

# Superior mineralization and neovascularization capacity of adult human metaphyseal periosteum-derived cells for skeletal tissue engineering applications

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**Abstract.** Bone tissue engineering is a promising cell-based strategy to treat bone defects. Mesenchymal stem cells from adult human bone marrow (hBMSCs) are a frequently used cellular source for bone tissue generation. However, the low frequency of these stem cells in adult bone marrow and their limited proliferation restrict their clinical utility. An alternative source of MSCs is the periosteum-derived cells, and these cells appear to be easy to harvest and expand *ex vivo*. We isolated human metaphyseal periosteum-derived cells (hMPCs) and hBMSCs from the same donors and compared their osteogenic capacity both *in vitro* and *in vivo*. After osteogenic induction in monolayer cultures, hMPCs resulted in more robust mineralization and expressed higher mRNA levels of BMP-2, osteopontin and osteocalcin than hBMSCs. Eight weeks after implantation of cellular- $\beta$ -TCP scaffolds in immunodeficient mice, hMPC implantation showed higher neovascularization and higher percentage of mature bone formation than hBMSC implantation. In conclusion, hMPCs represent a promising cellular candidate for bone tissue engineering.

## Introduction

Bone defects and deformities are commonly caused by inflammation, tumor, and trauma. The repair of bone defects remains an important clinical obstacle. The most well-regarded current

orthopedic surgical procedure for bone repair uses autologous bone grafts to stimulate bone growth and implant fixation. However, disadvantages of this procedure include a limited supply of graft material, persistent pain and nerve damage following engraftment and cosmetic damage at the donor site (1). Allografts from bone banks are readily available, but the complication rate following this procedure is high, with risk of graft-versus-host disease, infectious disease transmission and graft failure (2,3). Therefore, alternative methods are currently being investigated. Tissue engineering of the bone based on the combination of multi-potent mesenchymal stem cells (MSCs) and a scaffold represents a promising new approach for bone regeneration.

MSCs with osteogenic potential have been isolated from a diverse range of tissue types and ontogenies, including bone marrow, periosteum, adipose tissue and synovium (4,5). However, these different cells present diverse differentiation capacities both *in vitro* and *in vivo*. Bone marrow mesenchymal stem cells (BMSCs) have to date been considered the major cellular source for bone tissue engineering. In recent years, successful bone regeneration by BMSCs implantation has been performed in animal models (6) and humans (7,8). However, the percentage of BMSCs in bone marrow is only approximately 0.001–0.01% of cells, and this percentage decreases with age (9). In addition, adult BMSCs have high cellular senescence and limited proliferation capacity (10,11).

Another source of MSCs commonly used for bone tissue engineering is the periosteum. Cells isolated from the periosteum have multipotential capacity, are easily expanded in culture, and are phenotypically stable with a low morbidity rate at the site of harvest (4,12). The osteogenic potential of these cells is maintained even in elderly individuals (13). In addition, the proliferation potential of periosteum-derived cells (PCs) is greater than that of BMSCs. Thus, the use of PCs may shorten the time period necessary for cell culture, thereby reducing both the cost and the risk of contamination (14). However, wide variability has been reported in the osteogenic capacity of PCs from different donor sites (15) and different species (16). Anatomically, the periosteum contains two distinct layers: a thick, fibrous outer layer, which adheres

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to a thin inner cambium layer that is adjacent to the bone. MSCs reside in the cambium layer and participate in osteogenesis, chondrogenesis and adipogenesis. It has previously been demonstrated that the cambium layer thickness and the cell number in the metaphyseal periosteum were much higher than in the diaphyseal area, and that these do not significantly decrease with age (17). Therefore, we hypothesized that MSCs isolated from the metaphyseal periosteum might be a promising cellular source for bone tissue engineering.

In the current study, we isolated human BMSCs (hBMSCs) and human metaphyseal periosteum-derived cells (hMPCs) from the same human donors. The osteogenic capacity of the two different cell types was compared by means of biochemical and histological methods. *In vitro* osteogenic differentiation was evaluated based on alkaline phosphatase (ALP) activity, mineralized nodule formation and osteogenic specific gene expression. For *in vivo* assessment, the cells from both groups were seeded onto porous  $\beta$ -TCP scaffolds and implanted in the subcutaneous dorsum of athymic mice. The area of bone formation and degree of neovascularization were subsequently determined.

## Materials and methods

**Human tissue samples.** Human metaphyseal periosteum and bone marrow samples were obtained from patients undergoing lower limb amputation surgery following severe limb trauma. Samples were obtained from five healthy donors (four males and one female, 22 to 30 years of age) in accordance with the local ethics committee and following informed consent. Bone marrow was harvested from the inferior segment of the tibia. During the same surgical procedure, the metaphyseal periosteum was harvested from the distal part of the tibia.

**Cell culture.** The isolation of hBMSCs was performed as previously described (18). Briefly, single-cell suspensions were prepared by flushing bone marrow cells out of the tibia using a 22-gauge needle and then passing the cell suspension through an 80- $\mu$ m cell strainer. Cells were then plated in 25-cm<sup>2</sup> culture flasks and cultured in complete medium (CM) consisting of Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum (Gibco), 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin. Non-adherent cells were removed by changing the medium twice a week. When hBMSCs reached 80–90% confluence, adherent cells were detached with 0.25% trypsin/EDTA (Gibco) and subcultured at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> in 25-cm<sup>2</sup> culture flasks.

The culture of hMPCs was performed in a similar manner to that previously described (19). After rinsing the periosteum thoroughly with PBS containing 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin, tissue biopsies were minced into small pieces and digested in 0.2% type II collagenase (Sigma, St. Louis, MO, USA) for 4 h at 37°C. The isolated cells were centrifuged and resuspended in CM. The hMPCs were subcultured as described above for hBMSCs. All experiments were performed on passage 3 cells.

**Immunophenotypic analyses.** Cells were trypsinized and suspended in PBS, followed by incubation ( $4 \times 10^5$  cells)

on ice with fluorescein isothiocyanate (FITC)-conjugated antibodies specific for CD13, CD29, CD105, CD34 and CD45 (BD Biosciences, Shanghai, China) for 30 min. Mouse IgG FITC (Upstate Biotechnology, Lake Placid, NY, USA) was used as a negative control. After incubation, the cells were washed with PBS and resuspended in 1 ml PBS for analysis. Cell fluorescence was evaluated by flow cytometry using a FACScalibur (Becton-Dickinson, San Diego, CA, USA) and data were analyzed using the CellQuest software (Becton-Dickinson).

**Osteogenic assays.** Cells at a density of 3000 cells/cm<sup>2</sup> were seeded into 6-well dishes in CM. After 24 h culture, the medium was replaced with osteogenic medium (OM), consisting of CM supplemented with 10 nM dexamethasone (Sigma), 0.05 mM L-ascorbic acid 2-phosphate (Sigma) and 10 mM  $\beta$ -glycerophosphate (Sigma). The cells were cultured in OM for 3 weeks, with the medium changed twice a week. Samples were harvested in triplicate for the following assays. Mineralized extracellular matrix was detected by alizarin red S staining as previously described (20,21). ALP activity in cell lysates was measured using a PNPP ALP assay kit (Njcc-Biotechnology, Nanjing, China) according to the manufacturer's instructions and normalized to total protein content using a Bradford assay (Njcc-Biotechnology).

**Quantitative real-time PCR.** MSCs were cultured in osteogenic differentiation conditions as above for up to 21 days. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of RNA was determined by spectrophotometry. Complementary DNA (cDNA) was synthesized using a PrimeScript First Strand cDNA Synthesis kit (Takara Biotechnology, Dalian, China). Reactions were performed in a PTC 200 Thermal Cycler PCR machine (Bio-Rad, Waltham, MA, USA). Real-time PCR was performed using a quantitative real-time amplification system (Light Cycler 480, Roche, Switzerland). SYBR-Green Premix Ex Taq II (Takara Biotechnology) was used in each reaction. Reactions were performed with 40 cycles (95°C for 5 sec, 55°C for 30 sec and 72°C for 30 sec). The primers used for real-time PCR were as follows: BMP2, 5'-TGGAAGTGGCC CATTAGAG-3', 5'-TGACGCTTTCTCTGTTTGTG-3'; Collagen1 $\alpha$ 1 (COL1 $\alpha$ 1), 5'-CCTGCGTGTACCCCACTCA-3', 5'-ACCAGACATGCCTCTTGTCTT-3'; osteopontin, 5'-AC ACATATGATGGCCGAGGTGA-3', 5'-TGTGAGGTGATGC CTCGTCTGTAG-3'; osteocalcin, 5'-CAAAGGTGCAGCCT TTGTGTC-3', 5'-TCACAGTCCGGATTGAGCTCA-3'; GAPDH, 5'-GCACCGTCAAGGCTGAGAAC-3', 5'-ATGGT GGTGAAGACGCCAGT-3'. Results were normalized against the housekeeping gene GAPDH and relative gene expression was analyzed with the 2<sup>- $\Delta\Delta C_t$</sup>  method. The human osteoblasts were taken as the control cell types. Each measurement was assessed in triplicate.

**Cellular-scaffold construct preparation.**  $\beta$ -tricalcium phosphate scaffolds ( $\beta$ -TCP, Bio-lu Biomaterials Co., Shanghai, China) were used in this study. Cells ( $9 \times 10^5$  cells per scaffold) were resuspended in fibrin gel (Sigma) and loaded into porous  $\beta$ -TCP scaffolds (19) (2 mm height, 6 mm diameter). The

cell-fibrin-scaffold constructs were pre-differentiated in OM for 21 days and processed for *in vivo* implantation.

**Surgical procedures in mice.** After inducing general anesthesia, a midline longitudinal skin incision was made on the dorsal surface of nude mice (CD-1 nude/nude, Charles River) and subcutaneous pockets were created, into which the scaffolds were inserted. The skin was closed with 4-0 Vicryl sutures. A sample size of  $n=6$  in each group were examined. After 8 weeks, animals were euthanized and the implants were retrieved for histological analysis.

***In vivo* bone-forming ability assay.** To quantitatively determine the amount of newly formed bone, the harvested samples were fixed in 4% formaldehyde, decalcified, and embedded in paraffin wax. The middle sections ( $5\ \mu\text{m}$ ) of each implant were stained with hematoxylin and eosin (H&E) for total new bone formation and van Geison's for mature bone formation. The results were observed under a light microscope (magnification  $\times 100$ ), and at least 10 images were randomly obtained from each section. Using the image analytical software Image-Pro Plus (Media Cybernetics, USA), the total new bone formation was expressed as the percentage of newly formed bone area at a  $\times 100$  magnification area and the mature bone formation was calculated as a percentage of mature bone area of the total new bone tissue area.

***In vivo* neovascularization assay.** To determine the extent of blood vessel ingrowth, the middle section of the implants were immunostained for vWF (Biosynthesis Biotechnology, Beijing, China), a protein present in large quantities in sub-endothelial matrices such as blood vessel basement membranes (18). Blood vessels in the total scaffold area, as indicated by vWF staining (only circular vWF staining was taken to indicate a blood vessel subendothelium) were counted manually under  $\times 100$  magnification. The results were expressed as vessel number at a  $\times 100$  magnification area.

**Statistical analyses.** The data are expressed as the mean values  $\pm$  standard deviation. Statistical significance was analyzed using one-way analysis of variance. P-values  $<0.05$  were considered significant.

## Results

**Characterization of hBMSCs and hMPCs.** Immunotyping of hBMSCs and hMPCs confirmed that both cell types had an MSC marker profile: the cells remained positive for CD13, CD29 and CD105 and negative for CD34 and CD45 (Table I).

***In vitro* osteogenic differentiation.** When exposed to OM, both hBMSCs and hMPCs generated mineralized nodules after approximately 12 days in culture, and the number of nodules progressively increased in a time-dependent manner. At 21 days, a more robust mineralized extracellular matrix was visible in the hMPC culture than in the hBMSC culture, as demonstrated by light microscopy (Fig. 1a-d) and alizarin red S staining (Fig. 1e-h). However, ALP activity was not statistically different between the two cell types. ALP activity was expressed early in both cell cultures, peaked at 14 days

Table I. Comparison of immunophenotypes of hBMSCs and hMPCs ( $n=3$ ).

Markers	hBMSCs (%)	hMPCs (%)
CD 29	$93.6\pm 0.15$	$98.5\pm 0.12$
CD 105	$99.0\pm 0.12$	$98.8\pm 0.23$
CD 13	$29.5\pm 0.85$	$36.5\pm 1.20$
CD 34	$4.9\pm 0.06$	$1.5\pm 0.05$
CD 45	$0.1\pm 0.02$	$0.1\pm 0.03$

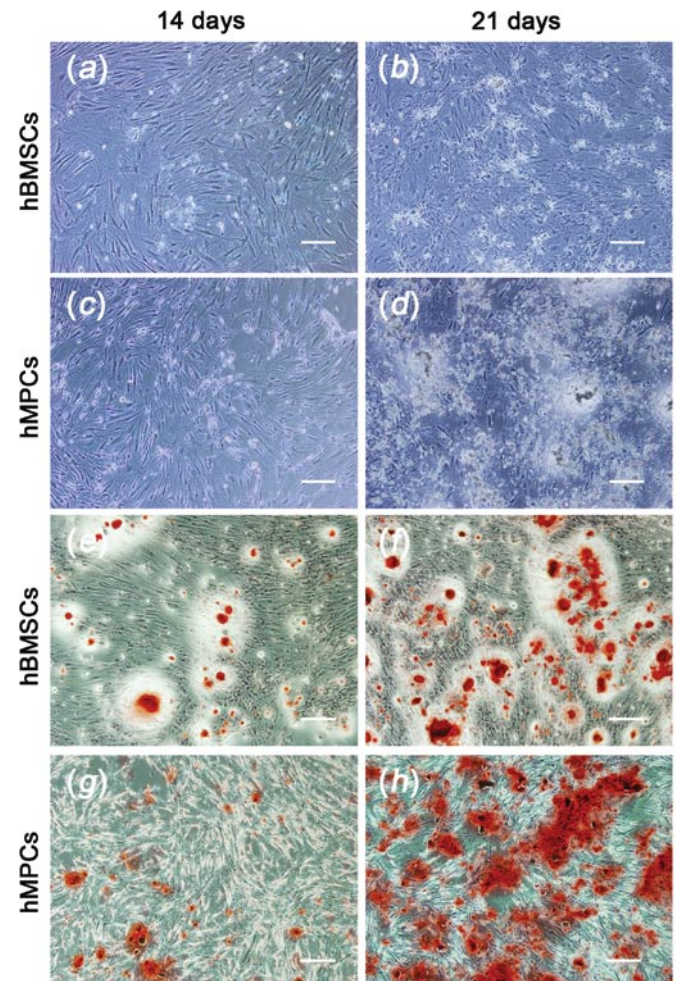


Figure 1. The mineralized nodule formation of two types of cells in the monolayer culture. Microscopic observation (a-d) and alizarin red S staining (e-h) was performed at days 14 and 21 following osteogenic induction (bar,  $200\ \mu\text{m}$ ).

and decreased somewhat over the remaining culture period (Fig. 2).

**Quantitative real-time PCR.** Following culture in osteogenic medium, mRNA derived from hBMSCs and hMPCs was assessed for expression of COL1 $\alpha$ 1, BMP-2, osteopontin and osteocalcin transcripts. The COL1 $\alpha$ 1 gene expression in both groups reached a peak value after 7 days in culture and then decreased slowly. The expression level of COL1 $\alpha$ 1 transcript in hBMSCs was significantly higher than in hMPCs at 7 days

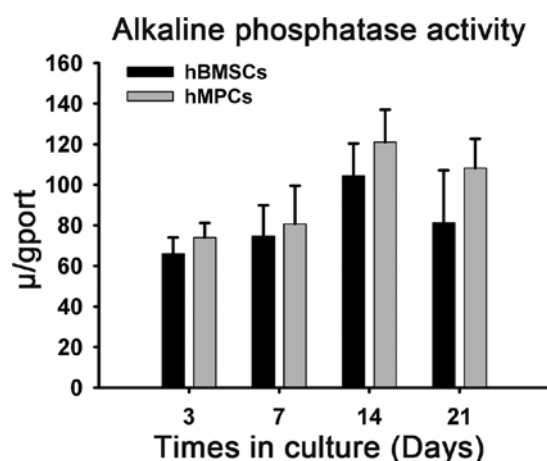


Figure 2. ALP activity of two types of cells in the monolayer cultures. The ALP activity was tested at days 3, 7, 14 and 21 of osteogenic induction.

(Fig. 3a). BMP-2 mRNA expression became detectable only after 14 days in both types of MSCs. On day 14 and day 21, BMP-2 transcript was expressed more highly in hMPCs than in hBMSCs (Fig. 3b). The peak of the osteopontin transcript expression occurred on day 7 and expression levels then slowly decreased in hBMSCs. However, osteopontin mRNA expression in hMPCs steadily increased with time, and by day 21 remained at a significantly higher level than measured in hBMSCs (Fig. 3c). Osteocalcin mRNA was highly expressed at day 14 in both cell types and progressively increased during the following culture periods. The osteocalcin transcript

expression level was markedly higher in hMPCs than in hBMSCs (Fig. 3d).

***In vivo new bone formation and neovascularization.*** Two months following implantation, the presence of both hBMSCs and hMPCs on the scaffolds had remarkably enhanced bone formation. Transplantation of osteogenically differentiated hBMSCs scaffolds and hMPCs scaffolds increased the bone formation area by 2.8-fold and 3.2-fold, respectively, as compared to transplantation of blank  $\beta$ -TCP scaffolds (Fig. 3a-c and j). Although the total new bone formation between transplanted hBMSCs and hMPCs was not statistically significant, hMPCs resulted in more mature bone formation than hBMSCs (Fig. 3d-f and k).

The degree of neovascularization was determined by immunostaining tissue sections for vWF. Two months after implantation, hBMSCs and hMPCs scaffolds had increased blood vessel number by 1.5-fold and 2.08-fold, respectively, as compared to transplantation of blank  $\beta$ -TCP scaffolds. In addition, hMPCs scaffolds exhibited significantly higher neovascularization than hBMSCs scaffolds (Fig. 3g-i and l).

## Discussion

In this study, we have performed the first isolation of PCs from the metaphyseal periosteum of the tibia and a comparison of the osteogenic capacity of these cells with hBMSCs from the same donors, in an attempt to determine whether hMPCs could be an alternative cellular source to hBMSCs in cell-based approaches to bone tissue engineering. We found that,

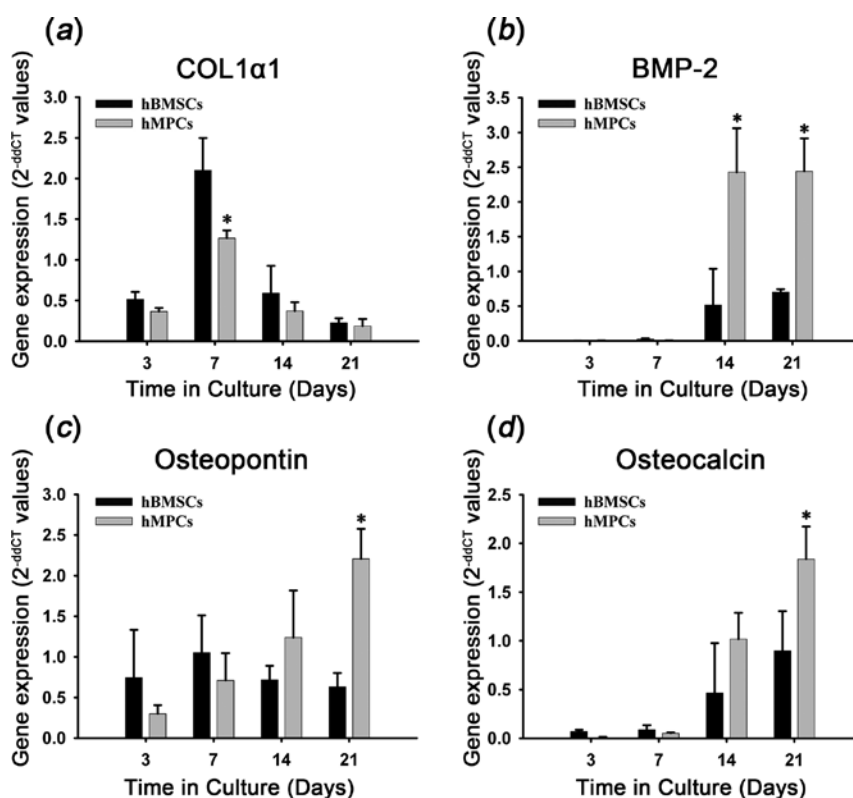


Figure 3. Expression of osteogenic genes in differentiating hBMSCs and hMPCs. Expression of COL1α1 (a), BMP-2 (b), osteopontin (c) and osteocalcin (d) mRNA transcripts were analyzed by quantitative real-time PCR. \*P<0.05 hMPCs as compared to hBMSCs.



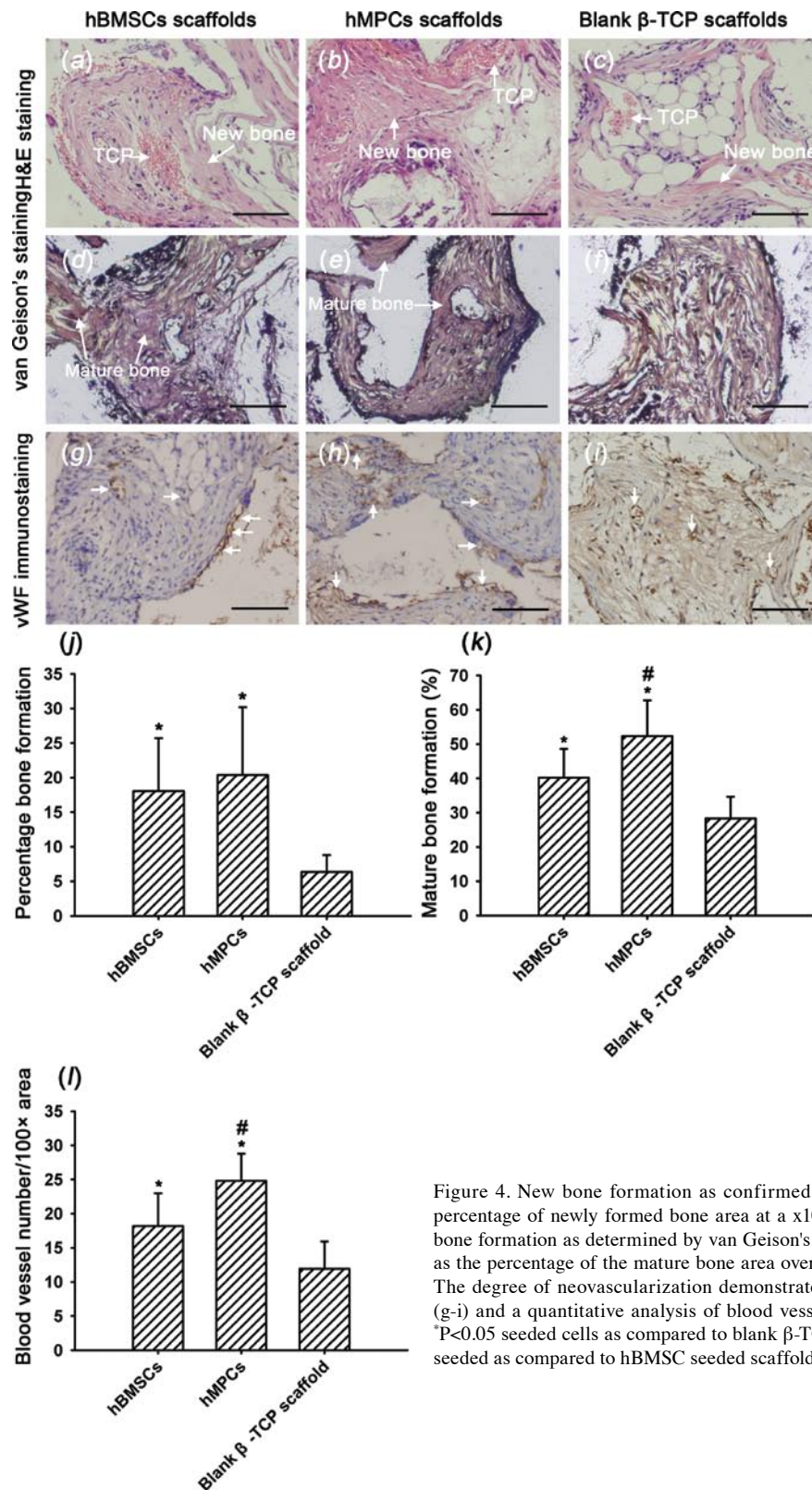


Figure 4. New bone formation as confirmed by H&E staining (a-c); the percentage of newly formed bone area at a x100 magnification (j). Mature bone formation as determined by van Geison's staining (d-f) and calculated as the percentage of the mature bone area over the total new bone area (k). The degree of neovascularization demonstrated by vWF immunostaining (g-i) and a quantitative analysis of blood vessel number (l). Bar, 200  $\mu$ m; \*P<0.05 seeded cells as compared to blank  $\beta$ -TCP scaffolds; #P<0.05 hMPC seeded as compared to hBMSC seeded scaffolds.

compared with hBMSCs, hMPCs not only exhibited more robust mineralization *in vitro*, but showed a high degree of neovascularization and a higher percentage of mature bone formation *in vivo* following the same test conditions.

The *in vitro* osteogenic potential of both types of cells was investigated in standard monolayer cultures. The results were corroborated by ALP activity assays, observation of

mineralized nodule formation and quantitative real-time PCR. ALP activity was used as a biochemical determinant of osteogenic phenotype, as it is considered an important factor in determining bone differentiation and mineralization (22,23). Thus, the expression of ALP activity is an indicator of osteogenic differentiation of MSCs. In addition, extracellular matrix maturation and mineralization are crucial steps in the

osteogenic cascade (24) and have been associated with the final differentiation phase (4). In the present study, we found that ALP activity was not significantly different between hBMSCs and hMPCs from the same donors. Both types of cells highly expressed ALP during early stages of culture and ALP expression decreased during the later differentiation stages. However, compared with hBMSCs, hMPCs showed a higher extent of mineralization in the later stages of differentiation, as indicated by alizarin red S staining. This difference between hBMSCs and hMPCs, may be accounted for by the notion that ALP activity is an early but unspecific marker of osteogenic differentiation (25), which is possibly not in accord with the late-stage mineralization observed in both hBMSCs and hMPCs. We also demonstrated that the PCs isolated from the metaphyseal periosteum displayed superior mineralization capacity *in vitro*.

To further assess osteogenic differentiation, the gene expression of osteogenic markers was characterized using quantitative real-time PCR. We evaluated the expression levels of COL1 $\alpha$ 1, BMP-2, osteopontin and osteocalcin transcripts in mRNA isolated from the two cell types. In general, collagen type I and osteopontin are early markers of osteoblastic differentiation, whereas osteocalcin is associated with the late phase of osteoblastic differentiation (26). It has been suggested that BMP-2 could serve as an osteoinductive signal to increase the infiltration and recruitment of repair cells surrounding the site of injury to further enhance bone regeneration and induce blood vessel ingrowth (27,28). In the data presented here, we show that, in the late osteogenic differentiation stage, hMPCs possess a remarkably higher expression of BMP-2, osteopontin and osteocalcin mRNA transcripts than hBMSCs. In comparison, during the early differentiation phase, a relatively low expression level of COL1 $\alpha$ 1 was recorded in hMPCs compared to hBMSCs.

According to our *in vitro* data, the peak expression of COL1 $\alpha$ 1 mRNA occurs during the early phase of differentiation, and expression then decreases with time. This may indicate that COL1 $\alpha$ 1 is associated with early-stage osteogenic differentiation, and this protein possibly does not directly participate in late-stage osteoblastic mineralization. Osteocalcin is the most abundant non-collagenous protein in the bone and acts as a bone-specific marker for terminal osteoblast differentiation. It has been reported that only MSCs that have differentiated to a mature osteoblast phenotype express osteocalcin and osteopontin (29). In our study, the high expression of osteopontin and osteocalcin transcripts is consistent with the abundant mineralization observed in hMPCs, strongly suggesting that hMPCs have a higher mineralization capacity than hBMSCs *in vitro*. In addition, it is believed that the expression of BMP promotes both osteogenesis and the vascularization processes (30). We found a relatively high expression of BMP-2 in hMPCs, and this could at least in part account for the abundant mineralization in monolayer culture and the high degree of neovascularization *in vivo* seen in these cells.

To evaluate the bone regeneration capacity of the two cell types *in vivo*, we analyzed the degree of new bone formation 2 months following ectopic implantation of cellular-scaffold constructs. Our data show that hMPCs seeded on  $\beta$ -TCP scaffolds resulted in a greater amount of mature bone formation

than hBMSCs, although a statistically significant difference of the total new bone formation was not found. The outstanding osteogenic ability of PCs has been confirmed by several previous investigations (5,31,32). In the present study, we also observed a small quantity of newly formed bone inside the porous section of the non-cellular  $\beta$ -TCP implantations. Similar findings have previously been published (33). This suggests that the  $\beta$ -TCP implants could also have osteoinductive capacity (34,35). Taken together, these findings suggest that extensive bone regeneration can be achieved by seeding hMPCs on  $\beta$ -TCP scaffolds.

Bone is a complex and highly vascularized tissue. Vascularization can enhance bone regeneration by accelerating the differentiation and maturation of infiltrating osteoblasts and osteoblast precursor cells during the development of new bone (36). Adequate oxygen tension and the supply of other nutrients result from neovascularization, which allows the direct formation of mineralized matrix in the interior of the scaffold and thereby facilitates bone generation (37). In the current study, the presence of hMPCs seeded onto scaffolds *in vivo* led to greater neovascularization than was observed when hBMSCs were seeded. The high extent of neovascularization in hMPCs implants possibly facilitated the delivery of oxygen and nutrients around the constructs and thus may have been beneficial to osteogenic differentiation and mineralization.

In conclusion, this study presents the first isolation of PCs from human metaphyseal periosteum and demonstrates the superior osteogenic potential of these cells both *in vitro* and *in vivo*. In a clinical setting, the main advantages of hMPCs over hBMSCs are their relatively easy procurement and expansion, which greatly increases the feasibility of conducting bone engineering. Based on our findings, hMPCs may be recommended as a promising cellular candidate for bone tissue engineering. Further studies are necessary to elucidate the clinical potential of this cell-based therapy, including assessing the osteogenic ability of hMPCs in a critical-sized bone defect model and conducting *in vivo* bone generation experiments in immune-competent animals to investigate the potential inflammatory reactions to hMPCs.

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