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Enhanced Bone-Forming Activity of Side Population Cells in the Periodontal Ligament

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Regeneration of alveolar bone is critical for the successful treatment of periodontal diseases. The periodontal ligament (PDL) has been widely investigated as a source of cells for the regeneration of periodontal tissues. In the present study where we attempted to develop an effective strategy for alveolar bone regeneration, we examined the osteogenic potential of side population (SP) cells, a stem cell-containing population that has been shown to be highly abundant in several kinds of tissues, in PDL cells. Isolated SP cells from the rat PDL exhibited a superior ability to differentiate into osteoblastic cells compared with non-SP (NSP) and unsorted PDL cells in vitro. The mRNA expressions of osteoblast markers and bone morphogenetic protein (BMP) 2 were significantly upregulated in SP cells and were further increased by osteogenic induction. To examine the bone-forming activity of SP cells in vivo, PDL SP cells isolated from green fluorescent protein (GFP)-transgenic rats were transplanted with hydroxyapatite (HA) disks into wild-type animals. SP cells exhibited a high ability to induce the mineralized matrix compared with NSP and unsorted PDL cells. At 12 weeks after the implantation, some of the pores in the HA disks with SP cells were filled with mineralized matrices, which were positive for bone matrix proteins, such as osteopontin, bone sialoprotein, and osteocalcin. Furthermore, osteoblast- and osteocyte-like cells on and in the bone-like mineralized matrices were GFP positive, suggesting that the matrices were directly formed by the transplanted cells. These results suggest that PDL SP cells possess enhanced osteogenic potential and could be a potential source for cell-based regenerative therapy for alveolar bone.

Keywords: Side population (SP) cells; Periodontal ligament (PDL) cells; Osteogenesis; Bone regeneration

INTRODUCTION

A sufficient amount of regeneration of alveolar bone is essential for the successful treatment of periodontal diseases and dental implants. To achieve better therapeutic results, several kinds of methods using various materials and techniques have been investigated. Among these trials, cell-based therapies, especially those using mesenchymal stem cells (MSCs), have generally produced acceptable results (1,6,19,20).

Since alveolar bone is originally formed by osteoblasts that had differentiated from periodontal ligament (PDL) cells, the PDL has been considered to be a potential source for cell-based regenerative therapy. In support of this notion, previous reports, including ours, showed that PDL cells have osteogenic potential (10,26). These observations, together with the results obtained with MSCs, led us to hypothesize that the stem cell population of PDL cells may have a higher ability to regenerate alveolar bone than other cells in the PDL population.

Efficient identification and isolation of an appropriate stem cell population are critical processes to develop stem cell-based regenerative therapies. The stem cells in the PDL have been identified by the detection of cell surface antigens, such as STRO-1, cluster of differentiation 44 (CD44), CD106, and CD146 (5,17), all of which are also used as markers of bone marrow-derived MSCs. The

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PDL stem cells showed the ability to differentiate into multiple cell types, including osteoblastic, adipocytic, and chondrocytic cells in vitro (29), and repaired periodontal defects by forming cementum-like and PDL-like periodontal tissue in vivo (26).

As an alternative method for isolating PDL stem cells, in the present study, we focused on side population (SP) cells. SP cells appear as a tail of dimly stained cells when PDL cells are stained with Hoechst 33342, a DNA-binding fluorescence dye, and examined by dual-wavelength flow cytometry (7). SP cells have been found in several kinds of tissues, including bone marrow and dental pulp (7,15), and shown to be highly enriched in stem cells. Although SP cells were also identified in the PDL (18), their ability to regenerate periodontal tissues remains to be elucidated.

To explore this issue, we isolated SP cells from rat PDL cells by using a fluorescence-activated cell sorter (FACS) system and examined their osteogenic potential in vitro and in vivo.

MATERIALS AND METHODS

Reagents

Rabbit polyclonal antibody against bone sialoprotein (BSP) was purchased from Alexis Corp. (Lausen, Switzerland), and that specific for osteopontin (OPN) was described previously (22). Goat polyclonal antibody against osteocalcin (OCN) was from Biomedical Technologies, Inc. (Stoughton, MA, USA). All other materials used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise specified.

Isolation of Rat PDL Cells

Rat PDL cells were isolated from 10 green fluorescent protein (GFP)-transgenic male rats (5 weeks old, Lewis strain) (8,13) as previously described (10). These rats were generously provided by PhoenixBio Co. (Tochigi, Japan). Litters of mutant rats were produced and raised in our animal facility. Before SP cell isolation, the PDL cells were cultured for 6 days in growth medium, α-minimum essential medium (αMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Biological Industries Ltd., Haemek, Israel) and antibiotic–antimycotic (Life Technologies Japan Ltd., Tokyo, Japan), in a humidified atmosphere of 5% CO_2 in air. All animal experiments were reviewed and approved by the Animal Management Committee of Matsumoto Dental University.

Identification and Isolation of SP Cells

Harvested PDL cells suspended at 1×10^6 cells/ml in Hanks' balanced salt solution (HBSS, Life Technologies Japan Ltd.) supplemented with 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and 2% FBS were incubated with 5 μg/ml Hoechst 33342 dye (Sigma-Aldrich) for 60 min at 37°C. Some cells were incubated in the presence of 50 μM verapamil (Sigma-Aldrich), an inhibitor of the ATP-binding cassette (ABC) transporter, especially P-glycoprotein/multidrug resistance 1 (MDR-1) (28). The cells were then washed with HBSS, analyzed, and sorted by the use of a FACS Vantage SE (BD Biosciences, San Jose, CA, USA). Cells in the typical main population and those in the population that disappeared in the presence of verapamil were isolated as non-SP (NSP) and SP cells, respectively.

Cell Proliferation

SP and NSP cells (5×10^2 cells/well) were plated in 96-well plates (BD Biosciences, Tokyo, Japan) and cultured for 24 h in growth medium. Cell proliferation was determined for the subsequent 6 days by using a nontoxic colorimetric-based Alamar blue assay (Biosource, Camarillo, CA, USA) according to the manufacturer's protocol.

Osteoblastic, Adipocytic, and Chondrocytic Differentiation In Vitro

Osteoblastic differentiation was determined as described previously (23). SP, NSP, and unsorted PDL cells were cultured with or without osteogenic induction for 5 or 10 days. Alkaline phosphatase (ALPase) activity and alizarin red staining were performed as described previously (16,23). To test the effects of Hoechst 33342 on osteoblast differentiation, unsorted PDL cells pretreated with Hoechst 33342 (5 μg/ml) for 60 min at 37°C were cultured for 7 or 21 days. Adipocytic differentiation was determined as described previously (24). The cells were cultured for 21 days and then stained with Oil Red O solution. Chondrocytic differentiation was determined as described previously (30). The cells were cultured for 14 days and stained with Alcian blue.

Microarray Analysis

Total RNA was extracted by using an RNeasy Plus Micro Kit (QIAGEN, Tokyo, Japan) and reverse transcribed with oligo(dT) primer containing the T7 promoter. Cy3- or Cy5-labeled cRNA was synthesized from a cDNA pool and purified with an RNeasy Mini Kit (QIAGEN). Labeled cRNA was then fragmented and hybridized to the microarray of a Whole Rat Genome Microarray Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) at 65°C overnight. A color-swap experiment was performed for the dye-specific effect and to ensure statistically relevant data. Hybridized microarray slides were scanned with an Agilent DNA Microarray Scanner (Agilent Technologies) at 5-μm resolution. The scanned images were analyzed numerically by using Agilent Feature Extraction Software (G256AA, Agilent Technologies).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated with the RNeasy Plus Micro Kit. The cDNA was synthesized by using PrimeScript Reverse Transcriptase (Takara Bio Inc., Shiga, Japan). The sequences of primers were as follows: *mdr-1*, 5′-att tggcaaagctggagaga-3′ (forward) and 5′-accctgtagccccttttc act-3′ (reverse); *glyceraldehyde-3-phosphate dehydrogenase (gapdh)*, 5′-atgggaagctggtcatcaac-3′ (forward) and 5′-gtggttcacacccatcacaa-3′ (reverse). PCR products were separated on 2% agarose gels containing ethidium bromide and visualized under ultraviolet light. The size of the fragments was confirmed by reference to a 100-bp DNA ladder.

Real-Time RT-PCR

SP and NSP cells (1×10^5 cells/well) were plated in six-well plates (BD Biosciences, Japan) and cultured with or without osteogenic induction for 5 days or with adipogenic induction as described above for 21 days. Real-time RT-PCR was performed by using One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara Bio) and a StepOnePlus Real-time PCR system (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol. The sequences of primers were as follows: *tissue-nonspecific alkaline phosphatase (tnap)*, 5′-gcacaacatcaaggacatcg-3′ (forward) and 5′-tcagttctgttcttggggtacat-3′ (reverse); *osterix (osx)*, 5′-ctgcacccccagaaaaag-3′ (forward) and 5′-cagatct ctagcaggttgctctg-3′ (reverse); *runt-related transcription factor 2 (runx2)*, 5′-cgtacccaggcgtatttcag-3′ (forward) and 5′-tgcctggctcttcttactgag-3′ (reverse); *bsp*, 5′-tcggaa gaaaatggagatgg-3′ (forward) and 5′-ttcctcttcatttgaagtctc ctc-3′ (reverse); *ocn*, 5′-atagactccggcgctacctc-3′ (forward) and 5′-ccaggggatctgggtagg-3′ (reverse); *opn*, 5′-cggtgaaag tggctgagttt-3′ (forward) and 5′-ggctacagcatctgagtgtttg-3′ (reverse); *bone morphogenetic protein 2 (bmp2)*, 5′-cgg actgcggtctcctaa-3′ (forward) and 5′-ggggaagcagcaacact aga-3′ (reverse); *peroxisome proliferator-activated receptor γ (pparγ)*, 5′-ccctggcaaagcatttgtat-3′ (forward) and 5′-ac tggcacccttgaaaaatg-3′ (reverse); *lipoprotein lipase (lpl)*, 5′-cagagaaggggcttggaga-3′ (forward) and 5′-ttcattcagc agggagtcaa-3′ (reverse); β-*actin*, 5′-cccgcgagtacaacct tct-3′ (forward) and 5′-cgtcatccatggcgaact-3′ (reverse). Values of mRNA were determined using an appropriate standard curve and were normalized with those of the internal control, β-*actin*. All assays were performed in triplicate.

BMP2 ELISA

SP and NSP cells (2×10^4 cells/dish) were plated in 35-mm dishes (BD Biosciences, Japan) and cultured in serum-free growth medium for 24 h. The BMP2 level in the culture supernatant was measured by the use of a BMP2 Quantikine ELISA Kit (R & D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

Cell Transplantation

SP, NSP, and unsorted PDL cells (5×10^5 cells/disk) were plated on five hydroxyapatite (HA) disks (CELLYARD HA scaffold, $\phi5\times2$ mm, 50% porosity, Pentax Corp., Tokyo, Japan) and then cultured in growth medium for 24 h. After having been rinsed with PBS, the disks were transplanted bilaterally under the fascia of the dorsal muscles of 6-week-old male wild-type Lewis rats (SLC Japan Co., Ltd., Shizuoka, Japan) under anesthesia with pentobarbital (0.05 mg/g body weight, IP; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan).

Histological Analysis

Unfixed, Frozen, and Nondecalcified Sections. After the rats had been sacrificed by an overdose of diethyl ether, the implanted HA disks and their surrounding tissues were dissected en bloc, embedded in 5% carboxymethyl cellulose gel, and immediately frozen in hexane at −80°C (11). Then, sections were made with a cryomicrotome (CM3050; Leica Microsystems K.K., Tokyo, Japan) and stained with 0.05% toluidine blue (pH 7.0) or 1% alizarin red S. GFP-positive cells were observed with a fluorescence microscope. The numbers of total pores in the disks, of pores with GFP-positive cells, and of pores with mineralized matrix were counted by examining seven randomized sections from each specimen. Total areas of GFP-positive pores and a mineralized matrix with osteocyte-like cells were measured by using analytic software (TRI/2D-BON, Ratoc System Engineering Co., Ltd., Tokyo, Japan).

Decalcified Paraffin Sections. Under anesthesia with pentobarbital (0.05 mg/g body weight), the rats were perfused through their left ventricle with 4% paraformaldehyde solution. The HA disks and surrounding tissues were dissected en bloc, immersed in the same fixative overnight, decalcified in 10% EDTA solution for 8 weeks at 4°C, and embedded in paraffin. Paraffin sections were made according to the conventional methods (31).

Immunohistochemistry. Immunohistochemical staining of BSP, OPN, and OCN was performed by using a Histofine Simple Stain Kit (Nichirei Biosciences Inc., Tokyo, Japan) according to the manufacturer's protocol. Chromogen was developed by the use of a diaminobenzidine (DAB) Liquid System (Dako Japan Inc., Kyoto, Japan). The slides were counterstained with hematoxylin.

Enzyme Histochemistry. ALPase was detected as described previously (16).

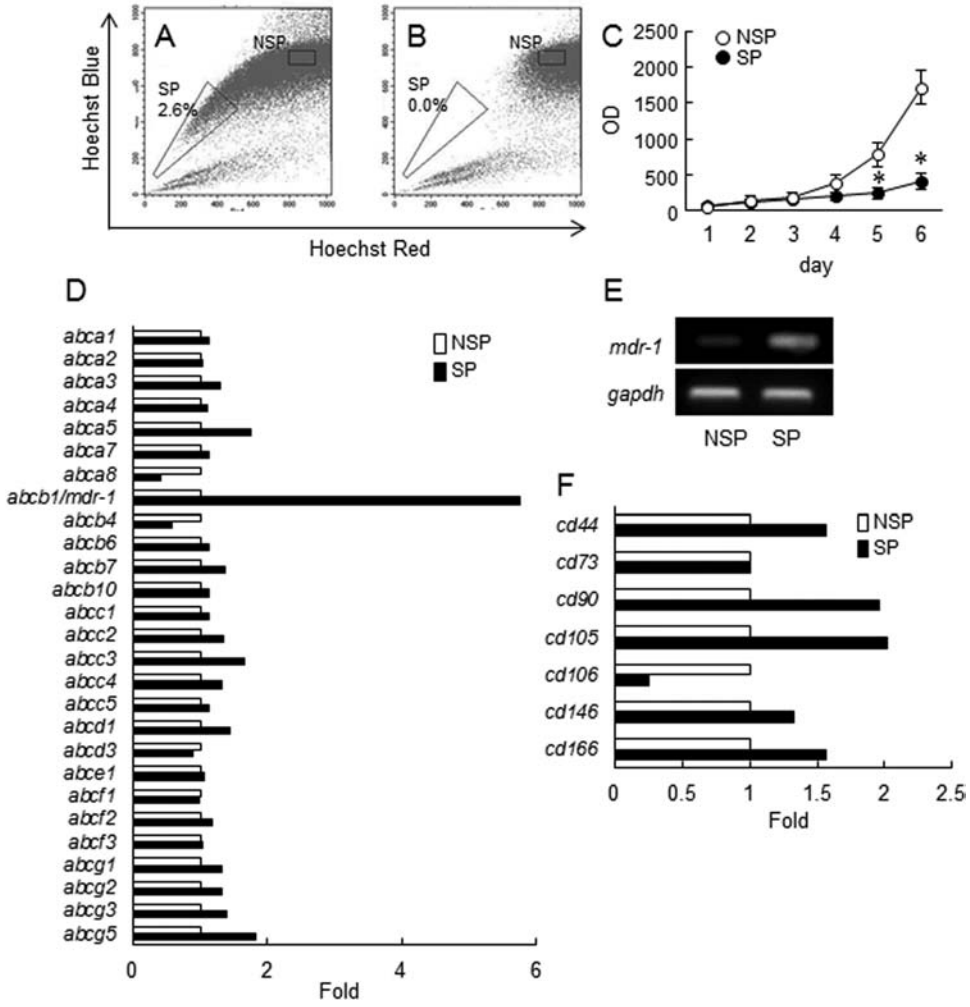
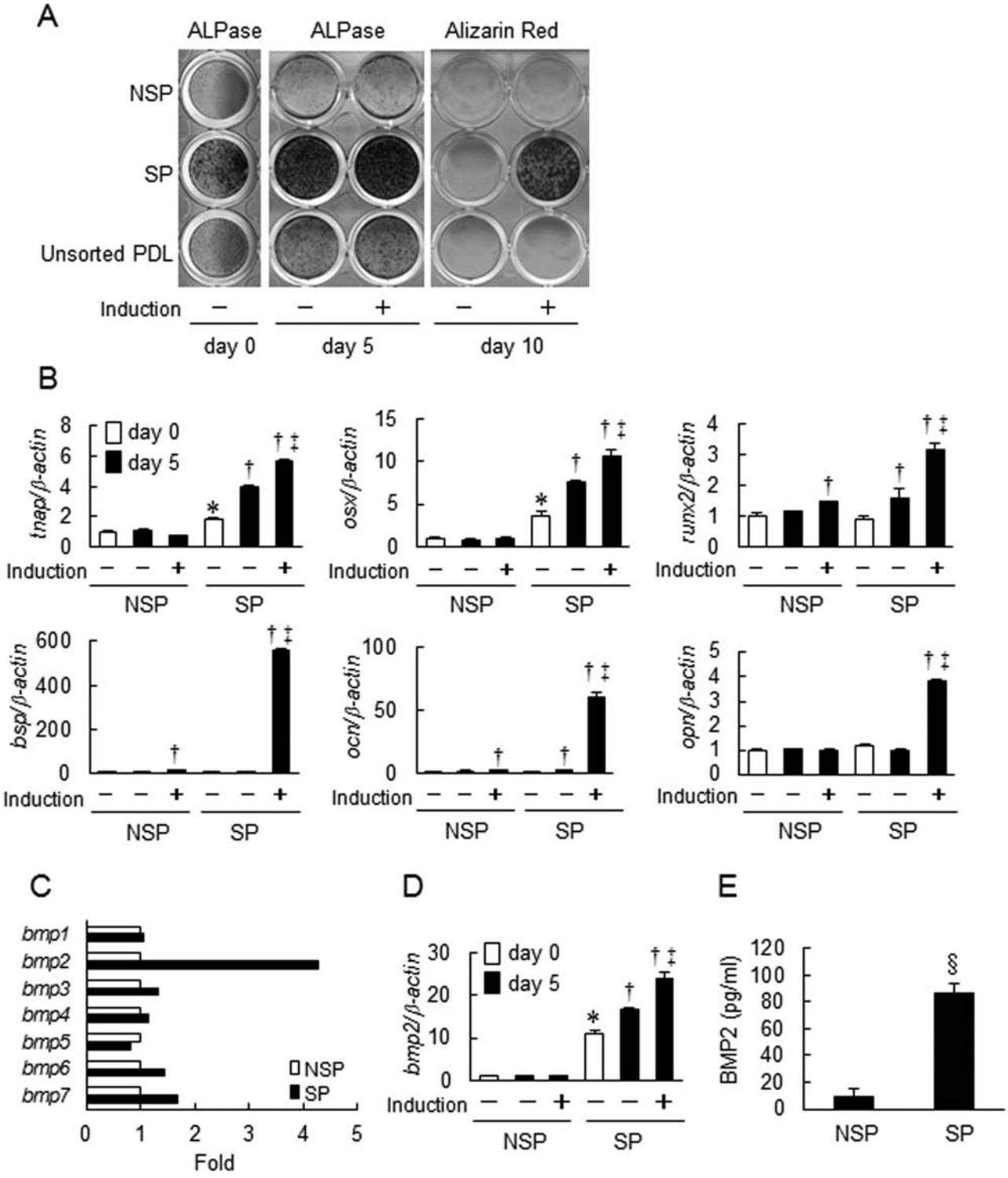


Figure 1. Identification and isolation of SP cells from rat PDL. (A, B) Representative dot plots of Hoechst 33342-stained periodontal ligament (PDL) cells in the absence (A) and presence (B) of verapamil. Side population (SP) and non-SP (NSP) cells in the gated regions were isolated and used for the experiments. Hoechst 33342 fluorescence is displayed at two emission wavelengths (red and blue) with ultraviolet (UV) excitation. (C) Cell proliferation examined by performing the Alamar blue assay ($n=3$). The data are presented as the mean ± SD. *$p<0.01$ compared with OD value for NSP cells. (D) Microarray analysis of the expression of ATP-binding cassette (ABC) transporter family members in SP and NSP cells. The data are expressed as fold increase compared with values for NSP. (E) RT-PCR analysis of multidrug resistance 1 (*mdr-1*) mRNA expression. Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) served as a control. (F) Microarray analysis of the expression of mesenchymal stem cell (MSC) markers in SP and NSP cells. The data are expressed as fold increase compared with values for NSP. Microarray analysis was performed twice. *cd44*: cluster of differentiation 44.

FACING PAGE

Figure 2. Osteoblastic differentiation of SP cells. (A) Alkaline phosphatase (ALPase) and alizarin red staining of SP, NSP, and unsorted PDL cells cultured for 5 or 10 days with or without osteogenic induction. (B) Real-time RT-PCR analysis of mRNA expression of osteoblast markers tissue-nonspecific alkaline phosphatase (*tnap*), osterix (*osx*), runt-related transcription factor 2 (*runx2*), bone sialoprotein (*bsp*), osteocalcin (*ocn*), and osteopontin (*opn*). SP and NSP cells were plated in six-well plates and cultured for 0 or 5 days with or without osteogenic induction (each $n=3$). (C) Microarray analysis of the expression of bone morphogenetic protein (BMP) family members in SP and NSP cells. The data are expressed as fold increase compared with values for NSP. Microarray analysis was performed twice. (D) Real-time RT-PCR analysis of *bmp2* mRNA expression. SP and NSP cells were plated in six-well plates and cultured for 0 or 5 days with or without osteogenic induction ($n=3$). (E) BMP2 production by SP and NSP cells determined by ELISA ($n=3$). The cells were cultured for 24 h, and the culture supernatant was then collected. In the various graphs, the data are presented as the mean ± SD. *$p<0.01$ versus NSP cells on day 0, †$p<0.01$ versus either cell population on day 0, ‡$p<0.01$ versus either cell population without osteogenic induction on day 5, §$p<0.01$ versus value for NSP cells.


A
ALPase
ALPase
Alizarin Red
NSP
SP
Unsorted PDL
Induction
day 0
day 5
day 10
B
tnap/β-actin
osx/β-actin
runx2/β-actin
bsp/β-actin
ocn/β-actin
opn/β-actin
day 0
day 5
NSP
SP
C
bmp1
bmp2
bmp3
bmp4
bmp5
bmp6
bmp7
Fold
D
bmp2/β-actin
E
BMP2 (pg/ml)

Statistical Analysis

Data were expressed as the mean and standard deviation (SD) for each group. Comparisons between SP, NSP, and unsorted PDL cell groups were performed by one-way analysis of variance (ANOVA) and the post hoc Tukey test. Values of $p < 0.05$ were considered significant.

RESULTS

Identification and Isolation of SP Cells From Rat PDL Cells

Flow cytometric analysis identified SP cells in the rat PDL cell population, in which cells comprised 2.6 ± 0.9% of the total PDL cells (Fig. 1A). The cells in this SP fraction disappeared in the presence of verapamil, an inhibitor of MDR-1 (Fig. 1B) (9). The in vitro cell proliferation of isolated SP cells, as determined by performing the Alamar blue assay, was significantly lower than that of the NSP cells (Fig. 1C). Microarray analysis showed that the mRNA expression of ABC transporters other than *mdr-1* was similar between SP and NSP cells and revealed that SP cells mainly efflux Hoechst dye by MDR-1 transporter (Fig. 1D). Semiquantitative RT-PCR analysis demonstrated that the mRNA expression of *mdr-1* in SP cells was higher than that in NSP cells (Fig. 1E). Microarray data showed that several markers of MSCs, such as *cd44*, *cd90*, *cd105*, and *cd166*, were upregulated in SP cells compared with their expression in NSP cells (Fig. 1F).

Abilities of SP Cells in Osteoblastic Differentiation and Calcification

SP cells expressed higher ALPase activity than did NSP cells at the basal level (Fig. 2A). Real-time RT-PCR analysis showed that mRNA expression of *tnap* and *osx* at day 0 was significantly upregulated in SP cells (Fig. 2B). SP cell cultures in osteogenic medium showed markedly increased ALPase activity and formation of a mineralized matrix, whereas these changes were not seen in the NSP cells (Fig. 2A). The ALPase activity of unsorted PDL cells at day 5 was slightly higher than that of NSP cells, but much lower than that of SP cells, in either growth medium or osteogenic induction medium (Fig. 2A). Mineralized matrix formation was not observed in unsorted PDL cells at day 10 (Fig. 2A). The expression of all osteoblast markers examined, that is, *tnap*, *osx*, *runx2*, *bsp*, *ocn*, and *opn*, was increased in SP cells by the osteogenic induction (Fig. 2B). Of interest, the mRNA expression of *tnap*, *osx*, and *runx2* in SP cells was elevated during culture even without the induction. Such upregulation in NSP cells was rarely observed (Fig. 2B).

Expression of BMPs in SP Cells

From microarray analysis, we found that BMP2 expression was upregulated in SP cells (Fig. 2C). BMP expression in SP and NSP cells was also confirmed by real-time RT-PCR (Fig. 2D). Steady-state secretion of BMP2 into the culture medium was also increased in the SP cells (Fig. 2E). Furthermore, mRNA expression of BMP2 in SP cells was spontaneously increased during culture and became more efficient with osteogenic induction (Fig. 2D). On the other hand, expression of BMP receptors and BMP2-induced intracellular signaling determined by the phosphorylation of small body size/mothers against decapentaplegic 1/5/8 (Smad1/5/8) was not different between SP and NSP cells (data not shown).

Multipotency of SP Cells

Since PDL cells have been shown to contain multipotent cells that have the ability to differentiate into multiple kinds of mesenchymal lineage cells, including osteoblasts and adipocytes (10), we examined whether PDL SP cells possessed these capacities. A small population of SP and NSP cells exhibited adipocytic differentiation when cultured for 21 days in the induction medium (Fig. 3A). Although the expressions of adipocyte markers, such as *pparγ* and *lpl*, in SP cells were upregulated by adipogenic induction, they were lower than those in NSP cells (Fig. 3B). Chondrocytic differentiation was not induced in either cell population (data not shown).

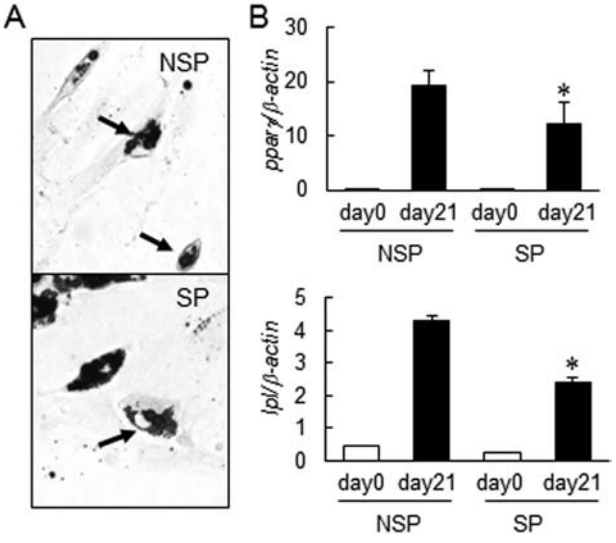


Figure 3. Adipogenic differentiation of SP cells. (A) Oil red O staining of SP and NSP cells cultured for 21 days with adipogenic induction. Arrow shows an adipocyte. (B) Real-time RT-PCR analysis of mRNA expression of adipocyte markers peroxisome proliferator-activated receptor γ (*pparγ*) and lipoprotein lipase (*lpl*). SP and NSP cells were plated in six-well plates and cultured for 0 or 21 days with adipogenic induction (each $n = 3$). The data are presented as the mean ± SD. *$p < 0.01$ versus NSP cells on day 21.

Effects of Hoechst 33342 on Proliferation and Differentiation of PDL Cells

It has been argued that the phenotypic differences between SP and NSP cells are a consequence of damage induced by Hoechst 33342, which is retained longer in NSP cells (7). To exclude this possibility, we tested the effects of the Hoechst dye on proliferation and osteoblast differentiation of unsorted PDL cells. The dye at a concentration employed for the detection of SP (5 μg/ml) did not affect the proliferation, differentiation, or mineralization of the PDL cells (Fig. 4), suggesting that the differences between SP and NSP cells were not attributable to the pharmacological effects of the Hoechst dye.

Formation of Mineralized Matrix by SP Cells In Vivo

To determine whether SP cells possessed the ability to form a bone-like matrix in vivo, we plated SP and NSP cells isolated from GFP-transgenic rats on HA disks and transplanted them into wild-type rats of the same strain (Fig. 5). At 4 weeks after the implantation, the pores in the HA disks were filled with GFP-positive PDL cells (Fig. 5A, C, D, F, G, I). Alizarin red staining showed mineralized matrix formation on the surface of the pores in the SP cell and PDL cell-seeded HA (SP-HA and PDL-HA, respectively) disks (Fig. 5B, H), the formation of which was not observed in the NSP cell-seeded HA (NSP-HA) disks (Fig. 5E). At 12 weeks, some of the pores in the SP- and PDL-HA disks were filled with an alizarin red-positive matrix (Fig. 5K, Q). The matrix formation was found in approximately 33% and 12% of the pores with SP and unsorted PDL cell infiltration, respectively, whereas no matrix formation was seen in the pores containing NSP cells (Fig. 5T). Likewise, SP cells induced a large amount of mineralized matrix compared with other cells (Fig. 5U). The ratio of cell infiltration into the pores was approximately 30% in each disk (Fig. 5S). Fluorescence microscopic observations showed that the matrix-embedded osteocyte-like cells were GFP positive (Fig. 5R). Immunohistochemical examination demonstrated that the matrices formed in the SP-HA disks were positive for bone matrix proteins, such as OPN, BSP, and OCN (Fig. 6B–D). ALPase-positive osteoblast-like cells were also seen on the formed matrices (Fig. 6E).

DISCUSSION

SP cells possess an efflux function of drug or dye via ABC transporters. The ABCG2/ breast cancer resistance protein 1 (BCRP1) transporter is especially expressed in SP cells of several tissues and is considered as a general stem cell marker (3,32). We determined the expressions of *abc* transporters in SP and NSP cells. However, there was not much difference between the *abcg2* expressions in these cells. On the other hand, the *abcb1/mdr-1* expression in SP cells was approximately sixfold higher level than that in NSP cells. In the flow cytometric analysis, the cells in the SP fraction disappeared in the presence of verapamil, an inhibitor of MDR-1. These findings revealed that verapamil is suitable for detection of SP fraction in PDL cells.

Several lines of evidence suggest that PDL cells have an osteoblast-like phenotype and osteogenic potential in vitro and in vivo (10,12). However, since PDL cells consist of heterogeneous cell populations, including mature fibroblasts and the precursor cells that can differentiate into fibroblasts, cementoblasts, and osteoblasts, which kind of cells are directly responsible for the osteoblastic phenotype of PDL cells must be determined. Establishment of methods to efficiently identify osteogenic cells is also important for potential clinical applications of PDL cells for regenerative therapy of alveolar bone. In the present study, we showed for the first time that rat PDL SP cells possessed a strong ability to differentiate into osteoblast-like cells in vitro and to form a bone-like mineralized matrix in vivo.

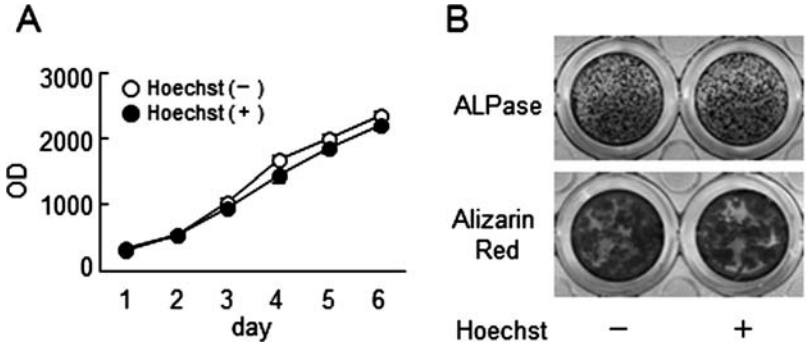
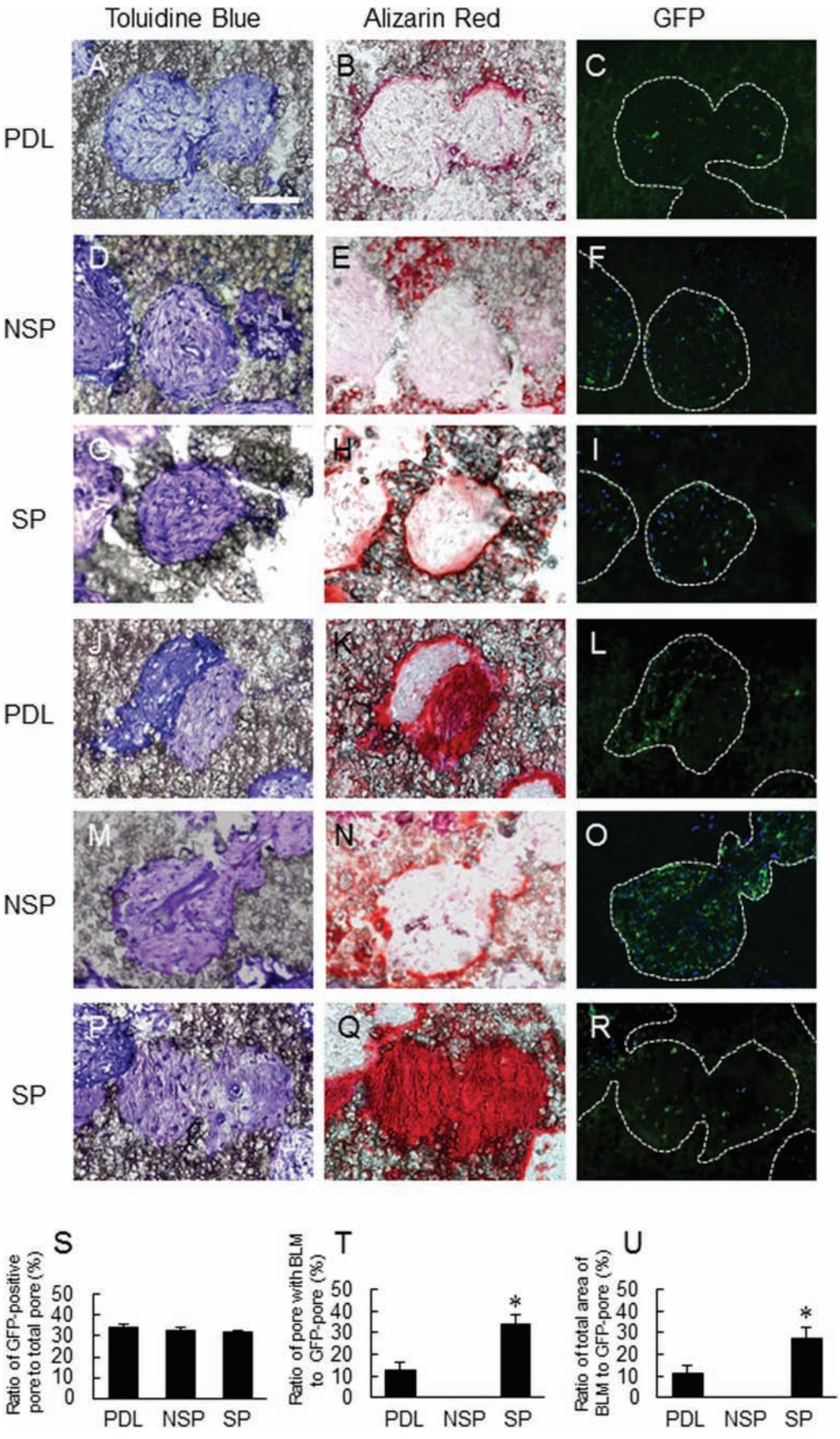


Figure 4. Effects of Hoechst 33342 on proliferation and osteoblast differentiation of PDL cells. (A) Unsorted PDL cells with or without treatment of Hoechst 33342 (5 μg/ml) were cultured in growth medium. Cell proliferation was determined by performing the Alamar blue assay. (B) Treated and nontreated PDL cells were cultured with osteogenic induction. Osteoblast differentiation was determined by ALPase and alizarin red staining at days 7 and 21, respectively (each $n = 3$).


Toluidine Blue
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Ratio of GFP-positive pore to total pore (%)
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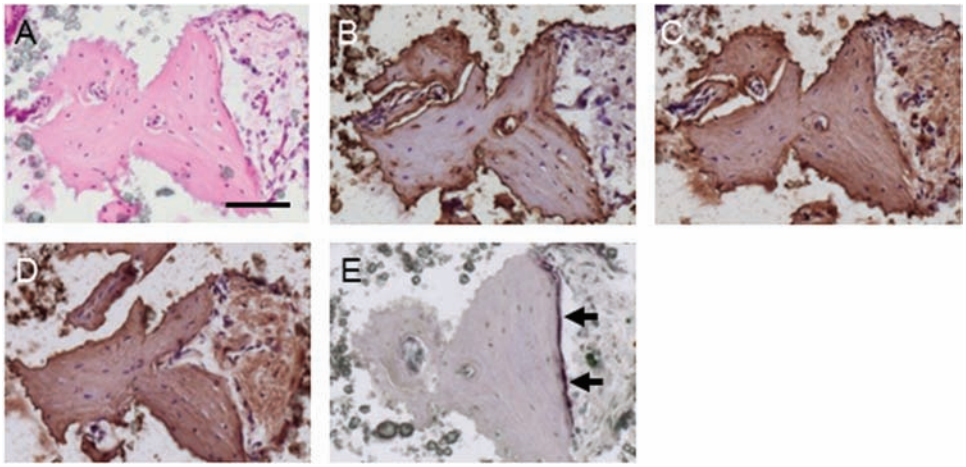

Figure 6. Histological analysis of the mineralized matrix in SP-HA disks at 12 weeks after the implantation. Decalcified paraffin-embedded sections were examined. The sections of SP-HA disks were stained with hematoxylin–eosin (A) and were examined by immunohistochemistry for OPN (B), BSP (C), and OCN (D), and by enzyme histochemistry for ALPase (E). Scale bar: 100 μm. Arrows in (E) indicate osteoblast-like cells.

It is of particular note that our in vitro and in vivo studies demonstrated that the abilities of PDL SP cells to differentiate into osteoblastic cells and to induce a bone-like mineralized matrix was superior not only to that of NSP cells but also to that of unsorted PDL cells. These findings prove our hypothesis that this predifferentiated population of PDL cells has the greater ability to regenerate bone.

Our results showed that PDL SP cells expressed higher baseline levels of *tnap* and *osx* mRNAs and ALPase activity than NSP cells and that these levels were further increased by the osteogenic induction. The other osteoblast differentiation markers, *runx2*, *bsp*, *ocn*, and *opn*, were also markedly upregulated in SP cells by the induction. Of interest, SP cells showed a spontaneous increase in the mRNA expression of these genes during culture. These results suggest the possibility that the population of SP cells contains a substantial subpopulation of cells that are committed to the osteoblast lineage.

Microarray analysis revealed that steady-state *bmp2* expression was elevated in SP cells, a finding that was confirmed by real-time RT-PCR and ELISA. However, the expressions of BMP receptors and the intracellular signals in response to BMP2 were not different between SP and NSP cells. Therefore, in addition to the differences in intrinsic properties, the autocrine actions of this increased amount of BMP2 may also account for the spontaneous differentiation of PDL SP cells into osteoblastic cells in vitro. It is also possible that these effects beneficially affect bone formation in vivo.

Since their original discovery (7), SP cells have been identified in a variety of tissues and recognized as a stem cell-enriched population (4). Consistent with this notion, microarray analysis showed the higher expression of MSC markers (2,17), including *cd44*, *cd90*, *cd105*, and *cd166*, in PDL SP cells. However, although PDL SP cells exhibited multipotency to differentiate into osteoblastic and adipocytic cells, the ability to differentiate into adipocytes was lower than that of the NSP cells. These data suggest that PDL SP cells possess some of the MSC-like phenotypes but do not completely maintain the MSC-like undifferentiated state. The results also suggest that PDL SP cells are not identical to the previously described PDL stem cells (17,26,29).

Immunohistochemical analysis demonstrated that the mineralized matrix formed in the SP-HA disks was positive for bone matrix proteins, that is, OPN, BSP, and OCN. These results indicate that the matrix was bone-like in nature. In addition, fluorescence microscopic observations

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Figure 5. Histological analysis of the mineralized matrix formation. Histological analysis of the mineralized matrix formation in unsorted PDL- (A–C, J–L), NSP- (D–F, M–O), and SP-hydroxyapatite (HA) disks (G–I, P–R) at 4 (A–I) and 12 (J–R) weeks after the implantation using undecalcified frozen sections. Dotted lines in the green fluorescent protein (GFP) images (C, F, I, L, O, R) indicate the border between pore and HA disks (scale bar: 100 μm). Ratio of GFP-positive pores to total pores (S), of pores with bone-like matrix (BLM) to GFP-positive pores (T), and of total BLM area to GFP-pores area (U) in unsorted PDL-, NSP-, and SP-HA disks are shown ($n = 5$). The data are presented as the mean ± SD. *$p < 0.01$ compared to unsorted PDL and NSP.

revealed the presence of GFP-positive cells on and in the bone-like matrix, suggesting that PDL SP cells directly contributed to the matrix formation.

While mesenchymal stem cells in PDL can differentiate into cementoblasts and osteoblasts, it raises a possibility that the mineralized matrix formed by SP cells may be cementum. Cementum contains cementocytes in their lacunae as with osteocytes in bone lacunae. Since the matrix proteins in cementum and bone tissue are very similar, the specific marker to distinguish them has not been discovered yet. Thus, it is extremely difficult to solve this possibility.

It has been reported that dental pulp-derived SP cells form a mineralized matrix in vivo (14). However, this matrix contains dentin sialophosphoprotein (14), suggesting that the matrix is a dentin-like but not a bone-like one. Thus, dental pulp-derived SP cells are unlikely to be a suitable source of cells for bone regeneration. SP cells isolated from adipocyte populations have also been shown to possess osteogenic potential in vitro and in vivo (25,27). Comparison between SP cells originating from different tissues needs to be performed in future studies.

It has been argued that the phenotypic differences between SP and NSP cells might be caused by Hoechst dye-induced damage because it is retained longer in NSP cells. In our study, the dye at the concentration (5 μg/ml) used for FACS affected neither the proliferation nor differentiation of the PDL cells. These results suggest that the differences between SP and NSP cells were not attributable to the toxic effects of Hoechst 33342, although it is difficult to completely exclude this possibility.

We found that the ABC transporter MDR-1 was specifically upregulated in SP cells, suggesting the possibility that MDR-1 can be used as a marker for SP cells. Since it may be possible that Hoechst 33342 exerts cytotoxic effects, as mentioned above, MDR-1 could perhaps be a better alternative marker to identify SP-like cells.

It has been concerned that the number of the stem cells in PDL is limited, and it may not be enough for clinical application (18,21,26). Our data that PDL SP cells isolated after expansion of cell number by culture for 6 days, comprised 2.6% total PDL cells and the proliferation of sorted SP cells lower than that of NSP cells, suggest that the preparation of PDL SP cells is difficult for clinical use. However, a ratio of PDL SP cells also did not alter in cultured PDL cells for 2 weeks (data not shown). This finding indicates that SP cells in a proper environment grow with the same proliferative rate to NSP cells. Therefore, the coculture system with any cells in PDL as feeder cells might expand PDL SP cells. Identification of feeder cells to PDL SP cells and osteogenic potentials of PDL SP cells in long-term culture should be determined in the future.

In conclusion, our results suggest that PDL SP cells possessed high osteogenic potential. These cells could thus be a potential candidate for a source for cell-based regenerative therapy of alveolar bone.

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