

# Pulsed Electromagnetic Fields Accelerate Proliferation and Osteogenic Gene Expression in Human Bone Marrow Mesenchymal Stem Cells During Osteogenic Differentiation

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Osteogenesis is a complex series of events involving the differentiation of mesenchymal stem cells to generate new bone. In this study, we examined the effect of pulsed electromagnetic fields (PEMFs) on cell proliferation, alkaline phosphatase (ALP) activity, mineralization of the extracellular matrix, and gene expression in bone marrow mesenchymal stem cells (BMMSCs) during osteogenic differentiation. Exposure of BMMSCs to PEMFs increased cell proliferation by 29.6% compared to untreated cells at day 1 of differentiation. Semi-quantitative RT-PCR indicated that PEMFs significantly altered temporal expression of osteogenesis-related genes, including a 2.7-fold increase in expression of the key osteogenesis regulatory gene *cbfa1*, compared to untreated controls. In addition, exposure to PEMFs significantly increased ALP expression during the early stages of osteogenesis and substantially enhanced mineralization near the midpoint of osteogenesis. These results suggest that PEMFs enhance early cell proliferation in BMMSC-mediated osteogenesis, and accelerate the osteogenesis. *Bioelectromagnetics* 31:209–219, 2010. © 2009 Wiley-Liss, Inc.

**Key words:** pulsed electromagnetic fields; osteogenesis; mesenchymal stem cells

## INTRODUCTION

The complex process of osteogenesis involves the differentiation of mesenchymal stem cells (MSCs) for the generation of progenitor cells including osteoprogenitor cells, osteoblasts, and finally osteocytes. MSCs are present in many tissues [Noth et al., 2002; De Ugarte et al., 2003; Fukuchi et al., 2004; Lee et al., 2004b] and are rich in adult bone marrow [Civin et al., 1984; Owen and Friedenstien, 1988; Caplan and Bruder, 2001; Tae et al., 2006]. It has been reported that bone healing is accelerated by the proliferation and differentiation of new osteoblasts from MSCs [Wlodarski and Galus, 2005; McFarlin et al., 2006].

Physical stimulation, such as mechanical stretching and exposure to the pulsed electromagnetic field (PEMF), can regulate bone marrow mesenchymal stem cell (BMMSC) proliferation [Song et al., 2007; Sun et al., 2009] or osteo-chondrogenesis [Cui et al., 2006; Friedl et al., 2007]. PEMFs have been used clinically for years to promote bone fracture healing [Friedenberg and Brighton, 1966; Heckman

et al., 1981; Yonemori et al., 1996]. There are four principal developmental periods of gene expression during osteogenesis [Stein et al., 1996]. In the early

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period, cell proliferation and cell cycle progression would be activated. In addition, cell proliferation raises the expression of collagen type 1 (COL I), a major bone extracellular matrix protein, at this time. In the second period, expression of genes associated with maturation, such as alkaline phosphatase (ALP), are up-regulated. In the third period, organization and maturation of bone-like tissue occurs, and osteopontin and osteocalcin (OC) are maximally expressed. Finally, the expression of collagenase is elevated and apoptotic activity occurs in the fourth developmental period. Diniz et al. [2002] showed that PEMF treatment of the osteoblast cell line MC3T3-E1 early during osteogenesis accelerated cell proliferation and enhanced cell differentiation and formation of bone-like tissue. Recently, Selvamurugan et al. [2007] showed that continuous treatment of primary osteoblastic cells with bone morphogenetic protein-2 (BMP-2) in conjunction with a PEMF significantly increased the expression of mRNAs, encoding ALP, OC, and  $\alpha 1$  (I) procollagen during the early stages of differentiation but not during later stages or the mineralization stage. However, there are conflicting data regarding the effect of PEMF stimulation on differentiation and proliferation of some osteogenic cell lines in vitro [De Mattei et al., 1999; Lohmann et al., 2000, 2003; Chang et al., 2004].

Osteoblasts in different stages, from BMMSCs to osteocytes, have been found in bone tissue, and they exhibit different cellular responses to cytokine stimulation [Gerstenfeld et al., 1988; Matsumoto et al., 1991; Harris et al., 1994; Rice et al., 2000; Fakhry et al., 2005]. We previously demonstrated that application of a PEMF to BMMSCs in medium containing human basic fibroblast growth factor (FGF-2) resulted in early onset of cell proliferation and, consequently, higher cell densities during the exponential growth phase [Sun et al., 2009]. However, information regarding the effects of PEMFs on BMMSCs during the early osteogenesis is limited, particularly with respect to gene expression and cell proliferation. Therefore, we investigated cell proliferation, gene expression, ALP activity, and extracellular matrix mineralization to determine how PEF exposure influences the osteogenic differentiation of BMMSCs (Fig. 1).

## MATERIALS AND METHODS

### PEMF Exposure

The PEF device (Biomet, Parsippany, NJ) used for exposure inside the culture plates or dishes was described previously by Sun et al. [2009]. Briefly, cells were placed in a CO<sub>2</sub> incubator, and a PEF was applied at a frequency of 15 Hz in 20 pulses of 4.5-ms bursts

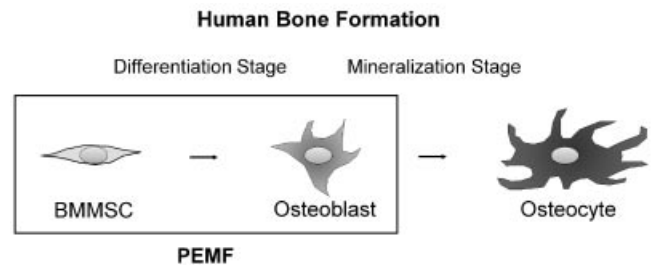


Fig. 1. Diagram of bone marrow mesenchymal stem cell (BMMSC) differentiation and mineralization during human bone formation. Culture of BMMSCs in the presence of dexamethasone,  $\beta$ -glycerol phosphate, and ascorbic acid induces differentiation into osteoblasts. The boxed region highlights the finding that osteoblast formation is stimulated by pulsed electromagnetic field (PEMF) treatment during osteogenic differentiation.

[Bassett, 1989]. During each pulse, the magnetic field increased from 0 to 1.8 mT in 200  $\mu$ s, and then decayed to 0 mT in 25  $\mu$ s. The cells were cultured in 35 mm dishes (Nunc, Rochester, NY) or six-well culture plates (BD Falcon<sup>TM</sup>, BD Biosciences, Mississauga, ON, Canada) and exposed to a PEF continuously for 8 h each day during the culture period. Control experiments were performed in a separate CO<sub>2</sub> incubator that had no PEF device. The incubators were alternated for the treatment and control experiments.

### BMMSC Culture and Osteogenic Differentiation

The human BMMSCs used in this study were isolated as reported by Lee et al. [2004a] using negative immunodepletion with a RosetteSep kit (StemCell Technologies, Vancouver, BC, Canada). Human BMMSCs between passages 10 and 15, with the potential for osteogenic differentiation in vitro as reported by Sun et al. [2009], were used for all experiments. To maintain and expand BMMSCs, the cells were cultured in BMMSC expansion medium consisting of Iscove's modified Dulbecco's medium (Gibco BRL, Grand Island, NY) and 10% fetal bovine serum (HyClone, Logan, UT) supplemented with 10 ng/ml FGF-2 (R&D Systems, Minneapolis, MN), 100 U penicillin, 1000 U streptomycin, and 2 mM L-glutamine (Gibco BRL). To study the osteogenesis, BMMSCs were seeded into 75 cm<sup>2</sup> T-flasks (BD Biosciences) containing osteogenic medium comprised of high-glucose Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 0.1  $\mu$ M dexamethasone (Sigma, St. Louis, MO), 10 mM  $\beta$ -glycerol phosphate (Sigma), 0.2 mM ascorbic acid (Sigma), 100 U penicillin, 1000 U streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum, to mimic the osteogenic microenvironment of the human body and to maintain

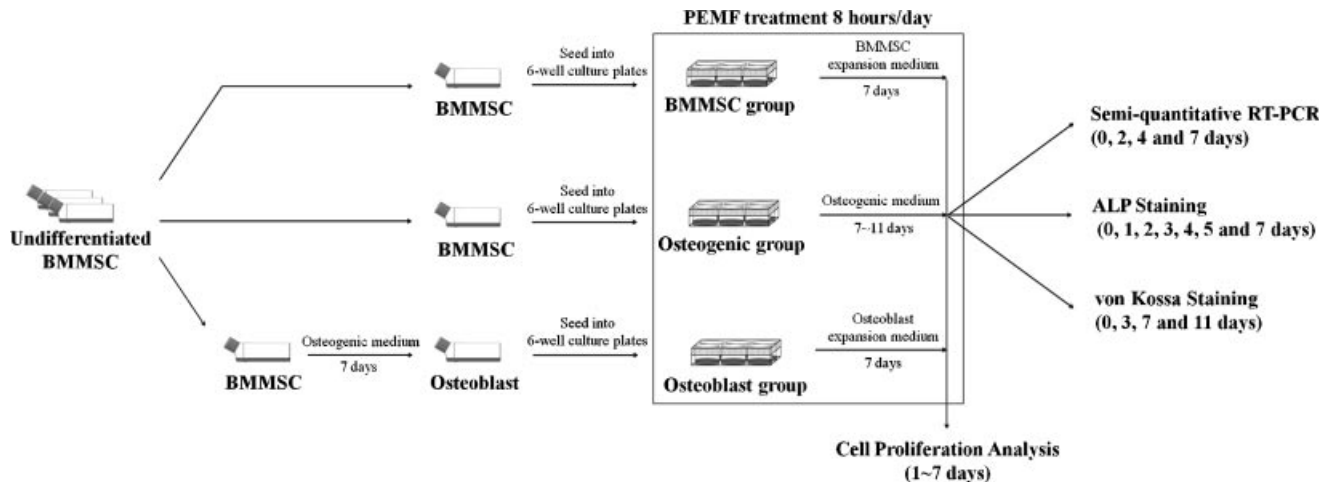


Fig. 2. Schematic of the culture process of the three groups of BMMSCs during the different osteogenic stages with PEMF treatment. Analysis of cell proliferation in the three groups was performed at days 1–7. Osteogenesis-related gene expression (semi-quantitative RT-PCR), ALP activity (ALP staining), and mineralization assay (von Kossa staining) of BMMSCs from the osteogenic group were performed on different days, as indicated.

the capability of cell proliferation during the process of differentiation.

### Cell Proliferation Analysis

To determine the effect of PEMF exposure on cell proliferation during osteogenesis, BMMSCs cultured in BMMSC expansion medium were seeded into six-well culture plates at an initial density of 3000 cells/cm<sup>2</sup> and grown for 1 day. Expansion medium was then removed, and osteogenic medium was added. These cells were defined as the osteogenic group. For comparison, BMMSCs cultured in BMMSC expansion medium containing FGF-2 were also seeded into six-well culture plates at 3000 cells/cm<sup>2</sup> and maintained in FGF-2-containing expansion medium (BMMSC group). For the other comparison, BMMSCs were seeded into 75 cm<sup>2</sup> T-flasks with osteogenic medium for 7 days to induce osteogenic differentiation, and the resulting human osteoblasts were named the osteoblast group. To maintain osteoblast expansion, cells were cultured in the osteoblast expansion medium containing high-glucose Dulbecco's modified Eagle's medium and 10% fetal bovine serum supplemented with 100 U penicillin, 1000 U streptomycin, and 2 mM L-glutamine. The medium was changed twice a week in all experiments. The differences in the culture process among the BMMSC, the osteogenic, and the osteoblast groups with PEMF treatment are shown in Figure 2. Plates of cells in the three groups were placed side by side in the PEMF device and exposed to PEMF for 8 h/day during the culture period. To determine cell growth kinetics, cells were detached using trypsin–EDTA (Gibco BRL)

and cell scrapers (BD Biosciences), and then counted in triplicate; cell counts were done twice.

### Total RNA Isolation and Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

To examine the expression levels of osteogenesis-related genes at different time points, cells were seeded into 35 mm dishes at 3000 cells/cm<sup>2</sup> and treated with or without PEMF on days 0, 2, 4, and 7 of osteogenic differentiation as described above [Sun et al., 2009]. Total RNA was isolated from cells using the RNeasy kit (Qiagen, Valencia, CA). RNA concentration and purity were estimated based on absorbance at 260 and 280 nm. cDNA was synthesized from 10 µg of total RNA using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). The resulting cDNA was subjected to PCR amplification using gene-specific primers (Table 1) including OC, parathyroid hormone receptor type 1 (PTH-R1), ALP, COL I, core binding factor alpha 1 (cbfa1), BMP-2, and β-actin.

The PCR amplification was done as follows: initial heating at 94 °C for 5 min, followed by 25–35 cycles at 94 °C for 40 s, 56 °C for 50 s, 72 °C for 60 s, and a final step at 72 °C for 10 min. PCR products were subjected to agarose gel electrophoresis followed by ethidium bromide staining and were quantified in triplicate using a GS-800 Calibrated Imaging Densitometer (Quantity One 4.0.3 software, Bio-Rad, Hercules, CA). Gene expression levels were normalized to amplification of the β-actin internal control.

TABLE 1. Primers used for RT-PCR

Primer	Sequence	Product (bp)
$\beta$ -Actin	S: 5'-CGCCAACCGCGAGAAGAT-3' A: 5'-CGTCACCGGAGTCCATCA-3'	168
PTH-R1	S: 5'-CACAGCCTCATCTTCATGG-3' A: 5'-GCATCTCATAGTGCATCTGG-3'	357
COL I	S: 5'-TGCTTGAATGTGCTGATGACAGGG-3' A: 5'-TCCCCTCACCTCCCAGTAT-3'	414
OC	S: 5'-ATGAGAGCCCTCACACTCCTC-3' A: 5'-GCCGTAGAAGCGCCGATAGGC-3'	294
ALP	S: 5'-TGGAGCTCAGAAGCTCAACACCA-3' A: 5'-ATCTCGTTGTCTGAGTACCAGTCC-3'	454
Cbfa1	S: 5'-CTCACTACCACACCTACCTG-3' A: 5'-TCAATATGGTCGCCAAACAGATTC-3'	320
BMP-2	S: 5'-AACACTGTGCGCAGCTTCC-3' A: 5'-CTCCGGTGTGTTTCCCAC-3'	74

PTH-R1, parathyroid hormone receptor type 1; COL I, collagen type 1; OC, osteocalcin; ALP, alkaline phosphatase; cbfa1, core binding factor alpha 1; BMP-2, bone morphogenetic protein-2; S, sense; A, antisense.

### ALP Staining

To determine ALP activity, BMMSCs were seeded into 35 mm dishes to obtain photomicrographs, and into 35 mm dishes with a 2 × 2 mm grid (Nunc) to count the number of ALP expressing cells upon seeding, and after PEMF treatment during osteogenesis. After fixation with citrate buffered acetone, the cells were stained for 1 h at 25 °C using Sigma kit #85L1 (Sigma) according to the manufacturer's instructions. Cells were protected from drying and direct light during incubation and were rinsed with deionized water and air-dried in the dishes prior to image analysis (days 0, 1, 2, 4, and 7) and cell counting (days 1–5). For each experiment, at least three dishes of ALP-stained cells (blue/purple) were counted.

### Mineralization Assay

BMMSCs were seeded into 35 mm dishes at 3000 cells/cm<sup>2</sup>, and calcium deposition was evaluated on days 0, 3, 7, and 11 after PEMF treatment using von Kossa staining. Briefly, cells were fixed with 10% formaldehyde in phosphate-buffered saline for 10 min, rinsed with water, and incubated with 2% silver nitrate (Sigma) under ultraviolet light for 60 min. Cells were then incubated with 3% sodium thiosulfate (Sigma) for 5 min, and counterstained with van Gieson stain (Sigma) for another 5 min. With this method, calcium deposits stain black, and the osteoid red.

### Statistical Analyses

Statistical analyses of cell proliferation, gene expression levels, and ALP-positive cell numbers in each group were carried out with the Microsoft Excel data analysis program for *t*-test analysis, and a

*P*-value < 0.05 was considered statistically significant. Experiments were performed at least twice. Results are expressed as the mean ± standard deviation (SD).

## RESULTS

### Effect of PEMF on Cell Proliferation During Osteogenesis

To investigate whether PEMF exposure affects BMMSC proliferation at different stages during osteogenesis, BMMSCs were grown in either the stem cell expansion medium or in osteogenesis stimulation medium. Osteoblasts derived from these BMMSCs were grown in osteoblast expansion medium and treated without PEMF or with PEMF for 8 h/day, and cell growth was examined over time.

As indicated in Figure 3, on day 1 the density of PEMF-treated cells was significantly higher than that of untreated cells. In the osteogenic group, the density of PEMF-treated cells increased from  $3.92 \times 10^3$  to  $5.44 \times 10^3$  cells/cm<sup>2</sup> on day 1, whereas the density of the control cells increased to  $4.65 \times 10^3$  cells/cm<sup>2</sup> over the same period. Likewise, in the BMMSC group, the density of PEMF-treated cells increased from  $3.00 \times 10^3$  to  $4.20 \times 10^3$  cells/cm<sup>2</sup> on day 1, whereas the density increased to  $3.20 \times 10^3$  cells/cm<sup>2</sup> in the control cells over the same period [Sun et al., 2009]. In summary, the densities of PEMF-treated osteogenic and BMMSC cell groups were 29.6% and 34.8% higher, respectively, than that of the untreated control group on day 1, and this difference remained through day 7 (Fig. 3). These findings reveal that the osteogenic and BMMSC groups have similar proliferation responses to PEMF stimulation in vitro.

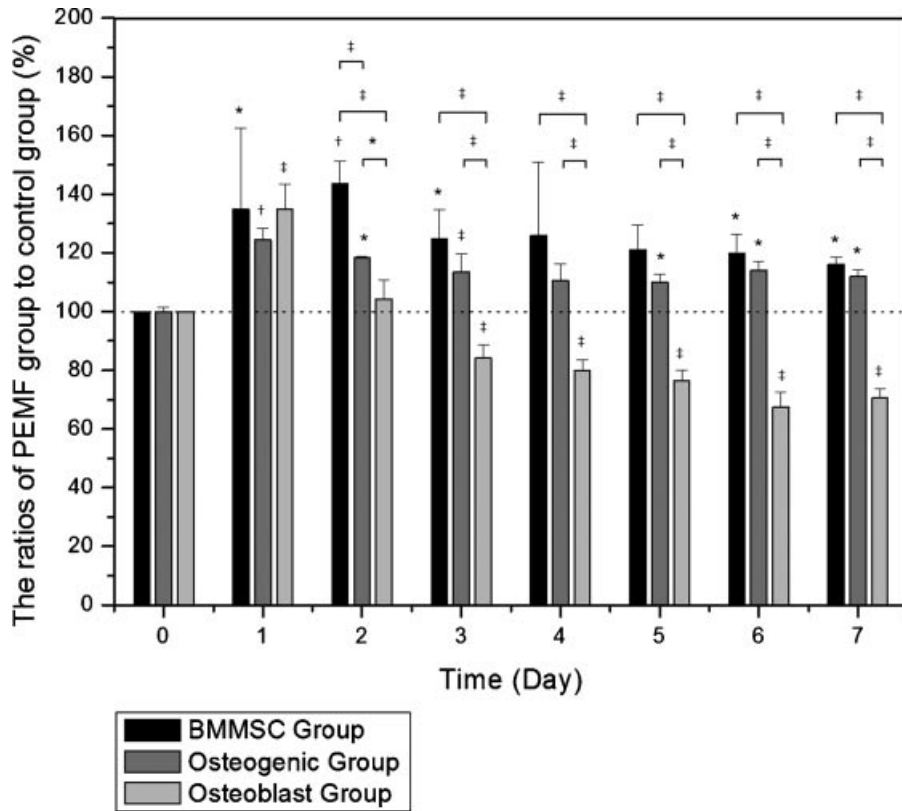


Fig. 3. Effect of PEMF exposure on cell proliferation. BMMSCs were cultured in osteogenic medium (Osteogenic Group) and exposed to PEMF for 8 h/day starting on day 0. Undifferentiated BMMSCs were cultured in BMMSC expansion medium (BMMSC Group), and human osteoblasts that resulted from BMMSCs after 7 days of osteogenesis cultured in osteoblast expansion medium (Osteoblast Group) under the same PEMF treatment were used for comparison. Cell populations were monitored over time, and data are presented as the relative ratio of PEMF-treated to untreated cell populations. The data points represent the mean cell number  $\pm$  SD ( $n = 3$ ). \*  $P < 0.05$ ; †  $P < 0.01$ ; ‡  $P < 0.005$ .

There were significant differences between the osteogenic and osteoblast groups after the first day. In the osteoblast group, the density ratio of PEMF-exposed to non-PEMF-exposed cells decreased and became  $<85\%$  after the third day (Fig. 3). In the osteogenic group, the cell density ratio of PEMF-exposed to non-exposed remained  $>110\%$  after the third day. These findings reveal that BMMSCs at different stages of osteogenesis have different proliferation responses to PEMF stimulation in vitro.

#### Effect of PEMF on the Expression of Osteogenic Genes

To study the effect of PEMF exposure on the expression of osteogenesis-related genes in BMMSCs during osteogenesis, total RNA was isolated on days 2, 4, and 7 from BMMSCs cultured in osteogenic medium in the absence or presence of PEMF, and then subjected to semi-quantitative RT-PCR analysis (Fig. 4).

After 2 days of osteogenesis with PEMF treatment, the mRNA expression of the osteogenesis master

regulatory gene *cbfa1* in the PEMF group was significantly higher, but *BMP-2* mRNA expression in the PEMF group was significantly lower, compared to the untreated control group (Fig. 4B). In addition, after 2 days of osteogenesis with PEMF treatment, *PTH-R1* and *COL I* mRNAs were slightly higher than the untreated control group, but these differences were not statistically significant. After 4 days of treatment, the expression of *cbfa1* and *COL I* mRNAs the PEMF group was significantly lower than the untreated group, and the expression of *OC*, *ALP*, and *BMP-2* mRNAs was higher in PEMF-treated cells compared to untreated control cells. After 7 days of treatment, the expression of *ALP* and *BMP-2* mRNAs in the two groups increased between days 4 and 7, and the expression of *ALP* and *BMP-2* mRNAs in the PEMF group was significantly higher than the untreated control group. Finally, after 7 days of PEMF treatment, the expression of other mRNAs did not differ significantly from untreated cells except for *COL I* mRNA, which increased with PEMF treatment. These

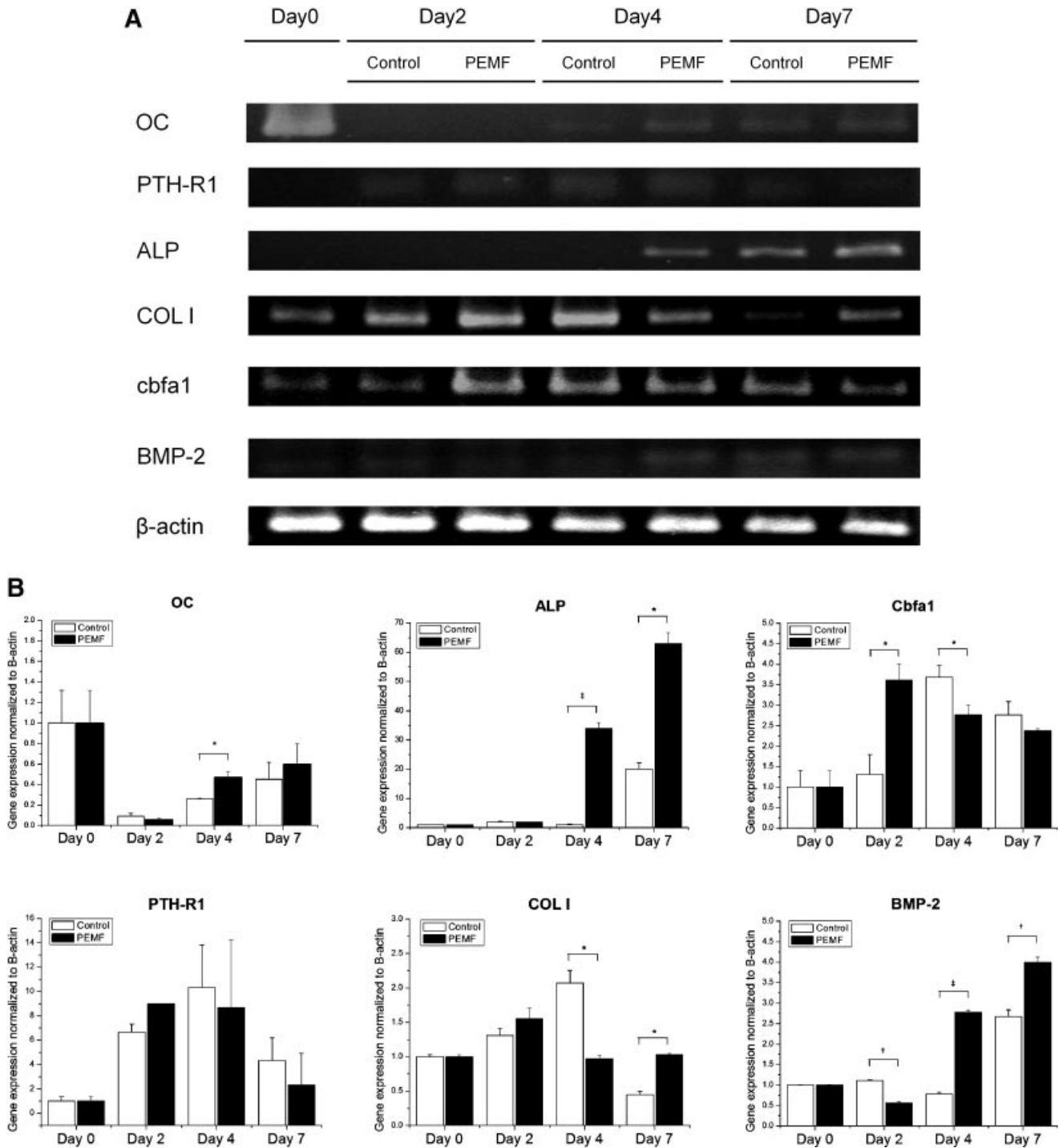


Fig. 4. Effect of PEMF exposure on osteogenic gene expression during osteogenic differentiation of BMMSCs. BMMSCs were cultured in osteogenic medium and exposed (or not, control) to PEMF for 8 h/day. **A:** Electrophoresis of reverse transcriptase-polymerase chain reaction (RT-PCR) productions. **B:** To examine changes in expression of osteogenic genes with time and with the duration of PEMF exposure, cells were harvested on the indicated days and subjected to semi-quantitative RT-PCR, followed by agarose gel electrophoresis and ethidium bromide staining.  $\beta$ -actin was amplified as an internal control. RT-PCR amplification products were quantified, and values represent fold change relative to day 0 (undifferentiated BMMSCs). The data points represent the mean value  $\pm$  SD ( $n = 3$ ). \*  $P < 0.05$ ; †  $P < 0.01$ ; ‡  $P < 0.005$ .

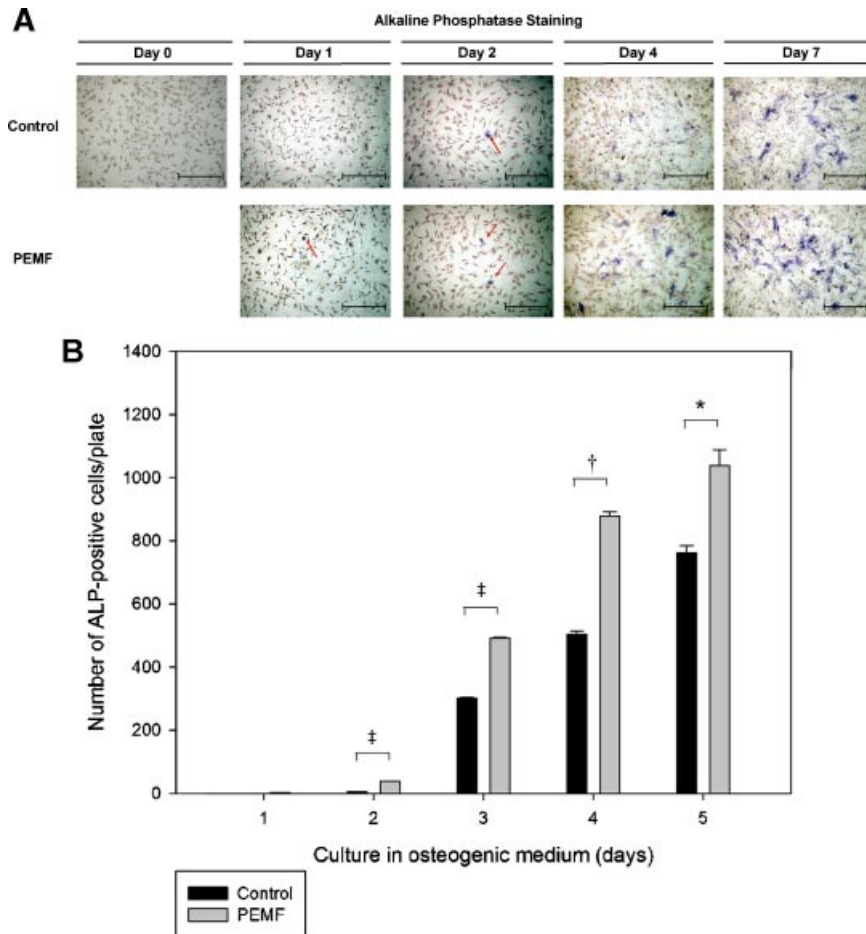


Fig. 5. Effect of PEMF exposure on alkaline phosphatase (ALP) expression during osteogenic differentiation. **A**: Representative images of ALP staining of BMMSCs on days 0, 1, 2, 4, and 7 of culture in osteogenic medium and exposure (or not, control) to PEMF (8 h/day). Bar = 500  $\mu$ m. Arrow indicates ALP-positive cells (blue/purple stain). **B**: ALP-positive cells in the cultures described in panel A were quantified, and data reflect average values ( $n = 3$ )  $\pm$  SD at each time point. \*  $P < 0.05$ ; †  $P < 0.01$ ; ‡  $P < 0.005$ .

results suggest that PEMF alters osteogenesis-related gene expression early during the osteogenic differentiation process in BMMSCs in vitro.

### Effect of PEMF on ALP Activity

The effect of PEMF exposure on cellular ALP activity during osteogenesis was examined by measuring ALP reaction products in PEMF-treated and untreated cells over time. Representative cultures are shown in Figure 5A. On day 1, several ALP-positive cells were observed in the PEMF-exposed cultures, but no ALP-positive cells were observed in the untreated control group. The number of ALP-positive cells in each plate was counted and determined to be  $\geq 30\%$  higher in the PEMF-treated group than in the untreated control group on days 1–5 (Fig. 5B). These results suggest that PEMF exposure caused ALP to be expressed earlier in BMMSCs during the osteogenesis,

where the total number of ALP expressing cells was greater than that in the control group at all times.

### Effect of PEMF on Mineralization

The effect of PEMF exposure on mineralization during osteogenesis was also studied. Mineralization of the extracellular matrix is typically observed near the midpoint of osteogenic differentiation [Stein et al., 1996]. Therefore, BMMSCs were cultured in osteogenic medium and were either exposed to PEMF for 11 days or not exposed to PEMF, and mineralization was detected by von Kossa staining (Fig. 6). Untreated and PEMF-treated BMMSCs growing in osteogenic medium exhibited a small amount of matrix mineralization on days 0, 3, and 7, but PEMF-stimulated BMMSCs exhibited stronger matrix mineralization compared to the untreated controls on day 11. These findings suggest that PEMF treatment substantially



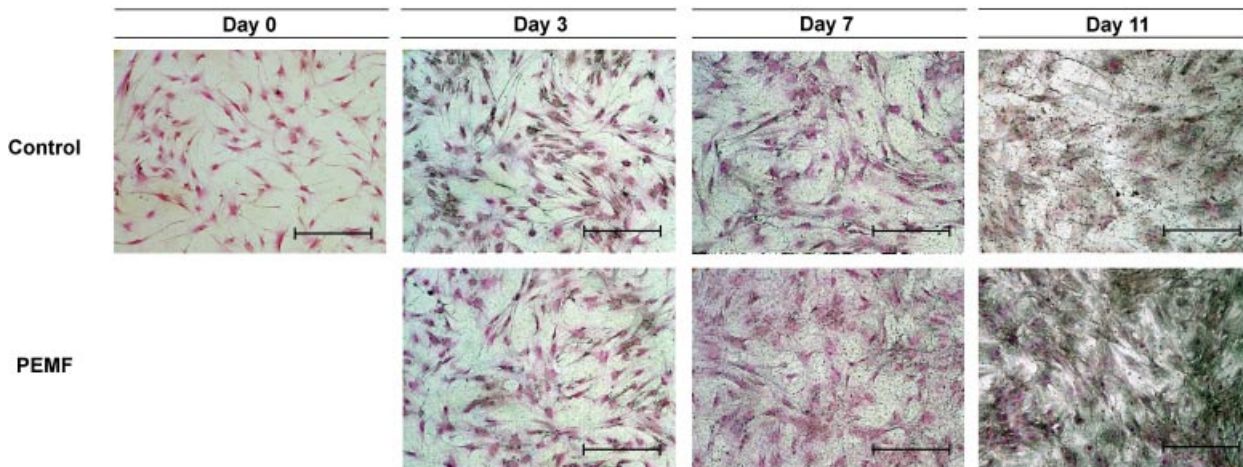


Fig. 6. Effect of PEMF exposure on mineralization during osteogenic differentiation of BMMSCs. BMMSCs were cultured in osteogenic medium and exposed (or not, control) to PEMF for 11 days. Mineralization was detected by von Kossa staining on days 0, 3, 7, and 11. Representative images are shown. Only the PEMF-exposed cells on day 11 displayed heavy matrix mineralization. Bar = 200  $\mu$ m.

increased mineralization near the midpoint of osteogenesis.

## DISCUSSION

In the human bone marrow stroma, a large percentage of BMMSCs differentiate into osteoblasts and participate in bone regeneration during the healing of bone fractures [Koc et al., 1999]. We previously reported that 40–59% more viable BMMSCs were obtained in the PEMF-exposed cultures with FGF-2, 24 h after plating, and that 20–60% higher cell densities were achieved during the exponentially expanding stage at different seeding densities [Sun et al., 2009]. These findings suggested that PEMF exposure *in vitro* increases the population of BMMSCs, the source of osteoblasts which likely contributes to the observed increase in bone fracture healing *in vivo* following PEMF treatment of patients. Our current data suggest that PEMF not only significantly induces increased or decreased cell proliferation depending on the osteogenic stage of cells at the time of exposure but also significantly modifies the expression of certain osteogenesis-related genes and enhances the level of ALP activity and mineralization at early and middle stages of BMMSC osteogenic differentiation.

Despite the fact that PEMF has been used clinically for years to promote bone fracture healing, *in vitro* data have always been inconsistent [De Mattei et al., 1999; Lohmann et al., 2000, 2003; Chang et al., 2004; Selvamurugan et al., 2007; Schwartz et al., 2008]. According to Diniz et al. [2002], PEMFs accelerate cell proliferation, enhance cellular differentiation,

and increase the formation of bone-like tissue from osteoblasts during the early stage of osteogenesis. Chang et al. [2004] reported that PEMF enhances osteoblast proliferation, and Selvamurugan et al. [2007] determined that BMP-2 and PEMF have an additive effect on rat osteoblast proliferation, which may be explained by the possibility that BMP-2 stimulates BMMSC differentiation into mature osteoblasts [Zachos et al., 2006]. Schwartz et al. [2008], however, reported a decrease in cell numbers when human BMMSCs were cultured in osteogenic medium. These results led us to hypothesize that osteoblasts at different stages of osteogenesis have different proliferative responses to PEMF. To test our hypothesis, we examined PEMF stimulation of BMMSCs at different stages of osteogenesis.

In our study, exposure to PEMF for 1 day led to increased cell proliferation in both the osteogenic and BMMSC groups, but PEMF exposure decreased cell proliferation in the osteoblast group during subsequent days of exposure. In addition, PEMF exposure also decreased proliferation of human MG63 osteoblast-like cells cultured in osteogenic medium without serum (data not shown), similar to what was reported by Lohmann et al. [2000] and Schwartz et al. [2008]. Finally, achievable cell densities in the BMMSC groups ( $5.4\text{--}5.8 \times 10^4$  cells/cm<sup>2</sup>) were higher by approximately ninefold compared with those of osteogenic groups ( $5.5\text{--}6.2 \times 10^3$  cells/cm<sup>2</sup>) with or without PEMF stimulation. The absence of a PEMF effect under these conditions is likely because osteoprogenitor cells exhibit greater contact inhibition than stem cells.



The reason(s) for the observed differences in cell proliferation responses to PEMF among BMMSCs, osteoprogenitor cells and osteoblasts remains unclear. However, osteoblasts at different stages of osteogenesis exhibit different cellular responses to cytokine stimulation [Gerstenfeld et al., 1988; Matsumoto et al., 1991; Harris et al., 1994; Rice et al., 2000; Fakhry et al., 2005], and electromagnetic fields induce osteoblasts to increase the expression of receptors for insulin-like growth factor II [Fitzsimmons et al., 1995], BMP-2 [Bodamyali et al., 1998], and transforming growth factor- $\beta$ 1 [Lohmann et al., 2000]. In addition, we previously reported that the early stages of PEMF exposure affect the cell cycle of BMMSCs and result in more newly divided cells compared to control [Sun et al., 2009]. Expression of functional ion channels may regulate proliferation, and voltage-gated delayed rectifier  $K^+$  current channels were up-regulated while  $Ca^{2+}$ -activated  $K^+$  current channels were down-regulated, during progression from  $G_1$  to S phase in undifferentiated rat MSCs [Deng et al., 2007]. Thus the different cell proliferation responses to the same PEMF treatment we observed between BMMSCs, osteoprogenitor cells and osteoblasts may have resulted from altered cell cycle progression caused by a different type or number of cytokine receptors or ion channels expressed on BMMSCs at different osteogenic stages.

Stein et al. [1996] proposed that there are four principal developmental periods of gene expression in the osteoblast developmental sequence and that specific genes are up-regulated or down-regulated at different times during the process. Among these periods, the process of osteoblast development involves a cascade of cellular signals. Expression of the master regulatory gene *cbfa1* is activated, which then regulates several bone-associated markers (i.e., bone sialoprotein, osteopontin, OC, ALP, and COL I) [Ducy et al., 1997; Harada et al., 1999; Karsenty, 2000]. Although the mechanism of *cbfa1* expression is not completely understood, it has been reported that BMP-2, BMP-4, and BMP-7 up-regulate *cbfa1* expression in osteoblasts [Yamaguchi et al., 2000]. In addition, Bodamyali et al. [1998] demonstrated that PEMF up-regulates transcription of BMP-2 and BMP-4 in rat osteoblasts in vitro. In our study, PEMF exposure resulted in an earlier appearance and disappearance of *cbfa1* transcripts during osteogenesis, but PEMF exposure resulted inversely in earlier disappearance and appearance of BMP-2 mRNA compared to the untreated control group. Finally, although the expression of maturation-associated genes such as those encoding OC and ALP was up-regulated at days 4 and 7 in the untreated control cells following osteogenic stimulation, expression of these genes in PEMF-treated cells was several times higher than the

control group at the same time points. This suggests that PEMF exposure modifies the expression of certain osteogenesis-related genes to enhance osteogenesis during BMMSC osteogenic differentiation.

ALP is required during the mineralization process and hydrolyzes phosphate-containing substrates to increase the local phosphate concentration [Robison, 1923]. It has been suggested that ALP activity in osteogenic cell lines increases after PEMF exposure [Lohmann et al., 2000]. However, Schwartz et al. [2008] reported that PEMF alone does not affect ALP activity in BMMSCs, but rather synergizes with BMP-2 to increase ALP activity in BMMSCs during osteogenesis. In our study, the number of ALP-positive cells in the PEMF-exposed group was significantly higher than in the untreated control group during early osteogenesis (Fig. 5B), and the level of mineralization in PEMF-treated cells was also higher than in the control group near the midpoint (day 11) of osteogenic differentiation (Fig. 6). On the other hand, PEMF treatment did not affect ALP activity and mineralization in differentiated osteoblasts (data not shown). These findings are consistent with the results of Schwartz et al. [2008]. Culturing BMMSCs with FGF-2 preserves their osteogenic differentiation potential [Martin et al., 1997], and the BMMSCs in our present study expressed BMP-2 transcripts after osteogenic induction (Fig. 4), suggesting that PEMF exposure and BMP-2 expression synergistically increase ALP expression in BMMSCs during osteogenesis.

In conclusion, our data provide information about the interaction between PEMFs and osteogenesis in BMMSCs. Our findings suggest that PEMF not only significantly increases cell proliferation in human BMMSCs during osteogenesis in the presence of serum but also significantly up-regulates the expression of the osteogenesis master regulatory gene *cbfa1* at early stages of the culture process. This is followed by the enhancement of other osteogenesis-related genes including those encoding OC, ALP, and BMP-2. In addition, PEMF increases ALP levels and mineralization at early and middle stages of BMMSC osteogenic differentiation.

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