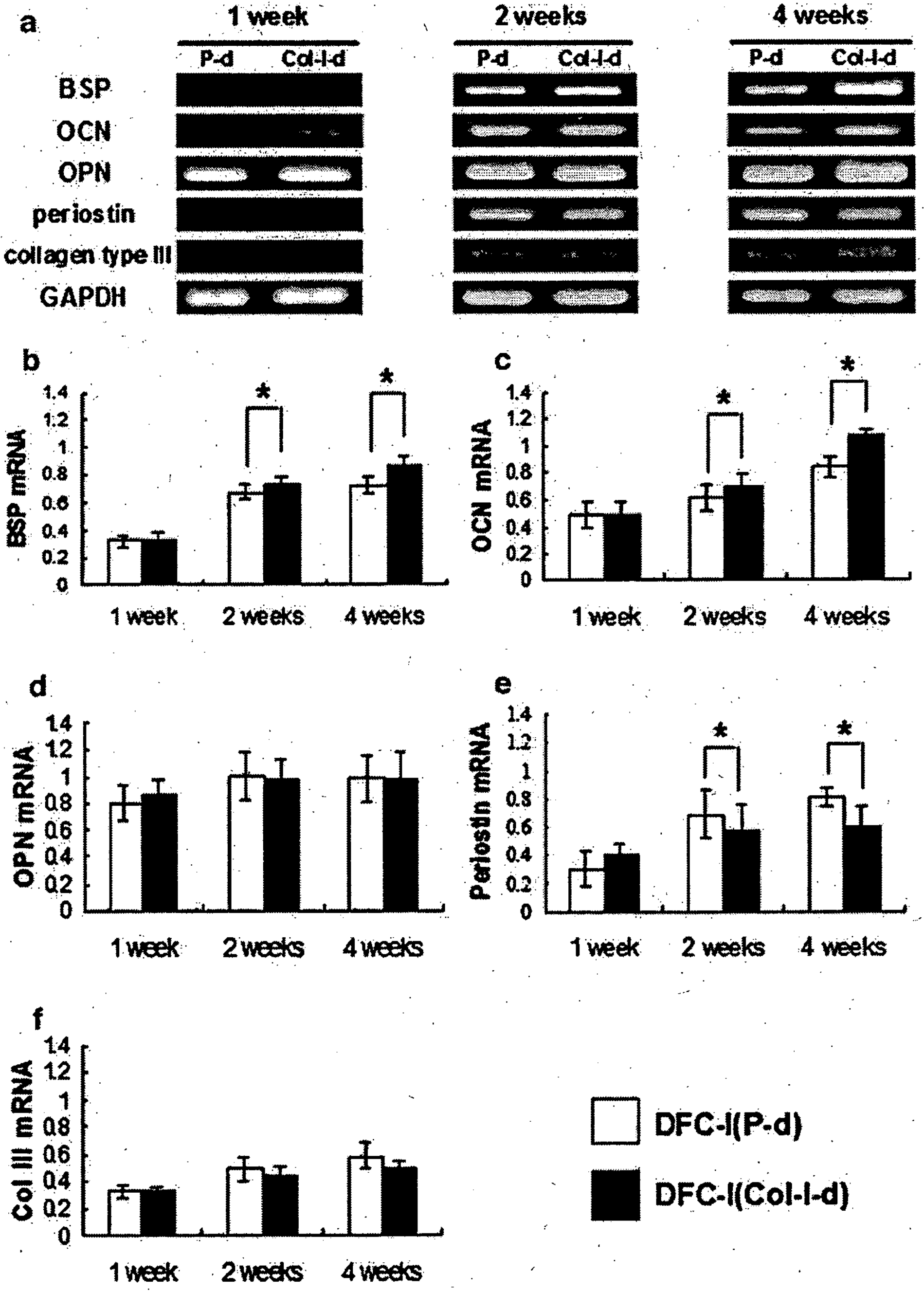
and OCN were expressed, albeit at a lower level in DFC-I than in PDLC and BMSC (Fig. 3b–f). These data suggested that DFC-I represented more immature cells compared with PDLC and BMSC.

Effect of Col-I matrix on cell proliferation and ALPase activity

After 1 and 7 days in culture, the cell proliferation of DFC-I cultured on Col-I-d was similar to that on P-d. After 14 days, DFC-I cultured on Col-I-d showed a significantly higher rate of cell proliferation compared with clones cultured on P-d (Fig. 4a). ALPase activity is a recognized mineralization marker in tissue (Alliot-Licht et al. 2005; Pavasant et al. 2003). The DFC-I cultured for 1, 7, and 14 days exhibited different and increasing levels of ALPase activity (Fig. 4b). At days 1 and 7 of culture, the ALPase activity change was the same regardless of culturing conditions, but at 14 days, DFC-I cultured on Col-I-d showed higher activity than those cultured on P-d (Fig. 4b). This

Fig. 6 Effects of Col-I matrix on gene expressions in DFC-I in vivo. **a** At 1 week, the BSP, periostin, and collagen type III genes were not expressed in either implant type but were clearly expressed at 2 and 4 weeks after transplantation. OCN and OPN genes were expressed strongly in both implant types at all time points. **b–f** Quantification of expression of BSP, OCN, OPN, periostin, and collagen type III genes by using Scion picture-imaging software. BSP and OCN genes were more highly expressed in the implants cultured on Col-I-d than in those on P-d at 2 and 4 weeks. In contrast, periostin expression was higher in implants on P-d at 2 and 4 weeks than in those cultured on Col-I-d. No significant difference in the expressions of OPN and collagen type III was seen for both implant types at all time points. *Statistically significant at $P<0.05$ (paired t-test)



result suggested that adhesion to Col-I promoted differentiation of the DFC-I.

Effect of Col-I on gene expressions in clone-DFC-I

We next investigated, by rt-PCR, the effect of Col-I culturing on gene expression in the DFC-I cells (Fig. 5). Putative markers of PDLC and cementoblasts/osteoblasts, such as periostin, biglycan, and OCN, were amplified more readily from DFC-I cultured on Col-I compared with DFC-I cultured on P-d. Periostin, biglycan, and OCN expression levels in DFC-I cultured on Col-I-d were approximately 100-fold, 6-fold, and 5-fold higher, respectively, than in DFC-I cultured on P-d. The expression levels of upregulated genes resembled levels of the same genes in PDLC, whereas the expression levels of Col-I and collagen type III showed no change, even in DFC-I cultured on Col-I. BSP was not expressed in DFC-I cultured on P-d or Col-I-d, or

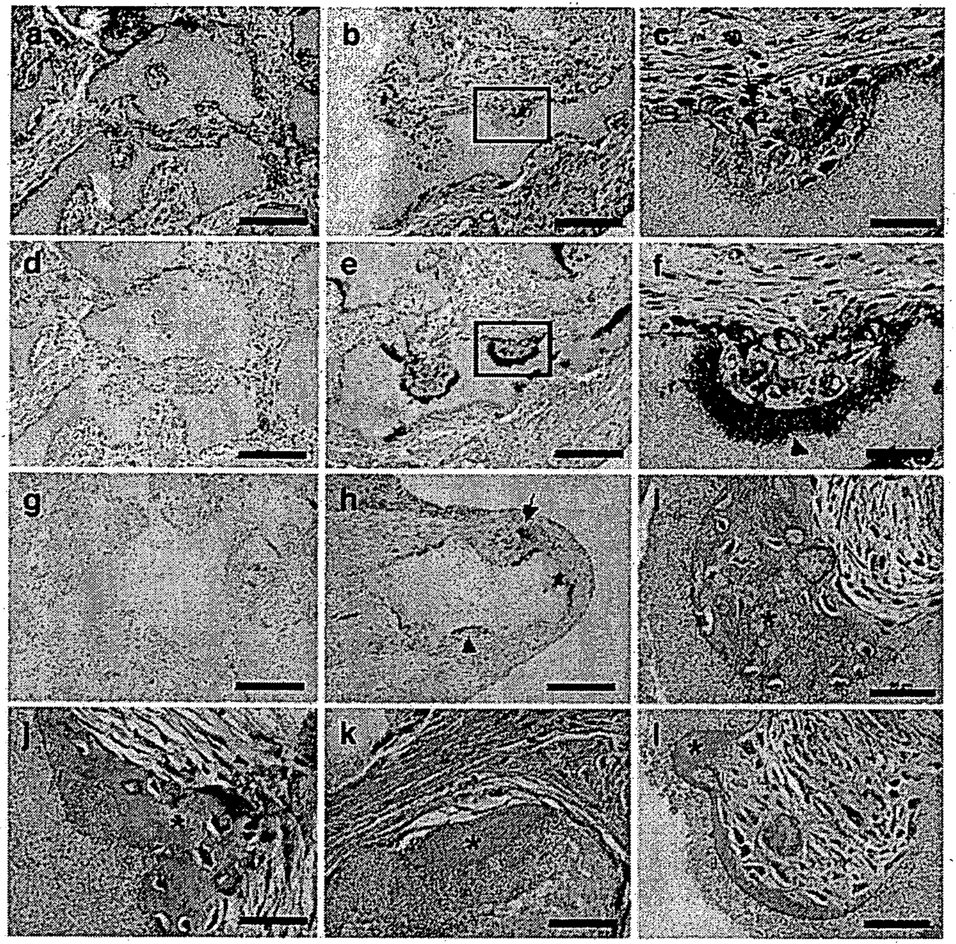


Fig. 7 **a–h** Morphology of representative implants of DFC-I cultured on P-d (**a**, **d**, **g**) or Col-I-d (**b**, **c**, **e**, **f**, **h**), at 4 weeks after transplantation. **a** No hard tissue formation was seen in the implants from P-d. Connective tissues were formed between the TCP particles. **b** A small amount of the hard tissue was produced in the implants from DFC-I cultured on Col-I-d. **c** Higher magnification of the *boxed area* in **b** showing the mineralized tissues on the surface of the TCP particles in the part of the implants cultured on Col-I-d. **d** No specific staining with the BSP antibody was observed in implants of DFC-I cultured on P-d. **e** Specific staining with BSP was clearly observed in the extracellular matrix of the cells lining the TCP particles from DFC-I cultured on Col-I-d. **f** Higher magnification of the *boxed area* in **e** showing specific staining for BSP in the cytoplasm of cells cultured on Col-I-d and on the surface of the TCP particles (*arrowheads*). **g** Implants of DFC-I cultured on P-d showed no positive Von Kossa staining. **h** Von Kossa staining showed clear mineral nodules (*arrow*) in the parts of implants of DFC-I cultured on Col-I-d and on the surface of the TCP particles (*arrowhead*). **i–l** Morphology of representative implants of DFC-I cultured on P-d (**i**, **k**) and Col-I-d (**j**, **l**), at 8 weeks after transplantation. **i** Hard tissue formation was clearly identified. Bone-like tissues were formed, and osteocyte-like cells were embedded in the part of implants of DFC-I cultured on P-d (*asterisk*). **j** The bone-like tissues were clearly visible in the implants from Col-I-d (*asterisk*). **k** Cementum-like tissues were formed and lined the TCP-particle surfaces (*asterisk*) in the part of implants cultured on P-d. **l** Cementum-like tissues were formed and lined the TCP-particle surfaces in the part of the implants from Col-I-d culture (*asterisk*). No clear differences were apparent in the amount of hard tissue formation in the implants from DFC-I cultured on Col-I-d compared with P-d. *Bars* 200 μm (**a**, **b**, **d**, **e**, **g**, **h**), 100 μm (**c**, **f**, **i**, **j**, **k**, **l**)

in PDLC. These results demonstrated that gene expression patterns in DFC-I cultured on Col-I resembled those of PDLC.

Developmental potential of clone-DFC-I in vivo

In vivo characterization of clone-DFC-I

To extend the comparison of DFC-I cultured on Col-I-d and on P-d, we investigated the potential for mineralization in vivo by transplanting DFC-I in conjunction with TCP into the subcutaneous tissue of immunocompromised scid mice as described previously (Handa et al. 2002; Saito et al. 2005). At 1, 2, and 4 weeks after transplantation, we examined the relevant gene expression in the implants by sq-PCR. No expression of the BSP or periostin genes was seen at 1 week after transplantation in either implant type (DFC-I cultured on Col-I-d versus P-d), but clear expression was detected at 2 and 4 weeks (Fig. 6a,b). Periostin expression was distinctly lower in Col-I-d-cultured implants than in P-d-cultured implants (Fig. 6a,e). OCN and OPN expression was strongly detected in both implants at 4 weeks, with higher levels being detected in the Col-I-d-cultured implants than in those cultured on P-d (Fig. 6a,c,d). Collagen type III expression in implants from both dish types was just detectable at 1 week and gradually increased at 2 and 4 weeks, with no differences in expression levels between culturing conditions (Fig. 6a,f).

In vivo histochemical analysis

At 4 weeks, no hard tissue was apparent in control implants (Fig. 7a), but a small amount of calcified tissue was observed in the experimental group (Fig. 7b,c). We therefore performed immunohistochemical staining on the complex of TCP and developing DFC to examine this possibility in more detail. Some of the cells lining the TCP particles were positive for BSP antibody staining in the experimental group (Fig. 7e,f), whereas no positive staining was detected in the control group (Fig. 7d). Furthermore, Von Kossa staining was performed to investigate whether DFC-I cells cultivated on Col-I-d would promote the formation of mineralized nodules. This staining revealed substantial mineralization of tissue in the implants with DFC-I cultivated on Col-I-d (Fig. 7h), but not in the implants with DFC-I cultivated on P-d (Fig. 7g). These results indicated that DFC-I under Col-I culture conditions had an advanced ability to induce hard tissue formation.

At 8 weeks, hard tissues were identified in both implants on the surface of TCP particles and in the cells (Fig. 7i–l), with no obvious difference between the two experimental groups.

Discussion

We have obtained one clonal cell population (DFC-I) from porcine cultured DFC isolated from developing teeth at the early crown-formation stage. Although previous studies have analyzed DFC from the root surface during root formation, no data exist for cells at the early crown-formation stage (Hakki et al. 2001; Handa et al. 2002; Jin et al. 2003; Saito et al. 2005). The isolation of progenitor cells from the DF may therefore help to identify the as-yet-unknown mechanism for differentiation into the cementoblasts, PDL fibroblasts, and osteoblasts that make up the periodontium (Lekic et al. 1996; Pitaru et al. 1994). The properties exhibited by a single cell clone population could possibly be used to elucidate the mechanisms of differentiation. Based on these ideas, we have propagated and characterized DFC-I with respect to the expression of several marker genes preferentially expressed in PDLC and BMSC (Table 1).

Characterization of the DFC-I gene expression pattern has revealed inconsistencies from the patterns among DF, nonclone-DFC, PDLC, and BMSC. Comparison of our DFC-I with PDLC and BMSC has shown that most osteogenesis-related genes including BSP, OPN, and CTGF are not detectable in DFC-I, although BSP, OPN, and CTGF have been detected in DF and BMSC. Normally, OPN is expressed followed first by BSP and subsequently by OCN, which characterizes the postproliferative phase (Aubin 2001; Liu et al. 2003). Biglycan is a small leucine-rich proteoglycan that binds to various ECM components and has a role in mineralization (Xu et al. 1998). With regard to PDLC, no clear differentiation marker specifically characterizes this type of cell, especially in the pig. In this study, periostin expression has been weakly detected in DFC-I, but strongly in PDLC. Taken together, our results demonstrate the low expression of genes related to osteogenesis in the DFC-I, despite the ECM being osteogenic at this stage of development. Although the DF consists of heterogeneous cell populations, our findings suggest that DFC-I is an immature cell type, distinct from both PDLC and BMSC. Recently, three types of DFC have been reported (Luan et al. 2006). Further examination of the other clone-cell populations obtained in the present study from one tooth bud at the early crown-formation stage is therefore required.

As a second aim of this study, we have evaluated the effect of a Col-I matrix on DFC-I. Col-I is well known to interact with $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ integrin receptors via the DGEA amino acid sequence (Staatz et al. 1991). A previous study has demonstrated that Col-I regulates OCN gene expression, and the mRNA level of OCN gene expression is upregulated in dental pulp cells (Mizuno et al. 2003). By immunohistochemistry, we have shown that

Col-I is a major component of the DF. We have further shown that ALPase activity is higher in Col-I-treated DFC-I, suggesting a role for Col-I in stimulating the differentiation of DFC-I. Sasaki et al. (1990) have shown intense ALPase activity along the plasma membranes of whole cell surfaces in cementoblasts of human deciduous teeth. Moreover, PDLC also express high ALPase activity (Giannopoulou and Cimasoni 1996; Ogata et al. 1995) in vitro in the presence of osteogenic medium (Nohutcu et al. 1997; Ramakrishnan et al. 1995). These findings are in agreement with our results.

We have further explored the expression of genes associated with the periodontium. DFC are similar to odontoblasts and osteoblasts in terms of mineralized tissue formation (Gronthos et al. 2000; Shi et al. 2001). However, the gene expression of BSP has not been found to be upregulated by Col-I in our study. Our results are therefore not consistent with expression patterns in dental pulp and MSCs. On the other hand, Col-I potently stimulates the expression of periostin, biglycan, and OCN in DFC-I. A recent study has shown that PDLC lines and osteoblasts share the expression of gene for periostin but not the genes for BSP or OCN (Saito et al. 2002). Biglycan facilitates the initiation of apatite formation and inhibits the growth of apatite in a gelatin gel system (Boskey et al. 1997). Our results are consistent with these observations and suggest that the expression pattern of DFC-I exposed to Col-I resembles those of PDLC. Together, the results of the ALPase activity and gene expression analyses therefore indicate that a Col-I matrix influences the differentiation of immature DFC along the osteogenic pathway, as for PDLC. However, the limited data obtained from one specific cell population in the present study may be insufficient to provide information on this issue. Therefore, further study is needed on the other clone populations.

Finally, we tested whether DFC-I have the capacity to produce hard tissue. Progenitor cells isolated from bovine DF at the root-formation stage have previously been shown to generate cementum (Handa et al. 2002); however, no data exist for DFC at the early crown-formation stage. Examination of the gene expression patterns related to osteogenesis has revealed the expression of BSP at 2 weeks after transplantation, and OCN, periostin, and collagen type III expressions gradually increase. OCN appears immediately before the start of mineralization (Nakashima 1994), and BSP mRNA is expressed almost exclusively in differentiated osteoblasts, odontoblasts, and cementoblasts (Bianco et al. 1991; Chen et al. 1991b, 1992). Significant differences occur in BSP and periostin expression at 2 and 4 weeks and in OCN at 4 weeks between implants cultured on Col-I-d versus P-d and suggest that Col-I promotes follicle cells in the TCP particles to differentiate along a mineralization pathway in vivo.

The implants in control groups at 4 weeks have no hard tissues, whereas the implants of the experiment group show a small extent of calcification by Von Kossa staining. In addition, a significant difference has been observed in BSP immunostaining. When DFC cultured on Col-I are implanted, the cells lining the TCP particles are BSP-immunostained, but this is not seen with P-d. At 8 weeks, hard tissue formation is apparent in both groups. These findings therefore suggest that the DFC-I under the TCP particles develop into a cementoblast/osteoblast lineage capable of forming a mineralized ECM, and that Col-I facilitates this differentiation in vivo. However, specific factors promoting this differentiation have not been identified in these studies.

Up to now, PDLC have been obtained from the tooth-root surface to establish periodontal-tissue engineering (Akizuki et al. 2005; Hasegawa et al. 2005). Thus, a tooth has to be extracted to obtain periodontal tissues. Most people have an impacted third molar that does not cause occlusion. The DF usually involves impacted third molars, which are often extracted for orthodontic therapy. The use of impacted third molars as a cell source for periodontal-tissue engineering might expand the avenues for treatment of disease in this field. The present DF study may be a promising first step toward complete periodontal-tissue engineering.

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REGULAR ARTICLE

Characteristic phenotype of immortalized periodontal cells isolated from a Marfan syndrome type I patient

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Abstract The periodontal ligament (PDL) is situated between the tooth root and alveolar bone, thereby supporting the tooth, and is composed of collagen and elastic system fibers. Marfan syndrome type I (MFS1, MIM #154700) is caused by mutations in *FBN1* encoding fibrillin-1, which is a major microfibrillar protein of elastic system fibers. MFS1 is characterized by tall stature, aortic/mitral valve prolapse, and ectopia lentis and is occasionally accompanied by severe periodontitis. Since little is known about the biological functions of elastic system fibers in PDLs and the pathogenesis of the periodontitis in MFS1, PDL cells were isolated from an MFS1 patient with a heterozygous missense mutation in a calcium-binding epidermal-growth-factor-like domain of *FBN1*. Isolated PDL cells were immortalized by transducing a retrovirus carrying genes for the human Polycomb group protein, Bmi-1, and human telomerase reverse transcriptase. Immortalized PDL cells from the MFS1 patient (termed M-HPL1) and those of a healthy volunteer (termed HPDL2) both expressed various PDL-related genes. The growth and attachment of M-HPL1 and HPDL2 to hydroxyapatite particles were comparable. However, when M-HPL1 were transplanted with hydroxyapatite particles into immunodeficient mice, disorganized cell alignment and irregular microfibril assembly were noted. The activation of the signaling of transforming grwoth factor-β (TGF-β) is thought to cause the pathogenesis for lung and cardiovascular abnormalities in MFS1. Interestingly, M-HPL1 shows a higher level of activated TGF-β than HPDL2. Thus, M-HPL1 represent a powerful tool for clarifying the biological roles of elastic system fibers in PDL and the pathogenesis of periodontitis in MFS1. Our findings also suggest that *FBN1* regulates cell alignment and microfibril assembly in PDLs.

Keywords Elastic fiber · Fibrillin-1 · Marfan syndrome · Periodontitis · Periodontal ligament · Human

Introduction

The periodontal ligament (PDL) is a specialized connective tissue situated between the cementum covering the root of teeth and the alveolar bone socket (Beertsen et al. 1997; Freeman 1998). PDLs consist of various kinds of cells and fibers. The cells include fibroblasts, epithelial cell remnants of Malassez, macrophages, undifferentiated mesenchymal cells, cementoblasts, osteoblasts, and osteoclasts. The fibers include collagen and elastic system fibers. PDLs are well adapted to support teeth in bone and to act as a sensory

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receptor. To support teeth, collagen fibers are embedded both in the cementum and alveolar bone, and each collagen fiber works as a spliced rope to withstand the considerable forces of mastication.

Elastic system fibers provide elasticity and resistance to stretch and expansion forces (Mecham 1991). They are widely distributed in various tissues, e.g., skin, lungs, eyes, and blood vessels. Three types of elastic system fibers (oxytalan and elastic and elaunin fibers) are known; they differ in the content of elastin (Kielty et al. 2002). Oxytalan fibers solely consist of bundles of microfibrils, which are predominantly composed of glycoproteins and fibrillin-1 and -2. The elastic fibers are constructed of bundles of microfibrils peripherally associated with elastin. In the elaunin fibers, bundles of microfibrils are intermingled with small amounts of elastin. In the PDL, the main elastic system fibers are oxytalan fibers oriented in an occluso-apical direction (Fullmer et al. 1974; Beertsen et al. 1997). A small amount of elaunin fibers is also found in the apical region (Staszyk and Gasse 2004; Sawada et al. 2006). In contrast to collagen fibers, the biological functions of the elastic system fibers in PDLs are still obscure.

Marfan syndrome type I (MFS1, MIM #154700) is an autosomal dominant disorder affecting the elastic system fibers. Its prevalence has been estimated to be 2–3 per 10,000 (Nollen and Mulder 2004). MFS1 is characterized by various clinical manifestations primarily in skeletal, ocular, and cardiovascular organs, e.g., tall stature, aortic dissection, mitral valve prolapse, and ectopia lentis (Pyeritz 2000). The responsible gene for this syndrome has been identified as *FBN1*, which encodes the major microfibrillar protein, fibrillin-1 (Dietz et al. 1991; Maslen et al. 1991). In addition to anomalies in skeletal, ocular, and cardiovascular systems, MFS1 exhibits characteristic oral features including maxillary protrusion (Westling et al. 1998), high palate, and crowding and fragility of the temporomandibular joint (Bauss et al. 2004). Severe periodontitis, which has a serious impact on the quality of life of MFS1 patients, is occasionally associated with this syndrome (Straub et al. 2002).

To clarify the biological functions of fibrillin-1 in PDLs and the pathogenesis of the periodontitis in MFS1, PDL cells have been isolated from an MFS1 patient. The prepared immortalized PDL cells, which have a mutation in a calcium-binding epidermal-growth-factor-like (cbEGF) domain of fibrillin-1, might be a powerful tool to help answer these questions.

Materials and methods

Subjects

The patient was a 46-year-old female. She was 172 cm tall, weighed 58 kg, and had arachnodactyly. Her father and younger brother were also diagnosed as having Marfan syndrome. She had previously had dissecting aneurysm of the aorta and had had a surgical replacement of the aortic root (Bentall operation) at 42 years of age. She had suffered from mitral valve prolapse and had had a replacement of the mitral valve at 46 years of age. Since she had severe periodontitis in all teeth, all teeth were extracted before the mitral valve replacement to avoid infective endocarditis. The patient kindly provided these teeth to us with consent.

Extracted teeth were also provided by three healthy volunteers (volunteer A, 15-year-old male; volunteer B, 15-year-old male; volunteer C, 21-year-old female) during the course of orthodontic treatment, with consent. The experimental protocol was approved by the Ethical Review Committee of Tokyo Medical and Dental University.

Isolation and culture of primary PDL cells

Isolation and culture of human PDL cells were performed as previously described (Kapila et al. 1996; Shiga et al. 2003). In brief, the extracted teeth from the MFS1 patient and healthy volunteers were washed with α-minimum essential medium (α–MEM; Kohojin Bio, Japan) containing Antibiotic-Antimycotic (GIBCO, Calif.). The PDL attached to the middle part of the root was isolated with a surgical scalpel. The PDL was minced and placed in 35-mm tissue culture dishes (SUMILON, Japan). The explants were then covered with sterile glass coverslips and incubated in α–MEM with 10% fetal bovine serum (FBS; Japan Bioserum, Japan) at 37°C under 5% CO_2 and 95% air until cells outgrew from the explants. After the outgrowth of cells, coverslips were removed from the culture dishes. The culture medium was changed every 3 days. PDL cells from passages 3–7 were used for examing mineralization, measuring alkaline phosphatase (ALP) activity, and transduction.

Mineralization of the primary culture of PDL cells

To determine the mineralization of cultured PDL cells, cells were plated at 2.0×10^4 cells/cm^2 and cultured in α–MEM containing 10% FBS. After cells became confluent, the medium was changed to α–MEM containing 10% FBS with 50 μg/ml ascorbic acid (Wako, Japan), 10 nM dexamethasone (Sigma, Mo.), and 10 mM β-glycerophosphate (Sigma) in some cultures, as previously described (Cho et al. 1992; Nohutcu et al. 1997; Chien et al. 1999). The medium was changed every 3 days, and the cells were cultured for 3 weeks. Mineralized matrix in the culture were stained by Alizarin Red S (Wako, Japan) at the end of the culture (Saito et al. 2002). All experiments were performed in triplicate wells.

ALP activity

Cells were cultured under the same conditions as described above for mineralization. ALP activity was assayed in cell lysates by enzymatic conversion of the p-nitrophenylphosphate substrate to p-nitrophenol by using the LabAssay ALP kit (Wako) according to the manufacturer's instructions. The activity was recorded as millimoles per milligram per 15 min. The total protein amount in the cell lysates was measured by using the Bradford microassay (Bio-Rad, Calif.) according to the manufacturer's instructions.

Immunohistochemical staining of cultured PDL cells

Cultured PDL cells were immunohistochemically stained by using anti-human periostin rabbit polyclonal antibody (BioVendor laboratory Medicine, N.C.), anti-bovine collagen type XII monoclonal antibody (Clone 378D5, Kamiya Biomedical, Wash.), anti-active transforming growth factor-β (TGF-β) rabbit polyclonal antibody (LC1-30, provided by K. Flanders, National Cancer Institute, Md.; Flanders et al. 1989), or anti-human latency-associated peptide-β1 (LAP-β1) goat polyclonal antibody (R&D systems, Minn.). Cells (2.0×10^4 cells/cm^2) were cultured on poly-L-lysine-coated glass (Iwaki, Japan). They were then fixed with 4% paraformaldehyde (PFA) for 30 min, blocked with 1% bovine serum albumin (BSA), and incubated with each antibody for 1 h. Sections were then treated with Alexa Fluor 594 goat anti-rabbit IG (H+L; Invitrogen, Calif.), Alexa Fluor 488 goat anti-mouse IG (H+L; Invitrogen), or Alexa Fluor 488 donkey anti-goat IG (H+L; Invitrogen). As negative controls, primary antibodies were replaced with normal rabbit serum (Vector Laboratories, Calif.), mouse IgG (Jackson Immuno Research Laboratories, Pa.), or normal goat serum (Vector Laboratories). After washes with phosphate-buffered saline, fluorescence was observed by means of a fluorescence microscope (AF6000, Leica, Germany).

Mutational analysis

DNA from the MFS1 patient and healthy volunteers was extracted by using a DNA extraction kit (Bio-Rad, Calif.). Extracted DNA was amplified by using specific primers for *FBN1* and *TGFBR2* (encoding TGF-β receptor II, which is the responsible gene for Marfan syndrome type II; MFS2, MIM #154705; Mizuguchi et al. 2004). Primer sequences and polymerase chain reaction (PCR) conditions were as given on the website of "Multiple Malformation Syndromes (http://www.dhplc.jp/genetics/frame.html)" provided by the Department of Pediatrics, Division of Medical Genetics, Keio University School of Medicine. Mutations in each amplicon were analyzed by denaturing high-performance liquid chromatography (DHPLC), as described in previous studies (Kosaki et al. 2005; Udaka et al. 2005).

After DHPLC analysis, PCR products were purified on a desalting column and were sequenced by a dideoxy-sequencing method (BigDye Dideoxy sequencing kit, Applied BioSystems, Calif.) and an automated sequencer (ABI3100, Applied Biosystems; Udaka et al. 2005).

Retroviral vectors and infection

Primary PDL cells, obtained from a healthy volunteer and the MFS1 patient, were transduced with genes for human Polycomb group protein, Bmi-1, and human telomerase reverse transcriptase (hTERT) by using retrovirus-mediated gene transfer. The production and infection of LXSN-Bmi-1 and MSCVpuro-hTERT retroviruses were performed as described previously (Kyo et al. 2003; Saito et al. 2005). The infected cells were selected in the presence of geneticin (125 μg/ml) or puromycin (0.5 μg/ml). For combined retroviral infection, cells were sequentially transduced with LXSN-Bmi-1 and then with MSCVpuro-hTERT. Stably transduced cells were maintained in the medium described above.

Detection of telomerase activity

After infection, telomerase activity was determined by a telomerase repeat amplification assay by using the TRAPeze Telomerase Detection Kit (CHEMICON International, Calif.), according to the manufacturer's instructions.

Cell proliferation in monolayer culture

To examine cell proliferation, cells were inoculated at 5.0×10^3 cells/cm^2 into 6-well dishes (Iwaki, Japan) and cultured in α–MEM containing 10% FBS. The medium was changed every 3 days, and cells were counted every 3 days up to day 15. All experiments were performed in triplicate wells.

Western blot analysis

The introduction of Bmi-1 was identified by Western blot analysis by using anti-human Bmi-1 monoclonal antibody (BD Pharmingen, San Diego, Calif.). Cells were cultured in α–MEM containing 10% FBS and lysed in buffer containing 50 mM TRIS-HCl (pH 7.4), 125 mM NaCl, 0.1% Nonident P-40 (NP-40; Sigma), and 1 mM each of EDTA and phenylmethylsulfonyl fluoride, followed by sonication. After electrophoretic resolution of the cell lysate (20 μg each protein) on 12.5% SDS-polyacrylamide gels, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham, N.J.). The subsequent detection procedure was performed as described previously (Saito et al. 2005).

RNA preparation and reverse transcription/PCR

Total RNA was isolated from primary PDL cells (both from healthy volunteer B and the MFS1 patient) and from immortalized PDL cells cultured in α–MEM containing 10% FBS, by using ISOGEN (Nippon Gene, Japan) according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA by QuantiTect Reverse Transcription (QIAGEN, Germany), and each cDNA was used as the template for subsequent PCR amplification. Amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems). The reaction conditions were 94°C for 1 min, 60°C for 30 s, and 72°C for 30 s. The sequences of the used primers were: *POSTN* encoding periostin, sense 5′-ATTGATGGAGTGCCTGTG-3′, antisense 5′-CCTTGGTGACCTCTTCTTG-3′; *ASPN* encoding asporin, sense 5′-CGATACAAAGAACTACAAAGGCTGG-3′, antisense 5′-GCATTTCCCAGTATTTCA CCG-3′; *COL12A1* encoding collagen type XII, sense 5′-CGGACAGAGCCTTA CGTGCC-3′, antisense 5′-CTGCCCGGGTCCGTGG-3′; *BGLAP* encoding osteoclacin, sense 5′-CCTTTGTGTCCAAGCAGGAG-3′, antisense 5′-TCAGCCAACTCGTCACAGTC-3′; *OPN* encoding osteopontin, sense 5′-TTGCAGTGATTTGCTTTTGC-3′, antisense 5′-TGTGGGG CTAGGAGATTCTG-3′; *BSP* encoding bone sialoprotein, sense 5′-GAACCACTTCCCCACCTTTT-3′, antisense 5′-TCTGACCATCATAGCCATCG-3′; *COL1A1* encoding collagen type I, sense 5′-CTGACCTTCCTGCGCCTGATGTCC-3′, antisense 5′-GTCTGGGGCACCAACGTCCAAGGG -3′; and a human gene encoding β-actin, sense 5′-ATGAGGATCCTCACCGAGCGCGGCTACAG C-3′, antisense 5′-ACACCACTGTGTTGGCGTACAGGTCTTTGC-3′. Optimization of PCR cycle number to allow semi-quantitative analysis was performed by generating saturation curves of amplified product against cycle number. Saturation was seen with 33, 34, 31, 34, 41, 41, 25, and 25 cycles for *POSTN*, *ASPN*, *COL12A1*, *BGLAP*, *OPN*, *BSP*, *COL1A*, and β-actin, respectively. Thus, the semi-quantitative gene expression analysis by reverse transcription/PCR (RT-PCR) was performed with 30, 31, 28, 31, 39, 39, 23, and 23 cycles for *POSTN*, *ASPN*, *COL12A1*, *BGLAP*, *OPN*, *BSP*, *COL1A*, and β-actin, respectively.

A 151-bp fragment of *POSTN* (2220–2370 in NM_006475), a 292-bp fragment of *ASPN* (1031–1322 in NM_017680) (Yamada et al. 2001), a 180-bp fragment of *COL12A1* (7041–7220 in NM_080645), a 151-bp fragment of *BGLAP* (122–272 in NM_199173), a 166-bp fragment of *OPN* (173–338 in NM_001040058), a 201-bp fragment of *BSP* (876–1076 in NM_004967), a 300-bp fragment of *COL1A1* (4180–4479 in NM_000088), and a 327-bp fragment of the gene encoding β-actin (641–967 in NM_001101) were separated on 2% agarose gels (Nippon Gene, Japan) by electrophoresis. The gels were stained with ethidium bromide, photographed under ultraviolet excitation, and analyzed by using picture-imaging software (Scion Image, Scion, Md.).

Cell adhesion assay

To examine the adhesion of PDL cells, viz., HPDL2 from healthy volunteer 2 and M-HPL1 from the MFS1 patient, to hydroxyapatite particles (size 300–500 μm; OSferion, Olympus, Japan), both types of cells were labeled by using the PKH26 Red Fluorescent Cell Linker Mini Kit (Sigma) and incubated with hydroxyapatite particles for 18 h in α–MEM containing 10% FBS. The attached cells were observed by using a fluorescence microscope (AF6000, Leica, Germany).

In vivo differentiation assay

Fiber formation in the HPDL2 and M-HPL1 cells was assessed as described previously (Handa et al. 2002; Saito et al. 2005; Yokoi et al. 2007). Briefly, 1.5×10^6 cells were incubated with 40 mg hydroxyapatite particles and fibrin clot (mixture of mouse fibrinogen and thrombin; Sigma). They were transplanted subcutaneously into 5-week-old male CB-17 *SCID/SCID* mice (Nihon Crea, Japan). Mice were sacrificed after 4 weeks and implanted tissues were collected. Three transplants were prepared for each group, and experiments were repeated in triplicate.

To examine human (not mouse) vimentin-positive cells in transplanted tissues, tissues were fixed in 4% PFA for 1 day, decalcified with 10% formic acid for 3 days, and embedded in paraffin, and 5-μm-thick sections were prepared. To avoid non-specific staining by mouse monoclonal antibodies, sections were blocked by using the M.O.M. kit (Vector Laboratories, Calif.) as previously described (Handa et al. 2002). Sections were incubated, for 1 h, with anti-human vimentin monoclonal antibody (V9, DAKO, Calif.), which recognizes human but not mouse cells. After being washed, sections were incubated with biotinylated secondary antibody (M.O.M. kit) and avidin-peroxidase conjugate (M.O.M. kit). The reaction was visualized by using diaminobenzidine.

To examine human fibrillin-1-positive cells, transplanted tissues were embedded in carboxymethyl cellulose compound (Finetec, Japan), and 5-μm-thick frozen sections were prepared (Kawamoto and Shimizu 2000). Frozen sections were incubated with anti-human fibrillin-1 rabbit polyclonal antibody (Elastin Products, Mo.) for 1 h. After being washed, sections were incubated with Alexa Fluor 594 goat anti-rabbit IG (H+L; Invitrogen), and fluorescence was observed with a fluorescence microscope (AF6000, Leica, Germany).

Statistical analysis

Student's *t*-test was used to analyze differences in cell numbers between M-HPL1 and HPDL2. Each difference was considered significant at a *P*-value of less than 0.05.

Results

MFS1 patient with a heterozygous mutation in cbEGF domain of FBN1

As shown in Fig. 1a–d, the 46-year-old female patient had severe periodontitis. All teeth had to be extracted before the surgical replacement of the mitral valve to avoid infective endocarditis. Mutational analysis of *FBN1* and *TGFBR2* was performed by using a genomic DNA sample. Since *FBN1* and *TGFBR2* have 65 and 7 exons, respectively, screening of gene mutations before direct sequencing was performed by DHPLC. In total, 65 and 8 amplicons of *FBN1* and *TGFBR2*, respectively, were amplified by PCR and subsequently analyzed by DHPLC. Among them, the peak in the amplicon of exon 52 in *FBN1* from the MFS1 patient shifted to the left compared with that of the wild-type sample (Fig. 1e). This demonstrated heteroduplex formation of the amplicon from the MFS1 patient. Direct sequencing of this product was performed (Fig. 1f). A heterozygous mutation (A to G) was seen at position 6431 (from the translation site in NM_000138). This missense mutation resulted in the replacement of Asn by Ser at amino acid position 2144 (N2144S) in the 32th cbEGF domain (Fig. 1g; see also in previous study of this MFS1 patient in Hewett et al. 1993). Thus, this is not a single nucleotide polymorphism (SNP) or a novel mutation. The elution profile of DHPLC for *TGFBR2* did not show differences between the wild-type sample and MFS1 patient.

Phenotype of primary PDL cells with N2144S mutation from MFS1 patient

Cells were isolated from the PDL of extracted teeth from the MFS1 patient and were cultured in vitro. In order to examine the cellular phenotype of these isolated PDL cells, ALP activity (Fig. 2a) and mineralization (Fig. 2b) were examined. In the cell differentiation medium containing

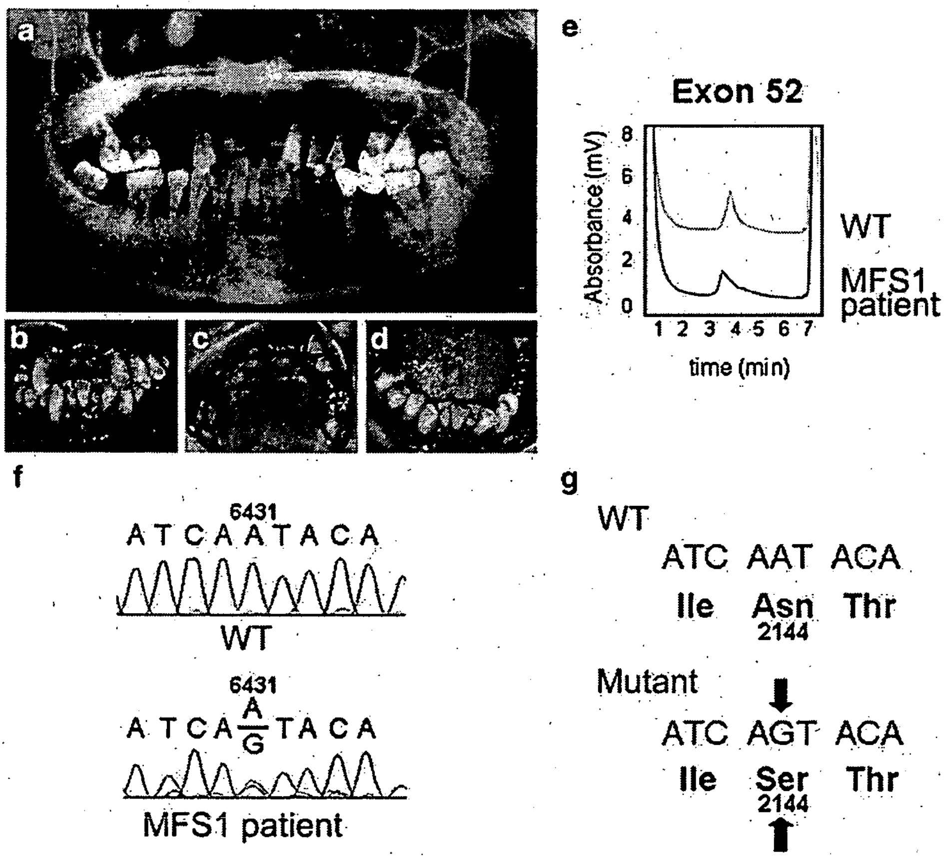


Fig. 1 Severe periodontitis and mutational analysis of the MFS1 patient. **a** In the panoramic X-ray, severe alveolar bone loss was observed around tooth roots. **b–d** Oral photographs showing the severe periodontitis of the patient. **e** DHPLC analysis of exon 52 in *FBN1*. Note, the peak in the elution profile of the MFS1 patient shifted to the left compared with that of the wild-type (WT), demonstrating heteroduplex formation. **f** Nucleotide sequence of exon 52 in *FBN1*. **g** Amino acid sequence of fibrillin-1. The 6431A→G change resulted in the heterozygous missense mutation of Asn to Ser (N2144S)

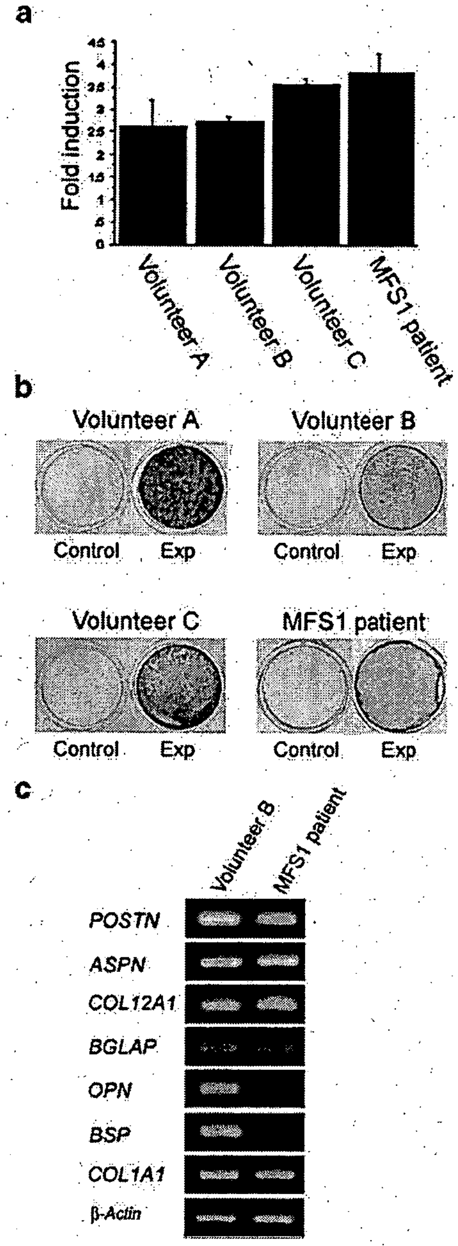


Fig. 2 **a** ALP activities of primary PDL cells isolated from three healthy volunteers (*volunteers A–C*) and the *MFS1 patient*. PDL cells were cultured in the cell differentiation medium containing ascorbic acid (50 μg/ml), β-glycerophosphate (10 mM), and dexamethasone (10 nM) for 3 weeks. Each ALP activity is represented as the ratio (*Fold induction*) to the value before culturing in the cell differentiation medium. PDL cells isolated from the MFS1 patient showed increased ALP activity as in healthy volunteers. **b** Mineralization of PDL cells cultured in the cell differentiation medium for 3 weeks. All PDL cells cultured in the cell differentiation medium were stained positively with Alizarin Red (*Exp*), but were negative when cultured in the medium containing 10% FBS solely (*Control*). **c** Expression of PDL-related genes, such as *POSTN* encoding periostin, *ASPN* encoding asporin, *COL12A1* encoding collagen type XII, *BGLAP* encoding osteocalcin, *OPN* encoding osteopontin, *BSP* encoding bone sialoprotein, *COL1A1* encoding collagen type I, and a human gene encoding β-actin, in cells from volunteer B and the MFS1 patient; reverse transcription/polymerase chain reaction (RT-PCR)

β-glycerophosphate, dexamethasone and ascorbic acid, these cells showed increased ALP activity, as did PDL cells of healthy volunteers (volunteers A–C) after a 3-week culture (Fig. 2a). The PDL cells from the MFS1 patient and healthy volunteers showed mineralization in the cell differentiation medium, but not in the medium only containing 10% FBS (Fig. 2b). The levels of the mineralization varied among cultures. Based on the similarities in the level of mineralization, PDL cells from volunteer B and MFS1 patient were selected for use in the further experiments.

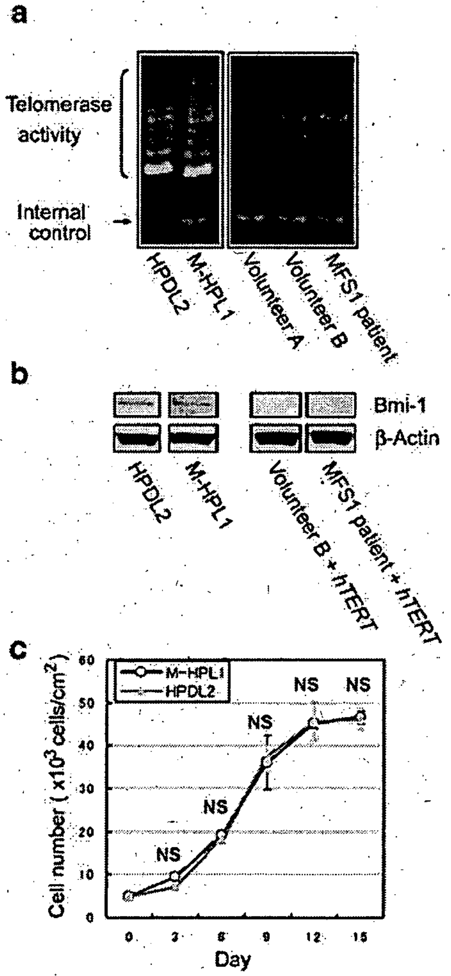
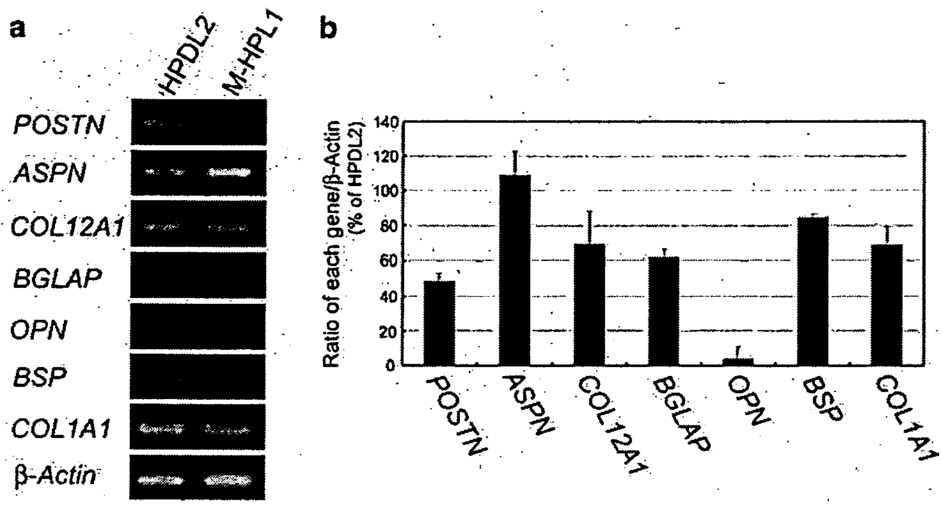


Fig. 3 **a, b** Telomerase activity and Western blot analysis of Bmi-1, respectively, in *hTERT*- and *Bmi-1*-transfected HPDL2 (originally from healthy volunteer B) and M-HPL1 (originally from the MFS1 patient). Note the characteristic ladder formation showing telomerase activity in HPDL2 and M-HPL1, but not in untransfected cells (volunteers A or B or MFS1 patient). Western blot analysis showing the expression of Bmi-1 in HPDL2 and M-HPL1, but not in cells transfected solely with *hTERT* (*Volunteer B* + *hTERT*, *MFS1 patient* + *hTERT*). **c** Proliferation of HPDL2 and M-HPL1 in culture. No significant difference occurs in the growth of the two types of cells at days 3, 6, 9, 12, 15 (*NS* not significant). Data represent means ± SD (n=3)

Fig. 4 **a** Expression of *POSTN* encoding periostin, *ASPN* encoding asporin, *COL12A1* encoding collagen type XII, *BGLAP* encoding osteocalcin, *OPN* encoding osteopontin, *BSP* encoding bone sialoprotein, *COL1A1* encoding collagen type I, and a human gene encoding β-actin in immortalized HPDL2 and M-HPL1; RT-PCR. **b** Densitometric data were normalized to β-actin in both types of cells. The bar graph represents the ratios of the expression of each gene in M-HPL1 (% of HPDL2). Data represent means ± SD (n=3)



The expression of various PDL-related genes was examined in cells from volunteer B and MFS1 patient by RT-PCR (Fig. 2c). Both types of PDL cells expressed *POSTN*, *ASPN*, *COL12A1*, *BGLAP*, *BSP*, and *COL1A1*. PDL cells from volunteer B expressed *OPN*. These results demonstrated that, induced by the culture conditions, the isolated PDL cells could differentiate into an osteoblastic phenotype.

Immortalization of isolated PDL cells

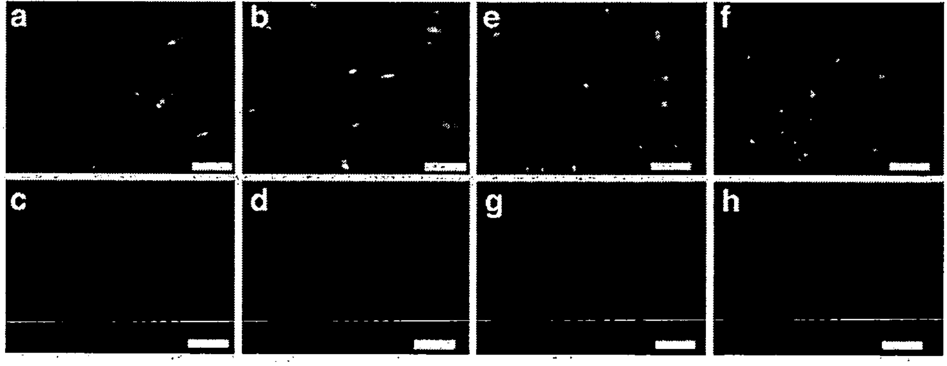
Since human cells have a limited life span (Sherr and DePinho 2000), and since the present PDL cells from the MFS1 patient with N2144S are valuable from a research viewpoint, the cultured cells (primary cells from volunteer B and MFS1 patient) were immortalized by retrovirus-mediated transduction. Non-transduced PDL cells and PDL cells transduced solely with *hTERT* showed senescence by passage 15. In contrast, PDL cells transduced with *Bmi-1* and *hTERT* did not show cellular senescence up to passage 20 as indicated by their cell morphology. Thus, we decided to transduce cells with both *Bmi-1* and *hTERT*. The transduced PDL cells from healthy volunteer B and the MFS1 patient were termed HPDL2 and M-HPL1, respectively.

Activation of telomerase by the transduction of *hTERT* was confirmed by the telomerase repeat amplification assay (Fig. 3a). Overexpression of Bmi-1 was confirmed by Western blot analysis (Fig. 3b). Bmi-1 was easily detected in M-HPL1 and HPDL2, but not in cells transduced solely with *hTERT*. No significant difference was seen in the cell growth between HPDL2 and M-HPL1 cultured in medium with 10% FBS up to day 15 (Fig. 3c). After day 12, the cell numbers of neither HPDL2 nor M-HPL1 increased extensively, suggesting that both types of cells had limited proliferation.

Phenotype of HPDL2 and M-HPL1

To characterize the phenotype of the immortalized PDL cells, expression of the reported PDL-related genes and β-actin was examined by semi-quantitative RT-PCR (Fig. 4a). HPDL2 and M-HPL1 both expressed *POSTN*, *ASPN*, *COL12A1*, *BGLAP*, *BSP*, and *COL1A1*. The relative expression of *POSTN*, *COL12A1*, *BGLAP*, and *COL1A1* was lower in M-HPL1 than in HPDL2 (Fig. 4b). *OPN* was expressed in HPDL2, but this was scarcely expressed in M-HPL1 (Fig. 4a, b). Immunohistochemistry with antibodies

Fig. 5 Positive immunohistochemical localization of collagen type XII in cultured HPDL2 (**a**) and M-HPL1 (**b**) and of periostin in cultured HPDL2 (**e**) and M-HPL1 (**f**). Primary antibodies were replaced with mouse IgG (**c** HPDL2, **d** M-HPL1) or normal rabbit serum (**g** HPDL2, **h** M-HPL1) for negative controls. *Bars* 100 μm



against collagen type XII (Fig. 5a, b) and periostin (Fig. 5e, f) showed numerous immunostained cells in cultures of HPDL2 and M-HDL1. Staining was scarcely seen in negative controls in which primary antibodies had been replaced with mouse IgG (Fig. 5c, d) or normal rabbit serum (Fig. 5g, h).

In the lung (Neptune et al. 2003) and cardiovascular (Ng et al. 2004) systems, gene mutation in *FBN1* has been suggested to be involved in the activation of TGF-β. To examine whether this is the case in M-HPL1, immunostaining with LC1-30, which only recognizes the active form of TGF-β, was performed. M-HPL1 showed more intense staining than HPDL2 (Fig. 6e, f), although a comparable reaction was seen in HPDL2 and M-HPL1 to the antibody against LAP-β1, which also forms complexes with TGF-β (Fig. 6a, b; Miyazono et al. 1993). Staining was scarcely seen in negative controls, in which primary antibodies were replaced with normal goat serum (Fig. 6c, d) or normal rabbit serum (Fig. 6g, h).

Cell and fiber alignments in tissues transplanted with M-HPL1

Ectopic fiber formation by M-HPL1 and HPDL2 in the subcutaneous tissues of SCID mice was examined by transplantation of these cells with hydroxyapatite particles. In this experiment, hydroxyapatite was chosen because it is the major inorganic component of teeth and bones (Ten Cate 1998). HPDL2 (Fig. 7a) and M-HPL1 (Fig. 7b) both attached to the hydroxyapatite particles 18 h after being mixed with the particles.

Four weeks after the transplantation of the cells with hydroxyapatite particles into SCID mice, sections of the cells were immunostained with anti-vimentin antibody recognizing only human but not mouse cells. HPDL2 aligned in parallel between the particles (Fig. 8a, c). In contrast, M-HDL1 were mainly located around the particles and were aligned irregularly (Fig. 8b, d). Similar observations were also seen in eight other transplants from a total of three SCID mice.

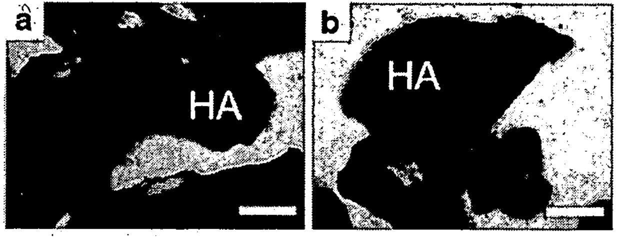


Fig. 7 Attachment of HPDL2 (a) and M-HPL1 (b) to hydroxyapatite particles (*HA*) after 18-h culture. *Bars* 100 μm

Transplanted tissues were immunostained with anti-human fibrillin-1 antibody. In contrast to the elaborate network of immunoreactive fibrillin-1 in HPDL2, M-HPL1 showed disorganized microfibril assembly (Fig. 9a, b). Staining was scarcely seen in the tissues in which the hydroxyapatite particles without cells were transplanted into SCID mice (Fig. 9c), demonstrating that the antibody only recognized human cells but not mouse cells.

Discussion

The present Japanese female patient had profound skeletal and cardiovascular symptoms including tall stature, arachnodactyly, aortic dissection, mitral valve prolapse, and severe periodontitis. Two types of Marfan syndrome (type I, MIM #154700; type II, MIM #154705) have been described so far. A large French family has been reported to exhibit the skeletal and cardiovascular features of Marfan syndrome in an autosomal dominant manner (Boileau et al. 1993). No mutation in *FBN1* has been seen in this family, and they have been classified as MFS2. Recently, *TGFBR2*

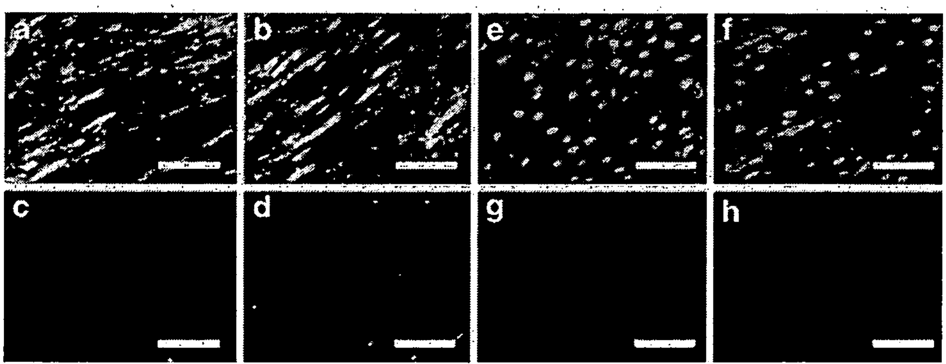


Fig. 6 Immunohistochemical localization of LAP-β1 in cultured HPDL2 (a) and M-HPL1 (b) and of LCI-30 in cultured HPDL2 (e) and M-HPL1 (f). Note that LCI-30, which recognizes only the active form of TGF-β, immunoreacts more abundantly in M-HPL1 than in HPDL2, whereas the level of LAP-β1 was comparable in the both types of cells. Primary antibodies were replaced with normal goat serum (c HPDL2, d M-HPL1) or normal rabbit serum (g HPDL2, h M-HPL1) for negative controls. *Bars* 100 μm

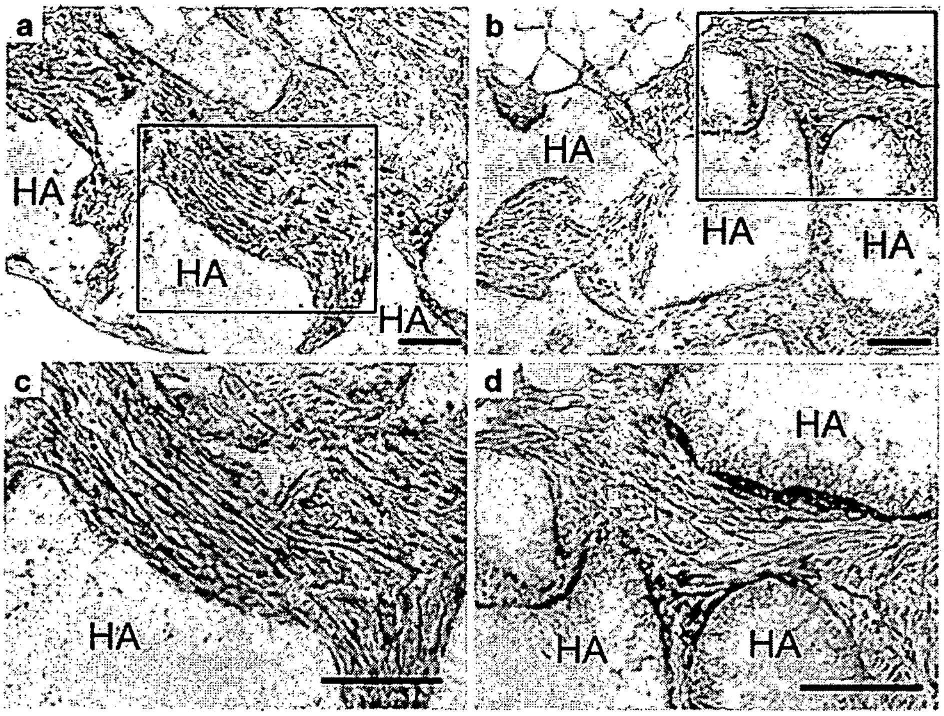


Fig. 8 Sections prepared from tissues in which HPDL2 (**a, c**) or M-HPL1 (**b, d**) were transplanted with hydroxyapatite particles (*HA*) into SCID mice for 4 weeks. Immunostaining with anti-human vimentin monoclonal antibody. The *boxed areas* in **a, b** are shown at higher magnification in **c, d**, respectively. Note that HPDL2 aligned in an organized manner, but M-HPL1 showed disorganized alignment. *Bars* 100 μm

has been identified as the responsible gene in MFS2 (Mizuguchi et al. 2004). The present patient has a heterozygous mutation in *FBN1* (Fig. 1f); this mutation results in a missense substitution (N2144S; Fig. 1g), clearly identifying the disease as MFS1.

Since *FBN1* is a large gene with 65 exons, direct sequencing of all the exons to identify mutations is time-consuming and costly. Therefore, we have performed DHPLC to screen for mutations in *FBN1*. In total, 65 amplicons for *FBN1* and 8 amplicons for *TGFBR2* have been synthesized by using specific primers by PCR. Heteroduplex formation has been identified in the product of exon 52 in *FBN1* by DHPLC (Fig. 1e). By using this method, systems have previously been developed to screen 20 congenital disorders (Kosaki et al. 2005). The present DHPLC method is a sensitive and powerful tool that allows the screening of gene mutations before direct sequencing; this is especially useful for large genes such as *FBN1*.

Cells isolated from the PDL of our MFS1 patient and of healthy volunteers have both shown increased ALP activity and mineralization in the cell differentiation medium. PDL cells are known to show an osteoblastic phenotype when cultured under these conditions (Cho et al. 1992; Giannopoulou and Cimasoni 1996; Nohutcu et al. 1997; Chien et al. 1999). After immortalization of cells by introducing *hTERT* and *Bmi-1*, both HPDL2 and M-HPL1 express PDL-related genes, viz., *POSTN* (Fujii et al. 2006), *ASPN* (Yamada et al. 2001), *COL12A1* (Fujii et al. 2006), *BGLAP* (Fujii et al. 2006), *BSP* (Yokoi et al. 2007), and *COL1A1* (Yokoi et al. 2007; Fig. 4a). These observations demonstrate that both types of cells have the characteristic phenotype of cultured PDL cells. However, *POSTN*, *COL12A1*, *BGLAP*, and *COL1A1*

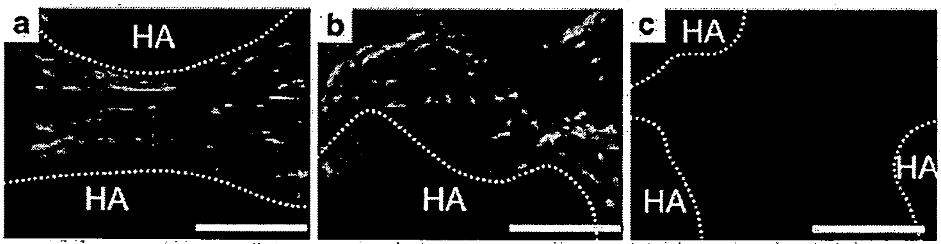


Fig. 9 Sections prepared from tissues in which HPDL2 (**a**) or M-HPL1 (**b**) were transplanted with hydroxyapatite particles (*HA*) into SCID mice for 4 weeks (*dotted lines* outline of HA). Immunostaining with anti-human fibrillin-1 antibody. Note the irregular microfibril assembly in the tissue implanted with M-HPL1 (**b**) but not in that with HPDL2 (**a**). **c** Sections prepared from tissues in which HA particles without cells were transplanted into SCID mice for 4 weeks. Note the absence of immunostaining with anti-human fibrillin-1 antibody (*dotted lines* outline of HA). *Bars* 50 μm

exhibit a lower expression in M-HPL1 than in HPDL2, and *OPN* is hardly expressed in M-HPL1 (Fig. 4a). We now need to examine whether PDL cells isolated from other MFS1 patients reveal a similar down-regulation of these genes.

Recently, attempts have been made to establish immortalized PDL cells by introducing *hTERT* (Berry et al. 2003; Kamata et al. 2004; Fujita et al. 2005; Saito et al. 2005; Fujii et al. 2006; Zhang et al. 2006). Cyclin-dependent kinase inhibitors $p16^{Ink4a}$ and $p21^{WAF1}$ induce premature senescence in human cells by telomere-independent mechanisms (Ramirez et al. 2001). As Bmi-1 can down-regulate the expression of $p16^{Ink4a}$ and $p14^{ARF}$ (Jacobs et al. 1999), it has been used to extend the life span of bovine and human cells (Dimri et al. 2002; Cudre-Mauroux et al. 2003; Itahana et al. 2003; Saito et al. 2005; Haga et al. 2007). Thus, in this study, we have immortalized human PDL cells with retrovirus-mediated transduction of both *hTERT* and *Bmi-1*. By using this method, both HPDL2 and M-HPL1 have been immortalized while maintaining their original gene expressions (Fig. 2c, Fig. 4a), as reported in cementoblast progenitor cells (Saito et al. 2005).

In MFS1, the activation of TGF-β signaling has been suggested as the pathogenesis for mitral valve prolapse and emphysema (Neptune et al. 2003; Ng et al. 2004). Mutations in *FBN1* alter or preclude matrix alteration of the latent complex of TGF-β, rendering TGF-β more accessible for activation (Neptune et al. 2003). In this study, activated TGF-β has been shown to be more abundant in M-HPL1 than in HPDL2 (Fig. 6e, f), suggesting that activated TGF-β signaling occurs in the PDL of our MFS1 patient.

N2144S in fibrillin-1 is predicted to alter one of the key calcium-binding residue ligands within the 32th cbEGF domain (Kettle et al. 1999; Yuan et al. 2002). This mutation is known to increase flexibility in the peptide backbone (Yuan et al. 2002). Attempts should be made to link this mutation and the disorganized cell alignment and microfibril assembly seen in this study.

OPN expression is lower in M-HPL1 than in HPDL2 (Fig. 4a). The exact reason for this difference is not known. However, TGF-β blockade has been reported significantly to enhance the BMP-2-induced upregulation of *OPN* expression, suggesting that TGF-β is a negative regulator on *OPN* expression (Shen et al. 2007). An examination is required of whether decreased expression of *OPN* (Fig. 4a) is mediated by the enhanced TGF-β activation in M-HPL1 (Fig. 6). Moreover, since no study has reported a relationship between fibrillin-1 and *OPN* expression, an investigation of *OPN* expression in M-HPL1 would be of interest after transfecting wild-type fibrillin-1 or during the culture of these cells on the fibrillin-1-coated dishes.

In summary, PDL cells have been isolated from an MFS1 patient with a heterozygous mutation in the 32th cbEGF domain (N2144S). These PDL cells have been immortalized by transducing human *mi-1* and *hTERT*. The present immortalized PDL cells show increased levels of activated TGF-β and should provide a powerful tool for the clarification of the biological roles of the elastic system fibers in PDLs and the pathogenesis of periodontitis in MFS1.

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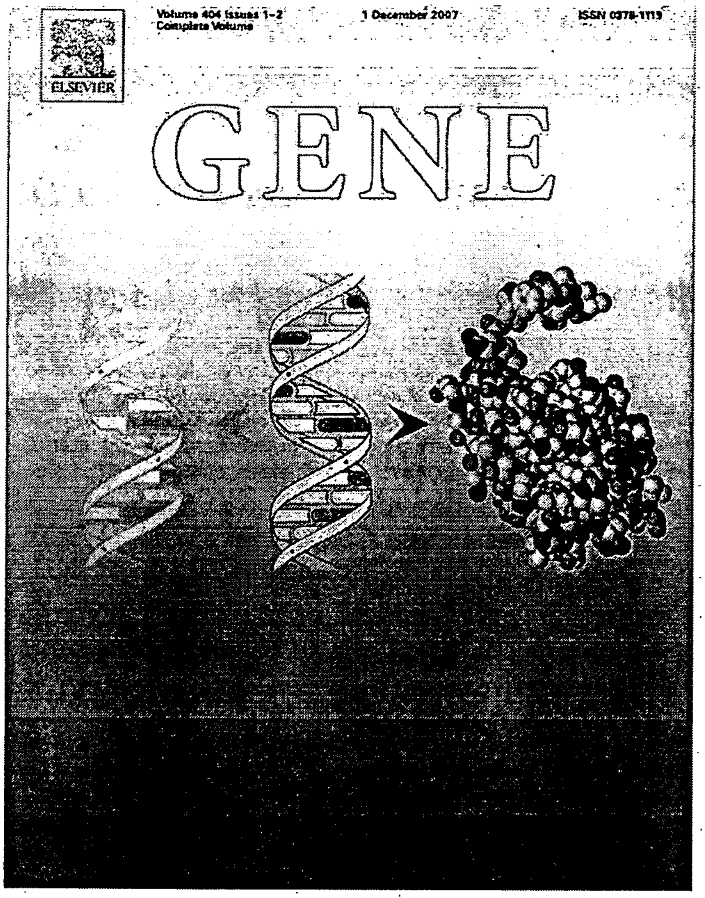
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Transcriptome database KK-Periome for periodontal ligament development: Expression profiles of the extracellular matrix genes

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Abstract

Specialized connective tissues such as tendon/ligament develop through a series of events that require temporal and spatial expression of numerous genes in mesenchymal progenitors. However, the genes required for tendon/ligament development have not been identified yet. To solve this problem, we made a cDNA library from periodontal ligament and sequenced 11,520 cDNA clones, as a model for investigating tendon/ligament development. The resulting sequence data was assembled to 617 expressed sequence tag (EST) clusters, and an EST database for human periodontal ligament (PDL) was constructed (designated as the KK-Periome database). In the KK-Periome database, the top 13 EST clusters were related to extracellular matrix (ECM) genes. The temporal and spatial expression patterns of these genes during mouse PDL development were examined by *in situ* hybridization. Among these genes, *F-spondin* was expressed specifically in dental follicle (DF) cells during tooth germ development, whereas *tenascin-N* was strongly expressed in the terminally differentiated PDL. This characteristic expression profile was confirmed by *in vivo* differentiation assay of human PDL (hPDL) cells in the mouse transplant. Thus, the KK-Periome database was proven to be a useful resource for PDL-derived ESTs (transcriptome), and in fact, initial evidence indicated that *F-spondin* and *tenascin-N* might serve as markers for DF and PDL, respectively.

Keywords: EST library; Database; Expression patterning; Connective tissue; Extracellular matrix; Periodontal ligament

1. Introduction

Periodontium is a tooth supporting tissue composed of periodontal ligament (PDL), cementum and alveolar bone. More specifically, the PDL is composed of densely packed collagen-rich connective tissue capable of withstanding occlusal force. Collagen type I is predominant, but collagen types III, IV, V, VI and XII and proteoglycans are also deposited into the PDL extracellular matrix (Huang et al., 1991; Karimbux et al., 1992; Lukinmaa et al., 1992; Ten Cate, 1994; Matheson et al., 2005; McCulloch, 2006). In periodontitis, a chronic inflammatory disease that affects the periodontium, the PDL is irreversibly damaged. Despite a number of novel approaches, it has not yet been possible to reliably regenerate the PDL (D'Errico et al., 1999). Hence, there is considerable interest in the developmental mechanisms of PDL.

PDL originates from dental follicle (DF) cells that form on the embryonic day 14 (E14) of tooth germ development. During tooth germ development, DF cells differentiate into progenitors of

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Abbreviations: DF, Dental follicle; ECM, Extracellular Matrix; EST, Expressed Sequence Tag; PDL, Periodontal Ligament.

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