Effect of F-spondin on cementoblastic differentiation of human periodontal ligament cells

Masae Kitagawa[a,b], Yasusei Kudo[a], Shinji Iizuka[a], Ikuko Ogawa[b], Yoshimitsu Abiko[c], Mutsumi Miyauchi[a], and Takashi Takata[a]

[a]Department of Oral and Maxillofacial Pathobiology, Graduate School of Biomedical Sciences, Hiroshima University, [b]Center of Oral Clinical Examination, Hiroshima University Hospital, Hiroshima, 734-8553; [c]Department of Biochemistry, School of Dentistry at Matsudo, Nihon University, 270-8587, Japan.

Keywords: F-spondin, Cementoblast, Periodontal ligament, Differentiation

Grant support: This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

Correspondence: Takashi Takata, Department of Oral and Maxillofacial Pathobiology, Division of Frontier Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku. Hiroshima 734-8553, Japan.

TEL: +81 82-257-5634, FAX: +81 82-257-5619; E-mail: ttakata@hiroshima-u.ac.jp

Abstract

Cementum is a mineralized tissue produced by cementoblasts covering the roots of teeth that provides for the attachment of periodontal ligament to roots and surrounding alveolar bone. To study the mechanism of proliferation and differentiation of cementoblasts is important for understanding periodontal physiology and pathology including periodontal tissue regeneration. However, the detailed mechanism of the proliferation and differentiation of human cementoblasts is still unclear. We previously established human cementoblast-like (HCEM) cell lines. We thought that comparing the transcriptional profiles of HCEM cells and human periodontal ligament (HPL) cells derived from the same teeth could be a good approach to identify genes that influence the nature of cementoblasts. We identified F-spondin as the gene demonstrating the high fold change expression in HCEM cells. Interestingly, F-spondin highly expressing HPL cells showed similar phenotype of cementoblasts, such as up-regulation of mineralized-related genes. Overall, we identified F-spondin as a promoting factor for cementoblastic differentiation.

Introduction

Cementum is a mineralized tissue produced by cementoblasts covering the roots of teeth that provides for the attachment of periodontal ligament to roots and surrounding alveolar bone [1]. Cementum contributes to the regeneration of the connective tissue attachment to root surface, denuded due to periodontal disease. Therefore, it is very important for studying the detailed mechanisms proliferation and differentiation of human cementoblasts to understand periodontal physiology and pathology, including periodontal tissue regeneration. Several attempts have been made to obtain makers of cementoblasts [2-5]. Recent studies have shown new makers of cementum or cementum-periodontlal ligament such as cementum-derived attachment protein (CAP) [2] and cementum-derived protein (CP-23) [5]. However, the detailed role of these molecules has not been revealed in the differentiation of cementoblasts.

We recently have established human cementoblast-like (HCEM) cell lines and human periodontal ligament (HPL) cell lines from same teeth by *hTERT* transfection to examine the molecule involving with the differentiation of cementobalsts [6]. HCEM cell lines obtained from teeth root lining cells showed high alkaline phosphatase (ALP) activity, calcified nodule formation and the expression of mineralized related genes, including type I collagen (COLI), ALP, runt related transcription factor 2 (Runx2), osteocalcin (OCN), bone sialoprotein (BSP) and CP-23. On the other hand, HPL cells from middle part of periodontal ligament showed low ALP and mineralization activity, and didn’t express OCN and BSP which are maker of genes showing the mature differentiation of cementoblasts. Here we compared the

transcriptional profiles of HCEM cells and HPL cells by microarray analysis in order to identify the genes that differ in their expression. We identified F-spondin as the gene demonstrating the high fold change expression in HCEM cells. F-spondin is an extracellular matrix protein required for pathfinding of commissural axons during floor plate development [7, 8]. In the present study, to know the role of F-spondin for cementoblastic differentiation, we transfected F-spondin into HPL cells, and examined the expression of the mineralized related genes, COLI, ALP, Runx2, OCN, BSP and CP-23 *in vitro*.

Methods

These studies were performed in compliance with regulations administered by the experimentation committee of the Graduate School of Biomedical Sciences, Hiroshima University.

Cell culture. HCEM cells which we previously established [6] and human osteoblasts (NHOst, OTT4 and Ost) provided by Dr. Tahara (Hiroshima University) were cultured in Minimum Essential Medium Alpha (α-MEM, Invitrogen, Grand Island, N.Y.) with 10% fetal bovine serum (FBS) plus penicillin G solution (10 U/ml) and streptomycin (10 mg/ml) in a humidified atmosphere of 5 % CO_2 at 37 ˚C. NHOst and Ost are normal human osteoblast cells, OTT4 is immortalized human osteoblast cell lines with *SV40-T* and *hTERT*. HPL cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Nissui Pharmaceutical Co. Ldt.).

Gene array analysis. The human focus array using the system containing 8500 genes probes was used for comparing the transcriptional profiles between HCEM cells and HPL cells. This array contains a broad range of genes derived from publicly available, well-annotated mRNA sequences. Total RNA was isolated from cultures of confluent cells using the RNeasy Mini Kit (Qiagen, K.K., Tokyo, Japan) according to the manufacture's instructions. Preparations were quantified and their purity was determined by standard spectrophometric methods.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cultures of confluent cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Preparations were quantified and their purity was determined by standard spectrophotometric methods. cDNA was synthesized from 1 μg total RNA according to the Rever Tra Dash (Toyobo Biochemicals, Tokyo, Japan). The oligonucleotide RT-PCR primers for human F-spondin, rat F-spondin, chemokine orphan recetpor (CMKOR1), phosphotriesterase related (PTER), solute carrier family membrane1(SLC14A1) and matrix metalloproteinase (MMP) 13 were listed in Table 1. Primers for COLI, ALP, Runx2, OCN, BSP, CP-23 and glyceraldehyde-3-phosphate (GAPDH) were described previously [6]. Aliquots of total cDNA were amplified with 1.25 U of rTaq-DNA polymerase (Qiagen), and amplifications were performed in a PC701 thermal cycler (Astec, Fukuoka, Japan) for 28-30 cycles after an initial 30 sec denaturation at 94 ˚C, annealed for 30 sec at 55-60 ˚C, and extended for 1 min at 72 ˚C in all primers. The amplification reaction products were resolved on 1.5 %

agarose/TAE gels (Nacalai tesque, Inc., Kyoto, Japan), electrophoresed at 100 mV, and visualized by ethidium-bromide staining.

Generation of F-spondin highly expressing HPL cells. Packaging GP-293 cells (Clontech, Palo Alto, CA) were transfected with retroviral plasmid encoding a rat F-spondin cDNA according to the manufacturer's instructions. A plasmid pMT21-FP5, encoding rat F-spondin cDNA, was kindly provided by Dr. Klar (Hebrew University). After 48 h of transfection, the virus-containing medium was collected and supplemented with 8 μg/ml polybrene (Sigma, St. Louis, MO). Then, the culture medium of the target cells was replaced with this viral supernatant for 24 h. This infection process was repeated a second time after a 12 h recovery in normal medium. The stable clones were obtained by puromycin selection (1 μg/ml) in the culture medium.

And clones were examined expressions of F-spondin by RT-PCR and Western blot. Four F-spondin-expressing clones were chosen for the subsequent experiments. A rat F-spondin cDNA was also cloned into pBICEP-CMV-2 (Sigma) and was transfected into 293T cells.

Western blot analysis. Subconfluent cells, in 90 mm culture dishes, were used for western blot analysis. Western blotting was carried out as we described previously [9]. Thirty μg/ml of protein was solubilized in Laemmli sample buffer by boiling, and subjected to 10 % sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting onto a nitrocellulose filter. The filter was blocked for 1h at 4 °C with phosphate-buffer saline (PBS) buffer containing 5 % nonfat dry milk powder. Western blot analysis was performed using an anti-SPON1

polyclonal antibody (ProSci, Flint Place Poway, CA), anti-FLAG M2 monoclonal antibody (Sigma) and ß-actin monoclonal antibody (Sigma) dissolved in PBS containing 5 % nonfat dry milk powder and incubating for 60 min at room temperature. Incubation with a secondary peroxidase-coupled goat anti-IgY Fc antibody (ProSci) and a secondary peroxidase-coupled goat anti-mouse antibody was performed under the same conditions. For detection of the immunocomplex, the ECL western blotting detection system (Amersham, Buckinghamshire, UK) was used.

Tissue samples. Tissue samples of periodontal tissues including tooth were retrieved from the Surgical Pathology Registry of Hiroshima University Hospital, after approval by the Ethical Committee of our institutions. 3.7 % buffered-formalin fixed, decalcified for 3 days and paraffin embedded tissues were used for immunohistochemical examination.

Immunohistochemical staining. To examine expression of F-spondin in 7 human periodontal tissues cases, the 4.5 μm sections were stained immunohistochemically with an anti-SPON1 antibody (Prosci). Endogenous peroxidase was quenched by incubating with 0.3 % H_2O_2 in methanol for 30 min. Nonspecific staining was blocked using Dako Protein Block Serum Free (Dako, Carpinteria, CA). The sections were incubated with the primary antibody (1:500) for overnight at 4 ˚C, and then incubated with a secondary peroxidase-coupled goat anti-IgY Fc antibody (ProSci) for 30 min. For visualization, they were treated with Liquid DAB (3,3'-diaminobenzidine) Chromogen Syatem (Dako) according to the manufacturer's protocol.

Measurement of ALP Activity. The quantitative analysis of ALP activity was performed biochemically by Bessey-Lowry enzymologic method using nitrophenyl phosphate as a substrate [10]. Cells were plated in 24 well culture plates ($1x10^5$ cells per well) and cultured in DMEM containing 10 % FBS, penicillin G sodium (10 U/ml) and streptomycin sulfate (10 mg/ml) for confluent that cells were plated for 1 week. The cells were washed with PBS and homogenized ultrasonically in 0.5 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 25 mM $MgCl_2$. Aliquots of the homogenates were used for quantification of ALP activity.

Collagen assay. The measurement of collagen concentration was performed using Sircol^{TM} Collagen Assay kit (Biocolor, Northern Ireland) according to the manufacture's instruction. Cells were plated in 24 well culture plates ($1x10^5$ cells per well) and cultured in DMEM containing 10 % FBS, penicillin G sodium (10 U/ml) and streptomycin sulfate (10 mg/ml) for confluent that cells were plated for 1 week. The culture media were used for measurement of collagen concentration.

Statistical analysis. The results of cell growth analysis and quantitative ALP activity were shown as mean ± SE, and analyzed for significance using Wilcoxon's test for non-paired examination. *P* values of less than 0.05 were judged to be statistically significant.

Results

Identification of F-spondin as a cementoblast specific highly expressed gene

We previously established a HCEM cell line from root surface and a HPL cell line from middle part of periodontal ligament of the same extracted human teeth [6]. In the present study, therefore, we thought that to compare the transcriptional profiles of HCEM cells and HPL cells could be a good approach to identify genes that influence the nature of cementoblasts (Fig.1A). By microarray analysis, several genes were selectively highly expressed in HCEM (Fig. 1A). Among these genes, one of the highly expressed genes was F-spondin. Highly expression of F-spondin in HCEM cells, but not in HPL cells was confirmed by RT-PCR (Fig. 1B). As cementoblasts share many characteristics to osteoblasts, we examined the expression of F-spondin in HCEM and human osteoblasts (NHOst, OTT4 and Ost). Interestingly, HCEM expressed F-spondin mRNA, but osteoblasts did not (Fig. 1C). On the other hand, CMKOR1, PTER, SLC14A1 were detected in both HCEM and NHOst (Fig. 1C). Thus, F-spondin is specifically expressed in cementoblasts among the cells, which compose the periodontal tissue.

F-spondin expression in human periodontal tissue

To confirm the specific expression of F-spondin in cementoblasts by immunohistochemical analysis, we firstly checked the accuracy of the antibody by western blotting. F-spondin antibody specifically recognized F-spondin expression in FLAG-F-spondin transfected 293T cells (Fig. 2A). Then, we immunohistochemically examined its expression in 7 normal periodontal tissues. As expected, F-spondin obviously expressed in the root lining cells (Fig. 2B).

High expression of F-spondin promotes the differentiation of HPL cells in vitro.

To know the role of F-spondin for cementoblastic differentiation, we stably transfected F-spondin into HPL cells, which is the poorly differentiated cells in comparison with cementoblasts. We obtained 4 stable clones of F-spondin highly expressing HPL cells (HPL-spondin) (Fig. 3A). High expression of F-spondin changed the morphology, showing short spindle shapes in comparison with control cells (Fig. 3B). Next, we examined the expression of mRNA for COLI, ALP, Runx2, OCN, BSP and CP-23 by RT-PCR in F-spondin highly expressing HPL cells. In addition, we examined collagen assay and ALP activity by biochemical methods. Higher expression of ALP, OCN and BSP and lower expression of COLI were observed in F-spondin highly expressing HPL-cells in comparison with control cells (Fig. 4A). Both HPL-spondin cells and control cells expressed Runx2 and CP-23 mRNA at the same levels (Fig. 4A). The findings that increased expression of ALP mRNA and decreased expression of COLI mRNA in F-spondin highly expressing HPL cells were confirmed by collagen assay (Fig. 4B) and ALP activity (Fig. 4C), respectively. To know the reason why type I collagen decreased in HPL-spondin, we examined MMP13 expression. It is known that MMP13 is involved in type I and II collagen degradation and expressed in both terminal hypertrophic chondorocytes and osteoblasts [11,12]. Interestingly, HPL-spondin cells expressed MMP13 mRNA at higher level in comparison with control cells (Fig. 4D).

Discussion

The periodontium is a complex structural and functional unit consisting of 4 different components, i.e., gingiva, alveolar bone, periodontal ligament, and cementum [13]. Cementum plays an important role of the attachment of periodontal ligament to roots. The progenitor cells in bone marrow spaces migrate into the perivascular area of the periodontal ligament and move to the bone and tooth surface, and then differentiate into osteoblasts or cementoblasts [14, 15]. In similar to osteoblasts, cementoblasts express noncollagenous bone matrix proteins, COLI, ALP, Runx2, OCN, BSP and so on [15, 16]. Recently, CAP and CP-23 has been identified as new makers for cementum or cementum-periodontal ligament [2, 5]. However, we still do not know the detailed mechanism of cementoblastic or osteoblastic differentiation from progenitor cells in bone marrow spaces. To study the detailed mechanism of cementoblastic differentiation and proliferation of cementoblasts, we recently established a human cementoblast-like cell line, HCEM from root lining cells and a human periodontal ligament cell line, HPL, from middle part of periodontal ligament of the same teeth by using enzymatic digestion method [6].

In the present study, to identify the molecules playing the role for cementoblastic differentiation, we compared gene expression profiles between HCEM and HPL by microarray analysis. As HCEM and HPL were established from same teeth, we thought that comparing the gene expression profiles between them could be a good approach for identifying the gene of cementoblastic differentiation. Here we found several genes highly expressed in HCEM cells in comparison with HPL cells, but

most genes except for F-spondin expressed in osteoblasts by RT-PCR (Fig. 1A and 1C). Therefore, in the present study, we focused on F-spondin. In fact, the expression of F-spondin mRNA was observed only in cementoblast, but not in osteoblasts and periodontal ligament cells (Fig. 1B and 1C). Moreover, F-spondin expression was observed only in the cells on the root surface (cementoblasts) by immunohistochemistry (Fig. 2B). These finding suggest that F-spondin may be a specific molecule for cementoblasts among the periodontal tissues.

F-spondin is an extracellular matrix protein and promotes neurite outgrowth of dorsal root ganglion cells [8, 17] and spinal cord neurons in cell culture [18]. F-spondin promotes the differentiation of neural precursor cells to cells with the characteristics of neurons [19]. This is the first report on the function of F-spondin in cementoblasts. It is considered that periodontal ligament consists of different cell populations in various differentiation stages according to the position in periodontal ligament. We previously reported that cell populations with larger growth potential were generally located in the middle position of periodontal ligament and cell populations with higher ALP and mineralization activities toward the surface of the root in rat periodontal ligament [20]. Therefore, we transfected F-spondin into HPL cells which are more poorly cell population than HCEM cells. Interestingly, high expression of F-spondin changed the morphology (Fig. 3C) and increased the expressions of ALP, OCN and BSP mRNA and ALP activity (Fig. 4A, C) in HPL cells, suggesting that F-spondin may influence on the differentiation of HPL cells. As cementoblasts and osteoblasts expressed ALP, OCN and BSP, which play an important

role for the mineralization [10], F-spondin promoted the differentiation of HPL cells toward the mineralization. Moreover, both HPL-spondin cells and control cells expressed CP-23 mRNA (Fig. 4A). CP-23 was expressed in mature cementum, cementoblasts and periodontal ligament cells but not osteoblasts [21]. Therefore, F-spondin promoted cementoblastic differentiation not osteoblastic differentiation. We also found that high expression of F-spondin decreased the expression of COLI mRNA and collagen concentration (Fig. 4A, B). This finding may be caused by increased expression of MMP13, because MMP13 is known to be a collagenase of type I collagen and to increase in the process of osteoblastic differentiation [22].

By microarray analysis, we could find a lot of the genes that highly expressed in HCEM. For instance, CMKOR1, PTER and SLC14A1 are also highly expressed in HCEM. Nuclear Factor I (NFIB), which is site specific DNA-binding protein [23], is also listed. In the future, we will examine if these genes are involved in cementoblastic differentiation. In summary, in the present study, we demonstrated a critical role of F-spondin for cementoblastic differentiation. Our findings provide new and important information for understanding the mechanism of cementoblastic differentiation. We suggest that F-spondin could be used for a novel molecular target for periodontal regeneration therapy.

Acknowledgements

We thank Dr. A. Klar for providing F-spondin vector. We also thank Dr. H. Tahara for providing human osteoblasts and Ms. A. Imaoka for supporting by microarray. This work was supported by in part by grants-in-aid from the Ministry of Education,

Science and Culture of Japan to M.K. (18791350), Y. A. (A1-16209063) and T. T. (B1-1739048600)

References

[1] Ten Cate AR. The periodontium: Oral Histology: Development, Structure and Function. Mosby: St. Louis. (2003) 276-279.

[2] Arzate H, Olson SW, Page RC, Gown AM, Narayanan AS. Production of a monoclonal antibody to an attachment protein derived from human cementum. FASEB J. 6 (1992) 2990-2995.

[3] Saito M, Iwase M, Maslan S, Nozaki N, Yamauchi M, Hanada K, Takahashi O, Sato S, Kawase T, Teranaka T, Narayanan AS. Expression of cementum-derivrd attachment protein in bovine tooth germ during cementogenesis. Bone. 29 (2001) 242-248.

[4] Hara R, Wato M, Tanaka A. Maker of cemento-peiodontal ligament junction associated with periodontal regeneration. J. Periodont. Res. 40 (2005) 231-238.

[5] Alvarez-Perez MA, Narayanan S, Zeichner-David M, Carmona BR, Arzate H. Molecular clonig, expression and immunolocalization of a novel human cementum-derived protein (CP-23). Bone. 38 (2006) 409-419.

[6] Kitagawa M, Tahara H, Kitagawa S, Oka H, Kudo Y, Sato S, Ogawa I, Miyauchi M, Takata T. Characterization of established cementoblast-like cell lines from human cementum lining cells *in vitro* and *in vivo*. Bone. in press.

[7] Feinstein Y, Klar A. The neuronal class 2 TSR proteins F-spondin and midin: a small family with divergent biological activities. Int.J. Biochem. Cell Biol. 36 (2004) 975-980.

[8] Klar A, Baldassare M, Jessekll T. M. F-spondin: a gene expressed at high levels in the floor plate encordes a secreted protein that promotes neural cell adhesion and neurite extension. Cell. 69 (1992) 95-110.

[9] Kitajima S, Kudo Y, Ogawa I, Bashir T, Kitagawa M, Miyauchi M, Pagano M, Takata T. Role of Cks1 overexpression in oral squamous cell carcinomas: cooperation with Skp2 in promoting p27 degradation. Am. J. Pathol. 165 (2004) 2147-2155.

[10] Bessey OA, Lowry OH, Brock MJ. A method for the rapid detemination of alkaline phosphatase with five cubic millimeter of serum. J. Biol. Chem. 164 (1946) 321-329.

[11] Ortega N, Behonick D, Stickens D, Werb Z. How proteases regulate bone morphogenesis. 995 (2003) 109-116.

[12] Stickens D, Bshonick D. J, Orthalie N, Heyer B, Hartenstein B, Yu Y, Fosang A. J, Schorpp-Kistner M, Angel P, Werb Z. Altered endochondral bone development in matrix metalloproteinase 13-deficient mice. Dvelopment. 131 (2004) 5883-5895.

[13] Bosshardt DD. Are cementoblasts a subpopulation of osteoblasts or a unique phenotype? J. Dent. Res. 84 (2005) 390-406.

[14] McCulloch CAG, Melcher AH. Cell density and cell generation in the periodontal ligament of mice. Am. J. Anat. 167 (1983) 43-58.

[15] McCulloch CAG, Nemeth E, Lowenberg B, Melcher AH. Paravascular cells in endosteal spaces of alveolar bone contribute to periodontal ligament cell population. Anat. Rec. 219 (1987) 233-42.

[16] D'Errico JA, MacNeil RL, Takata T, Berry J, Strayhorn C, Somerman MJ. Expression of bone associated markers by tooth root lining cells, in situ and *in vitro*. Bone. 20 (1997) 117-126.

[17] Burstyn-Cohen T, Frumkin A, Xu Y. T, Scherer S. S, Klar A. Accumulation of F-spondin in injured peripheral nerve promotes the outgrowth of sensory axsons. J. Neuron 23 (1998) 8875-8885.

[18] Burstyn-Cohen T, Tzarfaty V, Frurnkin A, Feinstein Y, Stoecki E, Klar A. F-spondin is required for accurate pathfinding of commissural axons at the floor plate. Neuron. 23 (1999) 233-246.

[19] Schubert D, Klar A, Park M, Dargusch R, Ficher W. H. F-spondin promotes nerve precursor differentiation. J. Neurochem. 10 (2005) 1111/j.1471-4159.

[20] Kaneda T, Miyauchi M, Takekoshi T, Kitagawa S, Kitagawa M, Shiba H, Kurihara H and Takata T. Characteristics of periodontal ligament subpopulations obtained by sequential enzymatic digestion of rat periodontal ligament. Bone. 38(2006) 420-426.

[21] Arzate H, Olson SW, Page RC, Gown AM, Narayanan AS. Production of a monoclonal antibody to an attachment protein derived from human cementum. FASEB J. 6(1992) 2990-2995.

[22] Tuckermann JP, Pittois K, Partridge NC, Merregaert J, Angel P. Collagenase-3 (MMP-13) and integral membrane protein 2a (Itm2a) are marker genes of chondrogenic/osteoblastic cells in bone formation: sequential temporal, and spatial expression of Itm2a, alkaline phosphatase, MMP-13, and osteocalcin in the mouse. J. Bone. Miner. Res. 15 (2000) 1257-1265.

[23] Gronostajski RM. Roles of the NFI/CTF gene family in transcription and development. Gene. 249 (2000) 31-45.

Figure legends

Figure1. F-spondin is identified as a cementoblast specific gene . (A) Human cementoblast-like (HCEM) cells and human periodontal ligament (HPL) cells were established from the same extracted teeth by using modified enzymatic digestion methods. The transcriptional profiles of HCEM cells and HPL cells were compared by microarray analysis. Highly expressed genes in HCEM cells are listed. Among these genes, F-spondin was identified the gene demonstrating the high fold change expression in HCEM cells. (B) The expression of F-spondin mRNA by RT-PCR. F-spondin mRNA expression is observed in HCEM cells, but not in HPL cells. (C) The expression of F-spondin mRNA is not observed in human osteoblast cell lines (NHOst, OTT4 and Ost). The expression of CMKOR1, PTER and SLC14A1 is observed in both HCEM and NHOst.

Figure2. F-spondin expression in human periodontal tissues. (A) Checking the accuracy of the anti-SPON1 antibody by Western blot. F-spondin antibody specifically recognizes F-spondin in FLAG-F-spondin transfected 293T cells. (B) Immunohistochemical staining with the anti-SPON1 antibody in human periodontal tissue. High expression of F-spondin was observed in cell located along the root surface (triangles). C:cementum, D:dentin. PL:periodontal ligament, AB: alveolar bone.

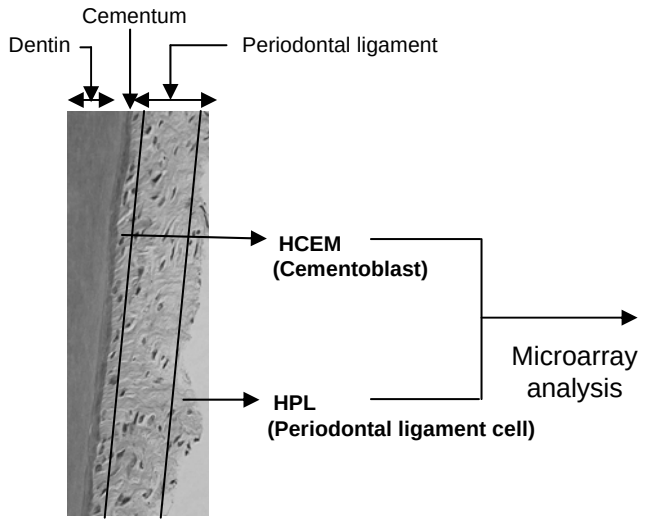
Figure3. High expression of F-spondin in HPL cells. (A) Stable clones of F-spondin highly expressing HPL-cells (HPL-spondin) expressed F-spondin mRNA and protein. GAPDH and ß-actin were used for loading control in RT-PCR and Western blot

analysis. (B) HPL-spondin cells shows shorter spindle shapes (b) in comparison with control cells (a). (scale bar = 100 μm)

Figure4. F-spondin promotes the differentiation of HPL cells in vitro. (A) Expression of mRNA for COLI, ALP, Runx2, OCN, BSP and CP-23 by RT-PCR in HPL-spondin. Higher expression of ALP, OCN and BSP and lower expression of COLI are observed in HPL-spondin in comparison with control cells. Both HPL-spondin and control cells express Runx2 and CP-23 mRNA at the same levels. (B) Mesurement of collagen concentration. Collagen concentration tends to decrease in HPL-spondin by collagen assay. (C) ALP activity by biochemical methods. Increased ALP activity is observed in HPL-spondin cells in comparison with control cells. *Significant difference ($p<0.05$). (D) Expression of MMP13 mRNA by RT-PCR. HPL-spondin cells express MMP13 mRNA at higher level in comparison with control cells.

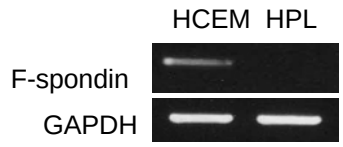
Figure1

A



Ratio(HCM/HPL)	Common	Gene Title_Affymetrix
819.1	CMKOR1	chemokine orphan receptor 1
646.6	SPON1	spondin 1, (f-spondin) extracellular matrix protein
359.1	KRT19	keratin 19 /// keratin 19
351.7	TM4SF1; L6; H-L6; M3S1; TAAL6	transmembrane 4 superfamily member 1
273.5	NFIB	nuclear factor I/B
268.0	C20orf103	chromosome 20 open reading frame 103
255.7	PTER	phosphotriesterase related
253.1	MEST	mesoderm specific transcript homolog (mouse)
241.2	TM4SF1	transmembrane 4 superfamily member 1
168.8	FOXG1B	forkhead box G1B
158.8		Homo sapiens transcribed sequences
114.2	GPC4	glypican 4
107.5	UCC1	upregulated in colorectal cancer gene 1
86.5	TM4SF1	transmembrane 4 superfamily member 1
71.2	LOC375295	hypothetical gene supported by BC013438
70.7	SLC14A1	solute carrier family 14 (urea transporter), member 1 (Kidd blood group)

B



C

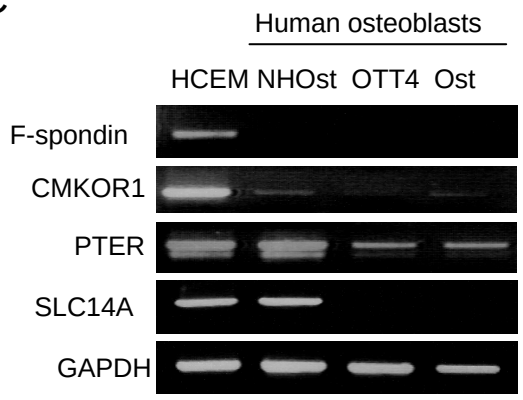
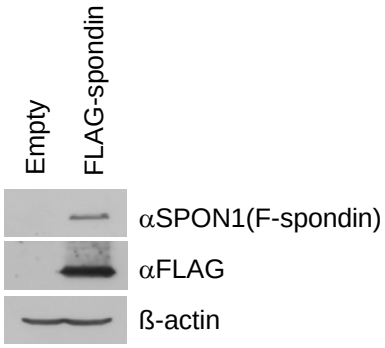


Figure2

A



B

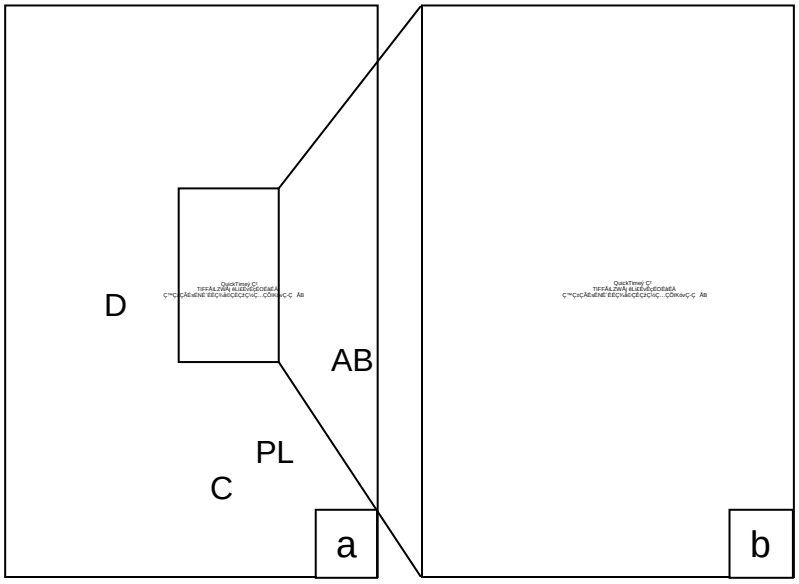


Figure3

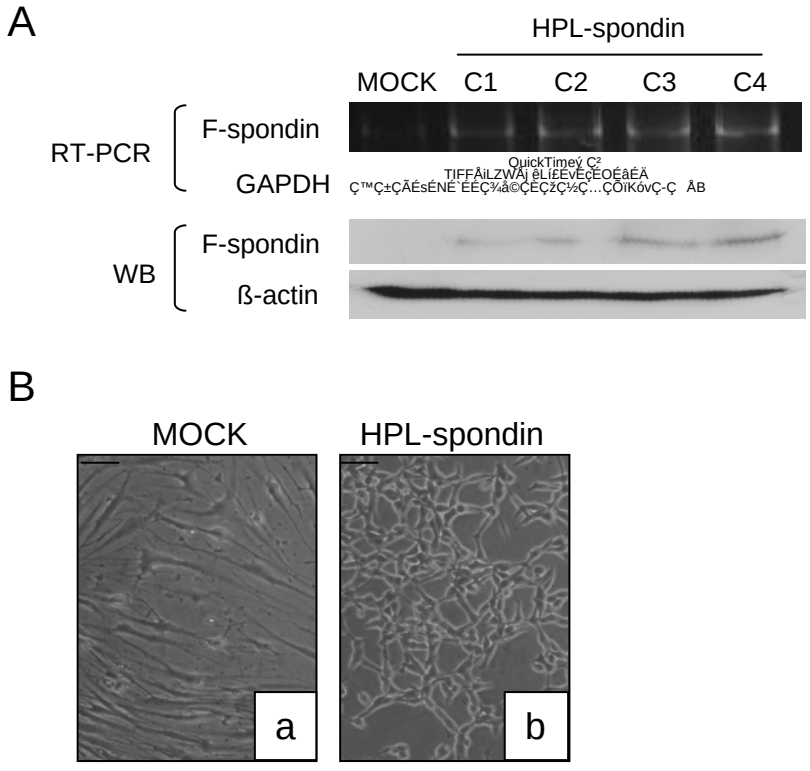
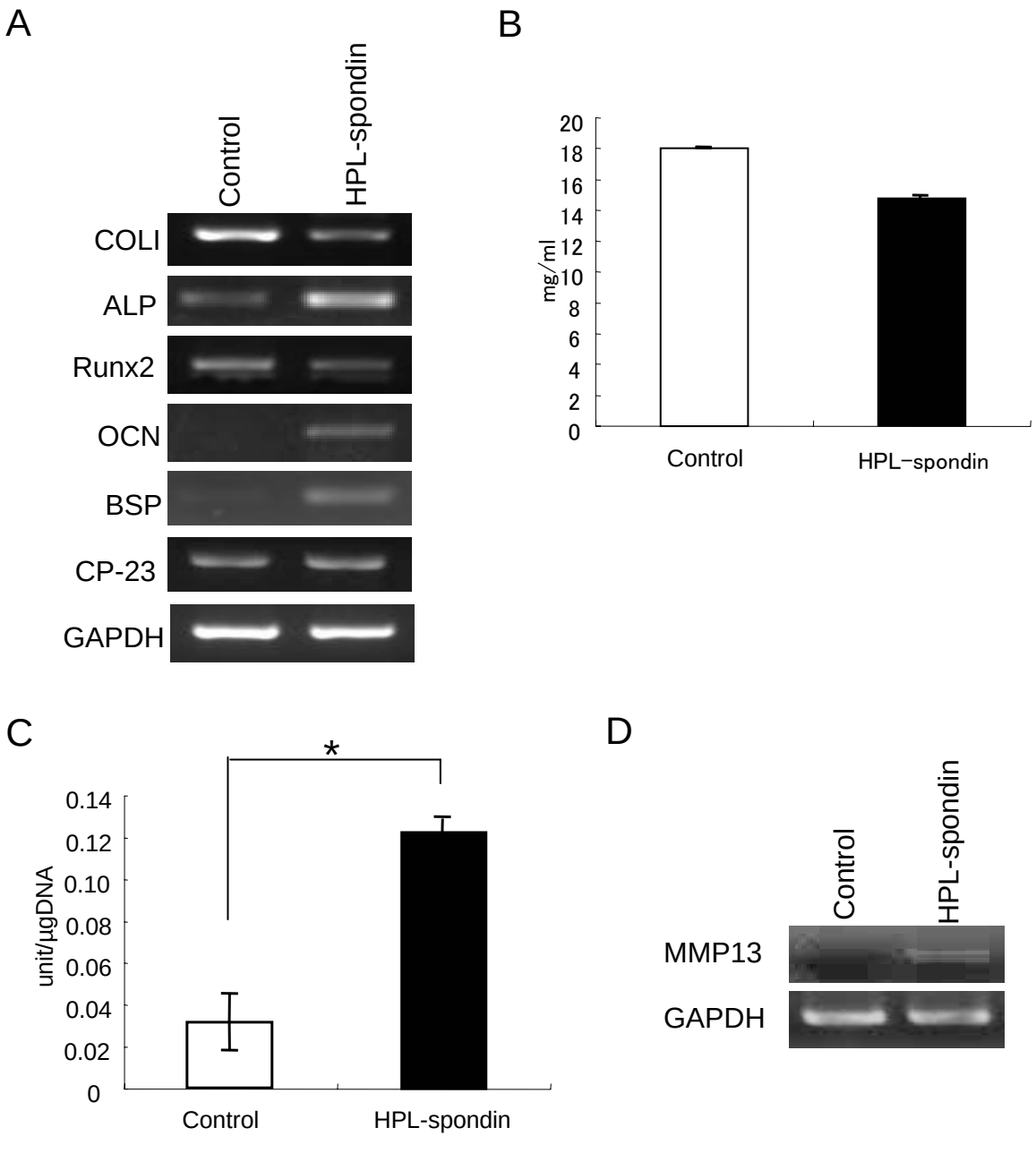

A
HPL-spondin
MOCK
C1
C2
C3
C4
RT-PCR
F-spondin
GAPDH
WB
F-spondin
ß-actin
B
MOCK
HPL-spondin
a
b

Figure4


A
Control
HPL-spondin
COLI
ALP
Runx2
OCN
BSP
CP-23
GAPDH
B
mg/ml
20
18
16
14
12
10
8
6
4
2
0
Control
HPL−spondin
C
*
unit/μgDNA
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Control
HPL-spondin
D
Control
HPL-spondin
MMP13
GAPDH