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Enhanced adipogenic differentiation and reduced collagen synthesis induced by human periodontal ligament stem cells might underlie the negative effect of recombinant human bone morphogenetic protein-2 on periodontal regeneration

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Background and Objective: Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a potent inducer for the regeneration of mineralized tissue, but has a limited effect on the regeneration of cementum and periodontal ligament (PDL). The aim of the present study was to determine the effects of rhBMP-2 on the *in vitro* and *in vivo* biologic activity of well-characterized human PDL stem cells (hPDLSCs) and to elucidate the underlying mechanism of minimal periodontal regeneration by rhBMP-2.

Material and Methods: hPDLSCs were isolated and cultured, and then transplanted into an ectopic subcutaneous mouse model using a carrier treated either with or without rhBMP-2. Comprehensive histologic, histometric and immunohistochemical analyses were performed after an 8-wk healing period. The effects of rhBMP-2 on the adipogenic and osteogenic/cementogenic differentiation of hPDLSCs were also evaluated. The effect of rhBMP-2 on both soluble and insoluble collagen synthesis was analyzed, and the expression of mRNA and protein for collagen types I, II, III and V was assessed.

Results: In the present study, rhBMP-2 promoted both adipogenic and osteogenic/cementogenic differentiation of hPDLSCs *in vitro*, and the *in vivo* potential of hPDLSCs to form mineralized cementum and organized PDL tissue was down-regulated following treatment with rhBMP-2. Collagen synthesis, which plays a crucial role in the regeneration of cementum and the periodontal attachment, was

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significantly reduced, with associated modification of the relevant mRNA and protein expression profiles.

Conclusion: In summary, the findings of the present study suggest that enhanced adipogenic differentiation and inhibition of collagen synthesis by hPDLSCs appear to be partly responsible for the minimal effect of rhBMP-2 on cementum and PDL tissue regeneration by hPDLSCs.

Recombinant human bone morphogenetic protein-2 (rhBMP-2) has been investigated as a possible therapeutic growth factor to promote the regeneration of mineralized tissue. Indeed, several studies have found that rhBMP-2 induces the osteoblastic differentiation of many types of cells *in vitro*, including cells from periodontal ligament (PDL) tissue (1–3), and provokes favorable regeneration of mineralized tissue *in vivo* in various bone-defect models (4–7). Although rhBMP-2 enhances significant new bone formation in bone defects, it was found that the application of rhBMP-2 into a periodontal defect induced some complications, such as root resorption and ankylosis, and conferred no beneficial effect for new cementum formation (8,9), which is essential for the functional attachment of the tooth to the alveolar bone. In addition, rhBMP-2-induced PDL tissue was shown to have a reduced number of collagen fibers and did not have the usual well-organized structure exhibited by normal PDL tissue, limiting its application in periodontal regeneration therapy (8,9). It was suggested that the biologic activities of the cells that are responsible for periodontal regeneration were somehow negatively modified by treatment with rhBMP-2. However, the precise mechanism underlying this effect remains to be established.

The first evidence for the existence of multipotent human PDL stem cells (hPDLSCs), which play a critical role in periodontal regeneration in PDL tissue, was reported in 2004 (10). The hPDLSCs isolated from PDL tissue have been shown to share the characteristics of other postnatal human mesenchymal stem cells (MSCs), including the formation of colony-forming units, characterization of cell-surface markers, and osteogenic, adipogenic and chondrogenic differentiation under specific conditions (10). When expanded *ex vivo* and transplanted with a biomaterial as a cell carrier into an ectopic site in immunodeficient mice, hPDLSCs can induce the formation of new cementum and well-organized PDL tissue with Sharpey's-fiber embedment. Therefore, hPDLSC technology might enhance the periodontal and bone regeneration induced by conventional periodontal therapy (11–13), and it may even be successfully applied to create a bio-tooth mimicking a natural tooth (14,15).

As the technology dealing with hPDLSCs and the current knowledge underlying their mechanism of action in periodontal regeneration has reached an advanced level, the observation of changes in the behavior of hPDLSCs following treatment with rhBMP-2 may help us to elucidate the possible cellular mechanisms underlying minimally beneficial effects of rhBMP-2 on periodontal regeneration. The aim of the present study was to determine the effects of rhBMP-2 on the *in vitro* and *in vivo* biologic activities of hPDLSCs.

Material and methods

hPDLSC culture

hPDLSCs were isolated and cultured according to a slight modification of previously reported protocols (10). Human premolars ($n = 10$), extracted for orthodontic reasons from seven systemically healthy adults (18–22 years of age) at the Department of Periodontology, Dental College, Yonsei University, were used under guidelines approved by the Yonsei Institutional Review Board. PDL tissues were separated from the root surface with the aid of a scalpel and were minced into the smallest size possible. The minced PDL tissues were digested five times at 30-min intervals in α-minimum essential medium (α-MEM; Gibco BRL, Grand Island, NY, USA) containing 100 U/mL of collagenase type I (Wako, Tokyo, Japan) and 2.5 U/mL of dispase (Gibco BRL) at 37°C. Single-cell suspensions were obtained by passing the mixture through a strainer with a pore size of 70 μm (Falcon; BD Labware, Franklin Lakes, NJ, USA), and 5×10^5 cells were seeded onto T75 cell-culture dishes containing α-MEM supplemented with 15% fetal bovine serum (FBS; Gibco BRL), 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St Louis, MO, USA), 2 mM L-glutamine (Gibco BRL), 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco BRL), and incubated at 37°C in an atmosphere containing 5% CO_2. Single-cell colonies were observed after 3–7 d, and hPDLSCs of passage P0 were cultured. Cells of passages P3–P5 were used for the study.

Characterization of isolated hPDLSC as MSCs

The basic characteristics of MSCs were demonstrated, showing the number of colony-forming units, osteogenic/adipogenic differentiation and cell-surface marker characterization (Fig. 1). First of all, single-colony formation was evaluated under the light microscope 14 d after seeding.

Then osteogenic and adipogenic differentiation procedures were applied, as follows: 1×10^5 cells were seeded into 35-mm culture dishes and cultured until they became subconfluent. The culture medium used for osteogenic differentiation comprised α-MEM containing 15% FBS (Gibco BRL),

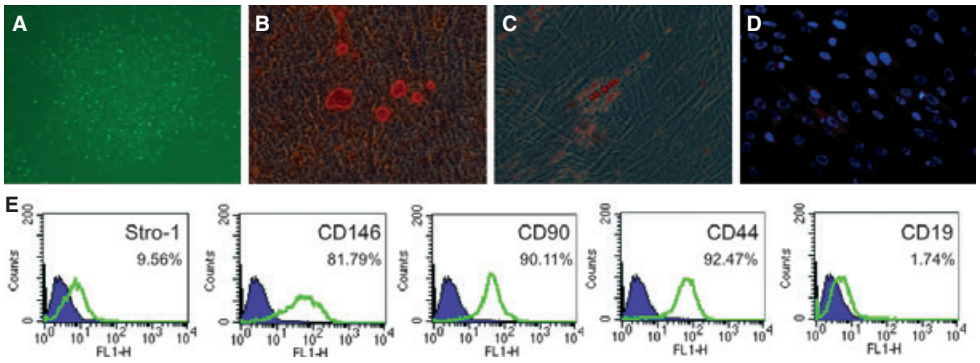

Fig. 1. Characterization of isolated putative stem cells from human periodontal ligaments. (A) Single cells formed colonies 14 d after seeding and were observed under a light microscope (original magnification ×40). (B, C) Mineralized nodules and adipocytes were formed after osteogenic induction (4 wk) and adipogenic induction (2 wk). (D) Immunocytochemical staining confirmed the expression of STRO-1 surface markers. (E) Surface marker characterization demonstrated that isolated putative stem cells were positive for STRO-1, CD146, CD90 and CD44 and negative for CD19.

2 mM L-glutamine, 100 μM L-ascorbic acid 2-phosphate, 1.8 mM KH_2PO_4, 10 nM dexamethasone (Sigma-Aldrich), 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco BRL). The culture medium for adipogenic differentiation comprised α-MEM containing 15% FBS, 2 mM L-glutamine, 100 μM L-ascorbic acid 2-phosphate, 0.5 mM isobutyl-methylxanthine, 60 μM indomethacin (Sigma-Aldrich), 0.5 μM hydrocortisone, 10 μM insulin (Sigma-Aldrich), 100 U/mL of penicillin and 100 μg/mL of streptomycin. After 2 wk (adipogenic differentiation) and 4 wk (osteogenic differentiation) of induction, the cells were stained with Oil Red O and Alizarin red, respectively (Fig. 1B and 1C).

For immunocytochemical staining, cells (of passage P3) were subcultured into two-chamber slides. The cells were fixed in 4% paraformaldehyde for 30–45 min and then blocked with blocking solution (Human Mesenchymal Stem Cell Characterization kit, cat. no. SCR067; Millipore, Billerica, MA, USA). They were then incubated with primary antibodies (anti-STRO-1; Human Mesenchymal Stem Cell Characterization kit) overnight at 4°C. After washing with phosphate-buffered saline (PBS), the cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody for 45 min at room temperature, then counterstained with the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI, 1 : 100 dilution; Vector Laboratories, Burlingame, CA, USA). Images were analyzed using a fluorescence microscope (Olympus BX41; Olympus Optical, Tokyo, Japan).

Cell-surface-marker characterization was performed using flow cytometry analysis, the procedure for which is described elsewhere (16). hPDLSCs in T75 flasks were treated with trypsin–EDTA. After the cells were harvested and transferred to a 1.7-ml tube (Oxygen, Union City, CA, USA), they were fixed by adding 4% paraformaldehyde for 15 min. The cells were then incubated for 1 h with 3% bovine serum albumin and another 1 h with primary antibodies raised against STRO-1, CD146, CD90, CD44 or CD19 (Human Mesenchymal Stem Cell Characterization kit), washed with wash buffer, and then the secondary antibody (conjugated to fluorescein isothiocyanate) was added for 45 min at room temperature. The cells were then washed three times and analysed using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Osteogenic and adipogenic differentiation following treatment with rhBMP-2

The effects of different concentrations of rhBMP-2 on osteogenic and adipogenic differentiation were assessed (Fig. 2). The cells (1×10^5) were seeded into 35-mm culture dishes and then cultured until they reached a subconfluent stage. The cells were then treated with 0, 10, 50, 100, 500 or 1000 ng/mL of rhBMP-2 for induction periods of 2 and 4 wk; during this time the osteogenic and adipogenic culture media were refreshed at intervals of 2- to 3 d in order to maintain the concentration of rhBMP-2.

After 2 wk (adipogenic differentiation) and 4 wk (osteogenic differentiation) of induction, the cells were stained with Oil Red O and Alizarin red, respectively, and evaluated by measuring the areas of adipocyte formation and newly formed nodules. Computer-assisted measurements using eight images from each sample, followed by mean measurements, were made using an automated image-analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA).

hPDLSC transplantation using a carrier treated with or without rhBMP-2 into an ectopic subcutaneous transplantation model

Specially designed disc-shaped (diameter 8 mm, height 3 mm) macroporous biphasic calcium phosphate (MBCP; Biomatlante Sarl, Vigneux de Bretagne, France) bone-graft material composed of hydroxyapatite and tricalcium phosphate was used as a carrier for the hPDLSCs. The rhBMP-2

expressed in *Escherichia coli* was produced at the Research Institute of Cowellmedi (Pusan, Korea) (7,17). The carrier was either pretreated or not pretreated with rhBMP-2 (100 μL of 0.05 mg/mL per carrier) at 4°C overnight before cell seeding. hPDLSCs (6×10^6 cells per carrier) were precultured for 1.5 h on a carrier before transplantation at 37°C in a 5% CO_2 atmosphere. The carriers with hPDLSCs were transplanted into a subcutaneous ectopic transplantation immunodeficient mouse model (10). Animal selection, management and surgical procedures followed a protocol that was approved by the Animal Care and Use Committee, Yonsei Medical Center (Seoul, Korea). The animals were treated under the following four experimental conditions: (i) MBCP carrier only (control, rhBMP-2$^-$/hPDLSC$^-$ group); (ii) MBCP carrier pretreated with rhBMP-2 (rhBMP-2$^+$/hPDLSC$^-$ group); (iii) hPDLSCs seeded onto an otherwise untreated MBCP carrier (rhBMP-2$^-$/hPDLSC$^+$ group); and (iv) hPDLSCs seeded onto an rhBMP-2-pretreated MBCP carrier (rhBMP-2$^+$/hPDLSC$^+$ group). Each group included five nude mice with four ectopic transplantations in each. The animals were allowed to heal for 8 wk and then they were killed and tissue analysis was performed (Fig. 3). For histometric analysis of new bone, cementum and adipose tissue formation, computer-assisted histometric measurements were made using an automated image-analysis system (Image-Pro Plus; Media Cybernetics), as described previously (5,18).

Histological and immunohistochemical analyses

Block sections were fixed in 4% formalin for 3 d. The specimens were decalcified with 5% EDTA (pH 8.0), dehydrated in ethanol and embedded in paraffin. The central sections were reduced to a thickness of 5 μm and then stained with hematoxylin and eosin. The histological analyses were performed using light and polarized-light microscopy (Olympus BX50; Olympus Optical). The formation and organization of the mineralized and related tissues were observed following Picrosirius staining.

For immunohistochemical analysis, deparaffinized sections were immersed in 0.3% hydrogen peroxide to block the endogenous peroxidase activity, and then incubated with primary antibodies diluted in PBS (final dilutions 1 : 200–1 : 500). Four types of primary antibody were used: mouse monoclonal antibody against osteocalcin (Millipore, Bedford, MA, USA), peroxisome proliferator-activated receptor gamma 2 (PPARγ2; Abcam, Cambridge, UK), human-specific mitochondria (hMito; Abcam) and proliferating-cell nuclear antigen (PCNA; Abcam). A commercially available kit was used for detection (Zymed SuperPicTure polymer detection kit; Zymed, Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. As negative antibody controls, some sections were treated in the same way but without incubation with the primary antibodies. Sections were subsequently counterstained with hematoxylin and then analyzed under a light microscope. The number of cells positive for immunohistochemical staining was counted using an automated image-analysis system (Image-Pro Plus; Media Cybernetics), and the results are illustrated in Fig. 4C.

Collagen synthesis analysis

Total soluble collagen in the cell supernatants was measured using the Sircol collagen assay kit (Biocolor, Carrickfergus, UK), according to the manufacturer's instructions. The cells were seeded into six-well multiwell plates (NUNC, Roskilde, Denmark) at a density of 5×10^4 cells per well and cultured until they reached a subconfluent stage. They were then treated with 0, 10, 50, 100, 500 or 1000 ng/mL of rhBMP-2. After 5 d of treatment, cell supernatants were collected by centrifugation at 260 g for 5 min. A 200-μL aliquot of the supernatant was added to 1 mL of Sircol dye and incubated for 30 min at room temperature, followed by further centrifugation at 9, 300 g for 10 min to collect the collagen–dye complex at the bottom of the tube. The resultant pellets were dissolved in 1 mL of Sircol alkali reagent and the relative absorbance was measured at 540 nm.

Collagen in the extracellular matrix (ECM), produced by hPDLSCs on the culture dishes, was evaluated by staining with Picrosirius red dye (Direct Red 80; Sigma-Aldrich). The cells were seeded into 48-well culture dishes at 3×10^4 cells/well, cultured until they reached a subconfluent stage and then treated with 0, 10, 50, 100, 500 or 1000 ng/mL of rhBMP-2 for induction. After 1 wk, cells were removed from the ECM by incubation with 2 mL of PBS containing 0.5% Triton X-100 (Sigma-Aldrich) and 20 mM NH_4OH for 5 min at 37°C. The remaining ECM was washed with PBS. Picrosirius red solution, containing 0.5 g of Direct Red 80 in 500 mL of 5% acidified water, was added to each well and incubated for 1 h at room temperature. The ECM was then carefully rinsed three times with PBS and examined by optical microscopy. Computer-assisted measurements using eight images from each sample, followed by mean measurements, were made using an automated image-analysis system (Image-Pro Plus; Media Cybernetics).

Total RNA extraction and RT-PCR

RT-PCR was conducted using samples from collagen assay experiments. Total RNA was isolated from *in vitro* culture specimens using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA). Specimens were lysed and homogenized with 1 mL of TRIzol reagent added to 200 μL of chloroform, and centrifuged at 13, 400 g for 15 min. The resulting RNA pellet was washed, dried and then dissolved in RNase-free water. RT was performed with 1 μg of RNA using a commercially available kit (iNtRon Biotechnology, Daejon, Korea). Synthesized cDNA was amplified by PCR using the primers listed in Table 1. The primers were designed according to the corresponding human genes. PCR products were separated by electrophoresis on agarose gels, visualized by ethidium bromide staining and analyzed using a gel-documentation system (Gel Doc XR; Bio-Rad, Hercules, CA, USA).

Table 1. Primer sequences and conditions for the RT-PCR

Gene	Primer sequence		Annealing temperature (°C)	GenBank no.	Product size (bp)
	Forward (5′–3′)	Reverse (3′–5′)			
Collagen type I	CCT GTC TGC TTC CTG TTA AC	AGA GAT GAA TGC AAA GGA AA	48	NM_000088	177
Collagen type II	GAA CAT CAC CTA CCA CTG CAA G	GCA GAG TCCTAG AGT GAC TGA G	50	NM_001844	500
Collagen type III	CTG CCA TCC TGA ACT CAA GAG TGG	CCA TCC TCC AGA ACT GTG TAG G	54	NM_000090	447
Collagen type V	GGA TGA GGA GGT GTT TGA	GCC CCT TGA CTG GTT TCA	60	NM_000093	387
PPARγ2	CAG TGG GGA TGC TCA TAA	CTT TTG GCA TAC TCT GTG AT	52	NM_015869	400
osteocalcin	ATG AGA GCC CTC ACA CTC CTC	CGG GCC GTA GAA GCG CCG ATA	58	X53698	297
β-actin	GGC GGA CTA TGA CTT AGT TG	AAA CAA CAA TGT GCA ATC AA	53	NM_007393	238

PPARγ2, peroxisome proliferator-activated receptor γ 2.

Western blotting assay

The cells from the collagen synthesis assay were used for a western blotting assay. Five days after initial treatment with rhBMP-2, expression of types I, II, III and V collagen were confirmed by western blotting, as reported, with slight modifications (19). Confluent monolayers of cells on 100-mm dishes were lysed in protein extraction reagent (M-PER, Mammalian Protein Extraction Reagent; Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail tablet (Complete EDTA-free; Roche, Roche Diagnostics Gmbh Mannheim, Germany) and were collected with a cell scraper. After lysis, the cells were incubated on ice for 1 h, centrifuged (270 *g*, 4°C, 15 min) and the supernatant was collected and stored at −20°C until used for protein-quantification purposes. After quantification of the amount of protein in each sample using the BCA® protein assay kit (Thermo™ Scientific, Rockford, IL, USA), cell lysates were solubilized with NuPAGE® LDS sample buffer (Invitrogen Corp.) at 70°C for 10 min, separated by SDS-PAGE (10%) and transferred to poly(vinylidene difluoride) membrane (Immobilon-P®; Millipore) using the Mini-PROTEIN® Tetra cell system (Bio-Rad Laboratories, Inc.). The blots were blocked with 0.5% (w/v) nonfat dried milk (Skim milk; BD Biosciences) and TBST [20 mM Tris–HCl, 137 mM NaCl, 0.1% (v/v) Tween 20] at 4°C overnight, followed by incubation for 2 h at room temperature with rabbit polycolonal antibodies [collagen type I (Abcam) at a dilution of 1 : 5000, collagen type III (Abcam) at a dilution of 1 : 1000, collagen type V (Abcam) at a dilution of 1 : 2000 and β-actin (Cell Signaling Technology, MA, USA)] and mouse polycolonal antibodies [collagen type II (AbD; Serotec, Oxford, UK) at a dilution of 1 : 2000]. The blots were incubated for 2 h at room temperature with anti-rabbit IgG conjugated to horseradish peroxidase (1 : 2000 dilution; Cell Signaling Technology®) and then treated with the Amersham ECL™ Western blotting detection reagent (GE Healthcare), as instructed by the manufacturer. The detected blot was exposed to X-ray film. The molecular weights of the proteins were estimated by comparison with the position of the standard (SeeBlue® Plus2 Pre-stained Standard; Invitrogen Corp.).

Statistical analysis

Each *in vitro* experiment was performed at least three times. All data are presented as mean and standard deviation. Statistical differences were determined by analysis of variance and the *post-hoc* *t*-test for multiple comparisons, and by the unpaired *t*-test for comparisons between two independent groups. The level of statistical significance was set at $p < 0.05$.

Results

Characterization of isolated cells as MSCs

Single-cell suspensions were isolated from PDL tissue to identify putative stem cells. The capacity to form adherent clonogenic cell clusters of fibroblast-like cells was demonstrated (Fig. 1A), and the cells were successfully differentiated into osteoblastic and adipogenic lineages *in vitro* (Fig. 1B and 1C). The isolated cells expressed the cell-surface molecule STRO-1, as observed by immunohistochemical staining, and the cells were consistently positive for STRO-1, CD146, CD90 and CD44 and were negative for CD19 (present on B lymphocytes). According to Dominici *et al.* (20) these are the basic characteristics of MSCs.

rhBMP-2 enhanced both the osteogenic/cementogenic and adipogenic differentiation of hPDLSCs: an *in vitro* study

We investigated the effect of rhBMP-2 on the osteogenic/cementogenic and adipogenic differentiation of hPDLSCs, because these represent the basic characteristics of MSCs. Previous findings have been controversial (21–24), but the osteogenic/cementogenic differentiation of hPDLSCs was found to be enhanced by rhBMP-2 in a dose-

dependent manner (Fig. 2). Interestingly, the adipogenic differentiation of hPDLSCs, as detected by the Oil Red O stain, was significantly up-regulated in a dose-dependent manner following treatment with rhBMP-2. The findings of the present study demonstrate that rhBMP-2 promotes both the adipogenic and osteogenic/cementogenic differentiation of hPDLSCs *in vitro*; it is this enhancement of the adipogenic differentiation that is suspected to underlie the rhBMP-2-induced modification of hPDLSC biologic activity.

rhBMP-2 down-regulated cementum and PDL tissue formation of hPDLSCs: a histologic analysis of an *in vivo* study

We were curious about the regenerative potential of hPDLSCs following treatment with rhBMP-2 and studied it in an ectopic transplantation model. We transplanted hPDLSCs, treated with or without rhBMP-2, using MBCP particles as carriers, into a subcutaneous pocket in immunodeficient mice. Clinical healing was generally uneventful, with material exposure and complications at the surgical site not being observed. Quantitative and qualitative results are shown in Fig. 3. In the control (rhBMP-2^-/hPDLSC$^-$) group, formation of ectopic mineralized tissue was rarely observed and no adverse reaction was noted.

The rhBMP-2^+/hPDLSC$^-$ group exhibited new ectopic bone formation, as previously reported (7,25). The new bone was in direct contact with the carrier, and differed markedly from the new cementum formed by the rhBMP-2^-/hPDLSC$^+$ group, as revealed using light and polarized-light microscopy. The newly formed bone was laminated and had woven bone featuring cemental lines; it was typically associated with a significant amount of newly formed adipose tissue. The area of newly formed bone and adipocytes in the rhBMP-2^+/hPDLSC$^-$ group was the highest among all four groups.

The successful regeneration of new cementum and PDL tissues was observed in the rhBMP-2^-/hPDLSC$^+$ group. The newly formed cementum comprised fibrous mineralized tissue that was in direct contact with the carrier and exhibited different depths of stain color with earlier and more recently deposited cementum. New cementum was formed along the surface of the pores of the carrier and there was evidence of relatively high cementogenic activity in the form of a cementoblast-like cell lining. Sharpey's fibers were observed running into the PDL tissue from the cementum. Dense and regularly organized collagen fibers with a high degree of cellular invasion were also associated in the newly formed cementum, as revealed by Picrosirius stain analysis. There was no evidence of adipose tissue formation in the specimens. Under polarized-light microscopy, the newly formed cementum exhibited characteristics of highly mineralized tissue.

The rhBMP-2^+/hPDLSC$^+$ group displayed combined findings of the rhBMP-2^-/hPDLSC$^+$ and rhBMP-2^+/hPDLSC$^-$ groups. The regenerated tissue was composed of new cementum, related PDL tissue, new bone and adipose tissue. Newly formed bone was observed in the peripheral area of the carrier, while new cementum and PDL tissue was formed in the macropores. However, the amount of newly formed cementum and related PDL tissue was significantly reduced compared with the rhBMP-2^-/hPDLSC$^+$ group, a

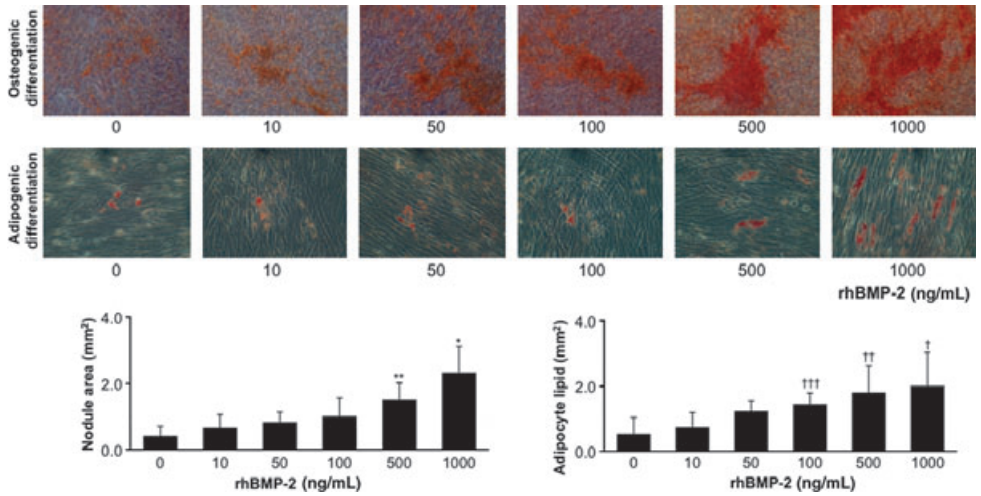

Fig. 2. In vitro osteogenic and adipogenic differentiation of human periodontal ligament stem cells (hPDLSCs) treated with recombinant human bone morphogenetic protein-2 (rhBMP-2). rhBMP-2 was applied at different doses to the hPDLSC culture, and the formation of mineralized nodules and adipocytes were observed by staining with Alizarin red and Oil Red O, respectively. The areas of both mineralized nodules and adipocytes gradually increased in a dose-dependent manner. *Statistically significant difference from 0, 10, 50 and 100 ng/mL of rhBMP-2. **Statistically significant difference from 0 and 10 ng/mL of rhBMP-2. †Statistically significant difference from 0 and 10 ng/mL of rhBMP-2. ††Statistically significant difference from 0 and 10 ng/mL of rhBMP-2. †††Statistically significant difference from 0 and 10 ng/mL of rhBMP-2 ($p < 0.05$: analysis of variance and the *post-hoc t*-test).

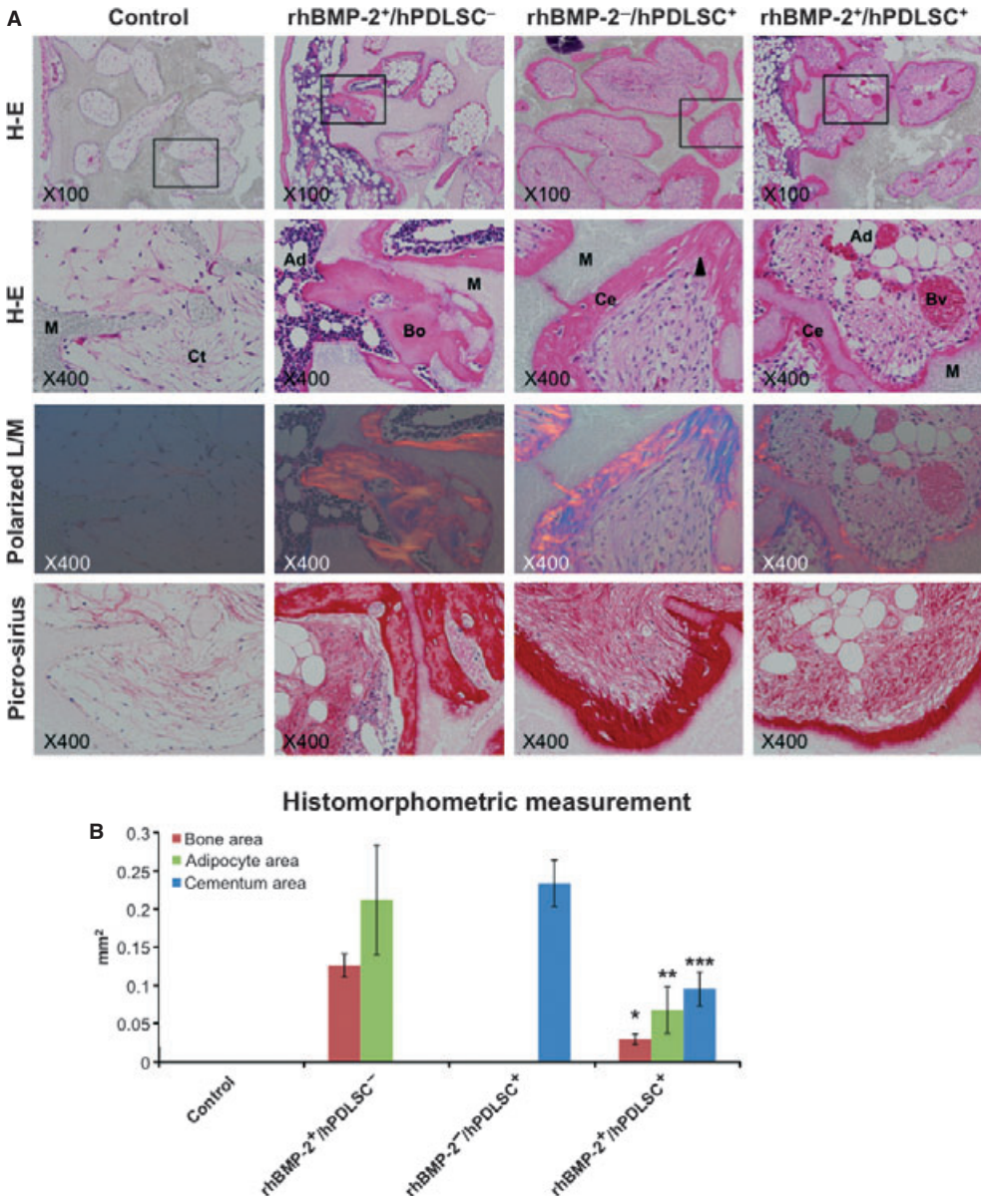

Fig. 3. Effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on human periodontal ligament stem cells (hPDLSC)-induced cementum and periodontal ligament (PDL) tissue regeneration. (A) hPDLSCs were transplanted using a macroporous biphasic calcium phosphate (MBCP) carrier, treated with or without rhBMP-2, into the subcutaneous pocket of immunodeficient mice, and histologic analysis was performed after 8 wk of healing. Inset boxes show areas at higher magnification. (arrowhead, Sharpey's fibers; M, material; Ct, connective tissue; Ad, adipocytes; Bo, bone; Ce, cementum;Bv, blood vessel.) (B) Histomorphometric measurements show new bone and adipocyte formation in the rhBMP-2^{+}/hPDLSC^{-} group. The rhBMP-2^{-}/hPDLSC^{+} group showed the largest amount of cementum and PDL tissue formation without the formation of bone or adipocytes. The rhBMP-2^{+}/hPDLSC^{+} group showed new bone, cementum and PDL fiber formation, but the amount was significantly reduced ($p < 0.05$). *Significant difference relative to the bone area in the rhBMP-2^{+}/hPDLSC^{-} group ($p < 0.05$). **Significant difference relative to the adipocyte area in the rhBMP-2^{+}/hPDLSC^{-} group ($p < 0.05$). ***Significant difference relative to the cementum area in the rhBMP-2^{-}/hPDLSC^{+} group ($p < 0.05$).

finding that has been reported consistently after the application of rhBMP-2 in periodontal regeneration experimental models (8,9). Adipose tissue formation was associated with the newly formed bone, but a particularly interesting finding was that adipose tissue formation was also found inside the PDL tissues. In that case, the amount of new cementum and PDL tissue formation would be significantly reduced, leading to a reduced cellular density. The Sharpey's fibers and PDL tissue also appeared to be much looser and more poorly organized than in the

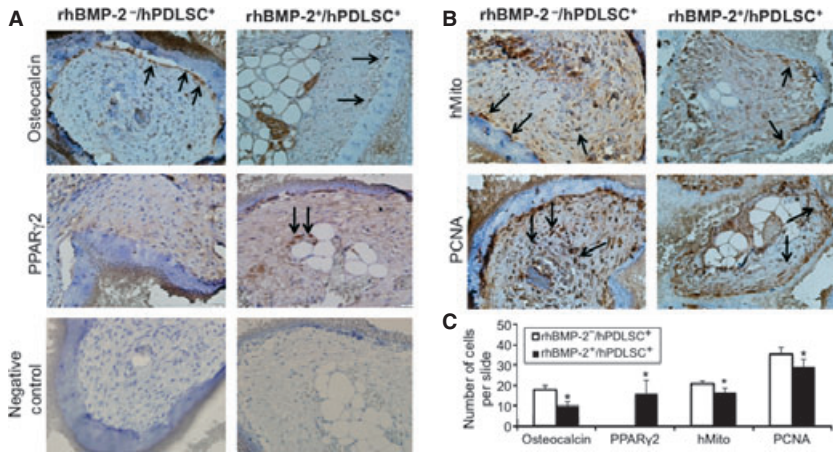

Fig. 4. Immunohistochemical staining showing reciprocal osteogenic/adipogenic regulation and decreased cell proliferation. Arrows indicate each specific marker. (A) Osteocalcin expression appeared down-regulated, while peroxisome proliferator-activated receptor gamma 2 (PPARγ2) expression was up-regulated following treatment of human periodontal ligament stem cells (hPDLSC) with recombinant human bone morphogenetic protein-2 (rhBMP-2). Sections incubated without the primary antibodies were the negative controls. (B) Staining intensities of human-specific mitochondria (hMito) and proliferating-cell nuclear antigen (PCNA) were also decreased in the rhBMP-2^+/hPDLSC^+ group, indicating that cellular proliferation of hPDLSC was inhibited by treatment with rhBMP-2. (C) Results of positively stained cell counting. *Statistically significant difference relative to the cell count in the rhBMP-2^-/hPDLSC^+ group ($p < 0.05$).

rhBMP-2^-/hPDLSC^+ group, as shown by Picrosirius stain analysis. Overall, polarized-light microscopy revealed that specimens exhibited a pattern of healing regeneration and consolidation that lacked maturity.

Immunohistochemical staining demonstrated increased adipogenic/decreased osteogenic/cementogenic differentiation and decreased proliferation of hPDLSCs following rhBMP-2 treatment

The results of the *in vivo* ectopic transplantation model were evaluated briefly using immunohistochemical staining in terms of cell proliferation and differentiation (Fig. 4). First, the reciprocal regulation between osteogenic/cementogenic and adipogenic differentiation of hPDLSCs following rhBMP-2 treatment was observed using immunohistochemical staining for osteocalcin and PPARγ2. Osteocalcin reportedly plays an important role in the mineralization of newly formed mineralized tissue (10,26), and in the present study its staining intensity was markedly reduced, whereas the expression of PPARγ2 – which is known to be expressed by a master regulatory gene for adipogenesis (27) – was enhanced in the rhBMP-2^+/hPDLSC^+ group. PPARγ2 was strongly expressed inside the PDL material containing adipose tissue, and even where adipose tissue formation was not observed, which indicates that adipogenic differentiation is an ongoing process, even in the late healing periods. Second, we also found that the staining intensity of hPDLSCs detected by staining for hMito and PCNA was significantly lower in the rhBMP-2^+/hPDLSC^+ group than in the non-rhBMP-2-treated (rhBMP-2^-/hPDLSC^+) group. This result is consistent with the aforementioned results evaluated by hematoxylin and eosin staining, showing decreased cellular density in the rhBMP-2^+/hPDLSC^+ group, and with previous reports indicating that rhBMP-2 might inhibit the proliferation of hPDLSCs (21,28).

rhBMP-2 reduced collagen synthesis by hPDLSCs and was associated with down-regulation of the mRNA expression of collagen types II and V

As the potential for PDL tissue formation and organization by hPDLSCs was markedly reduced in the present *in vivo* study, we evaluated the effect of rhBMP-2 on the synthesis of collagen by hPDLSCs *in vitro* (Fig. 5). It is well known that collagen plays a pivotal role in the formation and maturation of the PDL tissue and cementum (29). The results showed that the synthesis of soluble collagen (as measured by the Sircol collagen assay) and collagen synthesis in the ECM (as detected by Picrosirius staining) were reduced in a dose-dependent manner following treatment with rhBMP-2. Interestingly, the expression of mRNA for collagen types II and V was significantly down-regulated following treatment with rhBMP-2, while the effect of rhBMP-2 on the expression of mRNA for type I collagen was minimal and the expression of mRNA for type III collagen was enhanced in a dose-dependent manner by treatment with rhBMP-2 (Fig. 5C). This pattern of expression was corroborated by the results of the western blotting analysis showing similar changes in protein (Fig. 5D). These results suggest that the reduced potential of hPDLSCs for collagen synthesis is related to the reduced formation and maturation of PDL tissue and cementum. In addition, the reduced synthesis of collagen by hPDLSCs following treatment with rhBMP-2 seems to be somehow related to the reduced expression of the minor components of collagen types II and V rather than to the major components of collagen types I and III.

Discussion

Since publication of the finding that rhBMP-2 can induce significant alveolar bone regeneration, there have been numerous attempts to apply this biomodulator as a candidate for periodontal regeneration. However, even the most recent data can only tell us that the effects exerted by rhBMP-2 on alveolar bone regeneration are promising, but there is a critical limitation regarding its effects on cementum and PDL regeneration. In addition, the mechanisms underlying its effects have yet to be fully elucidated. We therefore studied its effects in further detail *in vitro* and *in vivo* by applying

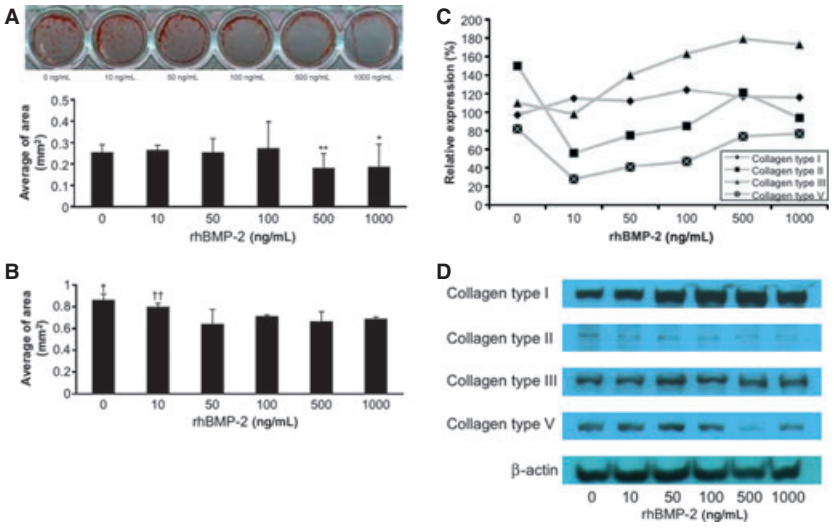

Fig. 5. Effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the synthesis of collagen by human periodontal ligament stem cells (hPDLSC) *in vitro*. (A) Collagen synthesis in the extracellular matrix decreased with increasing doses of rhBMP-2. (B) The amount of soluble collagen in the supernatant decreased in a dose-dependent manner. (C) Representative mRNA expression for collagen types I, II, III and V. mRNA expression for collagen types I and III was essentially unchanged. However, collagen types II and V were significantly down-regulated following treatment with rhBMP-2. (D) Western blotting analysis showed the similar changes of protein level in relation to mRNA expression. *Statistically significant difference from 0, 10, 50 and 100 ng/mL of rhBMP-2. **Statistically significant difference from 0, 10, 50 and 100 ng/mL of rhBMP-2. †Statistically significant difference from 0, 10, 50, 100, 500 and 1000 ng/mL of rhBMP-2. ††Statistically significant difference from 50, 100, 500 and 1000 ng/mL of rhBMP-2 ($p < 0.05$: analysis of variance and *post-hoc t*-test).

rhBMP-2 to hPDLSCs, which are regarded to be a source of periodontal regeneration.

With regard to the cellular mechanism, the findings of the present study suggest that it is the enhanced adipogenic differentiation and changes of collagen synthesis in hPDLSCs that underlies this negative effect of rhBMP-2 on periodontal regeneration. Enhanced *in vivo* adipogenic tissue formation was found in the tissue regenerated by hPDLSCs following treatment with rhBMP-2; similarly, rhBMP-2 enhanced the adipogenic differentiation of hPDLSCs *in vitro*. Although rhBMP-2 increased both the osteogenic/cementogenic and adipogenic differentiation potential of hPDLSCs *in vitro*, the *in vivo* results indicate that the osteogenic/cementogenic and adipogenic potential of these cells have a reciprocal relationship, as the treatment of hPDLSCs with rhBMP-2 induced a substantial amount of adipogenic tissue formation with a concomitant significant reduction in cementum regeneration. The most logical explanation would be that rhBMP-2 enhances both osteogenic/cementogenic and adipogenic potential *in vitro*, but it somehow interfered with collagen synthesis *in vivo*, which is essential for promoting osteoblastic differentiation (30,31) leading to the down-regulation of cementogenesis *in vivo*. Also, increased adipogenesis might have concomitantly down-regulated osteogenesis/cementogenesis in a reciprocal way (32). Nevertheless, further research is needed to explain the discrepancy of osteogenic/cementogenic activity between these *in vitro* and *in vivo* results.

It was previously confirmed that several osteogenic bone-morphogenetic proteins exert strong adipogenic effects on MSCs (27,33,34) and provoke enhanced adipogenic tissue formation at later healing stages when they are transplanted into rat models, despite the maturity and advanced amount of new bone formation (7). However, there is little, if any, information about how rhBMP-2 regulates the osteogenic/cementogenic and adipogenic differentiation of hPDLSCs. The present results provide the first evidence that the adipogenic differentiation of hPDLSCs can be enhanced by rhBMP-2, both *in vitro* and *in vivo*, in association with the reduced amount of cementum and PDL formation.

Other histologic modifications of hPDLSC activity exerted by rhBMP-2 were the poorly organized collagenous ECM in the PDL tissue, the reduction in the formation of Sharpey's fibers and collagen fiber density, and the increase in adipose tissue formation inside PDL. Also, collagen fibers between the rhBMP-2-induced newly formed bone and adjacent new cementum from the hPDLSCs were formed in a parallel orientation without perpendicular insertions into the new bone and cementum (data not shown). Several reports showed that rhBMP-2 has an inhibitory effect on collagen synthesis. For instance, exposure of cementoblasts and tendon fibroblasts to rhBMP-2 was reported to inhibit expression of the collagen type I gene and did not increase collagen synthesis (22,24,35). Moreover, newly formed rhBMP-2-induced collagenous PDL tissue did not exhibit the usual functionally oriented structure, and ankylosis associated with root resorption was frequently observed (9,36,37). The present *in vitro* experiments also showed that the reduction of collagen synthesis was associated with rhBMP-2-induced modification of the related mRNA expression profile.

It is well known that PDL tissues comprise mainly an abundance of collagens, with approximately 80% of the total collagen in PDL tissue being collagen types I and III (1). The PDL tissue also expresses collagen types II (38) and V (39,40) as minor components. Recent histological analysis using a nonhuman primate model demonstrated that the organization of the collagenous ECM and nucleation of the collagenic materials are critical early healing events before the induction of cementum and functionally oriented PDL tissue formation (29). It was suggested that MSCs migrate towards the

root surface, 'riding' along the functionally oriented collagen fibers, and then induce new cementum formation after repopulation along the exposed root surface. Based on the general consensus that collagen within the ECM plays an essential role in cellular migration (41) and osteoblastic differentiation (30,31), collagenous ECM formation by the hPDLSCs appears to play a pivotal role in periodontal regeneration. In this study, the mRNA expression of collagen types II and V was significantly reduced following treatment with rhBMP-2, whereas changes in expression of the major components of collagen types I and III were minimal, which was confirmed by the western blotting analysis showing similar changes in protein level. Taken together with the findings of previous studies, the present results suggest that the inhibition of collagen synthesis by rhBMP-2 is responsible for the hPDLSC-induced reduction in periodontal tissue regeneration, and that the inhibitory effect of rhBMP-2 on collagen synthesis is related to the down-regulation of the minor, rather than the major, components of collagen.

In summary, the findings of the present study suggest that enhanced adipogenic differentiation and inhibition of collagen synthesis by hPDLSCs are responsible for the minimal effect of rhBMP-2 on cementum and PDL tissue regeneration by hPDLSCs. Future research should include investigation of the precise regulatory mechanism underlying the effects of rhBMP-2 on hPDLSCs. The understanding of this mechanism may enable the maximization of periodontal regeneration by rhBMP-2 and allow the development of novel therapeutic strategies for periodontal regeneration.

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