ブタ歯髄由来SP細胞が間葉系幹細胞の性質を有し、骨形成能とともに血管再性能も有することが明らかになった。特に、$CD31^-$;$CD146^-$ SP細胞が有用であることが示された。

F．健康危険情報
なし

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庵原耕一郎、杉山昌彦、中村さやか、山田陽一、上田実、松下健二、中村洋、中島美砂子：乳歯由来歯髄細胞は血管新生・神経再生を促進する　第129回日本歯科保存学会　平成20年11月6日，富山，

H．知的財産権の出願・登録状況
（予定を含む。）

1．特許取得
なし

2．実用新案登録
なし

3．その他
なし

## III.　研究成果に関する一覧表

研究成果の刊行に関する一覧表

書籍

該当なし

雑誌

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# IV. 研究成果の刊行物・別冊

# The KK-Periome Database for Transcripts of Periodontal Ligament Development

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*ABSTRACT* The periodontal ligament (PDL) is a strong connective tissue that surrounds the tooth root, absorbs occlusal forces, and functions as a sense organ. PDL originated from dental follicle (DF), which possessed mesenchymal progenitors in the developing tooth germ. However, as specific marker genes for PDL and DF are currently unavailable, the molecular mechanisms of PDL development are yet to be clarified. To facilitate the identification of such genes, we have previously established a transcriptome database of the human PDL (the KK-Periome database) and screened for specific genes expressed during PDL development. Initial screening of the database revealed two marker genes for distinguishing DF and PDL. The KK-Periome database thus appears to offer a useful resource for investigating genes involved in PDL development. *J. Exp. Zool. (Mol. Dev. Evol.) 312B, 2009.* 

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The periodontium is a tooth-supporting tissue comprising gingiva, periodontal ligament (PDL), cementum, and alveolar bone (Ten Cate, '94). More specifically, the PDL is an element that connects the cementum covering the tooth root surface and the alveolar bone of the maxilla or mandible (Ten Cate and Mills, '72; Beertsen et al., '97; McCulloch et al., 2000). The PDL has the ability to absorb mechanical stress, and has thus been compared with tendon and ligament (McCulloch et al., 2000; Yoshizawa et al., 2004). Similar to tendons and ligaments, collagen type I is predominant, although other types of collagen (types III, V, VI, and XII) and proteoglycans that regulate collagen fibril formation are also deposited in the PDL (Lukinmaa and Waltimo, '92; Liu et al., '95; Everts et al., '98; MacNeil et al., '98). PDL cells also express genes involved in tendon and ligament formation, such as scleraxis and growth and differentiation factors-5, -6, and -7 (Sena et al., 2003; Seo et al., 2004; Yokoi et al., 2007). The cellular content of PDL is predominated by PDL cells, which can form Sharpey's fibers, collagen fibers embedded into the calcified matrix (McCulloch et al., 2000; Nanci and Bosshardt, 2006). Previous findings have suggested that although cultured PDL cells fail to form mineralized nodules, a partial resemblance to osteoblasts is apparent, such as high alkaline phosphatase activity and expression of osteoblast marker genes including RUNX2, bone sialoprotein, and type I collagen (Yamashita et al., '87; Nojima et al., '90; Lekic et al., 2001; Saito et al., 2002; Murakami et al., 2003). Recently, regulatory mechanisms preventing osteoblastic differentiation have been identified in cultured PDL cells, suggesting that the ability for suppressing osteoblast function may

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act to prevent PDL from mineralizing (Yoshizawa et al., 2004; Yamada et al., 2007).

The structure of the PDL is often irreversibly damaged when chronic inflammation in the form of "periodontitis" develops, suppressing the periodontium (Bartold and Narayanan, 2006). Although various treatments are available for periodontitis, reliable regeneration of the PDL is not yet possible (Melcher et al., '86; Somerman et al., '99; Bartold et al., 2000; Shimono et al., 2003). A basic understanding of PDL development at the molecular level is required to develop regeneration therapy of PDL, as the regeneration process needs to mimic the cellular events of PDL development (Grzesik and Narayanan, 2002). Recent advances have revealed the existence of progenitor or stem cells in adult PDL (Handa et al., 2002a; Seo et al., 2004; Fujii et al., 2008). To clarify the differentiation mechanisms of these cells, it is necessary to investigate the molecular basis of PDL development.

Progenitor cells are generated by multipotent stem cells and can differentiate into one specific type of cell depending on the cellular environment during organogenesis (Potten and Loeffler, '90). In the case of PDL development, dental mesenchyme generates the dental follicle (DF), which contains PDL cell progenitors that contribute to the formation of the PDL (Cho and Garant, 2000). However, the molecular aspects of PDL development are only vaguely understood owing to the limited information available on marker genes for PDL cell progenitors and PDL cells. Conversely, thanks to recent advances in the availability of genome sequence information for both human and mouse, transcriptomes for various tissues, and bioinformatics for database construction, the identification of genes potentially involved in the development of specialized tissues has become possible (Sunkin and Hohmann, 2007). We describe herein the initial screening of potential marker genes for PDL cell progenitor and PDL cells from the KK-Periome database, which is a collection of transcripts expressed during human PDL development. The significance of extracellular matrix (ECM) components as markers for PDL development is discussed.

## MOLECULAR MECHANISMS OF PDL DEVELOPMENT

The PDL originates from the DF formed during the cap stage of tooth germ development by an ectomesenchymal progenitor cell population originating from cranial neural crest cells (Chai et al., 2000; Cho and Garant, 2000). DF is formed from cranial neural crest-derived dental mesenchyme on embryonic day 15 (E15) mouse embryo at the stage of tooth germ development (Fig. 1). As DF contains three kinds of progenitor cells (cementoblast, PDL cell, and osteoblast progenitors), developmental events in the DF are of considerable interest (Morotome et al., '98; Hou et al., '99; Saito et al., 2005). At the cap stage in E15 mouse embryo, dental mesenchyme undergoes differentiation into two distinct types of cells: dental papilla (DP) cells and DF cells (Chai et al., 2000; Cho and Garant, 2000). At the E17 bell

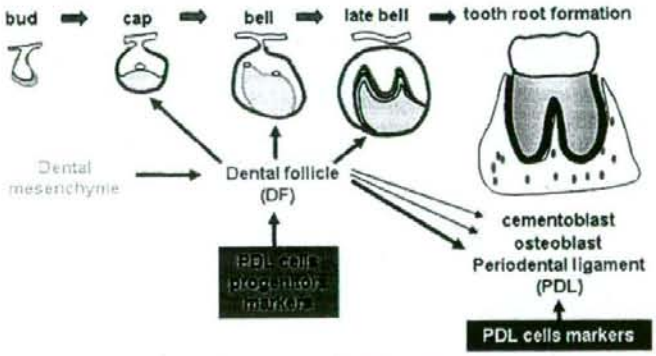


Fig. 1. Schematic of periodontal ligament development. The dental follicle (DF) is initially formed from dental mesenchyme in the cap stage of tooth germ, and differentiates to progenitors of the periodontal ligament (PDL), cementoblasts, and osteoblasts during the bell and late bell stages. After birth, differentiation of progenitors within the DF forms periodontium during the tooth root formation stage. Investigation of the molecular mechanisms underlying the PDL development requires the identification of specific marker for PDL cell progenitors and PDL cells.

stage, DP cells differentiate into cells of the odontogenic lineage, such as odontoblasts or dental pulp cells, eventually giving rise to the dentin–pulp complex in the tooth (Thesleff et al., 2001). After birth, differentiation begins during tooth root formation when cementoblast progenitors migrate to the surface of the tooth root and differentiate into cementoblasts to form cementum matrix (Bosshardt and Schroeder, '96; Saito et al., 2001). At almost the same time, PDL progenitors differentiate into PDL on cementoblasts, inserting collagen fibers known as Sharpey's fibers into the cementum matrix. Fiber insertion also takes place along the alveolar bone. Finally, both bone- and PDL-derived fibers coalesce in the PDL to form the intermediate plexus, which resembles tendinous tissue (Johnson, '87; Cho and Garant, '89, '96).

The fate of progenitor cells in the DF has been investigated using cultured DF cells. Because the experimental model for in vitro differentiation of PDL is not available, implantation analysis using immunodeficiency mice has been used for studying the potential of PDL formation. For instance, cultured DF cells do not form mineralized nodules in vitro even after treatment with mineralization-inducing medium, but are able to form PDL-like tissue and cementum-like structures after implantation into immunodeficient mice (Handa et al., 2002b; Yokoi et al., 2007). In addition, progenitor cell lines for cementoblasts and PDL have been established from immortalized DF cells using single cell cloning methods (Morsczeck et al., 2005; Saito et al., 2005; Luan et al., 2006). These findings support the notion that PDL cell progenitors are present in DF. Investigation of the differentiation of PDL cell progenitors into PDL has thus enabled clarification of transcription factors, signaling molecules, and specific niches involved in PDL differentiation. However, elucidation of the precise differentiation mechanisms for PDL cell progenitors in DF has been hampered by the paucity of specific marker genes for these cells (Pitaru et al., '94; Diekwisch, 2002; Nanci and Bosshardt, 2006). Identification of specific markers that can distinguish PDL cell progenitors and PDL cells is thus important (Fig. 1).

## FUNCTIONAL MOLECULES INVOLVED IN PDL DEVELOPMENT

Although no specific marker for PDL is available, several reports have shown that PDL development seems to be dependent on the ECM, which regulates collagen fibril formation and is mainly deposited in PDL (McCulloch, 2000). Type I and III collagens are the major components of collagen bundles and Sharpey's fibers in PDL, and fibril assembly is responsible for the structural stabilization and mechanical characteristics of the PDL (Ten Cate, '94; MacNeil et al., '98). Interactions between fibrillar collagen and small leucine-rich proteoglycans (SLRPs) such as lumican and decorin regulate flexible connective tissues such as tendons (Danielson et al., '97; Ezura et al., 2000; Matheson et al., 2005). Lumican- and decorin-deficient mice show abnormal collagen fibrils in PDL, indicating that these SLRPs regulate the organization of collagen fibrils during PDL formation (Matheson et al., 2005). Conversely, Yamada et al. (2001, 2007) found that PDL-associated protein (PLAP)-1/asporin, which belongs to a novel SLRP family, is specifically expressed not only in DF but also in adult PDL. PLAP-1/asporin directly interacts with bone morphogenetic protein-2 to inhibit the mineralization of the PDL. This finding strongly suggests that PDL synthesizes PLAP-1/asporin as a negative regulator of mineralization to prevent ankylosis. Noncollagenous ECM is also involved in the formation of PDL. Periostin is a secreted adhesion protein that displays homology with fasciclin I, an insect growth cone guidance protein (Horiuchi et al., '99). During tooth germ development, periostin is initially expressed in the DF, and then becomes restricted to postnatal PDL during tooth root formation (Kruzynska-Frejtag et al., 2004). Periostin-deficient mice show disorganization of the PDL and alveolar bone resorption, suggesting a critical role of this protein in the maintenance of PDL and onset of periodontal disease (Rios et al., 2005; Kii et al., 2006).

As PDL is morphologically similar to tendon and ligament, the suggestion has been made that tendon/ligament phenotype-related genes that are specifically expressed during the formation of tendons and ligaments are involved in the differentiation of PDL cell progenitors (Table 1) (Oliver et al., '95; Wolfman et al., '97; Brent et al., 2003; Salingcarnboriboon et al., 2003). Based on these observations, the ECM predominantly deposited in PDL and tendon/ligament-related genes that are expressed in PDL may contribute to the formation of PDL. However, it is difficult to investigate whether these factors are involved in PDL development owing to the lack of specific markers for PDL cell progenitors and PDL cells.

*TABLE 1. List of tendon- and ligament-related genes*

| Functional categorization | Gene name | References |
|---|---|---|
| Transcription factor | Scleraxis | Brent et al. (2003) |
| | Six1<br>Six2 | Oliver et al. ('95) |
| Plasma membrane protein | EphA4 | Patel et al. ('96) |
| Signaling molecules | GDF5 | Sodersten et al. (2005) and Wolfman et al. ('97) |
| | GDF6<br>GDF7 | |

Genes expressed in tendon and ligament are listed according to functional categorization. GDF, growth and differentiation factor.

## THE KK-PERIOME DATABASE AND SCREENING OF SPECIFIC MARKER FOR PDL CELL PROGENITORS AND PDL CELLS

To identify specific markers for PDL cell progenitors and PDL cells, we established the KK-Periome database as a collection of 617 clusters of expressed sequence tags (ESTs) highly expressed in human PDL (Nishida et al., 2007). These EST clusters were derived from short single-pass sequence reads of 11,520 randomly selected clones from the human PDL cDNA library and were considered invaluable for identifying particular genes and unique gene expression patterns during PDL development. For the identification of specific markers for PDL cell progenitors and PDL cells from the KK-Periome database, screening of candidate genes was performed using two different criteria of functional classification and expression pattern (Fig. 2). As ECM components are considered to be involved in the determination of cell specificity (Engler et al., 2006; Scadden, 2006), we hypothesized that the DF and the PDL are each composed of specific ECM proteins, and that these unique matrices could serve as specific markers for PDL cell progenitors or PDL cells. As a result of functional classification, we obtained 38 ECM clusters from KK-Periome database, which include type I collagen α 2, Secreted proteins acidic, cystein-rich (SPARC)/osteonectin, collagen type III, periostin, lumican, type I collagen α I chain, osteopontin, decorin, fibronectin, and PLAP-1/asporin as the ten most highly expressed transcripts. Most of the ECM proteins present here for the characterization of the PDL are ubiquitous molecules although the PDL has a unique structure and function (Yamada et al., 2001). Therefore, we next screened ECM proteins that had not previously been reported in PDL according to a PubMed search, and 13 ECM clusters were obtained as candidates, including the genes for collagen type XI α 1, SPARC-like 1, tenascin-N, collagen type XV α 1, hyaluronan and proteoglycan link protein 1, vitrin, type XVI collagen α 1, SPARC-related modular calcium binding 2, granulin, fibulin 5, F-spondin, nidogen 1, and leprecan 1. We next investigated temporal and spatial expression patterns of these 13 candidate genes using in situ hybridization analysis with mouse DF and PDL tissues. As a result, two genes

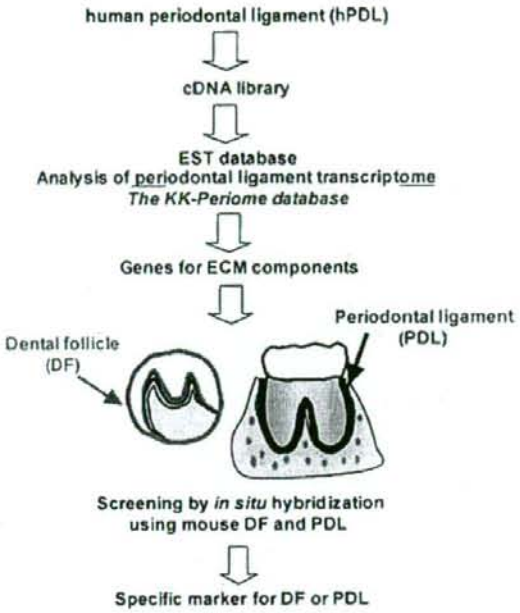


Fig. 2. Strategy for creating a transcriptome database for PDL (KK-Periome database) and a screening marker for PDL cell progenitors and PDL cells. The KK-Periome database was established following the expressed sequence tag (EST) sequence analysis of the human PDL cDNA library. Genes for ECM components including collagen type XI α 1, SPARC-like 1, tenascin-N, collagen type XV α 1, hyaluronan and proteoglycan link protein 1, vitrin, type XVI collagen α 1, SPARC-related modular calcium binding 2, granulin, fibulin 5, F-spondin, nidogen 1, and leprecan 1 were isolated and specific marker for PDL cell progenitors and PDL were screened by in situ hybridization analysis of mouse DF and PDL. PDL, periodontal ligament; ECM, extracellular matrix; DF, dental follicle.

(F-spondin and tenascin-N) were identified as candidate markers for the DF and the PDL, respectively. Interestingly, F-spondin was initially expressed in the DF at the cap stage and became progressively more evident in the DF at the bell stage. In addition, no expression of F-spondin was observed in other types of cells in the tooth germ, including DP, dental epithelium, odontoblasts, or ameloblasts. However, expression of F-spondin in DF was significantly down-regulated after birth and completely absent in the PDL of adult mice. Unlike F-spondin, expression of tenascin-N was not detected in DF cells of the developing tooth germ in the embryo, but was strongly induced in adult PDL. Although PDL contained several types of cells including PDL cells, epithelial cells of Malassez, endothelial cells, osteoblast progenitors, and cementoblast progenitors (McCulloch et al., 2000), the expression of tenascin-N is restricted to adult PDL cells. Based on these observations, F-spondin and tenascin-N may serve as specific markers for DF or PDL, respectively (Fig. 3).

## F-SPONDIN AND TENASCIN-N AS MARKERS FOR PDL CELL PROGENITORS AND PDL CELLS

F-spondin is a component of the ECM, which is known to be present in the embryonic floor plate of vertebrates (Klar et al., '92; Tzarfati-Majar et al., 2001) and the caudal somite of birds (Debby-Brafman et al., '99), apparently playing a dual role in the patterning of the nervous system. F-spondin promotes the adhesion and the outgrowth of axons, but inhibits the adhesion of neural crest cells. As F-spondin-knockout mice or transgenic mice expressing F-spondin specifically in PDL are not available, the precise function of F-spondin during PDL development is unclear. However, transient expression of F-spondin in DF strongly suggests the involvement of PDL development, rather than the maintenance of adult PDL function such as withstanding force of mastication. F-spondin was highly expressed in a human cementoblast cell line, and overexpression induced the up-regulation of cementoblast/osteoblast mar-

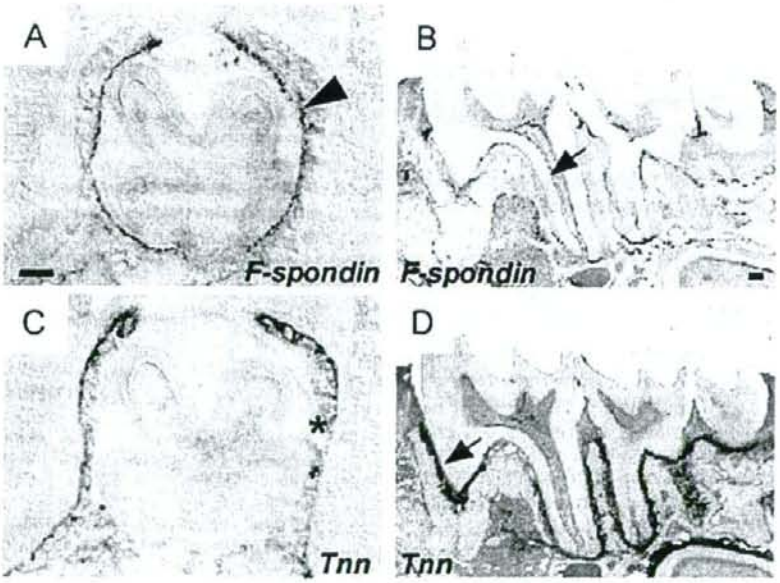


Fig. 3. Expression of F-spondin and tenascin-N during PDL development. In situ hybridization analyses of *F-spondin* (**A** and **B**) and *tenascin-N* (**C** and **D**) in the late bell stage of tooth germ (A and C) and adult molar (B and D) are shown. Note that *F-spondin* is intensely expressed in DF (A, arrowhead), whereas expression is significantly down-regulated in PDL (B, arrow). In contrast, *tenascin-N* (*Tnn*) is strongly expressed in the PDL (D, arrow), but not expressed in the DF (C, asterisk). Bar, 50 μm. Reprinted from Nishida et al. 2007. Transcriptome database KK-Periome for periodontal ligament development: expression profiles of the extracellular matrix genes. Gene 404:70–79 with permission from Elsevier. PDL, periodontal ligament; DF, dental follicle.

ker gene such as bone sialoprotein and osteocalcin (Kitagawa et al., 2006). Moreover, F-spondin protein was detected in the cementum matrix. Contrasting with these results, we found high levels of F-spondin mRNA in the DF and dramatic decreases in the adult PDL suggesting that F-spondin may involve in the formation of DF.

Tenascin-N is a novel member of the tenascin family of proteins, which are expressed in the brain, kidneys, and spleen of adult animals. Unlike other tenascins, tenascin-N is highly expressed in neurons of the central nervous system (Neidhardt et al., 2003). Interestingly, the expression of tenascin-N was strongly up-regulated in the adult PDL, whereas no expression was observed in the DF. Periostin has been used as a marker for distinguishing PDL from adjacent connective tissues such as bone and gingiva in adult tissue (Horiuchi et al., '99; Kruzynska-Frejtag et al., 2004). The expression of periostin was detected in both adult PDL and DF in the developing tooth germ. In contrast to periostin, tenascin-N was specifically expressed in adult PDL (Nishida et al., 2007). In addition, the expression patterning of PLAP-1/asporin resembled that of periostin during PDL development (Yamada et al., 2007). Tenascin-N was thus considered to serve as a distinct marker of differentiated PDL cells. Furthermore, tenascin-N was detected in the costal perichondrocytes that eventually form the ligament tissue suggesting that the expression may be associated with the formation of ligamentous tissues (Nishida et al., 2007).

## CONCLUSION

PDL development requires PDL cell progenitors present in the DF during tooth germ development (Cho and Garant, 2000). Although stem/progenitor cells capable of forming PDL have been identified (Seo et al., 2004; Yokoi et al., 2007; Fujii et al., 2008), the precise differentiation mechanisms of these cells remain unclear owing to the unavailability of markers for PDL cell progenitors and PDL cells. A combination of the transcriptome database for the human PDL (KK-Periome database) and in situ hybridization analysis helped to identify F-spondin and tenascin-N as specific markers for PDL cell progenitor or PDL cells, respectively (Fig. 4). Although functional roles of F-spondin and tenascin-N are unclear, development of the PDL is apparently associated with changes in the expression of these proteins. To investigate whether these molecules are involved in PDL formation, development of functional assay system either in vitro or using transgenic mice is necessary. However, further screening of the KK-Periome database may provide more novel marker genes that are useful for analyzing the molecular mechanisms of PDL development and identification of eventual therapeutic targets for the treatment of periodontal disease.

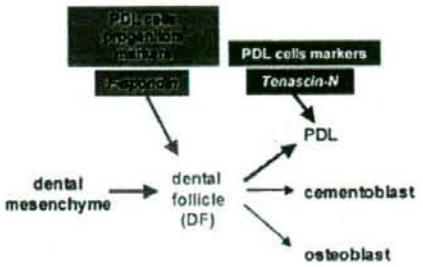


Fig. 4. F-spondin and tenascin-N as markers for PDL cell progenitors or PDL cells. F-spondin is specifically expressed in DF, whereas the expression is significantly down-regulated in PDL. In contrast, expression of tenascin-N is strongly induced in the PDL. We thus proposed F-spondin and tenascin-N as markers for PDL cell progenitors or PDL cells. PDL, periodontal ligament; DF, dental follicle.

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**REVIEW (Developmental Biology of HERS and Tooth Root Formation)**

# Comprehensive Analysis of Tissue-specific Markers Involved in Periodontal Ligament Development

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**Key words** : transcriptome/EST/periodontal ligament/extracellular matrix/development

**Abstract** : The periodontal ligament (PDL) is a tendon/ligament-like fibrous tissue that connects the tooth root surface and the alveolar bone. General interest has been expressed in PDL development as a model for connective tissue formation because of its ability to adapt to mechanical loading. PDL cell has its origin in the dental follicle (DF) cell, and it begins to differentiate during tooth root development. During apical development of the tooth root, PDL progenitors in DF differentiate into PDL cells to form the PDL ; however, the molecular mechanisms of PDL development have not yet been clarified, as PDL lineage-specific markers are not available. We recently established a transcriptome database for the human PDL (KK-Periome database), and screened the genes specifically expressed during PDL development. Initial screening of the database allowed us to identify F-spondin and tenascin-N, which are restricted to DF and PDL cells, respectively. Thus, these could serve as PDL lineage-specific markers that would be useful for investigating the molecular mechanisms of PDL development.

## Introduction

The periodontal ligament (PDL) is a connective tissue capable of withstanding mechanical loading, such as occlusal force. Like most connective tissues, collagen type I is predominant, but collagen types III, IV, V, VI and XII and proteoglycans, which regulate collagen fibril formation, are also deposited in the PDL extracellular matrix (ECM)[1—3]. The cellular content of PDL is predominated by PDL cells which can form Sharpey's fibers, collagen fibers embedded in the calcified matrix[4]. The structure of PDL is often irreversibly damaged by the chronic inflammatory disease "periodontitis", which affects the periodontium[5]. Although various treatments are available for periodontitis, it is not yet possible to reliably regenerate the PDL[6—8]. To develop methods to regenerate the damaged PDL, a basic understanding of PDL development at the molecular level is required, as the regeneration process must mimic the cellular events of PDL development[9,10]. Recent advances have revealed that progenitor or stem cells are present in the adult PDL[11—13]. Hence, it is important to clarify the molecular basis of PDL regeneration from these stem cells or progenitors.

The molecular mechanisms of PDL development have not yet been clarified, as there is limited information on the genes related to the differentiation and lineage commitment of PDL progenitors[14]. However, recent advances in transcriptome database technology and the availability of human and mice genome sequences allow unlimited access to information

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about genes potentially involved in the development of specialized tissue[15]. In this paper, we describe the methodology for screening PDL lineage-specific markers from the KK-Periome database, and discuss the significance of ECM components as markers for PDL development.

## Periodontal Ligament Development

PDL cells originate from DF cells that contain an ectomesenchymal progenitor cell population derived from cranial neural crest cells[16]. As DF contains three kinds of progenitors, including cementoblast progenitors, PDL progenitors, and osteoblast progenitor, the developmental events of DF are of considerable interest (Fig. 1). During the tooth root-forming stage, cementoblast progenitors migrate to the surface of the tooth root and differentiate into cementoblasts to form the cementum matrix[17,18]. Almost at the same time, PDL progenitors differentiate into PDL on cementoblasts, inserting collagen fibers known as Sharpey's fibers into the cementum matrix. Fiber insertion also takes place along the alveolar bone. Finally, both bone- and PDL-derived fibers coalesce in the PDL to form the intermediate plexus, which is similar to tendinous tissue[14,19].

A recent study demonstrated that cultured mouse DF cells are capable of forming PDL-like tissue that shows a high expression of PDL formation-related genes, such as periostin and type XII collagen, upon implantation into immunodeficient mice[20]. In addition, a PDL progenitor-like cell line that could not form mineralized nodules was isolated from cultured DF cells, thus suggesting that PDL lineage-committed progenitors are present among DF cells[21–23]. Although not well characterized at the molecular level, investigation of DF cell differentiation into PDL cells made it possible to clarify PDL development ; however, the precise differentiation mechanisms of DF cells remain poorly understood due to limited information on specific marker genes for PDL lineage commitment. Identification of PDL lineage-specific markers throughout the course of DF cell differentiation is thus important.

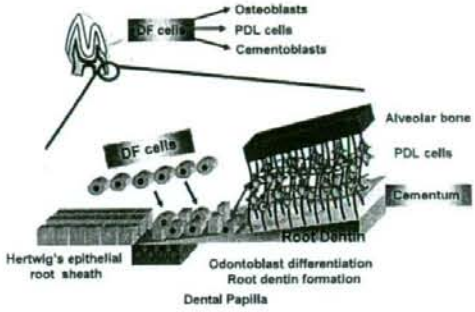


**Fig. 1** Schematic model of PDL development
DF cells are composed of ectomesenchymal progenitors for the periodontal ligament, cementoblasts and osteoblasts. After tooth root formation, cementoblast progenitors migrate onto the tooth root surface and then differentiate into cementoblasts. At the same time, PDL progenitors differentiate to form PDL cells, and Sharpey's fiber insertion into cementum occurs. Osteoblast progenitors also differentiate into osteoblasts to produce the bony socket wall. Finally, both bone- and PDL-derived fibers coalesce in the PDL to form the intermediate plexus.

## Establishment of KK-Periome Database

For identification of PDL lineage-specific markers, we established the KK-Periome database, which is a collection of expressed sequence tags (ESTs) that are strongly expressed in human PDL[24]. ESTs are short, single-pass sequence reads of randomly selected clones from a cDNA library, and are invaluable for identifying genes and gene expression patterns in particular types of tissue[25]. Thus, an EST database may provide a platform for identifying PDL specific-markers. It was previously reported that PDL possessed a stem cell population that can differentiate into PDL cells, osteoblasts and adipocytes[12]. We also reported that a progenitor able to form PDL tissue was present in DF and PDL[11,23]. From these findings, we hypothesized that both differentiated PDL-specific and immature PDL-specific markers could be identified from the EST database of PDL.

In order to construct an EST database for human

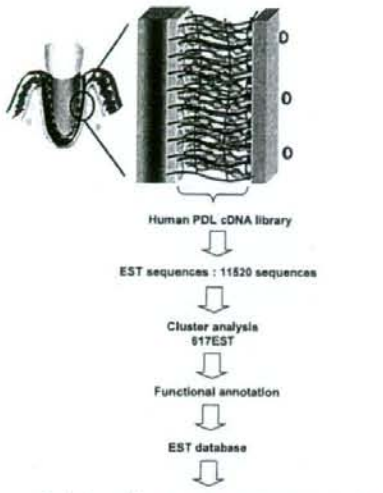


**Fig. 2** Strategy for construction of KK-Periome database

The KK-Periome database is a collection of 617 clusters of expressed sequence tags (ESTs) that are highly expressed in human PDL cells. These EST clusters were derived from short sequence analysis of 11,520 randomly selected clones from a human PDL cDNA library. The 617 ESTs were classified according to their ontological function.

PDL, we compiled a cDNA library from the PDL and sequenced 11,520 cDNA clones. The resulting sequence data was assembled into 617 EST clusters, and these comprised the KK-Periome database (Fig. 2). As expected, the most abundantly expressed genes in the KK-Periome database were type I collagen and type III collagen, which are the major fibrillar collagens in PDL[26]. Seven genes with particularly high expression in PDL are SPARC, periostin, lumican, osteopontin, decorin, fibronectin, and PLAP-1/Asporin[27–30]. This expression profile was similar to the previous PDL-EST database[29]. Thus, the KK-Periome database reflects the PDL phenotype.

| Gene symbol | DF | PDL |
|---|---|---|
| Col11a1 | | |
| Sparcl1 | | |
| * Tnn | | |
| Col15a1 | | |
| Hapln1 | | |
| Vit | | |
| ▲ Col16a1 | | |
| Smoc2 | | |
| Grn | | |
| Fbln5 | | |
| ● Spon1 | | |
| Nid1 | | |
| ▲ Lerpe1 | | |

**Fig. 3** Expression pattern analysis of 13 ECMs in DF cells and PDL cells

The expression patterns of 13 ECM clusters were investigated in DF cells and PDL cells by *in situ* hybridization analysis. Note that F-spondin (Spon1) is intensely expressed in DF cells (circle). In contrast, tenascin-N (Tnn) is strongly expressed in PDL cells (asterisk). Type XVI collagen (Col16a1) and leprecan (Lerpe1) are strongly expressed in PDL cells ; however, they are also expressed in odontoblasts (triangles). Black : strongly positive expression ; Gray : weakly positive expression ; White : no expression.

### Screening of PDL Lineage-specific Genes

From the KK-Periome database, PDL lineage-specific markers were screened by two different parameters, functional classification and expression pattern : (1) As ECM is involved in the specificity of PDL cells, a potential PDL-specific marker was obtained from ECM clusters in the KK-Periome database ; (2) Candidate genes that may play critical roles in the

process of PDL development were selected by expression pattern on *in situ* hybridization analysis in mouse DF cells and adult PDL cells. As a result of screening, 13 EST clusters were obtained as candidates. These included collagen type XI alpha 1 (*COL11A1*), SPARC-like 1 (*SPARCL1*), tenascin-N (*TNN*), collagen type XV alpha 1 (*COL15A1*), hyaluronan and proteoglycan link protein 1 (*HAPLN1*), vitrin (*VIT*), type XVI collagen alpha 1 (*COL16A1*), SPARC-related modular calcium binding 2 (*SMOC2*), granulin (*GRN*), fibulin 5 (*FBLN5*), F-spondin (*SPON1*), nidogen 1 (*NID1*), and leprecan 1 (*LERPE1*) (Fig. 3). In the final step, we examined and compared gene expression in DF cells during the late bell stage of the tooth germ, and in PDL cells of the adult periodontium by *in situ* hybridization analysis. Among the candidate genes, F-spondin was expressed specifically in DF cells, and no expression was seen in other cell types in the late bell stage of the tooth germ, thus suggesting that F-spondin could serve as a DF marker. Nidogen 1 was also intensely expressed in DF cells, as well as in odontoblasts. The remaining candidate genes showed weak or no expression in DF cells, while they were expressed in other cells of the tooth germ. In PDL cells, the expression patterns of candidate genes were significantly different from those in DF cells. Expression of F-spondin and nidogen 1 was markedly lower in adult PDL cells, whereas the expression of tenascin-N, type XVI collagen alpha 1, and leprecan was markedly up-regulated. Among the up-regulated genes, tenascin-N was highly restricted to PDL cells, indicating that it could serve as a PDL marker.

### F-spondin and Tenascin-N Serve as PDL Lineage-specific Markers

As a result of screening, F-spondin and tenascin-N were identified as candidate PDL lineage-specific markers. To test this possibility, we examined the expression patterns of F-spondin and tenascin-N during PDL development. F-spondin was initially expressed in the cap stage of DF cells and became progressively evident in the bell stage of DF cells ; however, the expression of F-spondin was significantly down-regulated in DF cells after birth, and no expression was observed in adult PDL cells. In contrast, the expression of tenascin-N could not be detected in the DF cells of developing tooth germs in the embryo, but was intensely up-regulated in adult PDL cells. Based on these observations, we believe that F-spondin and tenascin-N could serve as PDL lineage-specific markers for distinguishing DF cells from PDL cells (Fig. 4).

We then investigated whether F-spondin and tenascin-N are involved in the differentiation of PDL cells. Previous work has demonstrated that differentiation of cultured human PDL (hPDL) cells is induced upon implantation into immunodeficient mice[11,12]. Interestingly, tenascin-N expression was significantly induced in hPDL cells upon implantation while no expression was observed in *in vitro* cultured cells. These data suggest that hPDL cells express tenascin-N as a result of differentiation. Expression pattern analysis during PDL development agreed with the results of the *in vivo* differentiation study, thus suggesting that tenascin-N is a useful marker for differentiated PDL cells. In contrast to tenascin-N, the expression of F-spondin expression levels did not change after implantation into SCID mice. This is consistent with the notion that F-spondin may be involved in the initial process of PDL formation. Thus, F-spondin may play a role in the early stages of PDL formation, as demonstrated by *in situ* hybridization analysis during tooth germ development.

### F-spondin Serves as DF Marker

F-spondin is an ECM-attached protein observed in the embryonic floor plate of vertebrates[31,32] and the caudal somite of birds[33], playing a dual role in the patterning of the nervous system. It promotes the adhesion and outgrowth of axons but inhibits adhesion of neural crest cells. Recent evidence suggests that F-spondin may act as a regulator of amyloid precursor protein (APP) processing[34]. Specifically, F-spondin binds to the extracellular domain of APP and inhibits $\beta$-secretase cleavage. Thus, F-spondin serves as a regulator of neuronal development, and is related to the onset of Alzheimer's disease. F-spondin also

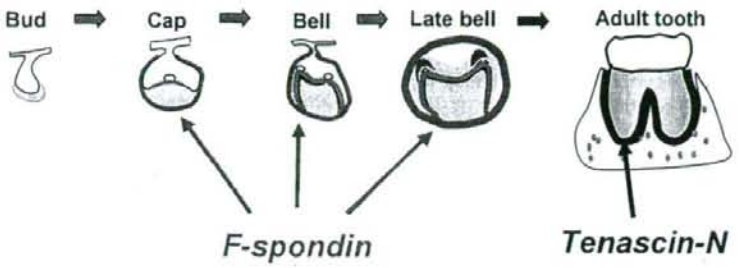


**Fig. 4** F-spondin and tenascin-N serve as PDL lineage-specific markers
F-spondin is specifically expressed in DF during the cap, bell and late bell stages of the tooth germ ; however, its expression was significantly down-regulated in PDL cells. In contrast, the expression of tenascin-N was strongly induced in PDL cells of adult teeth. Thus, F-spondin and tenascin-N may serve as PDL lineage-specific markers that can distinguish DF cells and PDL cells.

appears to be involved in the formation of the periodontium during tooth development. Kitagawa *et al.* reported that F-spondin is strongly expressed in an immortalized human cementoblast cell line, and an overexpression study revealed that it affected cementoblast/osteoblast differentiation[35]. Immunohistochemical staining revealed that F-spondin protein was deposited in the cementum matrix. In contrast to these results, we showed that F-spondin may play a role in the early stages of PDL formation, as demonstrated by the presence of high F-spondin mRNA levels in DF cells, which subsequently decreases dramatically in adult PDL cells (Fig. 4). Although the precise function of F-spondin during tooth germ development is not yet clear, F-spondin expression was observed in dermal papilla cells. Numerous ectodermal tissues, such as teeth and hair, share similar epithelial-mesenchymal interactions during early development[36]; therefore, the signaling molecules involved in ectodermal organogenesis may regulate the expression of F-spondin.

## Tenascin-N Is Expressed in Differentiated PDL Cells

Tenascin-N was identified as a novel member of the tenascin family that is expressed in the adult brain, kidney and spleen. Contrary to other tenascins, tenascin-N is strongly expressed in neurons rather than glial cells in the central nervous system[37]. Interestingly, tenascin-N is strongly expressed in PDL cells, while no expression was observed in DF cells (Fig. 4). Most ECMs reported for PDL cells are expressed in both DF cells and PDL cells. For instance, periostin and PLAP1/Asporin were strongly expressed in DF cells, as well as in PDL cells[38,39]. Thus, tenascin-N may serve as a marker for terminally differentiated PDL cells. Tenascin-N expression was also detected in costal perichondrocytes, which form ligament tissue, thus suggesting that tenascin-N is associated with ligament tissue formation[24].

## Concluding Remarks

In summary, a combination of the establishment of the KK-Periome database and *in situ* hybridization screening helped to identify PDL lineage-specific markers that would be useful for investigating PDL development. The development of PDL is associated with the change in the expression of ECM components. Among these, F-spondin and tenascin-N appear to be PDL lineage-specific markers. Further investigation is necessary to verify the actual function of these proteins/genes. Nevertheless, spatial and temporal expression analysis indicated that F-spondin and tenascin-N are related to the PDL differentiation process during development of the PDL lineage. Consequently, the molecular mechanisms of