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Original

The *in vitro* osteogenetic characteristics of primary osteoblastic cells from a rabbit calvarium

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Abstract: Previously, we showed that recombinant human bone morphogenetic protein-2 (rhBMP-2) increased bone augmentation beyond the skeletal envelope within a titanium cap in a rabbit calvarium; many cuboidal osteoblastic cells were observed histologically. These results suggested that the new osteoblastic cells might have differentiated and matured via stimulation by rhBMP-2. To date, however, no studies have reported the characteristics of osteoblastic cells derived from adult rabbit calvarium, after addition of rhBMP-2. To determine the effects of rhBMP-2 on osteoblastic cells, we observed morphological characteristics and alkaline phosphatase activity of osteoblastic cells from an adult rabbit calvarium. The expression of proteins in the BMP signaling pathway and extracellular matrix were analyzed, and mineralized nodule formation was assessed. The alkaline phosphatase activity increased significantly after rhBMP-2 stimulation. The protein levels of phosphorylated-Smad1, Runx2, osteocalcin, osteopontin, and type I collagen were augmented by rhBMP-2 stimulation using Western blotting or ELISA; rhBMP-2 also stimulated mineralized nodule formation with alizarin red staining. The results suggest that primary osteoblastic cells derived from a rabbit calvarium have osteogenetic characteristics *in vitro,* underscoring the potential use of these cells as a model for studying bone formation. These cells may play an important role in *in vivo* bone augmentation in a rabbit experimental model. (J. Oral Sci. 50, 427-434, 2008)

Keywords: rabbit calvarium; bone morphogenetic protein-2; bone augmentation.

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Introduction

The bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily and play important roles in osteoblast differentiation and maturation (1-5). Specifically, BMPs promote bone development by inducing bone formation and regeneration in adult vertebrates (6-8). More than 20 BMP-related proteins, including BMP-2, BMP-4, and BMP-7, have been identified. Recombinant human BMP (rhBMP-2) has a high osteoinductive capacity in various preclinical models and in humans (9-11) and can influence the differentiation of stem or stromal cells to osteoblasts (7,12-14). At the molecular level, BMPs bind to BMP type I and II serine/threonine kinase receptors. Upon ligand binding

and activation, the BMP type I and II receptors phosphorylate receptor-regulated Smads (R-Smads) such as Smad1, Smad5, and Smad8. These phosphorylated R-Smads form complexes with the common-partner Smad (Smad4 in mammals) and function as signal transducers in the BMP pathway (2,3,5). The complex translocates to the nucleus, where it accumulates and regulates the transcription of various target genes such as transcription factors related to osteoblast differentiation and maturation. One key transcription factor in the BMP pathway is runt-related transcription factor 2 (Runx2, also called Cbfa1). Runx2 is essential for osteoblast differentiation and bone mineralization (15,16); Runx2-knockout mice completely lack bone formation and die at birth. Other BMP-induced transcription factors, including distal-less homeobox 5 (Dlx5), msh homeobox homolog 2 (Msx2), and Osterix/Sp7, are closely related to osteoblast differentiation (17-22). The target genes of BMP-induced transcription factors include bone sialoprotein, galectin, TGF-β receptor I, dentin sialophosphoprotein-1, osteocalcin, and osteopontin (15,23-27), which regulate matrix mineralization and/or mineral deposition. Bone mineral is initially deposited at discrete sites in the matrix of type I collagen (28).

Our previous study showed that rhBMP-2 had a short-term effect on bone augmentation beyond the skeletal envelope within a titanium cap in a rabbit calvarium, and many cuboidal osteoblastic cells were observed in line beside the newly generated thin lamellar bone (8). From those observations, we speculated that rhBMP-2 may promote the differentiation and maturation of the new osteoblastic cells. However, to our knowledge, no studies have tested the hypothesis that osteoblastic cells derived from adult rabbit calvarium differentiate and mineralize upon stimulation with rhBMP-2. The objective of this study was to assess whether primary cultured osteoblastic cells could be established as an experimental model to test this hypothesis. We analyzed alkaline phosphatase activity as a potential calcium ion carrier (29) and assessed protein levels for the components of the BMP signaling pathway and of the extracellular matrix by Western blotting or enzyme-linked immunosorbent assay (ELISA). Mineralization was observed using alizarin red staining.

Materials and Methods

Isolation and culture of osteoblastic cells

Four-month-old male Japanese white rabbits weighing 2.5-2.8 kg were used in this study. The health of each rabbit was monitored for 2 weeks before the start of the experiment. The rabbits were kept in standard cages in an experimental animal room (24°C, 55% humidity, 1 atm, 12 h light/dark cycle) and were fed a standard laboratory diet and water *ad libitum*. This study was approved by the Animal Experimentation Committee of Nihon University School of Dentistry. All operations were conducted under sterile conditions. General anesthesia was administered by injecting pentobarbital sodium via an ear vein and was maintained by the inhalation of halothane. After an injection of approximately 1.8 ml of lidocaine HCl containing 1:80,000 epinephrine as a local anesthesia, a flap was made with a mid-sagittal incision and exfoliation from the forehead. The periosteum was incised and lifted to expose the calvarium on both sides of the midline. A circular groove with an inner diameter of 8 mm was cut on both sides of the midline, using a trephine drill (Bone trephine 131001; Technica, Tokyo, Japan). Calvaria were cut into small fragments that were dissociated to cell suspensions by enzymatic digestion with 0.1% collagenase-I (Wako Pure Chemicals, Osaka, Japan) for 30 min at 37°C. The bone pieces were washed repeatedly in Dulbecco's modified Eagle medium (DMEM; Gibco-BRL, Grand Island, NY, USA) without serum and then dipped in culture medium containing 15% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA) before they were equally seeded in 35-mm tissue culture dishes. The dishes were incubated in a humidified atmosphere with 5% CO_2 at 37°C for 4 h to hasten adhesiveness. This was followed by the addition of normal culture medium, which was DMEM supplemented with 10% FBS and penicillin/streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA), to the dish. The medium was changed twice per week. After 3 days, cells were observed emerging from the bone pieces. The cell layers around the bone tissue became 70-80% confluent after approximately 10 days. The cells were then transferred to 100-mm tissue culture dishes by using 0.05% trypsin-EDTA (Gibco-BRL), and the bone pieces were cultured continuously. The cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells that had been passaged fewer than three times were used for the following experiments.

Cell proliferation

The cells were placed into 96-well microplates at a density of 2×10^4 cells/cm^2 and cultured in DMEM containing 10% FBS with 0 or 100 ng/ml rhBMP-2 (R&D Systems, Minneapolis, MN, USA) for up to 14 days. The concentration of rhBMP-2 was followed as previously reported (30). At the noted intervals, the medium was replaced with fresh medium containing 10% cell-counting kit reagent (Wako Pure Chemical), and the cells were incubated for an additional 2 h. After incubation, the intensity of the reaction products was measured at 450 nm

using a microtiter plate reader. Relative cell numbers were calculated from the relative absorbance values on the basis of a standard curve.

Alkaline phosphatase (ALPase) activity

The cells were placed into 24-well microplates at a density of 2×10^4 cells/cm^2 and cultured in DMEM containing 10% FBS in the absence or presence of rhBMP-2 stimulation, for up to 14 days. To initiate the reaction, 100 µl of enzyme assay solution (8 mM *p*-nitrophenyl phosphate, 12 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ in 0.1 M glycine-NaOH buffer, pH 10.5) were added to the cells in each well, and the plate was incubated for several minutes at 37°C. The enzyme reaction was terminated by the addition of 100 µl of 0.2 M NaOH. The amount of *p*-nitrophenol released by the enzyme reaction was determined by measuring the absorbance at 405 nm using a microplate reader. One unit of ALPase activity was defined as the amount of enzyme required to liberate 1.0 µmol *p*-nitrophenol per minute. The enzyme activity was recorded as mU/10^4 cells.

SDS-PAGE and Western blotting from whole-cell extracts

To obtain whole-cell extracts, cells that had been incubated for up to 14 days in the absence or presence of rhBMP-2 were rinsed with PBS and lysed in buffer consisting of 50 mM Tris-HCl, 0.1% Triton X-100, 0.1 mM EDTA, and phosphatase inhibitor cocktail. Cells in the lysis buffer were sonicated three times for 20 s. Aliquots that contained equal amounts of protein in SDS-PAGE sample buffer were subjected to SDS-PAGE, followed by Western blotting.

Western blotting was performed using goat polyclonal anti-phospho-Smad1 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat polyclonal anti-Runx2 antibody (1:500; Santa Cruz Biotechnology), mouse monoclonal anti-β-Actin antibody (1:2000; Santa Cruz Biotechnology), and horseradish peroxidase-conjugated secondary antibody (anti-goat IgG antibody at 1:10,000 dilution, R&D Systems; or anti-mouse IgG antibody at 1:10,000 dilution, GE Healthcare, Buckinghamshire, UK). Immunoreactive proteins were visualized using a chemiluminescence kit (GE Healthcare) and X-ray film.

Enzyme-linked immunosorbent assay (ELISA)

The cells were cultured in DMEM containing 10% FBS with or without rhBMP-2 stimulation, for up to 14 days. Twenty-four hours before the measurements, the culture medium was replaced with DMEM containing 2% FBS with or without rhBMP-2. The amounts of extracellular matrix proteins in the culture medium were determined by standard procedures, using goat anti-human type I collagen polyclonal antibody, anti-osteocalcin antibody, and anti-osteopontin antibodies (all three from Santa Cruz Biotechnology); biotin-conjugated secondary antibody (Zymed Laboratories, San Francisco, CA, USA); and horseradish peroxidase-conjugated streptavidin (KPL, Gaithersburg, MD, USA). A colorimetric assay was performed using phenylenediamine, and the reaction was terminated by the addition of 100 µl of 8 M H_2SO_4. Quadruplicate assays were performed for each sample, and the absorbance at 492 nm was recorded.

Alizarin red staining

Primary calvarial osteoblastic cells were placed into 24-well tissue culture plates at a density of 2.0×10^4 cells/cm^2 and cultured for 14 days in DMEM containing 50 mM β-glycerophosphate and 50 µg/ml ascorbic acid, in the absence or presence of rhBMP-2. The culture medium was replaced with fresh medium every 3 days. Cell conditions and nodule formation were routinely checked by phase contrast microscopy. The presence of mineralized nodules was determined by staining with alizarin red (Wako Pure Chemical) as described previously (31).

Statistical analysis

All experiments were performed in quadruplicate. Each value represents the mean ± standard deviation (S.D.). Statistical significance between groups was determined using the unpaired Student's *t*-test. Differences with P values < 0.05 were considered significant.

Results

Peculiar morphology of isolated and cultured cells

During primary culture, round or polygonal cells were observed migrating from a few of the bone pieces on the 3rd day of culture (Fig. 1A and B). As the culture time increased, the cells became bigger and more triangular, short spindle-shaped, or polygonal. The cells formed nearly confluent cell layers around the bone tissue (covering 80% of the culture dish) after 10 days (Fig. 1C and D).

Cell proliferation and ALPase activity

Stimulation by rhBMP-2 did not affect the rate of cell proliferation for up to 14 days (Fig. 2). ALPase activity, which was measured for up to 14 days in culture, increased gradually during the experimental period regardless of rhBMP-2 stimulation (Fig. 3). However, at days 7 and 14 of culture, ALPase activity was significantly higher in the

BMP-2-treated cells than in the non-treated control cells ($P < 0.05$).

Effect of rhBMP-2 on expression of osteogenesis-related proteins in the BMP signaling pathway

Proteins in the BMP signaling pathway, specifically the levels of phosphorylated-Smad1 and Runx2, were examined in extracts from isolated primary calvarial osteoblastic cells treated with rhBMP-2 or control cells, by Western blotting. The amount of phosphorylated-Smad1 (Fig. 4, top panel) was substantially increased at day 3 of rhBMP-2 stimulation, whereas phosphorylated-Smad1 levels in untreated cells peaked at day 5. The expression of Runx2 was markedly increased at day 5 of rhBMP-2 stimulation (Fig. 4, middle panel).

Effect of rhBMP-2 on expression of extracellular matrix

To confirm that the osteoblastic cells produced extracellular matrix upon rhBMP-2 stimulation, the protein levels of three extracellular matrix proteins – type I collagen, osteocalcin, and osteopontin – were detected using ELISA. The protein level of type I collagen was significantly higher in rhBMP-2-treated cells at days 0, 7, and 14, compared with control levels (Fig. 5A, $P < 0.05$).

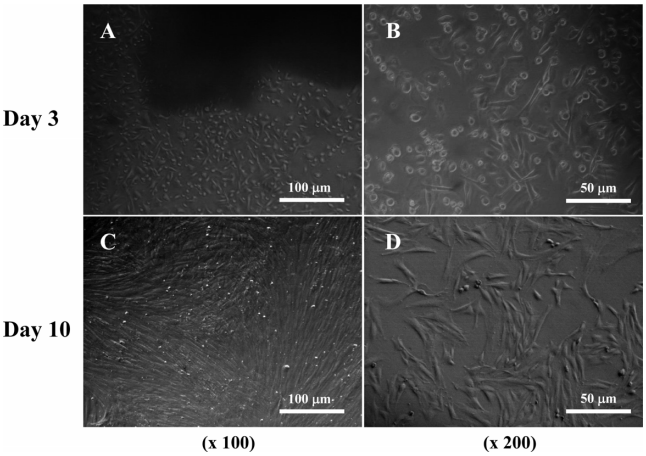

Fig. 1 Phase-contrast micrographs of osteoblastic cells from a rabbit calvarium. Primary isolated and cultured cells from bone pieces. The cells were cultured for up to 10 days. (A and B) Cells migrated from the bone pieces after 3 days of culture (magnification at ×100 and ×200, respectively). (C and D) Cells after 10 days of culture (magnification at ×100 and ×200, respectively).

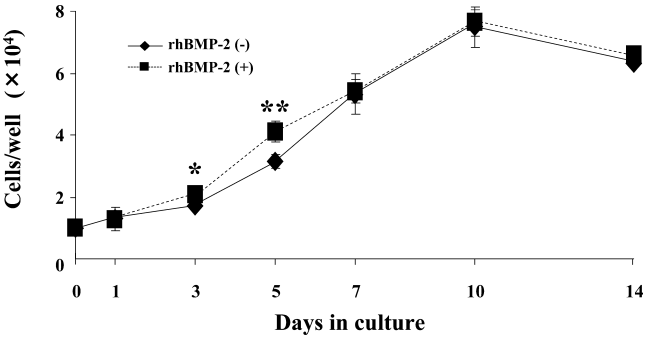

Fig. 2 Effect of rhBMP-2 stimulation on cell proliferation. The cells were cultured in the absence or presence of rhBMP-2, and cell numbers were determined at days 1, 3, 5, 7, 10, and 14 of culture. Each bar indicates the mean ± S.D. of three experiments. $^{**}P < 0.01$, $^{*}P < 0.05$, rhBMP-2 treatment versus control.

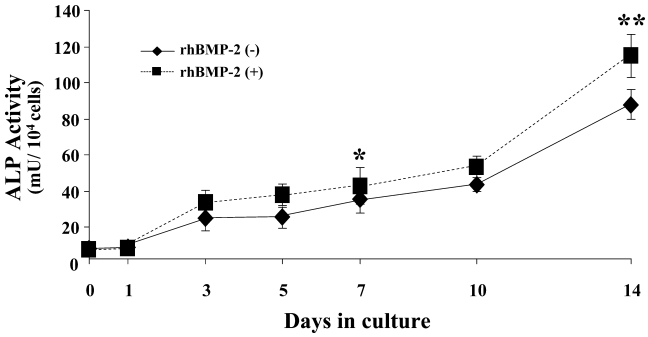

Fig. 3 Effect of rhBMP-2 stimulation on ALPase activity. The cells were cultured with or without rhBMP-2 stimulation, and ALPase activities were determined at days 1, 3, 5, 7, 10, and 14 of culture. Each bar indicates the mean ± S.D. of three experiments. $^{**}P < 0.01$, $^{*}P < 0.05$, rhBMP-2 treatment versus control.

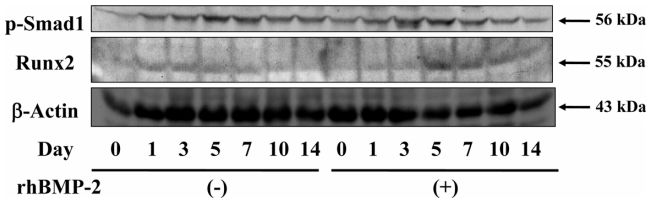

Fig. 4 Effect of rhBMP-2 on expression of osteogenesis-related proteins in the BMP signaling pathway. Rabbit primary osteoblastic cells were cultured in the absence or presence of rhBMP-2, and whole cell lysates were prepared after 1, 3, 5, 7, 10, and 14 days. The samples were normalized by the total protein concentration, and approximately 20 μg of total protein were subjected to SDS-PAGE and immunoblotted with anti-phosphorylated-Smad1 or anti-Runx2 antibodies. β-Actin served as a loading control. The blots shown are representative of at least three experiments.

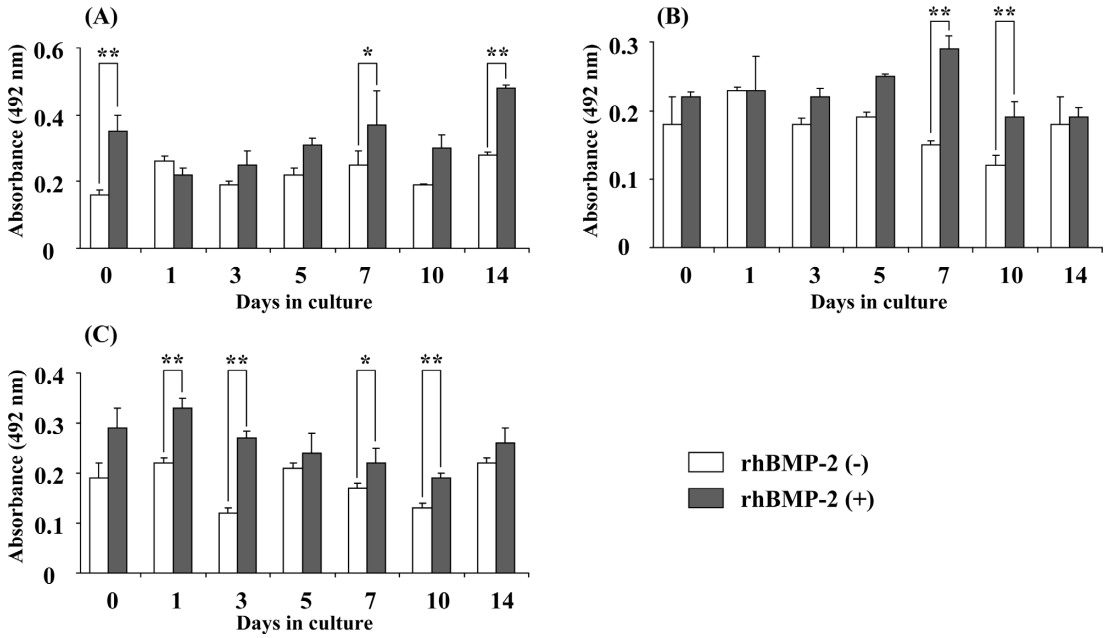

Fig. 5 Expression levels of (A) type I collagen, (B) osteocalcin, and (C) osteopontin were determined by ELISA. The cells were cultured in the absence or presence of rhBMP-2, and extracellular matrix protein expression was analyzed at 1, 3, 5, 7, 10, and 14 days. Each bar indicates the mean ± S.D. of three experiments; $^{**}P < 0.01$, $^{*}P < 0.05$, rhBMP-2 treatment versus control.

Osteocalcin and osteopontin protein levels were significantly increased at days 7 and 10, and days 1, 3, 7, and 10, respectively, in rhBMP-2-treated cells compared with control cells (Fig. 5B and C, $P < 0.05$).

Mineralized nodule formation

To examine the mineralization capacity of the osteoblastic cells, mineralized nodule formation was determined at day 14 of culture in cells that had been grown in the absence and presence of rhBMP-2. Alizarin red staining intensity of mineralized nodules was slightly higher in primary calvarial osteoblastic cells treated with 100 ng/ml rhBMP-2 at day 14, compared with control cells without rhBMP-2 stimulation (Fig. 6).

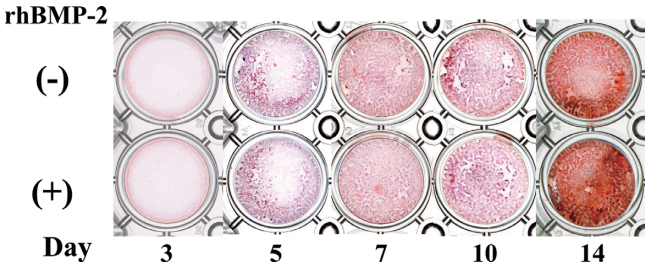

Fig. 6 Effect of rhBMP-2 on mineralized nodule formation. Primary calvarial osteoblastic cells were cultured in osteogenic medium in the presence or absence of rhBMP-2, and the formation of mineralized nodules was examined by staining with alizarin red at days 3, 5, 7, 10, and 14 after rhBMP-2 stimulation. (-): control, (+): rhBMP-2 stimulation.

Discussion

We isolated primary osteoblastic cells from a rabbit calivarium and assessed proliferation, ALPase activity, activation and/or expression of BMP pathway proteins and extracellular matrix proteins, and mineralized nodule formation. Cell proliferation was not changed by rhBMP-2, but ALPase activity was increased by rhBMP-2 stimulation. rhBMP-2 promoted an earlier peak of the phosphorylated-Smad1 protein level and dramatically increased Runx2 protein levels; the expression of the extracellular matrix proteins osteocalcin, osteopontin, and type I collagen was increased by rhBMP-2. Moreover, mineralized nodule formation, as determined by alizarin red staining, was slightly increased by rhBMP-2.

We previously developed a guided bone augmentation model in a titanium cap in rabbit calvarium (8,32-34) and histologically observed new bone beyond the skeletal envelope. However, a further understanding of the potential role of osteoblasts in bone augmentation is required to gain mechanistic insight. Moreover, an adequate experimental model for culturing osteoblasts from rabbit calvaria has not been developed and characterized. Only one study has reported the establishment of rabbit osteoblasts from

a calvarium (35), and this was performed in a young, rather than an adult, rabbit.

In the present study, we developed a method for culturing osteoblastic cells from an adult rabbit calvarium. During culture, the osteoblastic cells generally developed into triangular, short spindle-shaped, or polygonal cells, similar to the osteoblasts obtained from a young rabbit (35). This may be an important experimental model for bone augmentation therapy, which is often used to treat adult patients with severe periodontal disease or patients who have suffered bone loss due to dental implants.

The BMP signaling pathway, including the BMP receptor and some Smads, has a vital role in osteoblasts (1-6). The phosphorylation of Smad1 is required to up-regulate the pathway, and osteogenesis-related transcription factors, including Runx2, Dlx5, Msx2, and Osterix, are located downstream of Smad1 (17-22). The results of the present study showed that the peak level of phosphorylated-Smad1 was clearly accelerated by BMP-2 stimulation; Runx2 was also markedly increased at day 5 after BMP-2 stimulation. These data suggest that the BMP signaling pathway in osteoblastic cells is up-regulated by BMP-2 and that these cells may have the ability to differentiate and undergo osteogenesis. It is well known that extracellular matrix proteins such as type I collagen, osteocalcin, and osteopontin are produced via activation of BMP-related transcription factors (23-28). In our study, the presence of BMP-2 increased type I collagen, osteocalcin, and osteopontin, suggesting that these osteoblastic cells have the ability to produce osteogenesis-related extracellular matrix. Nonetheless, the extracellular matrix itself did not increase dramatically. Other transcription factors such as Msx2, which down-regulates type I collagen, osteocalcin, and osteopontin, may be needed for effective *in vitro* generation of extracellular matrix (20,36).

Alizarin red staining indicated that mineralization occurred in a time-dependent manner regardless of the presence of BMP-2. On day 14, mineralized nodule formation was slightly increased in cells treated with rhBMP-2. At the least, these results suggest that the osteoblastic cells have characteristics of osteogenetic cells.

In summary, the BMP signaling pathway appeared to be up-regulated by rhBMP-2 stimulation in primary osteoblastic cells obtained from a rabbit calvarium, and extracellular matrix protein levels were also increased. Moreover, these osteoblastic cells had the ability to form bone nodules, suggesting that osteoblastic cells derived from an adult rabbit calvarium have osteogenetic characteristics *in vitro* and could be used as an experimental model. These osteoblastic cells may also play an important role in *in vivo* bone augmentation beyond the skeletal envelope within a titanium cap in an adult rabbit calvarium. Further molecular studies are required to fully characterize these osteoblastic cells.

References

1. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. Science 242, 1528-1534
2. Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K (2002) Two major Smad pathways in TGF-β superfamily signaling. Genes Cells 7, 1191-1204
3. Shi Y, Massagué J (2003) Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113, 685-700
4. Chen D, Zhao M, Mundy GR (2004) Bone morphogenetic proteins. Growth Factors 22, 233-241
5. Miyazono K, Maeda S, Imamura T (2005) BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev 16, 251-263
6. Reddi AH (2005) BMPs: from bone morphogenetic proteins to body morphogenetic proteins. Cytokine Growth Factor Rev 16, 249-250
7. Gautschi OP, Frey SP, Zellweger R (2007) Bone morphogenetic protein in clinical applications. ANZ J Surg 77, 626-631
8. Hasegawa Y, Sato S, Takayama T, Murai M, Suzuki N, Ito K (2008) Short-term effects of rhBMP-2-enhanced bone augmentation beyond the skeletal envelope within a titanium cap in rabbit calvarium. J Periodontol 79, 348-354
9. Wang EA (1990) Recombinant human bone morphogenetic proteins induces bone formation. Proc Natl Acad Sci USA 87, 2220-2224
10. Wikesjö UM, Huang YH, Polimeni G, Qahash M (2008) Bone morphogenetic proteins: a realistic alternative to bone grafting for alveolar reconstruction. Oral Maxillofac Surg Clin North Am 19, 535-551
11. King GN, Cochran DL (2002) Factor that modulate the effects of bone morphogenetic protein-induced periodontal regeneration: a critical review. J Periodontol 73, 925-936
12. Peterson B, Zhang J, Iglesias R, Kabo M, Hedrick M, Benhaim P, Lieverman JR (2005) Healing of critically sized femoral defects, using genetically modified mesenchymal stem cells from human

adipose tissue. Tissue Eng 11, 120-129
13. Zeng Q, Li X, Beck G, Balian G, Shen FH (2007) Growth and differentiation factor-5 (GDF-5) stimulates osteogenic differentiation and increases vascular endothelial growth factor (VEGF) levels in fat-derived stromal cells *in vitro*. Bone 40, 374-381
14. Miyazaki M, Zuk PA, Zou J, Yoon SH, Wei F, Morishita Y, Sintuu C, Wang JC (2008) Comparison of human mesenchymal stem cells derived from adipose tissue and bone marrow for *ex vivo* gene therapy in rat spinal fusion model. Spine 33, 863-869
15. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89, 747-754
16. Komori T (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89, 755-764
17. Shirakabe K, Terasawa K, Miyama K, Shibuya H, Nishida E (2001) Regulation of the activity of the transcription factor Runx2 by two homeobox proteins, Msx2 and Dlx5. Genes Cells 6, 851-856
18. Lee MH, Kwon RG, Park HS, Wozney JM, Ryoo HM (2003) BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. Biochem Biophys Res Commun 309, 689-694
19. Tadic T, Dodig M, Erceg I, Marijanoric I, Mina M, Kalajzic Z, Velonis D, Kronenberg MS, Kosher RA, Ferrari D, Lichtler AC (2002) Overexpression of Dlx5 in chicken calvarial cells accelerates osteoblastic differentiation. J Bone Miner Res 17, 1008-1014
20. Kim YJ, Lee MH, Wozney JM, Cho JY, Ryoo HM (2004) Bone morphogenetic protein-2-induced alkaline phosphatase expression is stimulated by Dlx5 and repressed by Msx2. J Biol Chem 279, 50773-50780
21. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B (2002) The novel zinc finger-containing transcription factor Osterix is required for osteoblast differentiation and bone formation. Cell 108, 17-29
22. Lee MH (2005) Dlx5 specifically regulates Runx2 type II expression by binding to homeodomain-response elements in the Runx2 distal promoter. J Biol Chem 280, 35579-35587
23. Stock M, Schafer H, Stricker S, Gross G, Mundlos S, Otto F (2003) Expression of galectin-3 in skeletal tissues is controlled by Runx2. J Biol Chem 278, 17360-17367
24. Ji C, Casinghino S, Chang DJ, Chen Y, Javed A, Ito Y, Hiebert SW, Lian JB, Stein GS, McCarthy TL, Centrella M (1998) CBFa (AML/PEBP2)-related elements in the TGF-beta type I receptor promoter and expression with osteoblast differentiation. J Cell Biochem 69, 353-363
25. Javed A, Barned GL, Jassanya BO, Stein JL, Gerstenfeld L, Lian JB, Stein GS (2001) Runt homology domain transcription factors (Runx, Cbfa, and AML) mediate repression of the bone sialoprotein promoter: evidence for promoter context-dependent activity of Cbfa proteins. Mol Cell Biol 21, 2891-2905
26. Sato M (1998) Transcriptional regulation of osteopontin gene *in vivo* by PEBP2alphaA/CBFA1 and ETS1 in the skeletal tissues. Oncogene 17, 1517-1525
27. Chen S, Gu TT, Sreenath T, Kulkarni AB, Karsenty G, MacDougall M (2002) Spatial expression of Cbfa1/Runx2 isoforms in teeth and characterization of binding sites in the DSPP gene. Connect Tissue Res 43, 338-344
28. Glimcher MJ (1998) The nature of the mineral phase in bone: Biological and clinical implications. In Metabolic bone disease and clinically related disorders, 3rd ed, Avioli LV, Krane SM eds, Academic Press, San Diego, 23-51
29. Montessuit C, Caverzasio J, Bonjour JP (1991) Characterization of a Pi transport system in cartilage matrix vesicles. Potential role in the calcification process. J Biol Chem 266, 17791-17797
30. Roostaeian J, Carlsen B, Simhaee D, Jarrahy R, Huang W, Ishida K, Rudkin GH, Yamaguchi DT, Miller TA (2006) Characterization of growth and osteogenic differentiation of rabbit bone marrow stromal cells. J Surg Res 133, 76-83
31. Williams DC, Boder GB, Toomey RE, Paul DC, Hillman CC Jr, King KL, Van Frank RM, Johnston CC Jr (1980) Mineralization and metabolic response in serially passaged adult rat bone cells. Calcif Tissue Int 30, 233-246
32. Yamada Y, Nanba K, Ito K (2003) Effects of occlusiveness of a titanium cap on bone generation beyond the skeletal envelope in the rabbit calvarium. Clin Oral Implants Res 14, 455-463
33. Tamura T, Fukase Y, Goke E, Yamada Y, Sato S, Nishiyama M, Ito K (2005) Three-dimensional evaluation for augmented bone using guided bone regeneration. J Periodontal Res 40, 269-276
34. Min S, Sato S, Murai M, Okuno K, Fujisaki Y,

Yamada Y, Ito K (2007) Effects of marrow penetration on bone augmentation within a titanium cap in rabbit calvarium. J Periodontol 78, 1978-1984

35. Cao XY, Yin MZ, Zhang LN, Li SP, Cao Y (2006) Establishment of a new model for culturing rabbit osteoblasts *in vitro*. Biomed Mater 1, L16-L19
36. Dodig M, Tadic T, Kronenberg MS, Dacic S, Liu YH, Maxson R, Rowe DW and Lichtler AC (1999) Ectopic Msx2 overexpression inhibits and Msx2 antisense stimulates calvarial osteoblast differentiation. Dev Biol 209, 298-397