

Multipotency of clonal cells derived from swine periodontal ligament and differential regulation by fibroblast growth factor and bone morphogenetic protein

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Background and Objective: A blood supply is indispensable for the regeneration of damaged or lost periodontal ligament (PDL) tissue. Mesenchymal stem cell-like activity of cells derived from the PDL has been identified by their capacity to form fibrous and osseous tissue and cementum. However, it remains to be clarified whether the cells have an ability to build the capillary network of blood vessels. This study evaluated the potential of cells derived from the PDL to construct a blood vessel-like structure and examined how growth factors controlled the multipotency of the cells.

Material and Methods: The ability of a swine PDL fibroblast cell line, TesPDL3, to construct a blood vessel-like structure was evaluated on and in the self-assembling peptide scaffold, PuraMatrixTM. In addition, the ability of the cells to form mineralized nodules was evaluated on type I collagen-coated plastic plates. In some cases, fibroblast growth factor (FGF)-2 and bone morphogenetic protein (BMP)-2 were added to these cultures. The status of the expression of vascular and osteoblastic cell-specific markers in the cells was evaluated using reverse transcriptase-polymerase chain reaction (RT-PCR), Western blotting and immunofluorescence analyses.

Results: The TesPDL3 cells not only formed mineralized nodules in response to BMP-2 stimulation but also constructed tube-like structures in response to FGF-2 stimulation. Intriguingly, FGF-2 inhibited the BMP-2-induced formation of mineralized nodules. Conversely, BMP-2 inhibited the FGF-2-induced formation of tube-like structures.

Conclusion: Periodontal ligament fibroblasts have the potential to differentiate not only into osteoblastic but also into vascular cell lineages. The destiny of the cells was reciprocally regulated by BMP-2 and FGF-2.

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The periodontal ligament (PDL) is a fibrous connective tissue located between the tooth root and alveolar bone. The PDL contains heterogeneous types of cells including PDL fibroblasts, cementoblasts, osteoblasts, epithelial cells (rests of Malassez), vascular endothelial cells (ECs), smooth muscle cells (SMCs) and certain types of nerve cells (1). In response to oral pathological and physiological environmental alterations such as periodontitis, wounding and tooth movement by orthodontic treatment, PDL cells have the capacity to reconstruct the periodontal structure. For tissue reconstruction, it is necessary that multipotent progenitor cells or putative stem cells are present in the PDL. The paravascular zones in the adult PDL contain the progenitors for the fibroblastic lineages and mineralized tissue-forming cell lineages, i.e. the osteoblastic and cementoblastic lineages (2). Recently, several studies have indicated that PDL fibroblastic cells have biological characteristics in common with bone marrow mesenchymal cells, suggesting that the mineralized tissue-forming cell and fibroblastic lineages may originate from a common early progenitor cell (3–6). Seo *et al.* (2004) also demonstrated that the PDL cells showed cementoblastic/osteoblastic and adipogenic differentiation *in vitro* and had the potential to generate cementum/PDL-like tissue *in vivo*, suggesting that multipotent stem cells are present in the PDL (7). In contrast, it has been shown that bone marrow mesenchymal cells have the ability to give rise to both osteoblasts and SMCs (8). Thus, it seems reasonable to suppose that both osteoblasts and SMCs may develop from the same progenitor, and that multipotent stem cells in the PDL may differentiate not only into cementoblasts/osteoblasts but also into SMCs.

A blood supply is indispensable for the regeneration of damaged or lost PDL tissue (9). An understanding of how PDL tissue recruits the precursors of vascular cells and how the precursors differentiate into vascular cells is necessary for the establishment of a practical strategy for regenerative therapy for the damaged or lost PDL. However, it

remains to be elucidated whether the multipotent cells in PDL have an ability to build the capillary network of blood vessels. The inner wall of the blood vessel is constructed from two types of cells, ECs and SMCs. Recently, it has been identified that multipotent flk-1-positive embryonic stem cells (ESCs), pulmonary valve progenitor cells and 'side population' progenitor cells from arteries give rise to ECs and SMCs (10). In addition, transdifferentiation of ECs to SMCs and that of SMCs to ECs has been reported recently (11,12). Thus, both ECs and SMCs develop from the same progenitor.

These general views on a progenitor of osteoblasts, ECs and SMCs lead to the hypothesis that multipotent cells in the PDL may give rise to a vascular cell lineage other than osteoblastic/cementoblastic cell lineages. We have previously shown that the TesPDL3 cell line derived from swine PDL tissue shows fibroblastic morphology and expresses procollagen α (I), osteopontin, periostin and alkaline phosphatase (ALP) mRNA; these genes are expressed in PDL tissue (13). Intriguingly, the cell line has the ability to express EC-specific marker CD31, vascular endothelial (VE)-cadherin, von Willebrand factor (vWF) and osteoblast-specific marker osteocalcin (OCN), and to form extracellular mineralized nodules. Thus, our data indicated that TesPDL3 cells had unique properties in expressing several phenotypes of fibroblasts, vascular endothelial cells and osteoblasts in the cultures. Recently, Takahashi and Yamanaka reported the induction of pluripotent stem cells from mouse embryonic or adult dermal fibroblasts by introducing four transcription factors: Oct3/4, Sox2, c-Myc and Klf4 (14). These cells exhibited the morphology and growth properties of ESCs and expressed ESC marker genes. Subcutaneous transplantation of the cells into nude mice resulted in tumors containing a variety of tissues from all three germ layers. These data demonstrate that pluripotent stem cells can be directly generated from adult dermal fibroblast cultures by addition of only a few defined factors. From these results, it seems reasonable to suppose that fibroblastic

lineages may have the plasticity to differentiate into various cell lineages. However, it remains to be clarified whether the fibroblastic lineage in PDL tissue has the same plasticity. The purpose of the present study was to evaluate whether the cells derived from PDL have the ability to build a capillary network of blood vessels as ECs, and how the expressions of osteoblast and endothelial properties are affected by various stimulants such as growth factors identified in PDL.

Material and methods

Cell culture

Periodontal ligament fibroblasts from TesPDL3 and TesPDL4 Clawson miniature swine were isolated and established as cell lines as described previously (13). The TesPDL3 and TesPDL4 cells were cultured on cell culture plates coated with type I collagen (Sumilon Celltight multi-well plate; Sumitomo Bakelite Co., Tokyo, Japan) with F-12 Ham's medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Moregate Biotech, Bulimba, Australia), with 1 or 20 ng/mL fibroblast growth factor (FGF)-2 (R&D Systems Inc., Minneapolis, MN, USA), with or without 50 ng/mL bone morphogenetic protein (BMP)-2 (Astellas, Tokyo, Japan), with or without 5 μ M SU5402 (Calbiochem, Darmstadt, Germany), and with 2 mM L-glutamine and 200 μ g/mL kanamycin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

For two-dimensional cell culture on the PuraMatrix™ self-assembling peptide scaffold (15; 3-D Matrix Japan, Ltd, Tokyo, Japan), 30 μ L of the 1% peptide scaffold solution was dispensed into each well of a 96-well culture plate after supersonic treatment. Exposure of the peptide solution to the growth medium at pH 7.1, by dropping the growth medium gently onto the peptide scaffold, resulted in scaffold formation within 5 min. The TesPDL3 and TesPDL4 cells were subsequently seeded onto the scaffold at a cell density of 8×10^4 cells per well with the growth medium.

For three-dimensional cell culture in the peptide scaffold (16), TesPDL3 cells were suspended in one volume of 10% sucrose solution (to maintain osmolarity) and mixed with four volumes of 22.5% sucrose solution and five volumes of 1% peptide scaffold solution, so that the final peptide concentration was 0.5%, and the final cell concentration was 3×10^6 cells/mL. The cell-peptide suspension was subsequently loaded into a Millicell® culture plate insert (Millipore Corp., Bedford, MA, USA; 10 mm inner diameter, 0.6 cm² membrane area, pore size = 0.4 µm). The culture plate insert was immediately transferred into the well of 24-well plates and moistened with 500 µL of the growth medium, in order to initiate peptide gel formation via a self-assembling process.

Immunocytochemistry

The TesPDL3 cells were subcultured into each well of poly-D-lysine-coated eight-chamber slides (1×10^5 cells per well; BD Biosciences, Bedford, MA, USA). In some cases, the surface of the eight-chamber slide was firstly coated with the PuraMatrix™, and the cells were seeded on it. The cells were fixed in 4% paraformaldehyde for 30 min or in acetone for 10 min, and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS). After background inhibition in bovine serum albumin, cells were double-labeled with anti-vWF rabbit polyclonal antiserum (1:500; Dako, Carpinteria, CA, USA) and anti-calponin mouse monoclonal antibody (clone hCP; 1:500; Sigma) or anti-human osteopontin monoclonal antibody (1:750; Immuno-Biological Laboratories Co., Ltd, Gunma, Japan) at room temperature for 1 h. After washing off the primary antibodies with 0.2% Triton X-100 in PBS, the cells were incubated with Alexa Fluor® 568-conjugated goat anti-rabbit immunoglobulin G (IgG) or Alexa Fluor® 488-conjugated rabbit anti-mouse IgG (1:200; Molecular Probes, Leiden, The Netherlands) for 45 min at room temperature. The cells were washed three times with 0.2% Triton X-100 in PBS. The signals were

subsequently detected using a confocal laser scanning microscope (Nikon TE2000-E; Nikon Corp., Tokyo, Japan).

Transfection of green fluorescent protein (GFP) and Smad7 expression vectors into cells

The TesPDL3 and TesPDL4 cells were transiently transfected with GFP-expression vector pTracer™-CMV2 (Invitrogen, Grand Island, NY, USA) and TesPDL3 cells with Smad7 expression vector pcDNA3-FLAG-Smad7 (17) using FuGENE HD (Roche Diagnostics, Indianapolis, IN, USA) in 24-well cell culture plates coated with type I collagen in accordance with the protocol provided by the manufacturer. Firstly, FuGENE HD (2 µL) diluted in 25 µL of Opti-MEM® I medium (Invitrogen) was mixed with 1 µg of purified pTracer™-CMV2, pcDNA3 or pcDNA3-FLAG-Smad7. The DNA-FuGENE HD mixtures were vortex mixed and incubated for 15 min at room temperature. The cells were cultured for 48 h in a humidified atmosphere of 5% CO₂ and 95% air at 37°C and subsequently seeded on or in the peptide scaffold to form the capillary-like structure.

Evaluation of the ability of cells to form a lumen in the capillary-like structure

A cell-peptide scaffold complex of the three-dimensional cell culture was rinsed with PBS and then embedded in a Tissue-Tek® OCT compound (Sakura Finetek Co. Ltd, Tokyo, Japan) and frozen. Specimens were prepared as 10-µm-thick frozen sections using a microtome. The TesPDL3 cells in each specimen were subsequently fixed using acetone and observed under a light-field microscope (Nikon Eclipse-E600; Nikon Corp.) following hematoxylin and eosin staining or under the confocal laser scanning microscope (Nikon TE2000-E; Nikon Corp.).

Alizarin Red staining of mineralized extracellular matrix

The TesPDL3 cells were fixed with 4% paraformaldehyde in PBS (pH 7.2) at

room temperature for 30 min. The monolayers were washed with distilled water three times and treated with 40 mM Alizarin Red solution for 30 min. They were washed again with distilled water at least three times. Alizarin Red was extracted from the monolayer by incubation of the monolayers in 10 mg/mL cetylpyridinium chloride (CPC) buffer for 1 h. The dye was subsequently removed, and 150 µL aliquots were transferred to a 96-well plate prior to reading at 577 nm with a plate reader (Bio-Rad Laboratories, Boston, MA, USA).

Measurement of alkaline phosphatase (ALP) activity in TesPDL cells

The TesPDL3 cells were lysed with 0.2% IGEPAL CA-630 (Sigma) in 10 mM Tris-HCl (pH 7.5), and centrifuged at 4500 *g* at 4°C for 10 min. The lysate was added with buffered substrate (5 mM phenylphosphate-2-sodium, 2.5 mM 4-aminoantipyrine, 25 mM sodium carbonate and 20 mM sodium hydrogen carbonate). Total cellular ALP activity in the lysate was measured with *p*-nitrophenyl phosphate as the substrate. The amount of DNA in the lysate was measured with a DNA quantitative kit (Hokudo Co., Ltd, Tokyo, Japan) in accordance with the protocol provided by the manufacturer.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the TesPDL3 cells using Isogen (Nippongene Co., Toyama, Japan) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized with Omniscript reverse transcriptase (Qiagen, Valencia, CA, USA) using (dT)₁₅ primer (1 µM). Subsequent amplification for the detection of TesPDL3 cell cDNAs was performed using the requisite number of cycles, under the following conditions: 94°C for 30 s, annealing temperature optimized for each primer pair for 30 s, and 72°C for 90 s. The primer sequences used for PCR amplification (Table 1) were designed

Table 1. Primers used for RT-PCR

Specificity	Oligonucleotide sequence (5'-3')	Annealing temperature (°C)	Predicted size (bp)
vWF	GCTCTGGGTTCGTCAGAGTC CAGGCACCGTTATGGAGAAT	58	308
VE-cadherin	CGACTCATCCGACTCTGACA CCCAGACAGAACACCATCCT	58	355
CD31	GCAAGGTGGTCAAGAGAAGC GCCTGGGTGTCATTCAAAGT	55	493
Calponin	GGCTGAGGTCAAGAACAAGC CCAGTTCTGGGTGGACTCAT	55	202
α SM actin	ATCACCATTGGGACGACAT GGGACGTTGAAGGTCTCAAA	55	164
Smoothelin	GAAGCCACAGGAACAAGAGC CTTAGCACGGATAGGGAACG	55	435
Osterix	CAATGGGCTCCTGTCACCT CACTGGGCAGACAGTCAGAA	60	161
Runx2	GGTACCAGATGGGACTGTGG ATGCGCCCTAAATCACTGAG	58	315
β -Actin	CGCACCCTGGCATTGTCAT GCCGTGATCTCCTTCTGCAT	55	518

Abbreviations: vWF, von Willebrand factor; VE-cadherin, vascular endothelial-cadherin; α SM actin, α -smooth muscle actin; and Runx2, Runt-related transcription factor 2.

using cDNA sequences of swine mRNA for vWF, vascular endothelial-cadherin, CD31, calponin, α -smooth muscle actin, smoothelin, osterix, runt-related transcription factor (Runx) 2, and β -actin. All of the primers were synthesized by Hokkaido System Science (Sapporo, Japan). Amplified products were separated by agarose gel electrophoresis and stained with ethidium bromide. The PCR experiments were performed using samples from at least three different cell preparations and the results were confirmed by triplicate PCR experiments from the same cell samples.

Western blotting

The TesPDL3 cells were lysed in 25 mM Tris-HCl (pH 6.5), 1% sodium dodecyl sulphate (SDS) and 5% glycerol, and boiled for 5 min. Equal amounts of protein from each cell lysate were separated on a 4–8% tris-glycine SDS-polyacrylamide gel electrophoresis (PAGE) gel. The separated proteins were transferred onto PVDF membranes (Millipore Corp.) and blocked for 1 h at room temperature in 3% (w/v) skimmed milk. Anti-vWF rabbit polyclonal antiserum was added directly to the blocking solution

(dilution of 1:1000) at 4°C overnight. Membranes were washed and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG affinity-purified goat antibody (CHEMICON International Inc., Temecula, CA, USA) for 30 min at room temperature. Peroxidase activity on the membrane was visual-

ized by means of the ECL Western blotting detection system (Amersham Biosciences Corp., Piscataway, NJ, USA) in accordance with the protocol provided by the manufacturer.

Statistical analysis

Statistical differences were evaluated with respect to Alizarin Red staining and ALP activity between the various culture conditions using analysis of variance (ANOVA) with Tukey's HSD tests.

Results

TesPDL3 cells showed a unique property in simultaneously expressing several specific markers of osteoblasts, vascular ECs and SMCs

The TesPDL3 cells were found to express the vascular EC marker vWF (Fig. 1Aa,Ba), the SMC marker calponin (Fig. 1Ab) and the osteoblastic marker osteopontin (Fig. 1Bb). No fluorescence signal was detected from the TesPDL3 cells labeled with the second antibodies without the first antibodies against each cell-specific marker (data not shown). These cell-specific markers were expressed simultaneously in the TesPDL3 cells (Fig. 1Ad,Bd).

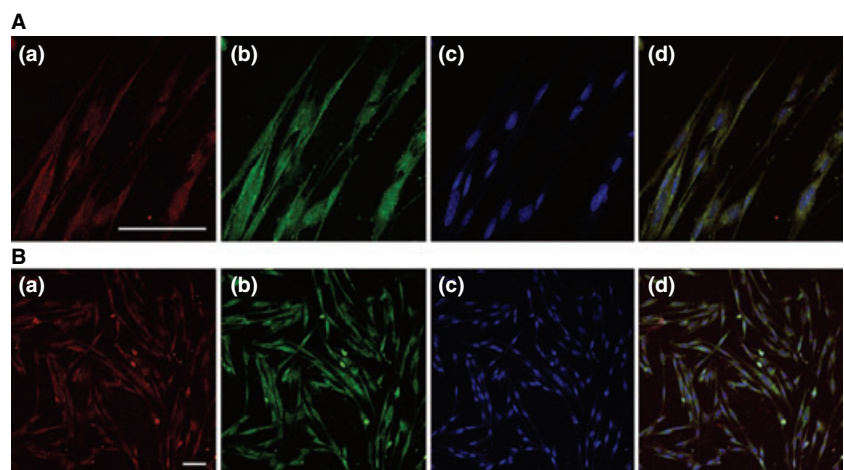


Fig. 1. The TesPDL3 cells were cultured on cell culture plates coated with type I collagen for 3 days and fluorescently labeled with antibodies for vWF (Aa,Ba; red), calponin (Ab; green) and osteopontin (Bb; green). The nuclei were counterstained with DAPI (Ac,Bc; blue). The images were merged (Ad,Bd). Signals were detected using a confocal laser scanning microscope. Magnification: $\times 400$ (A) and $\times 100$ (B); Bar, 100 μ m.

TesPDL3 cells expressed an ability to construct tube-like networks on stimulation with FGF-2

The TesPDL3 cells have a fibroblastic spindle shape on type I collagen-coated plastic culture dishes (data not shown). In contrast, TesPDL3 cells migrated actively, constructed many multicellular dense structures and formed networks with tube-like structures (Fig. 2Ba,Bb) on the peptide scaffold only with 20 ng/mL FGF-2 stimulation for 5 days (Fig. 2Aa,Ab). A specific inhibitor of the FGF receptor-1, SU5402 (5 μ M), suppressed the FGF-2-induced construction of multicellular dense structures and the formation of tube-like networks (Fig. 2Ca,Cb). The TesPDL4 cells did not share this ability of TesPDL3 cells (Fig. 2Da,Db,Ea,Eb).

Bone morphogenetic protein-2 inhibited the FGF-2-induced tube-like network formation by TesPDL3 cells

Bone morphogenetic protein-2 did not affect the FGF-2-induced construction of multicellular dense structures (Fig. 3Ba,Bb). However, it clearly inhibited the FGF-2-induced formation of tube-like networks (Fig. 3Aa,Ab). Overexpression of Smad7, a specific inhibitor of BMP-induced signal transduction, clearly suppressed the BMP-2-induced inhibition of

the tube-like structure formation (Fig. 3Ca,Cb).

TesPDL3 cells showed an ability to construct tube-like structures on stimulation with FGF-2

The TesPDL3 cells fluorescently labeled by transfection of GFP-expression vector were embedded in the peptide scaffold and cultured with FGF-2 (20 ng/mL) for 7 days. Tube-like structures with a lumen (red arrows) were observed in the section stained with hematoxylin and eosin (Fig. 4A). Various sizes of lumen were observed in the multicellular dense structure under the confocal laser scanning microscope (Fig. 4B).

Fibroblast growth factor-2 reduced BMP-2-induced mineralized nodule formation and Alizarin Red staining in TesPDL3 cells

Mineralized nodule formation was induced by BMP-2 (50 ng/mL) treatment (Fig. 5B) compared with the untreated control (Fig. 5A). Fibroblast growth factor-2 (20 ng/mL) reduced the BMP-2-induced nodule formation (Fig. 5C), while the addition of SU5402 suppressed the FGF-2-induced inhibition of the nodule formation (Fig. 5D). Mineralization of the extracellular matrix was measured with respect to intensity of Alizarin Red staining, and

the results confirmed the significant observations described above (Fig. 6).

Fibroblast growth factor-2 reduced the ALP activity enhanced by BMP-2 in TesPDL3 cells

Fibroblast growth factor-2 (20 ng/mL) revealed a tendency to reduce the BMP-2-enhanced ALP activity in TesPDL3 cells, while the addition of SU5402 significantly suppressed the FGF-2-induced reduction of ALP activity (Fig. 7).

Fibroblast growth factor-2 and BMP-2 controlled the expression levels of osteoblast and vascular cell-specific markers in TesPDL3 cells

Reverse transcriptase-polymerase chain reaction analysis revealed: (a) that the swine mRNA expression levels of the EC markers, vWF and VE-cadherin, were dose-dependently upregulated by FGF-2; (b) vWF was suppressed by BMP; (c) the vascular SMC markers, calponin and α SM-actin, were downregulated by FGF-2 or BMP-2; (d) the osteoblastic marker, osterix, was downregulated by FGF-2; and (e) the EC marker, CD31, the SMC marker, smoothelin, and the osteoblastic marker, Runx2, were not affected by FGF-2 or BMP-2 (Fig. 8A). Western blot analysis revealed that BMP-2 significantly suppressed the protein expression levels of vWF (Fig. 8B). Taking these results together, it is suggested that BMP-2 inhibits the differentiation of TesPDL3 cells into vascular cell lineages and that FGF-2 promotes the differentiation of TesPDL3 cells into ECs, but inhibits the differentiation into SMCs or osteoblasts.

Discussion

Therapies with biologically active, soluble factors such as cytokines and growth factors are being evaluated for clinical use in the regeneration of periodontal tissue damaged or lost as a result of periodontitis. Fibroblast growth factor-2 is a multifunctional growth factor which has a variety of effects, including induction of prolifer-

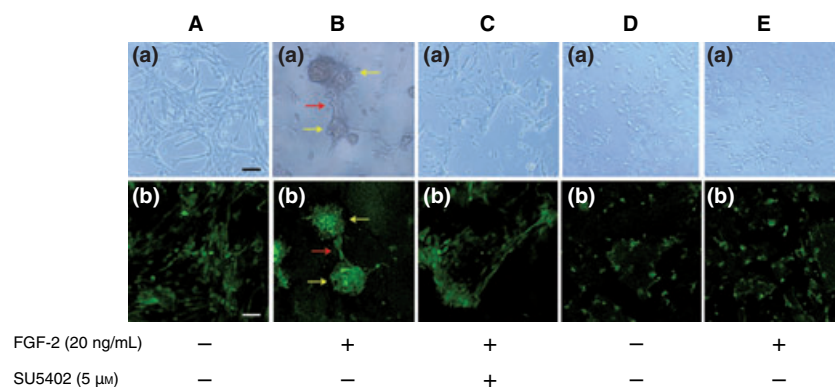


Fig. 2. TesPDL3 cells overexpressing GFP were cultured on the peptide scaffold for 5 days (A), with 20 ng/mL FGF-2 (B) and with 20 ng/mL FGF-2 and 5 μ M specific inhibitor of FGF receptor 1, SU5402 (C). TesPDL4 cells overexpressing GFP were cultured on the peptide scaffold for 5 days, with (E) or without 20 ng/mL FGF-2 (D). Yellow arrows indicate multicellular dense structures and red arrows tube-like structures. (a) Phase-contrast microscopy; (b) confocal laser scanning microscopy. Magnification $\times 100$; Bar, 100 μ m.

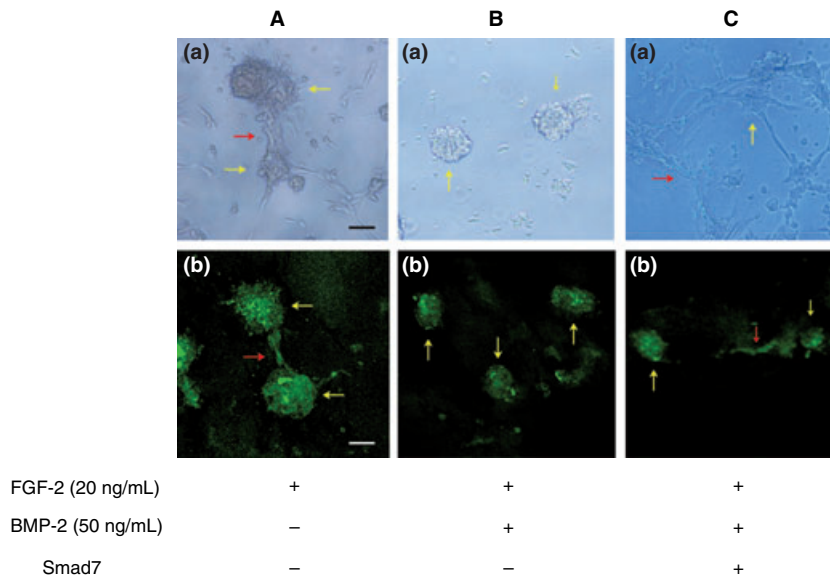


Fig. 3. TesPDL3 cells overexpressing GFP were cultured with 20 ng/mL FGF-2 (A) or with 20 ng/mL FGF-2 and 50 ng/mL BMP-2 (B), or TesPDL3 cells overexpressing Smad7 and GFP were cultured with 20 ng/mL FGF-2 and 50 ng/mL BMP-2 (C), on the peptide scaffold for 5 days. Yellow arrows indicate multicellular dense structures and red arrows tube-like structures. (a) Phase-contrast microscopy and (b) confocal laser scanning microscopy. Magnification $\times 100$; Bar, 100 μm .

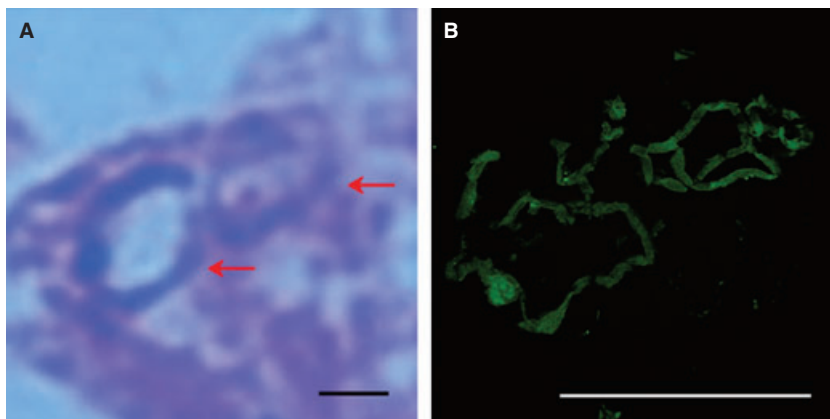


Fig. 4. TesPDL3 cells overexpressing GFP were cultured in the peptide scaffold with 20 ng/mL FGF-2 for 7 days. The frozen section was stained with hematoxylin and eosin. Tube-like structures (red arrows) were observed in the section under a light-field microscope (A). The GFP signal from frozen sections was detected using a confocal laser scanning microscope. Various sizes of lumen were observed (B). Magnification: $\times 1000$ (A) and $\times 200$ (B); Bar, 100 μm .

eration and morphogenesis in a wide range of mesodermal and neuro-ectodermal cells (18), and is considered to participate in the early stage of wound healing (19). A variety of intracellular signals induced by FGF-2 are expected to regulate migration, attachment, proliferation and differentiation of PDL cells. Scatchard analysis revealed

that expression of receptors for FGF-2 was detected on human PDL cells (20). An immunohistochemical analysis revealed that expression of FGF-2 was detected in the cytoplasm of PDL cells (21). These results suggest that the FGF-2–FGF receptor-mediated autocrine and paracrine loops play roles in the maintenance of homeostasis of

PDL tissue. In addition, FGF-2 seems to have a positive role in regenerating lost periodontal tissue, in that FGF-2 works as a potent mitogen for PDL cells *in vitro* (22). In a beagle dog model, where FGF-2 was applied to an artificial alveolar bone defect, significant PDL formation with new cementum deposits and new bone formation was observed in amounts greater than in control sites (23,24).

For PDL tissue reconstruction, it is likely that multipotent progenitor cells or putative stem cells proliferate and differentiate into heterogeneous types of cells, including PDL fibroblasts, cementoblasts, osteoblasts, epithelial cells (rests of Malassez), vascular ECs, SMCs and certain types of nerve cells in the PDL tissue. However, it remained to be clarified how FGF-2 affected the multifunctional ability of the multipotent progenitor cells or putative stem cells in the PDL. Here we showed, for the first time, that FGF-2 facilitates the cells to give rise to EC-like cells possessing the ability to construct the tube-like structures in culture (Figs 1 and 4). It is of interest that the other swine PDL-derived cell line, TesPDL4, did not construct any tube-like structures in response to FGF-2 stimulation (Fig. 2Ea,Eb). These results suggest that both terminally differentiated fibroblasts and mesenchymal stem cell-like fibroblastic cells reside in the PDL, and that fibroblasts from the tissues other than PDL do not have an ability to form tube-like structures as do ECs. It is generally known that FGF-2 is a potent angiogenic factor and that differentiated ECs have an ability to form a vascular tube-like structure in response to FGF-2 stimulation in culture (25). Moreover, FGF-2 facilitates neural stem cells to give rise to ECs and to form vessel-like tube structures consisting of EC marker-positive cells in collagen gel culture (26). It follows that it seems reasonable to conclude that the PDL-derived mesenchymal cells have an ability to form vascular tube-like structures in response to FGF-2 stimulation in culture as do differentiated ECs and neural stem cells.

Tsutsumi *et al.* (2001) demonstrated that FGF-2 was a potent mitogen for

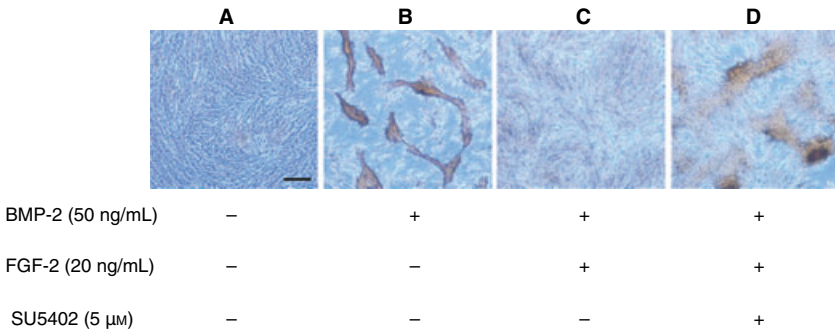


Fig. 5. TesPDL3 cells were cultured on type I collagen-coated plastic culture dishes for 7 days, without BMP-2 (A), with BMP-2 (50 ng/mL; B), with BMP-2 (50 ng/mL) and FGF-2 (20 ng/mL; C) or with BMP-2 (50 ng/mL), FGF-2 (20 ng/mL) and the specific inhibitor of FGF receptor 1, SU5402 (5 μM; D); Bar, 300 μm.

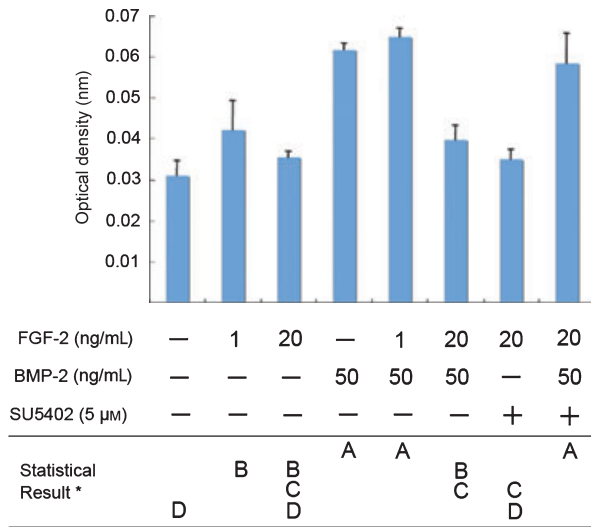


Fig. 6. TesPDL3 cells were cultured on type I collagen-coated plastic culture dishes without or with BMP-2 (50 ng/mL) for 7 days. Some cells were treated with FGF-2 (1 or 20 ng/mL) or the specific inhibitor of FGF receptor 1, SU5402 (5 μM). Alizarin Red staining was measured as described in the Material and methods. * Statistical differences were evaluated using analysis of variance (ANOVA) with Tukey's HSD tests. Measurement values with the same letter revealed no statistical differences ($p < 0.05$ for significance).

mesenchymal stem cells and that incubation with FGF-2 maintained the multilineage differentiation potential of mesenchymal stem cells throughout many mitotic divisions (27). We established the swine PDL fibroblast cell line, TesPDL3, under the condition that the cells were continuously stimulated with FGF-2 (1 ng/mL) in order to maintain the multilineage differentiation potential of the cells (13). In the present study, TesPDL3 cells simultaneously expressed EC markers, SMC markers and osteoblastic markers, indicating the multipotency of the cells.

In contrast, previous studies have shown that human PDL cells that are not stimulated with FGF-2 do not express EC markers such as CD31 (5,28). This discrepancy might be due to the differences in the cell culture conditions. Oswald *et al.* (2004) demonstrated that several populations of bone marrow-derived mesenchymal cells had the potential to differentiate into endothelial-like cells under the stimulation with vascular endothelial growth factor (VEGF; 29). We examined whether VEGF induced the endothelial differentiation of TesPDL3

cells. However, the cells did not respond to VEGF stimulation (data not shown). In addition, RT-PCR analysis revealed that TesPDL3 cells did not express a detectable level of VEGF receptor 2 (data not shown), sometimes referred to as KDR or FLK1. It could, therefore, be concluded that multipotent PDL fibroblasts may have a distinct way of expressing the EC-like character from the way used by bone marrow-derived mesenchymal cells. However, it remains to be clarified whether the discrepancy that the bone marrow-derived mesenchymal cells express KDR but the TesPDL cells do not might be due to the differences in the culture conditions.

Bone morphogenetic proteins are members of the transforming growth factor (TGF)- β superfamily, which mediate multiple biological processes including bone formation (30). Bone morphogenetic proteins play a pivotal role in the commitment and differentiation of cells of osteoblastic lineage (31). Bone morphogenetic protein-2 promotes osteoblast maturation by increasing the expression of the transcription factor Runx2, previously referred to as Cbfa1/Pebsp α A/AML3, and the expression of osteoblastic marker genes (32,33). Immunohistochemical and RT-PCR analyses revealed that expression of BMP-2 and BMP receptors (BMPRs) IA and II were detected in or on the surface of PDL fibroblasts (34). These results suggest that the BMP-2–BMPR-mediated autocrine and paracrine loops play roles in the maintenance of homeostasis of periodontal tissue. In addition, BMP-2 has an ability to promote the PDL cells along a cementblast/osteoblast pathway, in that BMP-2 induces expression of osteoblast-specific markers such as Runx2, ALP, OCN and bone sialoprotein in the cells and to form extracellular mineralized nodules in the cell culture (35). In the present study, BMP-2 induced extracellular mineralized nodule formation (Fig. 5) and enhanced ALP activity (Fig. 7) as described in the previous report (13). It should be noted that FGF-2 completely suppressed the BMP-2-induced

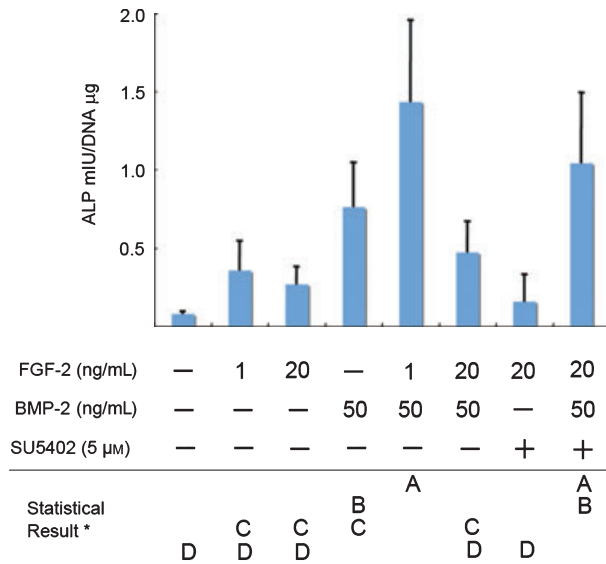


Fig. 7. TesPDL3 cells were cultured on type I collagen-coated plastic culture dishes without or with BMP-2 (50 ng/mL) for 7 days. Some cells were treated with FGF-2 (1 or 20 ng/mL) or the specific inhibitor of FGF receptor 1, SU5402 (5 µM). Alkaline phosphatase activity was measured as described in the Material and methods. *Statistical differences were evaluated using analysis of variance (ANOVA) with Tukey's HSD tests. Measurement values with the same letter revealed no statistical differences ($p < 0.05$ for significance).

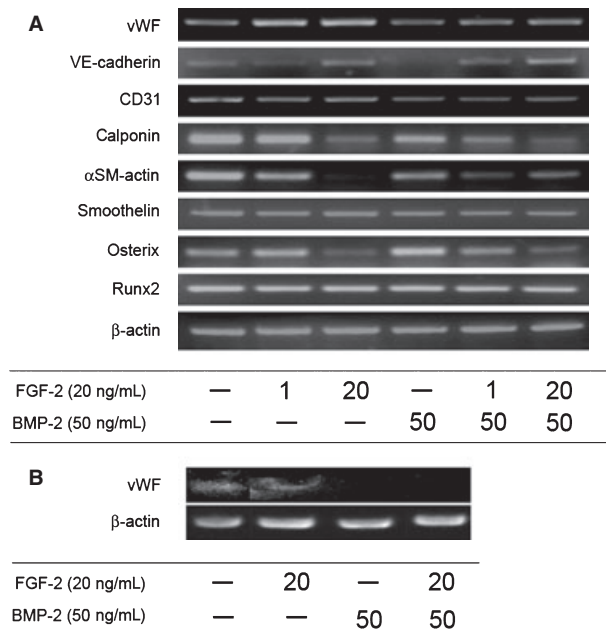


Fig. 8. TesPDL3 cells were cultured on type I collagen-coated plastic culture dishes without or with BMP-2 (50 ng/mL) for 7 days. Some cells were treated with FGF-2 (1 or 20 ng/mL) and their mRNA and protein expression levels were analysed. (A) RT-PCR analyses of vascular cell-specific and osteoblast-specific markers. The mRNA levels of EC markers (vWF and CD31) were upregulated by FGF-2 in a dose-dependent manner; vascular SMC and osteoblastic markers (calponin, α SM actin and osterix) were downregulated by FGF-2; and vascular EC and SMC markers (vWF, CD31, calponin and α SM actin) were downregulated by BMP-2. (B) Western blotting analyses; BMP-2 significantly suppressed protein expression of vWF.

formation of extracellular mineralized nodules in the PDL cell culture. In addition, FGF receptor antagonist, SU5402 suppressed nodule formation (Fig. 5). Thus, we showed that FGF has multifunctional abilities not only to induce vascular EC-like functions but also to suppress the BMP-induced cementoblast/osteoblast-like function in the PDL-derived fibroblast-like cells.

Although some studies have been done on the effect of BMPs on vascularization, there is little agreement as to the outcome. Raida and colleagues reported that BMP-2 promoted formation of tube-like structures by human dermal microvascular ECs in culture on growth factor-reduced Matrigel™ (36). In contrast, Smith and co-workers reported that BMP-2 had no effect on the vascularization in chorioallantoic membranes in organ culture (37). Here, we showed that BMP-2 suppressed the expression of EC-specific adhesion molecules CD31 and VE-cadherin (Fig. 8A) and partly inhibited the FGF-2-induced formation of vascular tube-like structures on the self-assembling peptide scaffold in culture (Fig. 3). It seems reasonable to suppose that these disagreements concerning the biological effects of BMP-2 on vascularization may depend on the differences in the culture systems or on the differences in cell types between various experiments.

As mentioned in the present study, TesPDL3 cells not only form mineralized nodules in response to BMP-2 but also construct tube-like structures in response to FGF-2 *in vitro*. Intriguingly, FGF-2 inhibits the BMP-2-induced formation of mineralized nodules. In contrast, BMP-2 inhibits the FGF-2-induced formation of tube-like structures. This is the first report to identify the multipotent abilities of PDL fibroblast-like cells to differentiate not only into osteoblastic but also into vascular cell lineages, which were reciprocally controlled by BMP-2 and FGF-2. The present findings regarding the differential control of FGF and BMP on EC-like and osteoblast-like differentiation of PDL-derived fibroblast-like cells can be a useful aid to understanding of how damaged or lost

periodontal tissue recruits the ECs and osteoblasts.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1. The TesPDL4 cells were cultured on cell culture plates coated with type I collagen for 3 days and fluorescently labeled with antibodies for von Willebrand factor (vWF; Ca,Da; red), calponin (Cb; green) and osteopontin (Db; green). The nuclei were counterstained with DAPI (Cc,Dc; blue). The images were merged (Cd,Dd). Signals were detected using a confocal laser scanning microscope (Magnification: C ×400 and D ×100).

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