in freshly isolated human periodontal ligament cells using expression profiling techniques. PLAP-1 mRNA expression was confirmed in vitro-maintained periodontal ligament cells. PLAP–1 was classified as a new member of the class I small leucine-rich proteoglycan family based on the alignment of its amino acid sequences. PLAP-1 is unique from other members of this group, however, in that it does not contain any glycosaminoglycan binding sites (making it not a real proteoglycan per se) and it has a characteristic stretch of 16 aspartate acid residues at the N-terminal that may contribute to its function, which is presently unknown.

1.5.2 Class II Small Leucine-Rich Proteoglycnas

Fibromodulin

Fibromodulin, a keratan sulfate proteoglycan, was first recognized as a 59-kDa glycoprotein in cartilage. It has since been located in cartilage, sclera, tendon (Plaas et al, 1990) and periodontal ligament (Watanabe et al, 1998) and is expressed by fibroblasts isolated from human periodontium (Hakkinen et al, 1996). Degradation products, although not found in normal tissues, are apparent in arthritic cartilage, with a greater extent in rheumatoid arthritis joints than in osteoarthritis (Neame & Kay, 2000). The fibromodulin gene is located on human chromosome 12q (Sztrolovics et al, 1994).

Structurally, fibromodulin appears in different forms that seem to be age and location dependent. The leucine-rich repeat region of fibromodulin has potential glycosaminoglycan binding sites allowing it to appear as a proteoglycan in immature cartilage. However, fibromodulin may also appear as a glycoprotein, in its unsulfated form, such as in the case of mature cartilage (Roughly et al, 1996). For this reason, fibromodulin has been described as a "part-time" proteoglycan. The molecule can contain up to 4 karatan sulfate chains (Plaas et al, 1990) and its structure bears much resemblance to lumican (Blochberger et al, 1992), another member of this group. Fibromodulin form is location-dependent as well. In tracheal cartilage, for instance, the glycosaminoglycan chains are 5-7 disaccharides long whereas in articular cartilage, they are 8-9 disaccharides in length (Lauder et al, 1996).

The function of fibromodulin is suspect but revolves around its collagen-binding capacity implicating it in collagen fibril formation (Blochberger et al 1992). Fibromodulin has been found to inhibit collagen fibrillogenesis (Hedbom & Heinegard, 1989) and has been shown to bind to collagen types I and II (Hedbom & Heinegard, 1993). Aside from its role in collagen binding and collagen fibrillogenesis, fibromodulin appears to play a role in cell migration (Wilda et al, 2000) perhaps via its an association with fibronectin. As well, fibromodulin has been shown to bind TGF-β via its protein core. In fact, this binding capacity appears to be stronger than the binding affinity between decorin or biglycan and TGF-β (Hidebrand et al, 1994).

Lumican

Lumican is a keratan sulfate proteoglycan (Iozzo, 1997) whose gene, like that of decorin, is found on the distal end of mouse chromosome 10 (Chakravarti & Magnusson, 1995) and on human chromosome 12q21.3-22 (Chakravarti et al, 1995). Aside from bovine cornea, where it was first localized (Axelsson et al, 1978), lumican is located in corneal stroma of chicken (Blochberger et al, 1992), heart valves (Funderburgh et al 1986), skin, skeletal muscle, intervertebral discs (Chakravarti & Magnuson, 1995), cartilage (Grover et al, 1995; Knudson & Knudson, 2001), cementum (Cheng et al 1996), predentin and dentin (Hall et al, 1997), bone (Raouf et al, 2002) but is scarce in or absent in brain, liver and spleen (Funderburgh et al, 1997).

Structurally, the amino acid terminals of lumican may be sulfated (Oldberg et al, 1989) and the leucine-rich repeat region possesses potential glycosaminoglycan binding sites. The molecule is very similar in sequence homology in bovine, human, chick and mouse (Funderburgh et al, 1995; Grover et al, 1995; Funderburgh et al, 1993; Blochberger et al, 1992; Ying et al, 1997). The presence of the sulfated form in the cornea implies that in this tissue it has specialized function while its role in other tissues seems elusive (Ying et al, 1997). Lumican exists as a small leucine-rich proteoglycan in the cornea but may also appear as a glycoprotein in other tissues (Grover et al, 1995).

Lumican plays an important role in the maintenance of corneal transparency, which is where it received its name (Blochberger et al, 1992). During mouse development, lumican appears at day 7 and subsequently appears in many organ systems with highest

concentration in the heart and eyes (Oldberg et al, 1989). Initially in the eye, the lumican is unsulfated. The appearance of sulfated forms of lumican in the eye is correlated with the development of corneal transparency at day 15 (Funderburgh et al, 1986). Studies in mice that are missing lumican show that the mice lose their corneal transparency at 10 weeks indicating that lumican is essential for the maintenance but not initiation of corneal transparency (Chakravarti et al, 1998). It is believed that lumican, like fibromodulin and decorin, acts via its collagen-binding capacity and its ability to control the collagen fibril diameters and interfibrillar spacing between them (Blochberger et al, 1992). In the cornea, the protein core of lumican acts to limit collagen fibril diameter while its glycosaminoglycan side chains act in the regular spacing of fibrils and the acquisition of corneal transparency (Funderburgh et al, 1986; Cornuet et al, 1994; Scott, 1991). Lumican also plays a role in corneal hydration due to its negatively charged carbohydrate components (Rawe et al, 1992; Bettelheim et al, 1975; Funderburgh et al, 1991).

In addition, lumican appears to play a role in the acquisition or maintenance of skin tensile strength. Lumican knockout mice (homozygous mutation) show an 86% decrease in tensile strength of skin, a finding that is correlated with irregular collagen fibril morphology and diameter, much the same as that seen in decorin knockout mice (Chakravarti et al, 1998).

Osteoadherin/Osteomodulin

Osteoadherin was first isolated from mineralized matrix of bovine bone (Wendel et al, 1998). Called osteoadherin in bovine tissue and osteomodulin in human and rat tissues, it

has actually been found to represent the same protein in different species (Buchaille et al, 2000). Since its discovery, osteoadherin has been found via in-situ hybridization studies in human and rat odontoblasts, in the ameloblastic layer of developing rat teeth and expressed by cells of alveolar bone surrounding the teeth (Buchaille et al, 2000). This finding was consistent with the finding of osteoadherin in bovine bone through in vitro hybridization (Sommarin et al, 1998).

Although structurally similar to fibromodulin, lumican, keratocan, and PRELP, osteoadherin is not closely related to them. It has a very acidic C-terminal peptide extension that clearly distinguishes it from the other mentioned proteoglycans above which may act to anchor the proteoglycan to the hydroxyapatite mineral in bone (Sommarin et al, 1998).

Although osteoadherin appears to be rather specific for bone, its function remains unclear. As previously stated, it is an acidic small leucine-rich proteoglycan that binds to hydroxyapatite and may play a role in bone mineralization (Sommarin et al, 1998). As well it appears to be involved in cell-matrix adhesion and binds to osteoblast integrin $\alpha_v\beta_3$ (Wendel et al, 1998).

1.5.3 Localization Of Small Leucine-Rich Proteoglycans In Soft Oral Tissues

Table 3 lists the components of soft dental tissues. Inspection of this table shows that each tissue contains cellular and acellular elements. The most predominant member of the acellular elements is collagen. Various collagen types exist in the various dental

tissues (Table 4). The type of collagen ultimately determines the nature of the tissue. Along with collagen, one finds various other components, notably proteoglycans.

Of the proteoglycans, a few small leucine-rich proteoglycans have been associated with oral tissues. Limited research exists in the field of small leucine-rich proteoglycans in oral tissues, but with advances in laboratory techniques, one predicts that several more will come to light. The following section discusses the small leucine-rich proteoglycans that have been found to be associated with soft dental tissues or their development to date.

Lining Mucosa and Gingiva

The gingiva is the soft tissue that covers the alveolar process and surrounds the necks of the teeth and hard palate while the lining mucosa is the soft tissue lining of the remainder of the oral cavity (except the tongue). While both the gingiva and lining mucosa are comprised of a top layer of epithelium and an underlying layer of connective tissue, they are unique oral tissues with different functions.

The gingiva can be divided into marginal, attached and interdental areas. The marginal gingiva is the unattached collar of tissue around the teeth and has an outer (oral) surface (facing the oral cavity), an inner (sulcular) surface (facing the tooth) and a joining (junctional) surface attached to the tooth. The attached gingiva is continuous with the marginal gingiva and is bound tightly to the underlying alveolar bone. Gingiva is comprised of a surface layer of stratified squamous epithelium that tends to be keratinized in the oral extent and non-keratinized in the sulcular and junctional areas.

While many cell types are found in the epithelium, the main cell type is the keratinocyte. Four cell layers (stratum) can be identified (basale, spinosum, granulosum, corneum) in keratinized epithelium, much the same as in skin, while nonkeratinized epithelium presents with slightly different cell layers superficially (basale, spinosum, intermedium and superficiale) (Ten Cate, 1994).

Immediately under the epithelium is the basal lamina, sometimes referred to as the basement mambrane. The basal lamina consists of two zones of relatively equal thickness (45-50nm), the lamina lucida and the lamina densa. The lamina lucida is closest to the epithelium and appears as a structureless band in H&E-stained sections but there are slight condensations of material present opposite the hemidesmosomes on the epithelial cell membrane called anchoring filaments as well as glycoproteins including bullous pemphigoid antigen. The lamina densa is between the lamina lucida and underlying connective tissue. The lamina densa consists of type IV collagen in a chicken wire configuration, laminin, entactin, and proteoglycan rich in heparin sulfate. The lamina densa has anchoring fibrils inserting into it made of type VII collagen which extend into the underlying connective tissue through which type I and III collagen run on the connective tissue side, anchoring the epithelim to the connective tissue. Fibronectin and type V collagen are sometimes associated with the connective tissue surface of the basal lamina. The primary role of the basal lamina is to provide physical support to the epithelium. It also provides a surface for cell attachment and around endothelium, provides a filtration role (Ten Cate, 1994).

Underlying the epithelium is a layer of connective tissue or lamina propria. The lamina propria can be divided into a more superficial papillary layer that is closest to the epithelium and a deeper reticular layer (so named for the netlike arrangement of the collagen fibers here) that is contiguous with the periosteum of the alveolar bone. The difference between these layers is poorly defined but is based on relative concentration and arrangement of the collagen fibers. In the papillary layer the connective tissue fibers are thin and loosely arranged and many capillary loops are present. The reticular layer, on the other hand, has thick collagen fiber bundles that lie parallel to the surface plane. The lamina propria is comprised of cells, blood vessels, neural elements, and fibers in an amorphous ground substance. The main cell type of the lamina propria is the fibroblast while the main extracellular matrix component is collagen type I (Ten Cate, 1994).

Type I collagen fibers, which account for approximately 60% of the gingival protein, although ultimately blended, can be divided into groups based on their location and orientation. They are named dentogingival, alveologingival, circular, periostogingival, transeptal group, transgingival, interpapillary, intercircular, and intergingival fibers (Fig. 4). These fiber groups act to attach the gingiva to the tooth and give rigidity and structure to the gingival margin to withstand forces of mastication. Other important components of the connective tissue layer include non-cellular elements such as: collagen type IV and V oxytalin fibers, eluanin fibers, elastin fibers, glycoproteins, glycosaminoglycans, proteoglycans, and cellular elements (8% by volume) like: fibroblasts (65% of the cellular component) and blood cells including inflammatory cells. A more extensive

review on the histology of the mucosa can be found in Ten Cate (1994), Itoiz & Carranza (1996) and Holmstrup(1996).

Small leucine-rich proteoglycans were first noted in gingival tissues by Pearson & Pringle (1986). Their work in bovine gingiva using chromatography and immunochemistry techniques determined that there were small and large proteoglycans in gingival tissues. The small one was a dermatan sulfate proteoglycan that later was determined to be decorin (Rahemtulla, 1992).

Later, research localized decorin in human gingival connective tissues. Hakkinen et al (1993) showed using immunohistochemical techniques to detect the protein core of decorin that it was found throughout the human gingival connective tissue. Decorin staining was more intense in the papillary regions of the connective tissue than the reticular regions and it was found to be associated with collagen fiber bundles. This finding is similar to those found in human dermis (Bianco et al, 1990; Voss et al, 1986; Fleischmajer et al, 1991) and is supported by studies showing that papillary fibroblasts secrete more decorin than reticular fibroblasts in vitro (Schonherr et al, 1993) and in vivo (Schonherr et al, 1995). The increased intensity of the decorin immunoreactivity in the subepithelial regions of the connective tissue is not surprising if one considers that decorin has been shown to bind to collagen types I-VI in vitro with the highest affinity for collagen type VI (Bidanset et al, 1992) which is highly concentrated in this subepithelial area (Chavrier et al, 1984; Becker et al, 1986; Rabanus et al, 1991). The localization of decorin and its ability to regulate collagen fiber assembly (Vogel et al,

1984) in other tissues, suggests that the decorin plays a similar role in gingival tissues. Decorin was also shown to stain intensely in the basement membrane zone, again similar to dermis (Voss et al, 1986) suggesting a role for decorin at this interface (Hakkinen et al, 1993).

Hakkinen et al (1993) showed in the same study that biglycan is weakly expressed in adult human gingival tissues showing some accumulation in the connective tissues under the epithelial rete ridges where it was localized as fine filaments on extracellular matrix fibers suggesting a role of biglycan in collagen fibrillogenesis. In fact, biglycan can potently interact with type VI collagen that in turn interacts with type I collagen (Wiberg et al, 2002). Although biglycan is found in developing epithelium of human dermis (Bianco et al, 1990) and in gastric epithlium (along with decorin) (Pohle et al, 2001), it was not (nor was decorin) localized in the epithelium of adult human gingival epithelium (Hakkinen et al, 1993). The role of biglycan in human periodontal tissues is not clear at present.

Periodontal Ligament

The periodontal ligament is that soft connective tissue found occupying the space between the cementum of the tooth roots and the alveolar socket wall. While its most obvious role is to attach the teeth to bone, it has several other functions that vary from physical to formative to nutritional and sensory. Physical functions of the periodontal ligament include transmission of occlusal forces to the surrounding bone, protection to the blood vessels and nerves within it from masticatory forces, formative functions

include normal remodeling of bone and cementum and regeneration, while it also plays roles in providing nutrition and lymphatic drainage to surrounding tissues and sensation in the way of proprioception, pain and pressure (Carranza & Ubios, 1996).

The periodontal ligament is comprised of cellular components, an extracellular matrix, blood vessels and nerves. The different cell types found in the periodontal ligament include fibroblasts, epithelial cells, undifferentiated mesenchymal cells, endothelial cells, cells associated with the sensory system, bone associated cells and cementoblasts. The predominant cell type is the fibroblast, which in rodent molars makes up approximately 35% of the periodontal ligament volume (excluding blood vessels) (Beertsen, 1975) and in sheep incisors comprises approximately 20% (Berkovitz & Shore, 1995) of the periodontal ligament volume. It is estimated that an adult human premolar would have a comparable cell density of 25% in the periodontal ligament (Beertsen et al, 1997). Fibroblasts are spindle-shaped and are oriented with their long axes parallel to the principle collagen fiber bundles. Their potential role includes the synthesis and degradation of collagen (Holmstrup, 1996) as well as the formation of other extracellular matrix components.

The extracellular matrix of the periodontal ligament is comprised of collagen fibers, elastic fibers (oxytalan and eluanin) and ground substance (Ten Cate, 1994). Type I collagen, which forms approximately 80% of the periodontal ligament collagen (Holmstrup, 1996) and type III collagen are the two most common types of collagen found in the periodontal ligament (Huang et al, 1991; Wang et al, 1980) in an

approximate 4:1 ratio (Butler et al, 1975) and they form banded fibrils. Collagen V is also found in minor amounts and is associated with collagen types I an III (Linsenmayer et al, 1983) found either buried within the fibril cores (Birk et al, 1988) or between the fibril bundle spaces (Becker et al, 1991). Several other minor collagens are found in the periodontal ligament including VI and XII (Becker et al, 1991; Bronckers et al, 1986; Dublet et al, 1988; Karimbux et al, 1992; Sloan et al, 1993).

The collagen fibers of the periodontal ligament, like those of the gingiva, are arranged in collagen fiber bundles and denoted the *principle fiber groups* (Fig. 5). These principle fibers occupy much of the space between the tooth root and alveolar bone and are inserted into the tooth as Sharpes' fibers. The principle fibers are wavy in their course and as such are able to absorb applied forces. The fibers can be divided into groups including alveolar crestal, oblique, transeptal, horizontal, interradicular, and apical groups (Holmstrup, 1996).

Among the principle fiber groups one finds loose connective tissue in which cells, fibers (secondary, reticular, elastic), vessels and nerves are embedded. Other proteins of the extracellular matrix include proteoglycans (Hakkinen et al, 1993; Embery et al, 1995) and glycoproteins (Zhang et al, 1993).

Among the proteoglycans found in the periodontal ligament, are the small leucine-rich proteoglycans. Few investigators have attempted to identify small leucine-rich proteoglycans in the periodontal ligament. Early research mainly focused on

glycosaminoglycan components of connective tissues with the most common glycosaminoglycans found in periodontal ligament being hyaluronan, chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate (Munemoto et al, 1970). The small leucine-rich proteoglycans that have been isolated thus far in the periodontal ligament include decorin, biglycan, fibromodulin, lumican and PLAP-1, although not all have been investigated.

Decorin was first localized in the periodontal ligament by Pearson and Gibson (1982) and later by Pearson & Pringle (1986) who in animal studies noted that decorin of the periodontal ligament is immunologically related to that of skin, pulp and gingiva. Human and animal studies using immunolocalization techniques determined that decorin is associated with periodontal ligament collagen fibers both in the periodontal ligament proper (Hakkinen et al, 1993; Cheng et al, 1999) and the periodontal ligament fibers (Sharpey fibers) that insert into cementum (Ababneh et al, 1999). The function of decorin in the periodontal ligament was investigated using a murine model where decorin knockout mice were compared to wild-type control mice. It was shown that mice deficient in decorin have increased numbers of fibroblasts and altered collagen fibrils characterized by heterogenous shape and irregular profiles (Hakkinen et al, 2000). Thus it appears that decorin plays a role in periodontal ligament collagen fibrillogenesis.

The other small leucine-rich proteoglycans mentioned above have been localized in the periodontal ligament but their functions have yet to be determined. Biglycan was localized in human periodontal ligament using immunolocalization techniques (Hakkinen

et al, 1996; Abebneh et al, 1999) but was found to be less abundant than decorin in this tissue (Hakkinen et al, 1996). Fibromodulin was first localized in the periodontal ligament in animal studies using ion-exchange chromatography and electrophoresis as well as immunostaining (Watanabe et al, 1998) confirming previous in vitro reports that showed fibroblasts of the periodontium express fibromodulin (Hakkinen et al, 1996). As well, lumican was localized in the human periodontal ligament using standard indirect immunoperoxidase techniques and it was found to be associated with the collagen fibers inserting into cementum (Ababneh et al, 1999). Another small leucine-rich proteoglycan that appears to be unique to the periodontal ligament was isolated in human tissue using gene-profiling techniques. This small leucine-rich proteoglycan, named periodontal ligament associated protein –1 (PLAP-1) was found to be structurally similar to the class I small leucine-rich proteoglycans and appears to play a role in mineralization through its role in cytodifferentiation processes (Yamada et al, 2001).

Pulp

The pulp is the soft connective tissue layer found in the center of the tooth which can be divided into four distinct zones at the histological level: the odontoblastic zone at the periphery, a cell free zone (the basal layer of Weil) under the odontoblastic zone, a cell-rich zone containing mainly fibroblasts, and the pulp core that containing the major blood vessels and nerves of the pulp (Torneck, 1994). The cells of the pulp include odontoblasts, fibroblasts, undifferentiated mesenchymal cells, as well as immunocompetent cells, while collagen and ground substance are found extracellularly. The collagen component of the pulp is concentrated greatly in the apical third and is

comprised mainly of types I and III in an approximate ratio of 55:45. The ground substance of the pulp is comprised of glycosaminoglycans, glycoproteins, water (Torneck, 1994) and proteoglycans. A more extensive review on the histology of the pulp can be found in Torneck, 1994.

Hall et al (1997) found using immunohistochemical techniques that lumican was weakly expressed throughout the pulp matrix and around odontoblasts in a fibrillar pattern. Later, Buchaille et al (2000) using in situ hybridization techniques, showed that osteoadherin (osteomodulin) mRNA is located in the pulp of developing rat teeth, more highly concentrated in the odontoblastic layer within it. Osteoadherin was also found in human odontoblasts lining the edge of the pulp chamber in partially developed human molars. It is believed from these studies that osteomodulin and lumican play a role in mineralization of hard dental tissues.

1.5.4 Localization Of Small Leucine-Rich Proteoglycans In Hard Oral Tissues

The role of proteoglycans in mineralized tissues has been underestimated in the past and it is only recently with the advent of new laboratory techniques and the use of genetically engineered mice that the importance of these molecules in mineralized tissues is being elucidated. Perhaps the reason for the underestimation in the past is due to the apparent negligible quantity of these molecules in the mineralized tissues (Jones and Leaver, 1972) and that *in vitro* studies showed that the amount of glycosaminoglycans decreases as mineralization progresses (Baylink et al, 1972). In vitro, proteoglycans have been shown to have an inhibitory effect on hydoxyapatite formation and growth. However, since then

it has been shown that *in solution*, proteoglycans *inhibit* mineral nucleation and growth whereas *on a surface*, the same molecules *promote* mineral formation (Linde et al, 1989). Table 5 lists components of dental hard tissues. A discussion of small leucine-rich proteoglycans in these tissues follows.

Dentin / Predentin

Dentin is the major mineralized tissue of teeth and is closely related to bone in both chemical composition and mineralization process. There are several types of dentin. The bulk of dentin in the mature tooth is primary dentin. It outlines the pulp chamber and is characterized by multiple closely packed dentinal tubules that traverse its entire thickness. Dentinal tubules contain the cytoplasmic processes of the odontoblast cells that line the pulp space. Mantle dentin is the first dentin deposited by odontoblasts during tooth development and is found lining the outermost portion of the primary dentin of mature teeth. Secondary dentin, which is less organized than primary dentin, develops upon the completion of root development. It is deposited at a slow rate by odontoblast cells lining the pulp chamber and results in a decreased pulp space. Tertiary dentin, also called reparative dentin, is produced in very localized areas in response to noxious stimuli. Its structure can be regular or irregular in nature depending on the nature and intensity of the stimulus. Tertiary dentin is comprised of collagen types I and III.

Mature dentin is made up of 70% inorganic material, 20% organic material and 10% water by weight (45%, 33% & 22% by volume). Table 5 shows that the inorganic component is comprised of hydroxyapatite while the organic portion is made up of

collagen (mainly type I), glycoproteins, proteoglycans, phosphoproteins, and plasma proteins. Approximately 56% of the mineralized phase is within the collagen. A more extensive review of the histology of dentin can be found in Torneck, 1994.

It has been suggested that there are two distinct groups of proteoglycans in dentin. The first one is in predentin where glycosaminoglycans function to move collagen toward the mineralization front and where fibrils are packed and undergo mineralization. It is here that the small leucine-rich proteoglycans perhaps aid in fibrillogenesis, linking subunits and orienting collagen fibrils (Scott, 1996). The second group is in the dentin proper where glycosaminoglycans are secreted distally by odontoblastic processes within dentinal tubules at some distance from the dentin/predentin junction (Scott, 1996).

In the 1970's and 80's, several glycosaminoglycans have been found to be associated with dentin and predentin including heparan sulfate, dermatan sulfate, chondroitin 6-sulfate, (Goldberg & Septier, 1986; Tenorio et al, 1990; Tagaki et al, 1990; Goldberg and Tagaki, 1993) chondroitin 4-sulfate (Embery, 1974; Smalley & Embery, 1980; Rahemtulla et al, 1984), hyaluronic acid (Clarke et al, 1965) and keratan sulfate (Linde, 1973). Only recently have studies been able to identify the actual proteoglycan to which these glycosaminoglycans probably belong.

Lumican was first discovered in human predentin and dentin by Hall et al (1997) using immunohistochemical procedures. Lumican was located in predentin and, to a lesser extent, dentin. It was suggested that lumican might play a role in the mineralization

process of dentin. Linde's finding of keratan sulfate in dentin supports these findings (Linde et al, 1973).

Embery et al (2001) found decorin and biglycan to be the most abundant small leucine-rich proteoglycans in dentin with lumican and fibromodulin found in lesser amounts. In this study, immunohistological techniques and electron microscopy were used to show that antibodies recognizing chondroitin sulfate showed a decreasing gradient from pulp to mineralization front of dentin with an opposite gradient occurring for keratan sulfate family of small leucine-rich proteoglycans. Anti-decorin antibodies showed an increasing gradient toward the mineralization front. It has been suggested that these proteoglycans act to organize the collagen network to receive phosphoproteins and phospholipids as well as the possibility of the spatially oriented glycosaminoglycan of decorin and biglycan binding Ca++ and directing mineralization initiation.

Studies have been carried out on mice that have been genetically engineered to be deficient in genes that code for one or two small leucine-rich proteoglycans. These studies suggest functional roles of small leucine-rich proteoglycans. Studies comparing biglycan knockout and wild-type mice show that collagen fibrils in predentin are decreased in the proximal third but increased in the central and distal third of predentin in the knockout mice compared to wild type mice. This, together with the knowledge that small leucine-rich proteoglycans control collagen fibril diameter and assembly, confirms the role of biglycan as a regulator of extracellular matrix organization (Goldberg et al, 2002).

Cementum

Cementum is a mineralized tissue that covers the roots of teeth which facilitates attachment of periodontal ligament collagen fibers to the tooth. Cementum can be divided into two types: acellular extrinsic fiber cementum and cellular mixed stratified cementum. The latter is composed of 3 intermingling types of tissues: cellular intrinsic fiber cementum, acellular intrinsic fiber cementum and acellular extrinsic fiber cementum. Cementum consists of mineralized organic matrix, the primary constituent of which is type I collagen with types III, IV and VI collagen present in minor amounts. As well, a number of non-collagenous proteins have been identified within cementum such as acidic glycoproteins, growth factors, attachment proteins and proteoglycans (Ababneh et al, 1999).

Early biochemical research by Bartold (1988) showed that the major glycosaminoglycan in human cementum was chondroitin sulfate while hyaluronic acid and dermatan sulfate were present in small amounts. Later, immunohistochemical research localized the chondroitin sulfate proteoglycan to the pericellular environment and the extracellular matrix of cementum (Bartold et al, 1990). As well, Rahemtulla et al (1984) noted the presence of two groups of proteoglycans in hard tooth tissue (dentin and cementum) consisting if chondroitin 4-sulfate and chondroitin 6-sulfate. No mention was made in these studies regarding the core proteins of the proteoglycans or the sulfation of the molecules. These proteoglycans were later identified as decorin and biglycan (Cheng et al, 1999).

Cheng et al (1996) showed using immunohistological techniques that fibromodulin and lumican core proteins are localized in cementum and precementum, on the walls of lacunae and on cementocytes of bovine teeth. Later, Cheng et al (1999) showed, using biochemical and immunochemical as well as immunostaining techniques, that biglycan core proteins are located in bovine cementoblasts and bovine precementum. Decorin was found to be mainly associated with bovine periodontal ligament collagen fibers and was also found in the bovine cementum matrix. The differential tissue distributions of the various small leucine-rich proteoglycans in cementum suggest that they play distinct roles in cementogenesis.

Cheng et al (1996, 1999) suggested that lumican, fibromodulin, biglycan and decorin play a role as inhibitors of the mineralization of cementum due to their specific location in the non-mineralized parts of cementum. This suggestion is supported by studies that showed mineralization taking place in dentin upon removal of the proteoglycans and those that show a decreasing proteoglycan content associated with mineralization and dentinogenesis (Tagaki et al, 1990; Hall et al, 1997). However, other studies have shown that when immobilized on a solid surface, some proteoglycans act as promoters of calcification (Lussi & Linde, 1993; Hunter & Weinert, 1996). In addition, it has been noted that the molecular weight of biglycan in cementum is significantly less in cementum and this decreased molecular weight is due to smaller glycosaminoglycan side chains, the significance of which remains to be determined (Cheng et al, 1999).

Human studies of small leucine-rich proteoglycans in cementum are few. Using a standard indirect immunoperoxidase technique on freshly extracted human permanent teeth, it was shown that decorin, lumican and biglycan are components of the extracellular matrix of cellular but not acellular cementum. They were found on cementocytes and at the borders and lumina of lacunae and canaliculi surrounding the cells as well as associated with periodontal ligament fibers entering cellular and acellular cementum and by cementoblasts lining the root surface. Fibromodulin, on the other hand, could not be demonstrated in either human cementum type, unlike in bovine cementum, except associated with Sharpes' fibers that were anchored in the cementum. Moreover, it was determined in this controlled comparative study between healthy and periodontally infected teeth that neither age nor periodontal disease appeared to qualitatively influence the proteoglycan population of cementum (Ababneh et al, 1999).

Bone

Holmstrup (1996) reviews alveolar bone microanatomy. Alveolar bone is that bone that surrounds and supports the teeth in the jaws. Like cementum, it contains blood vessels and nerves and is mineralized. Osteoblasts are the cells responsible for bone formation and they secrete osteoid, which later becomes mineralized. Upon mineralization, osteoblasts become surrounded by mineralized tissue and become osteocytes. Small canals called canaliculi connect osteocytes and osteoblasts to each other. Osteoid is the precursor form of mineralized bone and is comprised of collagen (mainly type I with small amounts of III and IV), glycoprotein and proteoglycans. Once secreted osteoid

becomes bone, nutrition is achieved through blood vessels that penetrate the bone through Haversian canals that are the center of osteons in bone.

Mature bone matrix is dominated by mineral in the form of hydroxyapatite (Table 5). The mineral crystals are aligned along the type I collagen fibrils, the predominant organic component in this tissue. The extracellular matrix of bone also contains small chondroitin sulfate proteoglycans like decorin and biglycan as well as non-collagenous proteins (Sommarin et al, 1998).

The first small leucine-rich proteoglycans to be localized in bone were decorin and biglycan (Franzen and Heinegard, 1984; Fisher et al, 1989). Very little information is known about the proteoglycans of alveolar bone as most research in bone has been done on long bones from various species (Rahemtulla, 1992). Animal studies in rabbit alveolar and basal bone have reported the presence of chondroitin 4-sulfate and keratan sulfate in both bone types with a 1:1 ratio in alveolar bone and 2:1 in basal bone (Waddington et al, 1988). In a later report, Waddington et al (1989) noted that chondroitin 4-sulfate made up 94% of human alveolar bone with hyaluronic acid, dermatan sulfate and heparan sulfate present in minor amounts. These findings were later confirmed by Bartold et al (1990) using immunohistochemical techniques on rabbit, pig and human alveolar bone.

Osteoadherin has been localized as a minor leucine- and argenine-rich keratan sulfate proteoglycan found in the mineralized matrix of bone. It has been shown to bind osteoblasts via the $\alpha v\beta 3$ integrin in a cation-dependent mechanism (Sommarin et al,

1998). Buchaille et al (2000) also found osteoadherin in cells of the alveolar bone surrounding developing rat teeth.

The importance of the small leucine-rich proteoglycans is highlighted in the recent studies of biglycan knockout mice. Xu et al (1998) developed a biglycan knockout mouse line to study biglycan's effect on development. Mice deficient for biglycan gene were born normally but displayed a phenotype characterized by a reduced growth potential and reduced bone mass due to decreased bone formation that worsened with age (discussed above). Goldberg et al (2002) found in a study of biglycan knockout mice that day-1 mandibles revealed the presence of large unmerged nodules with large interglobular spaces in the bone between them, supporting the findings of the osteoporosis phenotyope reported by Xu et al (1998). Deletions in two other connective tissue genes that are highly expressed in bone (decorin and osteonectin) failed to show similar phenotypes (Danielson et al, 1997; Gilmour et al, 1998) attesting biglycan's role in normal bone development. While the precise function of small leucine-rich proteoglycans in bone remains to be elucidated, it is clear from these gene knockout studies that they indeed play a role in bone development and mineralization.

Enamel

Enamel, the translucent hard tissue that covers the crowns of teeth, has no collagen component and as such has a matrix completely different than bone and dentin. Enamel is composed of unique proteins like amelogenin, ameloblastin, enamelin and tuftelin (Ten Cate, 1994).

Buchaille et al (2000) were the first to show a small leucine-rich proteoglycan associated with enamel when they showed that osteroadherin is located in the ameloblastic layer in developing rat teeth.

Goldberg et al (2002) in studies comparing biglycan knockout mice and wild-type mice noted that biglycan knockout mice had enamel that was 3-5x thicker than wild-type mice. Instead of the expected enamel rods, tubule-like structures were seen filled with stippled material and protracted Tomes' processes in the outer enamel. It was suggested by the authors, after immunostaining for amelogenin, that the biglycan acts as a repressor of amelogenin formation by ameloblasts and odontoblasts and this repression allows for normal enamel formation. Decorin and fibromodulin knockout mice did not develop the same abnormal enamel phenotype. Enamel formation in decorin knockout mice was repressed in the molars and aprismatic in the incisors while fibromodulin knockout mice appear to have normal enamel formation.

1.5.5 Localization Of Small Leucine-Rich Proteoglycans In Other Tissues

Temporomandibular Joint (TMJ) Disc

The TMJ disc is a fibrous connective tissue structure intervening between two bony components, the mandibular condyle and the glenoid fossa of the temporal bone. It accommodates the biomechanical forces evoked by orofacial functions and facilitates movement of the condyle-disc complex. Collagens and proteoglycans are prominent members of the extracellular matrix in the TMJ disc.

It has been shown previously that the distribution of decorin and biglycan is different in the peripheral and central areas of adult TMJ discs in both bovine (Scott et al, 1989, 1995b) and rat (Mizoguchi et al, 1998) models. More recently, decorin and biglycan were analyzed using biochemical and immunolocalization techniques in extracellular matrix of the TMJ disc of growing rats. It was shown that there are growth related-changes and regional differences in the expression of biglycan and decorin in the TMJ disc of growing rats. These changes may be a reflection of changes in the biochemical environment caused by the development of orofacial functions with decorin dramatically increasing with age and biglycan decreasing with age. It is interesting to note that during these changes in proteoglycans, there is no real change reflected in glycosaminoglycan content (dermatin sulfate in this case) in the TMJ disc. As well, decorin was shown to be more abundant in areas that undergo more tensile loading and biglycan more abundant in areas that undergo more compressive loading reflecting a difference in function of these two proteoglycans (Kuwabara et al, 2002).

1.6 Collagen: A Basic Constituent Of Oral Tissues

Collagen is a very important and ubiquitous structural component of connective tissue extracellular matrices and provides the shape and form to many tissues and a surface to which many macromolecules, glycoproteins, polymers, inorganic ions and cells attach (Kadler, 1995).

There are more than 20 distinct types of collagen in animal tissues (Kadler et al, 1996). The different collagens have different structures and can be generally classified into

fibrillar, non-fibrillar and FACIT (fibril-associated collagens with interrupted triple helices) types. The non-fibrillar types may occur as net-like sheets underlying epithelial and endothelial cells or as fine filaments that anchor basement membranes to specialized structures in the skin. They may also occur as hexagonal lattices in cartilage and Descement's membrane providing a substrate for cell differentiation, proliferation, migration and support (Kadler, 1995). However, fibrillar collagens (Types I, II, III, V, XI) make up the bulk of all connective tissues in animals (Kadler, 1995; Kadler, 1996; Corsi et al, 2002) and are the focus of the discussion on collagen herein. Table 6 lists the types of collagen and their distribution in dental and periodontal tissues.

The most abundant source of fibrillar collagen is the dense connective tissue of tendon, ligament, skin and bone. Here, fibrillar collagens are organized into fibrils, thicker fibers and fiber bundles. Small leucine-rich proteoglycans play a role in collagen fibrillogenesis. The rope-like fibers of fibrillar collagen help maintain the integrity of these tissues (Kadler, 1995). Type I collagen is a fibrillar collagen and is the most abundant type found in dental tissues (Ten Cate, 1994). Most research on collagen synthesis and fibrillogenesis is based on type I collagen.

1.7 Collagen Synthesis

Collagen synthesis and fibrillogenesis is a highly regulated process (Fig. 6) consisting of many steps and many players (Ten Cate, 1994; Kadler et al, 1995; Kadler et al, 1996). Collagen is comprised of three polypeptide chains (alpha chains) that form a triple helix (Fig. 7). The alpha chains are constructed from repeating Gly-X-Y triplets, where X and

Y can be any amino acid but are frequently proline and hydroxyproline respectively. Glycine in every third residue position of each chain is a prerequisite for folding of the three chains into the triple helix. The glycines are found facing the center of the helix. The triple helix forms is a right-handed super helix that repeats every 30 residues. A more extensive review on the biosynthesis and fibrillogenesis of collagen can be found in Kadler, 1995.

The synthesis of individual collagen alpha chains is thought to occur as a two-step process: the first step occurring intracellularly and the second step occurring outside the cell (Fig. 6). Translation of pre-procollagen molecules begins on free ribosomes, which later become associated with the endoplasmic reticulum in fibroblast cells. Amino acids on the ribosomes assemble to form the individual polypeptide chains. The chains first formed are substantially longer than those found in the final molecule because they contain amino acids at the N- and C- terminal ends that play important roles in the synthesis process and are later cleaved. Vitamin C-dependent enzymes act to hydroxylate the lysine and proline amino acids before helix formation begins. As hydroxylation ensues, glucose residues are added to the molecules, a process known as glycosylation, another enzymatically-driven process. Upon hydroxylation and glycosylation, the chains are aligned with the help of disulfide bonds at the C-terminal ends and the triple helix formation begins. Once formed, the triple helix molecule is transported to the golgi apparatus, where it further remodels to form the procollagen molecule. Procollagen molecules are aligned in a non-staggered configuration and are transported to the cell surface for excretion. The formation and excretion of the procollagen molecule takes

approximately 35-60 minutes. Fibrillar collagens are formed as procollagens, which are excreted from the cell where it undergoes enzymatic processing at the N- and C- terminal ends to form collagen. The fragments then undergo a process known as fibrillogenesis, a self-assembly process.

1.8 Collagen Fibrillogenesis

The process of collagen fibrillogenesis is not fully understood (Kadler, 1995; Kadler 1996). Since collagen fibrils ultimately determine the architecture, stability and mechanical attributes of tissues, the details of collagen fibrillogenesis are important. Determining how the process is regulated is important in understanding the assembly, function, pathology and healing of connective tissues.

Once secreted, procollagen molecules are aligned extracellularly to form the typical banded collagen fibril (Fig. 8). Assembly of fibrils is believed to be a self-assembly process driven by the amino acids that make up the molecule but is dependent on several types of other molecules including other collagen types and proteoglycans, particularly the small leucine-rich proteoglycans (Kadler, 1995; Kadler, 1996; Ezura et al, 2000).

It is believed that there may be selective removal of the C-terminal extension and partial removal of the N-terminal extension of the procollagen molecule that permits the alignment of a five unit staggered microfibril. These microfibrils in turn become aligned parallel to each other and staggered leaving a regular series of gaps in the molecule. The size and shape of the gaps is important since it is here that the minerals of hard tissues,

such as bone and dentin and cementum, reside (Ten Cate, 1994). Once aligned, the remaining portion of the N-terminus is lost and intermolecular bonding occurs that gives strength to the molecule. The bonding is comprised first of hydrogen bonds with the development of covalent bonds as the tissue matures (Ten Cate, 1994; Kadler, 1995).

Graham et al (2000) showed in a study of collagen fibril fusion in tendon that fibroblasts synthesize transient early fibril intermediates that fuse in an end-to-end fashion to generate long fibrils. The intermediates were of two types: unipolar fibrils, exhibiting a C- terminal and an N-terminal, and bipolar fibrils, exhibiting two N-terminals. End-to-end fusion required the C-terminal of the unipolar fibril intermediates. Small leucine-rich proteoglycans were noted to cover the long surfaces of these fibrils but not the tips of the fibrils, thus appearing to act as inhibitors of lateral fusion. When small leucine-rich proteoglycans were absent, the fibril intermediates aggregated laterally, thus demonstrating further that small leucine-rich proteoglycans promote end-to-end fusion of collagen fibril intermediates and inhibit lateral fusion.

It has been proposed that collagen fibril growth in length and diameter occurs by accretion of collagen fibril intermediates, the basic units in the growth of fibrils (Ezura et al, 2000). In this model (Fig. 9), it is believed that fibril intermediates are stabilized through their interactions with fibril-associated macromolecules, such as the small leucine-rich proteoglycans lumican and fibromodulin. The fusion of the fibril intermediates generates a mature fibril in a multi-step manner. Progression through the growth process would occur through additive and like fusion. In this model, based on

studies of mouse tendon in lumican and fibromodulin and double knockout mice, it seems that lumican and fibromodulin have temporal functions associated with this process whereby lumican functions together with fibromodulin in the early stages with progressively less lumican and progressively more fibromodulin as the tissue matures (Fig. 10).

1.9.1 Collagen And Small Leucine-Rich Proteoglycans: Interactions

The association of proteoglycans with collagen has long been known. In the early 1960's, dermatan sulfate was isolated from collagen and it was determined that its interaction with the collagen fibrils increased the stability of collagen and decreased its solubility (Toole et al, 1969). Since then, several small leucine-rich proteoglycans have been shown to bind collagen directly or be closely associated with collagen. It is through this interaction that they function in collagen assembly and fibrillogenesis. Table 7 lists the small leucine-rich proteoglycans that have thus far been shown to associate with fibrillar collagens. Other molecules, like link proteins (Chandrasekhar et al, 1984), glycosaminoglycans (Vogel et al, 1984; Vogel et al, 1987) and the N-terminus of small leucine-rich proteoglycans (Vogel et al, 1987), were found to have little or no effect on collagen fibrillogenesis. It should be noted that not all small leucine-rich proteoglycans have been investigated at this point, and there are probably more small leucine-rich proteoglycan / collagen interactions that have yet to come to light.

In vitro studies have shown a direct interaction between decorin and fibrillar collagen (Vogel et al, 1984; Uldebjerg and Danielson, 1988). Later investigations determined that

decorin binds to the d and e bands of collagen in bovine skin (Scott, 1990) and adult and embryonic human skin (Fleischmajer et al, 1991). The interaction of decorin with collagen is believed to be via binding of the protein core of the molecule (Vogel et al, 1984) at the 6^{th} leucine-rich repeat (Kresse et al, 1997). Chemical processing of the molecule to remove the disulfide bonds of decorin results in termination of its effect on fibrillogenesis (Scott et al, 1996) while removal of the N-terminus or glycosaminoglycans did not (Vogel et al, 1987). Noteably, the binding site of decorin was found to be different than that of lumican (Neame et al, 2000) and different than that of fibromodulin (Hedbom & Heinegard, 1993).

Early investigations of decorin / collagen interactions suggest that decorin may inhibit radial fibril growth by inhibiting cross-linking of the molecules and may inhibit calcification of mineralized tissues by occupying the gap regions where hydroxyapatite crystals are normally deposited (Scott and Orford, 1981). This inhibitory effect of decorin on collagen fibrillogenesis was later demonstrated at the electron microscope level in studies of bovine tendon and cartilage (Vogel et al, 1984; Vogel & Trotter, 1987). Later, it was shown that decorin might result in an increase in collagen fibril diameter (Kuc & Scott, 1997). Decorin's role in collagen fibrillogenesis is further substantiated by investigations showing that a decrease in decorin and decorin mRNA occurs at the time when collagen fibril growth is occurring. Collagen fibril growth appears to be in a length-wise fashion due to lateral fusion of the molecules at a time when decorin content is decreasing (Birk et al, 1995).

Decorin has also been shown to interact with other fibrillar collagens, like type V. Its binding is specific and saturable and is thought to be via its protein core (Whinna et al, 1993) while both its core protein (Ehnis et al, 1997) and glycosaminoglycan chains (Font et al, 1993) can bind to collagen type XIV, a non-fibrillar collagen found associated with collagen type I.

Biglycan has been reported in the past to be unable to bind fibrillar collagens (Neame & Kay, 2000). However, recombinant radiolabelled biglycan produced in a eukaryotic system binds to microtiter wells coated with collagen types I, II, III, V and VI approximately as well as decorin (Cox et al, 1970). While immunohistological evidence has indicated that biglycan may interact with fibrillar collagens with much less affinity than decorin (Schonnher et al, 1995b), other methods of binding cannot be ruled out. Binding of biglycan to collagen may occur by way of its glycosaminoglycan side chains (Midura et al, 1989) or via an ability to bind other collagens that are frequently found to be associated with collagen type I, such as collagen types V, VI and XIV (Hocking et al, 1998; Wiberg et al, 2002). Biglycan has been shown to bind other forms of fibrillar collagens, such as collagen type V, which it binds in a specific and saturable way (Whinna et al, 1993). In this situation, it is speculated that biglycan binds with collagen via its protein core and glycosaminoglycan side chains, although the exact interaction has yet to be elucidated.

Fibromodulin binds with collagen I and II at a site other than the decorin binding site (Hedbom & Heinegard, 1993). Fibromodulin has been shown to compete with lumican

for binding sites on type I collagen, however (Svensson et al, 2000). It has been suggested that fibromodulin affects collagen fibrillogenesis. In vitro studies on developing chick metatarsal tendon revealed a 6-8 fold increase in fibromodulin mRNA levels from days 14-19 of development when the metatarsal tendon is growing suggesting that fibromodulin regulates collagen fibril growth and matrix assembly (Nurminskaya & Birk, 1996). To determine what part of the fibromodulin molecule binds to the collagen I fibril and what part of the molecule is responsible for collagen fibrillogenesis inhibition, Font et al (1998) subjected fibromodulin to mild proteolysis to divide the molecule into its four domains. It was found that no single part of the molecule acts to inhibit fibrillogenesis thereby suggesting that its action requires binding at several sites.

Lumican has been found to bind fibrillar collagen in vitro (Rada et al, 1993). It competes for binding with fibromodulin (Svensson et al, 2000) at a site that is different than that of decorin (Hedbom & Heinegard, 1993). Like the other small leucine-rich proteoglycans mentioned above, lumican is able to affect collagen fibrillogenesis through this interaction. In the cornea for instance, it acts to maintain the transparency of the cornea by enabling the maintenance of thinner collagen fibers (Rada et al, 1993). Gene knockout studies (to be discussed in greater detail later) show that its absence, like decorin, increases skin fragility (Chakravarti et al, 1998), albeit by a different mechanism (Neame et al, 2000).

Other small leucine-rich proteoglycans that have so far not been investigated in oral tissues are also being investigated for their role in fibrillogenesis of collagen. Tasheva et

al (2002) in an investigation of mimecan knockout mouse skin and cornea showed using transmission electron microscopy that mimecan knockout mice have thicker collagen fibrils in the skin and cornea although these mice compared to wild-type mice displayed no evident pathological phenotype. No changes were apparent in corneal transparency though the skin of these animals was more fragile than in the wild-type mice. Further investigation is needed to determine if there is any functional overlap with other small leucine-rich proteoglycans in these mice.

Mansson et al (2001) investigated chondroadherin in vitro using recombinant chondroadherin and collagen type II. It was found that chondroadherin bonds to collagen type II. The significance of this interaction is not presently clear.

1.9.2 How Do Small Leucine-Rich Proteoglycans Interact With Collagen? The Binding Model

With the knowledge that has been derived thus far, estimations have been made as to the structure of the small leucine-rich proteoglycans. The current model of decorin structure is based on the structure of another protein that has a leucine-rich repeat region, the ribonuclease (RNAse) inhibitor (Kobe & Deisenhofer, 1993). Although the leucine-rich repeat of this molecule allows it to be compared to decorin and perhaps other small leucine-rich proteoglycans, it is a very different molecule. For this reason, the structural model of small leucine-rich proteoglycans should be treated with caution. It is believed that the leucine-rich repeat part of the protein core allows the molecule to bend or fold into a horseshoe shape (Fig. 11) with the concave portion of the molecule available for

interaction with collagen (Weber et al, 1996). Although the cys residues of the small leucine-rich proteoglycans are closer than those of the RNAse inhibitor, it is believed that if this bending occurs, there would be just enough space in the concave portion for a single tropocollagen triple helix to bind (diameter ~1.5nm) (Neame & Kay, 2000). It is speculated that the concave portion of the "horseshoe" binds to collagen and the convex portion binds to a different collagen fibril. The glycosaminoglycan(s) are believed to extend away from the convex side of the molecule to maintain interfibrillar space between the fibrils (Kobe & Deisenhofer, 1994; Reardon et al, 2000). The true structure will not be fully appreciated, however, until the crystal structure is obtained (Neame & Kay, 2000).

Scott (1996) showed using rotary shadowing-electron microscopy that decorin, lumican, and fibromodulin are horseshoe-shaped. It was suggested that decorin is a bidentate ligand attached to two parallel neighboring collagen molecules in the fibril, helping to stabilize fibrils and orient fibrillogenesis.

1.9.3 Small Leucine-Rich Proteoglycans In Collagen Fibrillogenesis: In Vitro Studies

As noted above, several in vitro studies have been done that attest to the role of small leucine-rich proteoglycans in collagen fibrillogenesis. Other studies are discussed below (Table 8).

Recently, Neame et al (2000) in a study using recombinant decorin and lumican in collagen fibrillogenesis assay based on turbidity, found that decorin and lumican act independently on collagen fibril formation where lumican accelerates and decorin retards initial collagen fibril formation. It was determined that lumican and decorin do not compete for binding sites on collagen fibrils and that lumican and decorin act to increase collagen fibril stability to thermal denaturation. Lumican and decorin result in reduced overall turbidity suggesting a lower collagen fibril diameter. The presence of both proteoglycans retarded fibril formation to a greater degree than either one alone – a synergistic effect.

Svensson et al (2000) in a study using recombinant lumican, fibromodulin and decorin proteoglycans and a collagen fibril formation / sedimentation assay, found that fibromodulin inhibits the binding of lumican and vice versa. Fibromodulin and lumican do not affect the binding of decorin to collagen or vice versa and fibromodulin binds to collagen with four times the affinity of that of lumican. Collagen was shown to have high and low affinity binding sites for fibromodulin and lumican (ie. each having two binding sites) with fibromodulin having the higher affinity.

Carlson et al (2002) showed in culture that mutant lumican cells produced an unorganized extracellular matrix with altered fibril packing and structure compared to wild cell lines and showed that the cysteine-rich domain of the lumican molecule to be an important factor in fibrillogenesis and stromal matrix assembly.

1.9.4 Small Leucine-Rich Proteoglycans In Collagen Fibrillogenesis: Genetic Evidence

Research in recent years using genetically engineered animal models have essentially confirmed that small leucine-rich proteoglycans play some role in collagen fibrillogenesis. Table 9 summarizes several of these studies. The evidence is seen at the electron microscope level in most cases while in other cases severe phenotypes are observed.

Danielson et al (1997) in a study of decorin knockout mice determined that they were viable but had fragile skin with reduced tensile strength. On a microscopic level, it was noticed that the expressed phenotype was accompanied by abnormal collagen morphology in skin and tendon with coarser and irregular fibril profiles. The fibrils had increases and decreases in mass along their length that may be the result of altered lateral fusion of the fibrils during fibrillogenesis due to the absence of decorin.

Xu et al (1998), in a study on biglycan knockout mice (discussed previously) found that mice with no biglycan expressed an osteoporotic phenotype characterized by reduced growth rate and decreased bone mass that became more obvious with age. High-resolution radiographic imaging showed that there was reduction in the amount and density of trabecular bone and reduced cortical thickness. It was determined that reduced bone mass was due to decreased bone formation rather than increased resorption.

Corsi et al (2002) studied the effect of biglycan and decorin deficiency in single and double knockout mice. It was determined that biglycan knockout mice expressed a phenotype characterized by abnormal collagen fibrils of bone, skin and tendon with thinning dermis occurring in the skin without overt skin fragility. The decorin knockout mice showed similar skin phenotype but bone seemed to be unaffected. The findings in the double knockout mice were found to be additive in dermis and synergistic in bone manifested as severe skin fragility and marked osteopenia. Ultrastructural analysis of collagen fibrils revealed a complete loss of basic fibril geometry characterized by rough fibril profiles.

Biglycan and fibromodulin deficiencies were studied by Ameye et al (2002). Mice deficient in biglycan and/or fibromodulin developed with gait impairment, ectopic tendon ossification and severe premature osteoarthritis. It was indicated that structurally weak tendons were responsible for the impairment, which in turn led to the ossification in the tendon as demonstrated by forced use of the joints.

Fibromodulin deficiency was studied by Svensson et al (1999) who found, using transmission electron microscopy, that fibromodulin knockout mice have altered collagen fibril morphology in tail tendon in that they were fewer in number, had rough, irregular outlines in cross-section. As well, fibromodulin knockout tendon had on average more thin fibrils. These mice also showed a four-fold increase in lumican deposition suggesting a functional overlap of these two small leucine-rich proteoglycans.

Ezura et al (2000) in a study of fibromodulin, lumican and double knockout mice determined that all three groups of mice exhibited altered collagen fibrils with the double knockout mice expressing a phenotype that was additive in nature – ie. worse than the single mutant phenotypes furthering the suggestion of functional overlap between these two small leucine-rich proteoglycans. Three distinct abnormalities aware found in collagen fibrils: an early presence of fibril diameter heterogenicity in the double knockout mice, an abnormally large amount of thin fibrils in later stages of development, and irregular profiles of the fibrils.

Chakravarti et al (1998) in a study on lumican knockout mice found that they too displayed skin fragility and laxity. In addition they expressed a phenotype characterized by corneal opacity. Ultrastructural analysis of skin and cornea revealed altered collagen fibril morphology characterized by a significant proportion of abnormally thick fibrils with altered interfibrillar spacing as well.

Jepsen et al (2002) in a study of lumican, fibromodulin and double knockout mice determined that double knockout mice were small in body size, displayed a gait impairment, joint laxity and age-dependent osteoarthritis characterized by extreme tendon weakness. These findings were attributed to altered collagen fibril morphology and a disproportionate increase in thin collagen fibrils.

1.10 Implications Of The Role Of Small Leucine-Rich Proteoglycans In Dental Disease

Since most dental tissues have fibrillar collagen as their major non-cellular element (Tables 3, 4, 5), and since small leucine-rich proteoglycans play a role in collagen fibrillogenesis, one would suspect that small leucine-rich proteoglycans would be necessary for normal development and maintenance of dental tissues.

Proteoglycans and collagen fibrils are the main constituents of the connective tissues (Wilda et al, 2000). While there has been no direct link of small leucine-rich proteoglycans in the formation or progression of periodontitis or caries, research indicates that there may be a role for these molecules in creating and maintaining the integrity of the dental soft tissues and development and mineralization of dental hard tissues (Tables 8 and 9). If they do in fact have a positive influence on the integrity of these tissues, one must assume that a defect in small leucine-rich proteoglycans would increase the risk of onset and progression of dental disease such as dental caries and periodontitis.

Marked loss of decorin and biglycan in chronic inflamed periodontal tissues has been noted in a human study (Oksala et al, 1997) suggesting a role of small leucine-rich proteoglycans in maintaining the tissue integrity of gingival tissues. As well, alterations have been detected in small leucine-rich proteoglycan distribution during human gingival wound healing again suggesting a role in organization and extracellular matrix integrity (Oksala et al, 1995). Moreover, a decrease or absence of small leucine-rich proteoglycans has been found to be associated with some connective tissue diseases. Decorin

deficiencies have been found associated with neonatal Marfan syndrome (Pulkkinen et al, 1990; Superti-Furga et al, 1992; Raghunath et al, 1993), infantile progeroid syndrome (Beavan et al, 1993), Ehlers-Danlos syndrome (Wu et al, 2001; Tajima et al, 1999; Quentin et al, 1990; Fushimi et al, 1989), and osteogenesis imperfecta (Dyne et al, 1996). Interestingly, these diseases have been shown to exibit dental manifestations including periodontitis (Straub et al, 2002; Pope et al, 1992; Reichert et al, 1999; Hartsfeld et al, 1990; McKusick, 1972). As well, research in gene knockout mice has shown that alterations in decorin, biglycan, lumican and fibromodulin may produce changes that mimic diseases like Ehlers-Danlos syndrome (Danielson et al, 1997; Chakravarti et al, 1998; Corsi et al, 2000; Jepsen et al, 2002). The role of small leucine-rich proteoglycans in maintaining tissue integrity has been inferred for years as evidenced by the use of proteoglycans and glycosaminoglycan detection methods in periodontal disease.

1.10.1 Proteoglycans And Glycosaminoglycans As Periodontal Disease Markers

Several studies have examined the occurrence of various extracellular matrix components in the gingival crevicular fluid in humans with various periodontal conditions including health. Most of these studies evaluate the occurrence of glycosaminoglycans and few are related to proteoglycans, especially small leucine-rich proteoglycans. It seems that the most valuable marker is chondroitin 4-sulfate, which is believed to originate in alveolar bone (Oksala et al, 1993) although it may also originate in the soft tissue. The general trend is for glycosaminoglycan levels of subjects with periodontal disease to be higher compared to those of healthy individuals.

The first report of glycosaminoglycan detection in the gingival crevicular fluid was by Embery et al (1982) who used electrophoresis to find sulfated glycosaminoglycans present in the gingival crevicular fluid of chronic periodontitis subjects. Last et al (1985) investigated glycosaminoglycans in the gingival crevicular fluid using electrophoresis as well. Gingival crevicular fluid was collected from subjects with chronic gingivitis, untreated advanced periodontitis, early periodontitis, juvenile periodontitis, teeth undergoing active orthodontic movement, teeth in traumatic occlusion, and healing extraction sockets. Chondroitin 4-sulfate was found in all situations where degenerative changes were taking place in the deeper periodontal tissues. Interestingly, these sulfated glycosaminoglycans could not be detected in areas of recent periodontal treatment consisting of periodontal surgery or daily subgingival irrigation with chlorhexidine.

Shibutani et al (1993) developed an ELISA test to detect chondroitin sulfate glycosaminoglycans in the gingival crevicular fluid of dogs with experimentally induced periodontitis. ELISA values for chondroitin 6-sulfate, chondroitin 4-sulfate and dermatan sulfate, although low, increased in proportion with the severity of inflammation present in these animals.

Giannobile et al (1993) investigated the quantities of chondroitin sulfate glycosaminoglycans in the gingival crevicular fluid in health, gingivitis, and adult periodontitis maintenance and adult periodontitis non-maintenance groups using a safranin O binding assay. It was found that the amount of glycosaminoglycans increased with severity and control of periodontal condition such that the levels of

glycosaminoglycans were approximately 4 ng GAG / sample in health, 15 ng GAG / sample in gingivitis, 23 ng GAG / sample in maintained adult periodontitis and 120 ng GAG / sample in unmaintained adult periodontitis.

Smith et al (1995) examined the hyaluronan and chondroitin 4-sulfate in the gingival crevicular fluid of patients with chronic adult periodontitis at diseased and healthy sites before and after treatment using electrophoresis. Significantly higher levels of chondroitin 4-sulfate were detected at diseased sites prior to treatment correlating with probing depth and attachment levels. Sites that responded well to treatment (oral hygiene instruction and root planing) had lower levels of chondroitin 4-sulfate than sites that did not respond well to treatment. Hyaluronan levels were less significantly associated with clinically successful treatment.

Hyaluronan being a rather ubiquitous component of gingival crevicular fluid samples represents approximately 40% of the total glycosaminoglycan content of human gingiva. Its levels are believed to be constantly high due to the constant turnover of the periodontal tissues and thus it is not a good disease marker (Embery et al, 1979). It tends to be absent in certain disease situations such as acute necrotizing ulcerative gingivitis (ANUG). Moreover, treatment of ANUG has resulted in an increase of hyaluronan to normal high levels (Last et al, 1987).

Heparan sulfate has been found in gingival crevicular fluid of orthodontic patients (Waddington et al, 1994).

Few studies have tried to detect parent proteoglycans in the gingival crevicular fluid. Waddington et al (1998) separated two proteoglycan species from gingival crevicular fluid. They were chondroitin sulfate-rich proteoglycans that reacted positively to anti-decorin and anti-biglycan antibodies.

Oksala et al (1997) examined basement membrane heparan sulfate proteoglycan, CD44, syndecan (three heparan sulfate proteoglycans), decorin and biglycan in chronically inflamed human periodontium using immunofluorescence microscopy of tissue sections. It was found that basement membrane heparan sulfate proteoglycan was decreased in subepithelial and subendothelial basement membranes while CD44 and syndecan were reduced in epithelial cells but increased in infiltrating lymphocytes. Decorin and biglycan levels were reduced in the periodontal connective tissue of chronically inflamed tissue with decorin localizing in connective tissue along short rod-like structures. The results of this study suggested that proteoglycan-dependent intercellular adhesion of keratinocytes is decreased in chronic inflammation while adhesion of infiltrating lymphocytes is increased in this state. Furthermore, the disappearance of adhesion modulating proteoglycans may regulate cell migration in inflamed periodontium.

These studies suggest that as periodontal tissues become inflamed, the proteoglycans (including the attached glycosaminoglycans) of the tissues decrease allowing for the inflammatory process to enfold and begin the battle against periodontal pathogens. The lost proteoglycans (and glycosaminoglycans) are released into the gingival crevicular

fluid, which can be detected by various methods including ELISA and electrophoresis. Chondroitin 4-sulfate seems to be a more sensitive marker for disease activity than chondroitin 6-sulfate, hyaluronan, or dermatan sulfate for determining active phases of periodontal destruction at individual sites. Further research is required to determine the value of this detection in the clinical setting. The virtual absence of soft tissue components in the gingival crevicular fluid may be a reflection of the very high turnover rate of the periodontal tissues. Periodontal ligament has a turnover rate of 15x that of skin and 5x that of alveolar bone (Sodek et al, 1977) with respect to its collagenous components. This high turnover rate may result in quick disappearance of tissue breakdown products rather than their elution into the gingival crevicular fluid (Embery et al, 2000).

Chapter Two – Aim of the Study

In the past, proteoglycans received little attention in tissue research since they comprised a relatively small proportion of tissue make-up. This small proportion, however, may not accurately reflect their importance. Over the last 30 years, methods of study have improved to include the use of immunohistochemical techniques and genetically engineered mice allowing new insights into the role and function of these important molecules.

Proteoglycans are an important and integral part of all periodontal tissues (cementum, periodontal ligament and bone especially). One must wonder, then, how these molecules influence the state of these tissues. Could their absence (partial or complete), for instance, influence the initiation or progression of periodontal disease or result in decreased regenerative ability of the periodontium? Could alterations of these molecules result in increased caries incidence? Only now, with the development and utilization of newer laboratory techniques and the investigation into genetically engineered mice can we begin to understand the importance of these molecules in the dental tissues.

Therefore this study will analyze the gross anatomy of dental and periodontal tissues as well as the structure and organization of these tissues at the light and electron microscope levels in fibromodulin, lumican and double knockout adult mice compared to age and sex-matched CD-1 wild-type mice. We hypothesize that the absence of lumican and / or fibromodulin will result in an altered morphology of the periodontal ligament collagen fiber bundles.

Chapter Three – Materials and Methods

3.1 Animals

8 lumican (LUM) knockout mice, 8 fibromodulin (FM) knockout mice and 8 lumican / fibromodulin (LUM/FM) knockout mice aged 4.5 to 8 months (gifts from Dr. Birk, Jefferson Medical college, Philadelphia, PA, Dr. Chakravarti, John Hopkins University School of Medicine, Baltimore, Maryland and Dr. Oldberg, University of Lund, Sweden) and 10 age and sex-matched CD-1 (wild-type) mice were used in this study (Table 10). The generation and characterization of the mice used in this study have been described in detail elsewhere (Svensson et al, 1999; Chakravarti et al, 1998; Jepsen et al, 2002).

Mouse heads were fixed in 4% formalin in neutral buffer for 3 weeks. The maxillae and mandibles were carefully 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, presence or absence of alveolar bone loss (see below), and to measure jaw sizes (see below). The mandibular samples were decalcified in 5% formic acid containing 0.9% sodium chloride for up to 7 weeks until no mineralized tissue was detected on radiographs. Both halves of the mandible were embedded in paraffin using standardized procedures (one half in a mesio-distal orientation, the other in a bucco-lingual orientation) and sectioned at 6μm. Standardized sections from the coronal and mid-third areas of the tooth root were chosen from each mouse such 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.

3.2 Morphometric Analysis of Jaw Size

One half of each defleshed maxilla was measured with a Boley Gauge. Only those jaws that remained intact after dissection were analyzed (Table 16). The tips of the calipers were placed at the alveolar margin facial to the erupted incisor, where it emerges from the alveolus and at the distal of the third molar (Fig. 12). The gauge was then read to 1/10 of a millimeter. Results were statistically analyzed with a student *t*-test to compare the single and double knockout mice to wild-type control mice and to compare the single knockout mice with the double knockout mice. Age and sex were considered in the statistical analysis.

3.3 Analysis of Alveolar Bone Loss

One half of all defleshed maxillae were analyzed under a dissecting microscope for bone loss using the classification system for bone loss in mice described by Wiebe et al (2001). Bone loss in this classification system was classified as follows: Grade I) horizontal component of bone loss in the furcation, Grade II) through & through furcations, and Grade III) through-and-through furcations with alveolar bone loss into the apical third of the tooth root (Table 11).

3.4 Histomorphometric Analysis

Paraffin sections from wild-type, fibromodulin, lumican, and double knockout mice were stained with phosphotungstic acid hematoxylin (PTAH) (Putchler et al, 1963) to study

collagen morphology and cells, hematoxylin and eosin (H&E) for general histology and picrosirius red (PSR) (see below) for analysis of collagen organization and fiber morphology. Table 12 lists the mice used for each stain. Stained sections were mounted with Entellan mounting medium. Two independent examiners (single blinded) analyzed the middle and coronal third areas of the H&E and PTAH-stained mandibular first molar sections using a light microscope (Axiolab E by Zeiss) equipped with a 20x and 40x objective. Images were recorded with a Nikon Coolpix 995 digital camera attached to a Nikon Eclipse TS 100 microscope equipped with a 20x objective. The coronal and middle third areas of the PSR-stained mandibular first molar sections were examined in the same manner using a polarizing light microscope (Carl Zeiss Jena Jenapol polarizing light microscope) equipped with a 20x objective. Angles on the polarizing light microscope were set so that at 0°, the sections were aligned with the long axis of the periodontal ligament spaces. Images were recorded at 0°, 45° and 90° using a Canon EOS D60 digital camera with the following settings (aperture priority at AE, shutter speed of 30, partial metering mode, 1.2 exposure compensation, ISO speed 100, manual focus).

Sections were analyzed under light and polarizing light microscopes and digital images of sections were also examined using Photoshop 6.0 software for morphologic changes in the dental and periodontal tissues, presence or absence of bone loss and cellular infiltrate indicative of inflammation. Photoshop alterations only included cropping and sharpening of photos such that unsharp mask was set at 15%, 0 threshold level and 16 pixel radius. Photoshop 6.0 was used to take advantage of the zoom feature.

PSR staining was carried out as follows. Deparaffinized section were immersed in picrosirius red (100ml saturated aqueous picric acid + .1g Gurr's Sirius red F3B) for 1 hour and then immersed in 1% acetic acid for 15 minutes (solution changed every 5 minutes) until solution was clear.

Parameters being examined were: gross morphology of bone, dentin and cementum, the presence or absence of external root resorption, the presence or absence of bone loss (Wiebe et al, 2001), the presence or absence of inflammatory cells in H&E sections, gingival fiber morphology and orientation. The following parameters were analyzed in the periodontal ligament: relative amounts of interfiber bundle space between collagen fiber bundles, relative thickness of collagen fiber bundles (homogeneity), relative length of collagen fiber bundles (whether or not it was difficult to trace a bundle's path from tooth to bone), fiber bundle orientation, and fiber bundle outline (smooth and distinct or hazy and indistinct). Relative amount of blood vessels were located in the periodontal ligaments of knockout mice and compared to those of wild type mice. Non-standardized, representative sections of 7 CD-1 wild-type mice, 5 fibromodulin knockout mice, 5 lumican knockout mice and 6 double knockout mice were analyzed for differences in numbers of blood vessels in the coronal two-thirds of the periodontal ligament. Blood vessels were recognized in PTAH and H&E-stained sections as circular or rounded voids within the ligament lined with endothelial cells and containing recognizable blood cells within it. The blood vessels were counted on the buccal and lingual surfaces and were analyzed together and separately as such. Tears in the periodontal ligament collagen fiber bundles were a relatively frequent finding in some samples. Therefore, the number of

breaks or disruptions were calculated from 6 CD-1 wild-type mice (mice 1-6), 5 fibromodulin knockout mice (mice 1-5), 5 lumican knockout mice (mice 1-5) and 5 double knockout mice (mice 1-5) (Table 10) under light microscope at 20x objective and statistically comparedd.

3.5 Immunohistochemical Analysis

For immunohistochemical localization of decorin, biglycan, lumican and fibromodulin deparaffinized sections of the mandibular first molars were incubated in a solution of 500μL 30% H2O2 + 50mL methanol for 30 minutes to block the endogenous peroxidase activity. Sections were rinsed and incubated with normal blocking serum (Vectastain; Vector Laboratories Inc., Burlingame, California) for 60 minutes at room temperature and then incubated with polyclonal antibodies against decorin (LF-113), biglycan (LF-106) (gifts from Dr. Larry Fisher, National Institutes of Health, MIDR, Bethesda, Maryland), lumican (gifts from Svensson et al, 1999, Dept of Molecular Biology, University of Lund, Sweden), fibromodulin (gifts from Dr. Plaas et al, 1997, Shriner's Hospital for Crippled Children, Tampa Fl.) or type I collagen (Chemicon, Tamecula LA) (Table 13) at 4°Celsius for 16 hours. After rinsing, sections were incubated with biotinylated anti-rabbit antibody for 1 hour and then reacted with ABC avidin/peroxidase reagent (Vectastain Elite kit, Vector Laboratories Inc.). Sections were then rinsed and reacted with the Vector VIP substrate for peroxidase for 1-4 minutes. For localization experiments, the reaction was stopped when the immunostain was detected in the extracellular matrix under light microscope by dipping the sections in distilled water for

at least 2 minutes. Bu/Li sections were used for localization of SLRP in the dental tissues (Table 12).

To determine if lack of one or two proteoglycans was compensated by increased expression of other members of the small leucine-rich proteoglycan family within the gingiva, dental and periodontal tissues, a standardized staining procedure was used on mesio-distal sections so that intensities of the various immunostains could be compared. Mesio-distal sections of each mouse group (Table 14) were divided into 4 groups of 4 (one mouse from each group) and the staining procedure was standardized as follows: one group was stained at a time and antibodies were applied to the groups of sections in the same sequence such that antibodies (anti-LUM, anti-DCN, anti-FM, anti-BGN) were applied to wild-type CD-1 mouse sections, followed by fibromodulin knockout mouse sections, lumican knockout mouse sections and double knock-out mouse sections. Reactions were stopped when immunostaining was visually apparent under light microscope in the extracellular matrix of wild-type mouse sections (approximately 2.5 minutes). Reactions were stopped in the same sequence as above to ensure equal exposure time to each group of mouse sections.

All sections were mounted with Entellan mounting medium before being examined by two independent (single blinded) examiners using a light microscope (Axiolab E by Zeiss) with 10x, 20x and 40x objectives for localization of proteoglycans within the gingiva, dental and periodontal tissues. The relative level of proteoglycan expression was

determined using a scale of 0-3 (0 = no stain; + = mild staining; ++ = moderate amount of staining; +++ = extreme or intense staining).

3.6 Scanning Electron Microscopy

Paraffin sections from the mid-root region of the periodontal ligament of mandibular first molars of CD-1 wild-type, fibromodulin, lumican, and double knockout mice were processed for scanning electron microscopy using standard procedures (Postek et al, 1980) and examined using a Cambridge 260 Stereoscan scanning electron microscope.

3.7 Statistical Analysis

Jaw size, numbers of blood vessels and tears in the periodontal ligament were compared using a student *t*-test assuming equal variance using the Microsoft Excel statistical package. Comparisons were made with pooled data and with age and sex-matched mice to control for these variables.

Chapter Four – Results

4.1 Immunohistochemical Analysis

In order to compare the expression and localization of fibromodulin and lumican to decorin and biglycan in periodontal and dental tissues of wild-type CD-1 mice, paraffin sections were stained with antibodies against decorin, biglycan, fibromodulin and lumican and analyzed under a light microscope using 5x, 10x, 20x and 40x objectives. All four proteoglycans localized abundantly in the mucosal, gingival and periodontal connective tissues (Table 15). Fibromodulin localized in the predentin, pulp, periodontal ligament and connective tissue of the gingiva and mucosa at similar intensities (Fig. 13-A). Slight immunoreactivity was detected in epithelial basal cells while no immunoreactivity was noted in cementum or bone. Lumican was localized in pulp connective tissue, periodontal ligament and connective tissue of gingiva and mucosa in increasing intensity in the order described (Fig. 13-B). No lumican reactivity was observed in bone, cementum, dentin or epithelium although a slight staining could be seen in the predentin layer. Biglycan immunoreactivity was located in pulp, predentin, periodontal ligament and gingival and mucosal connective tissue with the most intense staining occurring in the periodontal ligament. Moreover, gingival connective tissue appeared more intensely stained than mucosal connective tissue (Fig. 13-C). Decorin was located in pulp, predentin, periodontal ligament and gingival and mucosal connective tissue (Fig. 13-D). Most intense staining of decorin appeared to be in the connective tissue of the gingiva and mucosa with most intense staining occurring in the area immediate subjacent the epithelium.

The absence of fibromodulin and lumican in the gene knockout mice was confirmed using anti-fibromodulin and anti-lumican antibodies. Fibromodulin, lumican and double knockout mice showed no corresponding proteoglycans in any of the dental tissues (Fig. 14a – B, D, G, H) although a slight cross-reactivity was noted in the basal epithelial layer in the fibromodulin knockout sections stained with anti-fibromodulin (Fig. 14a – G).

To determine if targeted deletion of fibromodulin or lumican was associated with compensation upregulation of expression of other members of the small leucine-rich proteoglycan family, four groups of mesio-distal sections (one per mouse group) of fibromodulin, lumican, and double knockout and CD-1 wild-type mice were stained with anti-lumican, anti-fibromodulin, anti-biglycan or anti-decorin antibodies using a standardized VIP-developing time. The findings showed that the relative staining for fibromodulin was more intense in the periodontal ligament of lumican knockout mice than wild-type mice. Additionally, lumican reactivity was more intense in the connective tissue of fibromodulin knockout mice than wild-type mice, including that in the periodontal ligament (Fig. 14a - C, F). No notable differences in immunostaining intensity were noted with anti-decorin or anti-biglycan immunostaining between the knockout mice and wild-type mice (Fig. 14b – A-H).

4.2 Gross Morphological Analysis

The gross morphology of 8 fibromodulin knockout, 7 lumican knockout and 8 double knockout maxilla halves were analyzed and compared to those of 10 age and sex-matched CD-1 wild–type mice. All teeth, three molars and incisors, were present and

fully erupted in all specimens (Fig. 15). Class I furcation defects (Wiebe et al, 2001) were detected in all groups but there was no statistically significantly difference among the groups (data not shown). Only one specimen, fibromodulin knockout mouse #1, showed any appreciable amount of bone loss, exhibiting class II furcation defects according to Wiebe et al (2001).

In order to analyze if deletion of fibromodulin, lumican or both proteoglycans affected jaw size, the length of each maxilla was measured (Table 16). The mean jaw lengths of the fibromodulin knockout (10.85 mm), lumican knockout (10.90 mm) and double knockout (10.79 mm) mice were smaller than those of the wild-type mice (11.42 mm). The mean length of the double knockout mouse jaws was also slightly smaller than that of either of the single knockout mice as noted above. The differences between wild-type mice and fibromodulin knockouts as well as between wild-type and double knockouts were found to be statistically significant when data was pooled (Table 17). Other comparisons with pooled data were not found to be statistically significant.

In order to determine if age of the mice could explain the differences in jaw size, the jaw size of animals with different ages in each experimental group were compared (Tables 18, 19). The only statistically significant (P=.03) finding was found between the mean jaw lengths of wild-type 8 month old mice (11.7mm) compared to 8 month old double knockout mice (10.85 mm). Generally, the findings showed that as age increased, jaw length increased and generally these findings were found to be statistically insignificant.

Gender analysis was also carried out to determine if gender had an effect on jaw length. When males were compared to females within each group (Table 20), a statistically significant difference (P=.024) was found only within the fibromodulin group. Here, mean male jaw length (11.23 mm) was greater than mean female jaw length (10.62 mm). When males and females were compared between the groups (Table 21), the only statistically significant difference was found between the mean jaw lengths of wild-type females (11.40 mm) compared to mean jaw lengths of fibromodulin knockout females (10.62 mm) (P=.007). When age and gender were considered together (Tables 22, 23), a statistically significant result was seen between fibromodulin knockout 5.5 month old females (10.75 mm) and wild-type 6m female (11.4 mm) (P=.03). No other statistically significant difference was found between any of the groups.

4.3 Histomorphological Analysis

A total of 110 sections including the first molar, gingiva and periodontal ligament at the coronal and middle thirds of the root surface of the first molar were stained with phosphotungstic acid hematoxylin (PTAH), hematoxylin and eosin (H&E) and picrosirius red (PSR) and analyzed under light microscope for the presence of inflammatory cellular infiltrate in the connective tissues of the gingiva and relative alveolar bone loss as well as morphological characteristics of teeth, root cementum, gingiva, periodontal ligament and bone.

4.3.1 Teeth, Cementum, Alveolar Bone And Gingiva

Morphological and histological analysis of sections stained with H&E and PTAH show that the teeth of fibromodulin, lumican and double knockout mice developed normally and that there were no gross morphological differences in the dental and periodontal tissues of the wild-type mice compared to those of the knockout mice excluding enamel, which, due to processing, could not be compared. The periodontal tissues appeared healthy and there was no evidence of inflammatory cellular infiltrate in the connective tissues of any of the groups (Fig. 16).

Amounts of cementum differed from section to section in all animals and there was abundant cementum formed at the apical third of the roots in all groups. This finding appeared to be in keeping with the location of the section of the tooth and age of the specimen (data not shown). Bone lining the socket was found to be jagged in some areas and smooth in others, which appeared to be a random finding in all groups. No conclusive changes were noted in the pulp tissue, although some samples appeared to have an increased number of cells (data not shown). No gross morphological differences were noted in the gingival tissues of the different groups in that the amounts of connective tissue, number of cells, cellular organization, and thickness of epithelial layers seemed relatively proportional (Fig. 16).

4.3.2 Periodontal Ligament

In all animal groups, periodontal ligament fiber bundles on the buccal surfaces were different than the lingual surfaces with more unorganized fiber bundles found on the

lingual surface. As well, periodontal ligament fiber bundles were randomly organized in the apical third of all root surfaces in all four groups. This random organization increased in an apical direction in all groups and therefore, only the middle and coronal thirds of buccal surfaces were used to compare collagen organization and morphology in the periodontal ligament.

Sections stained with H&E (Fig. 17A), PTAH (Fig. 18A) and PSR combined with polarizing light microscope (data not shown) showed that the coronal third portion of the periodontal ligament of wild-type mice appeared to be filled with well-organized collagen fiber bundles with little space between them. The space between the fiber bundles appeared to be evenly distributed and homogenous in nature. The fiber bundles were homogenous in thickness relative to each other and for the entire length of the fiber from tooth to bone and were oriented, in a parallel fashion, at 45° to the root surface. Moreover, the fiber bundles could easily be traced from tooth to bone surface and appeared to have well defined, smooth edges and had dark bands associated with them as seen in the mid-third periodontal ligament area of PSR-stained sections analyzed under a polarizing light microscope (Fig. 19A).

Compared to wild-type mice, the periodontal ligaments of fibromodulin knockout mice stained with H&E (Fig. 17B), PTAH (Fig 18B) and PSR combined with polarizing light microscope (data not shown) displayed collagen fiber bundles in the coronal third area that were not homogenous in thickness along the length of the fiber bundles or among the different fiber bundles. There appeared to be a relative increase in the number of thick

fiber bundles in the ligament relative to all other groups, giving the ligament fiber bundles a *clumpy* or *curly* appearance. The fiber bundles had a less well-defined outline and often appeared *fuzzy*. Although the basic orientation of the periodontal collagen ligament fiber bundles was apparent, running at 45° from tooth to bone surface, it was difficult to consistently trace the fiber bundles from tooth to bone surface. As well, the spaces between the fiber bundles were not evenly distributed throughout the ligament with narrow and wide spaces scattered throughout. Overall, there appeared to be more spacing between the collagen fiber bundles than that found in wild-type mice, although this was not quantified. PSR-stained sections analyzed under polarizing light microscope shows that fiber bundles of the mid-third area of the periodontal ligament appear to have hazier outlines and relatively more dark bands associated with them compared to those of wild-type mice (Fig. 19B).

Lumican knockout mice expressed a periodontal ligament phenotype different from that of wild-type mice and fibromodulin knockout mice as seen in coronal third sections stained with H&E (Fig. 17C), PTAH (Fig, 18C) and PSR (data not shown). Collagen fiber bundles could usually be traced from tooth to bone surface but not in all cases. There appeared to be an increase in space between the fiber bundles compared to those of the wild-type mice and fibromodulin knockout mice. As well, the fiber bundles often appeared to be non-homogenous in thickness along the length of the fiber bundles or among fiber bundles within the ligament (although the fiber bundles were more homogenous in this regard than those of fibromodulin knockout mice). Unlike the fibromodulin knockout mice, there appeared to be a preponderance of *thin* fiber bundles

throughout the ligament giving the periodontal ligament fiber bundles a *stringy* appearance. Like the periodontal ligaments of fibromodulin knockout mice, lumican knockout mice displayed mid-third area collagen fiber bundles with hazy outlines and more dark bands associated with them than those found in wild-type mice (Fig. 19C).

The double knockout mice expressed a periodontal ligament phenotype that contained some of the attributes of both single knockout mice described above as seen in coronal third sections stained with H&E (Fig. 17D), PTAH (Fig. 18D) and PSR (Fig. 19D). There was usually increased space between collagen fiber bundles of the periodontal ligament compared to that found in either of the single knockout or wild-type mice. The collagen fiber bundles were non-homogenous in thickness along their lengths and among the different fiber bundles within the ligament with thick and thin fiber bundles readily apparent. As well, it was difficult to trace the fiber bundles from tooth to bone surface and the basic orientation of 45° from tooth to bone was altered. The combination of all the above findings gave the double knockout mouse periodontal ligament an overall *random* appearance, one that was notably worse than either of the single knockout mice. Mid-third PSR-stained sections of the periodontal ligament showed that the dark bands associated with the fiber bundles in the other groups were not readily apparent in these ligaments (Fig. 19D).

The above morphological findings were confirmed with analysis of mid-third root sections stained with H&E, PTAH and PSR (data not shown) as well as sections immunostained for type I collagen (data not shown). Analysis of sections stained with

antibodies against type I collagen showed a diffuse staining in the periodontal ligament of the wild-type mice and more discreet staining in the periodontal ligament of gene knockout mice. Different fiber bundle widths seemed to be apparent in the single and double knockout mice and fiber bundles appeared to be wavier than those of the wild mice.

4.3.2.1 Scanning Electron Microscopy Of Periodontal Ligament

Scanning electron microscopy was performed to analyze the morphology of the periodontal ligament fiber bundles in more detail. The periodontal ligament fiber bundles of wild-type CD-1 mice (Fig. 20-A, E) appear well organized with proper angulation traversing the space between tooth and bone. The fiber bundles appear to have smooth outlines and surface texture and the spacing between the bundles appears relatively even throughout the ligament. At 2.62kX magnification, the fiber bundles of wild-type mouse had a sheet-like appearance. By contrast, the fibromodulin knockout mice (Fig. 20-B, F) have periodontal ligament fiber bundles that appear unorganized, *clumpy,* of varying thickness and have many disruptions. As well, they appear to have a rough surface texture. The relative amount of spacing between the collagen fiber bundles is greater than in wild-type mice. The lumican knockout mouse periodontal ligament fiber bundles are different that that of wild-type and fibromodulin knockout mice (Fig 20-C, G). They are thinner than either wild-type and fibromodulin knockout mice, with a *stringy* appearance and have increased amounts of inter-fiber bundle spacing. As well, a roughened fiber bundle outline is apparent. The double knockout mouse ligament fiber bundles (Fig. 20-D, H) are of varying thickness and have altered surface texture compared to the other

groups. They have visible "crater-like" defects on the surface of the fiber bundles and more inter fiber bundle spacing than wild-type, like the other knockout groups. Another notable finding is that all three knockout groups display very thin filamentous structures branching out from the main fiber bundles of the periodontal ligament (Fig. 20-E-H). These thin branches were not apparent in the wild-type mouse ligament fiber bundles (Fig. 20 E).

4.3.2.2 Vasculature Of The Periodontal Ligament

Aside from the differences in collagen fiber bundle morphology, more blood vessels were located in the periodontal ligaments of knockout mice, compared to those of wild-type mice (Tables 24, 25; Fig 21). The number of blood vessels was higher on the buccal side of the periodontal ligament than the lingual side in all groups, except in the fibromodulin knockout mice where a more even distribution was found (discussed further in a later section). Relatively few, scattered small blood vessels were noted in the periodontal ligament of the wild-type mice. Overall, the mean number of blood vessels was higher in the fibromodulin knockout mice than any of the other groups. As well, the mean number of blood vessels was higher in the periodontal ligaments of double knockout mice than wild-type or lumican knockout mice. However, when total numbers and means of blood vessels were compared among the different groups, no statistically significant differences were noted.

No statistical significance was noted when comparing the blood vessels of the buccal aspects of the periodontal ligament. When lingual periodontal ligaments were compared,

however, fibromodulin knockout mice showed statistically significantly more blood vessels than wild-type mice, lumican knockout mice and double knockout mice (mean number of 2.2, 0, .25 and 1.2 respectively). Other blood vessel comparisons in the lingual periodontal ligament yielded statistically insignificant results (Table 25).

4.3.2.3 Tears In The Periodontal Ligament

Histological stained sections of periodontal ligaments showed that in many samples there were breaks or tears in the periodontal ligament collagen fiber bundles. Tears were identified as irregularly shaped voids within the ligament or between the ligament and bone or between the ligament and tooth characterized by absence of stain that contained no cells and often exhibited visibly torn tissue fragments within the void (Fig. 21). Tears were analyzed as pooled data only since some tears were quite large and wrapped around the apex of the tooth and thus appeared on both the buccal and lingual surfaces.

Virtually no tears were detected in the periodontal ligaments of the wild-type mice. Fibromodulin knockout mice exhibited a higher mean number of tears in the periodontal ligament than the other three groups. However, no statistically significant differences were noted when all groups were compared (Table 26).

Chapter Five – Discussion

Periodontal ligament collagen fiber bundles mediate the attachment of the tooth to the alveolar bone. Periodontal disease involves breakdown of this collagen fiber attachment. Certain inherited conditions, such as Ehlers-Danlos syndrome, involving altered collagen fibrillogenesis are associated with severe periodontal disease at an early age (Pope et al, 1992; Reichert et al, 1999; Hartsfeld et al, 1990; McKusick, 1972) suggesting that altered collagen fibrils may predispose to periodontal disease. The mechanisms that regulate collagen fiber organization in the periodontal ligament are poorly understood (Kadler, 1995). Fibromodulin and lumican belong to a family of small leucine-rich proteoglycans that associate with collagen fibrils (Scott, 1986; Pringle & Dodd, 1990; Hedlund et al, 1994; Birk et al, 1995), can modulate collagen fibril formation and inhibit lateral growth of fibrils in vitro (Vogel et al, 1984; Hedbom & Heinegard, 1993; Rada et al, 1993) and in vivo (Svensson et al, 1999; Ezura et al, 2000). They are expressed in a number of collagenous connective tissues and play a significant role in defining tissue integrity through their abilities to interact with other extracellular matrix molecules and potentially regulate collagen fibrillogenesis (Chakravarti et al, 1998; Svensson et al, 1999; Ezura et al, 2000; Ameye et al, 2002; Jepsen, 2002). Aims of the present immunohistochemical and scanning electron microscope study were to determine: 1) if fibromodulin and lumican are expressed in the dental and periodontal tissues of normal (wild-type) mice and how their distribution compares to that of the class I small leucine-rich proteoglycans, decorin and biglycan, 2) how the distribution of these four proteoglycans changes in mice that are missing lumican, fibromodulin or both of the genes that code for these proteoglycans, 3) if lumican and fibromodulin effect collagen fiber bundle

fiber bundle morphology and organization in the dental tissues. This study shows for the first time, the major roles of fibromodulin and lumican in regulation of periodontal ligament structure.

5.1 The Mouse Model

In order to study the function of fibromodulin and lumican, the periodontal tissues of adult fibromodulin, lumican and double knockout mice were analyzed in this study. Although the anatomical nature of mouse dentition is different from that of humans, the structure and organization of the periodontal tissues is similar. As a consequence, the essential features of periodontitis are held in common with humans (Page & Schroeder, 1982). Since the continuously erupting incisor teeth of mice are structurally and functionally different than human teeth (Page & Schroeder, 1982), only the molar teeth were included in this analysis. Mice have 3 molars in each quadrant. Upon reaching occlusion, which occurs at about 1 month of age, the molars continue to erupt in a bucco-occlusal direction. During continued eruption, cementum is deposited apically and cusp tips wear away to compensate (Page & Schroeder, 1982).

5.2 Fibromodulin And Lumican Are Abundantly Expressed In Oral Tissues

Fibromodulin, lumican, decorin and biglycan were all localized in the periodontal ligament of wild-type mice in the present study. This supports findings of previous studies (Hakkinen et al, 2000; Cheng et al, 1999; Ababneh et al, 1999; Hakkinen et al, 1996; Hakkinen et al, 1993; Watanabe et al, 1998). This study localizes for the first time in the healthy adult CD-1 wild-type mouse, fibromodulin and lumican to the gingival

connective tissue, and fibromodulin, decorin and biglycan to pulp. Fibromodulin was localized in the predentin, pulp, periodontal ligament and connective tissue of the gingiva and mucosa at similar intensities (++) and to the basal cells of the epithelium to a lesser degree (+) while no immunoreactivity was detected in cementum or bone. Lumican was localized in the connective tissue of the pulp (+), periodontal ligament (++), gingiva (+++) and mucosa (+++) in increasing intensity in the order described, a similar distribution to that of decorin. No lumican reactivity was observed in bone, cementum, dentin or epithelium, although a slight staining could be seen in the predentin layer. Biglycan immunoreactivity was located in the connective tissue of pulp (+), predentin (+), periodontal ligament (+++), gingiva (+++) and mucosa (++). Moreover, gingival connective tissue appeared more intensely stained than mucosal connective tissue. Decorin showed a similar distribution pattern to lumican with localization occurring in pulp (+), predentin (+), periodontal ligament (++) and gingival (+++) and mucosal (+++) connective tissues. The most intense staining of decorin appeared in the connective tissue of the gingiva and mucosa with most intense staining occurring in the area immediate subjacent the epithelium. Thus, decorin and lumican seem to be more strongly expressed in gingival and mucosal connective tissue as compared with periodontal ligament while biglycan is most abundantly expressed in periodontal ligament and fibromodulin shows relatively even expression level in various periodontal connective tissues.

This study did not localize biglycan in alveolar bone, although it is abundantly expressed in bone (Bianco et al, 1990). Furthermore, we did not localize the other small leucine-rich proteoglycans to the other dental hard tissues, although decorin, biglycan, fibromodulin

and lumican were expressed in the predentin to varying degrees. Previous studies have demonstrated the existence of these proteoglycans in mineralized tissues (Hall et al, 1997; Embery et al, 2001; Goldberg et al, 2002; Cheng et al, 1996; Cheng et al, 1999; Ababneh et al, 1999; Bartold et al, 1990). The lack of immunospecificity for these proteoglycans in the oral hard tissues in our study may be due to their loss during the demineralization treatment or the fixation, dehydration and embedding steps during processing (Goldberg et al, 2002). Although decorin and biglycan have been detected in epithelial cells of the gastric tissue using immunohistological techniques (Pohle et al, 2001) and biglycan has been detected in epithelium of developing human dermal tissue (Bianco et al, 1990), no decorin or biglycan was found in oral mucosal epithelium supporting findings of previous reports on dental tissues (Hakkinen et al, 1993; Hakkinen et al, 2000). Slight fibromodulin immunoreactivity was noted in the basal epithelial layers of fibromodulin knockout mice. Since these mice are lacking the fibromodulin genes necessary to synthesize fibromodulin, it is believed that the staining may be the result of cross-reactivity with other proteins in this tissue. The distribution of the small leucine-rich proteoglycans in different dental tissues would suggest that they have unique functional roles in the tissues where they accumulate. Several of the small leucine-rich proteoglycans show an overlap in distribution, suggesting a possible overlap in function in these tissues.

5.3 Fibromodulin And Lumican Compensate For Each Other

To determine if any compensation upregulation was occurring among the small leucine-rich proteoglycans in the knockout mice, mesiodistal sections of fibromodulin, lumican,

double knockout and wild-type mice were stained with anti-lumican, anti-fibromodulin, anti-biglycan and anti-decorin antibodies using standardized procedures. It was found that fibromodulin was more intensely stained in the periodontal ligament of lumican knockout mice than wild-type mice and lumican was more intensely stained in the periodontal ligament of fibromodulin knockout mice than wild-type mice. These findings may suggest a compensation mechanism between lumican and fibromodulin in single knockout mice.

Compensation between lumican and fibromodulin is an expected finding since both of these small leucine-rich proteoglycans share the same binding site on type I collagen with fibromodulin having a higher affinity (Svensson et al, 2000). Compensatory changes in expression between different small leucine-rich proteoglycans have already been *observed* using protein analysis (Svensson et al, 1999; Svennson et al, 2000; Ezura et al, 2000; Jepsen et al, 2002) or *suggested* based on severity of double knockout phenotypes compared to single knockout phenotypes (Ameye et al, 2002; Corsi et al, 2002). For lumican and fibromodulin, a compensation mechanism was noted previously. Jepsen et al (2002) and Svensson et al (1999) noted a 2-fold and 4-fold increase in fibromodulin respectively using lumican protein analysis in fibromodulin knockout mouse flexor-digotorum longus tendon of the limb and tail tendon respectively. Jepsen et al (2002) reported, however, that lumican knockout mice expressed *less* fibromodulin than wild-type mice, a contradiction to this report. One reason for this might be that different small leucine-rich proteoglycans, including fibromodulin, may play different roles in different tissues since Jepsen's study was on mouse tendon. In this study, we used

immunohistochemical stains to analyze the abundance of proteoglycans. This method is only semi-quantitative, and should be confirmed by biochemical analysis of these proteoglycans in the periodontal ligament. However, results of the staining intensities observed correlate with the severity of phenotypes observed in the knockout mice.

5.4 No Gross Morphologic Changes In Jaws And Teeth In Knockout Mice

The jaws of mice deficient in fibromodulin, lumican or both were intact and all teeth were present. A difference was noted in lengths of maxillary mouse jaws among the groups. The mean mouse jaw length of the single and double knockout mice were smaller than the that of the wild-type mice. The jaws of the double knockout mice, on average, were smaller than either of the single knockout varieties. Although these findings were not found to be statistically significant in this report, they do support previous findings that mice deficient in lumican, fibromodulin or both have smaller bodies than wild-type mice with double knockout mice being the smallest (Jepsen et al, 2002). Perhaps the reason for the lack of significance in this study is due to small sample size used. As well, a more accurate measure of jaw length may be reflected in mandibular jaw length measurements rather than maxillary jaw length as reported here. It should be noted, however, that a previous report noted that fibromodulin knockout mice developed with no gross anatomical defects and grew to normal size (Svensson et al, 1999) while lumican knockout mice were significantly smaller (70-80% body weight) than wild-type littermates at birth (Chakravarti et al, 1998).

Fibromodulin, lumican and double knockout mice showed no gross structural abnormalities in hard dental tissues, including bone, cementum and dentin (excluding enamel), compared to those of wild-type mice using the investigative techniques employed in this study. All teeth of the knockout and wild-type mice were present and fully erupted and there was no evidence of bone loss or inflammation that would indicate periodontitis in any of the mice studied. Periodontitis does not usually occur naturally in mice (Page & Schroeder, 1982), although some studies have reported it in some wild strains (Sheppe et al, 1965; Wiebe et al, 2001). To this extent, it is not surprising that no alveolar bone loss indicative of periodontal disease was found in any of the knockout mice. Bone loss was found associated with one fibromodulin knockout mouse as determined by the presence of class II furcation defects (Wiebe et al, 2001), which may be a result of hair impaction (Page & Schroeder, 1982). No bone loss was detected in any of the other mice.

5.5 Most Oral Soft Tissues Showed No Gross Morphologic Changes

No gross structural morphologic changes were noted among the dental soft tissues, including pulp, mucosal and gingival epithelium, mucosal and gingival connective tissues in any of the knockout mice compared to wild-type mice.

5.6 Collagen Fiber Bundles Of The Knockout Periodontal Ligaments Have Altered Morphology

Some general trends were noted in the periodontal ligament of all mouse groups. Collagen fiber bundles were more disorganized in the apical third of the tooth root, which

might be due to the continuing migration of these teeth and the constant, quick deposition of cementum at the apex (Page & Schroeder, 1982). For this reason, we used the coronal and middle third of the root to analyze collagen fiber bundle morphology and organization in the periodontal ligament. As well, collagen fiber bundles on the buccal surface of the root were more organized than those on the lingual surface of the root as seen in H&E, PTAH, PSR and immunostained bucco-lingual sections, which might be explained by the fact that the teeth erupt in an occluso-buccal direction (Page & Schroeder, 1982).

Wild-type mice displayed collagen fiber bundles in a sheet-like arrangement with smooth outlines, of relatively even thickness that traversed the space between tooth and bone such that fibers could almost be traced across the periodontal ligament space. As well, the spaces between the fiber bundles were evenly distributed. These findings support previous reports in the literature. Fiber bundles cross the entire width of the periodontal space but branch *en route* and anastomose with adjacent fiber bundles to form a complex three dimensional network (Sloan, 1978b; Sloan 1979b) and as a result, it may be impossible to trace an intact network of bundles across the periodontal space (Sloan & Carter, 1995). Collagen *fibrils* in the rat periodontal ligament (Berkovitz et al, 1981) and of the mouse tendon (Ezura et al, 2000) have been reported to have a unimodal distribution with collagen fibrils being small and of essentially equal diameter. However, these reports are based on studies of collagen *fibrils* (diameter ~50nm) using a transmission electron microscope, whereas this study analyzed collagen fiber *bundles* (diameter ~10μm) with a scanning electron microscope. The sheet-like arrangement of

collagen fiber bundles has been reported previously in scanning electron microscope study of continuously erupting rat incisors (Carter & Sloan, 1994). Molars were analyzed in this study confirming a three-dimensional model of the periodontal ligament (Sloan, 1978a). In this model, the bundles closest to bone and cementum are round with the alveolar bundles being larger than the latter. The middle zone of fiber bundles in this model are arranged into thin sheets that form a series of flattened compartments running along the axis of the tooth.

Fibromodulin, lumican, and double knockout mice displayed periodontal ligaments with abnormal morphology and organization of the collagen fiber bundles with a relatively increased amount of space between them. Although the basic fiber bundle orientation was apparent in many samples of all four groups of mice, the periodontal ligaments of the knockout mice were characterized by disorganized fiber bundles of varying widths as seen under light and scanning electron microscopy. All three knockout ligaments had relatively thinner bundles than wild-type. Fibromodulin knockout fiber bundles appeared *clumpy*, lumican knockout bundles appeared *stringy* and double knockout ligaments contained fiber bundles with both of these characteristics. Moreover, scanning electron micrographs of the periodontal ligaments revealed the presence of several small branches protruding from the main collagen fiber bundles present in all knockout groups.

Graham et al (2000) showed in a study of collagen fibril fusion in tendon that fibroblasts synthesize transient, early fibril intermediates that fuse in an end-to-end fashion to generate long fibrils. Small leucine-rich proteoglycans were noted to cover the long

surfaces of these fibrils but not the tips of the fibrils, thus appearing to act as inhibitors of lateral fusion. When small leucine-rich proteoglycans were absent, the fibril intermediates aggregated laterally, thus demonstrating that small leucine-rich proteoglycans promote end-to-end fusion of collagen fibril intermediates and inhibit lateral fusion. Ezura et al (2000) proposed that collagen fibril intermediates are stabilized through their interactions with fibril-associated macromolecules, such as the small leucine-rich proteoglycans lumican and fibromodulin while fusion of fibril intermediates occurred in a multi-step manner to generate a mature fibril. In this model, based on studies of mouse tendon in lumican and fibromodulin and double knockout mice, it seems that lumican and fibromodulin have temporal functions associated with this process whereby lumican functions together with fibromodulin in the early stages with progressively less lumican and progressively more fibromodulin acting as the tissue matures.

While these models offer information on the formation of collagen fibrils, they do not explain how suprafibrillar structures, like the collagen fiber bundles of the periodontal ligament, are formed. The collagen binding model of Weber et al (1996) and rotary shadowing-electron microscopy (Scott, 1996) suggest that small leucine-rich proteoglycans are horseshoe shaped which, based on decorin structure, would have just enough space in the concave portion for a single tropocollagen triple helix to bind (Weber et al, 1996). If this is so, then what role do small leucine-rich proteoglycans play in the formation of collagen fiber bundles of the periodontal ligament?

Different tissues have different suprafibrillar architectures (Giraud-Guille, 1996). For example, cornea, bone osteons, tendon and dermis have individual collagen fibril arrangements. Corneal fibrils are arranged in multiple layers or parallel lamellae, bone osteons have concentric lamellae, tendon fibers exhibit a crimp in their structure and dermis has a complex three-dimensional weave. These different suprafibrillar structures are characterized by distances on the scale of several micrometers, compared to the 0.3μm length of a single collagen molecule (Hulmes, 2002).

The periodontal ligament is comprised of parallel collagen fiber bundles (principle fibers) that extend across the periodontal space in a complex wavy course (Sloan, 1978b). They are named according to their location and direction. A "bundling mechanism" was described by Yamamoto & Wakita (1991) and is based on observations of the developing rat molar. This mechanism proposes that *loose* fibril bundles develop into *tight* fibril bundles as root formation takes place and involves the formation of compartments within the tissue by cellular processes. However, how the exact mechanism involved in the bundling of fibers remains unclear, but seems to most certainly involve the small leucine-rich proteoglycans, fibromodulin, lumican (as seen in this study) and decorin (Hakkinen et al, 2000). It is possible that the constituent collagen fibrils arrange themselves parallel to each other forming in essence, one large collagen fiber, in which case the so-called "collagen fiber bundle" would be more appropriately termed a "collagen *fibril* bundle" (Fig. 23). Alternatively, the fibrils may be arranged in smaller subunits (fibers) that come together to form bundles of collagen fibers (Fig. 24). The term "collagen fiber bundle" is frequently associated with the periodontal ligament in the literature and would certainly

imply the latter. This model is supported by the scanning electron microscope findings in this study using a micrometer scale since collagen *fibrils* have a reported diameter measured in nanometers.

Scanning electron microscopy of fibromodulin, lumican and double knockout mouse periodontal ligaments revealed the presence of very thin filamentous structures that appeared to be branching from the larger collagen fiber bundle. These branching structures were not present in wild-type mice. The filamentous structures seen may represent single collagen fibers that have broken away or failed to fuse with the rest of the collagen fiber bundle and is suggestive of altered lateral fusion of the fibers within the fiber bundle. It is clear that small leucine-rich proteoglycans play a role in the organization of the collagen fiber bundles of the periodontal ligament but seems likely that the mechanism by which they do so is different than that suggested at the molecular / fibril level.

Fibromodulin knockout mice in this study displayed periodontal ligament fiber bundles that were thinner than those of the wild-type but thicker than lumican knockout mice. Those of lumican knockout mice seemed relatively thinner as seen under light microscope in coronal and mid-root sections stained with H&E, PTAH, under polarizing light microscope with PSR stained sections and in scanning electron micrographs. Jepsen et al (2002) and Ezura et al (2000) reported in studies of mouse limb tendon that fibromodulin knockout mice had a higher number of thinner fibrils and lumican knockout mice had a higher number of thick fibrils compared to wild-type mice. Moreover, it

appears that lumican and fibromodulin have differential expression during mouse tendon development with lumican being expressed early and acting predominantly and fibromodulin expression increasing with age assuming a predominant role in collagen fibrillogenesis (Ezura et al, 2000). From this, it seems that lumican is required for early fibril development while fibromodulin in required for maturation of collagen fibrils. The different findings may be due to the different levels of analyzation since these studies looked at individual collagen fibrils using a 50 nm scale and this study looked at fiber bundles using a 10 μm scale. In the present study, we analyzed at histological and scanning electron microscope levels, the organization and morphologies of the periodontal ligament fiber bundles that are formed by collagen fibers that have joined together. We did not analyze the diameter of the individual collagen fibers. It is possible that the lack of proteoglycans may effect differently the formation of the collagen fibrils and how these fibers join to form highly organized fiber bundles typical of the periodontal ligament.

The rapid turnover of the periodontal ligament may result in phenotypes that differ from that in other tissues. It might also be possible that the constant renewal experienced in the periodontal ligament may alter the expression of lumican and fibromodulin in this tissue and the phenotypes expressed. The turnover rate of the periodontal ligament is very high and has been estimated to be 15x that of skin and 5x that of alveolar bone with respects to its collagenous components (Sodek et al, 1977). Turnover and remodeling in the periodontal ligament involve rapid synthesis and breakdown of matrix components most notably the meshwork of type I collagen fiber bundles that stretches out between

cementum and bone (Beersten et al, 1997). One might expect this turnover rate to be quite high in the mouse relative to other animal models especially humans considering their shorter life span and that their molars are continuously erupting (Page & Schroeder, 1982).

The findings of collagen fiber bundle morphology and organization were further substantiated with analysis of collagen fibers labeled with anti-type I collagen antibody. The periodontal ligament of the wild-type mice were found to have a diffuse staining pattern compared to those of knockout mice which demonstrated a more discreet staining of the fibers. This finding might suggest homogenous fiber bundle diameters in the periodontal ligament of the wild-type mice. The increased inter-bundle spacing and heterogenous nature of the fiber bundles of the knockout periodontal ligaments may make the staining appear more discreet. Hakkinen et al (2000) noted that decorin knockout mice, which were found to have altered collagen fibril diameters by transmission electron microscopy, had more diffuse staining of the periodontal ligaments than wild-type mice. The reason for this different collagen fiber organization at the light microscope level may be the effect of different small leucine-rich proteoglycans being investigated.

Picrosirius red stain and polarizing light microscopy was used in this study to investigate periodontal ligament collagen fiber bundle morphology. Picrosirius red is a strong anionic dye that stains collagen in such a way that the long axes of the elongated dye molecules are aligned with the collagen molecules resulting in an enhanced birefringency and is thus thought to be somewhat *specific* to collagen (Junqueira et al, 1979), although

Sirius red has been reported to bind to other basic amino acids via electrostatic forces (Nielsen et al, 1998). The enhanced birefringency of collagen can be appreciated with a polarizing light microscope.

Previous studies using this technique have reported that fibers of varying thickness show different colors under the polarizing light microscope. Some report that thick collagen fibers (1.6 – 2.4 μm) appear as yellow / green in color while thinner collagen fibers (<.8 μm) show a red / orange polarization color (Hirshberg et al, 1999). Other work determined that thin fibrils show a yellow / green polarization color and thick fibers show a red / orange polarization color (Dayan et al, 1989). The different collagen types being analyzed as well as perhaps the different tissues being examined can explain the discrepancy in these reports. The different colors have been attributed not only to varying collagen fiber thickness, but also to the tightness of the pack between collagen fibers as well as their alignment. Tightly packed, well-organized collagen fibers tend to have a polarization color of red / orange when stained with picrosirius red (Dayan et al, 1989). The different staining colors were not detected in this study presumably because the fibers were all within the "thin range" and all stained red / orange. Collagen fiber diameters of mammalian periodontal ligament are relatively small with mean diameters in the order of 45-55 nm with a unimodal distribution (Berkovitz et al, 1981; Luder et al, 1988). In other connective tissues, like tendon, fibril diameters may reach 250 nm. There is evidence that the diameter of collagen fibers increase with age and the small diameter of the periodontal ligament fibers could be the result of high turnover rate of this structure (Berkovitz et al, 1981) or a lack of mature collagen fibrils. Crocodile have been

reported to have large diameter fibril up to 250 nm, where collagen fiber turnover is slow (Berkovitz and Sloan, 1979). There are no significant differences in fibril diameter in continuously growing incisors (higher turnover rate) and non-continuously growing rat molars (lower turnover rate) (Berkovitz and Moxham, 1989). At the same time there is no increase in human fibril diameter in the maturing human periodontal ligament (Luder et al, 1988). There is no reported diameter for mouse periodontal ligament fiber bundles, although one would presume that it was smaller than those reported above given the smaller dimensions of the mouse dentition and the presumed rapid turnover of the fiber bundles around the continuously erupting teeth (Page & Schroeder, 1982).

Analysis of sections stained with picrosirius red revealed the presence of dark bands that appeared to be associated with the collagen fiber bundles of wild-type, fibromodulin and lumican knockout mouse periodontal ligaments. The banding is consistent with previous reports of crimping of collagenous tissues (Diamant et al, 1972; Keller & Gathercole, 1976). The "crimp" is a term that is used to describe the quantifiable periodicity of structure of various collagenous tissues. In polarized light, the crimp can be recognized as a regular banding of dark lines across a collagenous bundle. Polarized light analysis of the periodontal ligament fiber bundles has demonstrated this crimping arrangement previously (Diamant et al, 1972). The increased "banding" seen in the single knockout mice and relative absence in the double knockout mice further suggests alterations in the collagen fiber bundles of the periodontal ligament of these mice.

5.7 More Blood Vessels In Fibromodulin Knockout Periodontal Ligament

Using H&E and PTAH-stained sections, blood vessels appeared to be more numerous on the buccal side of the periodontal ligament than the lingual side in the wild-type, lumican knockout and double knockout groups while a more even distribution was found in the fibromodulin knockout group. Having a higher number of blood vessels on one surface than the other might be explained by the direction of eruption of the molar teeth. One would expect a higher number of blood vessels in mouse molars to be on the lingual / tension side of the periodontal ligament since the molars erupt in a buccal direction (Rygh & Brudvik, 1995) although the forces of eruption and migration cannot be compared to horizontal orthodontic forces. The significance of the more even distribution in the fibromodulin knockout mice is unclear from this study but may be due to altered eruption of the teeth due to alterations in the collagen fiber bundles of the periodontal ligament. Double knockout mouse periodontal ligaments had more blood vessels than the lumican knockout mice and wild-type mouse periodontal ligaments. Fibromodulin knockout mice, however, displayed more blood vessels in the periodontal ligaments that any of the other groups. These results were only found to be statistically significant when lingual surfaces were compared.

A case report of a type VIII Ehlers-Danlos syndrome patient receiving orthodontic therapy noted that the patient experienced severe apical root resorption and alveolar bone loss during the orthodontic therapy. The author suggested that these findings might be attributed to altered collagen fibers in the periodontal ligament making them weaker and unable to withstand routine orthodontic forces on the tension side of the periodontal

ligament. He suggested that this decreased structural integrity resulted in over-compression on the opposite side of the tooth which then led to increased hyalinization and circular disorders that were enhanced by vascular fragility leading to resorption of cementum and dentin (Karrer et al, 2000).

Previous in vitro and in vivo studies suggest that at least one small leucine-rich proteoglycan, decorin, is involved in angiogenesis (Jarvelainen et al, 1992; Schonnher et al, 1999; Gutierrez et al, 1997; Nelimarkka et al, 2001). In vitro studies suggest that decorin promotes blood vessel growth since: 1. large vessels express decorin when they begin to grow new capillaries (Jarvelainen et al, 1992) and 2. macrovascular endothelial cells form tubes in collagen lattices when they are transduced to overexpress decorin in vitro, while control cells do not (Schonnher et al, 1999). While the exact mechanism by which decorin regulates blood vessel growth is unclear, it may be due to its ability to bind collagen or interact with TGF-β or EGFR. The findings of the present study suggest that other small leucine-rich proteoglycans, such as fibromodulin, may also play a role in this process. The fact that fibromodulin and double knockout mouse periodontal ligaments had increased amounts of blood vessels suggest that presence of fibromodulin would down-regulate blood vessel growth.

H&E and PTAH-stained sections were analyzed and effort was made to count only those structures that contained a lumen, lined with endothelial cells and what appeared to be blood cells inside. In some sections the blood cells appeared to be stained of equal intensity to pulp cells (presumably blood cells) and darker than epithelial cells, but in

other sections both blood cells in the periodontal ligament and pulp stained with equal intensity as the epithelium raising questions to their true identity. To confirm these findings, further research should be employed using immunohistochemical techniques using antibodies against endothelial cells (antibodies for CD31 or von Willebrands Factor) to accurately identify blood vessels. One possibility is that with collection of the blood vessel data, what appears to be or what was counted as one blood vessel might actually be one blood vessel that was cut several times during sectioning. This would be dependent on the angulation of the cut or the curvature of the vessel. In any case, it is clear that the fibromodulin was different than the other mouse groups and further investigation is warranted to determine what these differences are.

5.8 More Disruptions In Fibromodulin Knockout Mouse Periodontal Ligaments

In histological sections, disruptions or breaks in the periodontal ligament appeared more frequently in the of fibromodulin knockout mice than the other mouse groups (not statistically significant). It is possible that the breaks are a result of sectioning procedures and represent artifacts in the periodontal ligament. Interestingly, however, the wild-type mice had very few breaks compared to any of the knockout mice. It is possible that the knockout mouse ligaments are lacking the structural integrity to resist these breaks during the processing of the samples. Small leucine-rich proteoglycans play a role in collagen fibrillogenesis and they also have been noted to provide the tendon with maintenance of hydration thereby increasing tissue strength to tensile stresses (Cribb & Scott, 1995). The existence of thinner fibrils due to decreased lateral fusion (Ezura et al, 2002) or reduced cross-linking of collagen molecules (Kadler, 1995) might also reduce the structural

integrity of this tissue. Other studies have shown that mice lacking lumican and decorin have increased skin fragility (Danielson et al, 1997; Chakravarti et al, 1998; Corsi et al, 2002) and those lacking lumican, fibromodulin or both (Jepsen et al, 2002) and biglycan, fibromodulin or both (Ameye et al, 2002) have dramatically less tendon stiffness. Certainly, the seemingly increased susceptibility of the periodontal ligament to tearing raises questions about the structural integrity of the periodontal ligament to periodontal breakdown and further analysis is required to determine the exact role of lumican and fibromodulin in providing strength to the periodontal ligament.

5.9 Fibromodulin Knockout, Lumican Knockout And Double Knockout Mice Express *Mild* Oral Phenotypes In Vivo

Mice deficient in a small leucine-rich proteoglycans exhibit microscopic and macroscopic phenotypes. Biglycan knockout mice show macroscopic phenotypes in that they exhibit decreased growth rate characterized by decreased bone mass (Xu et al, 1998). They also exhibit a microscopic phenotype characterized by thin skin but no macroscopic phenotype is seen in the skin of these mice (Corsi et al, 2002). Decorin knockout mice exhibit macroscopic changes in skin characterized by increased skin fragility associated with thinning of the skin (Danielson et al, 1997). They also exhibit microscopic changes in bone characterized by decreased average fibril diameter and size range compared to wild-type mice but no macroscopic phenotype as a result of this (Corsi et al, 2002). These findings might be explained by the relative abundance of biglycan in bone relative to decorin and decorin in skin relative to biglycan while ultrastructural changes noted may imply similar roles in these tissues. Similarly, lumican deficiency results in a

macroscopic phenotype characterized by decreased transparency of the cornea (Chakravarti et al, 1998), a tissue where it is abundant. Macroscopic effects of missing small leucine-rich proteoglycans seem to be more severe than microscopic ones that can only be observed at the microscopic level.

The apparent morphological changes in the periodontal ligament collagen fiber bundles seen in this study expressed by lumican, fibromodulin and double knockout mice appeared not to be associated with clear functional defects. This may be due to the existence of other small leucine-rich proteoglycans compensating for those that are missing thereby partially masking the effect of the missing small leucine-rich proteoglycans. It has been suggested that the small leucine-rich proteoglycans may substitute or compensate for each other in these defect states (discussed above). Our findings also suggested that fibromodulin and lumican compensated for each other when one of the proteoglycans was missing. However, the most likely explanation is that mice do not naturally get inflammatory periodontal disease and therefore, structurally abnormal periodontal ligament cannot contribute to increased susceptibility to the disease. Perhaps if the animals are induced to develop periodontal disease, the functional significance of the lack of certain proteoglycans could be better answered.

Chapter Six - Conclusions And Future Directions

6.1 Conclusions

1. Fibromodulin, lumican, decorin and biglycan are abundantly expressed in the connective tissues of the periodontal ligament, gingiva and pulp.
2. The absence of fibromodulin, lumican or both results in morphological alterations of the collagen fiber bundles in the periodontal ligament characterized by altered surface morphology, relative increase in inter-bundle spacing, increased random organization of collagen fiber bundles as seen under light, polarizing and scanning electron microscopy.
3. A more severe phenotype is seen in mice lacking both fibromodulin and lumican with regard to collagen fiber bundle morphology than single knockout or wild-type mice suggesting a synergistic effect.
4. Fibromodulin and lumican appear to compensate for each other in the periodontal ligament of mice.
5. The absence of fibromodulin and / or lumican may result in increased number of blood vessels in the periodontal ligament.
6. The absence of fibromodulin and / or lumican may result in decreased integrity of the periodontal ligament because there appeared to be an increased number of tears in the ligaments of these mice after sectioning.
7. This study suggests that collagen fibers bind together to form collagen fiber bundles and fibromodulin and lumican play a role in this process.

6.2 Future Directions

Although significant progress has been made in the membership, structure and function of small leucine-rich proteoglycans, much work remains to be done. As more members of this family are targeted for deletion in transgenic mice, and as we understand more about the molecular structure of the small leucine-rich proteoglycans we may start to approach an understanding of their role in oral tissues.

Future research with regards to fibromodulin and lumican can be done to determine if the alterations noted in this study would decrease the integrity of the periodontal tissues to the extent that it would lead to an increased susceptibility to periodontal destruction of the collagen fiber attachment of the teeth. This can be done by inducing periodontitis in transgenic mice. As well, further research is required to determine the nature and extent of the role of fibromodulin and lumican on the periodontal ligament vasculature. Do these small leucine-rich proteoglycans regulate angiogenesis in the ligament and if they do what are the repercussions of this? Studies on vasculature can be done with immunostaining techniques specific for the endothelial cells lining the blood vessels or for von Willebrand's factor that is associated with blood vessels. Alternatively, India Ink stain can be used to highlight the vessels or corrosion cast specimens created by injection of low viscosity acrylic resin into the vessels to allow for a three dimensional view of the periodontal ligament vasculature.

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Appendix I - Tables

Table 1. The small leucine-rich proteoglycans (SLRPs). Class I SLRPs (EMC2, Decorin, Biglycan, PLAP-1), Class II SLRPs (Osteoadherin, PRELP-1, Keratocan, Lumican, Fibromodulin), Class III SLRPs (Mimecan, Opticin, Epiphycan) and others (yet unclassified: Chondroadherin, Nyctalopin) are shown. (DS = dermatan sulfate; PG = proteoglycan; KS = karatan sulfate; CS = chondroitin sulfate; GAG = glycosaminoglycan; LRR = leucine-rich repeat) (Neame and Kay, 2000; Iozzo, 1998).

	Other names	GAG (#)	Chromosome	Exons	Cys Spacing	LRR
EMC2			Human 9q21.3	8	CX_3CXCX_6C	10
Asporin			Human 9q22-9q21.3	8	CX_3CXCX_6C	10
Decorin (36kDa)	DS-PG-II; PG II; PG-S2; PG40	DS/CS * (1**)	Human 12q21.3-23 Mouse 10	8	CX_3CXCX_6C	10
Biglycan (38kDa)	DS-PG-I; PG-I; PG-S1	DS/CS (1-2)	Human Xq28 Mouse X	8	CX_3CXCX_6C	10
PLAP-1 (periodontal ligament associated protein)				8	CX_3CXCX_6C	10
Osteoadherin	Osteomodulin	KS (2-3)	9q22-9q21.3	3	CX_3CXCX_9C	10
PRELP-1 (proline argenine-rich end leucine-rich repeat protein)		KS (2-3)	Human 1q32 Mouse I	3	CX_3CXCX_9C	10
Keratocan	KSPG 37A protein	KS (3-5)	Human 12q Mouse 10	3	CX_3CXCX_9C	10
Lumican (38kDa)	KSPG 37B	KS (3-4)	Human 12q21.3-q22 Mouse 10	3	CX_3CXCX_9C	10
Fibromodulin (42kDa)		KS (2-4)	Human 1q32 Mouse 10	3	CX_3CXCX_9C	10
Mimecan	Osteoglycin, KSPG25, OIF (osteoinductive factor)	KS (2-3)	Human 9q22-9q21.3	7-8	CX_2CXCX_6C	6
Opticin			Human 1q32 Mouse I	7-8	CX_2CXCX_6C	6
Epiphycan (36kDa)	PG-Lb, DSPG3	DS/CS (2-3)	Human 12q21.3 Mouse 10	7-8	CX_2CXCX_6C	6-7
Chondroadherin						
Nyctalopin						

* adult chick cornea contains keratan sulfate.

** avian decorin contains two glycosaminoglycan side chains.

Table 2. Glycosaminoglycans (GAG) of oral tissues (CS = chondroitin sulfate; DS = dermatan sulfate; HA = hyaluronic acid; HS = heparan sulfate; KS = keratan sulfate; PDL = periodontal ligament) (refs in text).

Dental Tissue	GAG
Bone	CS, DS, HA, HS, KS
Dentin	CS, HA, HS, KS
Cementum	CS, DS, HA, KS, HS
Gingiva	DS, HA, CS, HS, KS
PDL	DS, CS, HA, HS, KS
Pulp	DS, CS, HA, KS

Table 3. Components of dental soft tissues (PDL = periodontal ligament, GAGs = glycosaminoglycans; KT = keratinized tissue) (Ten Cate, 1994).

	Cellular	Acellular
PDL	Osteoblasts, osteoclasts, fibroblasts (35% in mouse molar), epithelial cell rests of malassez, macrophages, cementoblasts, undifferentiated mesynchymal cells	Collagen fiber bundles (51% in mouse molar), collagen types I, III, V, VI, VII, XII, ground substance (glycoproteins, GAGs, PGs, glycolipids), vessels, nerves
Connective Tissue (Lamina propria)	Fibroblast, histiocyte, macrophage, monocyte, mast cell, PMNs, lymphocyte, plasma cell, endothelial cell, inflammatory cells (lymphocyte, plasma cells)	Collagen I, III, IV, VII, V (in inflamed tissue), ground substance (PGs, glycoproteins), elastic fibers, fibronectin, vessels, nerves
Gingival Epithelium (KT)	Keratinocytes, nonkeratinocytes (melanocytes, Langerhans' cells, Merkel's cells, lymphocytes)	Hyaluronic acid
Basement Membranes		Collagen IV and VII, laminins, proteoglycans (perlecan), entactin, bullous pemphigoid antigen, other glycoproteins
Pulp	Odontoblasts, fibroblasts, undifferentiated mesenchymal cells, macrophages, other immunocompetent cells	Collagen I & III in a ratio of 55:45, ground substance (GAGs, glycoproteins, water), vessels, nerves

Table 4. Collagen composition in dental and periodontal tissues (Ten Cate, 1994; Ababneh et al, 1999).

Tissue	Collagen type	Location	References
Bone	I	Bone matrix, Sharpey's fibers	Takita et al, 1987
	III	Sharpey's fibers, endosteal spaces	Wang et al, 1980
	IV	Basement membranes of blood vessels and nerves	Romanos et al, 1991; Becker et al, 1986
	V	Bone matrix	Bronckers et al, 1986; Becker et al, 1986
	VI		Becker et al, 1986
	X		
	XI		
Cementum	I	Sharpey's fibers, fibrillar cementum	Becker et al, 1991
	III	Sharpey's fibers and cementum	Wang et al, 1980
	IV		
	V	Sharpey's fibers	
	VI		
PDL	I	Principle and secondary fibers	Takita et al, 1987; Becker et al, 1991; Butler et al, 1975; Huang et al, 1991
	III	Principle and secondary fibers	Wang et al, 1980; Takita et al, 1987; Becker et al, 1991; Butler et al, 1975; Huang et al, 1991; Wang et al, 1980
	IV	Basement membranes of blood vessels and nerves	Romanos et al, 1991
	V	Collagen fibers	Romanos et al, 1991; Becker et al, 1991
	VI	Fine fibers	Becker et al, 1991; Romanos et al, 1991; Sloan et al, 1993
	XII		Dublet et al, 1988; Karimbux et al, 1992
Gingiva	I	Lamina propria. Main component of all layers.	Chavrier et al, 1981; 1984; 1994; 1999; Takita et al, 1987
	III	Lamina propria. Mainly in upper layers under epithelium and within blood vessel walls.	Wang et al, 1980; Chavrier et al, 1981; 1984; 1994; 1999; Takita et al, 1987; Wang et al, 1980
	IV	Basement membranes	Chavrier et al, 1981; 1984; 1994; 1999; Becker et al, 1986
	V	Collagen fibers, blood vessels	Romanos et al, 1991; Rabanus et al, 1991; Becker et al, 1986; Schuppan et al, 1986
	VI	Microfibrils	Romanos et al, 1991; Rabanus et al, 1991; Becker et al, 1986
Dentin	I		Takita et al, 1987; Magloire et al, 1983
	V	Dentin matrix	Bronckers et al, 1986
Pulp	I	Course cross banded fibrils	Magloire et al, 1982
	III	Fine branched filaments associate with plasma membranes and fibroblasts	Martinez et al, 2000; Magloire et al, 1982; Wang et al, 1980

Table 4. Collagen composition in dental and periodontal tissues (Ten Cate, 1994; Ababneh et al, 1999).

Tissue	Collagen type	Location	References
Bone	I	Bone matrix, Sharpey's fibers	Takita et al, 1987
	III	Sharpey's fibers, endosteal spaces	Wang et al, 1980
	IV	Basement membranes of blood vessels and nerves	Romanos et al, 1991; Becker et al, 1986
	V	Bone matrix	Bronckers et al, 1986; Becker et al, 1986
	VI		Becker et al, 1986
	X		
	XI		
Cementum	I	Sharpey's fibers, fibrillar cementum	Becker et al, 1991
	III	Sharpey's fibers and cementum	Wang et al, 1980
	IV		
	V	Sharpey's fibers	
	VI		
PDL	I	Principle and secondary fibers	Takita et al, 1987; Becker et al, 1991; Butler et al, 1975; Huang et al, 1991
	III	Principle and secondary fibers	Wang et al, 1980; Takita et al, 1987; Becker et al, 1991; Butler et al, 1975; Huang et al, 1991; Wang et al, 1980
	IV	Basement membranes of blood vessels and nerves	Romanos et al, 1991
	V	Collagen fibers	Romanos et al, 1991; Becker et al, 1991
	VI	Fine fibers	Becker et al, 1991; Romanos et al, 1991; Sloan et al, 1993
	XII		Dublet et al, 1988; Karimbux et al, 1992
Gingiva	I	Lamina propria. Main component of all layers.	Chavrier et al, 1981; 1984; 1994; 1999; Takita et al, 1987
	III	Lamina propria. Mainly in upper layers under epithelium and within blood vessel walls.	Wang et al, 1980; Chavrier et al, 1981; 1984; 1994; 1999; Takita et al, 1987; Wang et al, 1980
	IV	Basement membranes	Chavrier et al, 1981; 1984; 1994; 1999; Becker et al, 1986
	V	Collagen fibers, blood vessels	Romanos et al, 1991; Rabanus et al, 1991; Becker et al, 1986; Schuppan et al, 1986
	VI	Microfibrils	Romanos et al, 1991; Rabanus et al, 1991; Becker et al, 1986
Dentin	I		Takita et al, 1987; Magloire et al, 1983
	V	Dentin matrix	Bronckers et al, 1986
Pulp	I	Course cross banded fibrils	Magloire et al, 1982
	III	Fine branched filaments associate with plasma membranes and fibroblasts	Martinez et al, 2000; Magloire et al, 1982; Wang et al, 1980

Table 5. Components of dental hard tissues (HA = hydroxyapatite; PGs = proteoglycans; wt = weight; vol = volume) (Ten Cate, 1994).

	Inorganic	Components	Organic	Components
Bone	67%	HA	33%	25% collagen (I, V, VI, X, XI), 5% noncollagenous material (osteocalcin, sialoprotein (also known as osteopontin), phosphoprotein, osteonectin, bone specific protein), osteoblasts, osteocytes, osteoclasts
Cementum	50%	HA	90%	Collagen (I, III, IV, VI), PGs, glycoproteins, phosphoproteins, cementoblasts, cementocytes
Dentin	70% (wt) 45% (vol)	HA	20% (wt) 33% (vol)	Collagen (I, V), glycoproteins, PGs, phosphoproteins, plasma proteins, odontoblasts
Enamel	96%	HA & minerals	4%	TRAP peptide sequence (Tyrosine rich enamel amelogenin protein) interlaced between the crystals

Table 6. Types and distribution of collagen. (FACIT = fibril-associated collagens with interrupted triple helices; PDL periodontal ligament) (Ten Cate, 1994; Bartold & Narayanan, 1996; Kadler et al, 1996; Kadler, 1995; Becker et al, 1991; Dublet et al, 1988; Butler et al, 1975; Huang et al, 1991; Sloan 1993; Karimbux et al, 1992; Rabanus et al, 1991).

Types of collagen		Location
Fibrillar	I	Skin, bone, gingiva, PDL, cementum, dentin, pulp, tendon, ligament, cornea, inter-vertebral disc, kidney, muscle, salivary gland, cartilage, menisci, artery, granulation tissue, intracellular fibrils, calvaria, most other connective tissues
	II	Cartilage, vitreous humor, notochord, intervertebral disc, ear, developing bone, cornea, placenta, menisci
	III	Widespread distribution particularly where type I collagen is found. Embryonic connective tissue, pulp, PDL, skin, gingiva, aorta, bone, cartilage, tooth, ligament, muscle, eye, tendon, salivary gland, dentin, cementum, placenta, lung, kidney, menisci, liver, artery, Schwann cells, granulation tissue
	V	Widespread distribution particularly where type I collagen is found. Basement membranes, blood vessels, ligament, skin, dentin, gingiva, PDL, most soft tissues, corneal stroma, bone, spleen, placenta, muscle, ear, vitreous humor, teeth, menisci, oral mucosa, mammary gland, cartilage, perichondrium, lung, granulation tissue, kidney, artery, uterus
	XI	Typically found associated with type II collagen predominantly in cartilage, heart, skeletal muscle, calvaria, skin, brain
FACIT	IX	Cartilage, vitreous and developing cornea
	XII	Soft tissue, including PDL
	XIV	All tissues
	XVI	
	XIX	
Basement Membrane Collagens	IV	Basement membranes
Short Chain Collagens	VIII	Descemet's membrane, fetal heart
	X	Cartilage, pericellular matrix
Domains – MACIT's	XIII	Fetal epidermis, intestinal mucosa
	XVII	
Multiplexins	XV	
	XVIII	
Others	VI	Soft tissues including the PDL, skin, cornea, tendon, gingiva as well as cementum (minor amounts)
	VII	Anchoring fibrils, calvaria, tendon, PDL, cartilage, cervix

Table 7. The small leucine-rich proteoglycans (SLRP) that have been shown to bind fibrillar collagen thus far (Schonnher et al, 1995; Hedbom & Heinegard, 1989; Hansson et al, 2001; Neame et al, 2000; Font et al, 1998; Rada et al, 1993; Vogel et al, 1987;Bidanset et al, 1992; Whinna et al 1993; Hedbom & Heinegard, 1993).

SLRP	Collagen
Decorin	I, II, III, V
Biglycan	I, V
Fibromodulin	I, II, XI
Chondroadherin	II
Lumican	I

Table 8. In vitro studies attesting the role of small leucine-rich proteoglycans (SLRP) in collagen fibrillogenesis (LUM = lumican; DCN = decorin; ECM = extracellular matrix; FM = fibromodulin; PGs = proteoglycans).

Study	Study design – SLRP studied	Findings
Carlson et al, 2002	Cultured mutant LUM cells	Mutant LUM cells produced an unorganized ECM with altered fibril packing and structure compared to wild type cells The cys domain of the LUM molecule is an important factor in collagen fibrillogenesis
Neame et al, 2000	Recombinant DCN and LUM PG in collagen fibrillogenesis assay based on turbidity.	DCN and LUM act independently on collagen fibril formation LUM accelerates initial collagen fibril formation DCN retards initial collagen fibril formation LUM and DCN do not compete for binding sites on collagen fibrils LUM & DCN act to increase collagen fibril stability to thermal denaturation LUM & DCN result in reduced overall turbidity suggesting a lower collagen fibril diameter. The presence of both PGs retarded fibril formation to a greater degree than either one alone – a synergistic effect.
Svensson et al, 2000	Recombinant LUM, FM and DCN PGs and a collagen fibril formation/sedimentation assay	FM inhibits the binding of LUM and vice versa. FM and LUM do not affect the binding of DCN to collagen or vice versa. FM binds to collagen with 4x the affinity of that of LUM. High and low affinity binding sites for FM and LUM (ie. each have two binding sites) with higher affinity for them being had by FM.

Table 9. In vivo studies attesting to the role of SLRP in collagen fibrillogenesis. For a more extensive review of these studies, see Ameye & Young, 2002 (DCN = decorin; KO = knockout; LUM = lumican; BGN = biglycan; FM = fibromodulin; PDL = periodontal ligament).

Study	SLRP studied	Findings
Danielson et al, 1997	DCN KO mouse skin	DCN KO mice have fragile skin with reduced tensile strength. Electron microscope showed irregular outline and size variability of collagen fibrils. Suspect uncontrolled lateral fusion of collagen fibrils.
Chakravarti et al, 1998	LUM KO mouse tail, skin, eyes	LUM is strongly expressed during development of the mouse LUM does not affect expression of DCN Loosely arranged fibril orientation in the homozygous mutants LUM KO mice had skin that was more compliant and weaker than normal Corneal transparency was reduced in LUM KO mice. Fibril morphology was uniformly thin in wild and single LUM KO (LUM -/+) LUM -/- mouse corneas showed three key differences: 1. thicker fibrils present in addition to normal diameter fibrils. 2. many irregular shaped fibrils 3. increased interfibrillar spacing. Similar findings were seen in mouse tail cross sections and back skin.
Xu et al, 1998	BGN KO mouse bone	BGN KO mice exhibit an osteoporotic phenotype characterized by reduced growth rate and decreased bone mass that becomes more obvious with age.
Svensson et al, 1999	FM KO mouse tendon	Altered morphologic phenotype in mouse tail tendon characterized by fewer and abnormal collagen fibrils. Fibers were disorganized and had irregular and rough outlines and more thin fibers were evident than normal.
Ezura et al, 2000	LUM KO, FM KO, and double KO mouse leg tendon	Premature presence of collagen fibril heterogeneity in 4d double KO tendon compared to wild type tendon. Abnormally large number of small diameter collagen fibrils in later stages of development especially at 3m. Collagen fibrils had irregular profiles
Hakkinen et al, 2000	DCN KO mouse PDL	Abnormal morphology and organization of collagen fibrils associated with increased numbers of fibroblasts.
Corsi et al, 2002	BGN and DCN and double KO mice skin and bone	In skin, BGN KO mice exhibited collagen fibrils that were irregular in profile, had a broader size range and were less tightly packed. Skin in these mice was thinner but not fragile. DCN KO mice have thin and fragile skin. Double KO mice show macroscopic and microscopic skin changes that are worse than either single KO mice (additive effect). In bone, BGN KO mice exhibit a larger average fibril diameter and an osteopenic phenotype while DCN KO mice show smaller average fibril diameter but no macroscopic phenotype. Double KO mice bone exhibits abnormally shaped fibrils and an osteopenic phenotype worse than either single KO mice – a synergistic effect.
Ameye et al, 2002	BGN and FM and double KO mice tendon	Both single KO and double KO mutants showed joint specific and age specific ectopic ossification in tendons but were larger and more numerous in double KO mice. Double KO mice also show gate impairment and severe osteoarthritis that occurs at a much earlier age than either single KO mice. This osteoarthritic change was found to be due to structurally weak tendons. Double KO mice were also smaller than normal and single KO mice.
Jepsen et al, 2002	LUM and FM and double KO mice tendon	LUM KO mice showed slightly smaller body weight and size, slight decrease in FM expression, increased average fibril size, and no tendon stiffness. FM KO mice showed slightly smaller body weight and size, decreased tendon stiffness, increased LUM expression, decrease in average fibril size. Double KO mice displayed considerably smaller body size and weight, severe gate abnormality, joint laxity, osteoarthritis, bowed legs and marked tendon stiffness decrease with increased average number of thin fiber subpopulations.
Goldberg et al, 2002	BGN KO dentin, enamel	BGN KO mice displayed altered enamel formation that was 3-5x thicker than normal, decreased collagen fibril diameter in proximal third of predentin, increased collagen fibril diameter in central and distal thirds of predentin.

Table 10. Sex (M=male; F=female) and age (months) of all wild-type CD-1, fibromodulin knockout (FM-/-), lumican knockout (LUM-/-) and double knockout (FM/LUM-/-) mice used in this study.

# Wild-type Mouse	Sex	Age (m)	# FM-/- Mouse	Sex	Age (m)	# LUM-/- Mouse	Sex	Age (m)	# FM/LUM-/- Mouse	Sex	Age (m)
1	M	5	**1**	F	5.5	**1**	M	4.4	**1**	F	8
2	M	5	**2**	F	5.5	**2**	M	4.5	**2**	F	8
3	M	5	**3**	F	5.5	**3**	M	6	**3**	F	8
4	F	6	**4**	F	5.5	**4**	M	6	**4**	F	8
5	F	6	**5**	F	5.5	**5**	F	6	**5**	F	8
6	F	6	**6**	M	5.5	**6**	F	6	**6**	M	8
7	F	8	**7**	M	5	**7**	F	6	**7**	M	8
8	F	8	**8**	M	5				**8**	M	4.5
9	M	8									
10	M	8									

Table 11. Classification of alveolar bone loss in mice as described by Wiebe et al, 2001.

Grade	Description
I	Exposure of the furcation and horizontal bone loss extending into the furcation.
II	Exposure of the furcation with a through-and-through defect from the buccal to the oral surface of the tooth.
III	Through-and-through furcation defect with horizontal bone loss extending into the apical third of the root.

Table 12. List of mice used for each staining and analyzed. Stains include: hematoxylin & eosin (H&E), phosphotungstic acid hematoxylin (PTAH), picrosirius red (PSR), anti-type I collagen antibody, anti-lumican (anti-LUM) antibody, anti-decorin (anti-DCN) antibody, anti-fibromodulin (anti-FM) antibody, anti-biglycan (anti-BGN) antibody. Stains were carried out on buccal-lingual (Bu/Li) sections and mesio-distal (M/D) sections where indicated. Each slide contained 4 sections per slide. Refer to table 10 for age and gender of mice.

Stain	CD-1#	FM-/-#	LUM-/-#	FM/LUM-/-#
H&E (Bu/Li)	1,2,3,4,5,6	1,2,4,5	1,3,4,5	1,2,3,4,5
H&E (M/D)	1,2,3,6	2,4,5	1,2,3,4,5	1,3,4,5
PTAH (Bu/Li)	1,2,4,5,6	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5,8
PTAH (M/D)	1,2,3,6	2,3,4,5	1,2,3,4,5	1,2,3,4,5
PSR (Bu/Li)	1,3,4,5,6	1,2,4,5	1,2,3,4,5	1,2,3,4,5
Anti-type I collagen (Bu/Li)	1,2,3,4,5,6	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5,8
Anti-LUM/Anti-DCN (Bu/Li)	1,2,3,4,5,6	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5,8
Anti-LUM/Anti-DCN (M/D)	1,2,3,6	2,3,4,5	1,2,3,4	1,2,3,4
Anti-FM/ Anti-BGN (Bu/Li)	1,2,3,4,5,6	1,2,3,4,5	1,2,3,4,5	1,2,3,4,5,8
Anti-FM/Anti-BGN (M/D)	1,2,3,6	2,3,4,5	1,2,3,4	1,2,3,4

Table 13. Dilution table of antibodies used. anti-decorin (anti-DCN), anti-biglycan (anti-BGN), anti-lumican (anti-LUM), anti-fibromodulin (anti-FM).

Antibody	**Source**	**Dilution**
Anit-mouse DCN	Rabbit	1/500
Anti-mouse BGN	Rabbit	1/500
Anti-human FM	Rabbit	1/1000
Anti-mouse LUM	Rabbit	1/1000
Anti-mouse collagen type I	Rabbit	1/500

Table 14. Mice used for compensation study. Mesio-distal sections of CD-1 wild-type mice, fibromodulin-deficient (FM-/-) mice, lumican-deficient (LUM-/-) mice, and fibromodulin & lumican-deficient (FM/LUM-/-) mice were chosen and divided into 4 groups as noted below. Each groups was stained separately. Each slide then received anti-lumican (anti-LUM), anti-decorin (anti-DCN), anti-fibromodulin (anti-FM) or anti-biglycan (anti-BGN) antibodies for an equal and standardized amount of time. Sections from each groups were then compared and intensities of immunostains were determined to be +++, ++, + or 0 stain.

	anti-LUM & anti-DCN antibody				anti-FM & anti-BGN antibody			
Group	CD-1	FM-/-	LUM-/-	FM/LUM-/-	CD-1	FM-/-	LUM-/-	FM/LUM-/-
A	1	2	1	1	1	2	1	1
B	2	3	2	2	2	3	2	2
C	3	4	3	3	3	4	3	3
D	6	5	4	4	6	5	4	4

Table 15. Localization of decorin, biglycan, lumican and fibromodulin in dental tissues of wild-type CD-1 mice using non-standardized sections. Intensity of immunostaining was noted on a relative basis as +++ (most intense), ++ (moderately intense) or + (least intense). – indicates no immunostaining detected. Intensity measures are relative to other tissues *within* the stain group and not relative to each other (CT=connective tissue).

Tissue	**Decorin**	**Biglycan**	**Fibromodulin**	**Lumican**
Periodontal ligament	++	+++	++	++
Gingival CT	+++	+++	++	+++
Mucosal CT	+++	++	++	+++
Gingival epithelium	-	-	+	-
Mucosal epithelium	-	-	+	-
Pulp	+	+	++	+
Bone	-	-	-	-
Cementum	-	-	-	-
Dentin/predentin (p)	+(p)	++ (p)	+++(p)	+(p)

Table 16. Maxillary jaw length measurements (millimeters), age (months) and sex (m = male; f = female) of wild-type (CD-1) mice, fibromodulin knockout (FM-/-), lumican knockout (LUM-/-), fibromodulin/lumican knockout mice. (NA=could not assess due to breakage).

CD-1	Length	Age	Sex	**FM-/-**	Length	Age	Sex	**LUM -/-**	Length	Age	Sex	**FM/LUM-/-**	Length	Age	Sex
1	10.9	5	M	1	10.3	5.5	F	1	10.2	4.5	M	1	11.0	8	F
2	NA	5	M	2	10.5	5.5	F	2	11.3	4.5	M	2	10.8	8	F
3	NA	5	M	3	10.4	5.5	F	3	NA	6	M	3	10.2	8	F
4	NA	6	F	4	10.9	5.5	F	4	10.1	6	M	4	11.3	8	F
5	11.6	6	F	5	11.0	5.5	F	5	11.8	6	F	5	10.8	8	F
6	11.2	6	F	6	11.4	5.5	M	6	11.0	6	F	6	NA	8	M
7	NA	8	F	7	11.3	5	M	7	11.0	6	F	7	11.0	8	M
8	11.5	8	F	8	11.0	5	M					8	10.4	4.5	M
9	NA	8	M												
10	11.9	8	M												

Table 17. Jaw length comparisons of pooled data. Pooled jaw lengths of wild-type (CD-1) mice (n=5, mean age 6.6 months, male & female), fibromodulin knockout (FM-/-) (n=8, mean age 5.4 months, male & female), lumican knockout (LUM-/-) (n=6, mean age 5.5 months, male & female) and fibromodulin/lumican knockout (F/L-/-) (n=7, mean age 7.5 months, male & female) mice were compared using the student t statistic assuming equal variance. P values given for those comparisons that were statistically significant (*). NS = not significant.

Groups Compared	P value	Significance
CD-1 vs FM-/-	P=.03	*
CD-1 vs. LUM-/-	>.05	NS
CD-1 vs. F/L-/-	P=.02	*
FM-/- vs. LUM-/-	>.05	NS
FM-/- vs. F/L-/-	>.05	NS
LUM-/- vs. F/L-/-	>.05	NS

Table 18. Jaw length comparison of different age groups (m = months) within the wild-type (CD-1), fibromodulin knockout (FM-/-), lumican knockout (LUM-/-) and fibromodulin/lumican knockout (F/L-/-) mouse groups. P value not shown for statistically insignificant comparisons. NS = not statistically significant.

Group	Ages compared	P value	Significance
CD-1	5m vs. 6m	>.05	NS
	5m vs. 8m	>.05	NS
	6m vs. 8m	>.05	NS
FM-/-	5m vs. 5.5m	>.05	NS
LUM-/-	4.5m vs. 6m	>.05	NS
F/L-/-	4.5m vs. 8m	>.05	NS

Table 19. Jaw length comparisons of similar ages (m = months) between different groups. Wild-type (CD-1), fibromodulin knockout, lumican knockout, fibromodulin/lumican knockout groups are compared. P value only shown for those comparisons that were statistically significant (*) using the student t-test assuming equal variance. NS = not statistically significant.

Groups compared	P value	Significance
CD-1 5m vs. FM-/- 5m	>.05	NS
CD-1 5m vs. LUM-/- 4.5m	>.05	NS
CD-1 5m vs. F/L-/- 4.5m	>.05	NS
FM-/- 5m vs. F/L-/-4.5m	>.05	NS
LUM-/- 4.5m vs. F/L-/- 4.5m	>.05	NS
FM-/- 5m vs. LUM-/- 4.5m	>.05	NS
CD-1 6m vs. FM-/- 5.5m	>.05	NS
CD-1 6m vs. LUM-/- 6m	>.05	NS
CD-1 6m vs. F/L-/- 4.5m	>.05	NS
CD-1 6m vs. F/L-/- 8m	>.05	NS
FM-/- 5.5m vs. LUM-/- 6m	>.05	NS
FM-/- 5.5m vs. F/L-/- 4.5m	>.05	NS
LUM-/- 6m vs. F/L-/- 4.5m	>.05	NS
LUM-/- 6m vs. F/L-/- 8m	>.05	NS
CD-1 8m vs. F/L-/- 8m	.03	*

Table 20. Jaw length comparisons of males vs. females within the wild-type (CD-1), fibromodulin knockout (FM-/-), lumican knockout (LUM-/-) and fibromodulin/lumican knockout (F/L-/-) groups. P value is given only for those comparisons that were statistically significant (*) using the student t-test assuming equal variance. NS = not statistically significant.

Groups compared	P value	Significance
CD-1 male vs. female	>.05	NS
FM-/- male vs. female	.02	*
LUM-/- male vs. female	>.05	NS
F/L-/- male vs. female	>.05	NS

Table 21. Jaw length comparisons of males and females between the wild-type (CD-1), fibromodulin knockout (FM-/-), lumican knockout (LUM-/-) and fibromodulin/lumican knockout (F/L-/-) groups. P value is given only for those comparisons that were statistically significant (*) using the student t-test assuming equal variance. NS = not statistically significant.

Groups compared	P value	Significance
CD-1 male vs. FM-/- male	>.05	NS
CD-1 male vs. LUM-/- male	>.05	NS
CD-1 male vs. F/L-/- male	>.05	NS
FM-/- male vs. LUM-/- male	>.05	NS
FM-/- male vs. F/L-/- male	>.05	NS
LUM-/- male vs. F/L-/- male	>.05	NS
CD-1 female vs. FM-/- female	.007	*
CD-1 female vs. LUM-/- female	>.05	NS
CD-1 female vs. F/L-/- female	>.05	NS
FM-/-female vs. LUM-/- female	>.05	NS
FM-/- female vs. F/L-/- female	>.05	NS
LUM-/- female vs. F/L-/- female	>.05	NS

Table 22. Jaw length comparisons of male mice of similar ages (m = months). Comparisons made between wild-type (CD-1), fibromodulin knockout (FM-/-), lumican knockout (LUM-/-) and fibromodulin/lumican knockout (F/L-/-) groups. P value is given only for those comparisons that were statistically significant (*) using the student t-test assuming equal variance. NS = not statistically significant.

Groups compared	P value	Significance
CD-1 5m male vs. FM-/- 5m male	>.05	NS
CD-1 5m male vs. LUM-/- 4.5m male	>.05	NS
CD-1 5m male vs. LUM-/- 6m male	?	NS
CD-1 5m male vs. F/L-/- 4.5m male	?	NS
FM-/- 5m male vs. LUM-/- 4.5m male	>.05	NS
FM-/- 5m male vs. LUM-/- 6m male	>.05	NS
FM-/- 5m male vs. F/L-/- 4.5m male	>.05	NS
LUM-/- 4.5m male vs. F/L-/- 4.5m male	>.05	NS
CD-1 8m male vs. F/L-/- 8m male	?	NS

Table 23. Jaw length comparisons of female mice of similar ages (m = months). Comparisons made between wild-type (CD-1), fibromodulin knockout (FM-/-), lumican knockout (LUM-/-) and fibromodulin/lumican knockout (F/L-/-) groups. P value is given only for those comparisons that were statistically significant (*) using the student t-test assuming equal variance. NS = not statistically significant.

Groups compared	P value	Significance
CD-1 6m female vs. FM-/- 5.5m female	.03	*
CD-1 6m female vs. LUM-/- 6m female	>.05	NS
CD-1 6m female vs.F/L-/- 8m female	>.05	NS
FM-/- 5.5m female vs. LUM-/- 6m female	>.05	NS
FM-/- 5.5m female vs. F/L-/- 8m female	>.05	NS
LUM-/- 6m female vs. F/L-/- 8m female	>.05	NS
CD-1 8m female vs. F/L-/- 8m female	>.05	NS

Table 24a. Table of number of blood vessels counted in the periodontal ligament of CD-1 wild-type, fibromodulin knockout (FM-/-), lumican knockout (LUM-/-) and double knockout (FM/LUM-/-) mice. Table shows mice from which data was collected and blood vessels counted on buccal and lingual sides. Blood vessels were counted only in the coronal two thirds of the periodontal ligament (#=mouse number; Bu=buccal; Li=lingual).

	Wild-type			FM-/-			LUM-/-			FM/LUM-/-		
	#	Bu	Li	#	Bu	Li	#	Bu	Li	#	Bu	Li
	1	4	0	1	2	1	1	3	0	1	2	1
	2	3	0	2	4	2	2	0	0	2	3	1
	3	2	0	3	4	3	3	1	0	3	3	2
	4	2	0	4	1	2	4	2	1	4	2	2
	5	1	0	5	0	3				5	2	0
										8	1	1
Means		2.4	0		2.2	2.2		1.5	.25		2.2	1.2

Table 24b. Table of number of tears counted in the periodontal ligament of CD-1 wild-type, fibromodulin knockout (FM-/-), lumican knockout (LUM-/-) and double knockout (FM/LUM-/-) mice. Table shows mice from which data was collected and tears counted in the entire periodontal ligament (#=mouse number).

	Wild-type		FM-/-		LUM-/-		FM/LUM-/-	
	#	Tears	#	Tears	#	Tears	#	Tears
	1	0	1	4	1	1	1	0
	1	0	2	0	2	3	2	1
	2	2	3	5	3	0	3	1
	3	0	4	2	4	2	4	4
	4	2	5	2	5	1	5	2
	5	1					8	0
	6	2						
Means		1		2.6		1.4		1.3

Table 25. Blood vessels of wild-type (CD-1), fibromodulin knockout (FM-/-), lumican knockout (LUM-/-), fibromodulin/lumican knockout (F/L-/-) mice are compared in total (pooled), as well as for buccal (Bu) and lingual (Li) periodontal ligament surfaces. P value is shown only for those comparisons that were statistically significant (*) with the student t-test assuming equal variance.

Groups compared	Pooled P value	Bu P value	Li P value
CD-1 vs. FM-/-	>.05	>.05	.0004*
CD-1 vs. LUM-/-	>.05	>.05	>.05
CD-1 vs. F/L-/-	>.05	>.05	.007*
FM-/- vs. LUM-/-	>.05	>.05	.005*
FM-/- vs. F/L-/-	>.05	>.05	>.05
LUM-/- vs. F/L-/-	>.05	>.05	>.05

Table 26. Tears of the periodontal ligament are compared between wild-type (CD-1), fibromodulin knockout (FM-/-), lumican knockout (LUM-/-), fibromodulin/lumican knockout (F/L-/-) mice are compared using pooled data. P value is shown only for those comparisons that were statistically significant (*) with the student t-test assuming equal variance.

Groups compared	P value	Significance
CD-1 vs. FM-/-	>.05	NS
CD-1 vs. LUM-/-	>.05	NS
CD-1 vs. F/L-/-	>.05	NS
FM-/- vs. LUM-/-	>.05	NS
FM-/- vs. F/L-/-	>.05	NS
LUM-/- vs. F/L-/-	>.05	NS

Appendix II - Figures

Figure 1. Basic proteoglycan structure can be seen in the schematics of decorin and biglycan small leucine-rich proteoglycans. Proteoglycans have a protein core with N- and C- terminal domains with glycosaminoglycans attached to it. In the case of biglycan and decorin, the glycosaminoglycan chains are either chondroitin sulfate (CS), as depicted here, or dermatan sulfate, as in the periodontal connective tissues, depending its tissue distribution. Larger proteoglycans have much more complex structures.

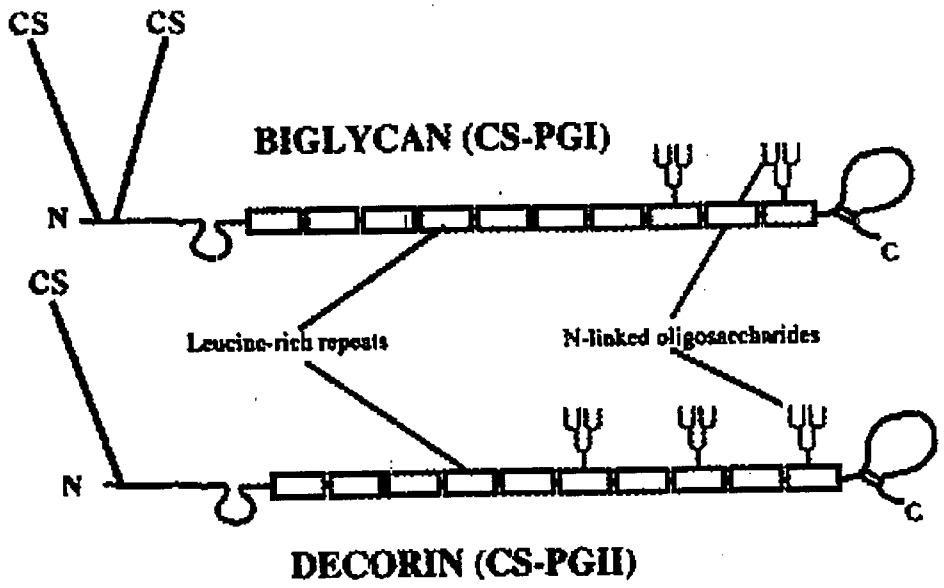


Figure 2: Basic structure of the small leucine-rich proteoglycan protein core (Neame & Kay, 2000).

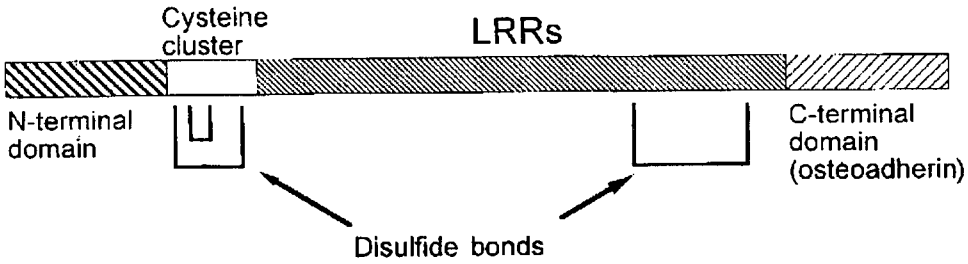


Figure 3. A schematic of the molecular organization of some representative small leucine-rich proteoglycans. Similarities can be seen among decorin and biglycan (two class I family members) and fibromodulin and lumican (two class II family members). The core proteins do not represent their actual lengths nor do the sites of glycosylation and leucine-rich repeats represent their actual location.

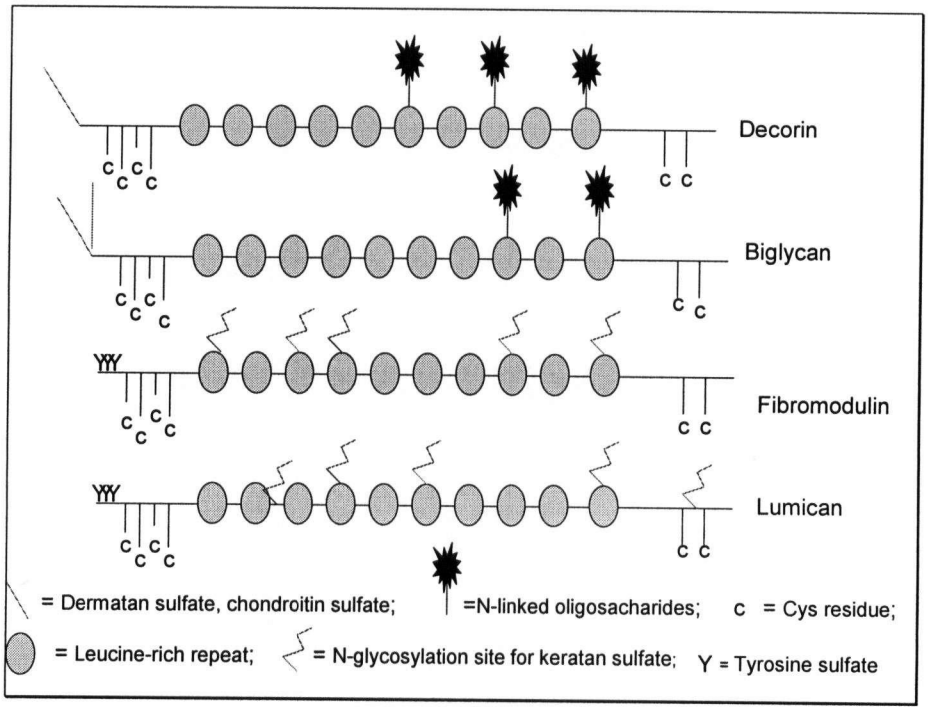


Figure 4. Gingival fibers. Type I collagen fibers of the gingiva have been classified according to their location and direction. There are four recognizable groups: the circular group, the dentogingival group, the dentoperiosteal group and the alveologingival group (Ten Cate, 1994).

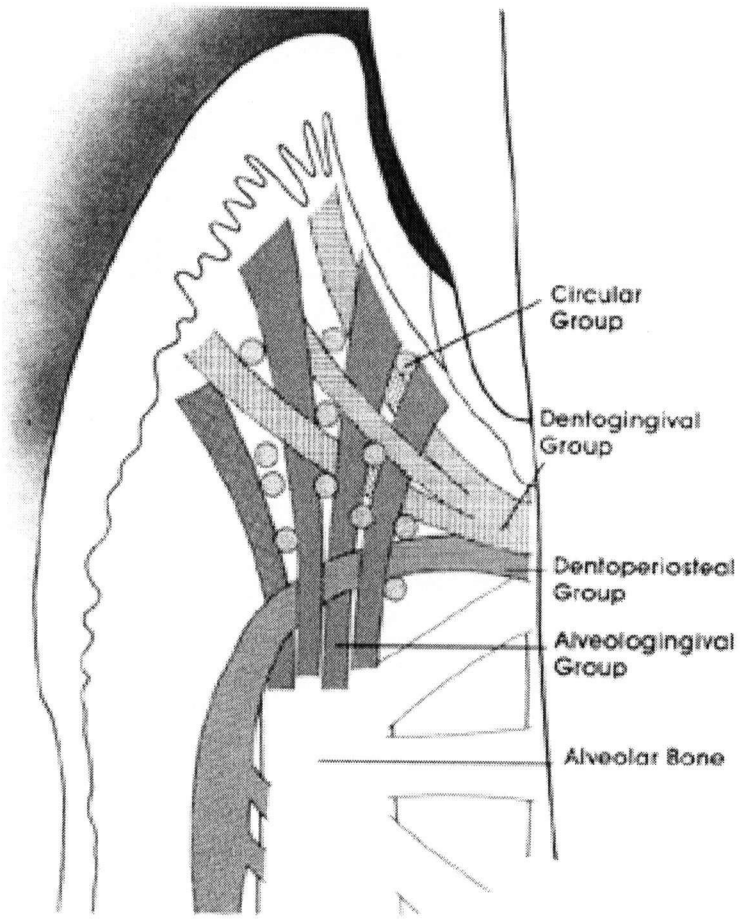


Figure 5. Principle fibers of the periodontal ligament. Type I collagen fiber bundles of the periodontal ligament have been divided into 6 groups based on their location and orientation. They include the transeptal, alveolar crest, horizontal, oblique, apical and interradicular fibers. They are highly organized fibers running parallel to each other in the ligament (Ten Cate, 1994).

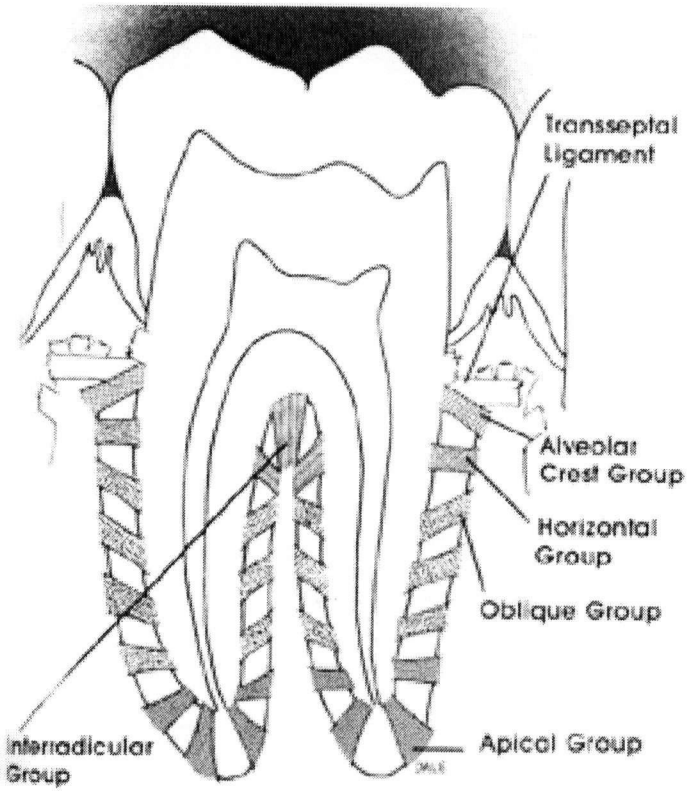


Figure 6. Collagen biosynthesis demonstrating intracellular (hydroxylation, glycosylation, nucleation and propagation) and extracellular events (peptide cleavage, fibril formation and cross-linking) that lead to the formation of a collagen molecule by fibroblasts. Specific amino acids on ribosomes form individual polypeptide chains that contain N and C terminal peptides. Some of the lysine and proline residues are hydroxylated (vitamin C dependent enzyme driven processes) forming hydroxylisine and hydroxyproline. Sugar residues are added, a process known as glycosylation (an enzymatically driven process). Peptide chains then form a triple helix and are transported to the golgi apparatus where the procollagen molecules is completed and excreted from the cell. Next fibrillogenesis takes place where the N and C termini are eventually cleaved and 5 unit staggered microfibrils are formed (Kadler, 1995)

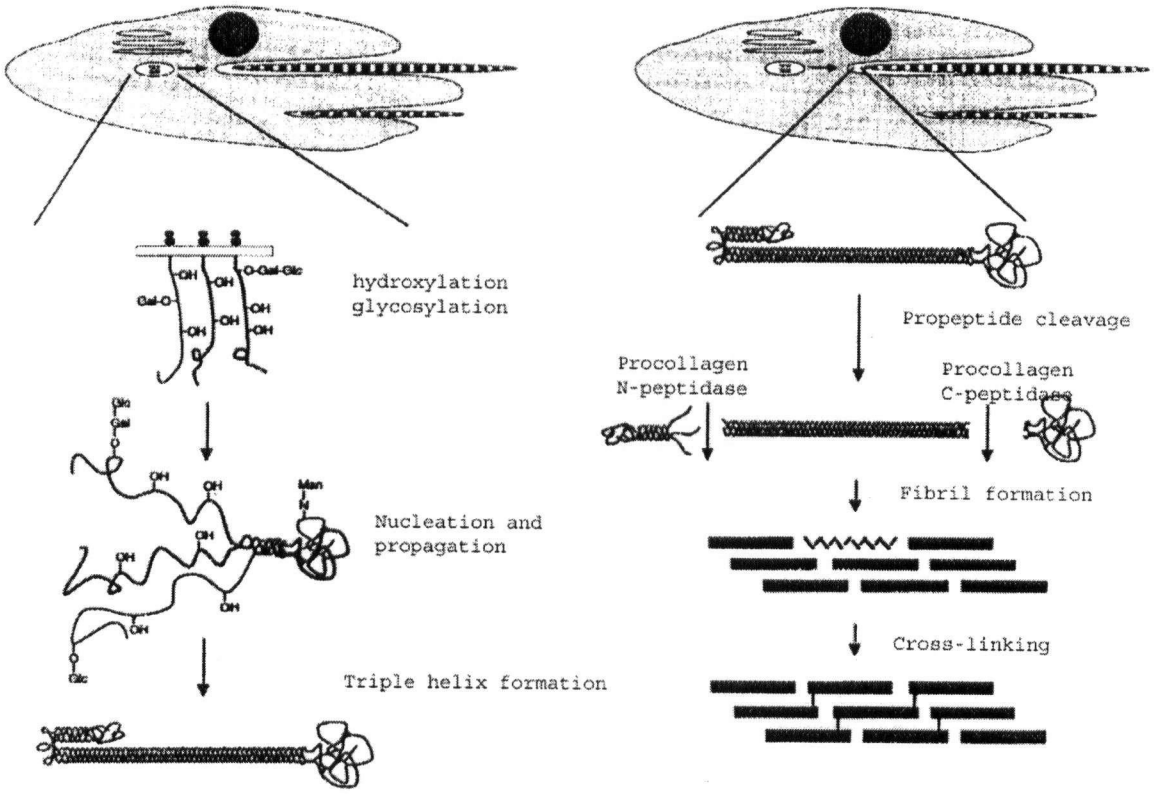
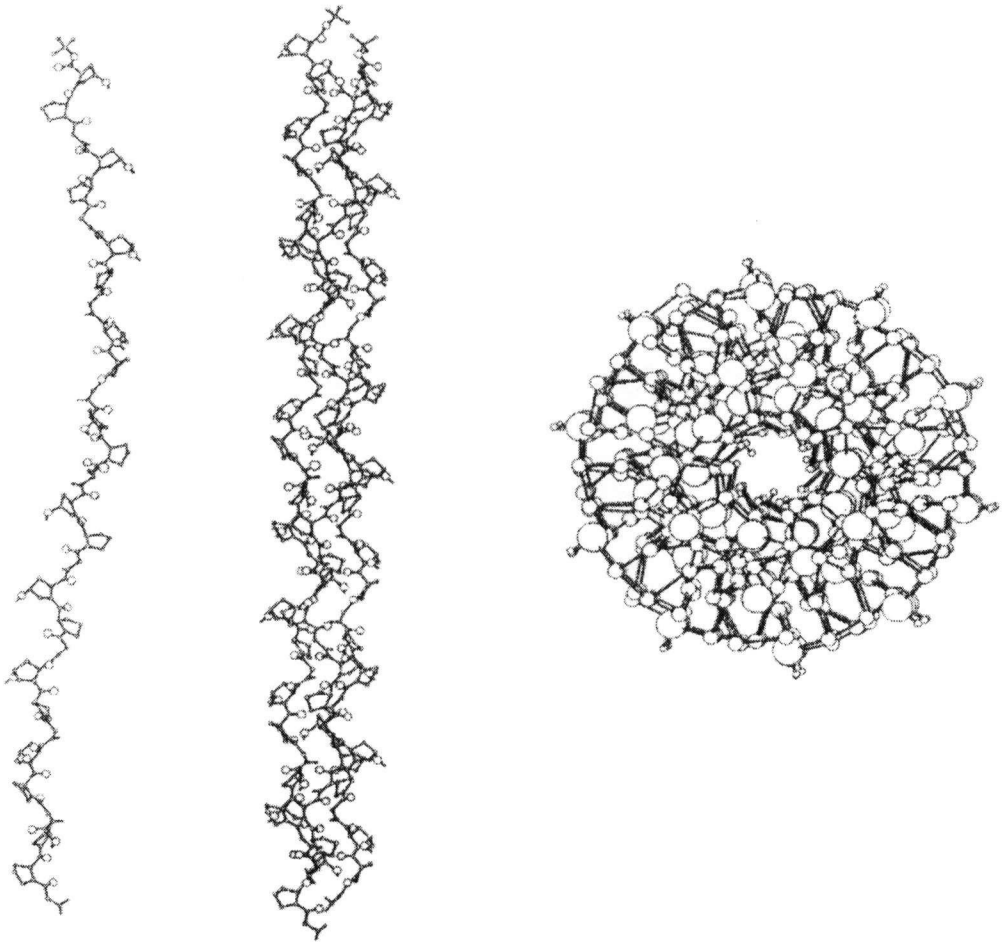


Figure 7. Triple helix of collagen. Schematic representation of collagen cased on a repeating triplet of Glycine-proline-4-hydrohyproline. (A) shows a single collagen alpha chain, (B) shows three alpha chains folded into a triple helix with Glycine residues in the center of the molecule and (C) shows a cross-sectional view of (B) looking down the axis of the triple helix molecule (Kadler, 1995).



A B C

Figure 8. Collagen fibril structure. Collagen molecules aggregate to form a banded fibril. Negative staining techniques allow gaps to contain more stain and show up as dark areas in electron microscope. Minerals in hard tissues accumulate in these gaps (Ten Cate, 1994).

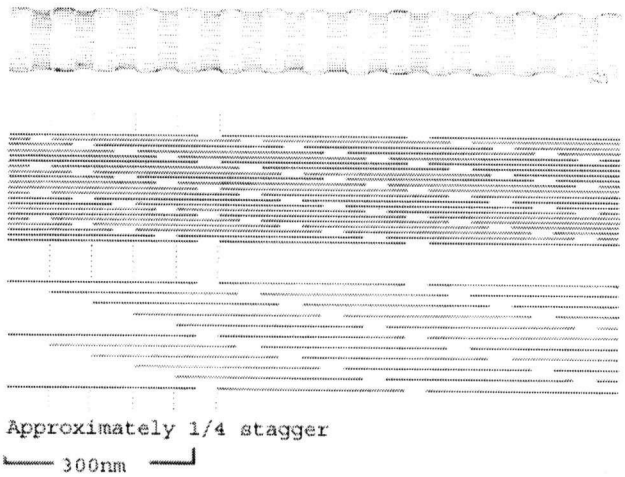


Figure 9. Model of lateral fusion of collagen molecules to form fibrils. Collagen molecules assemble into quarter-staggered arrays giving rise to fibril intermediates (65 nm in mouse tendon). These fibril intermediates are stabilized by fibril-associated macromolecules, such as fibromodulin and lumican, to allow for fusion of adjacent fibril intermediates in the formation of mature collagen fibrils. Model based on study in fibromodulin, lumican and double knockout mouse tendon (Ezura et al, 2000).

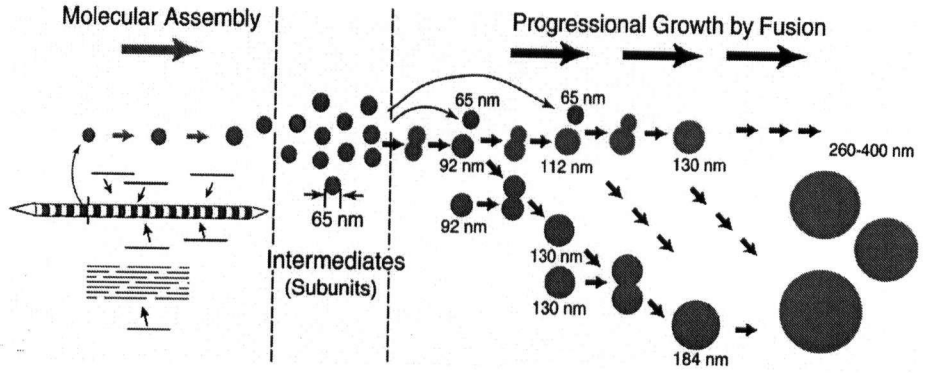


Figure 10. Contribution of fibromodulin and lumican during collagen fibrillogenesis. In the lateral fusion model described in figure 9, it is believed that lumican is expressed early in mouse tendon development suggesting a role in the initial stages of collagen fibrillogenesis. Fibromodulin expression increases during development suggesting a role in tendon fibril maturation (Ezura et al, 2000).

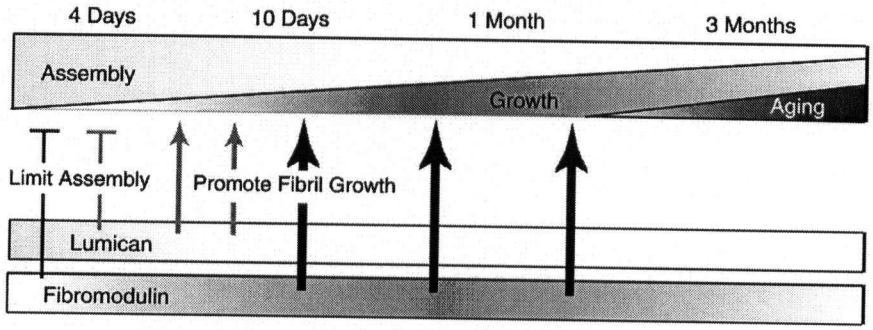


Figure 11: The binding model. Decorin, a class I small leucine-rich proteoglycan, is thought to be horseshoe-shaped and bind type I collagen fibrils on their concave side. Weber et al, 1996 estimate that each decorin molecule has enough space on the concave aspect of the arc to accommodate one collagen molecule.

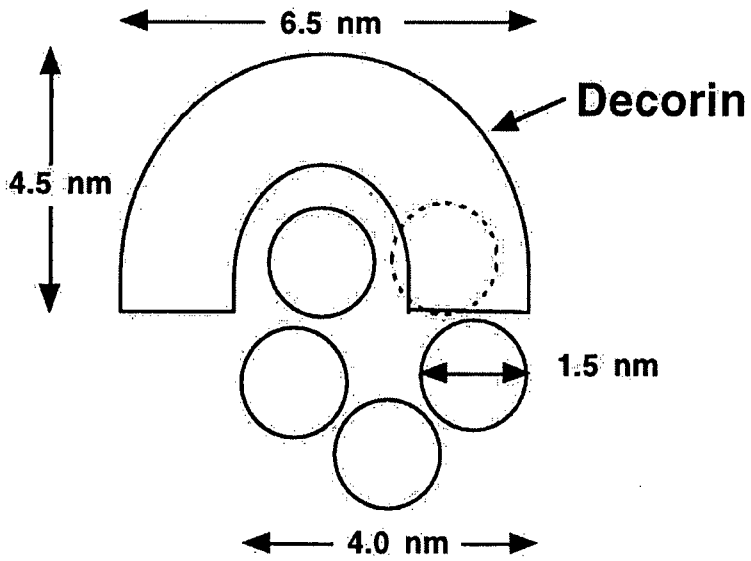


Figure 12: Jaw length measurements. Photograph of a defleshed, halved mouse maxilla showing where jaw length measurements were made using a Boley Gauge to the nearest tenth of a milliimeter. Measurements were made anteriorly at the osseous crest facial of the incisor at the point of incisor exit and posteriorly at the distal of the third molar (M1 = 1st molar; M2 = 2nd molar; M3 = 3rd molar).

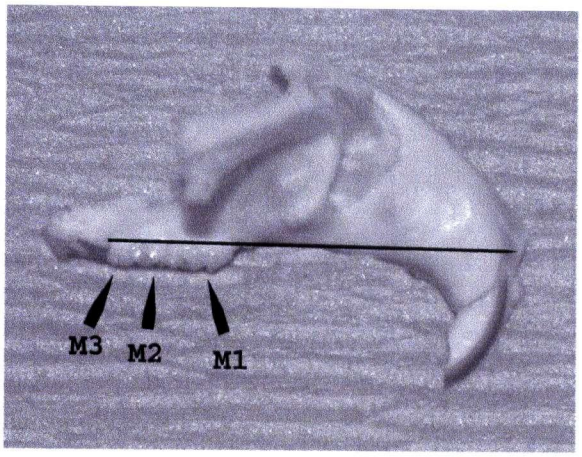
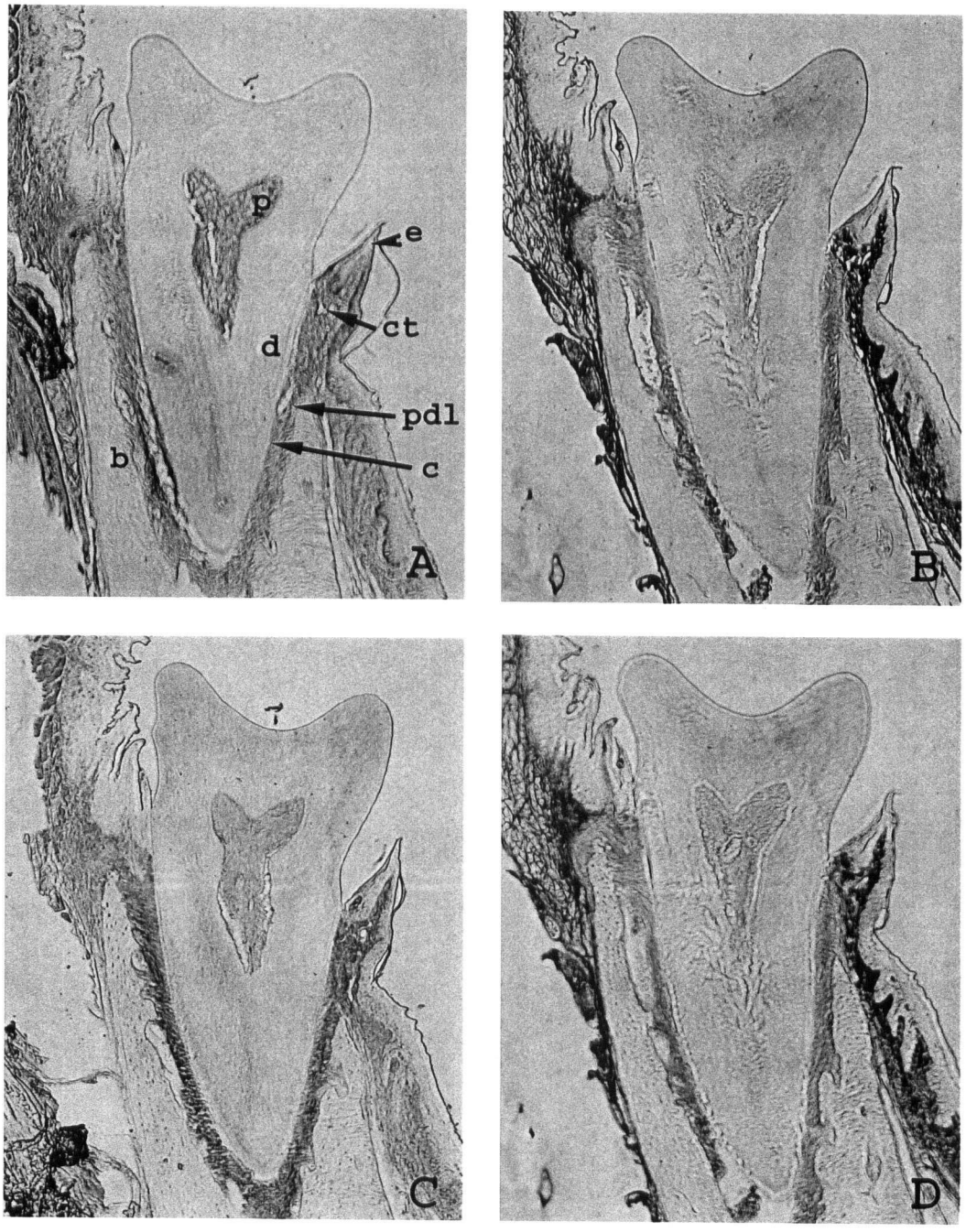


Figure 13. Localization of fibromodulin (A), lumican (B), biglycan (C) and decorin (D) in dental and periodontal tissues (p=pulp; d=dentin; b=bone; c=cementum; e=epithelium; ct=connective tissue; pdl=periodontal ligament) (immunostain intensity: mild = +, moderate = ++, intense = +++).



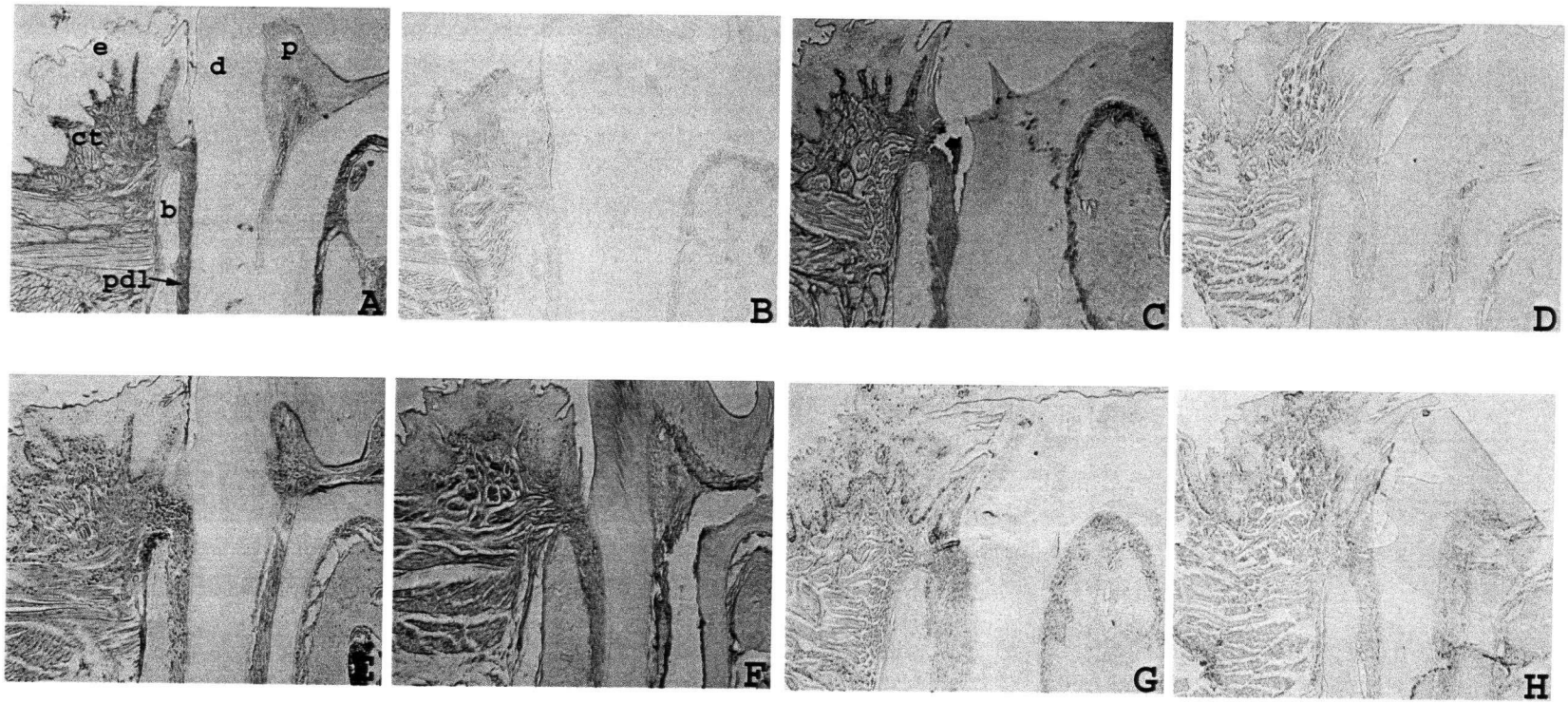


Figure 14a. Relative expression of fibromoduln and lumican in knockout mice. First molar sections immunostained with anti-lumican antibodies (A-D) and anti-fibromodulin anitbodies (E-H) of wild-type (A,E), lumican knockout (B, F), fibromodulin knockout (C, G) and double knockout (D, H) mice. Knockout mice are lacking immunostaining of the knockout proteoglycan in the dental and periodontal tissues (B, D, G, H). Lumican knockout mice have more intense fibromoduln immunostaining and fibromodulin knockout mice have more intense lumican immunostaining than willd-type mice (p=periodontal ligament; b=bone; ct=connective tissue; e=epithelium; d=dentin). (Note: nonspecific staining of nuclei and muscle fibers in G, H).

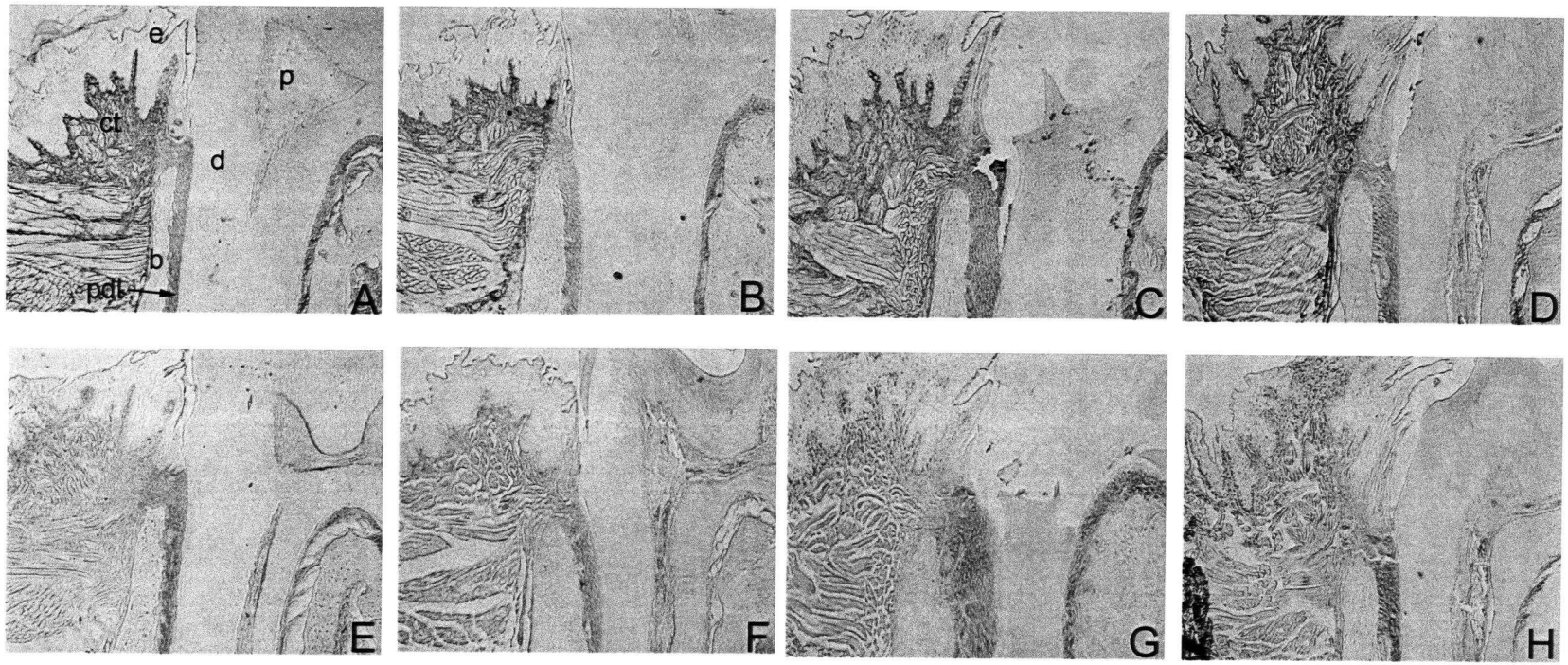


Figure 14b. Relative expression of decorin and biglycan in knockout mice. First molar sections immunostained with anti-decorin antibodies (A-D) and anti-biglycan antibodies (E-H) of wild-type (A,E), lumican knockout (B, F), fibromodulin knockout (C,G) and double knockout (D,H) mice. No marked differences in immunostain intensities are noted between any of the knockout mice and wild-type CD-1 mice (pdl=periodontal ligament; b=bone; d=dentin; p=pulp; ct=connective tissue, e=epithelium).

Figure 15: Defleshed maxillary jaw halves of wild-type (A),fibromodulin knockout (B), lumican knockout (D), and double knockout mice (C). All teeth are fully erupted (M1 = 1st molar, M2 = 2nd molar, M3 = 3rd molar).

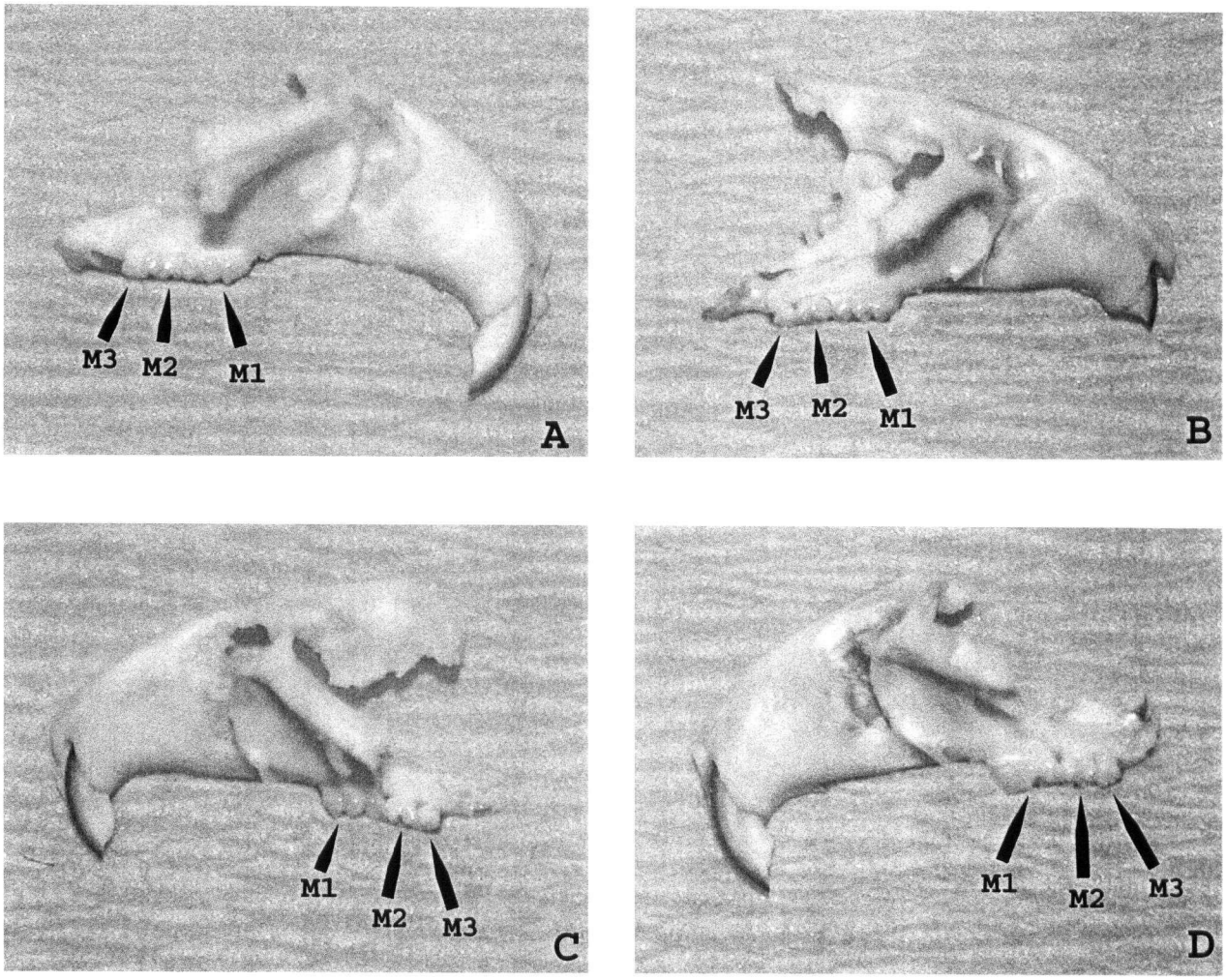


Figure 16. Sections of CD-1 wild-type (A), fibromodulin knockout (B), lumican knockout (C) and double knockout (D) mouse mandibular first molars stained with phosphotungstic acid hematoxylin. The first molars are fully erupted and have bone levels of similar height. Distal roots are missing due to sectioning angle. (b=bone; p=periodontal ligament; d=dentin; g=gingiva).

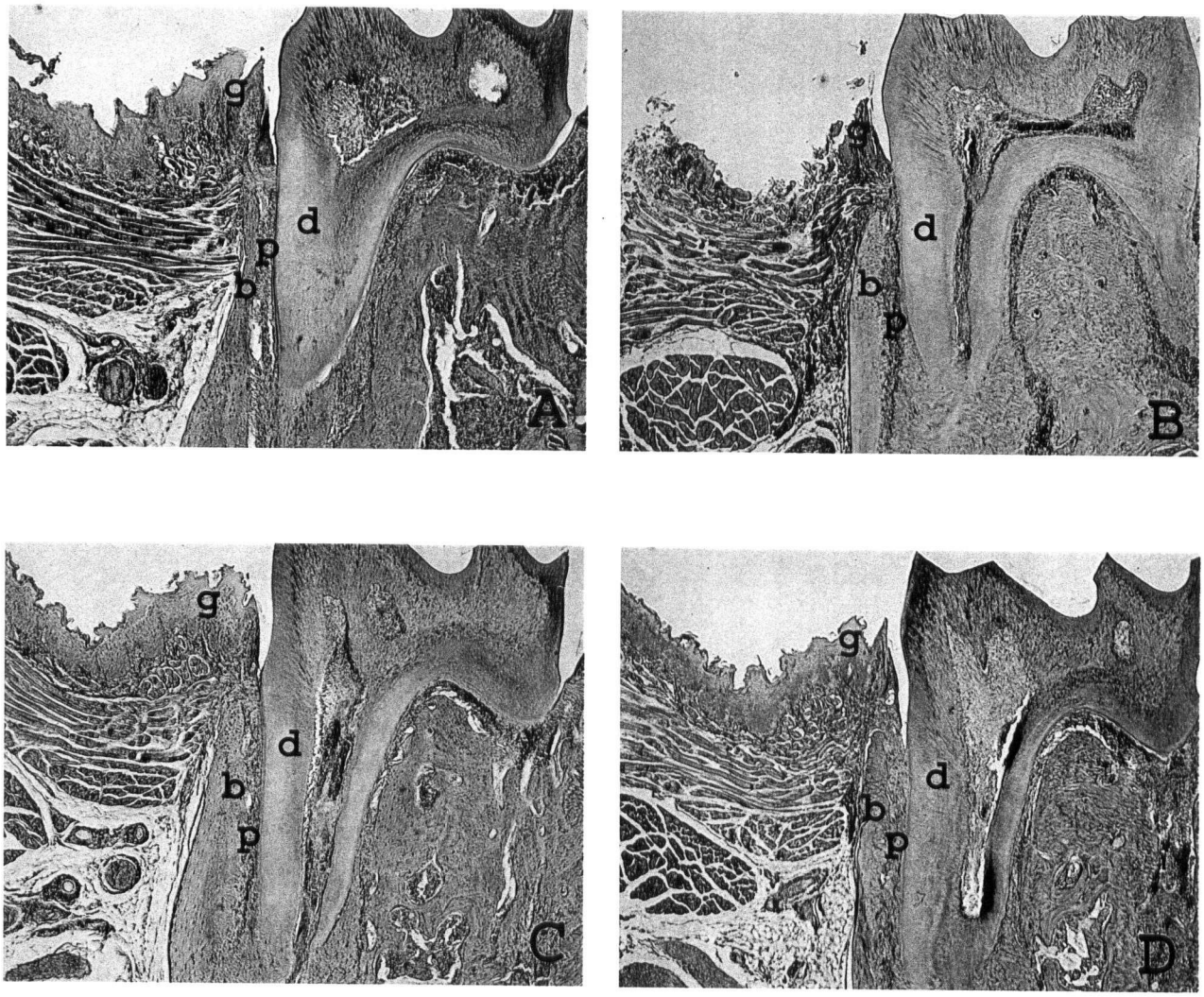


Figure 17. H&E stained sections of the coronal third of mandibular first molars of CD-1 wild-type (A), fibromodulin knockout (B), lumican knockout (C) and double knockout (D) mice. The periodontal ligament of wild-type mice are filled with type I collagen fiber bundles that are of relatively even thickness and properly oriented (arrow). The spacing between the bundles is evenly distributed. (arrowhead). Ligaments of the knockout mice have fiber bundles of uneven thickness (arrows) with uneven spacing between them (arrowheads). The double knockout mouse ligaments have the most random arrangement of collagen fiber bundles.

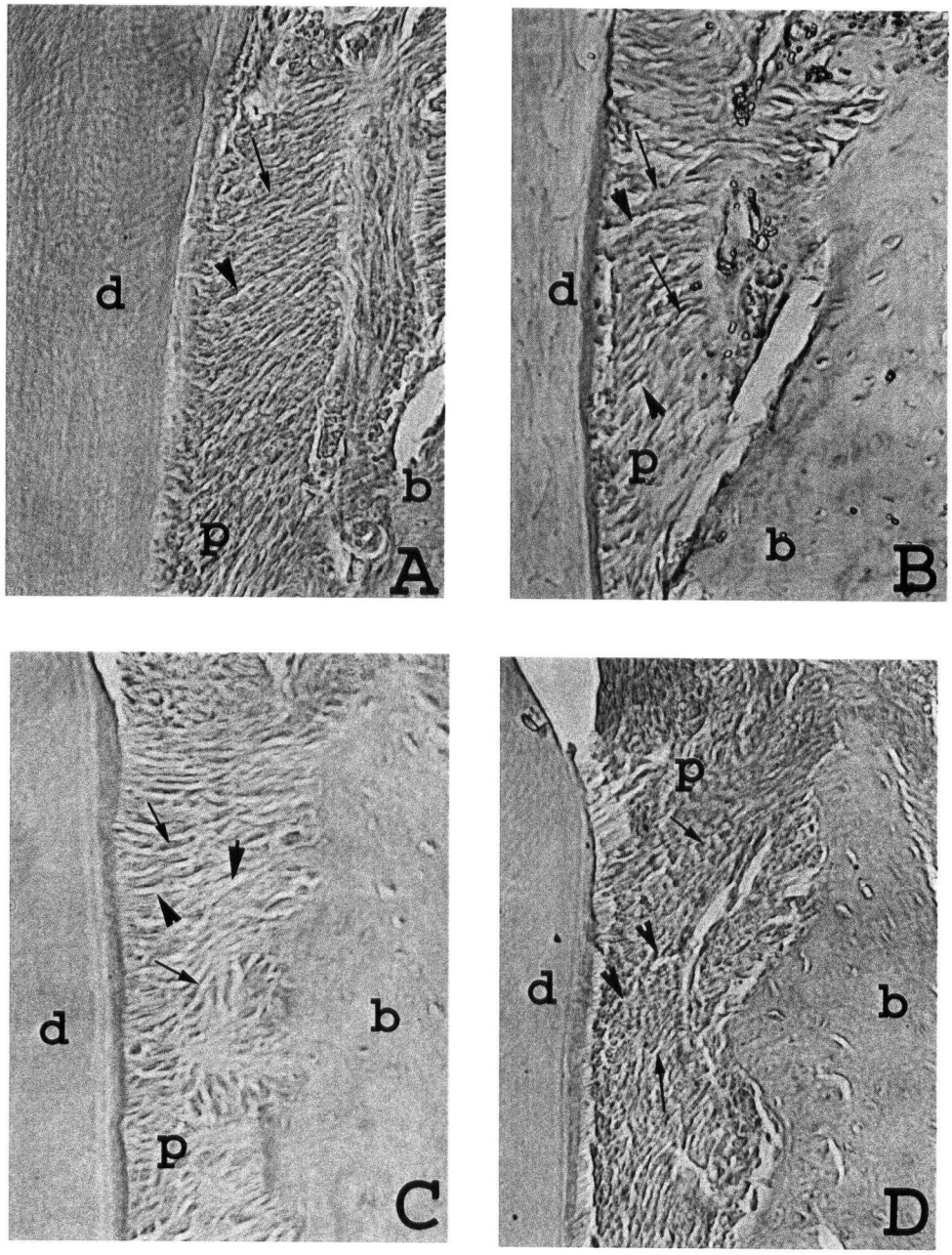


Figure 18. First mandibular molar sections stained with phosphotungstic acid hematoxylin of CD-1 wild-type (A), fibromodulin knockout (B), lumican knockout (C) and double knockout (D) mice. Small arrows point to fiber bundles of the knockout mice that are of variable thickness and arrowheads point to areas of increased inter fiber bundle spacing - features unlike wild-type. Large arrow points to area of external root resorption (B). (d=dentin; p=periodontal ligament; b=bone; c=cementum).

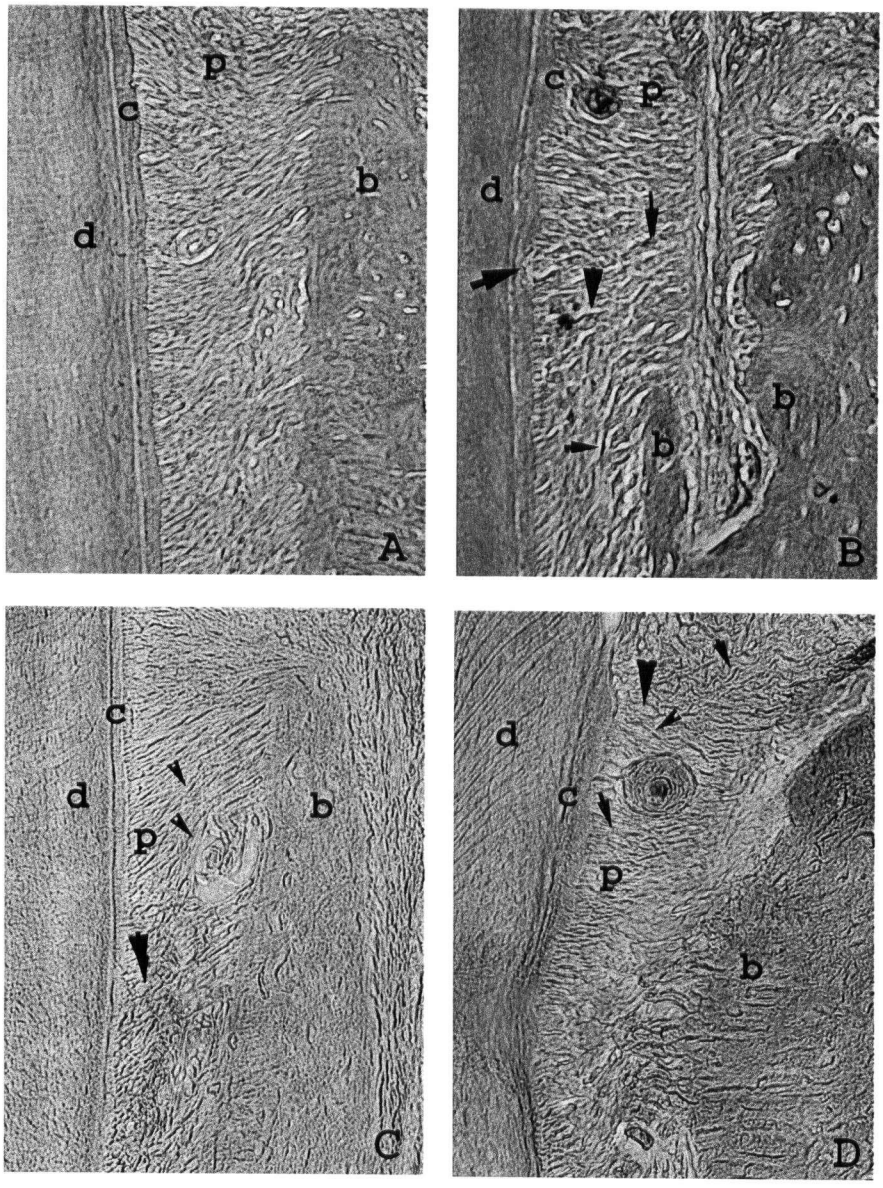


Figure 19. Sections of the mid-third mandibular molar periodontal ligament stained with picrosirius red and analyzed with a polarizing light microscope of CD-1 wild-type (A), fibromodulin (B), lumican (C) and double knockout (D) mice. A: Fiber bundles appear to fill the periodontal space (small arrow) and have smooth outlines (large arrows). B: fiber bundles are more unorganized and appear to have more stripes associated with them than wild-type (arrow). C: Stripes are apparent on fiber bundles (arrows) and bundles appear relatively thinner than wild-type. D: Fiber bundles have altered outlines (large arrows) and vertical fibers are seen (small arrows) (c=cementum; p=periodontal ligament; b=bone).

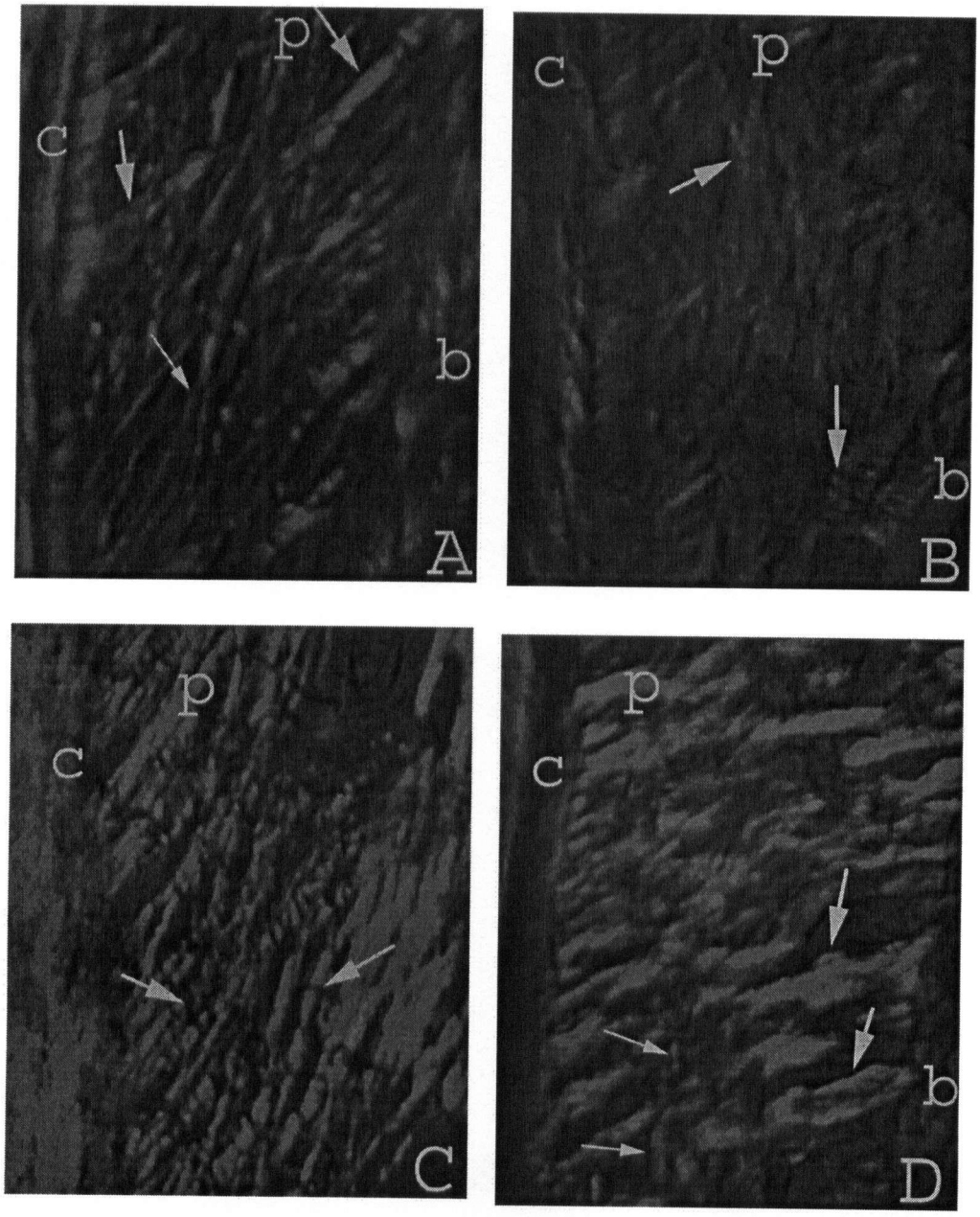
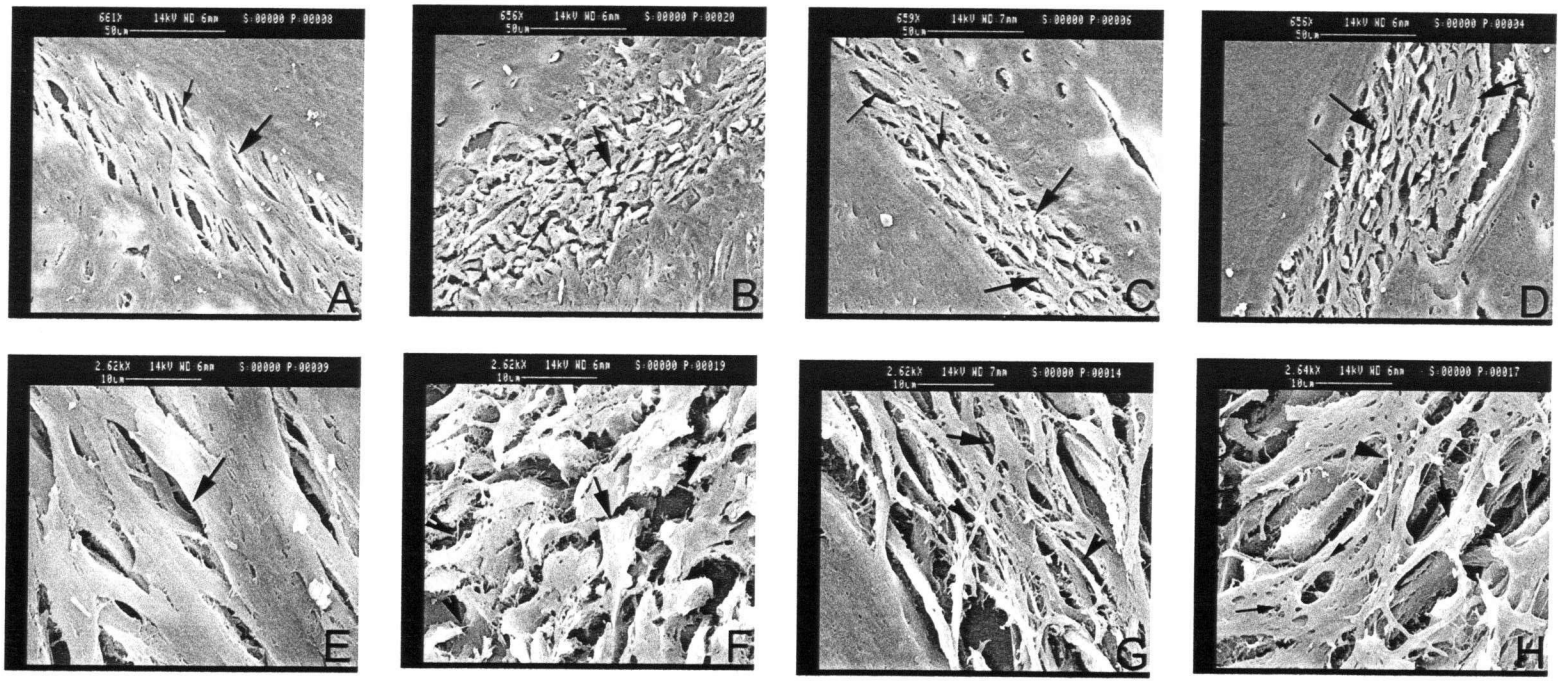


Figure 20. Scanning electron micrographs of CD-1 wild-type (A&E), fibromodulin (B,F), lumican (C,G) and double knockout (D,H) first molar mouse periodontal ligaments. A,E: Collagen fiber bundles of wild-type mice are smooth and even (E, arrow), well organized and traverse the periodontal space without disruption (A, large arrow). The inter-fiber bundle spacing in this group is relatively less than other groups and is evely distributed among the fiber bundles (A, small arrow). B,F: Collagen fiber bundles of fibromodulin knockout mice are of variable widths, have rough outlines (B, large arrow), are disorganized, have several interruptions along their length (F, large arrow), appear slightly thinner than wild-type and have thin branches protruding from them (F, arrowheads). There is more inter-fiber bundle space in these ligaments (B small arrows) and the basic orientation of the bundles is diminished. C,G: Collagen fiber bundles of lumican knockout mice are disorganized and thinner than wild-type and fibromodulin knockouts, have rough outlines (G, arrow) and many branches protruding from them (G, arrowheads). There is increased inter-fiber bundle spacing seen (C, small arrows). D,H: Collagen fiber bundles of double knockout mice are disorganized, of variable thickness (D, large arrows) and have rough outlines characterized by several small protruding branches (H, arrowheads) and holes (F, small arrow). The basic orientation of the fiber bundles is diminished.



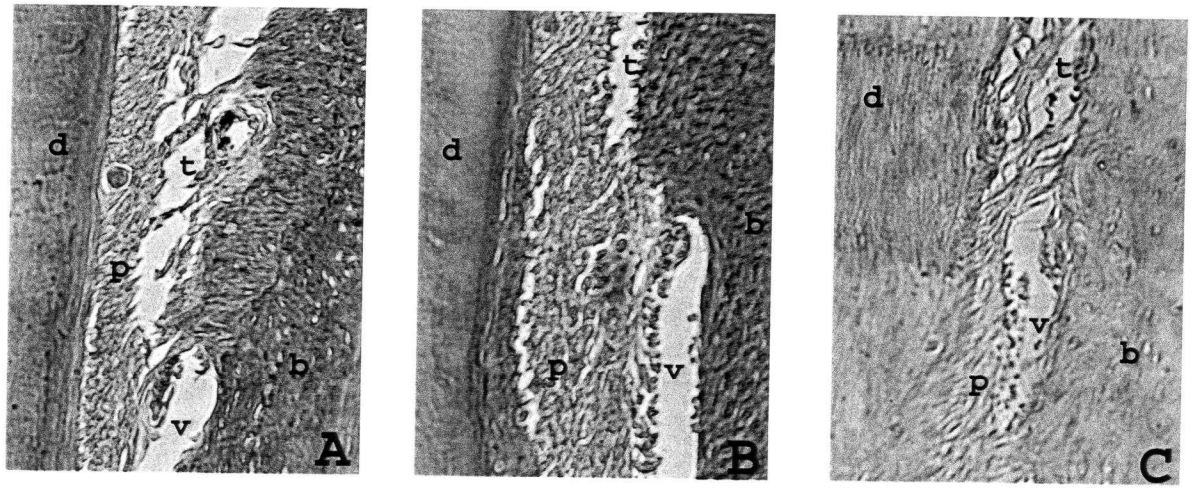


Figure 21. Examples of tears and blood vessels that were counted in this study. Blood vessels were recognized as voids in the ligament that had blood cells inside the lumen and endothelial cells lining the lumen. Tears were those voids with no cells associated with them and had a rough or stringy outlines. Both phosphotungstic acid hematoxylin and H&E stained sections were used for analysis (d-dentin; p=periododntal ligament; b=bone; v=vessel; t=tear).