Journal of
PERIODONTAL RESEARCH

J Periodont Res 2012; 47: 645–654

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2012.01477.x

# Dose- and time-dependent effects of recombinant human bone morphogenetic protein-2 on the osteogenic and adipogenic potentials of alveolar bone-derived stromal cells

**J-C. Park[1], J.C. Kim[1], B-K. Kim[1], K-S. Cho[1], G-I. Im[2], B-S. Kim[3], C-S. Kim[1]**

[1]Department of Periodontology, Research Institute for Periodontal Regeneration, College of Dentistry, Yonsei University, Seoul, Korea, [2]Department of Orthopedics, Dongguk University International Hospital, Goyang, Korea and [3]School of Chemical and Biological Engineering, Bio-MAX Institute, Institute of Chemical Processes, Engineering Research Institute, Seoul National University, Seoul, Korea

*Park J-C, Kim J.C, Kim B-K, Cho K-S, Im G-I, Kim B-S, Kim C-S. Dose- and time-dependent effects of recombinant human bone morphogenetic protein-2 on the osteogenic and adipogenic potentials of alveolar bone-derived stromal cells. J Periodont Res 2012; 47: 645–654. © 2012 John Wiley & Sons A/S*

*Background and Objective:* Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a well-known growth factor that can induce robust bone formation, and recent studies have shown that rhBMP-2-induced osteogenesis is closely related to adipogenesis. The aim of the present study was to determine the dose- and time-dependent effects of rhBMP-2 on the osteogenic and adipogenic differentiation of human alveolar bone-derived stromal cells (hABCs) *in vivo* and *in vitro*.

*Material and Methods:* hABCs were isolated and cultured, and then transplanted using a carrier treated either with or without rhBMP-2 (100 μg/mL) into an ectopic subcutaneous mouse model. Comprehensive histologic and histometric analyses were performed after an 8-wk healing period. To further understand the dose-dependent (0, 10, 50, 200, 500 and 1000 ng/mL) and time-dependent (0, 3, 5, 7 and 14 d) effects of rhBMP-2 on osteogenic and adipogenic differentiation, *in vitro* osteogenic and adipogenic differentiation of hABCs were evaluated, and the expression of related mRNAs, including those for alkaline phosphatase, osteocalcin, bone sialoprotein, peroxisome-proliferator-activated receptor gamma-2 and lipoprotein lipase, were assessed using quantitative RT-PCR.

*Results:* rhBMP-2 significantly promoted the osteogenic and adipogenic differentiation of hABCs *in vivo*, and gradually increased both the osteogenic and adipogenic potential in a dose- and time-dependent manner with minimal deviation *in vitro*. The expression of osteogenesis- and adipogenesis-associated mRNAs were concomitantly up-regulated by rhBMP-2.

*Conclusion:* The findings of the present study showed that rhBMP-2 significantly enhanced the adipogenic as well as the osteogenic potential of hABCs in dose- and time-dependent manner. The control of adipogenic differentiation of hABCs should be considered when regenerating the alveolar bone using rhBMP-2.

Professor Chang-Sung Kim, DDS, PhD, Department of Periodontology, Research Institute for Periodontal Regeneration, College of Dentistry, Yonsei University, Seoul 120-752, South Korea
Tel: +82222283186
Fax: +8223920398
e-mail: dentall@yuhs.ac

Key words: adipogenesis; osteogenesis; recombinant human bone morphogenetic protein-2; stem cells; tissue engineering

Accepted for publication February 19, 2012

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily and were discovered as a result of the bone-inductive activity concealed in the bone matrix (1). One of these proteins, recombinant human BMP-2 (rhBMP-2), has been evaluated in various studies of the augmentation of the alveolar process and the placement of dental implants (2–5). It has also been demonstrated that dental implants placed in bone induced by rhBMP-2 exhibited successful osseointegration and respond as well to functional loading as do dental implants placed in native alveolar bone (6).

While rhBMP-2 has yielded promising results regarding alveolar bone reconstruction, the quality of newly formed bone induced by rhBMP-2 appears to be poorer than native alveolar bone, having a significantly lower density and a larger area of bone marrow formation (7–9). Furthermore, bone voids, which are diagnosed as seromas, are typically observed both radiographically and histologically in newly formed bone induced by rhBMP-2 (4,6,9,10), and the cyst-like bone formation containing bone voids are observed particularly frequently when the high doses of rhBMP-2 required for human bone regeneration are applied (11). One recent study has identified the presence of adipose tissue in these bone voids (12), and further studies are continuing to elucidate the mechanism of the bone void formation. It appears that these seromas gradually resolve, becoming filled with bone, and thus enable successful osseointegration and functional loading of dental implants (11). However, the quality of new bone induced by rhBMP-2, with its low bone density and abundant bone voids, remains a matter of controversy.

In our previous study (13), we investigated the effects of rhBMP-2 on human periodontal ligament stem cells (hPDLSCs) to elucidate the mechanism of the minimally beneficial effect of rhBMP-2 on periodontal tissue regeneration. Interestingly, we observed significantly increased adipogenic differentiation of hPDLSCs that was driven by treatment with rhBMP-2, resulting in the formation of large amounts of adipose tissue, in line with the aforementioned studies. Consequently, we were curious as to whether rhBMP-2 would affect human alveolar bone-derived stromal cells (hABCs) in the same manner, and hypothesized that rhBMP-2 would also enhance adipogenic differentiation as well as osteogenic differentiation of hABCs, which are highly involved in the regeneration of alveolar bone tissue as a result of their innate potential for osteogenic differentiation (14). An understanding of the effects of rhBMP-2 on hABCs might elucidate the cellular mechanisms underlying rhBMP-2-induced alveolar bone tissue regeneration, and potentially suggest a method for improving the quality of that regenerated alveolar bone. To the best of our knowledge, no studies on the dose- and time-dependent effects of rhBMP-2 on the adipogenic or osteogenic differentiation of hABCs have been performed using an ectopic transplantation model, a well-established study model used to observe the behavior of transplanted cells in the absence of the innate healing factors present *in vivo* (15,16). Therefore, the aims of the present study were to determine the dose- and time-dependent effects of rhBMP-2 on hABCs in terms of both the osteogenic potential and adipogenic differentiation *in vivo* and *in vitro*.

## Material and methods

### Isolation of hABCs from bone chips acquired during minimally irrigated implant osteotomy

hABCs were obtained from the alveolar bone of 10 donors (29–63 years of age) during dental implant surgery, in accordance with a protocol approved by the Institutional Review Board of Yonsei University. Immediately prior to surgery, patients performed a 2-min oral rinse using chlorhexidine (Hexamedine, Bukwang, Seoul, Korea) to minimize the probability of bacterial contamination (17). Sequential osteotomies were performed using a lancet drill, a 2-mm-diameter twist drill and consecutively larger drills to the final size, according to the implant manufacturer's instructions. After drilling with a 2-mm-diameter twist drill, the drill speed was reduced to 50–200 rpm (KaVo Intrasurg 300 Plus system; Kavo, Lake Zurich, IL, USA), and cooling saline irrigation was minimized thereafter, in accordance with a previous report (18). The bone particles released during drilling were captured in the drill flutes. The bone chips thus obtained were placed in a 50-mL tube and immersed in 20 mL of α-minimal essential medium (α-MEM) containing 15% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 μg/mL of streptomycin and amphotericin B (all from Gibco, Invitrogen, Grand Island, NY, USA). The hABCs were then immediately isolated using a sequential digestion method with 2 mg/mL of collagenase (Wako Pure Chemicals, Osaka, Japan) and 1 mg/mL of dispase (Gibco, Invitrogen). Single-cell suspensions were obtained by passing the digested tissues through a strainer with a 70-μm pore size (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA), and the cells ($5 \times 10^5$) were seeded onto T75 cell-culture dishes containing α-MEM supplemented with 15% FBS (Gibco, Invitrogen), 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St Louis, MO, USA), 2 mM L-glutamine, 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco, Invitrogen), and incubated at 37°C in a 5% $CO_2$ atmosphere. Single-cell colonies were observed after 3–7 d and P0 passages of hABCs were cultured.

### hABC transplantation using a carrier, either pretreated or not pretreated with rhBMP-2, into an ectopic subcutaneous transplantation model

The cell carrier used for ectopic transplantation was 80 mg of hydroxyapatite/tricalcium phosphate ceramic powder [macroporous biphasic calcium phosphate (MBCP); Biomatlante, Vigneux, France] (19). hABCs ($6 \times 10^6$ cells per carrier, P5) were precultured for 1.5 h on a carrier

before transplantation at 37°C in a 5% $CO_2$ atmosphere. The rhBMP-2 expressed in *Escherichia coli* was produced at the Research Institute of Cowellmedi, Pusan, Korea, as described previously (20,21). The carrier was either pretreated or not pretreated with rhBMP-2 (100 μL of 0.05 mg/mL per carrier) at 4°C overnight before cell seeding to observe the effect of rhBMP-2 on hABCs, in an extension of a previous report studying the effect of rhBMP-2 on hPDLSCs (13). The carriers with hABCs were transplanted into a subcutaneous ectopic transplantation immunodeficient mouse model, as described previously (15,16). Animal selection, management and surgical procedures followed a protocol that was approved by the Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea. The animals were treated under one of the following four experimental conditions:

1 MBCP carrier only (control, rhBMP-$2^-$/hABC$^-$ group).
2 MBCP carrier pretreated with rhBMP-2 (rhBMP-$2^+$/hABC$^-$ group).
3 hABCs seeded onto an otherwise untreated MBCP carrier (rhBMP-$2^-$/hABC$^+$ group).
4 hABCs seeded onto an rhBMP-2-pretreated MBCP carrier (rhBMP-$2^+$/hABC$^+$ group).

Each group included five nude mice, each with four ectopic transplantations. The animals were allowed to heal for 8 wk, after which they were killed and tissue analysis was performed. The specimens were fixed with 4% formalin, decalcified with buffered 5% EDTA (pH 7.2–7.4) and then embedded in paraffin and sectioned at a thickness of 5 μm. The sections were deparaffinized, stained with hematoxylin and eosin and Masson's trichrome, and then observed with the aid of a light microscope (BX-50; Olympus Optical, Tokyo, Japan). Histometric analysis of the new bone and of adipose tissue formation was conducted with the aid of computer-assisted histometric measurements, which were made using an automated image-analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA), as described previously (13,22,23).

### Dose- and time-dependent rhBMP-2-induced osteogenic and adipogenic differentiation

While *in vivo* studies have been compelling, in order to better understand certain causal relationships among rhBMP-2 and hABCs, the *in vitro* effects of different doses of rhBMP-2 on osteogenic and adipogenic differentiation were assessed. The cells ($1 \times 10^5$, P5) were seeded into six-well culture dishes and then cultured until they reached a subconfluent stage. They were then treated with rhBMP-2 at 0, 10, 50, 200, 500 and 1000 ng/mL for an induction period of either 4 wk (osteogenic differentiation) or 2 wk (adipogenic differentiation). The dosages were determined based on the previous report to compare the dose-dependent effect of rhBMP-2 on hABCs with hPDLSCs (13). The osteogenic and adipogenic culture media described below were refreshed twice a week in order to maintain a constant concentration of rhBMP-2. Osteogenic and adipogenic differentiation of hABCs was determined according to a modification of procedures reported previously (13,22,23). Briefly, the culture medium for osteogenic differentiation comprised α-MEM containing 15% FBS, 2 mM L-glutamine, 100 μM L-ascorbic acid-2-phosphate, 1.8 mM $KH_2PO_4$, 100 U/mL of penicillin, 100 μg/mL of streptomycin (all from Gibco, Invitrogen) and 10 nM dexamethasone (Sigma-Aldrich). The culture medium for adipogenic differentiation comprised α-MEM containing 15% FBS, 2 mM L-glutamine, 100 μM L-ascorbic acid-2-phosphate, 500 μM isobutyl-methylxanthine, 60 μM indomethacin, 0.5 μM hydrocortisone, 10 μM insulin (all from Sigma-Aldrich), 100 U/mL of penicillin and 100 μg/mL of streptomycin.

After 2 wk (adipogenic differentiation) and 4 wk (osteogenic differentiation) of induction, the cells were stained with Oil Red O and Alizarin Red, respectively, and evaluated by measuring the areas of adipocyte formation and newly formed mineralized nodules. Computer-assisted measurements were performed on eight images of each sample, with mean values being obtained using an automated image-analysis system (Image-Pro Plus; Media Cybernetics).

After demonstration of the dose-dependent effect of rhBMP-2, the time-dependent effects of rhBMP-2 induction on *in vitro* osteogenic and adipogenic differentiation were also evaluated. Cells were treated with 200 ng/mL of rhBMP-2 during the induction period for both osteogenic and adipogenic differentiation, and the dose of rhBMP-2 was determined

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

| Gene | Primer sequence | | Annealing temperature (°C) | GenBank no. | Product size (bp) |
|---|---|---|---|---|---|
| | Forward (5′–3′) | Reverse (3′–5′) | | | |
| ALP | GGACCATTCCCACGTCTTCAC | CCTTGTAGCCAGGCCCATTG | 60 | NM_000478.4 | 137 |
| OCN | CAAAGGTGCAGCCTTTGTGTC | TCACAGTCCGGATTGAGCTCA | 60 | NM_199173.3 | 150 |
| BSP | CTGGCACAGGGTATACAGGGTTAG | ACTGGTGCCGTTTATGCCTTG | 60 | NM_004967.3 | 182 |
| PPARγ-2 | ACAGCAAACCCCTATTCCATGCTGT | TCCCAAAGTTGGTGGGCCAGAA | 64 | NM_015869.4 | 159 |
| LPL | TGGACTGGCTGTCACGGGCT | GCCAGCAGCATGGGCTCCAA | 64 | NM_000237.2 | 167 |
| β-Actin | CAT GTA CGT TGC TAT CCA GGC | CTC CTT AAT GTC ACG CAC GAT | 58 | NM_001101.3 | 249 |

ALP, alkaline phosphatase; BSP, bone sialoprotein; LPL, lipoprotein lipase; OCN, osteocalcin; PPARγ-2, peroxisome-proliferator-activated receptor gamma-2.

based on a previous study (13). The cells were stained with Alizarin Red and Oil Red O, 0, 3, 5, 7 and 14 d after culture initiation.

### Total RNA extraction and RT-PCR

Total RNA was isolated from *in vitro* culture specimens using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) and confirmed by quantitative RT-PCR (qRT-PCR), according to the manufacturer's instructions. hABCs that had not been exposed to rhBMP-2 for the induction of osteogenic or adipogenic differentiation were analyzed as controls. Isolated total RNA was used as a template for the synthesis of cDNA with CycleScript RT PreMix (Bioneer, Daejeon, Korea). The subsequent PCR-amplification reaction utilized the LightCycler480 II real-time PCR kit (Roche Diagnostics, Penzerg, Germany), the ABI 7500 real-time PCR system and associated software (Applied Biosystems, Foster City, CA, USA), and specific primers (as listed in Table 1). The relative levels of mRNA expression were quantified by comparison with those of an internal standard (β-actin). Each PCR was performed in triplicate with the same total RNA.

### Statistical analysis

Multiple comparisons were made using analysis of variance and post-hoc *t*-tests. Differences were considered to be statistically significant when $p < 0.05$.

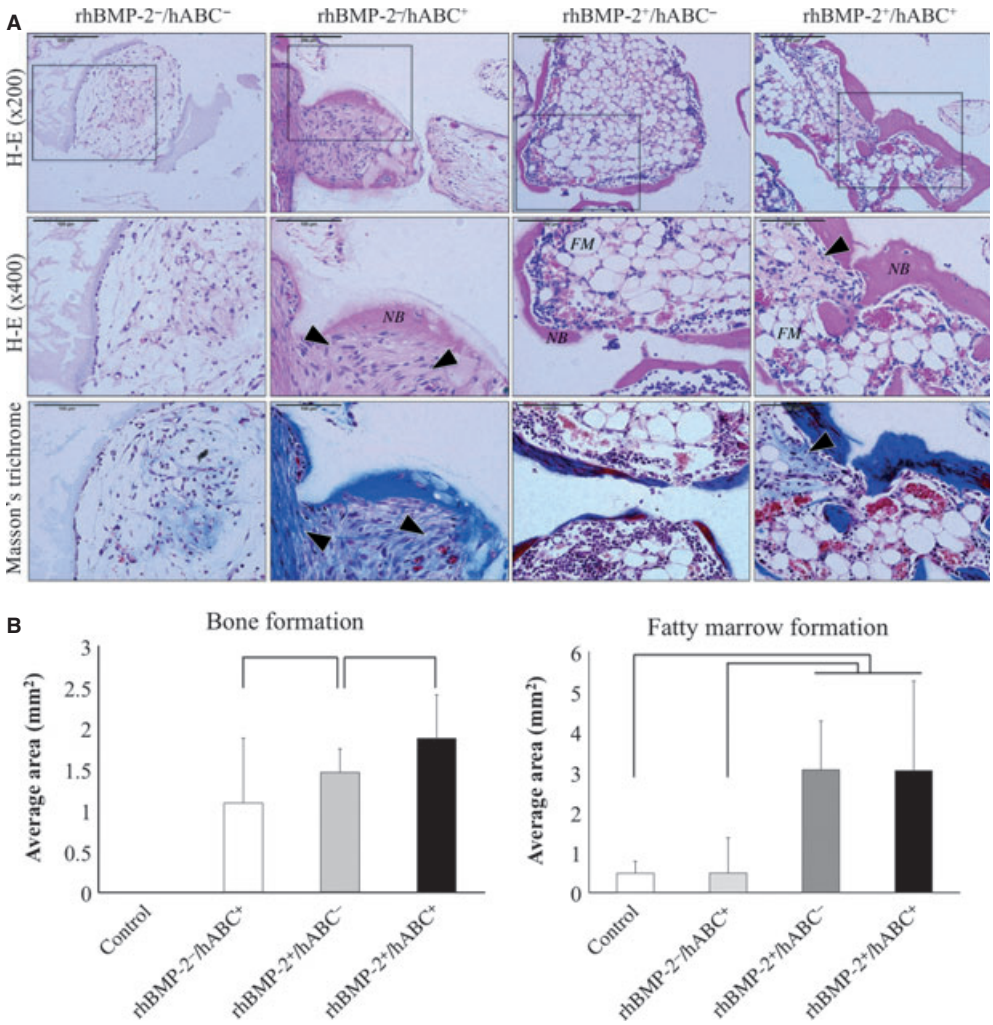


*Fig. 1.* Effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the induction of bone by human alveolar bone-derived stromal cells (hABCs). (A) hABCs were transplanted using a macroporous biphasic calcium phosphate (MBCP) carrier, treated with or without rhBMP-2, into the subcutaneous pocket of immunodeficient mice, and histologic analysis was performed after 8 wk of healing. Histological evaluations were performed by hematoxylin and eosin (H-E), and Masson's trichrome staining was performed to reveal collagen formation. Inset boxes show areas at higher magnification. Arrowheads show highly dense fibrous tissue with high cellularity. FM, fatty marrow; NB, new bone. (B) Histomorphometric measurements showing the area of new bone and fatty marrow formation. There was a statistically significant difference between the groups, and the rhBMP-$2^+$/hABC$^+$ group showed the largest amount of new bone formation. Both rhBMP-$2^+$/hABC$^-$ and rhBMP-$2^+$/hABC$^+$ groups showed the largest amount of fatty marrow formation. Solid lines: $p < 0.005$.

## Results

### Histology observations from the ectopic transplantation: an *in vivo* study

During the experiment, all animals experienced uneventful healing of the surgical area, and no postoperative infections were observed. At 8 wk postsurgery, the control group exhibited the formation of sparsely located loose connective tissue along the carrier materials (Fig. 1). Masson's trichrome staining revealed collagen formation (stained in blue); however, the amount and the degree of collagen fiber formation were minimal. Conversely, the rhBMP-$2^-$/hABC$^+$ group exhibited highly dense collagen tissue formation in association with newly formed bone tissue. The new bone was formed mainly along the surface of the carrier materials and within the macropores. Several osteocytes were entrapped in the newly formed bone and a few osteoblasts were observed aligned along the new bone surface. The rhBMP-$2^+$/hABC$^-$ group demonstrated significantly greater bone formation than the rhBMP-$2^-$/hABC$^+$ group. New bone formation was also observed mainly along the surface of the carrier materials, and several osteocytes were observed within the newly formed bone. Interestingly, marked formation of fatty marrow spaces was a unique histology finding in comparison with the rhBMP-$2^-$/hABC$^+$ group, and the total area of adipocytic fatty marrow was significantly greater in the rhBMP-$2^+$/hABC$^-$ and rhBMP-$2^+$/hABC$^+$ groups than in the others. Another unique histologic finding was that the formation of highly dense collagen fibers, which was observed dominantly in the rhBMP-$2^-$/hABC$^+$ group, was rarely found in the rhBMP-$2^+$/hABC$^-$ group, in which overwhelmingly dominant adipocyte formation was apparent. In the rhBMP-$2^+$/hABC$^+$ group, we could observe the concurrent formation of highly dense collagen tissue and fatty marrow, which were respectively observed in the rhBMP-$2^-$/hABC$^+$ and rhBMP-$2^+$/hABC$^-$ groups, and the formation of new bone was significantly enhanced in comparison with other groups ($p < 0.05$).

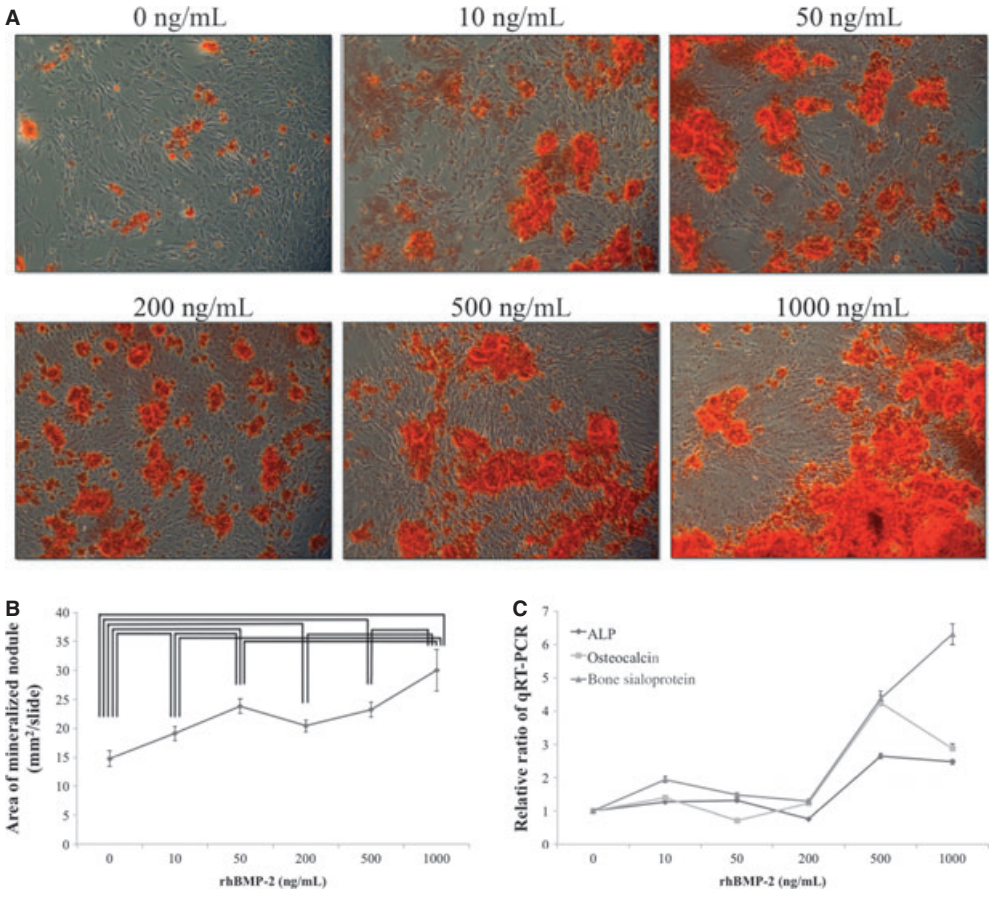


*Fig. 2. In vitro* osteogenic differentiation of human alveolar bone-derived stromal cells (hABCs) treated with different doses (0, 10, 50, 200, 500, and 1000 ng/mL) of recombinant human bone morphogenetic protein-2 (rhBMP-2). Original magnification: ×400. (A) The formation of mineralized nodules was observed by staining with Alizarin Red. (B) The areas of mineralized nodules gradually increased in a dose-dependent manner. Solid lines: $p < 0.05$. (C) Quantitative RT-PCR (qRT-PCR) results obtained for osteogenesis-related mRNAs, including those for alkaline phosphatase (ALP), osteocalcin and bone sialoprotein. High increases were observed at 500 and 1000 ng/mL of rhBMP-2.

### rhBMP-2 dose-dependently enhanced the osteogenic differentiation of hABCs: an *in vitro* study

Given the observation in this study that rhBMP-2 significantly enhanced the bone-forming activities of hABCs, we tested the *in vitro* effect of different doses of rhBMP-2 on this osteogenic potential. In the absence of rhBMP-2 (i.e. 0 ng/mL), the osteogenic medium only minimally enhanced the osteogenic differentiation of hABC, and a few mineralized nodules were observed (Fig. 2A). However, the area of mineralized nodules that stained positive for Alizarin Red increased significantly with the rhBMP-2 dose, although there were minimal deviations (Fig. 2B). Furthermore, the real-time PCR assay revealed a dose-dependent increase in the representative osteogenic markers for alkaline phosphatase (ALP), osteocalcin, and bone sialoprotein; Fig. 2C).

### rhBMP-2 dose-dependently enhanced the adipogenic differentiation of hABCs: an *in vitro* study

Following the demonstration of rhBMP-2-induced osteogenic differentiation by hABCs, the effects of different doses of rhBMP-2 on the adipogenic differentiation were evaluated using Oil Red O staining of lipid accumulations (Fig. 3A). A significant increase in adipogenic differentiation was observed for 1000 ng/mL of rhBMP-2. There was a general tendency toward an incremental increase of adipogenic differentiation in hABCs (Fig. 3B), and the 500- and 1000-ng/mL doses of rhBMP-2 had a notable effect on this parameter compared with the control (0 ng/mL) in the expression of adipogenesis-related mRNAs, including those for peroxisome-proliferator-activated receptor gamma-2 (PPARγ-2) and lipoprotein lipase (LPL) (Fig. 3C). Thus, it seemed that rhBMP-2 signaling generally induced adipogenic differentiation in a dose-dependent manner.

### rhBMP-2 time-dependently increased the osteogenic and adipogenic differentiation of hABCs: an *in vitro* study

After the demonstration of an rhBMP-2 dose-dependent increase of

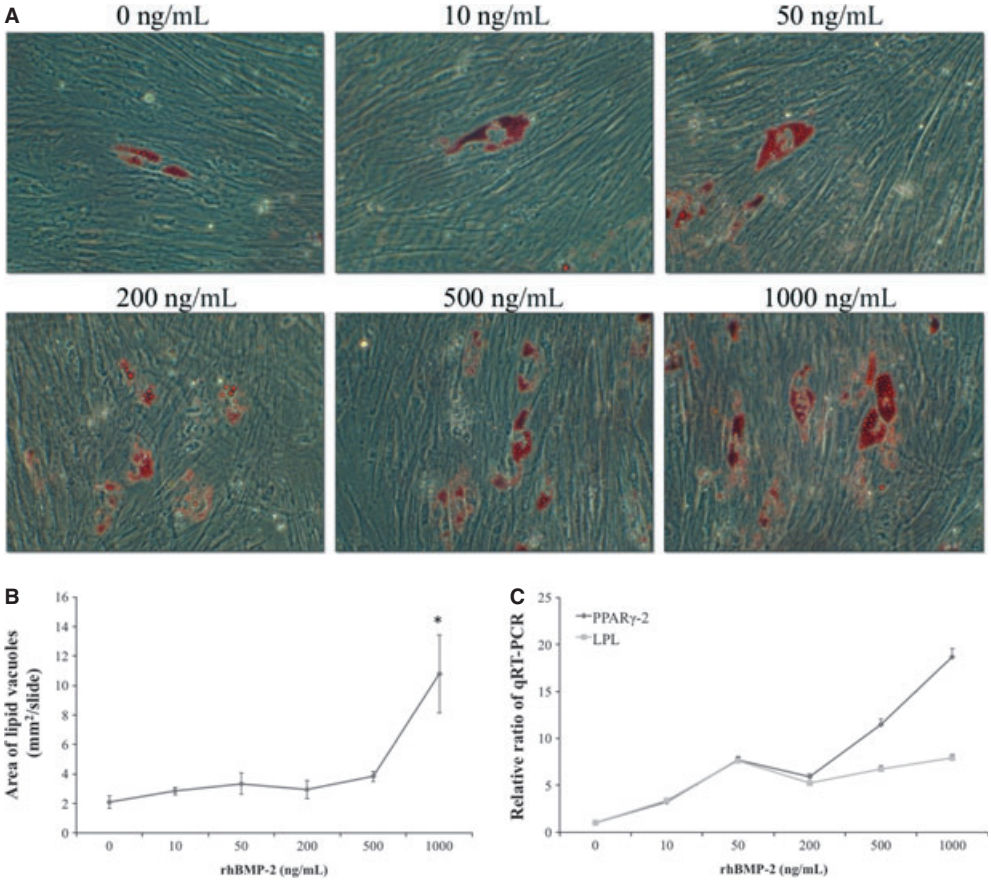


*Fig. 3. In vitro* adipogenic differentiation of human alveolar bone-derived stromal cells (hABCs) treated with different doses (0, 10, 50, 200, 500, and 1000 ng/mL) of recombinant human bone morphogenetic protein-2 (rhBMP-2). Original magnification: ×400. (A) The formation of lipid vacuoles was observed by staining with Oil Red O. (B) The areas of adipocyte formation gradually increased in a dose-dependent manner, especially at 1000 ng/mL of rhBMP-2. *Statistically significant difference from the other groups, $p < 0.05$. (C) Quantitative RT-PCR (qRT-PCR) results for adipogenesis-related mRNAs. Large increases were observed for peroxisome-proliferator-activated receptor gamma-2 (PPARγ-2) mRNA at 500 and 1000 ng/mL of rhBMP-2. LPL, lipoprotein lipase.

osteogenesis, a time-dependent effect of rhBMP-2 on hABCs was demonstrated regarding osteogenic and adipogenic differentiation. After induction by rhBMP-2 (200 ng/mL), the area of mineralized nodule and lipid vacuoles gradually increased in a time-dependent manner with minimal deviation (Figs 4 and 5). Osteogenic- and adipogenic-related expression of mRNAs, including those for ALP, bone sialoprotein, LPL and PPARγ-2, also exhibited a similar pattern, reaching a maximum at 14 d.

## Discussion

For the present study, we demonstrated that the bone-forming activity of hABCs was significantly increased following treatment with rhBMP-2 in the ectopic bone formation model, which is an ideal model for assessing the behavior of transplanted cells in the absence of the innate healing factors present *in vivo*. Interestingly, we could observe a marked formation of adipocytic fatty marrow after the application of rhBMP-2 with or without cell treatment around the newly formed bone, and the bone formation was significantly increased by the application of hABCs with rhBMP-2. We then established that both the *in vitro* osteogenic and adipogenic differentiation potentials were concomitantly increased in a dose- and time-dependent manner after treatment with rhBMP-2.

In the present study, we observed that the transplantation of hABCs induced ectopic bone formation in the rhBMP-$2^-$/hABC$^+$ group; however, the amount of newly formed bone was significantly smaller than that in the rhBMP-$2^+$/hABC$^-$ group. Although the alveolar bone marrow contained a population of potential mesenchymal pluripotent progenitor cells, which are capable of differentiating into various cell lineages under appropriate conditions, it seems that the degree of osteoinduction remained low in the absence of appropriate osteogenic signals. Clearly, treatment with osteogenic rhBMP-2 signals combined with simultaneous support from the mesenchymal stem cells in the rhBMP-$2^+$/hABC$^+$ group significantly induced the greatest amount of bone formation. hABCs stored within the carrier seem to serve as a reservoir of the inducible cell population, and result in better bone formation of superior

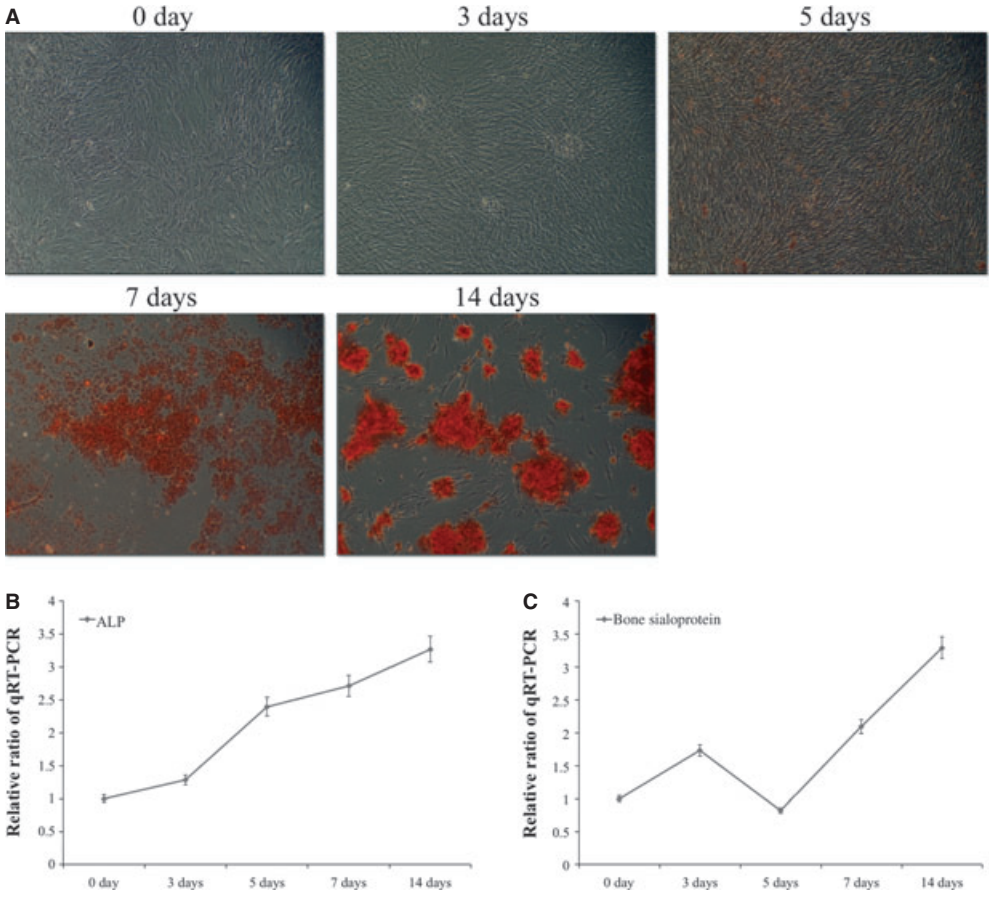


*Fig. 4.* Time-dependent increase in the osteogenic differentiation of human alveolar bone-derived stromal cells (hABCs) treated with 200 ng/mL of recombinant human bone morphogenetic protein-2 (rhBMP-2). Original magnification: ×100. (A) Formation of the mineralized area was observed by staining with Alizarin Red, and the positively stained area first appeared at 5 d after induction. (B, C) Quantitative RT-PCR (qRT-PCR) results for osteogenesis-related mRNAs, including alkaline phosphatase (ALP) and bone sialoprotein, showing a continuous increase of both in a time-dependent manner.

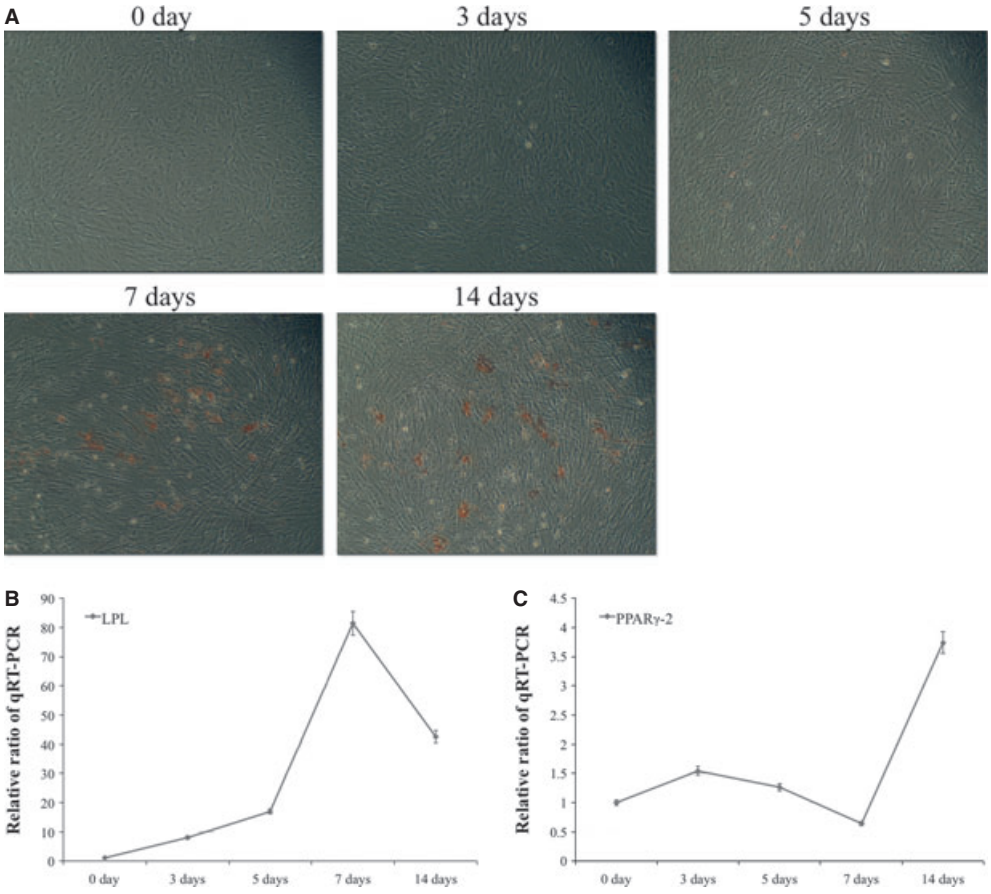


*Fig. 5.* Time-dependent increase of adipogenic differentiation of human alveolar bone-derived stromal cells (hABCs) treated with 200 ng/mL of recombinant human bone morphogenetic protein-2. Original magnification: ×100. (A) The formation of lipid vacuoles was observed by staining with Oil Red O, and the positively stained area first appeared at 7 d after induction. (B, C) Quantitative RT-PCR (qRT-PCR) results for adipogenesis-related mRNAs, including lipoprotein lipase (LPL) and peroxisome-proliferator-activated receptor gamma-2 (PPARγ-2).

performance by providing an acellular component (24).

These *in vivo* results are corroborated by those of our *in vitro* studies. In qRT-PCR analysis and in *in vitro* differentiation experiments, a general dose-dependent up-regulation pattern of ALP, osteocalcin and bone sialoprotein was observed, and the rate of mineralization confirmed a promotive effect of rhBMP-2 on hABCs. It is well known that rhBMP-2 induces the production of osteopontin in preosteoblasts, while osteocalcin is up-regulated in osteoblastic cells and bone sialoprotein is expressed in differentiated osteoblasts prior to mineralization (25). Collectively, our results confirm the previously demonstrated osteoinductive potential of rhBMP-2 (26,27).

There are several factors that affect the osteoinductive ability of rhBMP-2, including the possible presence of inhibitors, the type of carrier, the concentration and the duration of BMP action (27–29). A previous study found that low concentrations of BMP-2 (50 ng/mL) up-regulated the expression of the collagen II gene, whereas higher concentrations (100–400 ng/mL) inhibited its expression (30). Moreover, BMPs seem to have species-specific osteoinductive dose requirements, and it seems that rhBMP-2 induces cytodifferentiation in a specific way when permissive conditions for each cell type and the appropriate dose are provided. Therefore, the optimal dose for application in cells, animals and humans, or the application method for specific microenvironments, should be fully clarified to understand the role of rhBMP-2 in skeletal tissue engineering and to minimize the adverse reactions from using high doses of rhBMP-2, including cyst-like bone formation, severe tissue inflammation and cervical swellings.

One very interesting observation in the present study was that the newly formed tissue exhibited distinctive characteristics between the rhBMP-2-treated and hABC-regenerated tissues. In the rhBMP-$2^+$/hABC$^-$ group, dominant formation of adipocytic fatty marrow tissue was observed, while the rhBMP-$2^-$/hABC$^+$ group demonstrated the formation of highly dense collagen fibers in close contact with the newly formed bone. The rhBMP-$2^+$/hABC$^+$ group exhibited both histology characteristics equally and simultaneously, suggesting that there are two different pathways in new bone formation that are equally involved in this phenomenon. These results suggest

that newly formed bone tissue is also derived from two different pathways, but the distinction in histology was not achievable herein. Future studies should fully investigate this issue.

In our previous study (13), the transplanted hPDLSCs generated cementum-like tissue with numerous fiber insertion resembling Sharpey's fibers, and the application of rhBMP-2 inhibited the mineralization significantly. Although stem cells are supposedly multipotential and thus may differentiate into any cell type, it apparently seems that they are committed into certain lineages based on their origins. In the present study, hABCs mainly regenerated bone tissue and fatty bone marrow induced by rhBMP-2, unlike hPDLSCs. Our current knowledge limits us to elucidate these different reactions of hABCs and hPDLSCs to rhBMP-2; however, the understanding of this underlying mechanism could potentially explain why rhBMP-2 has not demonstrated favorable results when treated in the periodontal defects.

In the present study, bone formation was enhanced significantly by the combined application of hABCs and rhBMP-2; however, adipocytic fatty marrow formation was observed in the rhBMP-2-treated groups, regardless of simultaneous treatment with hABC, although we could not observe the significantly increased *in vivo* adipogenesis by rhBMP-2, unlike the results *in vitro*. Previous studies have shown that a reciprocal relationship exists between adipogenic and osteoblastic differentiation (31), and that the application of BMP-2 markedly promotes osteogenic differentiation in bone marrow stromal cells, while adipogenesisis was concomitantly inhibited (32–35). However, osteoblasts and adipocytes originate from a common source – mesenchymal progenitor cells – and their differentiation potential into osteoblastic and adipocytic lineages can be simultaneously induced by BMPs (32). Therefore, BMPs appear to play contradictory roles in adipogenesis, depending on the stage of the cell and the dose of BMP. For instance, a low dose of rhBMP-7 was found to modestly stimulate adipogenesis (32), while a high dose beyond a certain threshold seemed to increase adipogenesis. A potential mechanism underlying this phenomenon is that the adipogenic transcription factor, PPARγ-2, is readily up-regulated by BMP application in preosteoblast progenitor cells (36), and this leads bone marrow stem cells to differentiate into adipocytes rather than into osteoblasts (37–39). In addition, another recently proposed mechanism is the inhibition of the *Wnt* signaling pathway, which is involved in the down-regulation of adipogenesis (12). However, how osteogenic rhBMP-2 up-regulates PPARγ-2 and down-regulates the *Wnt* signaling pathway, and induces adipogenesis in a dose-dependent manner, remains to be established.

Collectively, increased bone formation was observed herein following the combined application of rhBMP-2 and hABC, both *in vivo* and *in vitro*, in a dose- and time-dependent manner. It is evident that certain doses of rhBMP-2 may result in favorable bone regeneration by hABCs. However, concomitantly increased adipose tissue formation was also observed, especially *in vitro*, and this unexpected phenomenon could produce abnormal bone tissue and compromise the mechanical stability of the newly formed bone tissue. A more detailed understanding of this mechanism might enhance the quality of alveolar bone regenerated by rhBMP-2.

## Acknowledgements

This study was supported by a Mid-career Researcher Program through an NRF grant funded by the MEST (No. 2009-0078884), and a grant of the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A100443). The authors would like to thank Ms J-M. Lee for providing excellent graphics.

## Conflicts of interest and source of funding statement

The authors declare that there are no conflicts of interest in this study.

## References

1. Urist MR. Bone: formation by autoinduction. *Science* 1965;**150:**893–899.
2. Wikesjo UM, Sorensen RG, Wozney JM. Augmentation of alveolar bone and dental implant osseointegration: clinical implications of studies with rhBMP-2. *J Bone Joint Surg Am* 2001;**83-A** (Suppl 1)**:**S136–S145.
3. Jung RE, Glauser R, Scharer P, Hammerle CH, Sailer HF, Weber FE. Effect of rhBMP-2 on guided bone regeneration in humans. *Clin Oral Implants Res* 2003;**14:** 556–568.
4. Sigurdsson TJ, Nguyen S, Wikesjo UM. Alveolar ridge augmentation with rhBMP-2 and bone-to-implant contact in induced bone. *Int J Periodontics Restorative Dent* 2001;**21:**461–473.
5. Hanisch O, Tatakis DN, Rohrer MD, Wohrle PS, Wozney JM, Wikesjo UM. Bone formation and osseointegration stimulated by rhBMP-2 following subantral augmentation procedures in nonhuman primates. *Int J Oral Maxillofac Implants* 1997;**12:**785–792.
6. Jovanovic SA, Hunt DR, Bernard GW *et al*. Long-term functional loading of dental implants in rhBMP-2 induced bone. A histologic study in the canine ridge augmentation model. *Clin Oral Implants Res* 2003;**14:**793–803.
7. Sigurdsson TJ, Fu E, Tatakis DN, Rohrer MD, Wikesjo UM. Bone morphogenetic protein-2 for peri-implant bone regeneration and osseointegration. *Clin Oral Implants Res* 1997;**8:**367–374.
8. Tatakis DN, Koh A, Jin L, Wozney JM, Rohrer MD, Wikesjo UM. Peri-implant bone regeneration using recombinant human bone morphogenetic protein-2 in a canine model: a dose-response study. *J Periodontal Res* 2002;**37:**93–100.
9. Wikesjo UM, Qahash M, Thomson RC *et al*. rhBMP-2 significantly enhances guided bone regeneration. *Clin Oral Implants Res* 2004;**15:**194–204.
10. Sigurdsson TJ, Lee MB, Kubota K, Turek TJ, Wozney JM, Wikesjo UM. Periodontal repair in dogs: recombinant human bone morphogenetic protein-2 significantly enhances periodontal regeneration. *J Periodontol* 1995;**66:**131–138.
11. Sciadini MF, Johnson KD. Evaluation of recombinant human bone morphogenetic protein-2 as a bone-graft substitute in a canine segmental defect model. *J Orthop Res* 2000;**18:**289–302.
12. Zara JN, Siu RK, Zhang X *et al*. High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. *Tissue Eng Part A* 2011;**17:**1389–1399.
13. Song DS, Park JC, Jung IH *et al*. Enhanced adipogenic differentiation and

reduced collagen synthesis induced by human periodontal ligament stem cells might underlie the negative effect of recombinant human bone morphogenetic protein-2 on periodontal regeneration. *J Periodontal Res* 2011;**46:**193–203.
14. Clausen C, Hermund NU, Donatsky O, Nielsen H. Characterization of human bone cells derived from the maxillary alveolar ridge. *Clin Oral Implant Res* 2006;**17:**533–540.
15. Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S. The efficacy of mesenchymal stem cells to regenerate and repair dental structures. *Orthod Craniofac Res* 2005;**8:**191–199.
16. Miura M, Gronthos S, Zhao M *et al*. SHED: stem cells from human exfoliated deciduous teeth. *Proc Nat Acad Sci USA* 2003;**100:**5807–5812.
17. Young MP, Korachi M, Carter DH, Worthington HV, McCord JF, Drucker DB. The effects of an immediately pre-surgical chlorhexidine oral rinse on the bacterial contaminants of bone debris collected during dental implant surgery. *Clin Oral Implant Res* 2002;**13:**20–29.
18. Flanagan D. Osteotomy irrigation: is it necessary? *Implant Dent* 2010;**19:**241–249.
19. Park JC, So SS, Jung IH *et al*. Induction of bone formation by *Escherichia coli*-expressed recombinant human bone morphogenetic protein-2 using block-type macroporous biphasic calcium phosphate in orthotopic and ectopic rat models. *J Periodontal Res* 2011;**46:**682–690.
20. Choi KH, Moon K, Kim SH, Cho KS, Yun JH, Jang KL. Production and purification of recombinant human bone morphogenetic protein-2 in a *E. coli* expression system. *J Korean Acad Periodontol* 2008;**38:**41–49.
21. Lee JH, Kim CS, Choi KH *et al*. The induction of bone formation in rat calvarial defects and subcutaneous tissues by recombinant human BMP-2, produced in *Escherichia coli*. *Biomaterials* 2010;**31:**3512–3519.
22. Park JC, Kim JM, Jung IH *et al*. Isolation and characterization of human periodontal ligament (PDL) stem cells (PDLSCs) from the inflamed PDL tissue: in vitro and in vivo evaluations. *J Clin Periodontol* 2011;**38:**721–731.
23. Park JC, Su C, Jung IH *et al*. Mechanism of alveolar bone loss in a collagen-induced arthritis model in mice. *J Clin Periodontol* 2011;**38:**122–130.
24. Lane JM, Yasko AW, Tomin E *et al*. Bone marrow and recombinant human bone morphogenetic protein-2 in osseous repair. *Clin Orthop Relat Res* 1999;**361:**216–227.
25. Cheifetz S, Li IW, McCulloch CA, Sampath K, Sodek J. Influence of osteogenic protein-1 (OP-1;BMP-7) and transforming growth factor-beta 1 on bone formation in vitro. *Connect Tissue Res* 1996;**35:**71–78.
26. Chen D, Harris MA, Rossini G *et al*. Bone morphogenetic protein 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts. *Calcif Tissue Int* 1997;**60:**283–290.
27. Wang EA, Rosen V, D'Alessandro JS *et al*. Recombinant human bone morphogenetic protein induces bone formation. *Proc Natl Acad Sci U S A* 1990;**87:**2220–2224.
28. Puleo DA. Dependence of mesenchymal cell responses on duration of exposure to bone morphogenetic protein-2 in vitro. *J Cell Physiol* 1997;**173:**93–101.
29. Raval P, Hsu HH, Schneider DJ *et al*. Expression of bone morphogenetic proteins by osteoinductive and non-osteoinductive human osteosarcoma cells. *J Dent Res* 1996;**75:**1518–1523.
30. Valcourt U, Ronziere MC, Winkler P, Rosen V, Herbage D, Mallein-Gerin F. Different effects of bone morphogenetic proteins 2, 4, 12, and 13 on the expression of cartilage and bone markers in the MC615 chondrocyte cell line. *Exp Cell Res* 1999;**251:**264–274.
31. Gimble JM, Robinson CE, Wu X, Kelly KA. The function of adipocytes in the bone marrow stroma: an update. *Bone* 1996;**19:**421–428.
32. Chen TL, Shen WJ, Kraemer FB. Human BMP-7/OP-1 induces the growth and differentiation of adipocytes and osteoblasts in bone marrow stromal cell cultures. *J Cell Biochem* 2001;**82:**187–199.
33. Gimble JM, Morgan C, Kelly K *et al*. Bone morphogenetic proteins inhibit adipocyte differentiation by bone marrow stromal cells. *J Cell Biochem* 1995;**58:**393–402.
34. Pereira RC, Delany AM, Canalis E. Effects of cortisol and bone morphogenetic protein-2 on stromal cell differentiation: correlation with CCAAT-enhancer binding protein expression. *Bone* 2002;**30:**685–691.
35. Song C, Guo Z, Ma Q *et al*. Simvastatin induces osteoblastic differentiation and inhibits adipocytic differentiation in mouse bone marrow stromal cells. *Biochem Biophys Res Commun* 2003;**308:**458–462.
36. Peng Y, Kang Q, Cheng H *et al*. Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signaling. *J Cell Biochem* 2003;**90:**1149–1165.
37. Takada I, Kouzmenko AP, Kato S. Wnt and PPARgamma signaling in osteoblastogenesis and adipogenesis. *Nat Rev Rheumatol* 2009;**5:**442–447.
38. Takada I, Kato S. Molecular mechanism of switching adipocyte / osteoblast differentiation through regulation of PPARgamma function. *Clin calcium* 2008;**18:**656–661.
39. Justesen J, Stenderup K, Eriksen EF, Kassem M. Maintenance of osteoblastic and adipocytic differentiation potential with age and osteoporosis in human marrow stromal cell cultures. *Calcif Tissue Int* 2002;**71:**36–44.