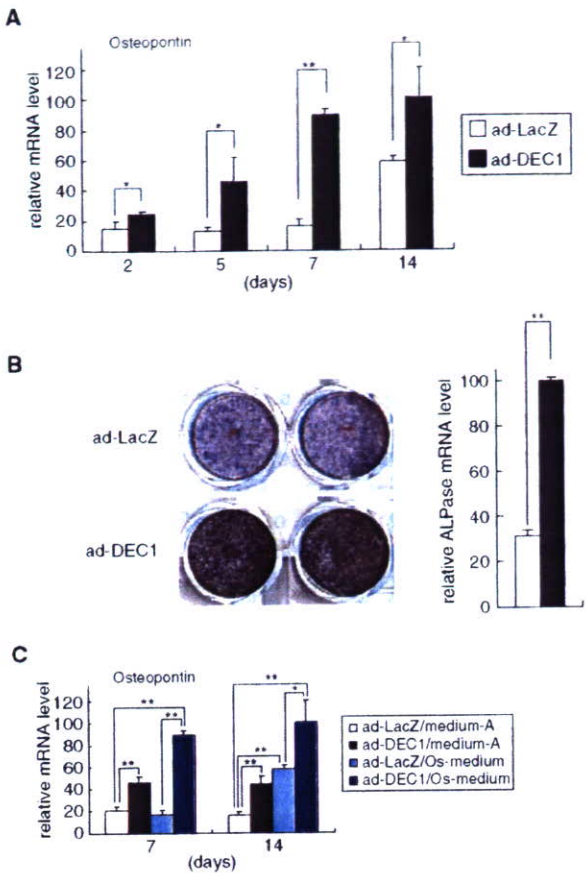



**Fig. 3.** Effect of Dec1 overexpression on osteopontin and ALPase expression in MSC in Os-medium. Six hours after MSC were infected with ad-Dec1 or control ad-LacZ at an MOI of 50, the culture medium was replaced with Os-medium. (A) Expression levels of osteopontin mRNA (mean ± SEM) on the indicated days were examined by real-time quantitative RT-PCR analysis. (B) ALPase activity on day 21 was examined by staining the cells with ALPase staining kit. Expression levels of ALPase mRNA (mean ± SEM) were also determined. (C) Comparison of osteopontin mRNA levels in Dec1-overexpressing MSC, MSC induced by Os-medium, and Dec1-overexpressing MSC induced by Os-medium. $^{**}P<0.01$, $^{*}P<0.05$ (Student's *t*-test).

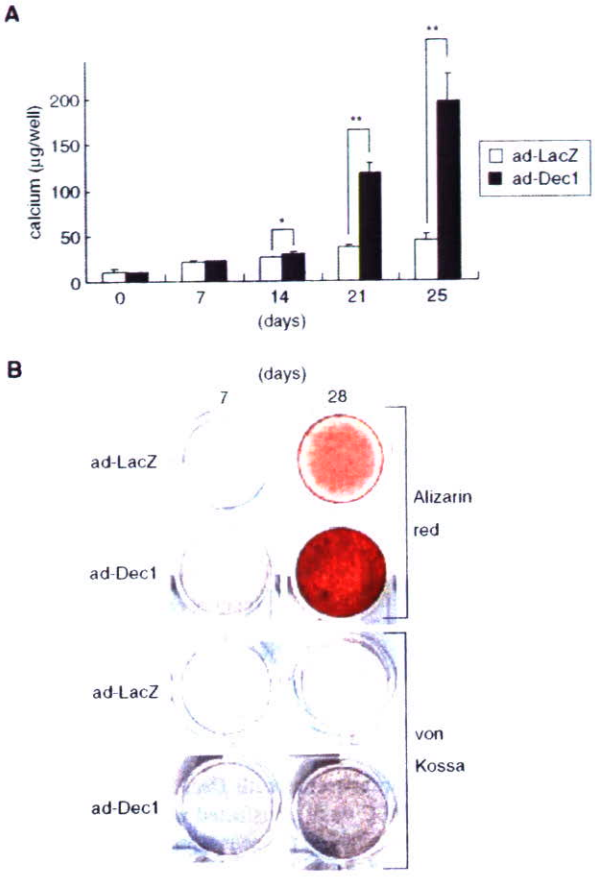



**Fig. 4.** Effect of Dec1 overexpression on matrix calcification in MSC cultures in Os-medium. (A) MSC infected with ad-Dec1 or ad-LacZ were cultured in Os-medium for the indicated days, calcium content (mean ± SEM) in the cell layers was determined. (B) Matrix calcification in MSC cultures was shown by Alizarin red or von Kossa staining on days 7 and 28. $^{**}P<0.01$, $^{*}P<0.05$ (Student's *t*-test).

times as high as that in control cultures expressing LacZ (Fig. 4A). The accelerated calcification was confirmed by Alizarin red or von Kossa staining (Fig. 4B). Moreover, PTH receptor expression induced by Os-medium was attenuated by *Dec1* siRNA on days 5 and 7 (Fig. 5), showing the involvement of Dec1 in the osteogenic differentiation process of MSC.

## Suppression of adipogenic differentiation of MSC by Dec1 overexpression

To explore the role of Dec1 in adipogenesis, expression levels of *Dec1* during adipogenic differentiation of MSC were examined: After adipogenic induction, mRNA levels for *Dec1* markedly decreased on days 3 and 7 (Fig. 6A), but not on day 14, which suggested that the expression of Dec1 at high levels may suppress adipogenic differentiation. To test this hypothesis, we looked at whether Dec1 overexpression would inhibit adipogenic differentiation of MSC: Infection with ad-Dec1 transiently lowered mRNA levels of PPARγ until day 7 (Fig. 6B), and the Dec1 overexpression also suppressed lipid accumulation on days 3 and 7, but not on day 14 (Fig. 6C), although Dec1 mRNA levels remained high on day 14 (Fig. 6D). These results indicate that Dec1 lowers the rate of adipogenic differentiation only in the early stage.

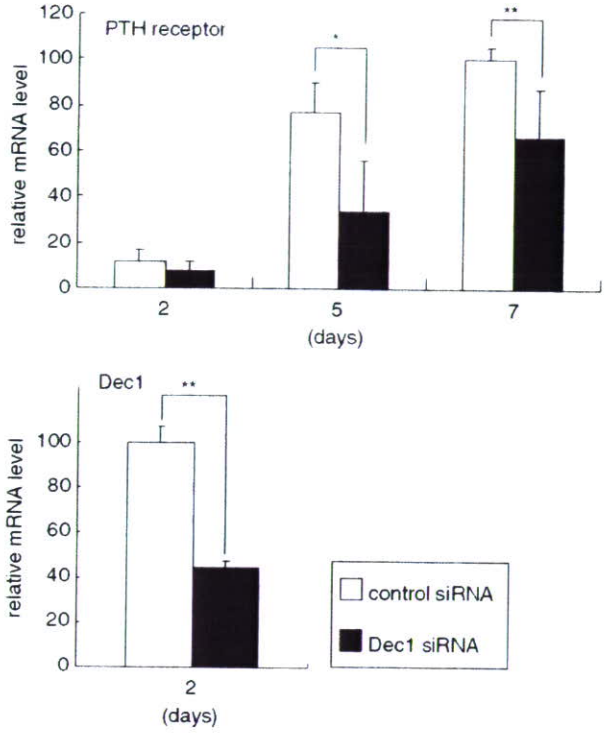



**Fig. 5.** Effect of RNA interference with *Dec1* in MSC cultures in Os-medium. After MSC were transfected with *Dec1* siRNA or non-silencing control siRNA, the cells were cultured in Os-medium for the indicated days. The values represent mRNA levels (mean±SEM) for *Dec1* and PTH receptor relative to GAPDH mRNA levels. $^{**}P<0.01$, $^{*}P<0.05$ (Student's *t*-test).

## Discussion

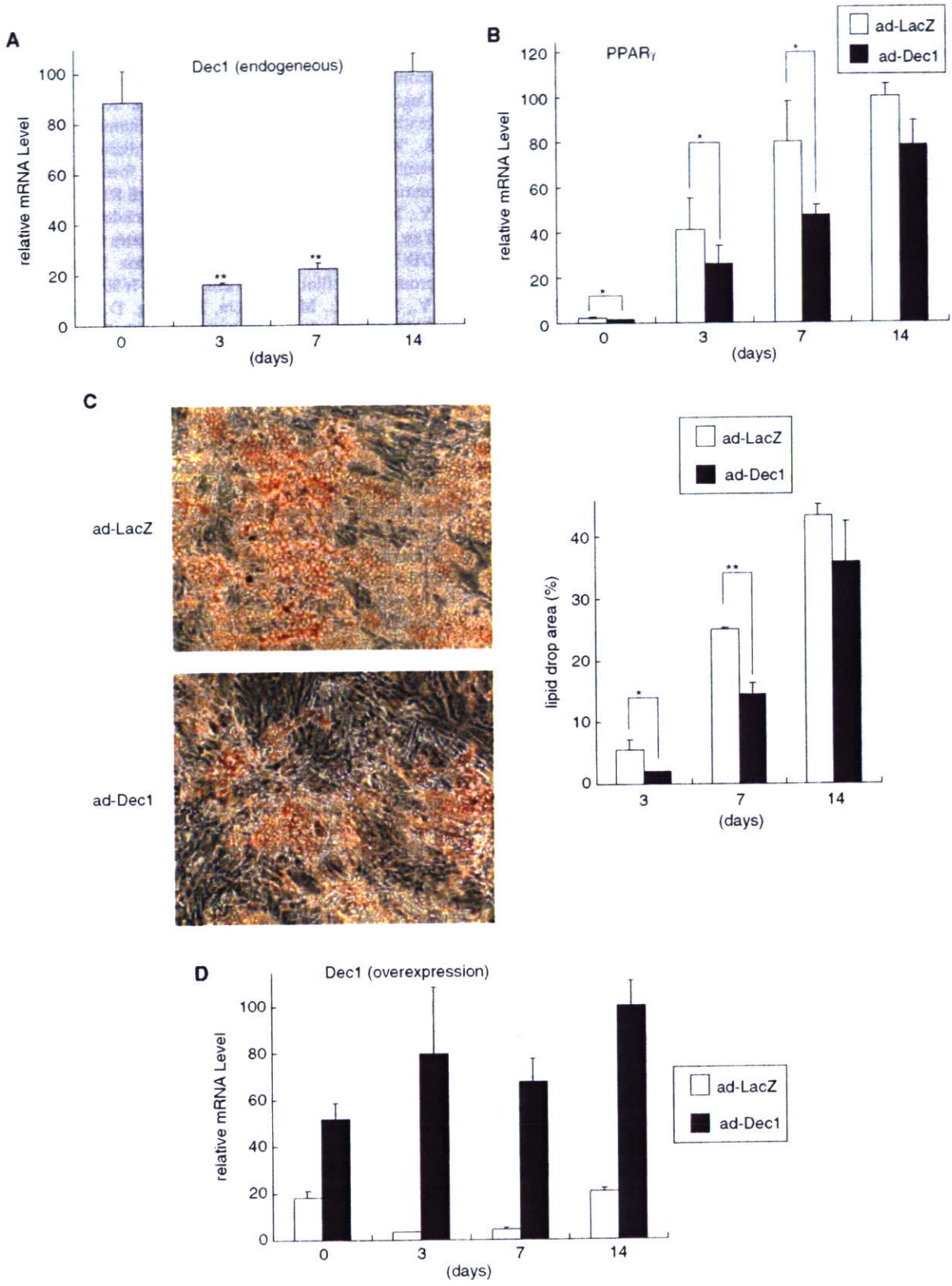
Osteogenic differentiation of MSC is inducible in Os-medium containing dexamethasone, β-glycerophosphate, and ascorbic acid (Matsubara et al., 2004; Pittenger et al., 1999; Tsutsumi et al., 2001): ALPase activity and calcium levels increase a week after osteogenic induction, and the differentiation continues to progress at least until day 21. In the present study, we demonstrated that expression of *Dec1* – as well as bone-related genes – was up-regulated in the induced MSC. Furthermore, forced expression of Dec1 in uninduced MSC up-regulated the expression of some bone-related proteins – such as osteopontin, PTH receptor, and ALPase – and accelerated the osteogenic differentiation and calcification in MSC cultures under osteogenic conditions. Decrease in the *Dec1* mRNA level by about 60% in the presence of siRNA resulted in the suppression of PTH receptor, but it did not decrease the level of osteopontin or ALPase under these culture conditions (data not shown). Furthermore, Dec1 overexpression alone did not induce calcification in MSC cultures. Taken together, these observations suggest that Dec1 is not essential for osteogenesis, but is involved in some aspects of the osteogenic differentiation process.

Since Dec1 overexpression had little effect on expression of Runx2 in MSC, the Dec1-induced osteogenesis could not be attributed to the induction of Runx2. In fact, in vivo studies using Runx2-transgenic mice showed that overexpression of Runx2 in osteoblasts inhibited their maturation (Liu et al., 2001), while Runx2 is essential for osteogenic differentiation in the early stage. These findings suggest that Runx2 stimulates or inhibits osteogenic differentiation of MSC stage-dependently. Some other transcriptional regulators must therefore be involved in the differentiation process of MSC: Dec1 could be one of these transcription factors, although Dec1 alone cannot induce the whole osteogenic differentiation program.

A previous study reported that Dec1 functions as an effector for hypoxia-mediated inhibition of adipogenesis via PPARγ suppression: Stable expression of Dec1 resulted in nearly complete inhibition of adipocyte differentiation of a mouse adipogenic cell line – 3T3-L1 cells (Yun et al., 2002). In the present study, we found that expression levels of *Dec1* decreased only in the initial stage of adipogenic differentiation of MSC, and that Dec1 overexpression suppressed PPARγ expression only in the initial stage. This result, obtained with the primary MSC, revealed the stage-dependent suppression of adipogenesis by Dec1.

Since Dec1 stimulates both osteogenesis and chondrogenesis (Shen et al., 2002), it is not involved in lineage determination, but Dec1 may increase or decrease the rate of differentiation when triggered by other transcription factors: Once the lineage is determined, increased Dec1 possibly enhances the differentiation of MSC into osteoblasts or chondrocytes while

**Fig. 6.** Effect of Dec1 overexpression on adipogenic differentiation of MSC. MSC were cultured in the adipogenic induction/maintenance medium as described in Materials and methods. (A) Total RNA was extracted from the cells on the indicated days and subjected to real-time quantitative RT-PCR analysis to determine the endogenous *Dec1* mRNA level. (B) Before adipogenic differentiation was induced, MSC were infected with ad-Dec1 or ad-LacZ at an MOI of 100. Relative mRNA levels (mean±SEM) for PPARγ on the indicated days were determined. (C) Lipid accumulation was analyzed using Oil red-O staining. Representative data on day 7 are shown. Percent (mean±SEM) of lipid drop areas stained with Oil red-O were also determined. (D) To confirm overexpression of Dec1 by ad-Dec1 infection, *Dec1* mRNA levels (mean±SEM) in MSC infected with ad-Dec1 or ad-LacZ were determined after the cells were cultured in the adipogenic medium for the indicated days. $^{**}P<0.01$, $^{*}P<0.05$ (Student's *t*-test).


A
Dec1 (endogeneous)
relative mRNA Level
100
80
60
40
20
0
**
**
0
3
7
14
(days)
B
PPARγ
ad-LacZ
ad-Dec1
relative mRNA Level
120
100
80
60
40
20
0
*
*
*
0
3
7
14
(days)
C
ad-LacZ
ad-Dec1
ad-LacZ
ad-Dec1
lipid drop area (%)
40
30
20
10
0
*
**
3
7
14
(days)
D
Dec1 (overexpression)
relative mRNA Level
100
80
60
40
20
0
0
3
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14
(days)
ad-LacZ
ad-Dec1

simultaneously inhibiting their differentiation into adipocytes, while decreased Dec1 may facilitate the onset of adipogenic differentiation in the presence of adipogenic induction factors. Thus, Dec1 may work in co-operation with several transcription factors to regulate the rate of osteogenic, adipogenic, or chondrogenic differentiation.

## Acknowledgments

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# Behavior of Transplanted Bone Marrow–Derived Mesenchymal Stem Cells in Periodontal Defects

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**Background:** Recently, there have been an increased number of basic and clinical reports indicating the superior potential of bone marrow–derived mesenchymal stem cells (MSCs) for tissue regeneration. In periodontal treatment, previous animal studies indicated that autotransplantation of bone marrow MSCs into experimental periodontal defects enhanced periodontal tissue regeneration. However, mechanisms for periodontal tissue regeneration with MSCs are still unclear. The purpose of this study was to elucidate the behavior of transplanted MSCs in periodontal defects.

**Methods:** Bone marrow MSCs were isolated from beagle dogs, labeled with green fluorescent protein (GFP), and expanded in vitro. The expanded MSCs were mixed with atelocollagen (2% type I collagen) at final concentrations of $2 \times 10^7$ cells/ml and transplanted into experimental Class III periodontal defects. Localizations of GFP and proliferating cell nuclear antigen (PCNA)-positive cells were evaluated by immunohistochemical analysis.

**Results:** Four weeks after transplantation, the periodontal defects were almost regenerated with periodontal tissue. Cementoblasts, osteoblasts, osteocytes, and fibroblasts of the regenerated periodontal tissue were positive with GFP. PCNA-positive cells were present in regenerating connective tissue.

**Conclusion:** These findings suggest that transplanted mesenchymal stem cells could survive and differentiate into periodontal tissue cells, resulting in enhancement of periodontal tissue regeneration. *J Periodontol 2006;77:1003-1007.*

KEY WORDS

**Atelocollagen; bone marrow cells; mesenchymal stem cells; regeneration.**

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Tissue regeneration requires three key elements: cells, scaffolds, and signaling molecules.[1] Considering current regenerative surgeries of periodontal tissue, scaffolds and signaling molecules are already used; however, the other key element, cells, has not yet been well established.

Bone marrow mesenchymal stem cells (MSCs) can easily be obtained and differentiated into osteoblasts, chondrocytes, tenocytes, adipocytes, muscle cells, or nerve cells in vitro and in vivo.[2-8] Thus, transplantation of bone marrow MSCs may provide a new method for treatment of osteoporosis, arthritis, cardiac diseases, and degenerative nerve diseases. Taking all of these findings into consideration, it is conceivable that MSCs might be useful for periodontal tissue regeneration.

We have focused on bone marrow MSCs and already succeeded in developing a new culture system with fibroblast growth factor-2 (FGF-2) to expand MSCs with multilineage differentiation potential.[8] To investigate the possibility of using MSCs in periodontal therapy, our previous animal study revealed that transplantation of bone marrow MSCs enhanced periodontal tissue regeneration.[9] Although it has been speculated that transplanted MSCs participate in periodontal tissue regeneration, the precise mechanism by which MSC transplantation enhances periodontal tissue

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regeneration is unknown. To elucidate the performance of the MSCs after transplantation, we transduced MSCs with DNA encoding for green fluorescent protein (GFP). GFP is widely used as a tracer and is well tolerated both in vitro and in vivo. The purpose of this study was to evaluate whether the resulting periodontal tissue regeneration originated from the transplanted MSCs.

## MATERIALS AND METHODS

After approval was obtained from the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine, six female beagle dogs weighing 10 to 14 kg and aged 12 to 20 months were used in this study. Good oral health was established by scaling and mechanical toothbrushing.

### *Isolation and Development of Bone Marrow MSCs*

Bone marrow aspirates of 1 ml were taken from the iliac crest of animals under sodium pentobarbital (40 mg/kg) anesthesia. Cell culture was performed in accordance with the technique described by Tsutsumi et al.[8] The cells were seeded at $2 \times 10^8$ cells/100-mm tissue culture dish and maintained in 10 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS),[¶] 0.05 U/ml penicillin,[#] and 0.05 mg/ml streptomycin.[**] Three days after seeding, floating cells were removed, and the medium was replaced by fresh medium. Passages were performed when cells were approaching confluence. Cells were seeded at $5 \times 10^3$ cells/cm$^2$ in 100-mm dishes and maintained in a medium containing 3 ng/ml FGF-2[††] for 2 weeks. The cells were harvested with trypsin plus EDTA, washed with phosphate-buffered saline, and stored in liquid nitrogen until use.

### *Transduction of MSCs With a Retrovirus Carrying GFP cDNA*

PtG-S2-pMZGFPIP (enhanced GFP packaging cells) and the adenovirus to transduce Cre recombinase were used.

### *Preparation of Vesicular Stomatitis Virus Glycoprotein (VSV-G) Pseudotype Retrovirus*

DMEM (high glucose) supplemented with 10% FBS and penicillin/streptomycin was used for retrovirus vector preparation. The VSV-G-pseudotyped vector was harvested every 24 hours from PtG-S2-pMZGFPIP at 3 to 7 days after introduction of Cre recombinase, as described previously.[10] The VSV-G-pseudotyped vector was concentrated by centrifugation at 6,000 × g for 16 hours at 4°C, and the pellet was suspended in DMEM without FBS (final ×100-fold concentration).

### *Retrovirus Transduction and Determination of Its Efficiency and Selection*

All transduction was carried out in the presence of 8 μg/ml polybrene[‡‡] after a 1-day passage. Three days after transduction, the titer of MSCs was determined by counting the numbers of clones with green fluorescence in cultures transduced with the vector in 10-fold serial dilutions made by adding fresh culture medium. We determined that the 1:40-fold dilution of the concentrated vector solution could transduce the MSCs effectively. To select the transduced cells, 4 μg/ml puromycin was added to eliminate any non-transduced cells. The transduced cells (>90% GFP-positive) obtained from cultures at passages 3 through 5 were used for transplants.

### *Creation of Class III Furcation Defects, Transplantation of MSCs, and Tissue Preparation*

Creation of Class III furcation defects and tissue preparation were performed according to the technique reported by Kawaguchi et al.[9] Mandibular second, third, and fourth premolars (P2, P3, and P4) in each dog were selected for experimentation. The MSCs cultured for 2 weeks were suspended, and then an MSC collagen gel material was prepared. A total of $2 \times 10^7$ MSCs were mixed with 1 ml atelocollagen (2% type I collagen, extracted from bovine calf skin by pepsin digestion).[§§] These MSCs in atelocollagen or atelocollagen only were transplanted. During the postoperative observation periods, good oral hygiene was maintained by brushing and swabbing with 0.2% povidone iodine.[||]

Four weeks after transplantation, anesthetized animals were sacrificed by vascular perfusion with 4% paraformaldehyde in a sodium cacodylate buffer containing 0.05% calcium chloride (pH 7.3). The mandibles were dissected and decalcificated with 10% EDTA for 2 weeks. They were dehydrated through graded ethanol, cleared with xylene, and embedded in paraffin. Serial sections (5 μm) were cut in the mesial-distal plane throughout the buccal-lingal extension of the tooth. The sections were stained with hematoxylin and eosin (H&E) and observed using a light microscope.

### *Immunohistochemical Procedures*

Av peptide antibody[¶¶] and mouse monoclonal anti-cell nuclear antigen[##] (proliferating cell nuclear antigen [PCNA]) were used as the primary antibodies. Av peptide antibody is a mixture of several rabbit

¶ Hyclone, South Logan, UT.
# Invitrogen, Carlsbad, CA.
** Invitrogen.
†† PeproTech EC, Rocky Hill, NJ.
‡‡ Sigma, St. Louis, MO.
§§ Koken, Tokyo, Japan.
|| Meiji-seika, Tokyo, Japan.
¶¶ Living Colors, BD Biosciences, Palo Alto, CA.
## DAKO, Carpinteria, CA.

anti-GFP antibodies. Sections were deparaffined with xylene, rehydrated through a descending ethanol series, and washed in distilled water. Endogenous peroxidase was blocked by incubating the sections with 3% hydrogen peroxide. After washing sections with Tris-buffered saline (TBS) (pH 7.2), the sections were treated with 0.1% bovine serum albumin*** to prevent non-specific binding, and the following incubations were performed. The primary antibodies were diluted in antibody diluent††† and incubated for ~1 hour at room temperature. The primary antibody dilutions were GFP (1:200) and PCNA (1:200). After incubation with the primary antibodies, sections were rinsed with TBS and incubated with an alkaline phosphatase-dextran complex‡‡‡ for 30 minutes in a moist chamber. These slides were rinsed in TBS. Antibody complexes were visualized with 3,3′-diaminobenzidine (DAB) substrate, washed in distilled water, and counterstained with hematoxylin. As controls, some sections were treated in the same way but without incubation with the primary antibodies. The differential labeling patterns obtained with various antibodies also served as internal controls.

## RESULTS

### *Morphological and Immunohistochemical Findings*

Morphological findings at 4 weeks after transplantation were almost the same as in the previous report. Most experimental specimens showed a significant amount of new bone, and an adequate width of periodontal ligament was observed (Fig. 1A).

The denuded root surface was almost completely covered with new cementum, and the regenerated periodontal ligament separated the new bone from cementum. However, complete alveolar bone reconstruction was not yet obtained (Fig. 1A). In contrast, periodontal tissue regeneration was insufficient in the control group compared to the experimental group (Fig. 2). Epithelial cells invaded into the top of the furcation, and cementum regeneration was not observed in the area (Fig. 2).

Immunohistochemical study showed that GFP-positive cells were present in the whole area of the defect. In the top area of the defect, abundant GFP-positive cells were observed in regenerating soft connective tissue and on the surface of regenerating alveolar bone (Figs. 1B and 1D).

Cementoblasts arranged along the denuded surface, osteoblasts and osteocytes of regenerated bone, and fibroblasts of the regenerated periodontal ligament were immunoreactive for GFP (Figs. 1C and 1E). In all control incubations, immunoreaction was not found over the tissue sections.

PCNA-positive cells were present in regenerating soft connective tissue (Figs. 1F and 1G). Cementoblasts, fibroblasts, osteoblasts, and osteocytes of regenerated periodontal tissue showed weak staining with PCNA (Fig. 1G).

## DISCUSSION

To elucidate the survivability and behavior of transplanted MSCs in periodontal defects, we used GFP-transduced MSCs and examined their localization in a regenerative procedure using an immunohistochemical method. Initially, we observed with a fluorescence microscope but could not detect any GFP-positive cells. Decalcification of the tissue during tissue preparation might influence GFP fluorescence. Therefore, GFP was visualized using anti-GFP monoclonal antibody immunostaining in this study.

Our histological analysis showed that the defects were almost regenerated with cementum, periodontal ligament, and alveolar bone 4 weeks after MSC transplantation. These results were consistent with our previous report.[9] Moreover, this study showed that osteoblasts and osteocytes in regenerated alveolar bone, cementoblasts on the denuded root surface, and fibroblasts in the soft connective periodontal tissue were GFP-positive. These anti-GFP monoclonal antibody immunostaining findings confirmed that some transplanted MSCs survived in periodontal defects at least 4 weeks after transplantation and differentiated into periodontal tissue-composing cells. It is also speculated that transplanted MSCs survive, differentiate into periodontal tissue cells, and release various kind of cytokines, all of which promote periodontal tissue regeneration.

The exact mechanism by which MSCs differentiate into cementoblasts, osteoblasts, and periodontal ligament fibroblasts is unknown. Previous in vivo studies revealed that host factors influence transplanted MSCs to differentiate into various connective tissue cells.[11,12] It has also been reported that the microenvironment and surrounding tissue provide the nutrients, growth factors, and extracellular matrices to support MSC differentiation.[13] It appears that when stem cells are removed to different locations, they undergo reprogramming of gene expression and cross lineage boundaries. For instance, brain stem cells give rise to hematopoietic cells, and bone marrow cells give rise to epithelial cells after transplantation.[14,15] In the periodontal field, bone marrow MSCs gain characteristics of periodontal ligament cells after co-culturing with periodontal ligament–derived cells.[16] The periodontal environment seemed to stimulate the transplanted MSCs to differentiate into specific periodontal tissue cells.

With regard to MSCs differentiated into cementoblasts, transplanted MSCs may promote their

*** Sigma.
††† DAKO.
‡‡‡ ENVISION, DAKO.

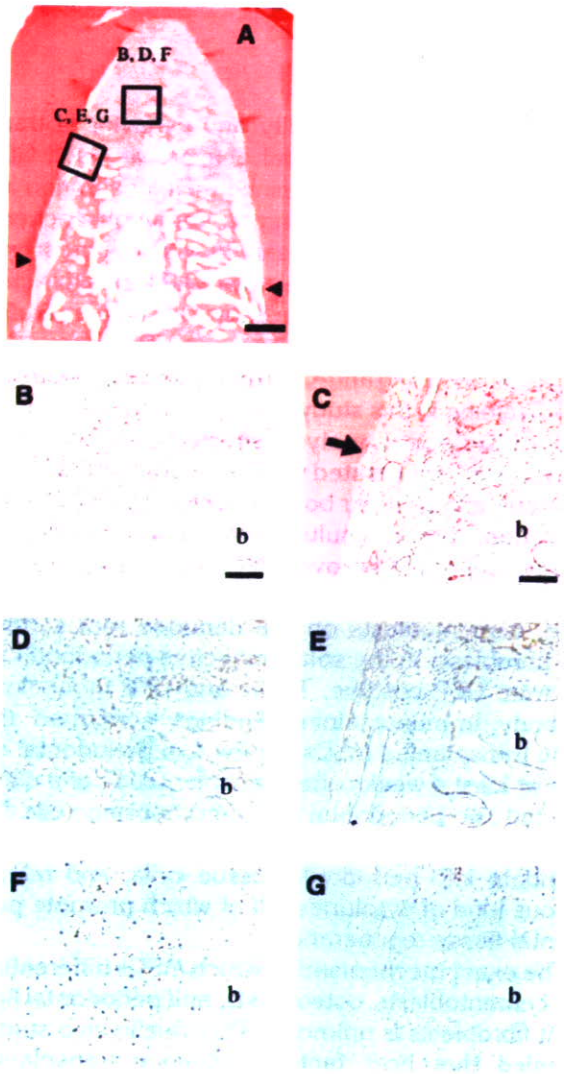


**Figure 1.**
*Four weeks after transplantation. **A)** New bone and an adequate width of periodontal ligament are observed in the experimental group. New cementum almost completely covers the denuded root surface. Regenerated periodontal ligament separates the new bone from the cementum. Arrowheads indicate the apical border of the denuded root surface. **B through G)** Higher magnification of square areas of A. B and D) In the top area of the defect, osteoblasts on regenerated bone (b) and fibroblasts in the surrounding connective tissue are GFP-positive. C and E) In the middle area of the defect, cementoblasts, fibroblasts, osteoblasts, and osteocytes of regenerated periodontal tissue are GFP-positive. F) Cells in connective tissue are PCNA-positive. G) Cementoblasts, fibroblasts, osteoblasts, and osteocytes show weak staining with PCNA. (B and C: H&E staining. D and E: immunohistochemical staining of GFP. F and G: immunohistochemical staining of PCNA. scale bars: A = 200 μm, B and C = 20 μm.)*

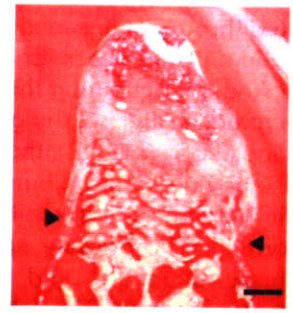

**Figure 2.**
*Four weeks after implantation of atelocollagen alone (control group). Epithelial cells invaded into the top of the furcation, and no cementum regeneration was observed in the area. Note less periodontal regeneration compared to Figure 1A. Arrowheads indicate the apical border of the denuded root surface. Bar = 200 μm.*

differentiation after attachment to the denuded dentin surface. Our in vitro study involving reverse transcription–polymerase chain reaction of MSCs showed that MSCs expressed non-collagenous bone proteins such as osteocalcin, osteopontin, and bone sialoprotein when MSCs were cultured on dentin blocks (data not shown). These findings suggest that MSCs attached on the root surface differentiate into cementoblasts in the early stage of the process. A variety of chemotactic factors, adhesion molecules, growth factors, and extracellular matrix macromolecules could participate together in inducing differentiation of MSCs into cementoblasts.[17] Once MSCs along the root surface differentiate into cementoblasts, these cells could release various kinds of cytokines, leading to a subsequent process of periodontal tissue regeneration.

The present study indicates that up to 4 weeks after transplantation, regenerating and regenerated periodontal tissue was derived largely from transplanted MSC cells. Furthermore, results of an immunohistochemical PCNA study suggest that MSCs are present at various differentiation stages. Nevertheless, the results in the control group showed that some of the defects were repaired by the invasion of cells from the surrounding tissue: regenerated periodontal tissue originates, at least partly, from host cells. It is not clear how long transplanted MSCs survive and take part in tissue formation. Transplanted MSCs in chondral defects showed that they decreased in number with time.[18] Although the cells might have lost the ability to produce GFP, their disappearance is more likely.[19] Other groups reported that transplanted cells survived for 10 days to 8 weeks in chondral defects.[20,21] Additional studies with short- and long-term observations will extend our knowledge on the exact fate of the transplanted MSCs.

## CONCLUSION

This study indicates that transplanted MSCs can survive and differentiate into periodontal tissue–composing

cells, resulting in enhancement of periodontal tissue regeneration.

## ACKNOWLEDGMENT

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# Molecular markers distinguish bone marrow mesenchymal stem cells from fibroblasts

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**Abstract**

To characterize mesenchymal stem cells (MSC), we compared gene expression profiles in human bone marrow MSC (11 lines) and human fibroblasts (4 lines) by RT-PCR and real time PCR. Messenger RNA levels of MHC-DR-α, MHC-DR-β, MHC-DR-associated protein CD74, tissue factor pathway inhibitor-2, and neuroserpin were much higher in MSC than in fibroblasts, even in the presence of large interindividual variations. Those of adrenomedullin, apolipoprotein D, C-type lectin superfamily member-2, collagen type XV α1, CUG triplet repeat RNA-binding protein, matrix metalloproteinase-1, protein tyrosine kinase-7, and Sam68-like phosphotyrosine protein/T-STAR were lower in MSC than in fibroblasts. FACS analysis showed that cell surface expression of MHC-DR was also higher in MSC than in fibroblasts. MHC-DR expression decreased after osteogenic differentiation, whereas the expression of adrenomedullin—a potent stimulator of osteoblast activity—along with collagen XV α1 and apolipoprotein D increased after osteogenic differentiation. The marker genes identified in this study should be useful for characterization of MSC both in basic and clinical studies.
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*Keywords:* Bone marrow; Marker genes; Mesenchymal stem cell; Regenerative medicine

Bone marrow mesenchymal stem cells (MSC), which are also called plastic-adherent marrow cells or bone marrow stromal cells, can differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes, and muscle cells in vitro and/or in vivo [1–11]. MSC can easily be isolated from adult bone, and can be expanded with serum ex vivo, so these cells are promising for regenerative medicine: they are already being used for treatment of osteogenesis imperfecta or bone/cartilage defects [7,8]. Nonetheless, MSC have not been fully characterized, and thus it is difficult to examine whether ex vivo expanded MSC population is free of fibroblasts. Bone marrow may contain fibroblasts or can be contaminated by fibroblasts during aspiration, and the fibroblasts—together with MSC—could possibly be expanded in the presence of serum ex vivo. However, the appearance

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of MSC is similar to that of fibroblasts. In addition, we found that several cell surface antigens, previously considered to be MSC markers, were also expressed in fibroblasts at similar levels. In other words, molecular markers for MSC remain unknown. To address this issue, we compared gene expression profiles between human bone marrow MSC and connective tissue fibroblasts—using DNA macroarrays, RT-PCR, and real time PCR. We identified 13 genes differentially expressed between these cells. Since contamination of transplantable cells by fibroblasts may delay regeneration, we used these markers to distinguish MSC from fibroblasts before transplantation at Hiroshima University Hospital.

## Materials and methods

*MSC and fibroblast cultures.* MSC were obtained from iliac crest or alveolar/jaw bone according to a protocol approved by Ethical Authorities at Hiroshima University. In addition, human iliac MSC were purchased from Bio-Whittaker (Walkersville, MD). For isolation of alveolar/jaw bone marrow, we selected patients whose bone marrow sites had been opened during oral surgery, and obtained marrow aspirates using routine syringes and needles [12]. Bone marrow cells including erythrocytes were seeded at a density of 0.1 ml aspirate per 35-mm tissue culture dish (Corning) and maintained in 2 ml DMEM supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin G, and 100 μg/ml streptomycin (medium-A). Three days after seeding, floating cells were removed and the medium was replaced by fresh medium-A. Thereafter, attached cells (plastic-adherent marrow cells) were fed with fresh medium-A supplemented with 1 ng/ml FGF-2. FGF-2 was added every other day. Passages were performed when cells were approaching confluence: the cells were seeded at a density of $5 \times 10^3$ cells/cm$^2$ on 100-mm tissue culture dishes (Corning) and maintained in 10 ml medium-A supplemented with 1 ng/ml FGF-2 [9]. To avoid direct actions of FGF-2 on gene expression, FGF-2 was removed from the culture medium of MSC or fibroblasts 72 h before isolation of RNA. Human skin fibroblasts were purchased from Kurabo (Tokyo, Japan), and human gingival fibroblasts were isolated as described previously [13]. Fibroblasts were also maintained in culture as described above.

*Osteogenic differentiation.* MSC or fibroblasts at passage fifth to eighth were seeded at $4 \times 10^4$ cells per 16-mm well in a 24-well plate, and maintained for 7–28 days in medium-A supplemented with 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbic acid-2-phosphate [1,9].

*Microarray analysis and RT-PCR.* Total RNA was isolated using TRIZOL reagent (Invitrogen), when the fifth to eighth passage cultures became confluent. Poly(A)$^+$ RNA was purified using Micro poly(A) purist (Ambion). DNA microarray analysis was performed with 0.5 μg poly(A)$^+$ RNA by Kurabo Life Array analysis service (Incyte Genomics; Lot # KL01081).

For RT-PCR, first-strand cDNA was synthesized with 1 μg of total RNA using SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Using the cDNAs as a template, PCR was carried out under the following conditions: denaturation at 94 °C for 30 s and primer extension at 65 °C for 1.5 min in 28 cycles for adrenomedullin; 30 cycles for matrix metalloproteinase-1 (MMP-1), tissue factor pathway inhibitor-2, apolipoprotein D, collagen type XV α1, CUG triplet repeat RNA-binding protein, serine (or cysteine) proteinase inhibitor clade-1 member-1 (neuroserpin), protein tyrosine kinase-7, Sam68-like protein, MHC-DR-α and MHC-DR-β; 33 cycles for C-type lectin superfamily member-2. The sequences of primers are shown in supplementary Table 1. Obtained PCR products were separated on 1% agarose gels,and stained with ethidium bromide.

*Real time PCR.* With the above cDNAs (1 μg) as a template, real time quantitative RT-PCR analyses were performed using an ABI Prism 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA). Sequences of the primers and probes are shown in supplementary Table 2. The primers and probes for CD73 (4331182-CD74) and GAPDH (4310884E) were purchased from PE Applied Biosystems. The mRNA level relative to that of GAPDH was calculated.

*FACS analysis.* Cells at passage fifth to eighth were harvested with trypsin and EDTA, centrifuged at 1500*g* for 5 min, and resuspended at $5 \times 10^6$ cells/ml in phosphate-buffered saline (PBS) containing 3% fetal bovine serum. Aliquots containing $10^5$ cells were incubated with individual primary antibodies or control IgG for 30 min at room temperature. The cells were washed in PBS containing 3% fetal bovine serum and incubated with a fluorescent conjugated secondary antibody for 30 min at room temperature. Samples were analyzed using a FACSCalibur cytometer (Becton Dickinson), and the data were analyzed using CELLQUEST software (Becton Dickinson). The following monoclonal antibodies (mAbs) were used: fluorescein isothiocyanate (FITC)-conjugated or *R*-phycoerythrin (PE)-conjugated antibodies against HLA-DR (MHC-DR), CD13, CD14, CD29, CD34, CD44, CD49b, CD54, CD56, CD71, CD90, CD105, CD106, CD117, CD124, CD138, CD144, MHC-DR, HLA-ABC, mouse-IgG1, mouse-IgG2a or mouse IgM (Immunotech Coulter Company); antibodies against MHC-DR, CD73, CD74, CD123, CD140b, CD166 or mouse-IgG3 (Pharmingen); antibodies against Flk-1 (Santa Cruz Biotechnology); antibodies against MT-MMP-1 (Sigma); antibodies against STRO-1 (Genzyme); antibodies against RANKL (R&D Systems), and anti-rabbit-IgG (Chemicon International).

*Statistical analysis.* Student's *t* test was used.

## Results

### *Differential expression of candidate marker genes between MSC and fibroblasts*

MSC or fibroblast lines were obtained from alveolar/jaw bone, ilium, gums or skin of young adults of similar age (Table 1). Under the osteogenic conditions, alveolar (MSC-1) and iliac MSC (MSC-9) induced matrix calcification—which was stained with alizarin red—on days 21 and 28 (supplementary Fig. 1). All of the other MSC lines—but none of the fibroblast lines—also induced calcification by day 28 (Table 1).

Gene expression profile was compared between MSC-1 and fibroblast-2 using DNA microarrays (9400 genes): many (~100) genes showed different signals (>2-fold) between these cells. To confirm the different expressions, we performed RT-PCR analysis for these genes with seven MSC and four fibroblast lines: many genes showed large interindividual variations or very low expression levels, so the comparison was difficult. Nonetheless, the following genes appeared to be expressed differently between MSC and fibroblasts: the mRNA levels of MMP-1 (Fig. 1A), adrenomedullin, (B), protein tyrosine kinase-7 (C), collagen type XV α1 (D), Sam68-like phosphotyrosine protein/T-STAR (E), C-type lectin superfamily member-2 (F), CUG triplet repeat RNA-binding protein

Table 1
Fibroblast and MSC lines used in this study

| Cell lines | Tissues | Age | Sex | OB |
|---|---|---|---|---|
| Fibroblast-1 | Gums | 17 | M | – |
| Fibroblast-2 | Gums | 18 | F | – |
| Fibroblast-3 | Skin | 29 | F | – |
| Fibroblast-4 | Skin | 33 | F | – |
| MSC-1 | Alveolar/jaw | 26 | M | + |
| MSC-2 | Alveolar/jaw | 24 | F | + |
| MSC-3 | Alveolar/jaw | 19 | F | + |
| MSC-4 | Alveolar/jaw | 23 | F | + |
| MSC-5 | Alveolar/jaw | 24 | F | + |
| MSC-6 | Alveolar/jaw | 19 | F | + |
| MSC-7 | Alveolar/jaw | 36 | M | + |
| MSC-8 | Ilium | 24 | F | + |
| MSC-9 | Ilium | 22 | F | + |
| MSC-10 | Ilium | 19 | F | + |
| MSC-11 | Ilium | 24 | M | + |

*Note.* OB, osteogenic differentiation.

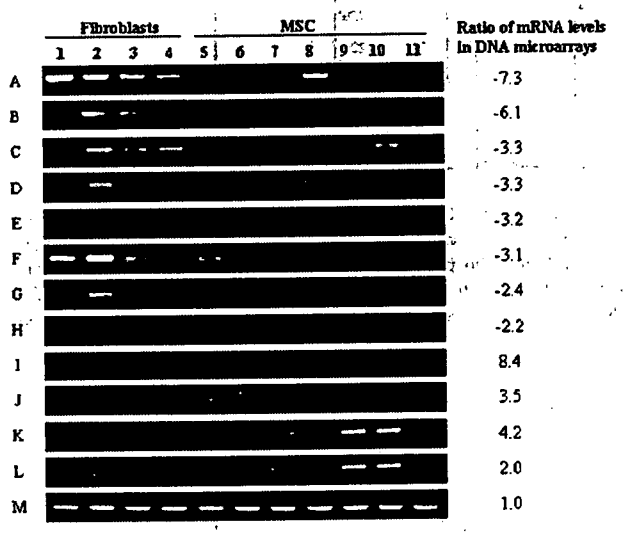


Fig. 1. RT-PCR analysis for genes expressed differently in fibroblasts and MSC. Fibroblast-1, -2, -3, and -4 (lanes 1–4, respectively) and MSC-1, -2, -3, -4, -9, 10, and -11 (lanes 5–11, respectively) were isolated from 11 different donors. The ratio of the mRNA level between MSC and fibroblasts in DNA microarray analysis is shown on the right of the panel. (A) MMP-1; (B) adrenomedullin; (C) protein tyrosine kinase-7; (D) collagen type XV α1; (E) Sam68-like phosphotyrosine protein; (F) C-type lectin superfamily member-2; (G) CUG triplet repeat RNA-binding protein; (H) apolipoprotein D; (I) tissue factor pathway inhibitor-2; (J) neuroserpin; (K) MHC-DR-α; (L) MHC-DR-β; (M) GAPDH.

(G), and apolipoprotein D (H) were lower in MSC than in fibroblasts. In contrast, mRNA levels of tissue factor pathway inhibitor-2 (I), neuroserpin (J), MHC-DR-α (K), and MHC-DR-β (L) were higher in MSC than in fibroblasts. The GAPDH mRNA level in MSC was equal to that in fibroblasts (M).

Real time PCR analysis showed that mRNA levels of these genes, relative to GAPDH, were statistically different ($P < 0.05$) between 11 MSC and 4 fibroblast lines (Fig. 2). Of the 12 genes, MHC-DR-α and -β showed differential expression between iliac and alveolar MSC (see below).

### *FACS analysis of MHC-DR expression*

Since MHC-DR-α and -β mRNA levels were higher in MSC than in fibroblasts, we examined the protein level of MHC-DR by FACS analysis (Fig. 3A): no positive cells were detected with fibroblast lines, whereas MSC lines showed MHC-DR expression at low or moderate levels.

### *Differential expression of CD74 mRNA between MSC and fibroblasts*

Since MHC-DR expression was higher in MSC than in fibroblasts, we compared the expression of MHC-DR-associated protein CD74 in MSC and fibroblasts. The mRNA level of CD74 in iliac MSC was higher than that in fibroblasts, although alveolar/jaw MSC showed CD74 expression at lower levels than did iliac MSC (Fig. 3B).

### *Changes in marker expressions after osteogenic differentiation*

We compared marker gene expressions before and after osteogenic differentiation. The MHC-DR-α and/or -β mRNA levels in five MSC lines decreased after osteogenic differentiation, and the MHC-DR-β mRNA level in alveolar MSC was lower than that in iliac MSC (Fig. 4A). In contrast, apolipoprotein D, adrenomedullin, and collagen type XV α1—which were suppressed both in iliac and alveolar MSC—increased after osteogenic differentiation (Fig. 4B). No changes in mRNA levels of tissue factor pathway inhibitor-2, neuroserpin, C-type lectin superfamily member-2, CUG triplet repeat RNA-binding protein, MMP-1, protein tyrosine kinase-7, or Sam68-like phosphotyrosine protein could be detected after osteogenic differentiation (data not shown). These findings suggest that MHC-DR is a positive marker for undifferentiated MSC, whereas the expression of apolipoprotein D, adrenomedullin, and collagen type XV α1 is temporarily suppressed at the undifferentiated stage.

### *FACS analysis of cell surface antigens*

Some cell surface antigens—in addition to MHC-DR—may be expressed selectively in MSC. SH2 (CD105), SH3 (CD73), ALCAM (activated leukocyte cell adhesion molecule/CD166), CD13, CD29 (integrin β-1), PDGF receptor, CD44 (hyaluronate receptor), and CD90 (Thy-1) were expressed in MSC and/or perichondrium mesenchymal stem cells

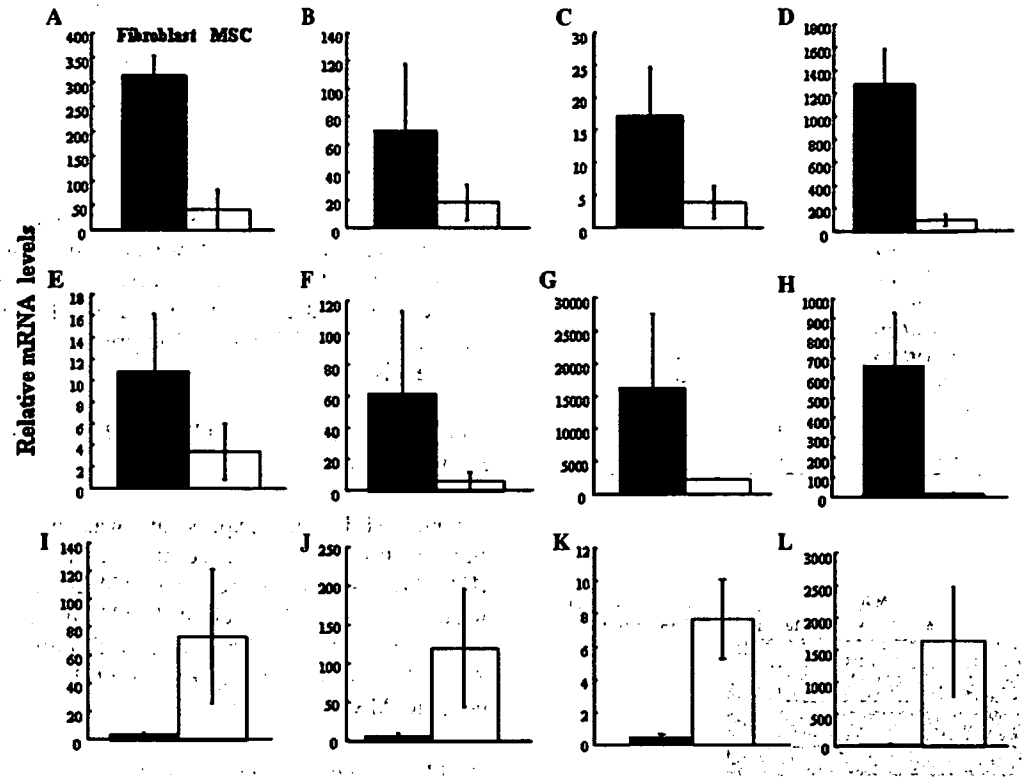


Fig. 2. Real time PCR analysis of candidate genes. Expressions of the candidate genes relative to GAPDH in confluent cultures of fibroblast-1, -2, -3, and -4 (closed columns) and MSC-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, and -11 (open columns) were examined by real time PCR. (A) MMP-1; (B) adrenomedullin; (C) protein tyrosine kinase-7; (D) collagen type XV α1; (E) Sam68-like phosphotyrosine protein; (F) C-type lectin superfamily member-2; (G) CUG triplet repeat RNA-binding protein; (H) apolipoprotein D; (I) tissue factor pathway inhibitor-2; (J) neuroserpin; (K) MHC-DR-α; (L) MHC-DR-β. Values are averages ± SD for 4 or 11 cultures. The mRNA levels of all examined genes were statistically significant between MSC and fibroblast lines ($P < 0.05$).

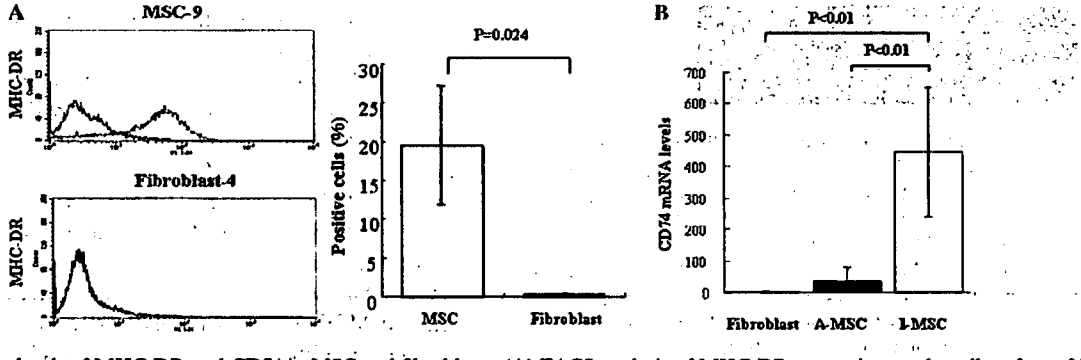


Fig. 3. Expression levels of MHC-DR and CD74 in MSC and fibroblasts. (A) FACS analysis of MHC-DR expression on the cell surface of MSC. MHC-DR expression in fibroblast-2, -3, and -4 (closed columns) and MSC-5, -6, -7, -8, -9, -10, and -11 (open columns) were examined by FACS analysis with anti-MHC-DR antibody (Immunotech Coulter). Values are averages ± SD for three fibroblast or seven MSC lines. (B) The expression of CD74 mRNA in alveolar/jaw MSC (A-MSC), iliac MSC (I-MSC), and fibroblasts. CD74 mRNA expression in fibroblast-1, -2, -3, and -4, alveolar MSC-1, -2, -3, -4, and -5, and iliac MSC-9, 10, and -11 in confluent cultures was examined by real time RT-PCR. Values are averages ± SD for three to five cultures.

[1,14–18]. SH2 and SH3 levels decreased after osteogenic differentiation [14], and ALCAM was expressed selectively in perichondrium mesenchymal cells [15]. However, it is unknown whether these cell surface markers were present or absent in fibroblasts. In this study, we compared the levels of these markers in human MSC and fibroblasts: overall, the surface marker expression levels in MSC in this study were consistent with those reported in the literature [1,14–16,18], but none of the cell surface antigens—including SH2,

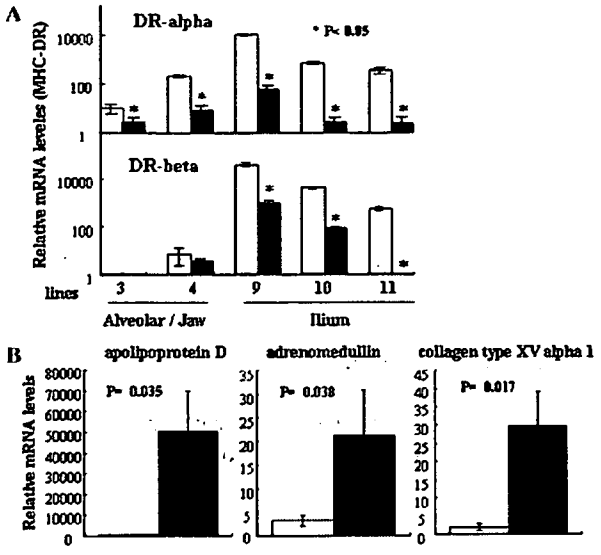


Fig. 4. Effects of osteogenic differentiation on gene expression in MSC. (A) Decrease in MHC-DR mRNA expression after osteogenic differentiation. MSC-3, -4, -9, -10, and -11 were maintained for 28 days in medium-A alone (open column) or in medium-A supplemented with 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbic acid-2-phosphate in the osteogenic status (closed columns). Values are averages ± SD for three cultures. (B) Increases in mRNA levels of marker genes in MSC after osteogenic differentiation. MSC-3, -4, -9, -10, and -11 were maintained for 28 days in medium-A alone (open columns) or in medium-A supplemented with 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbic acid-2-phosphate (closed columns). Values are averages ± SD for five cultures.

SH3, ALCAM, and STRO-1—differed between MSC and fibroblasts (supplementary Fig. 2). The levels of these cell surface markers in iliac MSC or fibroblasts were similar to those in alveolar MSC [12]. STRO-1 has been found in freshly isolated MSC [17], but in this study, MSC showed STRO-1 expression at a low level (supplementary Fig. 2). Previous studies have also shown that STRO-1$^+$ cells in human MSC population are only 7 ± 6% [18], so STRO-1 may be progressively lost with time in these cultures. In any case, our findings suggest that STRO-1 is not essential for the differentiation potential of MSC.

*Application of marker genes to regenerative medicine*

At Hiroshima University Hospital, we have commenced clinical studies on regenerative medicine for periodontal diseases, using autologous MSC. Before transplantation, we compared the marker gene expressions in patients' MSC (iliac MSC) with those in standard iliac MSC and fibroblast lines (Table 2). In the case of patient-1 (63, male), all examined genes in expanded plastic-adherent cells showed a similar expression pattern to that of standard MSC lines. Fibroblast contamination was unlikely, because MMP-1 and collagen type XV α1 mRNA levels in the marrow cells were only 1% of those in the fibroblastic lines (Table 2). In contrast, MHC-DR-α and -β mRNA levels in the marrow cells were 1000-fold greater than those in the fibroblast lines. Similar results were obtained with cells from patient-2 (39, male), patient-3 (64, female), patient-4 (46, female), patient-5 (25, male), patient-6 (56, female), and patient-7 (22, male) (data not shown). After the quality examination, we were able to transplant these cells with abundant self-confidence.

Table 2
Evaluation of ex vivo expanded MSC population before transplantation using marker genes

| Genes | Relative mRNA levels | | |
|---|---|---|---|
| | Patient-1 | Standard MSC | Standard fibroblasts |
| MMP-1 | 28 | 9 ± 10 | 1206 ± 1097 |
| Adrenomedullin | 3 | 1 ± 1 | 15 ± 5 |
| CUG triplet repeat, RNA-binding protein-2 | 7 | 6 ± 5 | 148 ± 107 |
| Collagen type XV α1 | 1 | No signal | 475 ± 163 |
| Sam68-like phosphotyrosine protein | No signal | No signal | 3 ± 2 |
| C-type lectin, superfamily member-2 | 3 | 5 ± 4 | 231 ± 191 |
| Apolipoprotein D | 14 | 3 ± 2 | 426 ± 326 |
| Tissue factor pathway inhibitor-2 | 9 | 23 ± 17 | 4 ± 4 |
| MHC-DR-β | 2121 | 563 ± 769 | 2 ± 1 |
| MHC-DR-α | 1561 | 582 ± 402 | 2 ± 2 |

Before transplantation, we examined the expression pattern of marker genes in patient's MSC expanded ex vivo, standard MSC, and standard fibroblasts. The patient's MSC were cultured, and total RNA in confluent cultures at passage 3 was isolated as described in Materials and methods. Gene expression profile in the patient's cells was compared with that in RNA samples of MSC-9, MSC-10, MSC-11, fibroblast-1, fibroblast-2, and fibroblast-3 by real time PCR. Values are averages of duplicate determinations (patient-1) or averages ± SD for three cultures (standard lines).

## Discussion

We identified several genes differentially expressed between MSC and fibroblasts, and the differential expression was unrelated to age, sex or culture conditions. We used MSC and fibroblasts from donors of similar age and cultured under similar conditions; sex did not affect the gene expressions (data not shown).

In addition, the difference was not due to in vivo location of the cell, since we used iliac and alveolar MSC, and skin and gingival fibroblasts. Alveolar MSC had potent osteogenic potential in vitro and in vivo, although their chondrogenic or adipogenic potential was less than that of iliac MSC [12]. Alveolar and iliac MSC shared many common marker genes, with a few genes (MHC-DR and CD74) being expressed at different levels.

MSC showed a lower level of MMP-1 and a higher level of its inhibitor—tissue factor pathway inhibitor-2—than did fibroblasts. Tissue factor pathway inhibitor-2 suppresses the activity of the collagenases—MMP-1 and MMP-13—as well as the gelatinases—MMP-2 and MMP-9 [19], so MSC may be less active in collagen-matrix breakdown. On the other hand, reduced expression of neuroserpin may increase activities of tissue-type plasminogen activator [20].

Type XV collagen occurs in basement membrane zones of tissues [21], and type XV collagen-derived endostatin has antiangiogenic actions [22]. Adrenomedullin—a member of the calcitonin family—stimulates osteoblastic activity and bone growth in vivo [23,24]. Accordingly, adrenomedullin mRNA expression increased after osteogenic differentiation of MSC. Protein tyrosine kinase-7 is essential for neural tube closure [25] and is involved in tumor metastasis [26]. Sam68-like phosphotyrosine protein (an Src substrate) is involved in cell proliferation [27,28]. Apolipoprotein D increases platelet-derived growth factor actions and synergistically stimulates migration of vascular smooth muscle cells [29]. Roles of the molecules in MSC remain unclear, but higher or lower levels of these molecules are characteristic of MSC. We are investigating the physiological roles of molecules in MSC or fibroblasts.

We also found that the expression of MHC-DR (class II) was higher in MSC than in fibroblasts, whereas MHC (class I)/HLA-ABC was expressed in MSC and fibroblasts at similar levels (supplementary Fig. 2). The expression level of MHC-DR-associated protein CD74 was also higher in MSC than in fibroblasts. CD74 binds to MHC-DR and this interaction involves the peptide-binding groove of MHC-DR [30]. MHC-DR-α and -β molecules are assembled with CD74 protein in the endoplasmic reticulum [31]. The physiological relevance of MHC-DR and CD74 expression in MSC is unknown, but MSC can modulate immune response [32].

Plastic-adherent marrow cells/MSC are often designated as a colony-forming unit-fibroblastic (CFU-F), and some CFU-F colonies do not show any differentiation potentials. Thus, ex vivo expanded adherent cell populations may contain fibroblast-like cells. In addition, bone marrow samples can be contaminated by connective tissue fibroblasts during surgery or aspiration. Contamination of MSC populations by fibroblasts delays regeneration and could be harmful, and thus we need to confirm the absence of fibroblasts before transplantation. Conventionally we have to incubate transplantable cells in differentiation-induction media for 21–28 days to examine the presence of MSC with multi-lineage differentiation potential, but this does not show the presence of fibroblasts directly. In the present study, we developed a method of distinguishing MSC from fibroblasts promptly before transplantation—using marker genes. This quality examination should be crucial to regenerative medicine with MSC. In addition, identification of marker genes will help us characterize MSC and examine their in vivo location.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.04.118.

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# Alveolar Bone Marrow as a Cell Source for Regenerative Medicine: Differences Between Alveolar and Iliac Bone Marrow Stromal Cells

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**ABSTRACT: We isolated and expanded BMSCs from human alveolar/jaw bone at a high success rate (70%). These cells had potent osteogenic potential in vitro and in vivo, although their chondrogenic and adipogenic potential was less than that of iliac cells.**

**Introduction:** Human bone marrow stromal cells (BMSCs) have osteogenic, chondrogenic, and adipogenic potential, but marrow aspiration from iliac crest is an invasive procedure. Alveolar BMSCs may be more useful for regenerative medicine, because the marrow can be aspirated from alveolar bone with minimal pain.
**Materials and Methods:** In this study, alveolar bone marrow samples were obtained from 41 patients, 6–66 years of age, during the course of oral surgery. BMSCs were seeded and maintained in culture with 10% FBS and basic fibroblast growth factor. In addition, BMSCs were induced to differentiate into osteoblasts, chondrocytes, or adipocytes in appropriate medium.
**Results and Conclusion:** From a small volume (0.1–3 ml) of aspirates, alveolar BMSCs expanded at a success ratio of 29/41 (70%). The success rate decreased with increasing donor age, perhaps because of age-dependent decreases in the number and proliferative capacity of BMSCs. The expanded BMSCs differentiated into osteoblasts under osteogenic conditions in 21–28 days: the mRNA levels of osteocalcin, osteopontin, and bone sialoprotein, along with the calcium level, in alveolar BMSC cultures were similar to those in iliac cultures. However, unlike iliac BMSC, alveolar BMSC showed poor chondrogenic or adipogenic potential, and similar differences were observed between canine alveolar and iliac BMSCs. Subsequently, human alveolar BMSCs attached to β-tricalcium phosphate were transplanted into immunodeficient mice. In transplants, new bone formed with osteoblasts and osteocytes that expressed human vimentin, human osteocalcin, and human GAPDH. These findings suggest that BMSCs have distinctive features depending on their in vivo location and that alveolar BMSCs will be useful in cell therapy for bone diseases.
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**Key words: bone marrow stromal cells, alveolar bone, osteogenesis, mesenchymal stem cells**

## INTRODUCTION

BONE MARROW STROMAL CELLS (BMSCs) can differentiate into a variety of tissues—bone, cartilage, tendon, muscle, adipose tissue, and neuronal tissue—and their transplantation promotes regeneration of various tissues.[1–4] BMSCs have been isolated from various bones, including the ilium, femur, tibia, and spine,[5–8] but whether their proliferative and differentiation potentials depend on their in vivo location is unknown. Furthermore, marrow aspiration from these bones is an invasive procedure. Considering these facts, we decided to try collecting BMSCs from alveolar bone during the course of dental surgery, because most young adults undergo wisdom tooth extraction. We examined whether BMSCs could be expanded ex vivo from a small volume of alveolar bone marrow aspirates, and we also examined the effects of age, sex, disease history, and the volume of aspirates obtained from patients on ex vivo expansion of alveolar BMSCs. Furthermore, we compared the proliferative and differentiation potentials of alveolar BMSCs with those of iliac BMSCs, using human and canine marrow aspirates.

Drs Kato and Tsuji own stock in Two Cells Co., Ltd. All other authors have no conflict of interest.

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Alveolar BMSC's were cultured with basic fibroblast growth factor (bFGF), because BMSC's maintained with bFGF retained their differentiation potentials throughout many mitotic divisions.[5,6] The use of bFGF allowed us to expand alveolar BMSCs from a small volume (0.1–3 ml) of marrow aspirates, although alveolar BMSCs were not obtained from all alveolar aspirates of patients >50 years of age. Although the alveolar BMSCs showed a proliferative capacity and a potent osteogenic potential, the cells—unlike iliac BMSCs—had a poor adipogenic or chondrogenic potential, suggesting that in vivo location of BMSCs modulates their differentiation potentials.

## MATERIALS AND METHODS

### *Cell cultures*

Human bone marrow was obtained from the alveolar bone or the ilium according to a protocol approved by ethical authorities at Hiroshima University. In this study, we selected patients whose bone marrow sites were opened during oral surgery to obtain marrow aspirates using routine syringes and needles without contamination by periodontal tissues. In extraction of impacted wisdom teeth or extirpation of cysts, alveolar bone around a tooth or a cyst was removed, and the bone marrow was exposed. Subsequently, the aspirate was obtained from the marrow site using an 18G injection needle (JMS, Hiroshima, Japan) connected to a disposable syringe (JMS). In cases of dental implant, the aspirate was obtained from drill holes in the alveolar bone. In cases of jaw deformities, the aspirate was obtained from the osteotomy groove along the anterior border of the mandibular ramus. In cases of mandibular fracture, the aspirate was obtained from the marrow site that was exposed by widening the gap between the fractured margins or from burr holes through which interosseous wire was passed. The aspirates were obtained from alveolar and/or jaw bones, but in this study, we refer to the BMSCs as alveolar BMSCs. In addition, alveolar and iliac bone marrow was obtained from 5- to 6-month-old female beagle dogs using a Komiya's puncture16G needle (1.5 × 25 mm; Kurita Injection Syringes, Tokyo, Japan). The puncture was made near lower molars under anesthesization. In pilot studies, a medium containing heparin was added to the marrow aspirates a few minutes after aspiration. However, this impaired ex vivo expansion of BMSCs, perhaps because of partial coagulation. Thus, in this study, marrow aspirates were mixed immediately with 1–3 ml of DMEM (Sigma), supplemented with 200 units/ml heparin. The cells were centrifuged at 500*g* for 5 minutes and resuspended with DMEM without heparin. Bone marrow cells including erythrocytes were seeded at a density of 0.1 ml aspirate/35-mm tissue culture dish (Corning) and maintained in 2 ml of DMEM supplemented with 10% FBS (Hyclone) and antibiotics (100 units/ml penicillin G and 100 μg/ml streptomycin; medium-A). Three days after seeding, floating cells were removed, and the medium was replaced by fresh medium-A. Thereafter, attached cells were fed with fresh medium-A supplemented with 1 ng/ml of bFGF, which was added every other day. Passages were performed when cells were approaching confluence: the cells were seeded at a density of $5 \times 10^3$ cells/cm$^2$ on 60- or 100-mm tissue culture dishes (Corning) and maintained in 4 or 10 ml of medium-A supplemented with 1 ng/ml of bFGF.

### *Differentiation potentials of BMSCs*

Osteogenic, chondrogenic, and adipogenic conversion of BMSCs was determined according to the procedures reported by Pittenger et al. with some modifications.[9] For osteogenic differentiation, cells were seeded at $4 \times 10^4$ cells/16-mm well ($2.3 \times 10^4$ cells/cm$^2$) and maintained for 21–28 days in DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbic acid-2-phosphate. For chondrogenic differentiation, cells were seeded at $2.5 \times 10^5$ cells/15-ml plastic centrifuge tube and maintained for 28 days in 0.5 ml of serum-free α-MEM (high glucose) supplemented with 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenite, 5.33 μg/ml linolate, 1.25 mg/ml bovine serum albumin, 10 ng/ml transforming growth factor-β3, 100 nM dexamethasone, and 50 μg/ml ascorbic acid-2-phosphate. The cultures were fed with 0.5 ml of the medium until 3 days after seeding. Thereafter, the cultures were fed with 1 ml of the medium every other day. Sections of these pellets were stained with toluidine blue on day 28. For adipogenic differentiation, cells were seeded at $2 \times 10^5$ cells/35-mm well ($2.3 \times 10^4$ cells/cm$^2$) and grown to confluence in medium-A. Adipogenic differentiation was induced by subjecting confluent monolayers to three rounds of adipogenic treatments. Each consisted of incubation with adipogenic induction medium (DMEM-high glucose, 10% FBS, 0.2 mM indomethacin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine, and 10 μg/ml insulin) for 72–96 h, followed by incubation with maintenance medium (DMEM-high glucose, 10% FBS, and 10 μg/ml insulin) for another 72–96 h.

### *Glycosaminoglycan, alkaline phosphatase activity, calcium, GAPDH activity, and DNA*

The glycosaminoglycan (GAG) content was determined using a sulfated GAG assay kit (Biocolor).[10] Alkaline phosphatase (ALP) activity was determined by the method of Bessey et al.[11] The calcium content was determined by the method of Gitelman.[12] GAPDH activity was determined using a GAPDH activity assay kit (Hokudo, Sapporo, Japan).[13] DNA was determined using a fluorescent DNA quantification kit (Bio-Rad).

### *RT-PCR analysis of BMSC cultures*

Total RNA was extracted from cultures using Isogen (Nippon Gene, Tokyo, Japan). The first-strand cDNA was synthesized from 1 μg of total RNA using the SUPERSCRIPT II RNase H$^-$ reverse transcriptase (Life Technologies). Using the cDNA as a template, PCR was carried out under the following conditions: denaturation at 94°C for 30 s and primer extension at 65°C for 1.5 minutes in 30 cycles. Pairs of nucleotides, 5′-GTCAAGGCCGAGAATGGGAA-3′ and 5′-GCTTCACCACCTTCTTGATG-3′ for GAPDH (GenBank Accession no., M33197, 613 bp), 5′-CATTTTGGGAATGGCCTGTG-3′ and 5′-

ATTGTCTCCTCCGCTGCTGC-3' for bone sialoprotein (J05213, 565 bp), 5'-CTAGGCATCACCTGTGCCA-TACC-3' and 5'-CAGTGACCAGTTCATCAGATT-CATC-3' for osteopontin (J04765, 331 bp), and 5'-CCACCGAGACACCATGAGAG-3' and 5'-CCATA-GGGCTGGGAGGTCAG-3' for osteocalcin (X53698, 419 bp) were used as primers for RT-PCR. Obtained PCR products were separated on 1% agarose gels and stained with ethidium bromide.

### *FACS analysis*

BMSCs at passages 3–4 were harvested with trypsin and EDTA, centrifuged at 1500*g* for 5 minutes, and resuspended at $5 \times 10^6$ cells/ml in PBS containing 3% FBS. Aliquots containing $10^5$ cells were incubated with individual primary antibodies or control IgG for 30 minutes at room temperature. The cells were washed in PBS containing 3% FBS and incubated with a fluorescent conjugated secondary antibody for 30 minutes at room temperature. Samples were analyzed using a FACSCalibur cytometer (Becton Dickinson), and the data were analyzed using the CELLQUEST software (Becton Dickinson). The following monoclonal antibodies (mAbs) were used: fluorescein isothiocyanate (FITC)-conjugated or R-phycoerythrin (PE)-conjugated antibodies against CD13, CD14, CD29, CD34, CD44, CD49b, CD54, CD56, CD71, CD90, CD105, CD106, CD117, CD124, CD138, CD144, HLA-ABC, HLA-DR, mouse-IgG1, mouse-IgG2a, or mouse IgM (Immunotech Coulter Company); antibodies against CD123, CD140b, CD166, or mouse-IgG3 (Pharmingen); antibodies against Flk-1 (Santa Cruz Biotechnology); antibodies against MT-MMP-1 (Sigma); antibodies against STRO-1 (Genzyme); antibodies against RANKL (R & D Systems), and anti-rabbit-IgG (Chemicon International).

### *Transplantation of BMSCs into mice*

The potential for cells to differentiate into osteoblasts after transplantation into immunodeficient mice was assessed as described.[14,15] Human alveolar BMSCs at passages 3–5 ($1.5 \times 10^6$ cells) in 1.0 ml of DMEM were mixed with 40 mg β-tricalcium phosphate (β-TCP) powder (OSferion; Olympus Co., Tokyo, Japan). After incubation at 37°C for 90 minutes, the mixture was centrifuged at 1500 rpm for 1 minute, and the supernatant was discarded. The pellet of β-TCP powder with adherent cells was mixed with 15 μl of mouse fibrinogen (3.3 mg/ml solution in PBS) and mouse thrombin (25 U/ml in 2% $CaCl_2$; both from Sigma) to form a fibrin clot. The fibrin clots transplanted into 5-week-old female CB-17 scid/scid (SCID; severe combined immunodeficiency) mice (Nihoncrea, Tokyo, Japan). After anesthetizing by intraperitoneal injection with 10% Nembutal (Dainihon Seiyaku Co., Osaka, Japan) in PBS, five skin incisions were made on the dorsal surface of each mouse, fibrin clots (five per mouse) were transplanted, and incisions were sutured. The transplants were harvested 8 weeks after transplantation.

### *Immunohistochemical and histomorphometrical analyses of transplanted sample*

The transplants were fixed in 4% paraformaldehyde for 1 day, decalcified with 10% formic acid for 3 days, and embedded in paraffin. Four-micrometer-thick sections were prepared in the middle of the transplants, collected on poly-L-lysine-coated slides, and stained using H&E for histochemical examination. To examine human vimentin expression immunohistochemically, endogenous peroxidase was quenched by incubating with 1% $H_2O_2$ and methanol. The sections were incubated with anti-human vimentin mAb (Dako; 50-fold dilution with Dako Antibody Diluent) for 1 h and treated with Envision (Dako). Color reaction was developed with diaminobenzidine (Dako). To examine osteocalcin expression, the sections were incubated with antihuman osteocalcin mAb (200-fold dilution; Biomedical Technologies, Stoughton, MA, USA) for 1 h, and color was developed with DAKO LSAB +System, HRP (Dako). Bone formation area in three fields (random sampling, 0.41 $mm^2$/field) in the middle sections—stained with H&E—of the transplants of alveolar BMSC (a9) plus β-TCP powder or β-TCP powder alone was captured by CCD camera (coolpix 4500; Nikon, Tokyo, Japan), and the bone area in the pictures was traced with Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA, USA); the bone area is expressed as the percentage of total area (0.41 $mm^2$).

### *RNA extraction from transplanted samples*

Total RNA was prepared from the transplants as described.[16] Briefly, extracted samples were snap-frozen in liquid nitrogen and pulverized for 1.5 minutes at 2000 rpm in a mixer mill. A 1-ml aliquot of TriReagent (Sigma) was added directly to the powdered samples and warmed to room temperature. Each sample was transferred to a 1.5-ml microcentrifuge tube and mixed by orbital rotation for 10 minutes at room temperature. After the addition of 0.2 ml of chloroform, samples were vortexed and allowed to sit at room temperature for 15 minutes, and then centrifuged for 20 minutes at 12,000 rpm. The upper aqueous phase was removed and mixed with an equal volume of 70% ethanol. Total RNA was isolated using RNeasy minicolumns and reagents (Qiagen). DNase I (Ambion) treatment was performed to remove genomic DNA from RNA samples according to the manufacturer's protocol.

### *PCR analysis of implanted samples*

First-strand cDNA was synthesized by Omniscript reverse transcriptase (Qiagen; 2 μg total RNA/20-μl reaction volume) using oligo dT primer (Promega). PCR amplification was performed (1 μl cDNA solution/25-μl reaction volume) using oligonucleotide primers corresponding to cDNA sequences for human-specific GAPDH (GenBank Accession no. M33197, 347 bp; sense 5'-CACCAGGTG-GTCTCCTCT-3', antisense 5'-GTACATGACAAGGT-GCGG-3'), human-specific osteocalcin (X53698, 315 bp; sense 5'-CATGAGAGCCCTCACA-3', antisense 5'-AGAGCGACACCCTAGAC-3'), mouse-specific osteocalcin (X04142, 443 bp; sense 5'-AACAGACAAGTC-CCACACAG-3', antisense 5'-GCTGTGACATCCATA-CTTGC-3'), and human and mouse GAPDH (M33197 and M32599, 268 bp; sense 5'-CACCAGGTGGTCTCCTCT-3', antisense 5'-GTACATGACAAGGTGCGG-3') using *Taq* polymerase (Promega). PCR was performed at 94°C/