Enhancement of *In Vivo* Bone Regeneration Efficacy of Human Mesenchymal Stem Cells

Kang, Sun-Woong[1], Jae-Sun Lee[2], Min Sun Park[1], Jung-Ho Park[2], and Byung-Soo Kim[3]*

[1]Department of Chemical Engineering, Hanyang University, Seoul 133-791, Korea
[2]Department of Orthopaedic Surgery, Ansan Hospital, College of Medicine, Korea University, Ansan 425-020, Korea
[3]Department of Bioengineering, Hanyang University, Seoul 133-791, Korea

We investigated whether transplantation of osteogenically differentiated bone marrow-derived mesenchymal stem cells (BMMSCs) and the use of an hydroxyapatite (HAp) scaffold can enhance the *in vivo* bone formation efficacy of human BMMSCs. Three months after implantation to the subcutaneous dorsum of athymic mice, transplantation of osteogenically differentiated human BMMSCs increased the bone formation area and calcium deposition to 7.1- and 6.2-folds, respectively, of those of transplantation of undifferentiated BMMSCs. The use of the HAp scaffold increased the bone formation area and calcium deposition to 3.7- and 3.5-folds, respectively, of those of a polymer scaffold. Moreover, a combination of transplantation of osteogenically differentiated BMMSCs and HAp scaffold further increased the bone formation area and calcium deposition to 10.6- and 9.3-folds, respectively, of those of transplantation of undifferentiated BMMSCs seeded onto polymer scaffolds. The factorial experimental analysis showed that osteogenic differentiation of BMMSCs prior to transplantation has a stronger positive effect than the HAp scaffold on *in vivo* bone formation.

Keywords: Bone formation, hydroxyapatite, mesenchymal stem cell, osteogenic differentiation

Bone marrow-derived mesenchymal stem cells (BMMSCs) are an attractive cell source for bone regeneration because of their ability of self-renewal with a high proliferative capacity and possession of osteogenic differentiation potential [6, 10, 12, 13, 22, 26, 27]. In recent years, successful bone regeneration by BMMSC implantation has been shown in animal models [1, 3, 17, 21, 24] and humans [18, 23].

There have been attempts to enhance the bone regeneration efficacy of BMMSCs. It has been reported that bioceramic substrate stimulates cell proliferation in human BMMSC cultures [19]. *In vitro* culture of BMMSCs prior to transplantation affected *in vivo* bone regeneration, as orthotopic transplantation of cultured sheep BMMSCs resulted in more extensive bone formation than that of freshly isolated, noncultured bone marrow cells [21]. BMMSCs genetically engineered to overexpress an osteogenic gene (*e.g.*, bone morphogenetic proteins) can enhance bone formation by inducing osteogenic differentiation of the engineered BMMSCs and recruiting host BMMSCs to the implantation site [11].

It is well known that a composite of BMMSCs and biocermic [*e.g.*, hydroxyapatite (HAp)] scaffold has *in vivo* osteogenic potential [9, 20, 28]. However, no study has directly compared the effects of a bioceramic scaffold and a non-bioceramic scaffold on human BMMSC transplantation-mediated bone formation. In addition, osteogenic differentiation of BMMSCs prior to transplantation could affect the bone formation efficacy of the transplanted BMMSCs. Therefore, in this study, we investigated whether transplantation of osteogenically differentiated BMMSCs and the use of a HAp scaffold can enhance the *in vivo* bone formation efficacy of human BMMSCs. To investigate this, human BMMSCs cultured in a non-osteogenic medium or an osteogenic medium were seeded onto PLGA scaffolds or PLGA/HAp composite scaffolds and implanted to the subcutaneous dorsum of athymic mice. Two and three months after implantation, the bone formation area and calcium deposition were determined. In addition, a 2-factor (osteogenic differentiation prior to transplantation and HAp scaffold), 2-level factorial experimental approach was used to quantitatively evaluate the effects of osteogenic differentiation prior to the transplantation and HAp scaffold on *in vivo* bone regeneration.

*Corresponding author
Phone: 82-2-2220-0491; Fax: 82-2-2291-0838;
E-mail: bskim@hanyang.ac.kr

MATERIALS AND METHODS

Preparation of Biomaterial Films and Scaffolds

75:25 poly(lactic-co-glycolic acid) [PLGA, molecular mass= 110,000 Da; Birmingham Polymers, Birmingham, AL, U.S.A.; 10% (w/v)] dissolved in methylene chloride was cast on a glass plate at room temperature [25]. PLGA/HAp films were fabricated by spraying HAp nanoparticles (diameter=approximately 100 nm; Berkeley Advanced Biomaterials Inc., Berkeley, CA, U.S.A., 0.1 g/cm^2) homogeneously on the polymer films. The solvent was allowed to evaporate, and the films were lyophilized to remove residual solvent for 3 days. PLGA films without HAp were also fabricated and used as a control. Porous PLGA/HAp composite scaffolds were fabricated with a previously described gas-forming and particulate-leaching (GF/PL) method [14–16]. PLGA scaffolds without HAp were also fabricated with the GF/PL method and used as a control.

Isolation and Culture of Human BMMSCs

Bone marrow aspirates were obtained from informed and consenting patients undergoing routine total hip replacement surgery. Only tissues that would have been discarded were used for the bone marrow isolation with the approval of the Korea University Hospital Ethics Committee. Primary cultures of bone marrow cells were established as previously described [12]. In brief, bone marrow aspirate was mixed with an equal volume of phosphate-buffered saline (PBS; Sigma, St. Louis, MO, U.S.A.). The mixture was centrifuged on a Ficoll-Paque density gradient (Amersham Biosciences, Arlington Heights, IL, U.S.A.) for 20 min at 1,500 rpm, and bone marrow mononuclear cells (BMMNCs) were isolated from the buffy coat layer between the Ficoll-Paque reagent and blood plasma component. BMMNCs were washed three times in PBS and plated in culture dishes. BMMSCs were obtained by culturing the BMMNCs in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, U.S.A.) containing 10% (v/v) fetal bovine serum (FBS; Gibco BRL) and 1% (v/v) penicillin-streptomycin (Gibco BRL) in humidified air with 5% CO$_2$ at 37°C. Non-adherent cells were removed during the culture. Adherent cells (BMMSCs) were cultured and expanded for further experiments. BMMSCs with the passage number 1 were used in the experiments.

Cell Culture Experiment

PLGA or PLGA/HAp films coated on glass dishes were sterilized using 70% (v/v) ethanol and washed with sterile distilled water. BMMSCs (5×10^3/cm^2 cells) were seeded onto the PLGA or PLGA/HAp films and cultured in non-osteogenic medium [DMEM containing 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin] or osteogenic medium [DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate (Sigma), 50 μg/ml L-ascorbic acid (Sigma), 100 nM dexamethasone (Sigma), and 1% (v/v) penicillin-streptomycin] [22] for 2 weeks (n=6 films per group). The culture medium was changed every other day. Analytical assays for osteogenic differentiation were performed at one and two weeks.

Cell Transplantation

Osteogenically differentiated and undifferentiated BMMSCs were suspended in DMEM at a concentration of 2×10^7 cells/ml and seeded onto porous PLGA and PLGA/HAp composite scaffolds (5 mm× 5 mm, 1.7 mm in thickness, n=6 per group). Athymic female mice (6 weeks old; Japan SLC, Inc., Japan) were anesthetized with intramuscular injection of ketamine hydrochloride (50 mg/kg; Yuhan Corporation, Seoul, Korea) and xylazine hydrochloride (5 mg/kg; Bayer, Seoul, Korea). After the mice were anesthetized, small incisions were made on the dorsal skins of the mice, and cell-seeded scaffolds were immediately implanted subcutaneously. Subsequently, the skin was closed with 5–0 Vicryl sutures (Ethicon, Lenneke Marelaan, Belgium). The implants were retrieved for analysis at two and three months after implantation.

Scanning Electron Microscopy

The cross-sectional morphologies of the cell-scaffold constructs were examined one day after cell seeding using a scanning electron microscope (SEM; JEOL, Tokyo, Japan). The samples were washed twice with PBS, prefixed in 1% (v/v) buffered glutaraldehyde for 1 h, and fixed in 0.1% (v/v) buffered formaldehyde for 24 h. The fixed samples were dehydrated in ascending grades of ethanol, dried, and mounted on aluminum stubs using double-sided carbon tape. The specimens were coated with gold using a Sputter Coater (Cressington 108; Cressington Scientific Instruments, Cranberry, PA, U.S.A.) and examined with an SEM at an acceleration voltage of 10 kV.

Histological and Immunohistochemical Analyses

Two and three months after implantation, the implants were retrieved and analyzed histologically. For histological analyses, specimens were fixed in 10% (v/v) buffered formalin, dehydrated with a series of graded alcohol, and embedded in paraffin. Tissue sections, 4 mm thick, were stained with Masson's trichrome for cross-linked collagen and von Kossa for calcium. The von Kossa-stained mid-portion sections were examined with a microscope for histomorphometry. The percentage of bone occupying space within the implants was measured using an image analysis system (KS400; Zeiss, Munich, Germany) coupled to a light microscope. The bone formation area was expressed as the percentage of bone area in the available pore space (bone area/pore area×100%). To measure the bone area formed by seeded cells, the calcium area of the scaffolds itself was also measured and subtracted from the total bone area. Tissue sections were stained immunohistochemically with antibodies against human nuclear antigen (HNA; Chemicon, Temecula, CA, U.S.A.) to detect implanted human cells. The antibody staining was visualized with an avidin-biotin complex immunoperoxidase kit (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, U.S.A.) and 3,3'-diaminobenzidine substrate solution kit (Vector Laboratories) and counterstained with Harris hematoxylin (Sigma).

Calcium Assay

The calcium content of the implants was quantified by a colorimetric endpoint assay that measures the amount of blue-purple colored calcium-Arsenazo^{2+} complex formed when Arsenazo III binds to free calcium in an acid solution [2]. Retrieved implants (n=3) were lyophilized for 3 days and weighed. Dried samples were placed in 6 N HCl overnight on a shaker table to dissolve the deposited calcium. Twenty-μl aliquots of each sample were added to 2 ml of Arsenazo III reagent (Diagnostic Chemicals Ltd., Oxford, CT, U.S.A.) in individual quartz cuvettes and the absorbance at 650 nm was read on a spectrophotometer (Agilent Technologies Inc., Palo Alto, CA, U.S.A.). Samples were compared against CaCl$_2$ standards (Sigma). To measure the calcium produced by cells, the calcium content of the scaffold itself was also measured and subtracted from the total calcium content of the implants.

Table 1. Nucleotide sequences of human-specific primer sets used for alkaline phosphatase (ALP), collagen type I (Col I), bone sialoprotein (BSP), osteocalcin (OC), bone morphogenetic protein (BMP)-6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in RT-PCR assays.

Primer	Sequences	Product size (bp)
ALP		
Sense	5'-GGACATGCAGTACGAGCTGA-3'	357
Antisense	5'-GCAGTGAAGGGCTTCTTGTC-3'	
Col I		
Sense	5'-CTG ACC TTC CTG CGC CTG ATG TTC-3'	599
Antisense	5'-TTG GAC GTT GGT GCC CCA GAC-3'	
BSP		
Sense	5'-GCT CAG CAT TTT GGG AAT GGC-3'	614
Antisense	5'-CTG CAT TGG CTC CAG TGA CAC-3'	
OC		
Sense	5'-ATG AGA GCC CTC AGA CTC CTC-3'	297
Antisense	5'-CGG GCC GTA GAA GCG CCG ATA-3'	
BMP-6		
Sense	5'-GCA GAA GGA GAT CTT GTC GG-3'	629
Antisense	5'-GTC TCT GTG CTG ATG CTC CT-3'	
GAPDH		
Sense	5'-ACAGTCAGCCGCATCTTCTT-3'	312
Antisense	5'-TTGATTTTGGAGGGATCTCG-3'	

bp: base pair.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay

BMMSCs cultured on PLGA or PLGA/HAp films were lysed by an addition of 0.5 ml of Trizol reagent (Invitrogen), and total RNA was extracted and reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen). Primers and PCR conditions for alkaline phosphatase (ALP), collagen type I (Col I), bone sialoprotein (BSP), osteocalcin (OC), bone morphogenetic protein (BMP)-6, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as shown in Table 1. PCR was performed under the following conditions: up to 35 cycles of denaturing at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 90 sec with a final extension at 72°C for 7 min. The PCR products were analyzed using 1.5% (w/v) agarose gel electrophoresis with ethidium bromide staining. Gels were read using a gel photodocumentation system (Gel Doc 1000; Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Factorial Experiments

A full 2^2 factorial experimental design with two variables was applied to investigate the effects of variables and interaction between the variables on the responses (Table 2). Osteogenic differentiation prior to the transplantation and containment of HAp in scaffold were chosen as the variables. The newly formed bone area and calcium

Table 2. Experimental groups used in the factorial design experiments.

Group	Type of scaffold	Type of BMMSCs for transplantation
1	PLGA	Undifferentiated
2	PLGA	Osteogenically differentiated
3	PLGA-HAp	Undifferentiated
4	PLGA-HAp	Osteogenically differentiated

deposition in bone tissues regenerated by human BMMSC transplantation were chosen as the responses. To evaluate the effects of each variable and the interaction between the variables on the response, Design-Expert software (version 7, Stat-Ease Inc.) was used [5].

Statistical Analysis

Quantitative data were expressed as the mean±standard deviation. Statistical comparisons were carried out using two-way ANOVA (SAS software; SAS Institute Inc., Cary, NC, U.S.A.). A probability level of less than 0.05 was considered to be statistically significant.
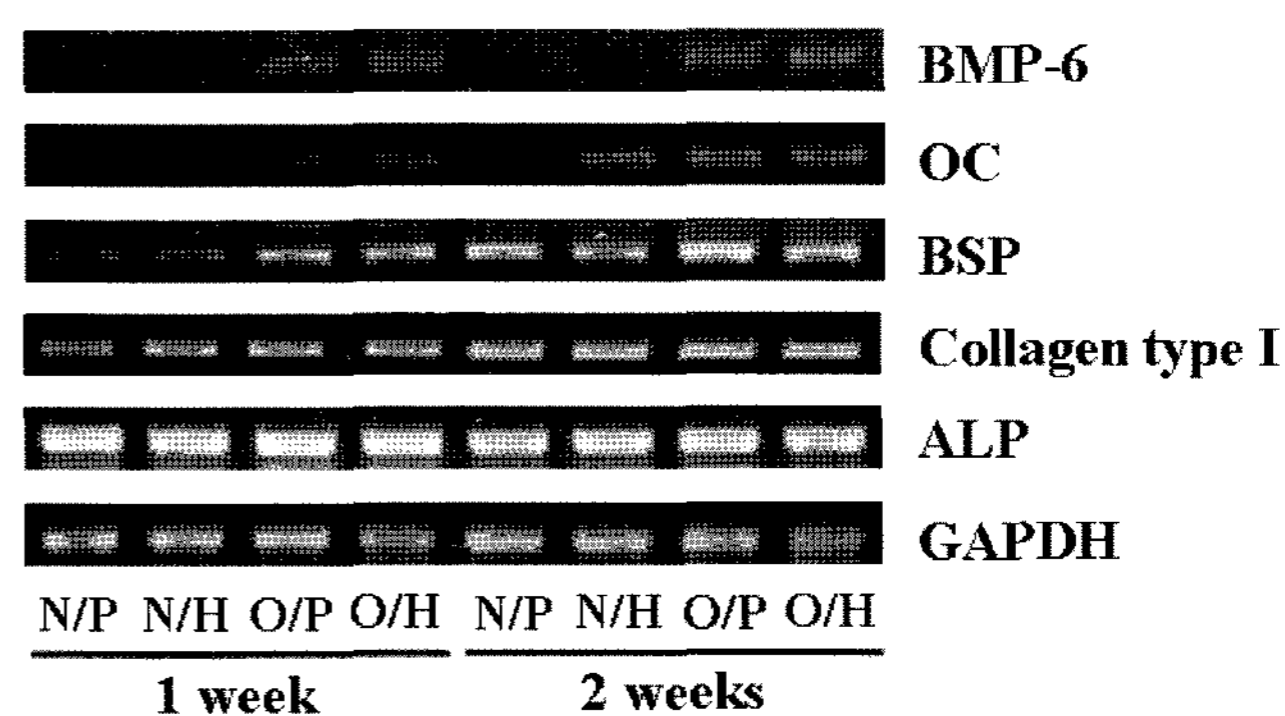

Fig. 1. RT-PCR analysis using human-specific primers to detect bone marker mRNA expression of human BMMSCs cultured on PLGA or PLGA/HAp films in non-osteogenic or osteogenic media for one or two weeks.

N/P, culture on PLGA films in non-osteogenic medium; N/H, culture on PLGA/HAp films in non-osteogenic medium; O/P, culture on PLGA films in osteogenic medium; O/H, culture on PLGA/HAp films in osteogenic medium.
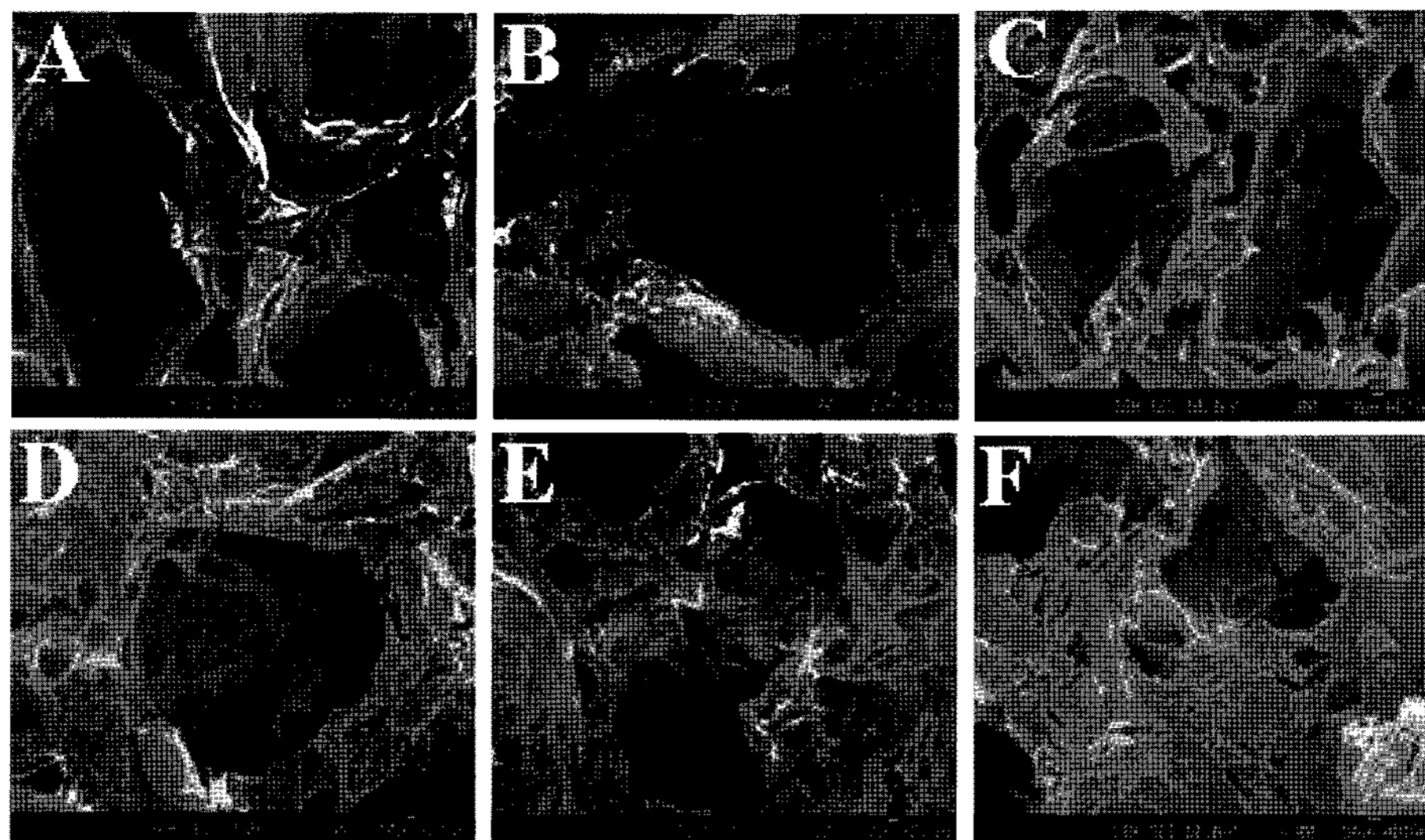
Fig. 2. Scanning electron microscopic photographs of (**A**) PLGA and (**B**) PLGA/HAp scaffolds with no cell seeding and PLGA and PLGA/HAp scaffolds seeded with human BMMSCs that had been cultured in (**C, D**) non-osteogenic medium or (**E, F**) osteogenic medium one day after seeding.

RESULTS AND DISSCUSION

To determine whether culture with an osteogenic medium and culture on a HAp biomaterial can induce osteogenic differentiation of the cultured human BMMSCs, the expression of mRNA for ALP, Col I, BSP, OC, and BMP-6 of human BMMSCs cultured on PLGA or PLGA/HAp films in osteogenic or non-osteogenic media was determined
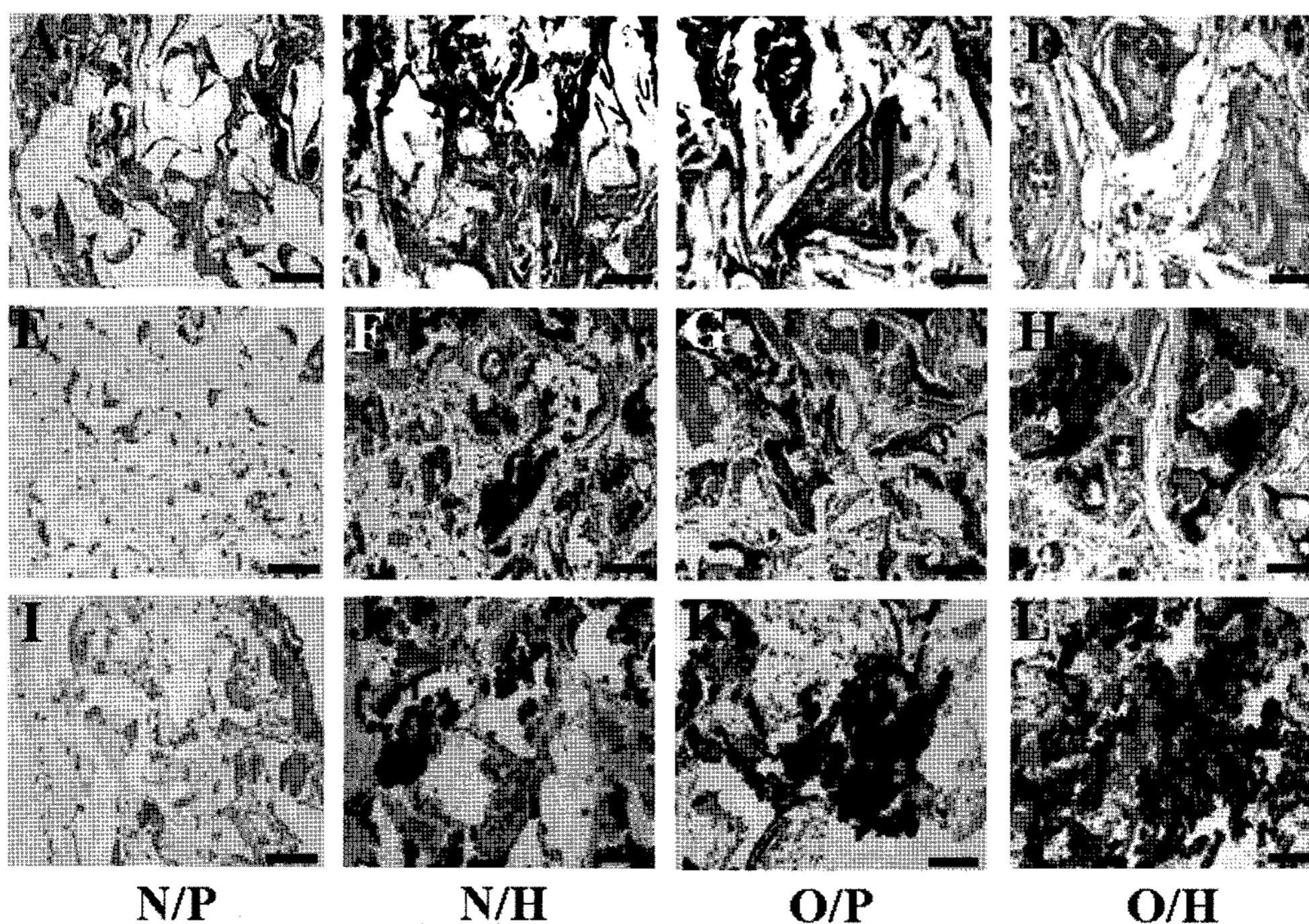

N/P   N/H   O/P   O/H

Fig. 3. Histological analyses of tissues formed by implantation of (**A, C, E, G, I,** and **K**) PLGA or (**B, D, F, H, J,** and **L**) PLGA/HAp scaffolds seeded with human BMMSCs that had been cultured in (**A, B, E, F, I,** and **J**) non-osteogenic medium or (**C, D, G, H, K,** and **L**) osteogenic medium into the subcutaneous dorsum of mice for (**A–H**) two or (**I–L**) three months.
(**A–D**) Masson's trichrome and (**E–L**) von Kossa staining. N/P, PLGA scaffolds seeded with BMMSCs that had been cultured in non-osteogenic medium; N/H, PLGA/HAp scaffolds seeded with BMMSCs that had been cultured in non-osteogenic medium; O/P, PLGA scaffolds seeded with BMMSCs that had been cultured in osteogenic medium; and O/H, PLGA/HAp scaffolds seeded with BMMSCs that had been cultured in osteogenic medium. Black color corresponds to calcified areas in von Kossa staining. All scale bars indicate 100 μm.
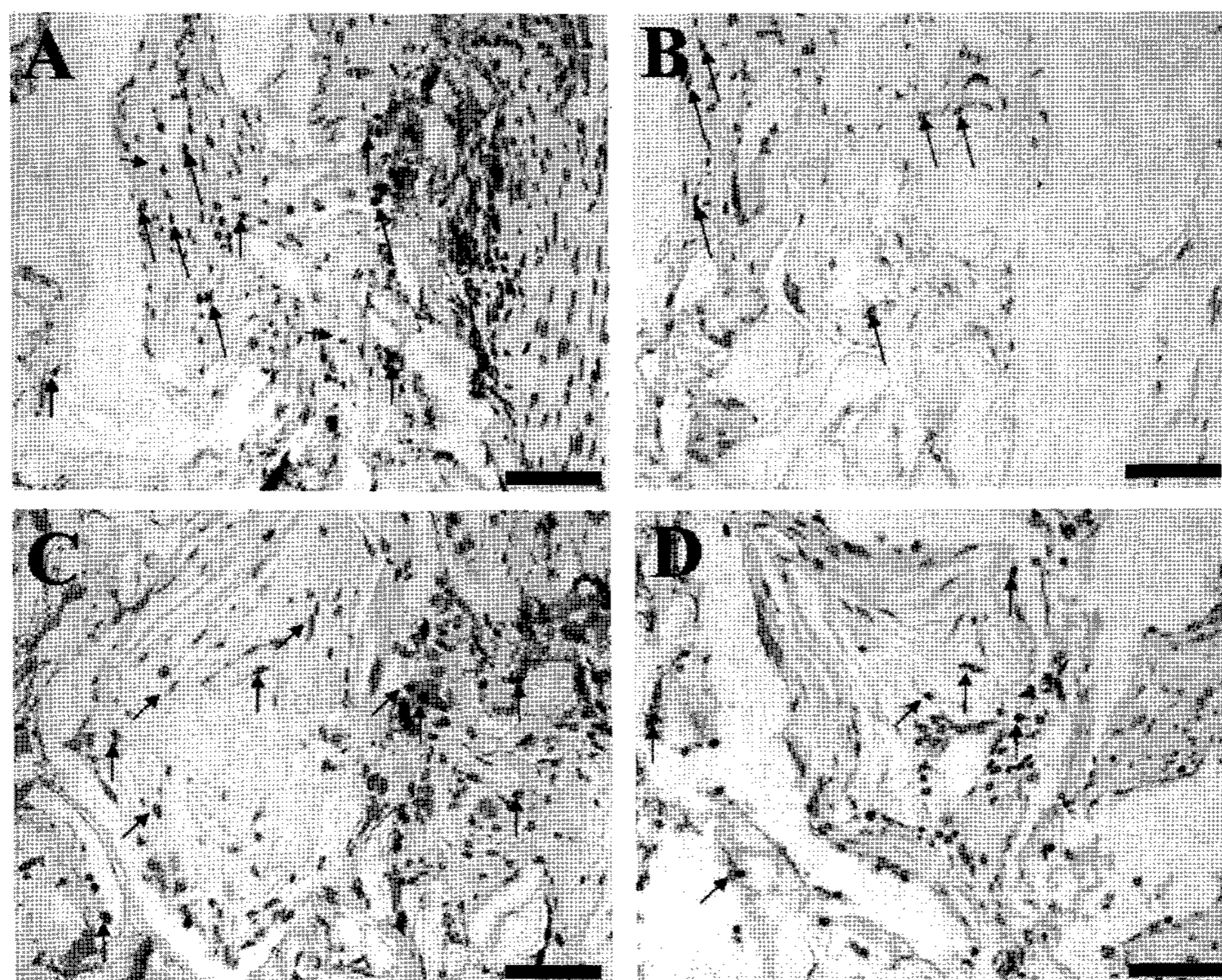
Fig. 4. Immunohistochemical staining for human nuclear antigen (HNA) to detect transplanted human BMMSCs in tissues formed by transplantation of BMMSC-scaffold constructs for two months.

The arrows indicate HNA-positive human BMMSC nuclei. The tissue sections were counterstained with Harris hematoxylin. **A.** N/P, PLGA scaffolds seeded with BMMSCs that had been cultured in non-osteogenic medium. **B.** N/H, PLGA/HAp scaffolds seeded with BMMSCs that had been cultured in non-osteogenic medium. **C.** O/P, PLGA scaffolds seeded with BMMSCs that had been cultured in osteogenic medium. **D.** O/H, PLGA/HAp scaffolds seeded with BMMSCs that had been cultured in osteogenic medium. All scale bars indicate 100 μm.

using RT-PCR (Fig. 1). The culture with osteogenic medium induced the expression of OC and BMP-6. In culture with non-osteogenic medium, the culture on PLGA/HAp films induced OC expression. The pattern of BMP-6, OC, BSP, Col I, and ALP expression was similar between the osteogenic medium-PLGA group and osteogenic medium-PLGA/HAp group and also between one and two weeks for all the groups. Importantly, these data report, for the first time, on the effect of culture on a HAp biomaterial with an osteogenic medium on *in vitro* osteogenic differentiation of human BMMSCs.

The seeding of human BMMSCs on three-dimensional scaffolds resulted in the formation of three-dimensional cell-scaffold constructs *in vitro* (Fig. 2). The porous scaffolds provide a substrate for cell adhesion and three-dimensional space for bone tissue formation upon implantation. One day after seeding, SEM examination of the BMMSC-seeded scaffolds revealed that the BMMSCs adhered well on the scaffolds (Figs. 2C–2F).

The ectopic implantation of the human BMMSC-seeded scaffolds resulted in new bone formation *in vivo* at two and three months after implantation (Fig. 3). Importantly, the bone formation extent varied depending on the cell differentiation status and scaffold type. In the implants

of undifferentiated BMMSCs seeded on PLGA scaffolds, most of the void spaces in the scaffolds were filled with fibrous connective tissue without evidence of bone formation. Mineral deposition was assessed with von Kossa staining (Figs. 3E–3L). When PLGA/HAp composite scaffolds were used as cell transplantation vehicles, calcium deposition was detected in either differentiated or undifferentiated BMMSC transplantations. In contrast, calcium deposition in the PLGA scaffolds rarely occurred when undifferentiated BMMSCs were transplanted. Immunohistochemical staining for HNA in the regenerated tissues showed that the transplanted human BMMSCs survived *in vivo* (Fig. 4). HNA-positive cells were detected in all the groups.

Histomorphometric analyses of the mid-portion sections of the regenerated tissues showed that both the HAp scaffold and osteogenic differentiation prior to transplantation remarkably enhanced *in vivo* bone formation (Fig. 5). Three months after implantation, transplantation of osteogenically differentiated human BMMSCs increased the bone formation area and calcium deposition to 7.1- and 6.2-folds, respectively, of those of transplantation of undifferentiated BMMSCs. The use of the HAp scaffold increased the bone formation area and calcium deposition to 3.7- and 3.5-folds, respectively,
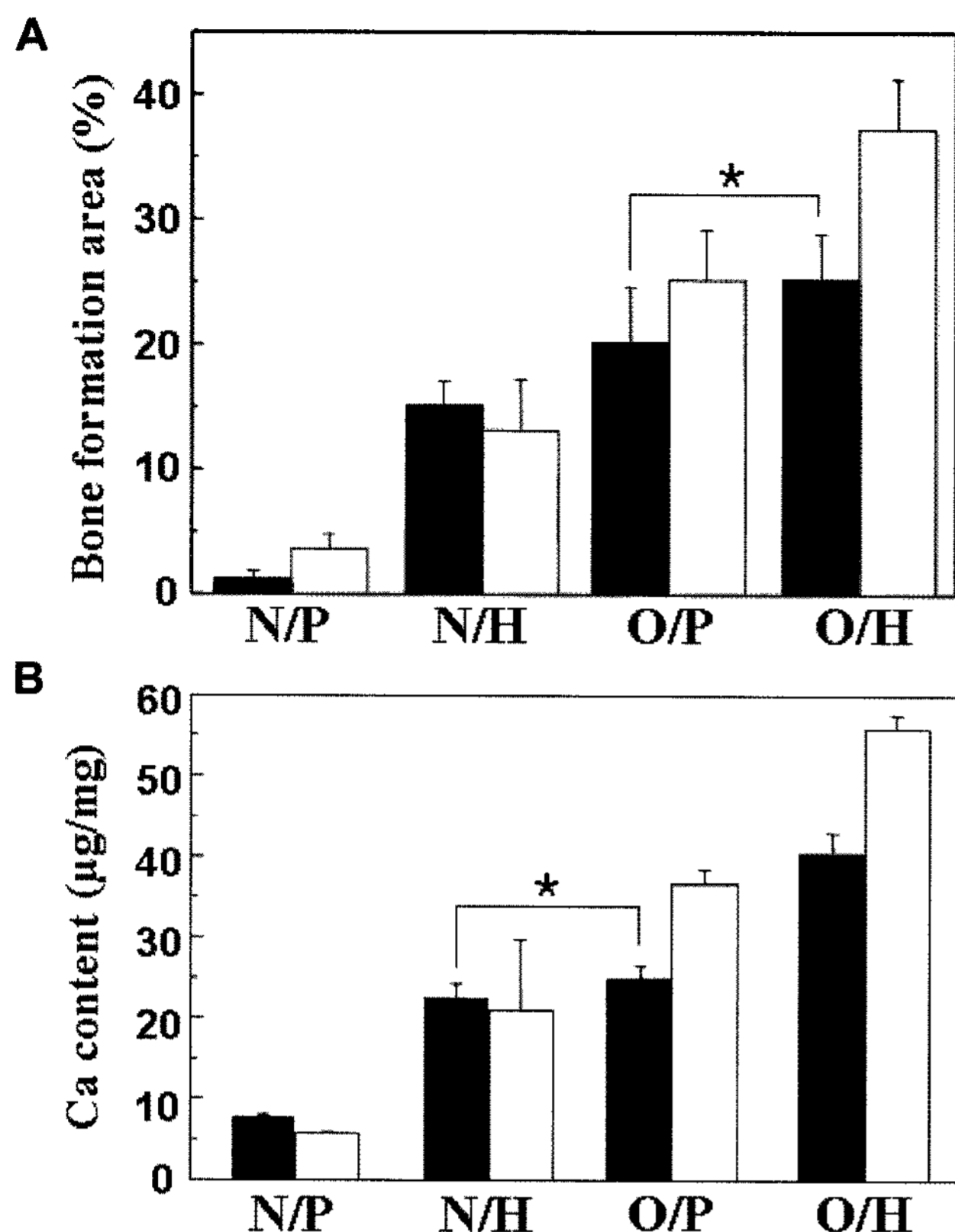
Fig. 5. (A) Bone formation area and (B) calcium deposition amount in tissues formed by implantation of BMMSC-scaffold constructs for two (black bars) and three (white bars) months.

N/P, PLGA scaffold seeded with BMMSCs cultured in non-osteogenic medium; N/H, PLGA/HAp scaffold seeded with BMMSCs cultured in non-osteogenic medium; O/P, PLGA scaffold seeded with BMMSCs cultured in osteogenic medium; and O/H, PLGA/HAp scaffold seeded with BMMSCs culture in osteogenic medium. $*p > 0.05$, otherwise $p < 0.05$ for any two groups at the same time points.

of those of polymer scaffolds. Moreover, a combination of transplantation of osteogenically differentiated BMMSCs and the use of HAp scaffold further increased the bone

Table 3. The effects of the variables and interaction between the variables on the bone formation area and calcium deposition content of implants at two and three months.

Variables	Two months		Three months	
	Bone formation area	Calcium content	Bone formation area	Calcium content
O	385.1	17.7	21.9	13.7
H	190.8	15.1	11.8	7.3
OH	2.1	0.5	1.2	0.1
Standard error	1.4	3.9	2.7	2.1

The effect of variable or interaction whose value is higher than the standard error is considered statistically significant.

O: Osteogenic differentiation of BMMSCs prior to transplantation.
H: Hydroxyapatite scaffold.
OH: Interaction between O and H.

formation area and calcium deposition to 10.6- and 9.3-folds, respectively, of those of transplantation of undifferentiated BMMSCs seeded onto polymer scaffolds.

The effects of the variables (*i.e.*, HAp scaffold and osteogenic differentiation prior to transplantation) and interaction between the variables on the *in vivo* bone regeneration were evaluated using the factorial experimental analysis (Table 3). The results indicate that the HAp scaffold and osteogenic differentiation of BMMSCs prior to transplantation had strong positive effects on both *in vivo* bone formation area and mineralization at two and three months. Osteogenic differentiation prior to transplantation had a stronger effect than HAp scaffold on both the bone formation area and mineralization. The positive effects of the variables on bone regeneration decreased at three months compared with that at two months. This was likely caused by decreased interaction between transplanted BMMSCs and HAp at three months, as the BMMSCs were embedded in extracellular matrices produced by the cells and by osteogenic differentiation of undifferentiated BMMSCs after transplantation. The interaction between HAp scaffold and osteogenic differentiation prior to transplantation had no significant effect on the bone regeneration three months after implantation (Table 3). The factorial experimental analysis may provide a means of rationally predicting and determining the optimal cell condition and scaffold type for bone tissue engineering.

It has been reported that bioceramic substrates (*e.g.*, HAp) stimulate expression of the osteoblast phenotype in cultures of osteoblasts and osteoblast precursors [7, 8]. It is well known that a composite of BMMSCs and HAp scaffold has *in vivo* osteogenic potential [19, 20, 28]. However, no study has directly compared the effects of bioceramic scaffolds and non-bioceramic scaffolds on human BMMSC transplantation-mediated bone formation. In the present study, a composite of BMMSCs and PLGA/HAp scaffold has a higher *in vivo* osteogenic potential than a composite of BMMSCs and PLGA scaffold. This clearly indicates that HAp scaffolds enhance the *in vivo* osteogenic potential of human BMMSCs.

The condition of the BMMSC culture prior to transplantation affected the *in vivo* osteogenic potential of the BMMSCs. Our results showed that human BMMSCs cultured with osteogenic medium supplemented with glycerophosphate, ascorbic acid, and dexamethasone underwent osteogenic differentiation *in vitro* and showed more extensive *in vivo* bone formation upon transplantation than those cultured with non-osteogenic medium. In previous studies, rabbit [9] or rat [28] BMMSCs cultured with medium supplemented with dexamethasone, an osteogenic inducer [4], showed a higher *in vivo* bone formation than those cultured without dexamethasone. Our results and the previous studies indicate that the *in vivo* osteogenic potential of BMMSCs can be enhanced by osteogenic differentiation of the

BMMSCs prior to transplantation. Moreover, the present study shows that osteogenic differentiation of human BMMSCs prior to the transplantation and HAp scaffold have an additive positive effect on the *in vivo* osteogenic potential of BMMSCs.

In summary, this study, for the first time, shows that the *in vivo* bone regeneration efficacy of human BMMSCs can be greatly enhanced by a combination of a HAp scaffold and osteogenic differentiation of human BMMSCs prior to transplantation. This study could have a great impact on stem cell therapy for bone regeneration in orthopedics and dentistry. However, further studies are necessary to assess the therapeutic potential of this method. Immunohistochemical and biomechanical analyses of the tissue-engineered bone are needed to determine the quality of the tissue-engineered bone. Additional research on long-term *in vivo* bone formation, bone regeneration experiment in orthopedic disease animal models, and inflammatory reactions of the PLGA/HAp scaffold in immune-competent animals would more fully elucidate the therapeutic potential of this method.

Acknowledgment

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