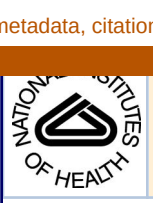

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Mechano-regulation of Collagen Biosynthesis in Periodontal Ligament

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Abstract

Purpose—Periodontal ligament (PDL) plays critical roles in the development and maintenance of periodontium such as tooth eruption and dissipation of masticatory force. The mechanical properties of PDL are mainly derived from fibrillar type I collagen, the most abundant extracellular component.

Study selection—The biosynthesis of type I collagen is a long, complex process including a number of intra- and extracellular post-translational modifications. The final modification step is the formation of covalent intra- and intermolecular cross-links that provide collagen fibrils with stability and connectivity.

Results—It is now clear that collagen post-translational modifications are regulated by groups of specific enzymes and associated molecules in a tissue-specific manner; and these modifications appear to change in response to mechanical force.

Conclusions—This review focuses on the effect of mechanical loading on collagen biosynthesis and fibrillogenesis in PDL with emphasis on the post-translational modifications of collagens, which is an important molecular aspect to understand in the field of prosthetic dentistry.

Keywords

Periodontal ligament; Mechanical loading; Collagen; Fibrillogenesis; Post-translational modification

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Conflict of interest statement

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1. Introduction

Periodontal ligament (PDL) is a specialized soft connective tissue that attaches the tooth to the alveolar bone socket. This fibrous tissue is very dynamic with high cellularity and vascularity, and plays critical roles in the development and maintenance of periodontium. These include: tooth support, regulation of tooth eruption, dissipation of masticatory forces, neurological feedback and orthodontic tooth movement. The mechanical properties of PDL are, thus, very important for these functions and they are mainly derived from the primary extracellular matrix protein; fibrillar type I collagen. One of the major characteristics of PDL collagen is its exceptionally high rate of turnover [1], which could be critical for tooth eruption and orthodontic tooth movement. One of the intriguing features of PDL is its ability to maintain the tissue without being mineralized despite the fact that it is connecting two specialized mineralized tissues, alveolar bone and cementum. Although this tissue is highly adaptive to external forces by temporarily changing the tissue space [2], the width remains relatively constant throughout its lifetime.

In daily prosthodontic practice, occlusion needs to be adjusted when a dental prosthesis is installed. The acceptable range of occlusal adjustment in natural teeth is generally considered to be ~30 μm because of the pressure displacement of PDL [3]. If the occlusal adjustment is performed inappropriately, it could cause widening of PDL space and increment of tooth mobility [4]. This clinical observation underscores the significance of optimum mechanical loading in the tissue maintenance of PDL. The expansion of PDL space and subsequent increase in tooth mobility are not only due to the expansion of PDL fibers, but also to the accelerated tissue turnover in response to mechanical loading [5]. Since fibrillar collagen is the predominant extracellular matrix (ECM) component of this tissue, it is important to understand how mechanical loading affects cells, subsequent collagen biosynthesis and tissue construction. Owing to recent advances in molecular and cellular biology and analytical technologies, it is now clear that collagen post-translational modifications are highly regulated by groups of specific enzymes, these modifications change in response to mechanical forces and ultimately affects collagen fibrillogenesis, stability and tissue mineralization [6-12]. This review focuses on the effects of mechanical loading on collagen biosynthesis and fibrillogenesis in PDL with emphasis on the post-translational modification of collagens.

2. Mechanical loading in PDL

The PDL is subject to various mode of mechanical loading in different clinical circumstances. For instance, occlusal loading is the intermittent jiggling force and orthodontic tooth movement is the continuous static force. Thus, when the effect of mechanical loading on PDL is investigated, it is utmost important to carefully consider the loading conditions (e.g. mode, magnitude and duration) and interpret the data. Kang *et al*. reported that 2D and 3D cultured PDL-derived cells showed different gene expression profiles in response to similar mechanical loading [13]. This indicates that the culture environment could also influence on cellular response. To analyze the effect of mechanical loading on PDL-derived cells *in vitro*, a number of investigators have used commercially available loading apparatus, such as Flexcell (Flexcell International Co., Hillsborough, NC)

[14-19], Strex (STREX Inc., Okayama, Japan) [20,21] and general laboratory centrifuge [17,22,23], while others fabricated their own loading devices [24-27]. Since optimal mechanical loading varies depending on cell type, culture condition and loading mode, it is important to use well defined loading regimen with a thoroughly characterized loading apparatus. However, unfortunately, such characterization of loading apparatus has been often overlooked [28].

In animal studies, models such as excessive occlusal loading and orthodontic tooth movement are frequently used to analyze the effect of mechanical loading in PDL at the tissue level. Excessive occlusal loading condition can be created by bite-raising as reported by many groups [29-32]. However, with this model, loading conditions such as magnitude, frequency and profile of wave cannot be controlled. To overcome this limitation, a motor-controlled device has recently been developed [33]. Using this device, the recruitment of TRAP-positive osteoclasts and the increment of RANKL/OPG ratio, which illustrates the osteoblast-mediated osteoclast recruitment, were confirmed in a magnitude- and time-dependent manner. The orthodontic tooth movements have been simulated by inserting elastic rubber band between molars (Waldo method)[34] or by installing coil spring between incisor and molar [35]. In these models, the loading condition can be manipulated in a relatively well-controlled manner. Histological studies demonstrated that compression side of PDL showed destructive changes, while tension side revealed additive changes [35].

Though *in vitro* studies provide valuable insights as to how certain PDL-derived cells respond to the external stress at the molecular level, they cannot replicate the changes *in vivo* as the PDL consists of a variety of cells and extracellular matrices. Thus, in addition to an in vitro study, it is indispensable to characterize histological and biochemical changes of PDL in response to the mechanical loading by using a well characterized animal model.

3. Collagens in PDL

The major component of PDL is fibrillar collagens including types I, III and V, accounting for ~75%, 20% and 5% of collagens, respectively [36,37]. In addition to the fibrillar collagens, non-fibrillar collagens such as types IV, VI, XII and XIV are also present as minor components in PDL [38,39] (Table 1). Microarray and expressed sequence (EST)-tag database studies have indicated that more collagen types, such as type II, XI, XV and XVI, are present in PDL [40,41]. Fibrillar collagens are the scaffold that provides tissue with form, connectivity and tensile strength; thus, genetic disorders in these collagens can result in severe connective tissue-related diseases [42]. While the tensile strength of PDL is provided primarily by fibrillar collagens, resistance against compressive forces in this tissue is likely carried out by water, hyaluronic acid and various proteoglycans [43]. The diameter of PDL collagen fibrils is relatively smaller than those of other connective tissues, likely due to the high rate of collagen turnover [1] and the presence of non-collagenous components that regulate collagen fibrillogenesis [44]. These fibrillar collagens, i.e. principal fibers in PDL, are not mineralized and appear to be highly glycosylated. On the other hand, fibrils of the Sharpey's fibers that are embedded in bone and cementum have a larger diameter and are partially mineralized. The site-specific composition and structural characteristics of

collagens and non-collagenous components could be an important factor for the function of PDL, and to prevent or facilitate proper mineralization.

4. Type I collagen

Type I collagen is the most abundant type of collagen among the collagen superfamily, comprising 29 members encoded by at least 44 genes, and is the structural basis for the form and mechanical properties in most tissues and organs. It is a heterotrimeric molecule composed of two α1 chains and one α2 chain, approximately 300 nm in length and 1.5 nm in thickness. The biosynthesis of type I collagen is a long, complex process that includes gene transcription, post-translational modifications of proα chains, formation of a triple-helical procollagen molecule, secretion to ECM, enzymatic processing to form a collagen molecule, self-assembly into a fibril and stabilization by covalent intra- and intermolecular cross-linking (for details, see recent reviews [6,45,46]) (Fig. 1). Intra- and extracellular post-translational modifications during biosynthesis are critical for the structural function of collagen fibrils. A number of enzymes, their binding molecules and molecular chaperones are involved in such modifications and most of these enzymes are collagen specific.

4-1. Epigenetic control of type I collagen

One of the first molecular mechanisms that regulate the gene expression of collagens is epigenetic modification, modulating transcription factor accessibility in an inherited manner without changing genomic DNA. The main epigenetic mechanisms of gene regulation are DNA methylation and histone modification [47]. Several studies have suggested that methylation of the cytosine residue at the CpG sequence in the promoter region suppresses gene expression, and that demethylation re-activates gene expression. It has reported that the age-associated decrease in type I collagen production in PDL cells is partly due to hyper-methylation in the promoter region of the *COL1A1* gene [48,49]. Arnsdolf *et al*. reported that 3 hours of oscillatory fluid flow reduced the DNA methylation of *Col1a1* gene promoter and associated increase in the expression of *Col1a1* gene on mouse bone marrow stromal cells [50]. It is thus possible that mechanical loading regulates the gene expression of type I collagen in PDL in an epigenetic manner.

4-2. Expression of type I collagen genes

Numerous studies have demonstrated that the gene expression of type I collagen (i.e., *COL1A1 & COL1A2* in human and *Col1a1 & Col1a2* in mice) are altered by mechanical loading in PDL-derived cells; however, the results are not consistent. Many have reported that the gene expression is up-regulated with mechanical loading [16,17,22,25,51], while others have reported it is unchanged or decreased [14,20,26,27,52]. Such inconsistent outcomes are likely due to differences in loading regimen (i.e., compression vs. tension, cyclic vs. static, frequency, duration.) and culture conditions. Comparative studies have been performed in order to analyze the effects of different loading conditions on the gene expression of type I collagen in PDL. A recent study by Chen *et al.* showed that 3% cyclic stretching increased the gene expression of *COL1A1* but decreased by 10% cyclic stretching on human PDL-derived cells [19]. Another study, by He *et al.* compared the effect of cyclic equibiaxial tension and compressive forces on the expression of type I collagen by using

human PDL-derived cells [24]. In this study, they reported that ten hours of 10 % tension force increased the expression of *COL1A1* gene, however, same magnitude of compressive force decreased the expression of *COL1A1* gene. These data suggest that the effect of mechanical loading on type I collagen gene expression in PDL cells is magnitude-, duration- and mode-dependent.

4-3. Post-translational modifications of type I collagen

It has been reported that there is a discrepancy between the expression of genes encoding type I collagen (i.e., *COL1A1* and *COL1A2*) and the production of type I collagen protein [53] (Fig. 2). Such discrepancies occur, in part, due to the complex biosynthesis process [46] including the sequential and multiple processes of post-translational modifications necessary for proper proα chain folding into triple helix, fibrillogenesis and stabilization of fibrils. Thus, to characterize the response of PDL cells to mechanical loading, it is necessary to analyze not only the gene expression of type I collagen itself, but also the expression of collagen modifying enzymes, their associated molecules and molecular chaperons.

4-3-1. Prolyl Hydroxylases—The majority of proline (Pro) hydroxylation of collagen (~99%) is in the form of 4-hydroxyproline (4-Hyp). It occurs in the sequence of -X-Pro-Gly (glycine)- being catalyzed by prolyl-4-hydroxylase (P4H). This modification is critical for the stabilization of triple helix conformation [54]. A very small number of Pro hydroxylation (~1%) occurs in form of 3-Hyp at Pro in the sequence of –Pro-4Hyp-Gly- catalyzed by the collagen prolyl 3-hydroxylation complex (P3H). In type I collagen, the major target residues for P3H are α1-Pro986 and α2-Pro707 [55,56]. Recently, much attention has been paid on this particular modification as defects in the genes encoding the components of the P3H complex cause recessive osteogenesis imperfect [55]. The complex is composed of prolyl 3-hydroxylase 1, cartilage-associated protein and cyclophilin B, residing in the endoplasmic reticulum. It is still not clear, however, how the lack of 3-hydroxylation in Pro causes such severe connective tissue phenotypes. Possibly, defects in this complex may also affect proper Lys modifications as some of them interact with lysyl hydroxylases [57], which could lead to defective collagen cross-linking [58]. The extent of 3-Hyp in PDL type I collagen is unknown. The expression of P4H in PDL cells was reported previously [59], however; there have been no reports on the expression of P3H, and the mechano-responsiveness of these genes in PDL.

4-3-2. Lysyl Hydroxylases—Specific lysine (Lys) residues of collagen can also be hydroxylated in the form of 5-hydroxylysine (Hyl). This modification is catalyzed by lysyl hydroxylases (LHs) encoded by procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD) genes. Lys hydroxylation occurs both in the helical-, and C- and N-telopeptide domains of type I collagen molecule. In the helical domain, it if formed in the sequence of -X-Lys-Gly (glycine)-, and in the telopeptide domains in -X-Lys-Ala (alanine)- and -X-Lys-Ser (serine) - sequences. To date, three isoforms of LHs have been identified (LH1-3) and partially characterized.

The substrate specificities of these isoforms *in vivo* are still not clearly established; however, substantial evidence indicate that LH1 primarily hydroxylates Lys residues in the helical

domains of fibrillar and non-fibrillar collagens [60]. For LH2, two alternatively spliced isoforms were identified, i.e. LH2a or LH2 (short) and LH2b or LH2 (long), respectively. The latter (LH2b) includes an additional 21 amino acids (exon 13A) and appears to be the major form of LH2 in most tissues [61]. Several studies indicate that LH2 (LH2b) functions as a telopeptidyl LH [10,62-64]. LH3 is a multifunctional enzyme possessing LH, GT (hydroxylysyl galactosyltransferase) and GGT (galactosylhydroxylysyl glucosytransferase) activities [65]. However, for type I collagen, the main function of LH3 appears to be GGT not LH or GT [7,8](see section 4-3-3 for GTs and GGTs).

It has also been reported that various factors influence the expression of LHs and subsequent Lys hydroxylation, including growth factors [66] and vitamin D [67], as well as mechanical loading [11,68,69]. Saito *et al.* reported that twenty-gram of gravitational force increased the gene expression of *LH2* in an osteoblastic cell culture, while simulated zero-gravity increased the gene expression of *LH1* [69]. In PDL-derived cells, it was reported that the static compressive force induces the expression of *LH2* [70]. We also confirmed that *LH2* responded to both static and cyclic compressive force in human PDL-derived cells and the PDL of excessively occluded molars in rat (Fig. 3, Kaku M, unpublished data). The *LH2* is a causative gene in Bruck syndrome, which is characterized by osteoporosis, joint contracture at birth, fragile bones and short stature, and exhibits under hydroxylation of Lys residues in telopeptides of type I collagen in bone; however, cartilage and ligament collagen shows normal hydroxylation of telopeptidyl Lys and normal patterns of cross-linking [64,71]. These results suggest that tissue-specific changes in Lys hydroxylation in type I collagen by mechanical loading may contribute to the tissue-specific collagen cross-linking pattern (see section 4-3-6).

4-3-3. Molecular Chaperones and Peptidyl-proryl cis-trans isomerases—Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone that inhibits collagen aggregation in the endoplasmic reticulum by binding a proα chains, facilitating the correct folding into the triple helix [72,73]. Transgenic mouse studies revealed that mutations in *HSP47* cause collagen-related genetic disorders such as osteogenesis imperfecta [74,75]. As the nature of heat shock protein, HSP47 is responsive to various stimuli, including heat stress, growth factors and mechanical loading, HSP47 rapidly detects mechanical loading and affects type I collagen fibrillogenesis [76,77].

Secreted Protein Acidic and Rich in Cysteine (SPARC)/osteonectin was originally identified as a collagen-binding glycoprotein, playing an extracellular role in collagen fibrogenesis [78]. SPARC is preferentially expressed in tissues with a high rate of collagen turnover, including PDL. SPARC-null mice demonstrated that the number of cells and collagen volume were markedly diminished in PDL, indicating crucial roles in PDL homeostasis [79]. Recent studies have shown that secreted SPARCs are internalized to cells and act intracellulary as molecular chaperones in concert with HSP47 [80-82]. Since both SPARC and HSP47 are known to be expressed in response to various stresses [82], these molecules may have some roles in the collagen fibrillogenesis in PDL in response to mechanical loading.

Proline isomerization is the rate-limiting step during triple helical formation of procollagen and this is catalyzed by a group of isomerases, Peptidylprolylisomerase (PPIase). Among seven PPIases residing in rough endoplasmic reticulum, three of them have been shown to be involved in procollagen biosynthesis, i.e. cyclophilin B, FK506-binding protein (FKBP) 65 and 22 [57]. The absence of these proteins lead to a recessive form of osteogenesis imperfecta or kyphoscoliotic type of Ehlers-Danlos Syndrome (type VI). PPIases form complexes with many collagen related proteins and play important roles in the collagen fibrillogenesis, however, to date, there are no reports regarding the expression and mechano-responses of PPIases in PDL cells.

4-3-4. Glycosyl transferases—Type I collagen glycosylation is *O*-linked glycosylation occurring at specific Hyl residues in the helical domain of the molecule, thus, Lys hydroxylation catalyzed by LHs is prerequisite for this modification. Structurally, galactose is attached to the hydroxyl group of Hyl by a β-glycosidic bond, while glucose is linked by an α-glycosidic bond to C-2 of the galactose [6]. These modifications are catalyzed by two groups of collagen glycosyltransferases i.e. hydroxylysyl galactosyltransferase (GT) and galactosylhydroxylysyl glucosyltransferase (GGT) (see section 4-3-2) producing galactosylhydroxylysine (G-Hyl) and glucosylgalactosylhydroxylysine (GG-Hyl), respectively [6]. With regard to GT, GLT25D1 and GLD25D2 have been identified [83]. The *Gld25d1* showed broad expression in several tissues, while *Glt25d2* was expressed in only a limited number of cell types, suggesting that GLT25D1 is the major isoform [83]. As for GGT, recent studies have indicated that LH3 is the major GGT enzyme for type I collagen [7,8,83]. The level of glycosylation differs among different types of collagen and, even within the same type of collagen, it differs from tissue to tissue. It has been reported that collagen glycosylation may contribute to structural and biological functions, such as control of collagen fibrillogenesis [84-87], collagen cross-linking [37,88,89] and collagen-cell interaction [90,91]. It has been reported that altered collagen glycosylation is associated with bone disorders, such as osteogenesis imperfecta [92-94], postmenopausal osteoporosis [95,96] and osteosarcoma, osteofibrous dysplasia [97], suggesting the significant roles of collagen glycosylation in mineralization. The most predominant glycosylated site of type I collagen, α1/2 Hyl-87, is one of the major helical cross-linking sites [98,99]. A recent study demonstrated that the glycosylation pattern is significantly different between immature and mature cross-links in types I and II collagen [100]. This, together with a report by Srichiolpech *et al.* suggest that di-glycosylation negatively controls the process of cross-link maturation [7]. It is interesting to note that, in PDL type I collagen, the cross-links involving α1/2 Hyl-87 PDL are mostly di-glycosylation forms, and they are predominantly immature cross-links [37]. This specific glycosylation pattern in PDL type I collagen may be associated with the smaller diameter of PDL collagen fibrils and possibly contribute to maintenance of non-mineralized state of this tissue.

4-3-5. Lysyl Oxidases—Once a triple-helical procollagen is formed, the molecules are packaged and secreted to the extracellular matrix (ECM) through the Golgi apparatus. In the ECM, the N- and C-terminal propeptide extensions are cleaved by procollagen proteinases generating a mature type I collagen molecule. These molecules are then self-assemble to form a fibril; a process called “fibrillogenesis”, and stabilized by the formation of covalent

intra- and intermolecular cross-linking (see section 4-3-6). In order to initiate cross-linking, the telopeptidyl Lys or Hyl need to be converted to the respective aldehyde forms, Lys^{ald} and Hyl^{ald}, by oxidative deamination catalyzed by an enzyme, lysyl oxidase (LOX). Once aldehyde is formed, the rest of the condensation reactions are spontaneous. These aldehydes spontaneously react with other aldehydes or ε-amino groups of unmodified Lys and Hyl residues to form a variety of intra- and intermolecular cross-links, which are critical for the formation of mechanically functional collagen fibrils. Together with the LOX, several isoforms of LOX, i.e. LOX-like proteins (LOXL1-4) have been identified. Recent findings revealed that LOX and LOXL proteins could be involved in various molecular functions other than collagen and elastin cross-linking, including chemotactic responses, tumor suppression and controlling growth factor activity. [101,102]. Increases in LOX expression in response to mechanical loading have been reported in bone marrow stromal cells [103], dermal fibroblast [104] and PDL-derived cells [19]. LOX is synthesized as proLOX and is processed by BMP1/Tolloid-like proteinases, the same proteinases that cleave the C-propeptide of type I procollagen, to form mature and active LOX [105]. It has been reported that the BMP1-mediated proteolytic activation of LOX is coordinately regulated by periostin which is a secretory matricellular protein, expressed in collagen-rich fibrous connective tissues, including PDL [106,107]. The PDL in periostin-null mice exhibits irregular collagenous fibrils and changes in the organization of major ECM proteins such as type I collagen, fibronectin and tenascin-C [108-110]. Orthodontic tooth movement increased the expression of periostin in wild type mice but resulted in the expansion of PDL width with the decreased immunolocalization of cathepsin K, MMP1 and MMP2 in periostin null-mice [109]. These results suggest that proteolytic regulation of LOX activity could be different in PDL.

4-3-6. Collagen cross-links—The final step of collagen biosynthesis is the formation of covalent intra- and intermolecular cross-links (for a review, see [6,111,112]). The importance of collagen cross-linking cannot be over-emphasized, as it is the molecular basis for the tissue stability. As described above, the formation of Lys^{ald} or Hyl^{ald} in the telopeptides by the action of LOX initiates the cross-linking process, and the rest of the condensation reactions are non-enzymatic. Many factors determine cross-linking pattern including: the initial aldehyde form (Lys^{ald} or Hyl^{ald}), extent of hydroxylation of the juxtaposed Lys residues on a neighboring molecule, glycosylation of Hyl involved in the reaction and microenvironment such as mechanical loading, mineralization and turnover rate [6]. Since these modifications and microenvironment vary among different cell types and tissues, collagen cross-linking pattern is highly tissue-specific and reflects tissue's physiological state and function. For instance, it has been reported that PDL type I collagen possesses abundant amounts of immature cross-links (three major reducible cross-links) with only small amounts of mature, stable cross-links [37]. Such pattern would allow the tissue to have high tensile strength, but at the same time to be readily turned over. Interestingly the bi-functional immature cross-links in PDL are mostly di-glycosylated which may also contribute to the negative control of collagen maturation. Though what precisely controls this specific cross-linking pattern in PDL is not clearly understood, specific microenvironment including constant mechanical loading would certainly be a contributing factor.

To the best of our knowledge, only one paper reported the direct measurement of the changes in collagen cross-linking in response to mechanical loading in PDL. Plecash *et al.* analyzed the composition of two immature, bi-functional cross-links in the occluded and non-occluded PDL of dogs (2, 4 and 6 weeks) and observed no significant difference among the different groups, concluding that a high rate of collagen turnover in PDL is intrinsic but not as a result of external or eruptive force [113]. However, the data need to be interpreted carefully as the number of analysis is very limited, i.e. single analysis per time point, use of a different type of dog at each time point, and lack of quantitative analysis for other cross-links in PDL. In another study, it has been reported that the bovine PDL exhibits no significant changes in the quantities of the two immature, bi-functional collagen cross-links during development and maturation [114]. But similar limitations may apply to this study. Further studies are needed to elucidate the effect of mechanical loading on the collagen cross-linking in PDL.

Effect of mechanical loading on the collagen cross-linking in bone has been relatively well investigated. Shiiba *et al.* reported that, employing a rat tail suspension model, mechanical unloading significantly changed the composition of collagen cross-links likely due to an increase in Lys hydroxylation [11,68]. Saito *et al.* reported that hyper-gravitational force enhanced not only the total reducible and non-reducible cross-link contents and the rate of cross-link maturation in an osteoblast culture system [69].

5. Other types of collagen

Type III collagen is a fibrillar collagen comprising three α1 chains and is typically co-localized with type I collagen within the same fibril [115]. The PDL contains a considerable amount of type III collagen (~20%) as compared with bone (~1%), cementum (~5%) and gingiva (~10%), however, the functional significance of the type III collagen in PDL is poorly understood. This amount of type III collagen is relatively high for mature connective tissue and is more characteristic of fetal connective tissue. Deficiency in *Col3a1* gene, which encodes type III collagen, in mice results in shorter lifespan due to the rupture of major blood vessels; therefore, type III collagen is considered to be essential for normal type I collagen fibrillogenesis [116]. In humans, patients with type IV Ehlers-Danlos syndrome, which shows fragile and inextensible connective tissues, lack the expression of type III collagen [117]. Increases in type III collagen were observed in early phases of wound healing and were eventually replaced with type I collagen [118]. In particular, in the early healing process, Sharpey's fiber-like structure in the tendon-bone interface express type III collagen [119-121]. Furthermore, increases in type III collagen expression in response to mechanical loading has been reported in dermal fibroblasts , mesenchymal stem cells [122], anterior crucial ligament cells [123] and medial collateral ligament [124]. Expression of type III collagen in PDL-derived cells is reported to increase at lower-magnitude of loadings, but decrease at higher-magnitude of loadings [19]. The abundance of type III collagen in PDL may therefore be related to a key function in PDL, such as the integrity of the PDL-bone and -cementum interface, or is a consequence of the PDL's rapid turnover [1].

Type XII collagen is a member of fibril-associated collagens with interrupted triple helices (FACIT), originally identified in bovine PDL [125]. The type XII collagen is a homo-trimer

of α1 chains and each chain has two triple helical collagenous domains (COL1 and 2) with three separate noncollagenous domains (NC1-3). The collagenous domains, which comprise only ~7.7% of the molecule, have an affinity for fibrillar collagen (i.e., type I and III collagen in PDL) [126,127] and non-triple-helical domains provide sites for interaction with other extracellular matrix such as fibromodulin, decorin and tenascin-XB [128,129]. Type XII collagen has been shown to be mainly expressed in the dense collagenous connective tissues of tendons, ligaments, dermis, cornea blood vessel walls [130] and to be distributed in well-organized mature fibrils [131]. In PDL, type XII collagen is expressed in a mature/functional stage, as compared with developing stages, while the expression of type I collagen decreases with maturity [39,132]. Tzortzaki *et al.* speculated that type XII collagen transiently stabilizes the type I collagen fibrils until LOX catalyzes the inter-molecular collagen cross-linking [133]. Type XII collagen-expressing cells were localized on the alveolar bone side of PDL, where mature collagenous fibers are accumulated in comparison with the cementum side [134]. A transgenic mouse line carrying a dominant interference mutation of the type XII collagen gene demonstrated disorganized collagen fibers associated with internal porosity in PDL [135]. Type XII collagen is mainly present in two splicing variants, a large form (XIIA), and a small form (XIIB) [136,137], and these are generally co-expressed in a mutually exclusive manner. Only the XIIA isoform contains sulfated glycosaminoglycans in an NC3 domain and is therefore a proteoglycan. XIIA is generally found in the developing tissue, whereas type XIIB is expressed in the mature tissue; therefore, it is anticipated that adult PDL predominantly contains XIIB [138].

The expression and alternative splicing of type XII collagen is known to be regulated by mechanical loading in various cell, such as fibroblasts [139,140], trabecular meshwork cells [141], vascular endothelial cells [142] and osteoblasts [143], as well as PDL-derived cells [20,138]. The promoter activity of type XII collagen is directly stimulated by mechanical loading, thus suggesting the conservation of a stress-response element [143,144]. Upregulation of type XII collagen occurs during orthodontic tooth movement in the cells on the tension side, where there is an abundance of mature collagen fibers [138]. Taken together, these observations suggest that type XII collagen is responsible for the organization of collagenous fibers in response to mechanical loading in mature PDL.

6. Small leucine-rich proteoglycans (SLRPs)

Small leucine-rich proteoglycans (SLRPs) belong to the LRR superfamily of proteins, constituting a network of signal regulation mostly at the extracellular level [145,146]). SLRPs regulate collagen fibrillogenesis by binding to specific sites of collagen molecules. Several SLRP family members have been identified in PDL [147,148]. The role of SLRPs in collagen fibrillogenesis and subsequent tissue conformation have been extensively studied in either single- or double-mutant transgenic mice [149]. Targeted deletion of decorin, fibromodulin, lumican or both lumican and fibromodulin resulted in the formation of abnormal collagen fibrils and fiber organization was evident in the PDL [148]. The decorin-null mice displayed larger diameter collagen fibers with randomly arranged orientation in PDL. In addition to collagen phenotype, the number of fibroblasts in the PDL is doubled in decorin-deficient mice, indicating hyper-cellularity due to increased proliferation in the

absence of inhibitory signals from decorin [150,151]. Some SLRPs, (e.g., decorin and biglycan) are reported to be responsive to mechanical loading in PDL-derived cells [51,152].

Another SLRP member, asporin, is predominantly expressed in PDL. Unlike other SLRP family members, asporin does not possess a glycosaminoglycan chain, thus, not a proteoglycan. Asporin is associated with various bone and joint diseases, including osteoarthritis, rheumatoid arthritis and lumbar disc disease, and it binds to collagens to complement decorin, and it appears to regulate collagen fibrillogenesis and biomineralization [153]. Asporin also binds to TGF-beta [154] and BMP-2 [155] and it is thought to be negatively regulating their activities to prevent non-physiological mineralization of PDL such as in ankylosis.

7. Future Directions

The biosynthesis of type I collagen is a complex process, involving several post-translational modifications. These modifications are functionally important and are in part regulated by mechanical loading at different stages (Summarized in Fig. 4). Thus, with the tissue's exposure to constant mechanical loading, it is likely that PDL collagen has unique molecular and structural characteristics that have been just partially elucidated.

To analyze the changes in post-translational modifications of collagen, high performance liquid chromatography (HPLC)-based biochemical analysis have been the gold standard [37,156]. By the HPLC-based analysis, collagen contents and the extent of post-translational modifications are determined in a quantitative manner [156]. Recently, liquid chromatography-tandem mass spectrometry (LC/MC) has become a powerful tool to characterize the post-translational modifications of collagens in a molecular site-specific and semi-quantitative fashion [58,157]. Data obtained from such analytical methods in various experimental models would provide valuable insights into the biochemical characteristics of PDL collagen, its response to mechanical loading and their biological significance.

However, due to the thin, small, membrane-like structure of PDL, it is still technically challenging to collect sufficient amount of tissue samples for biochemical analysis. To characterize the spatial differences of collagen organization and maturation in PDL, polarized light-based birefringence analysis have been used [158]. More recently, microscope-equipped vibrational spectroscopy, including Fourier transform infrared (FTIR) and Raman techniques, has been developed to characterize the chemical composition and bonding microenvironment of the tissue constituents [159,160]. FTIR analysis could be used to characterize the extent of collagen cross-link maturation at the tissue level [161], thus, utilization of such imaging techniques could be useful to characterize the effects of mechanical loading on the collagen maturation, fibrillogenesis and matrix organization of PDL in appropriate animal models.

In order to identify the mechano-responsive molecules in PDL, microarray technology has been utilized over the last decade; however, the data have been inconsistent [13,20,40,51,52,70,162-168] likely due to the differences in culture conditions, loading regimens and the nature of primary cells used. It should be kept in mind that PDL harbors many cell types, e.g., fibroblasts, osteoblasts, cementoblasts, endothelial cells, epithelial cell

rests of Malassez and osteoclasts. Furthermore, fibroblasts, the principal cells in PDL, can be further classified into sub-populations with different functional characteristics [169]. It is evident that, significant number of peri-vascular stem cells are present in PDL [170,171]. These cells can, in theory, differentiate into any types of cells in PDL, however, the factors that control the fate of PDL stem cells are still poorly understood. Possibly, site-specific mechanical loading and microenvironment of stem cells such as the mechanical property of ECM are contributing factors [172,173].

As summarized in this review, mechanical loading regulates the collagen biosynthesis in a tissue specific manner that could directly affect the mechanical function of PDL. The expression of collagen post-translational enzymes and their associated molecules (Table 2), and the response of these molecules to mechanical loading as a function of anatomical location in PDL will be an important subject to study in the field of prosthetic dentistry.

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Abbreviations

PDL	Periodontal ligament
ECM	Extracellular matrix
Pro	Proline
P4H	Prolyl-4-hydroxylase
P3H	Prolyl-3-hydroxylase
Lys	Lysine
LH	Lysyl hydroxylase
GGT	Galactosylhydroxylysine-glucosyl transferase
GT	Hydroxylysyl galactosyl transferase
LOX	Lysyl oxidase
FACIT	Fibril-associated collagens with interrupted triple helices
SLRPs	Small leucine-rich proteoglycans
HPLC	High performance liquid chromatography
LC/MC	Liquid chromatography-tandem *mass spectrometry*
FTIR	Fourier transform infrared

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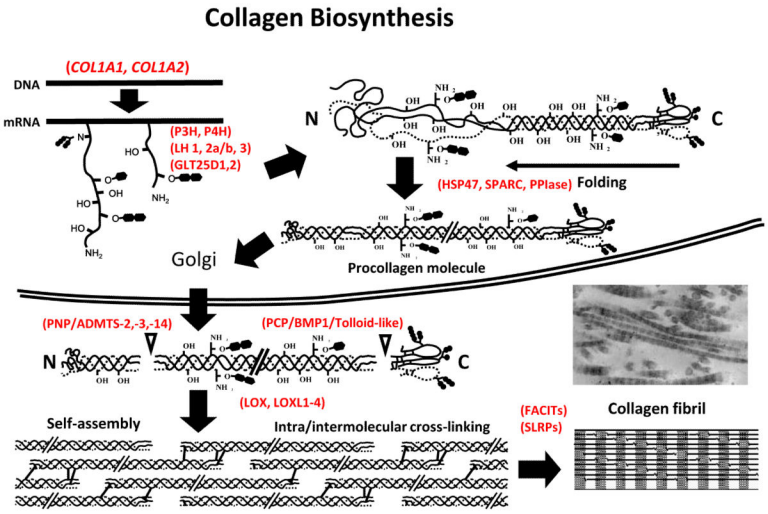

Fig. 1. Schematic image of biosynthesis of type I collagen

A series of collagen-modifying enzymes, molecular chaperones and associated molecules participate in the normal secretion and proper function of type I collagen. After or during the translation of α-chains of type I collagen, specific proline and lysine residues are hydroxylated by PHs and LHs, respectively. Following the hydroxylation of lysine residue, GLT25D serves as a GT and LH3 serves as a GGT. HSP47 and SPARC facilitate the folding of three α-chains into triple helical structures as molecular chaperones. The triple helical procollagen molecules are secreted to the extracellular space and then both N- and C-ends are cleaved by PNP and PCP to form mature type I collagen. LOX catalyzes aldehyde formation in the telopeptide domains of mature type I collagen. These aldehydes spontaneously react with other aldehydes or unmodified lysine and hydroxylysine residues to form various intra- and intermolecular cross-links. The FACITs and SLRPs bind to the surface of type I collagen fibrils and regulate fibrillar growth. PH, Prolyl hydroxylase; LH, Lysyl hydroxylase; GT, Hydroxylysine galactosyl transferase; GGT, Galactosylhydroxylysine-glucosyl transferase; PPIase, Peptidylprolylisomerase; PNP, Procollagen N proteinase; PCP, Procollagen C proteinase; LOX, Lysyl oxidase; FACITs, Fibril associated collagens with interrupted triple helices; SLRPs, Small leucine-rich proteoglycans. See text for details. Modified from (Yamauchi, 2002)[45].

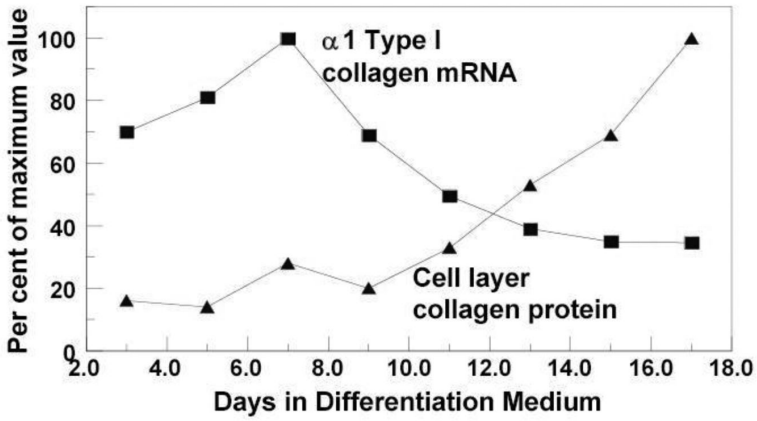

Fig. 2. Relationship between type I collagen mRNA and cell layer collagen protein accumulation

MC3T3-E1 osteoblastic cell line was cultured in differentiation medium, and gene expression of Col1a1 and cell layer type I collagen contents were analyzed by Northern blot and amino acid analysis, respectively. Col1a1 gene expression was highest at day 7 and decreased gradually thereafter, while extracellular collagen accumulation became evident after 9 days of culture. Such discrepancies occurs, in part, due to the complex biosynthesis process including post-translational modifications (shown in Fig.1) Modified from (Hong et al., 2004)[53].

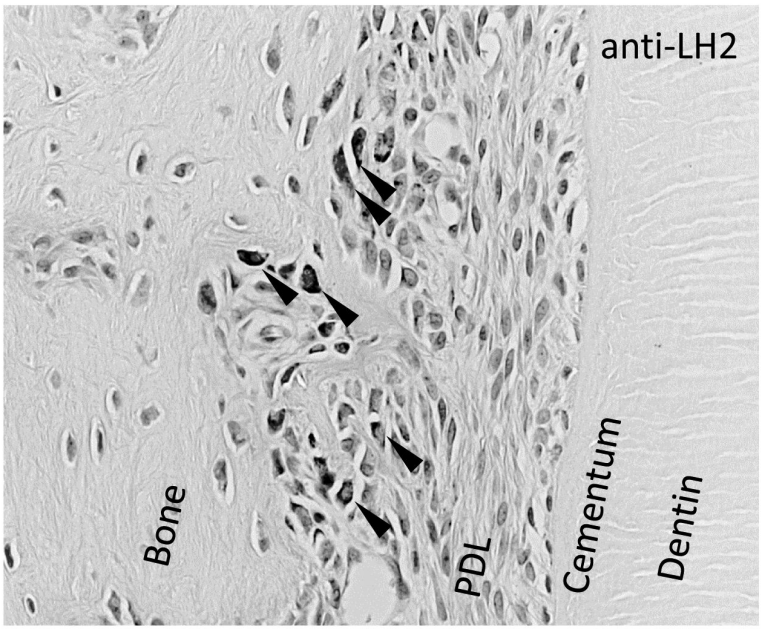

Fig. 3. Mechanical occlusal loading induces LH2 expression on alveolar bone side of PDL

Eight-week-old male SD rats were subjected to 3 days of excessive occlusal loading (Kaku et al., 2005)[29]. Decalcified, paraffin embedded histology samples were prepared and the distribution of LH2 was analyzed by means of immunohistochemistry. Anti-LH2-positive cells were detected only at the bone side PDL in the experimental group (arrow heads).

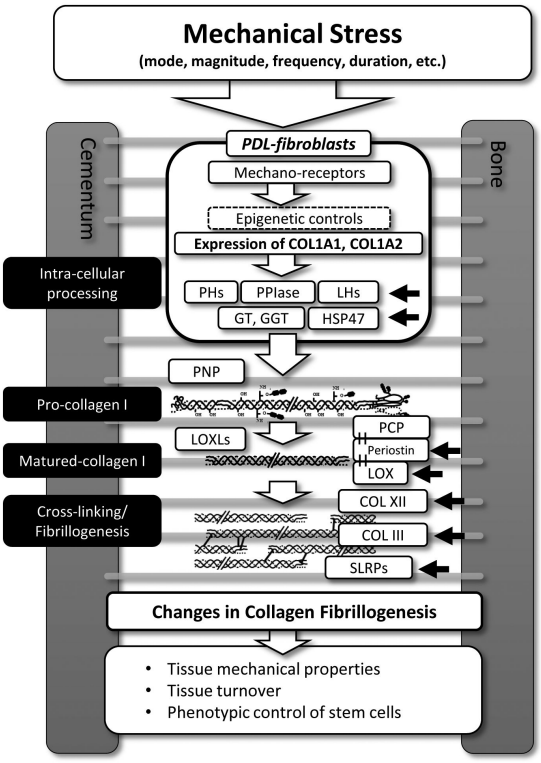

Fig. 4. Mechanical loadings affects the expression of type I collagen and its post-translational modifications in PDL at multiple steps

The collagen-modifying enzymes and -associated molecules which are crucial for collagen fibrillogenesis are summarized in this figure. Various factors of mechanical loading, such as mode, magnitude, frequency and duration, affect the response of PDL-fibroblasts. Black arrows indicate the known mechano-responsive collagen-related molecules in PDL. Other molecules could be affected by mechanical loading in PDL, but not have been tested. The changes in collagen fibrillogenesis exert influence on the tissue mechanical properties, tissue turnover and most likely phenotypic control of stem cells in PDL. PH, Prolyl hydroxylase; LH, Lysyl hydroxylase; GT, PPIase, Peptidylprolylisomerase; Hydroxylysine galactosyl transferase; GGT, Galactosylhydroxylysine-glucosyl transferase; PNP, Procollagen N proteinase; PCP, Procollagen C proteinase; LOX, Lysyl oxidase; SLRPs, Small leucine-rich proteoglycans. See main text for detail.

Table 1

Collagens found in Periodontal Ligament

Collagen	Form	Gene	Human disease	Mechano-response	References
Type I	Fibrillar	COL1A1, COL1A2	OI, EDS type VIIA and VIIB	◯	[38,138]
Type II	Fibrillar	COL2A1	Chondrodysplasia, Osteoarthrosis	◯	[40]
Type III	Fibrillar	COL3A1	EDS type IV	◯	[19,38,40]
Type IV	Basement membrane	COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6	Alport syndrome	ND	[174]
Type V	Fibrillar	COL5A1, COL5A2, COL5A3, COL5A4	EDS type I and II	◯	[38,175]
Type VI	Beaded filament	COL6A1, COL6A2, COL6A3	Bethlem myopathy, Ullrich muscular dystrophy	◯	[38,175]
Type XII	FACIT (PG)	COL12A1	Unknown, Disruption of PDL in KO mice	◯	[38,138]
Type XI	Fibrillar	COL11A1, COL11A2	Chondrodysplasias Osteoarthrosis	◯	[40,41]
Type XIV	FACIT (PG)	COL14A1	Unknown	ND	[38]
Type XV (EST-base)	Basement membrane (PG)	COL15A1	Unknown	ND	[41]
Type XVI (EST-base)	FACIT	COL16A1	Unknown	ND	[41]

UN; Unknown

ND; Not determined

PG; Proteoglycan

FACIT; Fibril associated collagens with interrupted triple helices

EST-base; Detected only in EST-base analysis

EDS; Ehlers-Danlos syndrome

OI; Osteogenesis imperfecta

Table 2

Collagen-modifying enzymes and molecular chaperones in PDL

Protein name (gene symbol)	Function	Human disease	Expression in PDL	Mecliano-response in PDL	References
Prolyl-4-hydroxylases	PH	UN	◯	ND	[59]
Prolyl-3-hydroxylase	PH	UN	ND	ND	
LH1 (*PLOD1*)	LH	EDS type VIA	◯	-	(Kaku, unpublished)
LH2 (*PLOD2*)	LH	Bruck syndrome	◯	◯	[70](Kaku, unpublished)
LH3 (*PLOD3*)	GGT	Connective tissue disorder	◯	-	(Kaku, unpublished)
GLT25D1	GT	UN	ND	ND	
GLT25D2	GT	UN	ND	ND	
HSP47	Molecular chaperone	OI type X	◯	◯	[176]
SPARC	Molecular chaperone	UN	◯	ND	[79]
Cyclophilin B (*PPIB*)	PPIase	OI type IX	ND	ND	
FKBP65 (*FKBP10*)	PPIase	OI type XI, Bruck syndrome	ND	ND	
FKBP22 (*FKBP14*)	PPIase	EDS type VIA and VIB	ND	ND	
ADAMTS-2	PNP	EDS type VIIC	ND	ND	
ADAMTS-3	PNP	UN	ND	ND	
ADAMTS-14	PNP	UN	ND	ND	
BMP1/TLD	PCP	OI type XIII	◯	ND	[177]
LOX	LO	Lathyrism	◯	◯	[19,178]
LOXL1	LO	Exofoliation syndrome	◯	ND	[178]
LOXL2	UN	UN	◯	ND	[178]
LOXL3	UN	UN	ND	ND	
LOXL4	UN	UN	ND	ND	

PH; Prolyl hydroxylase

GT; Hydroxylysine galactosyl transferase

GGT; Galactosylhydroxylysine-glucosyl transferase

FKBP; FK506 binding protein

PNP; Procollagen N proteinase

LOX; Lysyl oxidase

OI; Osteogenesis imperfecta

UN; Unknown

LH; Lysyl hydroxylase

PPIase; Peptidylprolylisomerase

PCP; Procollagen C proteinase

EDS; Ehlers-Danlos syndrome

ND; Not determined