TISSUE ENGINEERING: Part A
Volume 19, Numbers 21 and 22, 2013

DOI: 10.1089/ten.tea.2012.0648

Dual Delivery of rhPDGF-BB and Bone Marrow Mesenchymal Stromal Cells Expressing the *BMP2* Gene Enhance Bone Formation in a Critical-Sized Defect Model

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Bone tissue healing is a dynamic, orchestrated process that relies on multiple growth factors and cell types. Platelet-derived growth factor-BB (PDGF-BB) is released from platelets at wound sites and induces cellular migration and proliferation necessary for bone regeneration in the early healing process. Bone morphogenetic protein-2 (BMP-2), the most potent osteogenic differentiation inducer, directs new bone formation at the sites of bone defects. This study evaluated a combinatorial treatment protocol of PDGF-BB and BMP-2 on bone healing in a critical-sized defect model. To mimic the bone tissue healing process, a dual delivery approach was designed to deliver the rhPDGF-BB protein transiently during the early healing phase, whereas BMP-2 was supplied by rat bone marrow stromal cells (BMSCs) transfected with an adenoviral vector containing the *BMP2* gene (AdBMP2) for prolonged release throughout the healing process. In *in vitro* experiments, the dual delivery of rhPDGF-BB and *BMP2* significantly enhanced cell proliferation. However, the osteogenic differentiation of BMSCs was significantly suppressed even though the amount of BMP-2 secreted by the AdBMP2-transfected BMSCs was not significantly affected by the rhPDGF-BB treatment. In addition, dual delivery inhibited the mRNA expression of BMP receptor type II and Noggin in BMSCs. In *in vivo* experiments, critical-sized calvarial defects in rats showed enhanced bone regeneration by dual delivery of autologous AdBMP2-transfected BMSCs and rhPDGF-BB in both the amount of new bone formed and the bone mineral density. These enhancements in bone regeneration were greater than those observed in the group treated with AdBMP2-transfected BMSCs alone. In conclusion, the dual delivery of rhPDGF-BB and AdBMP2-transfected BMSCs improved the quality of the regenerated bone, possibly due to the modulation of PDGF-BB on BMP-2-induced osteogenesis.

Introduction

Growth factors are known to mediate wound healing and to regulate critical cellular activities, such as cellular recruitment, proliferation and differentiation of cell processes necessary for tissue regeneration.[1–3] The platelet-derived growth factor (PDGF) is released from aggregated platelets during the early healing phase at the wound site and exerts chemotactic and mitogenic effects on inflammatory cells and undifferentiated mesenchymal cells.[4] Although the osteogenic effects of PDGF *in vitro* are still controversial, regenerative therapy using rhPDGF-BB in preclinical and clinical studies has been reported to enhance bone regeneration, particularly in periodontal tissues.[5–8] Bone morphogenetic proteins (BMPs) regulate differentiation, chemotaxis, growth and apoptosis of osteogenic cells and induce significant bone regeneration both orthotopically and ectopically.[9–10] Among them, BMP-2 is one of the most potent osteoinductive proteins affecting osteoblast differentiation.[11] Therefore, many studies have investigated bone regeneration in craniofacial and periodontal defects through the *in vivo* application of rhBMP-2 or the *BMP2* gene.[12–15]

Bone formation is achieved through a sequential cascade of events relying on chemotaxis and mitosis of mesenchymal cells and differentiation of mesenchymal cells into osteoblasts.[16] This process is directed by the coordinated expression of growth factors, including BMPs and PDGF, to regulate osteogenic differentiation in the proper sequence and time.[17] PDGF-BB has a strong chemotactic effect on osteoblasts and acts to recruit mesenchymal cells into the wound site during bone formation.[18,19] BMP-2 can direct these cells to undergo osteogenic differentiation into

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osteoblasts and to form bone nodules.[20] Bone tissue engineering studies have also demonstrated that the combined therapy with PDGF-BB and BMP-2 induced more bone regeneration than either factor alone.[21–23] However, the control over their release is one of the major concerns in growth factor delivery because each growth factor has distinct actions in bone formation.

In this study, we hypothesized that the dual delivery of PDGF-BB and BMP-2 could enhance bone regeneration and better simulate the bone healing process; we further hypothesized that this delivery would increase the number of cells capable of differentiating into osteoblasts and subsequently differentiate these cells into osteoblasts. This delivery strategy was accomplished using rhPDGF-BB protein delivery for its transient actions in the early healing phase and the *BMP2* gene delivery to promote prolonged, sustained action. Therefore, rat bone marrow stromal cells (BMSCs) were transfected with adenoviral human *BMP2* and delivered with rhPDGF-BB into a critical-sized defect in a rat calvarium. Before their *in vivo* application, the effects of the dual delivery of rhPDGF-BB and *BMP2* on BMSCs were examined *in vitro*.

Materials and Methods

In vitro *experiments*

Cell isolation and culture. Rat BMSCs were harvested from both tibias of rats under general anesthesia of ketamine (90 mg/kg; Yuhan Co.) and xylazine (10 mg/kg; Bayer). Briefly, blood was collected from the tibial bone marrow.[24] BMSCs were then isolated by centrifugation and suspended in the α-minimum essential medium (MEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and a 1% penicillin–streptomycin solution (Gibco). The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed twice per week. Cells from passages two to three were used for transfection.

***BMP2* gene transfection and rhPDGF-BB treatment.** The construction of the adenovirus encoding human *BMP2* (AdBMP2) has been previously described.[25] BMSCs were plated at a density of 1×10^4 cells/well on a 24-well plate (for the alkaline phosphatase [ALP] activity assay, BMP-2 expression quantitation assay and the mineralization assay) and at 5×10^3 cells/well on a 96-well plate (for the cell proliferation assay). BMSCs were treated with AdBMP2 at a multiplicity of infection of 100 pfu for 4 h. The cells were serum-starved in a medium containing 0.5% FBS overnight and were subsequently treated with rhPDGF-BB (30 ng/mL[26]; R&D System) for 6, 12, 24, or 48 h.

Cell proliferation assay. Cell proliferation was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma). The MTT solution was added to each cultured sample on a different day, and the solution was further incubated for 4 h to promote MTT formazan formation. The formazan was solubilized in dimethyl sulfoxide (DMSO; Sigma), and the absorbance of the solution was measured at 540 nm with a microplate reader (Molecular Devices). Triplicate readings were taken for each sample.

Quantitation of BMP-2 expression. The culture medium from the BMSCs in each well of the 24-well plates was sampled at 3, 7, 10, 14, 16, and 21 days after the rhPDGF-BB treatment and frozen at −80°C for later analysis. The amount of BMP-2 in the collected medium on each examination day was measured using a commercial enzyme immunoassay kit (R&D System). All of the experiments were performed in triplicate.

ALP activity assay. The ALP activity was measured at 7 and 14 days after rhPDGF-BB treatment according to the manufacturer's instructions (Anaspec Co.). The cell lysates were transferred to 96-well plates and incubated with the ALP substrate at 37°C for 30 min; the reaction was then halted by the addition of a stop buffer. The p-nitrophenol (pNP) product formed by the enzymatic hydrolysis of p-nitrophenylphosphate (pNPP) was measured at 405 nm using an absorbance microplate reader. The protein concentrations of the samples were measured using a protein assay kit (iNtRON Biotechnology). The ALP activity was expressed as the concentration of pNP transformed per microgram of protein.

Mineralization assay. For the mineralization assay, both AdBMP2-transfected BMSCs and control BMSCs were treated with rhPDGF-BB (according to the treatment time protocol) and cultured in osteogenic media (α-MEM containing 15% FBS supplemented with 0.2 mM ascorbic acid [Sigma] and 10 mM β-glycerolphosphate [Sigma]) for 28 days. The cells were then fixed in 95% cold ethanol and stained with a 1% Alizarin Red S solution (Wako Chemicals) for 5 min. Mineralization was examined and imaged. Finally, quantitation was performed using an eluting procedure with cetylpyridinium chloride in 10 mM sodium phosphate.

Real-time PCR assay. BMSCs were plated in a 92-mm-diameter dish at a density of 1×10^6 cells and transfected with AdBMP2. As described above, rhPDGF-BB was applied to the AdBMP2-transfected BMSCs. Total RNA was isolated from the cells at 0, 6, 12, 24, and 48 h after rhPDGF-BB treatment using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. To remove any residual genomic DNA in the RNA solutions, an RNase-free DNase (Qiagen) treatment was performed. The complementary DNA (cDNA) that was synthesized from 5 μg of RNA using a Superscript III First-Strand Synthesis System (Invitrogen) was used as a polymerase chain reaction (PCR) template. Real-time PCR (qPCR) was performed using a real-time PCR system (Applied Biosystems); the reactions contained cDNA, primers (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/tea) and SYBR Premix Ex Taq II (Takara Bio Co.). The thermal cycling conditions were as follows: one cycle at 95°C for 15 s and 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 33 s. Post-PCR melting curves confirmed the specificity of single-target amplification, and the fold change of the gene of interest relative to *Gapdh* was determined. All of the reactions were performed in triplicate.

In vivo *experiments*

Animals. Seventy-two male Sprague-Dawley rats (8 weeks old) were used in this study. The care and treatment of the animals were conducted in accordance with guidelines

established by the Seoul National University Institutional Animal Care and Use Committee. This study conformed to Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines for preclinical studies. The animal research protocol was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-090410-1).

Preparation of autologous AdBMP2-transfected BMSCs and rhPDGF-BB delivery. To implant autologous BMSCs into defect sites, BMSCs were first harvested from both tibias of each animal under general anesthesia 3 weeks before calvarial surgery. The harvested BMSCs were cultured in the α-MEM supplemented with 10% FBS and an antibiotic–antimycotic solution. The cells from passages two and three were seeded at a density of 1×10^6 cells/dish and transfected with AdBMP2 according to their designated experimental group. BMP-2 expression was confirmed by ELISA before surgery.

Cells were labeled with chloromethylbenzamido-1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (CM-DiI; Invitrogen) to track the transplanted cells according to the manufacturer's instructions.[25] Briefly, cells were incubated with 5 μM CM-DiI for 5 min at 37°C and additionally for 15 min at 4°C.

BMSCs and 10 μg of rhPDGF-BB were delivered using 200 μL of 1% collagen hydrogel, which was prepared from purified porcine skin-derived type I atelocollagen, sterile lyophilized collagen (Matrixen-PSP; Bioland), 0.001 N HCl, 26 mM $NaHCO_3$, 20 mM HEPES, and 0.025 N NaOH at pH 7.4.

Surgical procedure. The surgical procedure was performed under general anesthesia. A midline incision was made over the calvarium, and a full-thickness flap was elevated. An 8-mm critical-sized calvarial defect was created using a trephine bur (3i Implant Innovation) under sterile saline irrigation. The animals were divided into the following six experimental groups: (1) negative control group—empty; (2) vehicle group—collagen hydrogel alone; (3) BMSC group—collagen hydrogel containing autologous BMSCs; (4) rhPDGF-BB group—collagen hydrogel mixed with rhPDGF-BB; (5) BMSC/*BMP2* group—collagen hydrogel containing autologous AdBMP2-transfected BMSCs; and (6) BMSC/*BMP2*/rhPDGF-BB group—collagen hydrogel containing both rhPDGF-BB and autologous AdBMP2-transfected BMSCs.

The incisions were sutured in layers with 5-0 chromic gut and 4-0 silk. All of the animals received a single intramuscular injection of cefazolin (30 mg/kg) 2 days after surgery. Six rats from each group were sacrificed 2 weeks after surgery, and the remaining six rats from each group were sacrificed 4 weeks after surgery.

Microcomputed tomography analysis. Tissues, including the surgical sites, were harvested and fixed in 10% neutralized buffered formalin, and microcomputed tomography (micro-CT) images were taken using a SkyScan 1172 (SkyScan) scanner. The percentage of mineralized bone volume of the defect tissue volume (BV/TV) was measured using a computer program (CT-analyzer; SkyScan) with a lower gray threshold level of 65.[27] The bone mineral density (BMD; g/cm^3) of the regenerated bone in the defect site was measured with the same program.

Histological and histomorphometric analyses. The specimens were decalcified with a 10% EDTA solution for 2 weeks before they were dehydrated through a series of ethanol solutions of increasing concentrations and embedded in paraffin. Five-micrometer-thick coronal sections through the center of the circular defects were obtained and stained with hematoxylin and eosin. The prepared specimens were examined by light microscopy.

After microscopic examination, a photograph of each slide was taken using a digital camera, and the resulting images were saved to a computer for histomorphometric analysis. The newly formed bone area (mm^2) within the defect was measured using an automated image analysis system (Tomoro Scope Eye 3.5 Image Analyzer; Techsan Digital Imaging). Defect closure (percentage) was measured as the ratio of the area of newly formed bone divided by the area of the entire defect.

Statistical analysis. Triplicate results were analyzed for each *in vitro* experiment using two-way analysis of variance (ANOVA) with Tukey's *post hoc* tests. ANOVA with Tukey's *post hoc* tests was performed to evaluate the differences among the experimental groups at each interval in the *in vivo* experiment. A p-value less than 0.05 was considered statistically significant.

Results

Results of the in vitro *experiment*

The effects of the dual delivery of rhPDGF-BB and *BMP2* on cell proliferation and osteogenic differentiation of BMSCs. In the proliferation assay, rhPDGF-BB significantly enhanced cell proliferation of both BMSCs and AdBMP2-transfected BMSCs at days 3 and 7 ($p < 0.001$ and $p < 0.001$, respectively; Fig. 1A). The AdBMP2-transfected BMSCs significantly induced BMP-2 overexpression during a 21-day period, which was not affected by the duration of the rhPDGF-BB treatment (Fig. 1B). However, osteogenic differentiation of the AdBMP2-transfected BMSCs was significantly suppressed by the rhPDGF-BB treatment. The rhPDGF-BB treatment of the AdBMP2-transfected BMSCs significantly decreased their ALP activity at both days 7 and 14 ($p < 0.001$ and $p < 0.001$, respectively; Fig. 1C), which was similar to other groups in which *BMP2* was not delivered. In addition, mineralized nodule formation was negatively affected by the rhPDGF-BB treatment of the AdBMP2-transfected BMSCs at day 7 ($p = 0.001$; Fig. 1D).

Gene expression patterns of AdBMP2-transfected BMSCs following the rhPDGF-BB treatment. The rhPDGF-BB treatment significantly suppressed the mRNA expression of osteogenic factors in AdBMP2-transfected BMSCs; the mRNA expression of runt-related transcription factor 2 (Runx2), distal-less homeobox 5 (Dlx5), ALP, and integrin-binding sialoprotein (Ibsp) of AdBMP2-transfected BMSCs were significantly suppressed by rhPDGF-BB over 48 h; the suppression levels were the highest at 24 h (Fig. 2A–D).

The mRNA expression of BMP receptor type II (BMPRII), a major receptor of BMP-2, was also significantly inhibited by the rhPDGF-BB treatment in the AdBMP2-transfected BMSCs, while the mRNA levels of BMP receptor type Ia (BMPRIa) were not significantly

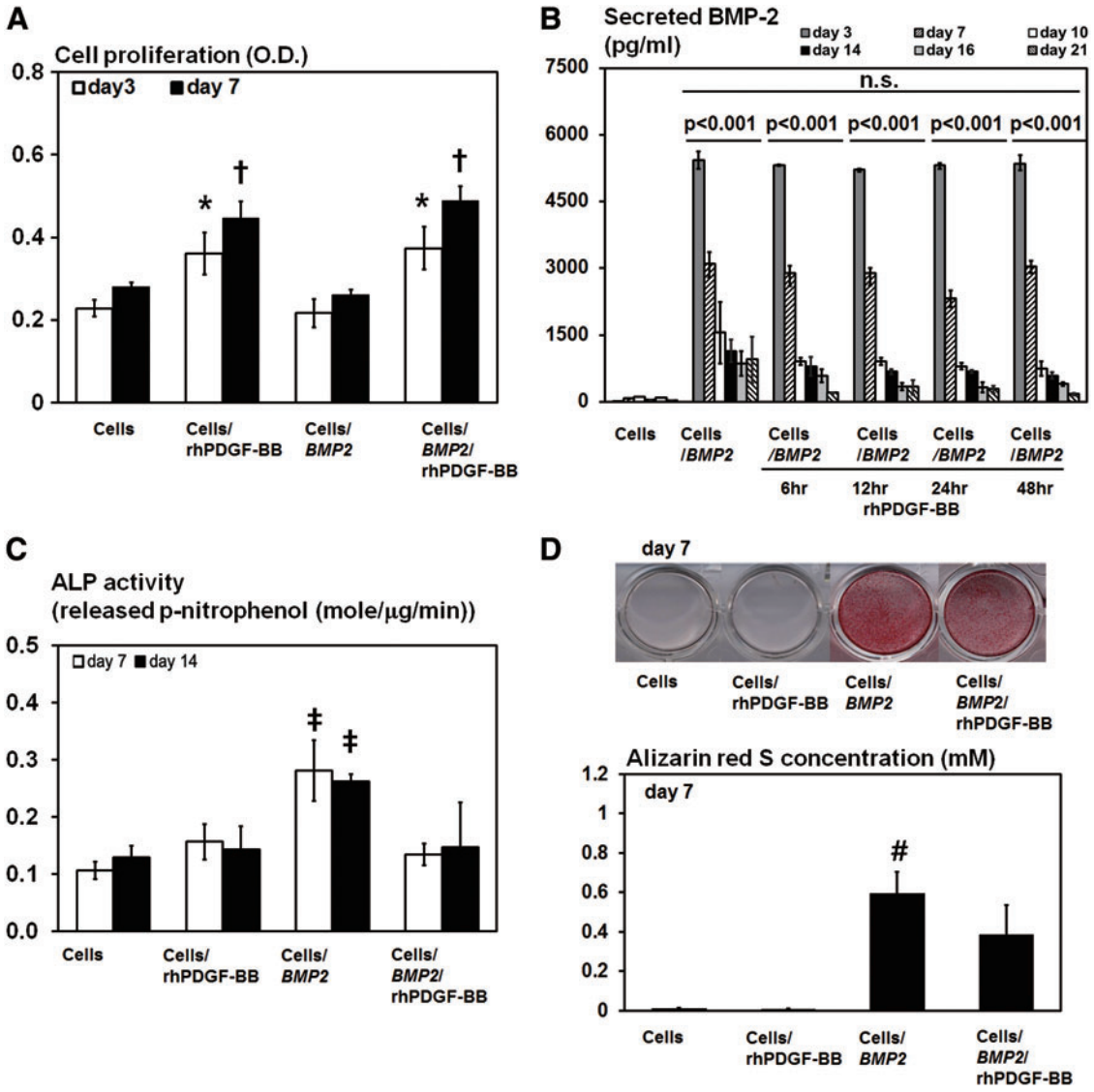

FIG. 1. The *in vitro* effects of rhPDGF-BB on AdBMP2-transfected BMSCs. **(A)** Effects of rhPDGF-BB treatment for 48 h on the proliferation of AdBMP2-transfected BMSCs (5×10^3 cells/well). The cell proliferation of both nontransfected BMSCs and AdBMP2-transfected BMSCs was significantly increased by the rhPDGF-BB treatment at days 3 and 7 (ANOVA, $p<0.001$ and $p<0.001$, respectively). **(B)** The amount of BMP-2 produced by AdBMP2-transfected BMSCs (1×10^4 cells/well), as determined by ELISA. Although BMP-2 was overexpressed over a 21-day period in AdBMP2-transfected BMSCs, BMP-2 expression was significantly decreased after 21 days compared to the initial levels (ANOVA, $p<0.001$). Furthermore, the rhPDGF-BB treatment did not significantly influence BMP-2 expression at each interval. **(C)** Effects of rhPDGF-BB on ALP (1×10^4 cells/well). The ALP activity was significantly increased by *BMP2* gene delivery, which was suppressed by the rhPDGF-BB treatment for 48 h after 7 and 14 days (ANOVA, $p<0.001$ and $p<0.001$, respectively). **(D)** Effects of rhPDGF-BB on the mineralization of AdBMP2-transfected BMSCs (1×10^4 cells/well). AdBMP2-transfected BMSCs formed mineralization nodules at day 7, which was significantly suppressed by the rhPDGF-BB treatment for 48 h (ANOVA, $p=0.001$). *Statistically significant difference from the BMSCs and AdBMP-2-trasnfected BMSCs at day 3. †Statistically significant difference from the BMSCs and AdBMP-2-trasnfected BMSCs at day 7. ‡Statistically significant difference from the other groups. #Statistically significant difference from the other groups. Values represent mean ± SD of three samples. OD, optical density. BMP-2, bone morphogenetic protein-2; BMSC, bone marrow stromal cell; ALP, alkaline phosphatase; ANOVA, analysis of variance; PDGF-BB, platelet-derived growth factor-BB; AdBMP-2, adenoviral vector containing the *BMP2* gene. Color images available online at www.liebertpub.com/tea

affected (Fig. 3A, B). In addition, the mRNA expression of Noggin, an inhibitory molecule of BMP-2 signal transduction, was significantly suppressed by the rhPDGF-BB treatment of the AdBMP2-transfected BMSCs compared to the AdBMP2-transfected BMSCs (Fig. 3C). The AdBMP2-transfected BMSCs highly overexpressed Noggin mRNA over the course of 48 h, whereas the dual delivery of *BMP2* and rhPDGF-BB maintained Noggin expression at low levels. Gremlin, another inhibitory molecule, was also slightly decreased in the rhPDGF-BB-treated AdBMP2-transfected BMSCs; however, the decrease was not statistically significant (Fig. 3D).

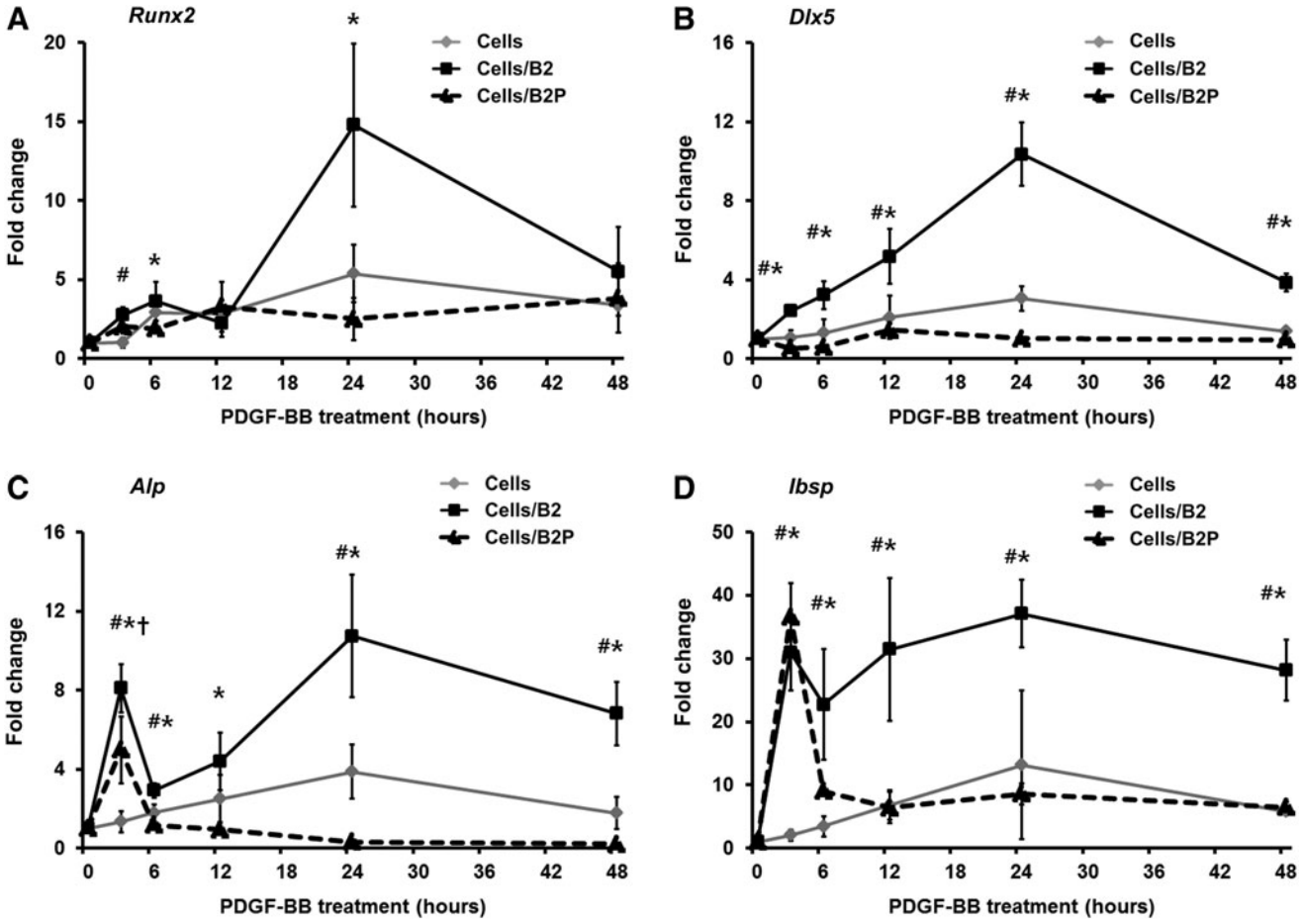

FIG. 2. Gene expression patterns of osteogenic markers in AdBMP2-transfected BMSCs following rhPDGF-BB treatment. The rhPDGF-BB treatment significantly suppressed the mRNA expression of **(A)** Runx2, **(B)** Dlx5, **(C)** Alp, and **(D)** Ibsp induced by the AdBMP2-transfected BMSCs, especially at 24 h. The mRNA expression of each osteogenic factor was normalized against *Gapdh*. *Statistically significant difference between AdBMP2-transfected BMSCs and AdBMP2-transfected BMSCs/rhPDGF-BB (ANOVA, $p < 0.05$). #Statistically significant difference between nontransfected BMSCs and AdBMP2-transfected BMSCs (ANOVA, $p < 0.05$). †Statistically significant difference between nontransfected BMSCs and AdBMP2-transfected BMSCs/rhPDGF-BB (ANOVA, $p < 0.05$). Cell, nontransfected BMSCs; cells/B2, AdBMP2-transfected BMSCs; cells/B2P, AdBMP2-transfected BMSCs/rhPDGF-BB. Values represent mean ± SD of three samples.

Results of the in vivo *experiment*

Histological findings and histomorphometric analysis. The implanted cells on the calvarial defects were detected in the regenerative bone tissue on histological sections at 2 and 4 weeks after surgery (Supplementary Fig. S1). When nontransfected BMSCs alone or rhPDGF-BB alone was applied into a critical-sized defect, a limited amount of bone regeneration was observed along the margin of the defects at 2 and 4 weeks after surgery (Fig. 4A). However, both the BMSC/*BMP2* and the BMSC/*BMP2*/rhPDGF-BB groups showed extensive immature woven bone formation at 2 weeks; these formations were replaced with rich bone marrow at 4 weeks. Moreover, it was found that bone bridging connected the central region of the defect in the BMSC/*BMP2*/rhPDGF-BB group. The negative and the vehicle groups showed minimal bone regeneration (Supplementary Fig. S2 and Supplementary Table S2).

In the histomorphometric analysis, the most new bone area at 2 weeks was observed in the BMSC/*BMP2*/rhPDGF-BB group (Fig. 4B). Both the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups showed a significantly greater new bone area than the other groups at 2 weeks after surgery ($p = 0.003$). However, no significant difference was observed between the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups. At 4 weeks, the BMSC/*BMP2*/rhPDGF-BB group showed significantly more new bone area than other groups ($p < 0.001$).

The defect closures of the critical-sized calvarial defects in both the BMSC/*BMP2* and the BMSC/*BMP2*/rhPDGF-BB groups (48.60% ± 30.90% and 63.75% ± 22.94%, respectively), were significantly greater than those of the other groups at 2 weeks ($p < 0.001$; Fig. 4C). However, no significant difference was observed between the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups. At 4 weeks, the BMSC/*BMP2*/rhPDGF-BB group showed significantly greater defect closure rates (79.68% ± 17.64%) than the other groups ($p < 0.001$).

Micro-CT findings and BMD. The newly formed BV at each defect site was evaluated using three-dimensional reconstructed micro-CT images (Fig. 5). Both the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups showed extensive

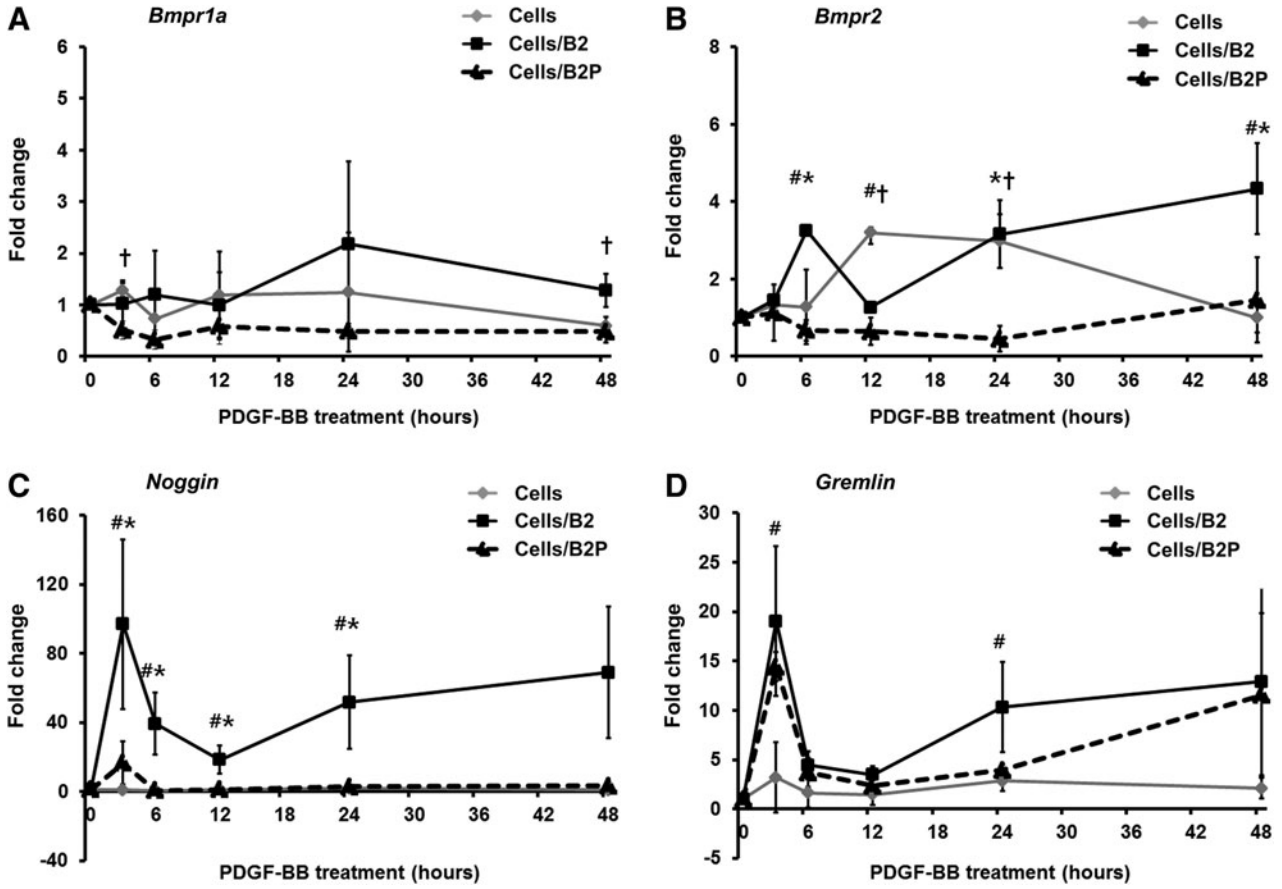

FIG. 3. Gene expression patterns of BMP-2 related factors Bmpr1a **(A)**, Bmpr2 **(B)**, Noggin **(C)**, and Gremlin **(D)** in AdBMP2-transfected BMSCs following the rhPDGF-BB treatment. The mRNA expression of both BMPRII and Noggin in the AdBMP2-transfected BMSCs was significantly decreased by the rhPDGF-BB treatment. The mRNA expression of each factor was normalized against *Gapdh*. *Statistically significant difference between AdBMP2-transfected BMSCs and AdBMP2-transfected BMSCs/rhPDGF-BB (ANOVA, $p < 0.05$). #Statistically significant difference between nontransfected BMSCs and AdBMP2-transfected BMSCs (ANOVA, $p < 0.05$). †Statistically significant difference between nontransfected BMSCs and AdBMP2-transfected BMSCs/rhPDGF-BB (ANOVA, $p < 0.05$). Cell, nontransfected BMSCs; cells/B2, AdBMP2-transfected BMSCs; cells/B2P, AdBMP2-transfected BMSCs/rhPDGF-BB. Bmpr1a, BMP receptor type Ia; Bmpr2, BMP receptor type II. Values represent mean ± SD of three samples.

new bone formation, although the newly formed bone had low radiopacity at 2 weeks after surgery. At 4 weeks, the radiopacity of the newly formed bone was enhanced in both the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups compared to the 2-week samples.

For quantitative analysis, the ratio of mineralized BV/TV was measured using micro-CT images. Both the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups showed significantly higher BV/TV values compared to the other groups at 2 weeks ($p = 0.026$). However, no statistical significance was observed between the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups. The BMD of the newly formed bone at 2 weeks was similar in all the groups.

At 4 weeks, BV/TV was significantly greater in the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups ($p = 0.001$). However, there was no statistical significance between the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups. The BMD of all of the experimental groups was higher at 4 weeks compared to 2 weeks except for the BMSC/*BMP2* group. In addition, the BMD of the BMSC/*BMP2* group was significantly less than the other groups ($p = 0.009$).

Discussion

Bone healing is a highly coordinated process that is orchestrated by various growth factors and cells. Tissue engineering mimics these healing process using exogenous or endogenous growth factors, the required cells and scaffolds. Many studies have described the effects of single and multifactor application on bone healing. Our previous work showed that the transplantation of autologous BMSCs and PDL cells with HA/TCP was more effective than cell-free HA/TCP in a canine model.[28] We also demonstrated that the combined therapy of *BMP2* with a collagen sponge or *BMP2* with bone graft materials promoted bone regeneration in animal studies.[25,29] Although combined therapies of BMP-2 and BMSC or carrier enhanced bone healing, these therapies have distinct mechanisms in bone formation. This study designed a system of combined and concerted delivery of BMP-2 and PDGF-BB using BMSCs and collagen gel to mimic the concomitant interactions among the various factors involved in bone regeneration; PDGF-BB was delivered in protein form to act transiently during early healing, whereas BMP-2 was produced from BMSCs transfected with *BMP2* to

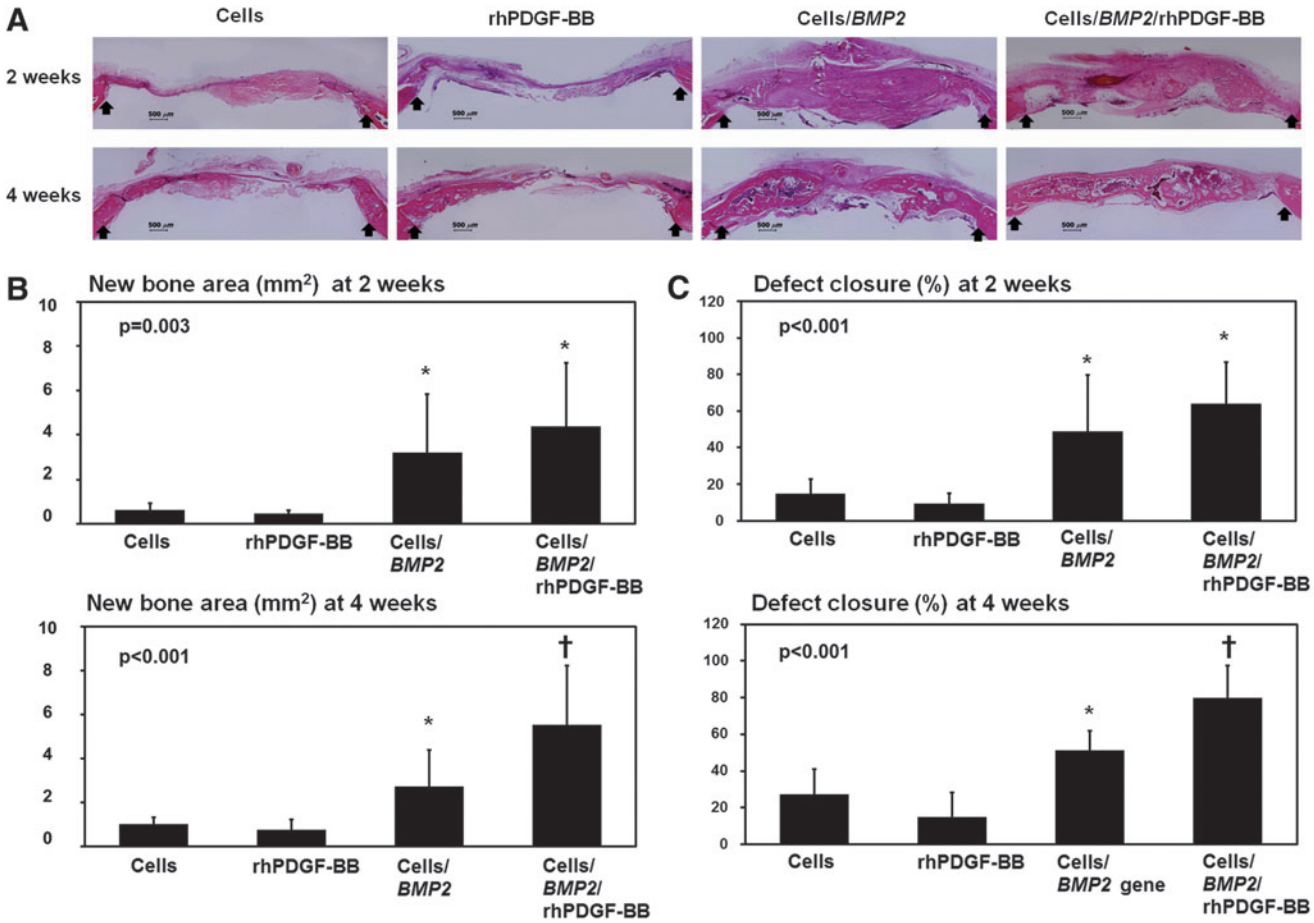

FIG. 4. *In vivo* histology and histomorphometric analysis. **(A)** Representative histology at 2 and 4 weeks postsurgery (decalcified; H&E stain; original magnification 2×). Both the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups showed extensive new bone formation at 2 and 4 weeks. In the BMSC/*BMP2*/rhPDGF-BB group, bone bridging that covered the central region of the defect area was observed. Arrow: margin of defect. **(B)** New bone area (mm^2) at the defect site. Both the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups showed significantly higher amounts of new bone area at 2 weeks after surgery (ANOVA, $p=0.003$). However, there was no significant difference between the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups. At 4 weeks, the BMSC/*BMP2*/rhPDGF-BB group had significantly higher levels of new bone area compared to the other groups (ANOVA, $p<0.001$). **(C)** Defect closure (%) within the defects. Both the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups produced a significantly higher percentage of defect closure at 2 weeks (ANOVA, $p<0.001$). However, there was no significant difference between the BMSC/BMP2 and BMSC/BMP2/rhPDGF-BB groups. At 4 weeks, the defect closure of the BMSC/*BMP2*/rhPDGF-BB group was significantly higher than the other groups (ANOVA, $p<0.001$). Defect closure was measured as the ratio of the area of newly formed bone divided by the area of the whole defect. *Statistically different from the BMSCs and rhPDGF-BB groups. †Statistically different from the other groups. Values represent mean ± SD of six samples.

provide BMP-2 during the entire wound-healing period, thereby mimicking natural healing events. Therefore, the present study demonstrated that the dual delivery of rhPDGF-BB and *BMP2*-transfected BMSCs improved bone formation in terms of bone quantity and quality.

When *BMP2*-transfected BMSCs were applied alone, new bone formation increased after 2 weeks at levels similar to a dual-delivery group. However, the BMD did not improve after 4 weeks when compared to the dual-delivery groups. The BMD of the newly formed bone is one of the critical parameters of bone quality because BMD indicates bone strength and maturation.[30,31] Similar to this study, it has been reported that the single application of BMP-2 as a protein or gene does not lead to bone healing in some critical-sized bone defects.[32–36] Virk et al.[37] proposed that the long-term application of BMP-2, such as more than 8 weeks of BMP-2 using a lentivirus, was required to stimulate mechanically superior bone formation. However, lentiviruses may be inserted into the host genome, whereas adenoviruses can only be inherited by daughter cells of previously transfected cells. Accordingly, additional signal transduction is needed to facilitate the maturation of bone in *BMP2*-transfected animals, and PDGF-BB may represent one of these signaling molecules. In fact, some studies have reported superior biomechanical properties in bone treated with rhPDGF-BB. In the ovariectomized osteoporotic rat model, the systemic administration of PDGF-BB prevented bone loss and increased bone density and skeletal strength.[38] Moreover, in geriatric osteoporotic rat models, a PDGF-BB treatment accelerated fracture healing; the rhPDGF-BB-treated tibias had

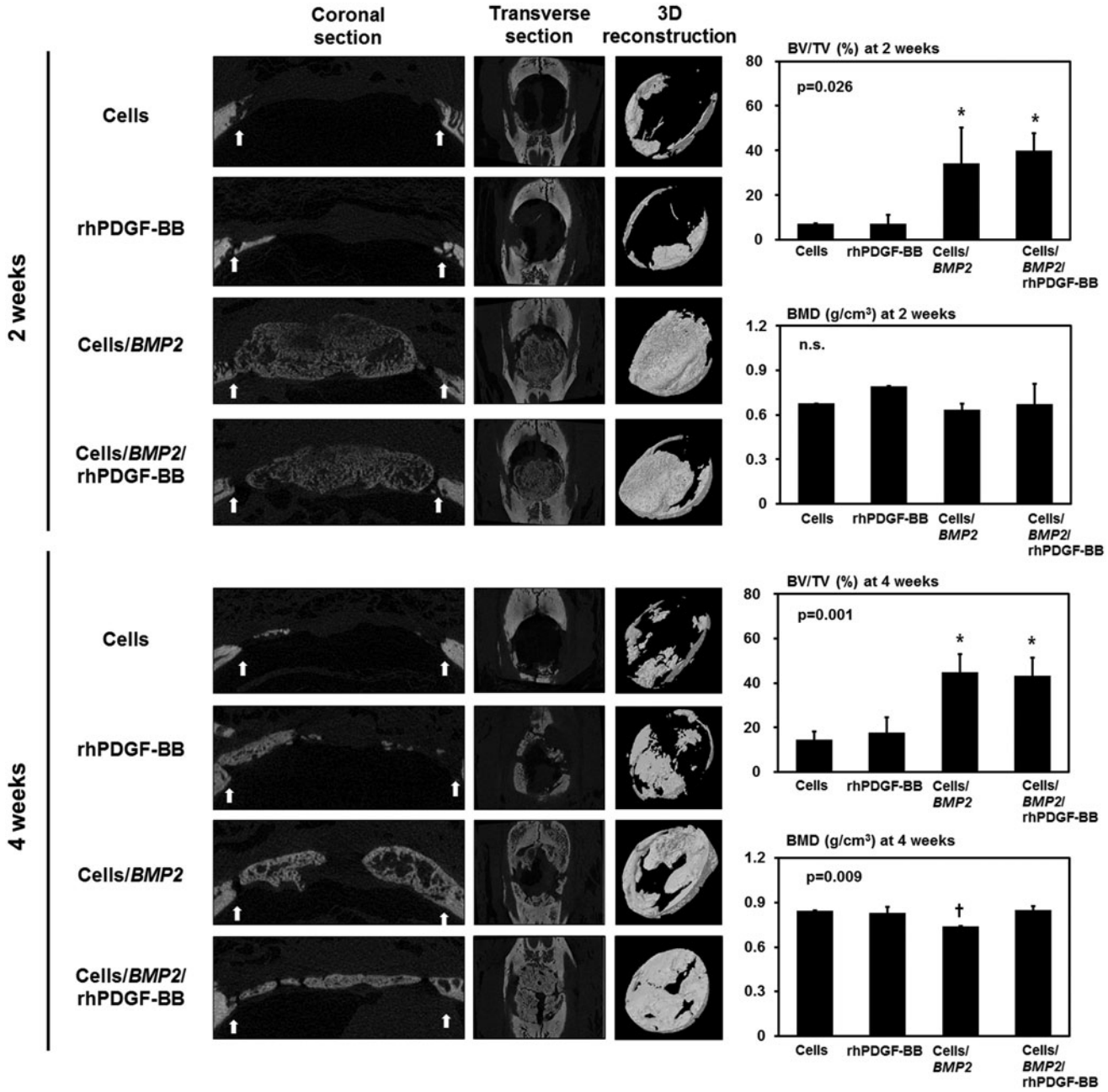

FIG. 5. Micro-CT analysis and quantitative measurements of calvarial defects at 2 and 4 weeks. Both the BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups showed extensive new bone formation, in which the new bone had low radiopacity at 2 weeks. The new bone at 4 weeks was more mature and had enhanced radiopacity compared to the bone at 2 weeks. Both BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups showed significantly higher levels of mineralized BV percentage to TV of defect at 2 and 4 weeks (ANOVA, $p=0.026$ and $p=0.001$, respectively). However, there was no significant difference between BMSC/*BMP2* and BMSC/*BMP2*/rhPDGF-BB groups. In terms of BMD, there was no statistical significance at 2 weeks. At 4 weeks, the BMSC/*BMP2* group showed significantly less BMD of newly formed bone than other groups, including BMSC/*BMP2*/rhPDGF-BB groups (ANOVA, $p=0.009$). *Statistically different from the BMSCs and rhPDGF-BB groups. †Statistically different from the other groups. Micro-CT images: left, coronal section view; middle, transverse view; right, 3D reconstruction of newly formed bone in the defect site; arrow, margin of defect; BV/TV, the percentage of mineralized bone volume of the defect tissue volume; BMD, bone mineral density. Values represent mean ± SD of six samples. micro-CT, microcomputed tomography.

equivalent torsional strengths compared to the contralateral nonoperated site.[39] The PDGF-BB treatment in a rabbit tibial osteotomy site increased the callus density and volume and bone strength; the resulting values were similar to non-operated contralateral bones.[40] Tokunaga et al.[41] also reported that PDGF receptor-β-depleted mice showed an increased immature woven bone ratio even though PDGF receptor-β signaling inhibited the osteogenic differentiation of mesenchymal stem cells. Similar to previous studies, rhPDGF-BB application in the current study also resulted in

higher BMD increases compared to the *BMP2* treatment alone.

However, some inconsistencies were apparent between the *in vitro* and *in vivo* studies. The *in vitro* results demonstrated that rhPDGF-BB suppresses osteogenic differentiation, while the *in vivo* results demonstrated that rhPDGF-BB cannot affect the amount of bone formation. The mitogenic effects of rhPDGF-BB were not hindered by *BMP2* delivery, and rhPDGF-BB did not interfere with BMP-2 secretion in AdBMP2-transfected BMSCs. However, osteogenic differentiation with BMP-2 was affected by rhPDGF-BB. This result may be attributed to the reduced gene expression of BMP-2 signaling-related proteins in the present study. The mRNA expression levels of Runx2 and Dlx5, a mediator between BMP-2 and Runx2,[42] were significantly suppressed by rhPDGF-BB. In addition, BMPRII mRNA expression was suppressed by rhPDGF-BB. Consequently, it seems that rhPDGF-BB interferes with BMP-2-dependent osteogenic differentiation *in vitro* via the suppression of BMPRII, which is required to initiate BMP-2 signaling, resulting in the downregulation of Runx2 and Dlx5.[43–45] The suppressive effect of PDGF-BB on osteogenic differentiation has also been reported in previous studies.[46,47] Gruber et al.[48] also reported that PDGF decreased osteogenic differentiation of mesenchymal cells, but increased cellular migration and proliferation. Recently, Gharibi et al.[49] suggested that PDGF-BB regulates proliferation and differentiation in relation to self-renewal in mesenchymal cells, thereby maintaining their multipotency through PDGFRβ-induced Akt and Erk pathways. This result may help to explain the synergistic or additive effects of rhPDGF-BB and AdBMP2-transfected BMSCs in this *in vivo* study. In conclusion, rhPDGF-BB may modulate differentiation *in vivo* and suppress osteogenic differentiation *in vitro*. Kratchmarova et al.[50] also reported that PDGF increased new bone formation *in vivo*, although PDGF minimally influenced osteogenic differentiation *in vitro*.

Some authors have reported that Noggin can negatively affect bone formation. Gazzerro et al.[51] reported that Noggin overexpression arrested stromal cell differentiation and prevented cellular maturation. Additionally, murine stromal cells, which overexpressed Noggin, showed a delayed appearance of mineralized nodules and an absence of osteocalcin in their study. The overexpression of Noggin has also been reported to decrease trabecular BV and impair osteoblastic function, leading to osteopenia and fractures.[52,53] In fact, significant increases of Noggin mRNA have been reported in nonunion human samples compared with healing bone tissue, and this imbalanced Noggin expression was proposed to be the reason for nonunion fracture healing.[54–56] Furthermore, Jin et al.[57] reported that periodontal bone repair was significantly inhibited by adenoviral Noggin gene delivery. The present study demonstrated that rhPDGF-BB suppressed *Noggin* expression (Fig. 3C). *Noggin* expression was significantly increased in AdBMP2-transfected BMSCs. However, *Noggin* expression was not increased following the rhPDGF-BB treatment in the AdBMP2-transfected BMSCS. The inhibitory effect of PDGF-BB on *Noggin* expression may also positively affect bone maturation of newly formed bone induced by BMP-2.

Taken together, BMP-2 expression through *ex vivo* adenoviral *BMP2* gene delivery was maintained for a 21-day period and was not influenced by rhPDGF-BB. rhPDGF-BB increased the number of cells capable of differentiating into various cell types, including osteoblasts needed for bone tissue healing. rhPDGF-BB demonstrated a modulatory effect on BMP-2-induced osteogenesis through the inhibition of the expression of both *BmprII* and *Noggin*. Although rhPDGF-BB suppressed the BMP-2-induced osteogenic differentiation of BMSCs *in vitro*, the dual delivery of rhPDGF-BB and AdBMP2-transfected BMSCs induced excellent new bone formation and significantly increased BMD compared to the *BMP2*/BMSC group.

Acknowledgments

The authors would like to appreciate the careful reading and helpful advice of Professor W.V. Giannobile (Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, USA; Michigan Center of Oral Health Research, Ann Arbor, USA). The authors thank Ms. Kwak, Eun-Hye for her technical support. This study was supported by grant no 03-2009-0018 from the SNUDH Research Fund.

Disclosure Statement

The authors have no competing financial interests.

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Received: October 31, 2012
Accepted: June 19, 2013
Online Publication Date: July 30, 2013